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The mechanism(s) of post-transcriptional regulation of the human constitutive endothelial nitric oxide synthase gene by tumor necrosis factor-alpha.

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A Thesis

in

The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy at
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ABSTRACT

The mechanism(s) of post-transcriptional regulation of the human constitutive endothelial nitric oxide synthase gene by tumor necrosis factor-alpha.

Farida Mohamed, PhD
Concordia University, 2000

Endothelial nitric oxide synthase (eNOS) is an enzyme that catalyzes the formation of nitric oxide (NO) from the amino acid L-arginine and molecular oxygen, with the help of cofactors and a heme iron. It is one of three isoforms discovered as yet, and was first identified in endothelial cells lining the blood vessels. Its product, NO, plays a crucial role in the maintenance of vascular tone and homeostasis. Being a gas, NO can diffuse out of the endothelial cells into the underlying smooth muscle cells to cause vasodilation and into the lumen to prevent the blood platelets and leukocytes from clumping and adhering to the vessel wall.

Since eNOS plays a vital role in cell biology, it needs to be tightly regulated. It is expressed constitutively in normal physiological conditions, but may be deregulated in pathophysiological conditions. The purpose of this thesis is to investigate the role of the inflammatory cytokine, tumor necrosis factor-alpha (TNFα), in the down-regulation of eNOS expression. It demonstrates that this process occurs at the post-transcriptional level, where TNFα is shown to affect the stability of the eNOS transcript, in that it reduces the half-life of the otherwise stable eNOS mRNA. Mechanisms of down-regulation by TNFα are explored, involving proteins that bind to the 3' untranslated region of the eNOS message.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
ARD-1	activation of RNA decay-1
ARDS	adult respiratory distress syndrome
ARE	AU-rich element
ARM	arginine-rich motif
AS	antisense
ATP	adenosine triphosphate
AU	adenine-uridine
AUBF	AU-binding factor
BAEC	bovine aortic endothelial cell
bFGF	basic fibroblast growth factor
bp	base pair
BH_4	tetrahydrobiopterin
Ca^{2-}	calcium
CA	cytidine-adenosine
CAT	chloramphenicol acetyl transferase
cGMP	cyclic guanosine monophosphate
CHX	cycloheximide
CL	cardiolipin
CMV	cytomegalovirus
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
CO	carbon monoxide
CPR	cytochrome P-450 reductase
CPSF	cleavage and polyadenylation specificity factor
CRD-BP	coding region determinant-binding protein

CUG-BP	CUG repeat mRNA-binding protein
DAG	diacylglycerol
DAN	deadenylation nuclease
db-cAMP	dibutyryl cyclic adenosine monophosphate
Dex	dexamethasone
DM	myotonic dystrophy
DMA	dimethyl arginine
DNA	deoxyribonucleic acid
DSE	downstream sequence element
DSRM	double-stranded RNA-binding motif
DTT	dithiothreitol
dTMP	deoxyribosylthymine monophosphate
dUMP	deoxyuridine monophosphate
EC	endothelial cell
EDRF	endothelial-derived relaxing factor
ELAV	embryonic lethal abnormal visual
ENAP-1	endothelial nitric oxide synthase-associated protein-1
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FAS	fatty acid synthase
Fe	iron
FMN	flavin mononucleotide
FMR1	fragile mental retardation-1
FMRP	fragile mental retardation-1 gene product
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	guanylate cyclase
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor

GTP	guanosine triphosphate
Hb	hemoglobin
HDL	high density lipoprotein
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HO	heme oxygenase
HONO	nitrous acid
hnRNP	heterogeneous nuclear ribonucleoprotein
HSA	heat stable antigen
hsp	heat shock protein
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
ICU-BP	IGF-II cleavage unit binding protein
IFNγ	interferon-gamma
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
IPTG	isopropyl-1-thio-β-D-galactoside
IRE	iron-responsive element
IRP	iron-regulatory protein
K^-	potassium
KH	hnRNP K homology
LDH	lactate dehydrogenase
LDL	low density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide

LRBP-1	lutropin receptor-binding protein-1
Lyso-PC	lysophosphatidylcholine
MBP	myelin basic protein
Mg	magnesium
m^7G	7-methylguanosine
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
Mt-PK	myotonin protein kinase
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NMD	nonsense-mediated decay
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NO_2	nitrogen dioxide
NO_2^-	nitrite
NO_3^-	nitrate
N_2O_3	dinitrogen tri-oxide
N_2O_4	dinitrogen tetra-oxide
nNOS	neuronal nitric oxide synthase
O_2	oxygen
$OONO^-$	peroxynitrite
ORF	open reading frame
PA	phosphatidic acid
PABP	poly(A)-binding protein
PAI-1	plasminogen activator inhibitor-1
PAN	poly(A) ribonuclease
PARS	poly (ADP-ribose) synthetase
PC	phosphatidylcholine
PCBP	poly(C)-binding protein

PCR	polymerase chain reaction
PDBu	phorbol-12,13-dibutyrate
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PMA	phorbol myristate acetate
PNPase	polynucleotide phosphorylase
PPAEC	porcine pulmonary artery endothelial cell
PS	phosphatidylserine
PTB	pyrimidine tract-binding protein
PTH	parathyroid hormone
PUB1	poly(U)-binding protein-1
qk(v)	Quakingviable
RGG	arg-gly-gly
RNA	ribonucleic acid
RRM	RNA recognition motif
SCLC	small-cell lung carcinoma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLBP	stem loop-binding protein
SM	sphingomyelin
SMC	smooth muscle cell
SNAP	S-nitroso acetylpenicillamine
SNP	sodium nitroprusside
snRNP	small nuclear ribonucleoprotein
SOD	superoxide dismutase
SSC	saline-sodium citrate

SSRE	shear stress-response element
SV40	Simian virus 40
TAR	trans-activating region
TBE	tris-borate-EDTA
TCA	tricarboxylic acid cycle
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCR	T cell antigen receptor
TGFβ	transforming growth factor-beta
TIS	transcription initiation site
TNFα	tumor necrosis factor-alpha
TPA	12-O-tetradecanoylphorbol-13 acetate
TPO	thrombopoietin
TRBP	TAR RNA-binding protein
TS	thymidylate synthase
3'UTR	3' untranslated region
5'UTR	5' untranslated region
uPA	urokinase-type plasminogen activator
URBP	U-rich binding protein
VEGF	vascular endotheial growth factor

1. INTRODUCTION

1.1. Brief history of nitric oxide:

Nitric oxide (NO) has long been considered to be one of the main ingredients of smog and other pollutants, such as car exhaust. It was believed to have no beneficial effects up until 1987 (Ignarro et al., 1987) when it was characterized as being endothelial-derived relaxing factor (EDRF), a factor first described by Furchgott et al. in 1980 (Furchgott and Zawadzki, 1980), who made the observation that acetylcholine stimulated the endothelium to release EDRF that then diffused into the underlying smooth muscle cells (SMCs), causing them to relax. Moncada of Wellcome Research Laboratories went on to demonstrate by chemiluminescence that endothelial cells (ECs) really did release NO (Palmer et al., 1987), which was EDRF. Since then, nitric oxide has gone on to be the molecule of the year in 1992 (Koshland, Jr., 1992) and has been implicated in a number of important biological functions.

One of its major roles is to act as a signalling molecule in the central and peripheral nervous systems; studies by Garthwaite and colleagues (Garthwaite et al., 1988) showed that rat cerebellum released a labile substance upon activation of the *N*-methyl-D-aspartate (NMDA) receptor, that led to a rise in cyclic guanosine mono-phosphate (cGMP) and could be blocked with N^G-monomethyl-L-arginine (L-NMMA). As far back as 1981, Tannenbaum and his associates at the Massachusetts Institute of Technology (M.I.T.) noted that humans and rats fed low-nitrate diets still excreted substantial amounts of nitrates (Green et al., 1981). He also found very high levels of urinary nitrates from a man who had infectious diarrhea (Tannenbaum et al., 1978) and noted that nitrate excretion in rats was increased after

injections of bacterial endotoxin (Wagner et al., 1983). Marletta, a former student of his at M.I.T., found that mice with a genetically determined macrophage deficiency excreted few nitrates (Stuehr and Marletta, 1985) and he therefore established an association between macrophages and nitrates. To probe further, he discovered that administering endotoxin and cytokines to cultured macrophages caused them to produce nitrates, and that removal of the amino acid arginine from the medium blocked the production of nitrates (Marletta et al., 1988). He proved that a specific enzyme in the macrophages was converting arginine into an intermediate chemical, which turned out to be NO, that very quickly got transformed into nitrites (NO_2^-) and nitrates (NO_3^-). The production of NO is the only known route to form nitrite and nitrate in mammals (Beckman, 1995). Microbes can generate NO via reduction of nitrite or oxidation of ammonia (Feldman et al., 1993).

Meanwhile Hibbs, of the University of Utah, working independently, demonstrated the ability of macrophages to kill cultured tumor cells, which disappeared when arginine was removed from the medium. He showed that arginine got converted into NO_3^- and the amino acid citrulline, and provided evidence that a specific enzyme produced NO from arginine (Hibbs, Jr. et al., 1987). He not only went on to demonstrate that NO gas was toxic to the tumor cells, but identified the first inhibitor of the enzyme that was synthesizing it by showing that a methyl derivative of arginine blocked the formation of nitrates and the macrophages' tumor-destroying capacity (Hibbs, Jr. et al., 1987).

1.2. Nitric oxide synthases:

NO is generated by nitric oxide synthase (NOS), of which three known isoforms have

been characterized: the neuronal form (NOS I or nNOS), the inducible form (NOS II or iNOS), and the endothelial form (NOS III or eNOS). Upon treatment with carbon monoxide (CO), NOS shows a λ_{max} of ~ 450 nm (White and Marletta, 1992); this rare spectral characteristic is relegated to cytochrome P-450, a large family of enzymes found in the liver, that oxidizes a wide variety of synthetic and natural products. The P-450 spectrum is derived from the ligation of a cysteine thiolate to a heme iron. The amino acid sequence of NOS was found to have similarity to the mammalian cytochrome P-450 reductase (CPR), which serves as an electron donor for the P-450 enzymes. Usually the microsomal P-450 enzymes require a flavoprotein reductase, i. e. a CPR, and an iron-sulfur protein (a cytochrome P-450 drug-metabolizing enzyme) to transfer electrons into the heme prosthetic group that is responsible for the oxidative catalysis, with the exception of the *Bacillus megaterium* $P\text{-}450_{BM3}$, which has both the flavoprotein reductase and the heme within the same polypeptide (Narhi and Fulco, 1986).

Since the C-terminal of NOS shows a significant homology to cytochrome P-450 reductase (Bredt et al., 1991a), and since the N-terminal contains a heme site, which is a cytochrome P-450 type iron-protoporphyrin IX prosthetic group (White and Marletta, 1992), NOS was thought of to be a self-sufficient mammalian P-450. But the substrates for the P-450 enzymes are very hydrophobic compared to the hydropilic NOS substrate arginine. Therefore NOS may lie outside the P-450 superfamily, but it uses the P-450 chromopore for its chemistry. Thus it is likely that NOS and the cytochrome P-450 enzymes were linked in evolution.

Besides cytochrome P-450 enzymes, CPR donates electrons to heme oxygenase (HO),

an enzyme that cleaves heme to form biliverdin and CO (Ishikawa et al., 1991). This relationship suggests that CO may serve functions resembling NO. So far, HO-1 has been found to be highly expressed in liver and spleen, induced by heme and oxidative stress (Maines, 1988). HO-2, the non-inducible form, is found in the brain (Sun et al., 1990) and is localized to endothelial cells and adventitial nerves of blood vessels, as well as to neurons in autonomic ganglia (Zakhary et al., 1996). CO can elicit vascular relaxation by stimulating soluble guanylate cyclase (GC), but is considerably less potent than NO (~ 0.1%) (Zakhary et al., 1996). The speculative role of CO is that it may function as an endogenous vasodilator when its concentration is sufficiently high or when endogenous NO production is low.

NOS enzymes contain a reductase domain, which has binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), similar to the enzyme cytochrome P450 reductase, and an oxygenase domain that harbors a heme, a tetrahydrobiopterin (BH_4), and the substrate (L-arginine) binding sites, as well as a consensus sequence for phosphorylation by cAMP-dependent protein kinase (PKA) (Figure 1.1A). These two domains are linked by a calmodulin-binding site, which consists of a basic amphiphilic α-helix, with hydrophobic residues on one side of the helix and positively charged amino acids on the opposing face (Venema et al., 1996a). The oxygenase domain of the different NOS isoforms does not share significant amino acid sequence with any other known proteins, but within this domain, a stretch of about 320 amino acids is highly conserved among the NOS isoforms, which are presumed to be binding sites for heme, BH_4, and L-arginine (Forstermann et al., 1991).

Glu-361 in human eNOS has been shown to be specifically involved in the interaction

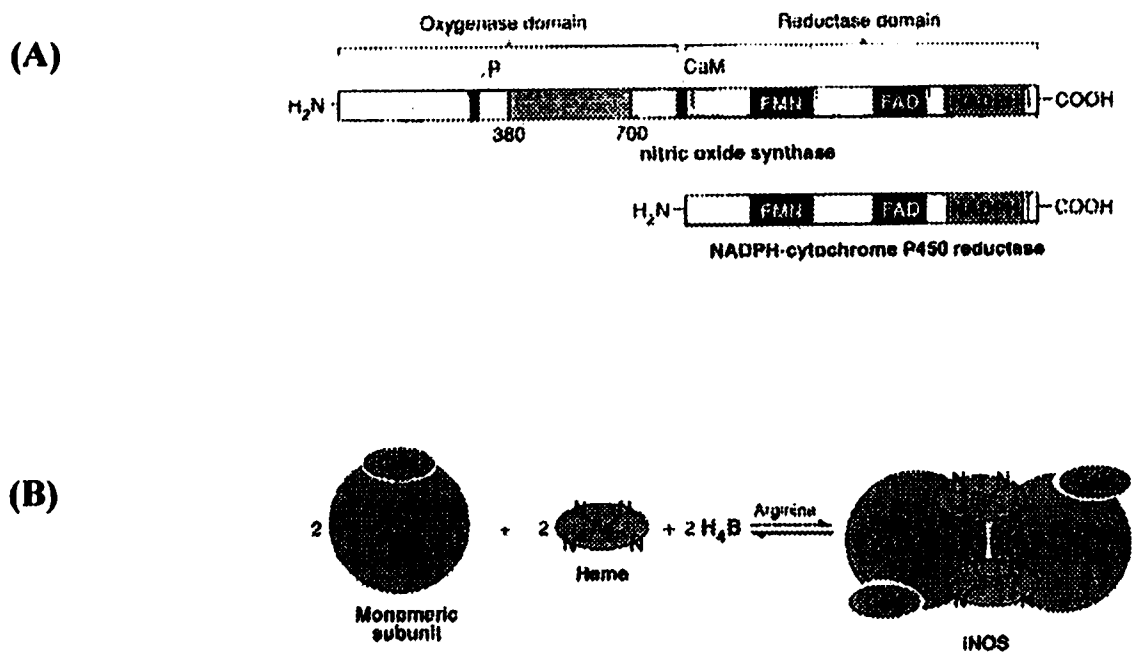


Figure 1.1 (A) The nitric oxide synthase (NOS) protein is compared to the cytochrome P450 reductase in that they both share the NADPH, FAD and FMN binding sites. The NOS protein also contains an oxygenase domain, harboring sites for binding the substrate L-arginine, the cofactor tetrahydrobiopterin and a heme iron, as well as a consensus sequence for phosphorylation by PKA (P). The calmodulin-binding site bridges the two domains. **(B)** The dimerization of the isoforms of NOS is necessary for their activity.

with L-arginine, since mutation of this amino acid resulted in a complete loss of L-citrulline formation and since L-arginine binding could not be detected by electron spin resonance spectroscopy (Chen et al., 1997). Asp-369 mutants also exhibit low citrulline formation activity, having high Kd values for L-arginine (1-10 mM) compared to wild-type eNOS (0.4 μM) (Chen et al., 1998). They display an unstable heme-CO complex and a very low BH_4 content. Thus the binding sites for L-arginine and BH_4 are in proximity. Furthermore, Asp-369 and Arg-372 mutants have been shown to be defective in dimer formation (Chen et al., 1998). Therefore these two residues must play a critical role in oxygenase domain active-site structure and activity. The crystal structure of eNOS has revealed that the small substrate L-arginine is hydrogen-bonded to a conserved glutamate (Glu-361) and binds in a narrow cleft within the larger active-site cavity containing heme and BH_4 (Fischmann et al., 1999). The crystal structure of the heme domain of the eNOS protein has revealed that a zinc ion is tetrahedrally coordinated to pairs of symmetry-related cysteine residues (Cys-$(X)_4$-Cys) at the dimer interface, maintaining the integrity of the BH_4 binding site (Raman et al., 1998). Cys-184 of eNOS is involved in heme coordination and catalytic activity (Chen et al., 1994). Cys-99 is critical for BH_4-dependent eNOS stability and activity (Chen et al., 1995). NOS and NADPH-cytochrome P-450 reductase may be the only two mammalian enzymes with recognition sites for both FMN and FAD. Sulfite reductase, a bacterial enzyme, also has recognition sites for FMN and FAD and displays substantial amino acid sequence homology to NOS (Ostrowski et al., 1989).

McMillan and Masters have expressed both domains of nNOS in *Escherichia coli* (E. coli) which exhibited activities associated with individual domains, i. e. the NOS hemoprotein

exhibited a ferrous-CO spectrum with a wavelength maximum at 445 nm, and spectral perturbations with L-arginine and BH_4 elicited a spectrum confirming the presence of binding sites for these molecules within the N-terminal part of the NOS polypeptide (McMillan and Masters, 1995). The C-terminal part or the reductase domain was competent in NADPH-dependent electron transfer to cytochrome c. Chen et al. have also successfully expressed the two separate domains of eNOS in a baculovirus system, purified them, and characterized their spectral and catalytic properties (Chen et al., 1996). They not only showed that eNOS has a bidomain structure, but that they could reconstitute the catalytic activity of the enzyme when they mixed the two domains together. They were also able to show that the oxygenase domain bound BH_4 but no flavins, whereas the reductase domain bound FAD and FMN but no heme or BH_4. Neither domain could catalyze the formation of L-citrulline from L-arginine on its own, but the reductase domain exhibited cytochrome c reduction activity which was equivalent to that of the intact eNOS.

1.3. Reaction catalyzed by NOS:

NOS catalyzes the conversion of L-arginine to L-citrulline and NO in a two-step reaction (Figure 1.2) involving first the oxidation of one of L-arginine's two equivalent guanidino nitrogens to form an intermediate N^G-hydroxy-L-arginine. This step requires two electrons, which are supplied by NADPH (Figure 1.3); the first electron binds to the ferric iron of the enzyme's heme prosthetic group to reduce it, enabling it to bind oxygen (O_2). Another electron breaks the oxygen-oxygen bond, with one atom of oxygen released as water and the other inserted into one of the terminal guanidino nitrogen-hydrogen bonds of L-

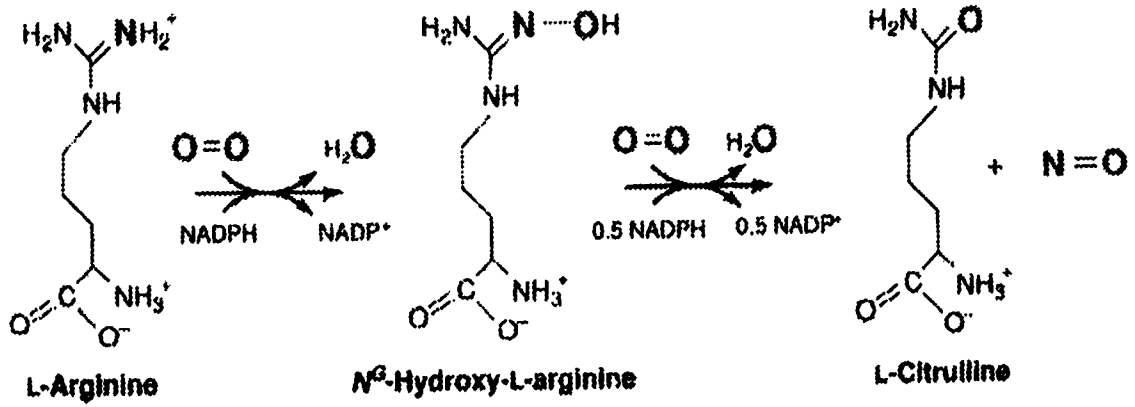


Figure 1.2 The reaction catalyzed by nitric oxide synthase. The substrate L-argignine is first converted into an intermediate product, hydroxy-L-arginine, via a two-electron oxidation and subsequently into L-citrulline by a three-electron oxidation step.

arginine, which binds NOS near the heme group. Thus the ferric iron is reformed and the hydroxyarginine intermediate remains bound to the active site of the enzyme.

The second step involves a third NADPH-derived electron that binds to ferric iron, again enabling it to bind a second molecule of O_2 (Figure 1.3). From then on, the electrons come from the substrate, which involve oxygen insertion and carbon-nitrogen bond scission to form L-citrulline and NO. The overall reaction involves 5 electrons, two of which are supplied by the substrate and three from NADPH. Therefore for each molecule of NO produced, one arginine, two O_2 and one and a half NADPHs are consumed. However, NOS transforms substrates with even numbers of electrons (L-arginine, oxygen, and NADPH) into products with even numbers of electrons (L-citrulline, water, and $NADP^+$) plus the free radical $NO^{\cdot}$. Since NADPH is a two-electron carrier, the enzyme NOS must have a means to store extra electrons; it contains two flavin cofactors which are able to hold one electron each. Thus over the course of two catalytic cycles, three NADPH molecules would transfer six electrons to NOS that would be used to reduce four O_2 molecules consumed in the oxidation of two arginines to two NOs and two citrullines.

Without NADPH or calcium (Ca^{2+})/calmodulin, enzyme activity is undetectable and without BH_4, 80% of enzyme activity is lost (Chen et al., 1996). As found for the neural and endothelial isoforms of NOS, reduction of cytochrome c by the flavoprotein or reductase domain is stimulated seven-fold by the addition of calmodulin and Ca^{2+} (Abu-Soud and Stuehr, 1993). Therefore electron transfer from the reductase to the oxygenase domain is very important and appears to be modulated by calmodulin. Electron transfer likely occurs from NADPH via FAD, which gets reduced, in turn leading to the reduction of FMN, which then

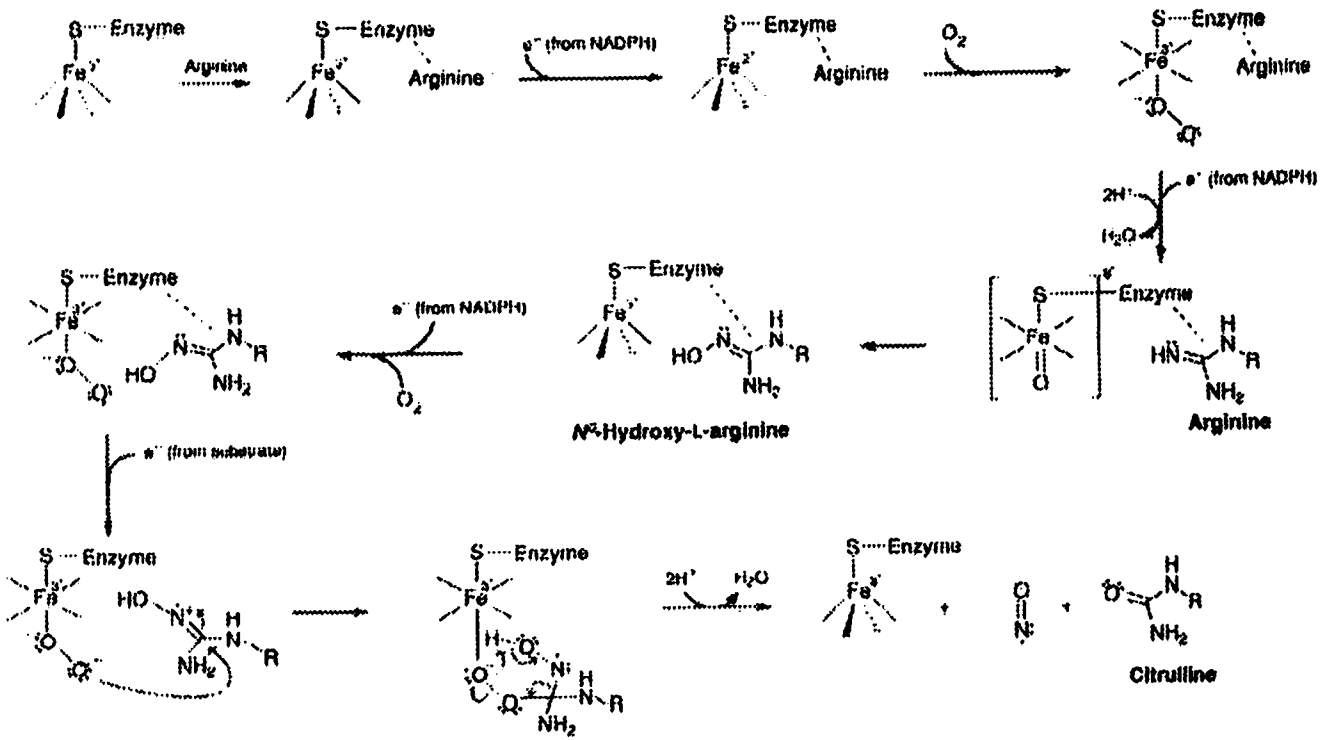


Figure 1.3 Schematic diagram outlining electron transfer from NADPH to the heme iron throughout the reaction catalyzed by nitric oxide synthase

transfers electrons to the ferric heme, promoting interaction with molecular O_2. The NOS isoforms are homodimeric proteins (Figure 1.1B) (Hellermann and Solomonson, 1997; List et al., 1997), and BH_4, which remains tightly bound to NOS, may play a role in this dimerization. Stuehr's group found that BH_4 is required for the formation of the functional dimer of iNOS (Baek et al., 1993). BH_4 directly increases the production of NO by stabilizing eNOS in its dimeric form, suggesting that an effect on allosteric conformation could be involved in this effect on NO production (Wever et al., 1997). BH_4 may also be modulating the heme environment of NOS, as shown by spectral analysis (Rodriguez-Crespo et al., 1996), as well as the binding of substrate; the spectroscopically determined binding constants for L-arginine are 1.9 μM in the presence and 4.0 μM in the absence of BH_4 (Rodriguez-Crespo et al., 1996).

Wever et al. has demonstrated that recombinant eNOS has the potential to generate superoxide as well as NO, a process that is tightly controlled by BH_4 (Wever et al., 1997). This appears to occur primarily at the heme center of its oxygenase domain. They were able to show superoxide dismutase (SOD)-inhibitable superoxide production, which was not affected by administration of L-arginine, but could be inhibited dose-dependently by BH_4, and could be completely inhibited by diphenyleneiodonium, an inhibitor of the flavin moiety of the enzyme. eNOS thus appears to be a superoxide generating enzyme probably through its flavin moiety, as well as a BH_4-dependent NO producing enzyme (Wever et al., 1997). The concentration of BH_4 regulates the ratio of superoxide to NO generated by eNOS.

1.4. Occurrence of NOS:

Ever since the neuronal form of NOS has been isolated from the cerebellum of the brain, the endothelial isoform from ECs, and the inducible NOS from macrophages, the occurrence of NOS has become more and more visible in other tissues. nNOS is also found in skeletal muscle, in locations where mitochondria are distributed (Frandsen et al., 1996). Since it is known that NO can suppress mitochondrial respiration through competitive interaction with the oxygen-binding site of cytochrome oxidase (Brown and Cooper, 1994; Cleeter et al., 1994), then NO in skeletal muscle would cause decreased adenosine triphosphate (ATP) generation with a consequent attenuation in muscle oxygen consumption and muscle contractility. NOS is found in the neurons that innervate the penis since these can cause NO-mediated relaxation of the smooth muscle of the corpus cavernosum, the major erectile tissue. Thus nNOS or rather NO acts as a normal regulator of penile erection, leading to increased blood flow into the penis and hence erection (Burnett et al., 1992). nNOS occurs throughout the lungs and gut, in autonomic nerve fibers, where NO carries out its duties as a neurotransmitter. During digestion, a series of systematic wavelike contractions (peristalsis) occurs to move food through the stomach and intestines; NO triggers the relaxation component (Lowenstein and Snyder, 1992). NO also controls the movement of the sphincter separating the stomach from the intestine, i. e. the pyloric sphincter (Oliveira et al., 1992), as well as the anal sphincter (Tottrup et al., 1992). nNOS as well as eNOS have been identified in brain microvascular ECs, namely mouse brain surface arterioles (Rosenblum and Murata, 1996). Cardiomyocytes possess a Ca^{2+}-dependent NOS activity that plays a role in the physiological modulation of myocardial contractility in an autocrine fashion (Schulz et al., 1991). nNOS is also found in the ß cells of pancreatic islets (Worl et al., 1994).

iNOS can be induced in a variety of other cell types within the body other than in activated macrophages, as for example, hepatocytes (Geller et al., 1993), SMCs (Wong et al., 1996), and ECs (Gross et al., 1991). It has even been found to be induced in the myocardium in response to inflammatory cytokines (Schulz et al., 1992). Since NOS is associated with reduced contractility, then the enhanced production of NO by the inducible isoform may account for the depression of myocardial contractility seen in septic shock, in allograft rejection, as well as during anti-tumour therapy with cytokines.

eNOS has been found in platelets (Sase and Michel, 1995), in the endometrium (Tseng et al., 1996) and in the placenta in pregnant females, where it presumably regulates blood flow, fetal nutrition, and growth (Garvey et al., 1994). With uterine contraction on parturition, NOS expression decreases to allow for delivery (Yallampalli et al., 1993) . NOS has been found to be regulated in the rat fallopian tube during the oestrous cycle (Bryant et al., 1995). NOS appears early in the fetal stage; eNOS has been detected as early as 29% gestation in the developing capillaries coursing through fetal mesenchyme in the developing ovine lung. After birth and in the distal pulmonary arteries of adult animals, eNOS decreases rapidly (Halbower et al., 1994). This may suggest a role for endogenous NO activity in angiogenesis, the sprouting of new vessels from already existing ones, in the developing pulmonary circulation. However, nNOS mRNA abundance has been seen to decline during late fetal life, but to rise again postnatally (North et al., 1994). NOS activity also occurs in the venules and arterioles pervading the intestine (Nichols et al., 1994).

1.5. Genetic structure of NOS:

Though these enzymes all perform the same function, i. e. they all produce NO from L-arginine, they differ slightly in molecular weight, with nNOS having a M_r of 155-kDa, iNOS being 130-kDa, and eNOS possessing a mass of 135-kDa. They also share about 50% amino acid sequence homology (Lamas et al., 1992). The transcript size of the three isoforms varies widely, even though the proteins themselves do not. nNOS boasts a messenger ribonucleic acid (mRNA) of approximately 10.4 kilobases (Kb), whereas iNOS mRNA is about 4.15 Kb and eNOS mRNA is 4.1 Kb in length. The three known NOS isoforms are the products of different genes; the human endothelial isoform, comprising 26 exons and spanning 21 Kb, lies on chromosome 7 at 7q35-36 (Marsden et al., 1993), whereas the human inducible form, also 26 exons but spanning 37 Kb, maps to chromosome 17 at 17cen-17q11 (Xu et al., 1994). The 28-exon human neuronal isoform is found on chromosome 12, precisely at the 12q24.2 region, and extends over 100 Kb (Kishimoto et al., 1992; Xu et al., 1993). The rat eNOS maps to chromosome 4 though (Hubner et al., 1995), while the mouse eNOS is found on chromosome 5 (Gregg et al., 1995). Interestingly, the human gene for the related protein cytochrome P_{450} oxidoreductase has been localized to 7q11.2 (Shephard et al., 1989). Sequence identity between the endothelial NOS from various mammals is quite high, for example, the coding regions of the bovine, human, and porcine eNOS exhibit 90% homology (using DNASIS, Hitachi).

The mouse iNOS promoter contains a TATA box 30 bases upstream of the mRNA transcription initiation site (TIS), as well as containing *cis*-elements homologous to consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines or bacterial products (Lowenstein et al., 1993; Xie et al., 1993). The human

eNOS promoter is "TATA-less" and exhibits proximal promoter elements consistent with constitutively expressed genes in ECs (Zhang et al., 1995). Therefore it is most likely the Sp1 site, situated -104 upstream of the TIS and immediately following a GATA site, that is responsible for the transcription of eNOS. The 105-kDa Sp1 transcription factor is a Cys-His zinc-finger protein that binds to guanine-rich (G + C) sites (Kuwahara et al., 1993). Sp1 may be co-operating with other transcription factors, like the GATA element found at -108. GATA-binding proteins are a zinc-finger family of deoxyribonucleic acid (DNA)-binding proteins that participate as cell-specific transcription factors (Ko and Engel, 1993). There are a few *cis*-regulatory elements in the eNOS promoter, which might be responsible for the constitutive expression of the gene, for example a shear stress response element (SSRE) has been identified at -985, which probably regulates the NOS gene in response to fluid mechanical forces at the transcriptional level in the vascular endothelium (Miyahara et al., 1994).

The neuronal NOS gene may be TATA-less, thus it is possible that there may be more than one transcriptional start site, as has been described for TATA-less promoters (Sharp, 1992). Analysis of 88 nNOS complimentary DNAs from adult and fetal human brain, heart, skeletal muscle, and kidney revealed developmental stage- and tissue-dependent transcriptional heterogeneity generated by the use of nine different first exons, followed by splicing within the 5'-untranslated region (5'UTR) to a common second exon (Wang et al., 1999a). That means that each exon 1 had its own distinct 5' flanking region and it is therefore likely that each is controlled by its own promoter.

A number of variable tandem repeats and dinucleotide repeats $(CA)_n$ have been

identified in the eNOS gene localized to intron 13 (Miyahara et al., 1994; Nadaud et al., 1994). $(CA)_n$ repeat loci are frequently polymorphic; the extent of allelic polymorphism is positively correlated with the size of a $(CA)_n$ repeat. Twenty-eight different alleles have been identified so far, containing 17-44 CA repeats. The presence of alleles containing 38 CA repeats or more in intron 13 of the eNOS gene has been associated with an excess risk of coronary artery disease (Stangl et al., 2000). A 27-base pair (bp) repeat is found closer to the 5' end of the eNOS gene, i. e. in intron 4 (Miyahara et al., 1994). Two alleles of eNOS intron 4, labeled a and b, have been detected; a has four and b has five tandem 27-bp repeats. In a normal healthy human population, 81% has eNOS4 b/b, 19% has eNOS4 b/a and 0% has eNOS4 a/a, whereas the frequency of the 4a allele is higher in cases with end-stage renal failure, 72.7% b/b, 25.1% b/a and 2.2% a/a (Wang et al., 1999b), as well as in patients with abdominal aortic aneurysm (Kotani et al., 2000). Subjects homozygous for the a allele were shown to have plasma nitrate and nitrite levels that were 20% lower than those with the b allele (Tsukada et al., 1998). In current and ex-cigarette smokers, but not nonsmokers, there is a significant excess of homozygotes for this rare 4a allele in patients with severely stenosed arteries, compared to those with no or mild stenosis (Wang et al., 1996). This genotype was also associated with a history of myocardial infarction. This smoking-dependent excess coronary risk in eNOS4a homozygotes is consistent with predisposition to endothelial dysfunction (Wang et al., 1996).

A common polymorphism in exon 7 of the eNOS gene (894G –> T), resulting in Glu-298 –> Asp mutation in the protein, has been linked to coronary artery disease (Hingorani et al., 1999) and has been associated with myocardial infarction in the Japanese population

(Shimasaki et al., 1998) as well as with hypertension (Shoji et al., 2000). This mutation, i. e. eNOS with aspartate at position 298, can result in the cleavage of the enzyme, resulting in 100- and 35-kDa products in human hearts and ECs, suggesting that this polymorphism has a functional effect on the eNOS protein (Tesauro et al., 2000). This marker-disease association may be due to the impaired effects of NO on the cardiovascular system: dysregulation of vascular tone, platelet aggregation and leukocyte adhesion and smooth muscle cell proliferation, all of which promote coronary atherosclerosis and thrombosis.

1.6. Targeting or localization of NOS:

Whereas cytochrome P-450 has a transmembrane domain at its N-terminal end, the NOS isoforms do not; nNOS and iNOS (the neuronal and inducible forms) are mostly cytosolic proteins, whereas the endothelial form, eNOS, is mostly membrane-associated (Pollock et al., 1991). Though it should be mentioned that nNOS has also been recovered in the particulate fraction upon isolation, bound mostly to the endoplasmic reticulum (Hecker et al., 1994). eNOS is targeted to Golgi as well as to plasma membranes (Liu et al., 1997a; Sessa et al., 1995). Targeting of eNOS to endothelial membranes is dependent upon two disitnct modifications, i. e. the co-translational addition of the 14-carbon fatty acid myristic acid to an amino-terminal glycine residue (Busconi and Michel, 1993) and the post-translational addition of the 16-carbon fatty acid palmitic acid to a cysteine residue (Robinson et al., 1995).

The myristoylation and palmitoylation motif Met-Gly-Cys-X-X-Ser/Cys is usually found at the N-terminal of some of the Src family of proteins as well as the α subunits of G

proteins (Mumby et al., 1994). For these proteins, the palmitoylation site, cysteine-3, is adjacent to the myristoylation site, glycine-2. In eNOS, the cysteine closest to the myristoylation site, glycine-2, is located at position 15. Hence it is possible that N-myristoylation is needed for the proper conformation of the enzyme for subsequent palmitoylation, or it is also possible that N-myristoylation allows the protein to associate weakly with the membrane, permitting access to a membrane-bound palmitoyltransferase (Gutierrez and Magee, 1991). Palmitoylation is a reversible process, involving a thiol ester bond, unlike myristoylation, which is irreversible (Gordon et al., 1991). It seems likely therefore that palmitoylation/depalmitoylation could regulate the amount of eNOS found in membranes and cytosol. For example, depalmitoylation and translocation of eNOS could influence NO signalling in the vasculature by removing the enzyme from proximity to membrane receptors, thereby modulating the response to extracellular signals.

Sessa's group investigated the role of palmitoylation by mutating the first two cysteines in the amino terminus of eNOS into serines (C15S and C26S respectively) and found 95% less incorporation of [^{3}H]palmitic acid than in wild-type eNOS (Liu et al., 1995). Surprisingly though, most of the mutated eNOS proteins were found in the membrane fractions of the transfected cells. Thus it seems that palmitoylation is not absolutely required for membrane association; hydrophobic amino acids in eNOS could most likely contribute to its interaction with the membrane. When they compared the palmitoylation of wild-type to the nonmyristoylated mutant eNOS, they found that the mutant could not be labeled with [^{3}H]palmitic acid; thus myristoylation of eNOS is necessary for palmitoylation to occur (Liu et al., 1995).

The same group went on to further investigate the role of palmitoylation in the localization of eNOS and discovered that it is necessary for the targeting of eNOS into caveolae (Garcia-Cardena et al., 1996). Caveolae are plasma membrane invaginations composed primarily of glycosphingolipids, cholesterol, and the integral membrane protein, caveolin, found mostly in microvascular endothelium and less in large vessel endothelium. In living cells, eNOS and caveolin form a heteromeric complex that undergoes cycles of dissociation and re-association modulated by calcium-mobilizing agonists (Feron et al., 1998). Not surprisingly, the site of caveolin binding on eNOS has been localized between amino acids 310 and 570 (Garcia-Cardena et al., 1997), which coincides with the calmodulin binding site situated between amino acids 493 and 512 in the bovine eNOS (Venema et al., 1996a). The binding of calcium/calmodulin to eNOS disrupts the association of eNOS from the scaffolding protein caveolin and thus the enzyme is subsequently translocated from plasmalemmal caveolae. As calcium returns to basal levels, eNOS re-associates with caveolin, and the inhibited enzyme is then restored to caveolae, a process accelerated by palmitoylation of the enzyme. Thus NO may modulate signal transduction through these plasma membrane compartments.

Palmitoylation-deficient forms of eNOS release less NO from the cells than do the wild-type enzyme (Liu et al., 1996). Since palmitoylation is necessary for localization of eNOS in caveolae, this would suggest that localization regulates the frequency and magnitude of NO release in response to stimuli in vivo. Numerous signalling molecules known to modulate NOS are concentrated in caveolae; they include G-protein-coupled receptors such as the muscarinic acetylcholine receptor, a plasma membrane Ca^{2+} pump, an inositol

phosphate (IP_3)-sensitive Ca^{2+} channel, and protein kinase C (PKC) (Anderson, 1993; Smart et al., 1995). The caveolar localization of eNOS may be necessary for acute regulation of the enzyme by intralumenal hormones. Therefore intracellular targeting of eNOS is critical for optimal NO production, since catalytically active eNOS is located on the Golgi complex and in the caveolae of endothelial cells, and since mutation of the N-myristoylation site, Gly-2, which prevents its association to Golgi membranes, impairs the production of NO (Liu et al., 1997a).

Myristoylation may not be the only mechanism responsible for membrane attachment of eNOS. The Gibbs free energy for binding of a myristoylated protein to a phospholipid is 8 kcal/mol, equivalent to an apparent K_D of 100 μM (Peitzsch and McLaughlin, 1993). So it is probably not sufficient for a protein to be myristoylated to be stably anchored to a cellular membrane. It is likely that not only hydrophobic interactions between protein and membrane fatty acyl chains are involved, but also interactions of positively charged protein residues with negatively charged membrane phospholipids. To this end, Venema and colleagues formed various phospholipid vesicles from each of the eight most abundant membrane phospholipids (Venema et al., 1995). These included the anionic or negatively charged lipids phosphatidylserine (PS), phosphatidic acid (PA), cardiolipin (CL) and phosphatidylinositol (PI), and the neutral lipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and lysophosphatidylcholine (lyso-PC). Vesicles were mixed with purified eNOS and centrifuged at 100,000 x *g*, after which supernatant (cytosolic) and pellet (membrane) fractions were analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. eNOS was

recovered in the pellet fractions of the anionic vesicles and in the supernatant fractions of the neutral vesicles. Thus eNOS selectively bound to phospholipids that were anionic. Since the calmodulin-binding domain of NOS is basic, its potential role in membrane association was investigated by the same group. The deletional mutation of the eNOS calmodulin-binding domain resulted in a loss of binding capacity for PS vesicles and removal of that domain converted the enzyme from a membrane to a cytosolic protein when expressed in insect Sf9 cells (Venema et al., 1995). Thus it appears that the calmodulin-binding domain of eNOS is involved in membrane association.

Venema and his colleagues, doing *in vitro* studies with cultured bovine aortic ECs (BAEC), have also identified an eNOS-associated protein, which they have termed ENAP-1 (for endothelial nitric oxide synthase-associated protein 1). This 90-kDa detergent-insoluble, cytoskeletal protein becomes tyrosine phosphorylated upon stimulation of the BAEC with bradykinin, a stimulator of PKC, and then associates with eNOS, translocating it to the cytoskeleton (Venema et al., 1996b). Thus tyrosine phosphorylation-dependent association of eNOS with the cytoskeleton may have a role in targeting NO production to specific subcellular compartments. It is possible that this protein is actually a heat shock protein (hsp90) acting as a chaperone, since increased association of eNOS to hsp90 has been observed following activation of eNOS (Gratton et al., 2000).

The enzyme eNOS can also be found in the cytosol, but in an inactive and phosphorylated state. It has been found that the enzyme can be phosphorylated both by PKC and PKA, thus there might be recognition sequences on the enzyme for both kinases. When purified eNOS was incubated with either one of these two kinases and [γ-^{32}P]ATP, label was

incorporated into NOS in a stoichiometric fashion with one molecule of ^{32}P/NOS monomer (Hirata et al., 1995). When enzyme activity was measured in homogenates of cultured ECs treated with dibutyryl cyclic adenosine mono phosphate (db-cAMP), a stimulator of PKA, or with 12-*O*-tetradecanoylphorbol-13 acetate (TPA) or phorbol 12,13-dibutyrate (PDBu), stimulators of PKC, NOS activity decreased by 30% with the PKC stimulators, but not with db-cAMP, even though the amount of enzyme remained the same (Hirata et al., 1995). Therefore it is likely that NOS might be phosphorylated at two different sites by PKC and PKA, as suggested above, since activation of PKC in intact cells led to a dramatic decrease in NOS activity, whereas activation of PKA did not modulate NOS activity. Indeed Bredt's group showed that PKC produced a phosphopeptide that migrated toward the cathode, compared to the one produced by PKA that migrated more toward the anode (Bredt et al., 1992). Many vasoactive substances are known to activate phospho-inositide hydrolysis through G protein-coupled receptors, which results in the production of IP_3 and diacylglycerol (DAG). Elevated intracellular calcium by IP_3 stimulates NO production through NOS, and PKC activation by DAG diminishes NO generation by NOS phosphorylation. Therefore, receptor-coupled second messenger systems may regulate NOS acitvity and NO production in opposite directions in vascular ECs. In contrast, phosphorylation of nNOS does not affect enzyme activity (Bredt et al., 1992).

1.7. Signal transduction mechanisms of NO:

Numerous mammalian cell types synthesize and release NO; studies indicate that the principal action of NO is on nearby target cells. eNOS is membrane-bound and can be found

on both the lumenal and ablumenal side of ECs, on the inner surface of the cell membrane (O'Brien et al., 1995) and can thus respond to mechanical stimuli, like shear stress, as well as to chemical stimuli. It is activated by Ca^{2+} influx, which in turn is controlled by Ca^{2+} channels in the plasma membrane. These channels open in response to a number of hormones, autacoids and drugs, like for example bradykinin, acetylcholine, adenosine diphosphate (ADP), ATP, and histamine (Figure 1.4). The flow of blood, exerting a shear force on the plasma membrane lining the blood vessels, also causes Ca^{2-} channels to open, admitting Ca^{2-} into the ECs, where it activates eNOS in a calmodulin-dependent fashion. Therefore shear stress on ECs causes them to release NO, which diffuses out in all directions, into the underlying SMC and into the lumen of the blood vessels (Figure 1.4). In SMC, NO binds to the heme group of soluble GC and activates that enzyme to produce cGMP from guanosine triphosphate (GTP) (Figure 1.4), which then acts as a secondary messenger to initiate cellular function, most likely via cGMP-dependent kinases, such as PKG. How would cGMP elicit its biological effects? Increased cGMP may cause Ca^{2-} channels to open in order to extrude intracellular Ca^{2+}, or it could actually activate a protein kinase that phosphorylates Ca^{2-} transporters, causing Ca^{2-} to be sequestered in intracellular structures in the muscle cells. It could perhaps activate a specific protein kinase that would phosphorylate and inactivate myosin light chain kinase, thereby resulting in the dephosphorylation of myosin light chain and therefore smooth muscle relaxation (Draznin et al., 1986). Or maybe cGMP could bind to calcium-binding proteins and thus maintain a very low intracellular concentration of free Ca^{2-} (Adams et al., 1989). Therefore muscle contraction would be greatly reduced since it is a process that requires Ca^{2-}. Whatever the cGMP-dependent mechanism is, NO induces the

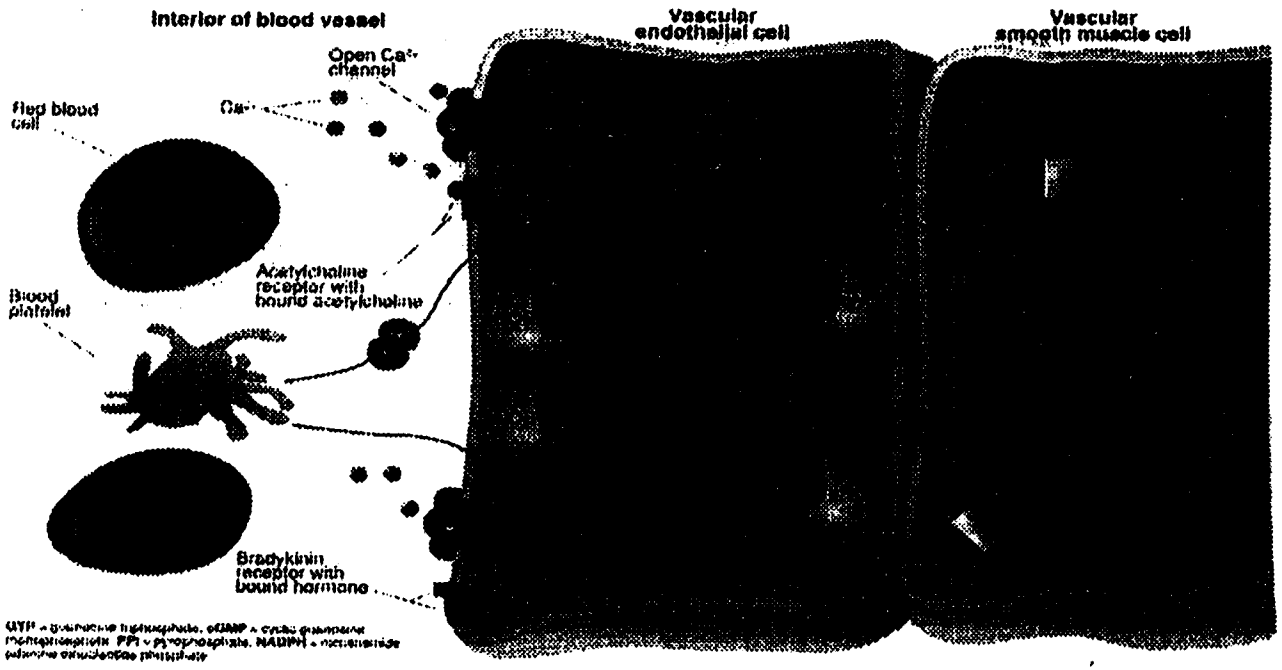


Figure 1.4 Diagram showing the activation of endothelial nitric oxide synthase (eNOS). Chemical and mechanical stimuli lead to the opening of calcium channels. The Ca^{2+} ions bind to calmodulin, which then activate eNOS, bound to the inner surface of the plasma membrane. Nitric oxide (NO) released diffuses out into the lumen and into platelets to decrease their aggrgation with each other and their adhesion to the endothelium. In the underlying smooth muscle cells, NO binds to the heme iron of guanylate cyclase, causing it to release cGMP from GTP. cGMP decreases the amount of free Ca^{2+} in the muscle cells, causing them to relax.

underlying smooth muscle to dilate. As the vessel dilates, shear stress is reduced, and the production of NO decreases. The local action and lifetime of NO, relative to rapid changes in blood flow, modulate the responses of a blood vessel, thereby minimizing turbulence.

In the lumen, NO acts to prevent platelet aggregation and adherence to the endothelial vessel wall, quite likely through mechanisms involving GC and cGMP (Sly et al., 1995), without excluding the possibility that other mechanisms may also be involved (Yin et al., 1995). How cGMP acts to achieve this is not fully elucidated yet. Vascular NO may also prevent leukocyte adhesion to the endothelium by down-regulating the leukocyte adhesion glycoprotein complex CD11/CD18 (Forstermann et al., 1993). Therefore NO helps to maintain a non-thrombogenic surface on the vascular endothelium. The NOS in platelets also contributes to this anti-thrombotic action in an autocrine manner (Chen and Mehta, 1996). In the same vein, NO also exerts anti-atherosclerotic actions by preventing leukocyte and macrophage adherence to injured endothelium, and by inhibiting SMC mitogenesis and migration (Garg and Hassid, 1989). Indeed NO also prevents EC proliferation, as indicated by decreased eNOS mRNA levels in proliferating endothelium (Flowers et al., 1995). Maintaining normal blood pressure seems to require vascular ECs to constantly synthesize NO, therefore eNOS is an enzyme that is constitutively expressed.

The interaction of NO and GC involves the heme group of the enzyme and this has been demonstrated in crude and in purified enzyme preparations from lung (Ignarro et al., 1982), liver (Ohlstein et al., 1982), and platelets (Mellion et al., 1983). It is likely that the nitric oxide radical, $NO^{\cdot}$, may alter the conformation of the heme by pulling the heme iron Fe^{2+} away from the enzyme protein (Figure 1.5), thereby breaking the axial ligand between Fe^{2+}

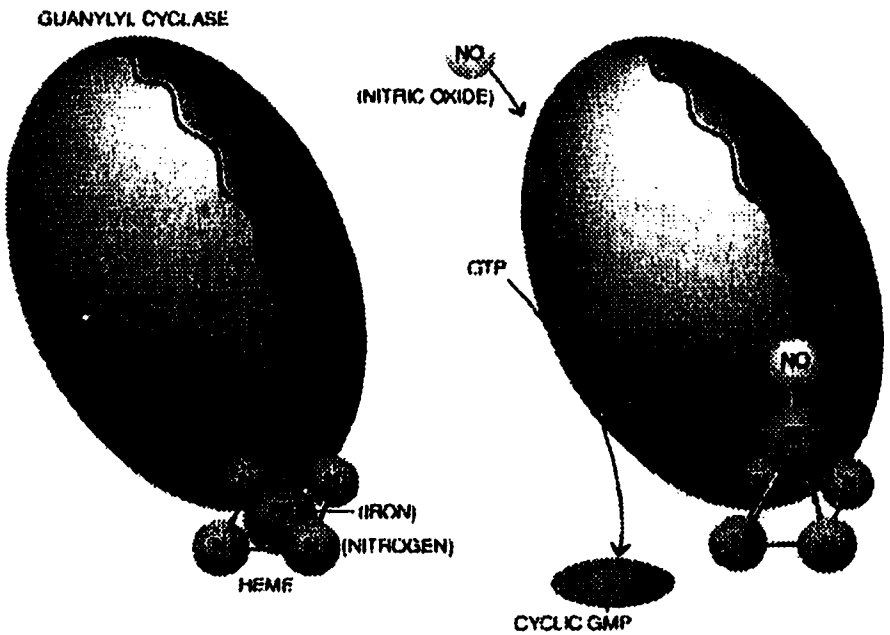


Figure 1.5 Nitric oxide (NO) binds to the heme iron, causing it to undergo a three-dimensional change by pulling it away from the plane of the porphyrin ring into a tetrahedral shape, thus activating guanylate cyclase and causing it to release cGMP from GTP.

and protein without altering the binding of the porphyrin ring (Ignarro et al., 1984). Thus the plane of NO-heme that binds to GC superficially resembles protoporphyrin IX (Figure 1.5). It has been proposed that binding of NO to the heme in GC weakens the affinity to a distal histidine, which then allows activation of the enzyme (Ignarro, 1989). GC activation is characterized by increased affinities of the enzyme for magnesium (Mg)GTP substrate and excess uncomplexed Mg^{2+}, and an increased V_{max} (Wolin et al., 1982). The signal transduction process is terminated upon re-establishment of the heme Fe^{2+} axial ligand as the labile NO-heme complex decomposes with the liberation of NO_2^- and NO_3^-.

The neuronal form nNOS, on the other hand, is believed to be cytosolic. In neurotransmission, glutamate released from the stores of a stimulated neuron binds to NMDA receptors on an adjacent neuron, causing a channel to open in the receptors, thereby admitting Ca^{2+} into the neuron (Figure 1.6). Ca^{2+} binds to calmodulin, allowing it to associate with NOS, and thus activating the enzyme to produce NO and L-citrulline from L-arginine, NADPH and O_2. nNOS is also constitutively expressed, but is not always active; an influx of Ca^{2+} leads to its activation to produce a relatively small amount of NO, what has been termed a "puff". It is believed that NO can act as a retrograde messenger in that it diffuses back into the presynaptic neuron (Figure 1.6) and binds to the iron of the heme cofactor of GC, causing increased synthesis of cGMP. This means that NO could play a role in long-term potentiation and therefore in memory (Schuman and Madison, 1991). NO differs from other neurotransmitters in that it is not synthesized and kept in storage vesicles, ready for release into the synapse to be picked up by the receiving cell, when a neuron fires. It is made when and where needed and simply diffuses out of the producing cell. Furthermore most

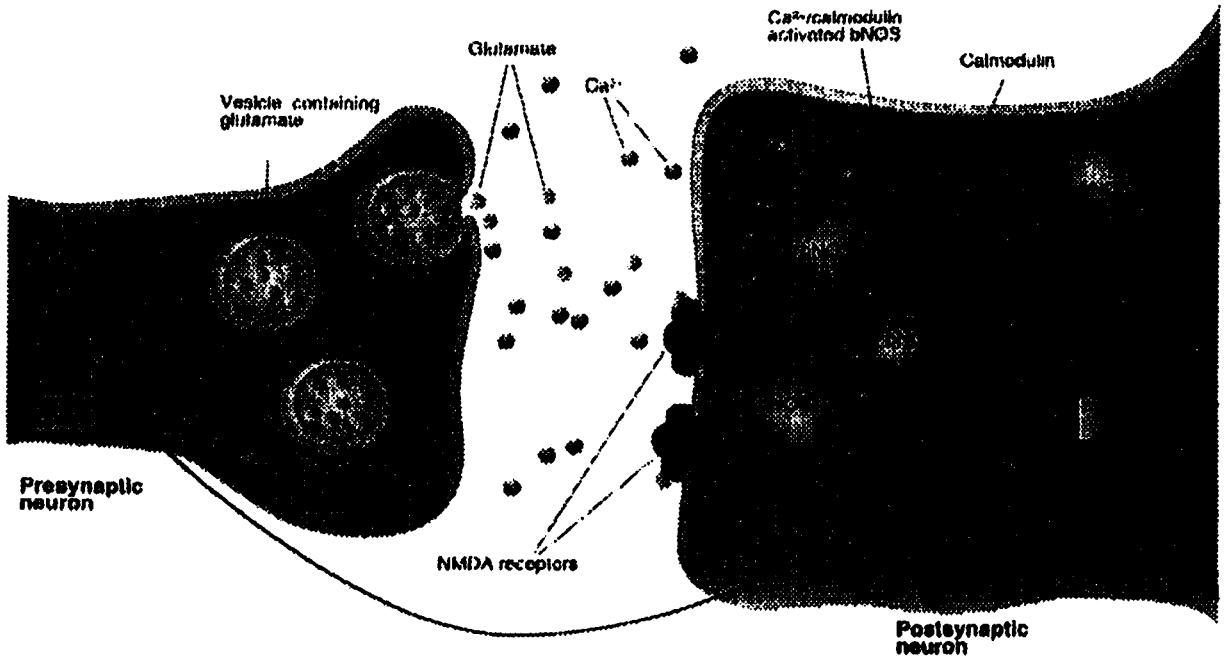


Figure 1.6 Diagram depicting the activation of the neuronal nitric oxide synthase (nNOS). Glutamate released from presynaptic neurons binds to N-methyl-D-aspartate (NMDA) receptors on an adjacent neuron, causing calcium channels to open. Ca^{2+} ions entering the cell bind to calmodulin, activating nNOS, which is mostly cytosolic. The nitric oxide (NO) formed from the catalytic reaction of nNOS can either activate guanylate cyclase (GC) within the neuron or it can diffuse back to the presynaptic neuron to activate GC in that cell as well. In this way, NO plays a role in conditioning processes, i. e. in potentiating memory.

neurotransmitters are composed of amino acids or peptides which couple with precisely configured receptors on the surface of cells. NO is a gas that needs no receptor gates, diffusing through membranes and binding to enzymes deep within cells. Even if the half-life of NO is short, for example, either one or a few seconds, this is still enough for it to spread through several cell diameters, with 37% of the NO produced by a point source spreading further than 57 μm in one second (Lancaster, Jr., 1994).

The inducible form iNOS is not constitutively expressed and was first discovered to play a role in the immune system, synthesized by activated macrophages, that have microbicidal and tumoricidal activities. This enzyme always binds calmodulin tightly, so that it is active at basal levels of Ca^{2+}. Transcription of iNOS is controlled by a number of biological response modifiers, like cytokines such as tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), and the interleukins 1 and 2 (IL-1 and IL-2 respectively), and also by lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria (Nunokawa et al., 1994; Xie et al., 1993). Once iNOS is induced, it can produce large amounts of NO that will diffuse across into tumor cells or bacterial cells and shut off their respiratory cycle by binding to iron-containing enzymes and inactivating them (Figure 1.7). This enzyme is rapidly induced, but is also rapidly down-regulated. The mechanism of cytotoxic action of macrophages is proposed to be NO-mediated nitrosation of key metabolic iron-containing enzymes or iron-sulfur proteins in the target cells (Stuehr and Nathan, 1989). Such proteins would be the ones involved in the mitochondrial electron transport as well as enzymes like aconitase, involved in the tricarboxylic acid (TCA) cycle which supports high energy phosphate metabolism, and nitrosation would inactivate them and provoke killing of

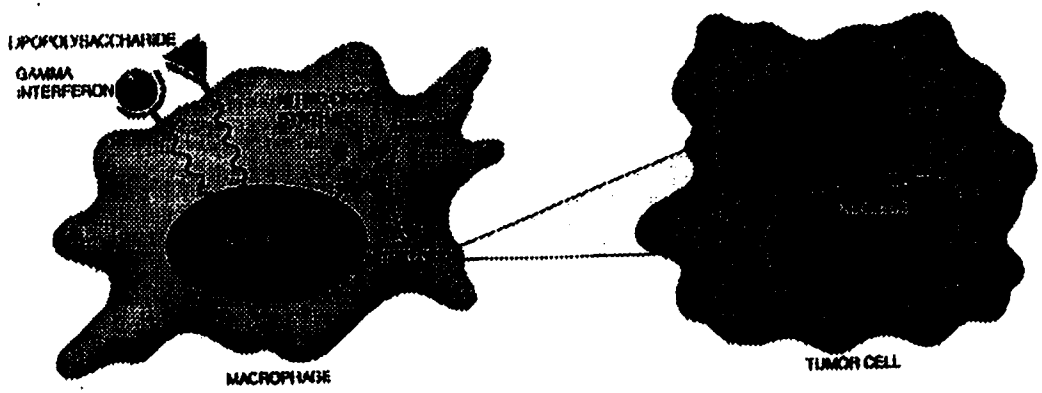


Figure 1.7 The inducible nitric oxide synthase (iNOS) does not require Ca^{2+} for activation. It is induced in macrophages by inflammatory cytokines and releases large amounts of nitric oxide (NO). The NO diffuses out, but the close proximity of macrophages to an invading cell, like a tumor or a bacterial cell ensures that a significant amount of NO enters that cell to bind to their heme-containing enzymes involved in the tricarboxylic acid cycle and in the electron transport system, thereby inactivating them and killing the cell. NO also inhibits ribonucleotide reductase, interfering with the formation of deoxyribonucleotides necessary for DNA synthesis and cell division.

target cells (Gardner et al., 1997). The cytotoxic effects of cytokine-activated macrophages are accompanied by the formation of iron-dinitrosyl-dithiolate complexes (Lancaster, Jr. and Hibbs, Jr., 1990). Why the cells of origin of NO, the macrophages, are not killed by the NO generated within, is a bit of a mystery. One plausible explanation is that nitrosation reactions cannot occur at pH values of neutrality and higher, whereas NO will rapidly form nitrous acid (HONO), a strong nitrosating agent, at acidic pH associated with digestive vacuoles, lysosomes and damaged cells (Ignarro, 1991).

NO also inhibits ribonucleotide reductase, thereby interfering with the formation of deoxyribonucleotides necessary for DNA synthesis and cell division (Lepoivre et al., 1991). One pathway towards neuronal cell death may involve NO-induced DNA damage overactivating the nuclear enzyme poly (ADP-ribose) synthetase (PARS), thereby depleting the cells of NAD followed by depletion of ATP (Dawson et al., 1993). PARS catalyses the transfer of ADP ribose units from NAD to nuclear proteins, and for each mole of ADP ribose transferred from NAD, the free energy equivalents of four moles of ATP are consumed in the regeneration of NAD. NO also enhances the ADP-ribosylation of certain platelet proteins through the activation of an ADP-ribosyltransferase (Brune and Lapetina, 1990). In the brain and red blood cells, the target of NO-stimulated ADP-ribosylation is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme with a crucial role in glycolysis (Zhang and Snyder, 1992). Since ADP-ribosylation of the enzyme involves the cysteine to which NAD binds in GAPDH catalysis, ADP ribosylation inhibits GAPDH activity. Therefore NO-stimulated ADP-ribosylation of GAPDH could also be a target to mediate NO neurotoxicity. GAPDH is also involved in gluconeogenesis, which is inhibited by NO in hepatocytes (Stadler

et al., 1995).

In neutral physiologic conditions, a variety of nitroso-compounds form effectively, for example, metal-nitrosyl complexes as mentioned above, thionitrites or nitrosothiols (RS-NO), nitrosamines (RNH-NO), alkyl and aryl nitrites (RO-NO), and dinitrogen tri- and tetra-oxides (N_2O_3 and N_2O_4) (Stamler et al., 1992a). When covalently bound to a carbon, nitrogen, oxygen or sulfur atom, the incorporated NO is referred to as a nitroso group; when bound to a transition metal, it is referred to as a nitrosyl group. NO· reacts with the superoxide anion O_2·- in aqueous solution to form peroxynitrite (OONO-), which is a highly reactive species, that can then form the hydroxyl radical (Beckman et al., 1990). SOD, the principal scavenger of superoxide *in vivo*, reacts with superoxide at a rate of about 2×10^9 $M^{-1}.s^{-1}$, which is the fastest rate of any enzyme, but still three-fold slower than superoxide reacting with NO (Huie and Padmaja, 1993). Such secondary oxidants, i. e. peroxynitrite or the hydroxyl radical, rather than NO, are responsible for the toxicity of the molecule, as in oxidizing iron/sulfur centres (Castro et al., 1994) and protein thiols (Radi et al., 1991a), as described above. Peroxynitrite is also involved in the nitration of protein tyrosine residues, where it reacts with the phenolic ring of tyrosine, to yield nitrotyrosine, a process that is actually catalyzed by SOD (Beckman et al., 1992).

NO also readily forms complexes with transition metal ions, including those regularly found in metalloproteins, for example, with non-heme iron, in most cases to inactivate them, as described above. It binds to hemoglobin (Hb), another heme-containing protein, to form a nitrosyl Fe(II)-hemoglobin adduct, at a much greater ratio and rate of uptake and release than oxygen, about five to six orders of magnitude higher (Doyle and Hoekstra, 1981). It is

thought that Hb binds to NO· in order to inactivate it. In fact, the major route for eliminating NO *in vivo* is by reaction with oxy-hemoglobin; since only one atom of O_2 binds to the ferrous heme of Hb, the other one is available with an unpaired electron for reaction with NO. The reaction results in the formation of met-hemoglobin and nitrate (Doyle and Hoekstra, 1981). The met-Hb is recycled by reductases, and the nitrate is secreted in the urine(Doyle and Hoekstra, 1981).

$$Hb\text{-}Fe^{2+}\text{-}O\text{-}O\cdot + \cdot NO \longrightarrow Hb\text{-}Fe^{2+}\text{-}OONO \longrightarrow Hb\text{-}Fe^{3+} + NO_3^-$$

Under anaerobic conditions (< 1% O_2), deoxy Hb readily forms HbNO by binding free NO without producing metHb (Kruszyna et al., 1993).

The predominant redox forms of NO in plasma are S-nitrosothiols, mostly as S-nitrosoproteins, for example, serum albumin, formed by the oxidation of NO to NO^+, which then reacts with thiol residues. They are thought to serve as a source and sink of NO, buffering the concentration of free NO (Stamler et al., 1992b). NO in this form exhibits a relative lack of reactivity towards O_2 and reactive oxygen species, therein limiting the generation of toxic NO_x. Thus the formation of RS-NO may provide a means to control the toxicity of NO·. RS-NO have been identified in human airway-lining fluid, under the high ambient concentrations of O_2 and $O_2^{\cdot-}$ in the lung. In addition, modulation of thiol groups on the NMDA-type glutamate receptor by RS-NO can afford protection from receptor-mediated neurotoxicity that is dependent on NO· generation (Lei et al., 1992). In other words, it is a mechanism that is responsible for the inhibition of NMDA receptors. Peroxynitrite can react with sugars, like glucose, or other compounds containing an alcohol group to form NO donors (Moro et al., 1995). This may represent a further detoxification pathway for

peroxynitrite *in vivo*.

The form in which NO is delivered and transported may determine its toxic potential. The appropriate packaging of NO might serve to facilitate its transport, prolong its life in the blood and tissues, target its delivery to specific effectors, and mitigate its adverse cytotoxic potential. The existence of various types of NO, determined by pH and redox potential of the microenvironment, provides a means through which NO can be tailored to evoke specific biolgical responses. Thus NO has a role as being cytotoxic as well as being cytoprotective.

1.8. Pathology of NO related to diseases:

Too much of a good thing can be a bad thing. In the gas phase, pure NO can exist at a concentration of 40 mM, but in saturated solutions of water, its concentration is 1.9 mM (Beckman, 1995). The physiological levels of NO range from 10 nM, based upon the levels needed to cause vasodilation, to as high as 1-10 μM near fully activated macrophages (Beckman, 1995). Given many minutes of exposure, even 1 μM of NO will react with O_2 to form nitrogen dioxide (NO_2), a strong oxidant and thus a toxic molecule. Because of its small size and lypophilic nature, diffusion through tissues to a blood vessel and reaction with Hb will occur in seconds *in vivo* and thus safely remove NO produced at the levels used for signal transduction. But in a state of up-regulated production of NO, as in infections, like sepsis or endotoxemia, causing the sustained induction of iNOS, the excess amount of NO can contribute to hypotension, leading to septic shock, and eventually resulting in high-output cardiac failure (Titheradge, 1999; Vallance and Moncada, 1993).

As mentioned above, NO is highly reactive and can adopt many forms, depending on

what it binds to. In oxidative stress, where there is an excessive production of superoxide anion and other oxygen-derived radicals, and therefore the potential formation of more peroxynitrite and hydroxyl radical as well, endothelial dysfunction accompanies cardiovascular risk factors, like hypercholesterolemia, hypertension and cigarette smoking, which likely evolve in the manifestation of clinical conditions, such as atherosclerosis, diabetes and heart failure (Zalba et al., 2000). Nitration, i. e. peroxynitrite reacting with tyrosine residues of proteins to form nitrotyrosyl residues (as mentioned in Section 1.7), has been observed in lung biopsy and autopsy samples from humans with sepsis, pneumonia or adult respiratory distress syndrome (ARDS) (Haddad et al., 1994; Kooy et al., 1995). Myocytes in human heart from patients with sepsis or myocarditis show extensive nitration (Chantler and Gratzer, 1975). Nitration of neurofilament L is the cause of amyotrophic lateral sclerosis (ALS), a motor neuron disease (Brady, 1993). Neurofilaments are intermediate filaments related to keratins that provide structural stability and control diameter in motor neurons. The head domain of human neurofilament L contains ten tyrosines in the first ninety amino acids and nitration of these residues change a normally hydrophobic residue into a negatively charged hydrophilic residue, thereby disrupting the assembly of these proteins into a long polymeric structure. Atherosclerotic lesions develop at sites subjected to frequent turbulence, which might be related to the prolonged exposure to NO and its secondary oxidants in these regions. Extensive nitration has been observed around foamy macrophages in human atherosclerotic lesions and in early fatty streaks (Beckmann et al., 1994). Peroxynitrite may cause lipid peroxidaion of cell membranes, though it can only do so at high concentrations of 200 μM to 1 mM (Radi et al., 1991b). Iron-nitrosyl complexes, associated with loss of protein activity,

have been detected in tissues undergoing rejection (Lancaster, Jr. et al., 1992).

NO may contribute to the development of apoptosis, an endogenous process of initiating cell death that may limit viral replication and the development of cancer. The host defence against tumor cells is in part based on the production of NO by iNOS in activated macrophages; cells of the blood vessels that pervade the tumor can also participate in antitumor defence responses by producing NO from eNOS, although an increased blood supply to the tumor might enhance its growth by delivering nutrients and growth factors. Apoptotic death of human leukaemic cells has been observed *in vitro* when co-cultured with vascular SMCs or ECs and exposed to cytokine treatment, like TNFα and IFNγ (Geng et al., 1996). In diabetes, apoptosis of the ß cells in the pancreatic islets by NO, is thought to be the pathologic mechanism (Mauricio and Mandrup-Poulsen, 1998).

nNOS occurs in discrete neuronal populations throughout the brain (Bredt et al., 1991b). In some areas, it resides in all neurons of a class, such as in the cerebellum. In other areas, such as the hippocampus and the cerebral cortex, it is localized to about 2% of the neuronal population. During a stroke, the NOS neurons become overstimulated by the excess neurotransmitter (glutamate) released, and therefore flood nearby cells with toxic amounts of NO. Thus excessive NMDA receptor activation contributes to glutamate neurotoxicity by enhanced production of NO. NOS neurons though are notable for their resistance to destruction in neurodegenerative conditions (Ferrante et al., 1985), after the ischemic damage of strokes (Uemura et al., 1990) and after NMDA neurotoxicity in primary brain cultures that mimic stroke damage (Choi, 1988). A reason for that may be that the NO produced is released from the NOS neurons in a directional manner and kills adjacent cells, most likely

due to ADP-ribosylation of certain proteins by peroxynitrite, as mentioned in Section 1.7. How NOS neurons are able to kill a lot of other neurons when they form only 2% of the neuronal population is because they are extensively branched with their fibres contacting a lot of other neurons in brain sections or primary cultures (Dawson et al., 1993). Overproduction of NO may also play a role in the aetiology of inflammatory disorders of the central nervous system (CNS). Induction of human iNOS in demyelinating regions of multiple sclerosis brains has been observed (Bo et al., 1994). High amounts of NO have been linked to migraine (Olesen et al., 1994). High levels of cGMP, due to NO, has been suggested to have a role in seizures (Ferrendelli et al., 1980). Superfusion of cGMP analogues onto grafts of hippocampus triggers prolonged epileptiform activity in the neurons (Freedman et al., 1979). High amounts of cGMP also cause destruction of photoreceptor cells in the retina, where NOS has been demonstrated by immunocytochemistry (Ross et al., 1990).

Too little NO is not good for the physiology either. When the nNOS gene was targeted for disruption, as in a "knockout" model in mouse, the mutant mice were viable and did not exhibit any abnormaliites in the CNS. But they developed grossly enlarged stomachs with hypertrophy of the pyloric sphincter and the circular muscle layer, which resembles the human disorder, infantile hypertrophic pyloric stenosis, a potentially lethal condition in which gastric outlet obstruction is associated with the lack of NOS neurons in the pylorus (Huang et al., 1993). Other "knockout" models of nNOS should probably be investigated, since only exon 1 was targeted in disrupting the neural gene in the model above. If nNOS may be transcribed from alternative first exons, then the possibility exists that nNOS may have still been expressed during fetal development in these "knockout" mice. Mice with the eNOS

"knockout" model exhibited shorter limbs than normal mice (10% in null mice) (Gregg et al., 1998) and 40% developed bicuspid instead of tricuspid aortic valves (Lee et al., 2000). They were also found to be hypertensive (Huang et al., 1995) as well as developing larger infarcts than the wild-type strain when assessed 24 hours after middle cerebral artery occlusion (Huang et al., 1996).

Experiments in rats have shown that if NO synthesis is blocked, the penis stays limp (Burnett et al., 1992). Endothelial functions are impaired in vascular diseases, such as hypertension, atherosclerosis, diabetes, and in other vasculopathies associated with bypass surgery, angioplasty, and transplantation (Busse and Flemming, 1993) and it is believed that a lack or a decrease of NO release or action plays a part in this impairment.. Indeed in patients suffering from pulmonary hypertension, the expression of eNOS is greatly diminished, as assessed by immunohistochemical analysis, *in situ* hybridization, and Northern blot analysis (Giaid, 1998). In ischemia-reperfusion injury, NO formation is initially increased and protective, e. g. by inducing collateral perfusion, but after time NO formation ceases. Upon reoxygenation, NO formation again recovers, but is then a cause of tissue damage due to the presence of reactive oxygen intermediates and peroxynitrite formation (Isobe et al., 1999).

1.9. Therapeutic usage of NOS/NO:

In hypertensive patients, pharmacological agents able to release NO, such as nitroglycerin or isosorbide dinitrate, are used as vasorelaxant therapy. Direct administration of NO has been attempted to treat vasoconstriction that is confined to the lungs. Infants with persistent pulmonary hypertension of the newborn (Pepke-Zaba et al., 1991) and older

patients with ARDS (Rossaint et al., 1993) have been given low doses of NO through respiratory ventilators. Reversal of pulmonary hypertension has been good in these studies and the procedure has not caused an unwanted drop in blood pressure in other parts of the body, due to NO's short half-life. But still the long-term toxicity of this procedure has to be evaluated, since NO can nitrosylate important cell proteins and since it can form toxic nitrogen oxides by reaction with O_2, although one would expect that inspired NO would quickly be scavenged by hemoglobin to prevent systemic events.

NO could also be a therapeutic solution for male impotence if non-invasive means of applying NO-releasing substances to the penis could be used as a novel procedure. Indeed, the arrival of a new drug Viagra, meant to treat angina (chest pain), has proved more successful in treating erectile dysfunction (Webb et al., 2000). Sildenafil, or Viagra, is a selective cGMP-specific phosphodiesterase type 5 inhibitor rather than an NO donor. In hypercholesterolemia, when endothelium-dependent vasodilation is impaired even before the development of atherosclerosis, infusion of L-arginine in the forearm of patients improves and augments the vasodilation (Creager et al., 1992). Since the discovery that NO or its derivatives have tumoricidal activity, the possibility exists that NO and NO donor complexes could be used as potential therapeutic agents in the treatment of cancer.

On the other hand, when there's too much NO, NOS inhibitors could be used as therapy to reduce nerve cell damage in strokes or to restore normal blood pressure in sepsis. Care has to be taken to ensure that the inhibitors would not cause hypertension in the general circulation. Vallance's group successfully treated two patients with life-threatening septic shock with NOS inhibitors, like L-NMMA and N^G-nitro-L-arginine methyl ester (L-NAME).

The inhibitors caused a dose-dependent increase in blood pressure and systemic vascular resistance (Petros et al., 1991). An alternative treatment for hypotension would be to administer glucocorticoids, anti-inflammatory steroids, which inhibit the expression of iNOS responsible for the high amounts of NO, but have no effect on eNOS (Radomski et al., 1990). Even agents that block the synthesis of BH_4 may offer an approach as treatment in conditions characterized by NO overproduction, since it is an essential cofactor for NOS. Transforming growth factor-β1 (TGFβ1) can also suppress NO release from iNOS since it acts by down-regulating the mRNA as well as by accelerating the degradation of the protein, even though the transcriptional activity is not affected (Vodovotz et al., 1993). Transgenic mice, whose TGFß1 gene has been knocked out, develop lethal, multifocal inflammatory disease, underlining the importance of TGFß1's role in immunogenicity (Kulkarni et al., 1993).

Future therapeutic strategies would involve gene transfer to the appropriate region of the body. For example, adenoviral-mediated transfer of the eNOS gene has already been attempted in rat lungs and its effect on pulmonary hypertension assessed (Janssens et al., 1996), as well as in balloon-injured arteries to restore NO production and inhibit neointima formation (Janssens et al., 1998). Expression of eNOS was found to reduce hypoxic pulmonary vasoconstriction. But such strategies activate the immune system, so that this kind of therapy remains a short-term solution. The packaging of NOS genes will become important in targeting to specific tissues, for example, using tissue-specific promoters to drive the gene of interest, or stably transfecting the gene into cells that can be re-injected back into the body, specifically into the tissue of interest. Injection of non-reactive polymers, to which the DNA of interest is attached, could be another strategy.

Antisense oligonucleotides to iNOS have been used successfully in an *in vitro* system, namely with cultured rat pulmonary artery SMCs, to inhibit the synthesis of the protein and thus NO production (Thomae et al., 1993). One limitation with using oligonucleotides is that they are rapidly degraded by nucleases in serum. That could be prevented by modifying the oligonucleotides, for example, replacing one of the oxygen atoms at each interbase phosphorus with a sulfur atom to create a phosphorothioate linkage, which would render the oligonucleotide nuclease-resistant and would increase its therapeutic potential. Ultimately the delivery of oligonucleotides to specific tissues, such as the lung, would be the most desirable strategy. A possible system would involve the encapsulation of oligonucleotide within cationic liposome carriers to protect against degradation in the circulation and to facilitate fusion with plasma membranes, as well as antibody coating to facilitate cell-specific targeting.

1.10. Regulation of eNOS:

Expression of the inducible isoform of NOS needs to be tightly regulated, since it can produce large amounts of NO, being Ca^{2+}/calmodulin-independent, which can prove toxic to various organs if the gene is transcriptionally active for long. Therefore the mRNA as well as the protein are as rapidly degraded as the gene is rapidly transcribed. On the other hand, the two constitutive forms of NOS are produced constantly in small amounts, so that they are always present in the cells of their origin, so as to be functionally active when needed. Do these constitutive genes still undergo some form of regulation of gene expression? This Section will briefly explore the effects of various physiological agents on the NOS enzymes at the transcriptional, post-transcriptional and post-translational levels. Not much was known

about the regulation of the constitutive endothelial NOS gene until recently.

In vitro studies have indicated that 8-bromo-cGMP may up-regulate the expression of eNOS, as shown by increased mRNA and protein levels, as well as by increased production of NO (Ravichandran and Johns, 1995). eNOS is defined as being strictly dependent on Ca^{2+}/calmodulin for activity, but it can be rapidly and strongly activated and phosphorylated on both Ser and Thr in the presence of cGMP-dependent protein kinase II (PKG II) and the catalytic subunit of PKA *in vitro* (Butt et al., 2000). Phosphopeptide analysis by mass spectrometry and phosphoamino acid analysis have identified Ser1177, Ser633, and Thr495, situated in the calmodulin-binding domain, as the phosphorylation sites (Butt et al., 2000). Both PKA and PKG phosphorylation of eNOS were able to cause a highly reproducible partial (up to 20%) eNOS activation which was independent of Ca^{2+}/calmodulin, and as much as a 4-fold increase in V_{max} in the presence of Ca^{2+}/calmodulin (Butt et al., 2000). Thus there exists an intercellular positive feedback mechanism for NO/cGMP signalling in the regulation of vascular tone.

Shear stress can increase eNOS expression in ECs, as mentioned in Section 1.7; it elevates eNOS mRNA levels by a pathway which is more dependent on potassium (K^+) channel opening than on PKC activation (Ranjan et al., 1995; Uematsu et al., 1995). Even though the eNOS promoter contains putative binding domains for AP-1 complexes, potentially responsive to activation of PKC, the selective PKC inhibitor calphostin C (100 nM) did not inhibit eNOS induction by shear stress, but the K^+ channel antagonist tetraethylammonium chloride (3 mM) did (Uematsu et al., 1995). The aorta of female rabbits has been shown to release a greater amount of NO than that of ovarectomized females and

male rabbits (Hayashi et al., 1997). Estrogen has been shown to up-regulate eNOS mRNA and thus eNOS protein expression by a receptor-mediated mechanism, both *in vitro* and *in vivo* (Hayashi et al., 1995; Van Buren et al., 1992). Estrogen not only achieves this in ECs (MacRitchie et al., 1997), but also in human osteoblast-like cells (Armour and Ralston, 1998), suggesting that the protective effects of estrogen, mediated in part by NO, act on bone as well as on the cardiovascular system. Estrogen-stimulated release of NO can be abrogated by herbimycin and geldanamycin, benzoquinone ansamycins that are potent inhibitors of hsp90 function (Russell et al., 2000). Thus estrogen also modulates eNOS activition by inducing an hsp90-eNOS association. Lyso-PC increases eNOS transcription by augmenting Sp1-binding activity to the promoter region of the eNOS gene, precisely at Sp1 sites -104 to -90 (Cieslik et al., 1998).

Insulin enhances eNOS gene expression in ECs and microvessels, an effect mediated by the activation of phosphatidylinositol-3 (PI-3) kinase (Kuboki et al., 2000). In pathologic conditions, as in insulin resistance and diabetes, eNOS expression may be greatly decreased, leading to endothelial dysfunction. 5-methyltetrahydrofolate, the active form of folic acid, can enhance NO production and reduce superoxide production by eNOS, but the mechanism by which it acts remains to be elucidated (Stroes et al., 2000). Vascular endothelial growth factor (VEGF), an endothelium-specific secreted protein that potently stimulates vasodilation, microvascular hyperpermeability, and angiogenesis, increases NO production by eNOS in ECs when it binds to its receptor KDR/Flk-1, but not when it binds to its other receptor Flt-1 (Kroll and Waltenberger, 1999; Shen et al., 1999). Treatment of bovine aortic endothelial cells (BAECs) with VEGF or insulin-like growth factor-1 (IGF-1) stimulates the

phosphorylation of eNOS on serine 1177 and activates the enzyme, leading to NO production (Michell et al., 1999). Phosphorylation at this site is believed to occur by the serine/threonine protein kinase Akt/PKB (Fulton et al., 1999). Both Akt and eNOS are localized to, and activated at, the plasma membrane (Michell et al., 1999). VEGF has also been shown to increase eNOS expression by increasing the stability of the eNOS mRNA (Bouloumie et al., 1999; Hood et al., 1998). Other growth factors, like basic fibroblast growth factor (bFGF) (Kostyk et al., 1995) and TGFβ1 (Inoue et al., 1995) also up-regulate eNOS mRNA and protein, as well as eNOS activity. TGFβ1's effect on eNOS expression is due to enhanced promoter activity. Deletion of a putative NF-1 binding site in the bovine eNOS promoter abolished this activity (Inoue et al., 1995). David Harrison's group has reported that proliferating ECs show enhanced eNOS expression due to increased stability of the eNOS mRNA compared to growth-arrested confluent cells (Arnal et al., 1994), although another group has reported the exact opposite (Flowers et al., 1995), as mentioned in Section 1.7.

Hypoxia (1% O_2) has been shown to increase the expression of eNOS in non-pulmonary ECs, although the total activity of the enzyme is unchanged even after induction (Arnet et al., 1996). Lowenstein's group was able to show that hypoxia activated the transcription of a reporter gene, namely luciferase, under the control of the eNOS promoter (Arnet et al., 1996). This would suggest that the eNOS gene possesses hypoxia-response element(s). However, the human eNOS promoter contains no homology to the published binding sequence of the hypoxia-inducible transcription factor HIF-1 (Forstermann et al., 1998); therefore hypoxia induces an increase in transcription of eNOS in non-pulmonary ECs by undefined transcription factors.

In pulmonary ECs though, hypoxia has been shown to down-regulate eNOS expression, by a mechanism which is attributed to both a decreased rate of transcription and a destabilization of the eNOS mRNA (Liao et al., 1995a). Smoking impairs the endothelium-dependent relaxation of arteries and veins, and this has been attributed to a reduction in the activity of eNOS caused by an inadequate supply of the coenzyme BH_4 (Higman et al., 1996). NO itself plays a direct role in inhibiting the activity of the NOS enzymes (Assreuy et al., 1994; Ravichandran et al., 1995; Rengasamy and Johns, 1993). Experiments have shown that NO, and not any NO-derived species, is the cause of NOS inhibition. The direct mechanism may involve binding of NO at a selective site, most likely to the heme iron, on the NOS protein to form an inactive complex. While NO acts to regulate iron intake by cells, iron acts to down-regulate the expression of eNOS by decreasing the transcription of the eNOS gene (Weiss et al., 1994) rather than by destabilizing the eNOS mRNA. Thus there exists a regulatory loop between iron metabolism and the NO/NOS pathway.

Low-density lipoproteins (LDL) have been shown to inhibit the early transcription of eNOS, down-regulate its message (Liao et al., 1995b), and even inactivate NO (Chin et al., 1992). The oxidized, rather than the native form, of LDL elicits these responses. The lipid, and not the protein, component of oxidized LDL inactivates NO after its release from ECs (Chin et al., 1992). In hyperlipidaemia or familial hypercholesterolaemia, macrophages and ECs take up native or modified forms of LDL indiscriminately via a scavenger receptor pathway (Baker et al., 1984). Since LDL is a known atherogenic risk factor and is present in high concentrations in hyperlipidaemia, and has been shown to inhibit endothelium-dependent relaxation in rabbit aorta (Andrews et al., 1987), then it can be concluded that the atherogenic

effects of LDL may be mediated by its effects on endothelial-derived NOS and NO. Since endothelium-dependent vasodilation is altered in atherosclerosis, enhanced monocyte/endothelial interactions are also implicated in early atherosclerosis (Marczin et al., 1996). Oxidized LDL displaces eNOS from plasmalemmal caveolae and impairs eNOS activation (Blair et al., 1999). Oxidized LDL, and not native LDL or high density lipoprotein (HDL), can deplete caveolae of cholesterol by serving as an acceptor of cholesterol. Since membrane cholesterol is essential for normal caveolar function (Chang et al., 1992), pathologic alterations in cholesterol balance may adversely influence endothelial cell NO production through effects on caveolae, thereby contributing to atherosclerotic processes.

Cytokines, like TNFα, and LPS, an endotoxin found in gram-negative bacteria, are known to induce iNOS in macrophages and other cell types (Xie et al., 1993), but they have also been shown to down-regulate eNOS (Yoshizumi et al., 1993). Therefore inflammatory cytokines can impair endothelium-dependent dilation and the endothelium may lose its ability to respond to hormones and autacoids. This effect could lead to a predisposition to vessel spasm, thrombosis, or atherogenesis. Surprisingly, in the face of falling enzyme, eNOS activity has been shown to be increased, and this could be possibly due to an increase in endogenous BH_4 levels (Rosenkranz-Weiss et al., 1994).

1.11. Hypotheses and Objectives of thesis:

The first hypothesis of this thesis is that the constitutively expressed endothelial nitric oxide synthase gene undergoes some form of regulation. This was tested by subjecting cultured ECs, namely human umbilical vein endothelial cells (HUVECs), to various

pharmacological agents, such as lymphokines like TGFβ, inflammatory cytokines like TNFα, bacterial endotoxin like LPS, and growth factors like bFGF, all factors that ECs might encounter in their lifetime. Since TNFα elicited a down-regulation of eNOS expression, the mechanism(s) by which TNFα effected this were explored.

The second hypothesis is that since TNFα is known to induce the production of NO through the induction of iNOS in various cell types, then NO would play a role in the action of TNFα in down-regulating the expression of eNOS. The induction of iNOS is known to release large amounts of NO, even above physiological levels. Therefore this hypothesis was tested by using exogenous NO donors, like sodium nitroprusside and S-nitroso-acetylpenicillamine, on cultured ECs to reproduce the effect of iNOS. Cells were also subjected to NOS inhibitors, like L-NAME and L-NMMA, to investigate whether this effect of down-regulation of eNOS by TNFα occured via NO. The level at which TNFα exerted its action on eNOS expression was also investigated, i. e. whether TNFα acted at the transcriptional level to prevent the transcription of the eNOS gene, or at the post-transcriptional level to desatbilize the eNOS mRNA or prevent its translation, or at the post-translational level to down-regulate the activity of the eNOS enzyme. Chapter 2 addresses both hypotheses and provides answers to the questions raised above.

TNFα was found to act at the post-transcriptional level. Many genes undergo some form of post-transcriptional regulation, where their transcripts are either stabilized to get translated further into more protein, or destabilized and degraded so that the cell can rid itself of any unwanted gene products, such as the oncogenes, which are transcribed only when needed. The 3' untranslated region (3'UTR) of many genes have been implicated in the

regulation of their mRNAs, and motifs that constitute an AU-rich element (ARE) have been found in those of rapidly degraded genes (Sachs, 1993). Whereas stable transcripts, like α-globin among others, contain cytidine (C)-rich elements in their 3'UTR, that play a role in the message stabilization. Those *cis*-acting motifs are thought to recruit *trans*-acting factors, mostly proteins, which bind to the transcripts to effect their mode of action. The regulation of mRNA stability is reviewed in Chapter 3 of the thesis.

The third hypothesis therefore is that the cytokine TNFα induces protein factors that bind to known "destabilizing" motifs in the 3'UTR of the eNOS mRNA, thus targeting the mRNA for degradation. This was tested by performing experiments, like gel mobility shift and label transfer assays, that visualize protein binding to the 3'UTR of the eNOS mRNA under control and TNFα-treated conditions. The question that arose was whether different proteins bind to the eNOS 3'UTR in the presence of TNFα, when compared to control conditions, or whether the proteins that bind under control conditions undergo differential binding in the presence of TNFα. The investigation of this is outlined in Chapter 4.

The fifth chapter of the thesis deals with the preliminary characterization of the eNOS 3'UTR-binding proteins, since it was of interest to know whether they were novel proteins or already existing proteins for which a new function would be defined. Chapter 6 includes a general conclusion and suggestions for future study.

2. LACK OF ROLE FOR NITRIC OXIDE IN THE SELECTIVE DESTABILIZATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE MESSENGER RNA BY TUMOR NECROSIS FACTOR-ALPHA

2.1. Summary:

The constitutive expression of eNOS is essential for the physiological regulation of vascular tone and structure. The mechanism of down-regulation of steady state eNOS mRNA in human umbilical vein endothelial cells exposed to TNFα was investigated by using Northern blot analysis of total cellular RNA. TNFα produced a dose- and time-dependent decrease in eNOS mRNA expression that was near maximal at 10 U/mL and 6 hours of exposure, respectively. In contrast, steady state expression of endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PAI-1) mRNA was up-regulated by TNFα. The pharmacological generation of NO using sodium nitroprusside (10 μmol/L) and S-nitroso-acetylpenicillamine (100 to 400 μmol/L) had no effect on eNOS mRNA levels, and TNFα-induced down-regulation of eNOS was not prevented by coincubation with the inhibitors of NO synthesis L-NAME (1 mmol/L) and L-NMMA (10 mmol/L). Under control conditions, eNOS and PAI-1 mRNA were stable after treatment with actinomycin D for periods greater than 24 hours, whereas ET-1 message was rapidly degraded (half-life < 1 hour). Pretreatment with TNFα (30 U/mL) selectively reduced the half-life of eNOS mRNA to less than 12 hours without altering the stability of PAI-1 message. TNFα-induced destabilization of eNOS mRNA could be partially prevented by co-incubation with cycloheximide (1 μmol/L) but was not reproduced by addition of sodium nitroprusside. These findings indicate that TNFα down-

regulation of eNOS expression in human endothelial cells results predominantly from the selective destabilization of the mRNA by a mechanism involving the synthesis of new protein. However, NO production by a TNFα-inducible isoform of NOS did not appear to contribute either to the decrease in steady state eNOS mRNA levels or the shortening of its half-life. **(Arterioscler Thromb Vasc Biol. 1995; 15:52-57).**

2.2. Introduction:

ECs express a "constitutive" isoform of NOS that catalyzes the oxidation of L-arginine to produce citrulline and NO (Moncada et al., 1991). The activity of eNOS is tightly regulated by a calmodulin-dependent mechanism (Lamas et al., 1992; Nishida et al., 1992). A wide variety of receptor agonists, including acetylcholine, bradykinin, and serotonin, have the ability to elevate intracellular Ca^{2-} concentrations, thereby increasing NO release from ECs (Furchgott and Vanhoutte, 1989). As a result of intimal shear stress associated with blood flow in the vascular lumen, high basal levels of NO production are also maintained under physiological conditions (Griffith et al., 1988). NO acts by stimulating soluble guanylate cyclase and the resulting increases in cGMP concentration mediate its any important physiological actions (Furchgott and Vanhoutte, 1989; Griffith et al., 1988), including vasodilation, prevention of smooth muscle cell proliferation, and inhibition of platelet and monocyte adhesion (Moncada et al., 1991).

Loss of endothelium-derived NO might therefore lead to abnormalities in smooth muscle tone or growth and allow adhesion of blood elements to the vessel wall (Stewart and Monge, 1993). Recently, several groups have demonstrated (Marsden et al., 1992; Nishida

et al., 1992; Yoshizumi et al., 1993) a potent down-regulation of steady state eNOS mRNA expression in ECs exposed to cytokines, in particular TNFα, by a mechanism involving destabilization of eNOS mRNA (Yoshizumi et al., 1993). Thus, decreased expression of endothelial NOS could contribute to the development of vascular disorders, such as atherosclerosis, in which increased production of TNFα (Barath et al., 1990) and impaired endothelium-dependent dilator responses have been reported (Ludmer et al., 1986; Stewart and Monge, 1993).

However, cytokines may also cause the expression of an inducible NOS isoform (iNOS) (Dinerman et al., 1993; Nathan, 1992) and increased NO production. Although identified in macrophages (Ewenstein et al., 1991; Lowenstein et al., 1992; Stuehr et al., 1991), it is now recognized that many cell types, including some EC lines (Gross et al., 1991; Lamas et al., 1991; Radomski et al., 1990), may have the potential to express iNOS. The functional relevance of iNOS expression in endothelium is unknown. Unlike the endothelial NOS, the inducible enzyme is not regulated by Ca^{2+}; rather, it produces large amounts of NO in a continuous manner. Such high levels of NO generation may have direct effects on cellular function, e. g. by promoting ADP ribosylation (Dinerman et al., 1993), and by inhibiting mitochondrial respiration (Stadler et al., 1991). NO has also been shown to alter gene expression, down-regulating monocyte chemoattractant protein-1 (Zeiher et al., 1993) and increasing levels of transferrin receptor mRNA (Drapier et al., 1993). Interestingly, this latter effect was mediated by an action of NO on the stability of the mRNA (Drapier et al., 1993). However, whether excessive endothelial production of NO by iNOS might contribute directly to the cytokine-induced down-regulation in eNOS expression, perhaps also mediated by an

effect on mRNA stability, is not known.

Therefore, the aim of the present study was to determine the mechanism of TNFα-induced decreases in eNOS expression in human ECs. We now report that TNFα reduced the steady state levels of eNOS mRNA, in large part due to a post-transcriptional mechanism involving destabilization of the mRNA and requiring de novo protein synthesis. However, this process was independent of endogenous NO production and was not reproduced by the pharmacological generation of exogenous NO.

2.3. Methods:

2.3.1. EC Culture: HUVECs were obtained from the American Type Culture Collection (ATCC) or isolated from fresh umbilical veins by 0.2% collagenase digestion (Jaffe et al., 1973). HUVECs were grown to confluence in T75 flasks (Fisher Scientific) equilibrated with 95% air and 5% CO_2 at 37°C in Ham's/F12 medium (GIBCO) and supplemented with 10% fetal calf serum, penicillin (500 U/mL), streptomycin (50 μg/mL), and heparin (100 μg/mL) (all from GIBCO) and EC growth factor (20 μg/mL, Boehringer Mannheim). Confluent ECs between the fifth and 18th passages were washed with Hank's buffered salt solution and preincubated in 10 mL medium supplemented with 10% fetal calf serum, 500 U/mL penicillin, and 50 μg/mL streptomycin for 2 hours. For experiments on steady state mRNA expression, fresh medium was added containing the following agents singly or in combination: human recombinant TNFα (Sigma), L-NAME (1 mmol/L, Sigma), L-NMMA (10 mmol/L, Sigma), dexamethasone (1 μmol/L, Sigma), sodium nitroprusside (SNP, 10 μmol/L, Roche Pharmaceuticals), and S-nitroso-acetylpenicillamine (SNAP, Biomol). Unless otherwise

specified, cells were incubated under the above conditions for 24 hours. For experiments on the stability of mRNA, actinomycin D (Sigma) was added to the cells after a 3-hour preincubation period in the presence or absence of TNFα (30 U/mL), cycloheximide (CHX, 1 μmol/L), or SNP (10 μmol/L) singly or in combination.

2.3.2. RNA Extraction and Northern Blot Analysis: RNA was isolated by a modified guanidinium thiocyanate-phenol-chloroform method using RNAzol B (Tel-Test) according to the manufacturer's recommendations. For the Northern blots, total cellular RNA (20 μg) samples were separated by electrophoresis on a 1.2% agarose gel containing 2 mol/L formaldehyde, 20 mmol/L 3-(N-morpho)propane sulfonic acid, 8 mmol/L sodium acetate, 1 mmol/L EDTA, and 5 mmol/L NaOH and transferred by capillary blotting to Hybond-N membranes (Amersham) in 20x saline-sodium citrate (SSC; 3 mol/L sodium chloride and 0.3 mol/L sodium citrate). The membrane was optimally cross-linked (120 mJ/cm^2) with UV light (UVXL-1000, Fisher Scientific). Membranes were prehybridized for 4 hours at 42°C in 50% formamide, 5x SSPE (0.75 mol/L NaCl and 0.05 mol/L NaH_2PO_4), 5x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), 0.5% SDS, and 100 μg/mL herring sperm DNA and hybridized overnight (42°C) with specific cDNA probes that had been radiolabeled with [α-^{32}P]dCTP (Amersham) by the random primer technique to a specific activity of at least 1x10^9 dpm/μg (Feinberg and Vogelstein, 1983). Membranes were then washed twice in 2x SSC and 0.1% SDS for 15 minutes at room temperature and then once in 1x SSC and 0.1% SDS for 15 minutes at 65°C. Autoradiography was performed by using double intensifying screens (Cronex) and Kodak XAR film at -80°C. Signal intensity

was quantified as integrated areas by using scanning densitometry.

2.3.3. Preparation of the cDNA Probes: A cDNA probe for human eNOS was prepared by polymerase chain reaction (PCR) of a 10 μL aliquot of a HUVEC lambda gt-11 cDNA library (provided by Dr. Morag Park, McGill University). The primers were designed on the basis of the sequence retrieved from the Genbank database (accession No. M93718) (Janssens et al., 1992) and were as follows: sense 5'-TTCCGGGGATTCTGGCAGGAG-3', antisense 5'-GCCATGGTAACATCGCCGCAG-3'. Amplification was as described (Friedman et al., 1990) with some modifications. Briefly, PCR was performed for 30 cycles with denaturation at 94°C for 1 minute and 20 seconds, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes. The PCR product was analyzed in a 2% agarose gel, revealing a band of the predicted size (299 bp). This product was then ligated directly into the PCR II vector (Invitrogen), and the NOS sequence was confirmed by sequencing with T7 DNA polymerase.

A full-length human prepro-endothelin-1 (ET-1) cDNA probe was prepared by screening a HUVEC cDNA library in lambda gt-11 with a synthetic 33-base oligonucleotide complementary to the mRNA region encoding for residues Met-59 to Leu-69 of human prepro-ET-1 (5'-CAGGTGGCAGAAGTAGACACACTCTTTATCCAT-3') (Bloch et al., 1989). The oligonucleotide was 5' end labeled with T4 polynucleotide kinase and [α-^{32}P]dATP. Bacteriophages were plated and transferred to nylon membranes (Hybond N, Amersham, Canada). Filters were hybridized with radiolabeled oligonucleotide, washed, and autoradiographed as described (Duby, 1987); hybridization and washing were performed at 50°C. DNA from hybridizing recombinant bacteriophages was prepared by the methodof

QIAGEN, Inc. Inserts from the recombinant bacteriophages were recovered by EcoRI digestion, ligated into the plasmid pGEM-3Z, and sequenced with T7 DNA polymerase.

GAPDH cDNA, a constitutively expressed gene, was obtained (ATCC No. 57091) (Tso et al., 1985), and a 0.78-Kb Pst 1/Xba I fragment was used as a cDNA probe. A full-length cDNA probe for plasminogen activator inhibitor-1 (PAI-1) was a generous gift from Dr. David Ginsburg, University of Michigan, Ann Arbor (Ginsburg et al., 1986) , and a 1.1-Kb fragment was used that had been generated by digestion with EcoRI and Ava I.

2.3.4. Nitrite Measurement: Nitrite was determined by incubating 0.25 mL of the EC-conditioned medium with the Griess reagent (0.025 mL of 6.5 mol/L HCl and 0.025 mL of 37.5 mmol/L sulfanilic acid) for 10 minutes at room temperature (Schini et al., 1992). Ethylenediamine (0.025 mL) was added, and the absorbance at 540 nmol/L was determined 30 minutes later by using a spectrophotometer (Milton Roy). The concentrations were calculated from a standard curve derived from prepared solutions of $NaNO_2$ (0 to 25 μmol/L).

2.3.5. Western Blot Analysis: Western blot analysis was performed by using the ECL system (Amersham). Crude cytoplasmic extracts were prepared from HUVECs that were either incubated under control conditions or exposed to TNFα (30 U/mL) for 24 hours, by lysis in hypotonic buffer containing 0.2% Nonidet P-40, 40 mmol/L KCl, 10 mmol/L HEPES, pH 7.9, 3 mmol/L $MgCL_2$, 1 mmol/L DTT, 5% glycerol, 8 ng/mL aprotinin, 2 ng/mL leupeptin, and 0.5 mmol/L phenylmethylsulfonyl fluoride (Sigma). The nuclei were removed by centrifugation at 14 000g for 2 minutes at 4°C. Cytoplasmic extracts were immediately frozen

on dry ice and stored at -80°C. Sample protein (25 μg) was then loaded on a 10% to 20% gradient SDS-tricine gel (Novex), and electrophoresis was performed at 125 V for 90 minutes. The gel was then electrotransferred to a polyvinylidene difluoride membrane (Novex) using 30 V for 90 minutes. After overnight blocking in 5% nonfat milk in Tris-buffered saline at 4°C, the filter was incubated for 1 hour at room temperature with a primary antibody (diluted 1:1000), either a polyclonal raised against human eNOS (Dr. David Harrison, Emory University, Atlanta, Ga) or a monoclonal raised against mouse iNOS (Transduction Laboratories, Lexington, Ky). The membrane was then incubated with the secondary antibody (anti-mouse or anti-rabbit immunoglobulin G conjugated with horse radish peroxidase, 1:1000) following the manufacturer's recommendations and autoradiographed for 10 to 15 seconds.

2.4. Results:

Incubation of human ECs with TNFα markedly down-regulated the expression of eNOS. As shown in Figure 2.1, steady state mRNA levels of eNOS were profoundly reduced relative to GAPDH, even at concentrations as low as 10 U/mL and after an incubation period of less than 6 hours. In contrast, the expression of PAI-1 and ET-1 was up-regulated by TNFα (Figure 2.2). The decrease in levels of eNOS mRNA induced by TNFα could not be prevented by L-NAME, an inhibitor of NO synthesis, or dexamethasone, which inhibits the induction of iNOS by cytokines (Radomski et al., 1990).

Figure 2.3 shows the effect of L-NMMA, another inhibitor of NOS, on TNFα-induced down-regulation of eNOS expression. Even at a relatively high concentration 10 mmol/L),

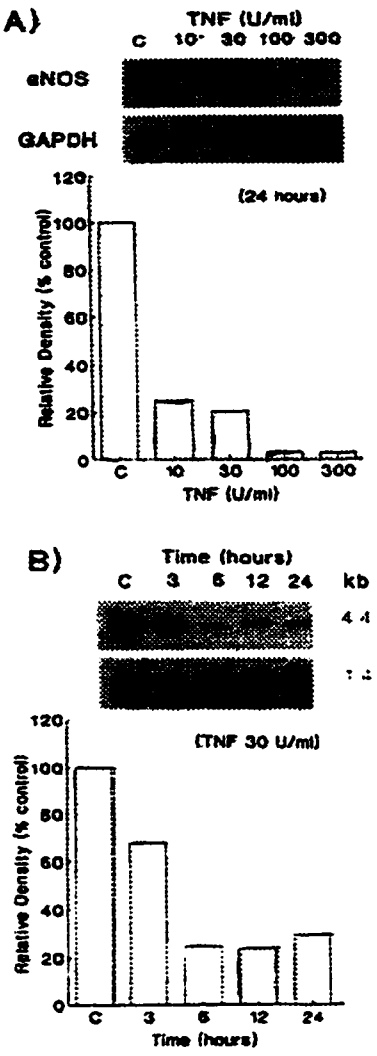


Figure 2.1. Concentration-response relation **(A)** and time course **(B)** of tumor necrosis factor (TNF)-induced downregulation of steady-state constitutive nitric oxide synthase (eNOS) mRNA levels in human endothelial cells (ECs). Northern blots hybridized with radiolabeled cDNA probes for eNOS and GAPDH to control for total RNA loaded in each of the lanes are shown in upper part of each panel. Histograms (lower part of each panel) show density of the eNOS hybridization signal relative to the GAPDH signal and are expressed as a percentage of control values. **(A)** ECs were incubated for 24 hours with varying concentrations of TNF; **(B)** ECs were incubated for varying time periods as shown with a single concentration of TNF (30 U/mL).

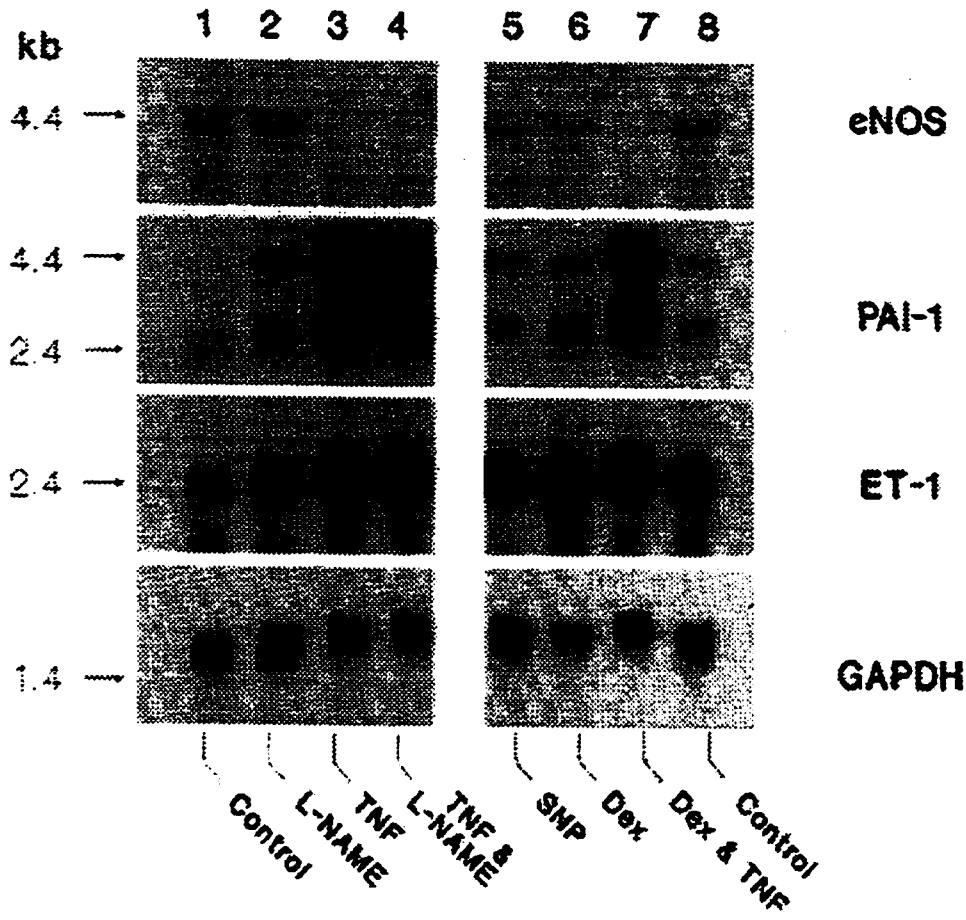


Figure 2.2. Northern blot analysis of total cellular RNA hybridized with specific radiolabeled cDNA probes for endothelial constitutive nitric oxide synthase (eNOS), plasminogen activator inhibitor-1 (PAI-1), endothelin-1 (ET-1), and GAPDH. RNA was extracted from endothelial cells incubated for 24 hours with control medium alone (lanes 1 and 8) or with the addition of the following agents: N^{w}-nitro-L-arginine methyl ester (L-NAME, 1mmol/L, lane 2); tumor necrosis factor (TNF, 300 U/mL, lane 3); TNF and L-NAME (lane 4); sodium nitroprusside (SNP, 10 µmol/L, lane 5); dexamethasone (Dex, 1 µmol/L, lane 6); and TNF and Dex (lane 7).

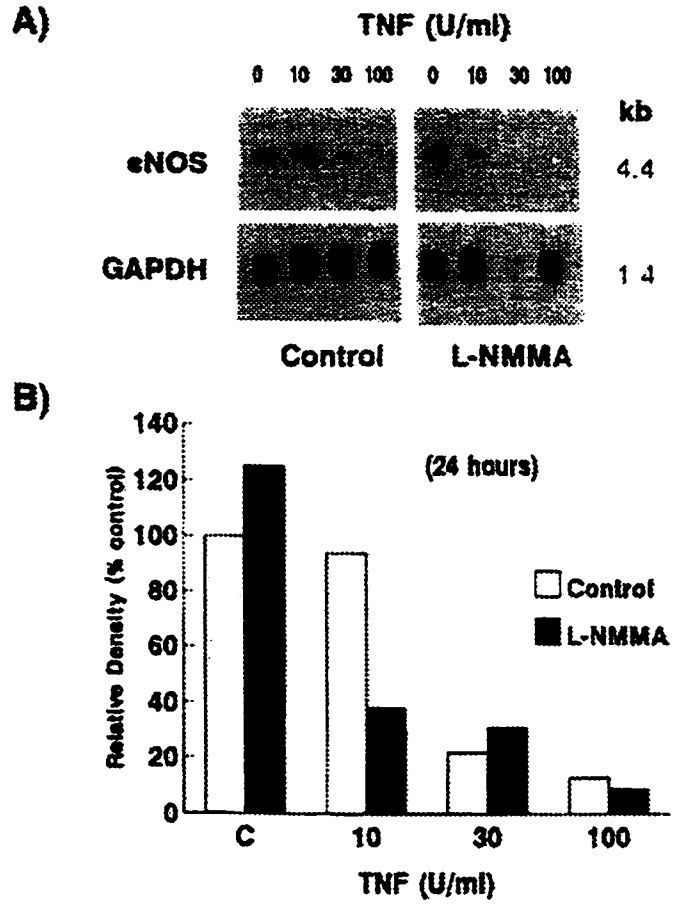


Figure 2.3. Concentration-response relations of tumor necrosis factor (TNF)-induced downregulation of steady-state constitutive nitirc oxide synthase (eNOS) mRNA levels under control and in the presence of 10 mmol/L L-NMMA. **(A)** Northern blots hybridized with cDNA probes for eNOS and GAPDH to control for total RNA loaded in each lane. **(B)** Histograms showing the density of the eNOS hybridization signal relative to GAPDH expressed as a percentage of the control value at TNF 0 mmol/L in the presence ( ) and absence ( ) of L-NMMA. In lane 3 of the L-NMMA-treated cells (TNF, 30 U/mL) substantially less total RNA was loaded; however, the eNOS hybridization signal was still reduced relative to GAPDH to a similar extent as under control conditions.

L-NMMA did not prevent the down-regulation of eNOS mRNA by TNFα. To further exclude a role for NO in this action of TNFα, the effect of pharmacological generation of NO was examined by using SNP (1 μmol/L, Figure 2.2) or SNAP (100 and 400 μmol/L; Figure 2.4A). These NO donor compounds failed to substantially reduce the expression of eNOS in HUVECs.

The effect of TNFα on the expression of eNOS and iNOS proteins in HUVECs was studied by Western blot analysis. As shown in Figure 2.4B, pretreatment of HUVECs for 24 hours with TNFα markedly reduced the amount of eNOS protein, while iNOS protein was not detected in HUVEC cytoplasmic extracts either under control conditions or following pretreatment with TNFα. In addition, nitrite levels were not different in control (0.38±0.05 μmol/L) and TNFα-treated (0.38±0.03, 0.46±0.06, and 0.44±0.04 μmol/L for 10, 30, and 100 U/mL, respectively; n=3) cells. In contrast, substantial increases in nitrite could be demonstrated following incubation of cells with 100 and 400 μmol/L SNAP (8.04 and 66.55 μmol/L, respectively).

Figure 2.5 compares the stability of various EC mRNAs following the complete inhibition of transcription by the administration of actinomycin D. There was no loss of the hybridization signal for eNOS over 24 hours; indeed there was an apparent increase in eNOS mRNA. However, when corrected by normalization with GAPDH mRNA, the relative density of the eNOS hybridization signal was constant (Figure 2.6), as was that for PAI-1. In contrast, ET-1 mRNA was rapidly degraded with a half-life of less than 1 hour.

The effect of TNFα on the stability of eNOS mRNA in the presence and absence of cycloheximide is presented in Figure 2.7. TNFα alone destabilized eNOSmessage, while that

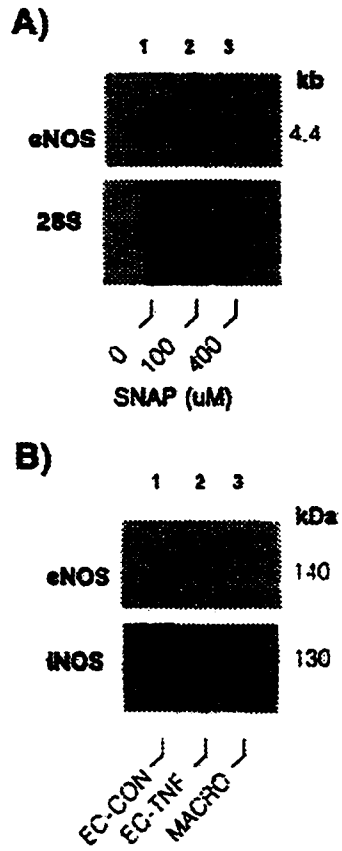


Figure 2.4. (A) Northern blot showing the effect of *S*-nitroso-acetylpenicillamine (SNAP) on the expression of endothelial constitutive nitric oxide synthase (eNOS) mRNA, hybridized as in Fig. 2.1. Ribosomal RNA (28S) was used to control for total RNA loading. **(B)** Western blot showing the expression of eNOS protein compared with that of the inducible form of NOS (iNOS). Lanes 1 and 2 represent 25 μg cytoplasmic protein extracted from human umbilical vein ednothelial cells cultured under control conditions (EC-CON) or in the presence of tumor necrosis factor (EC-TNF, 30 U/mL), respectively, for 24 hours. Lane 3, 2.5 μg of a murine macrophage lysate (MACRO; Transduction Laboratories) induced by endotoxin and interferon gamma.

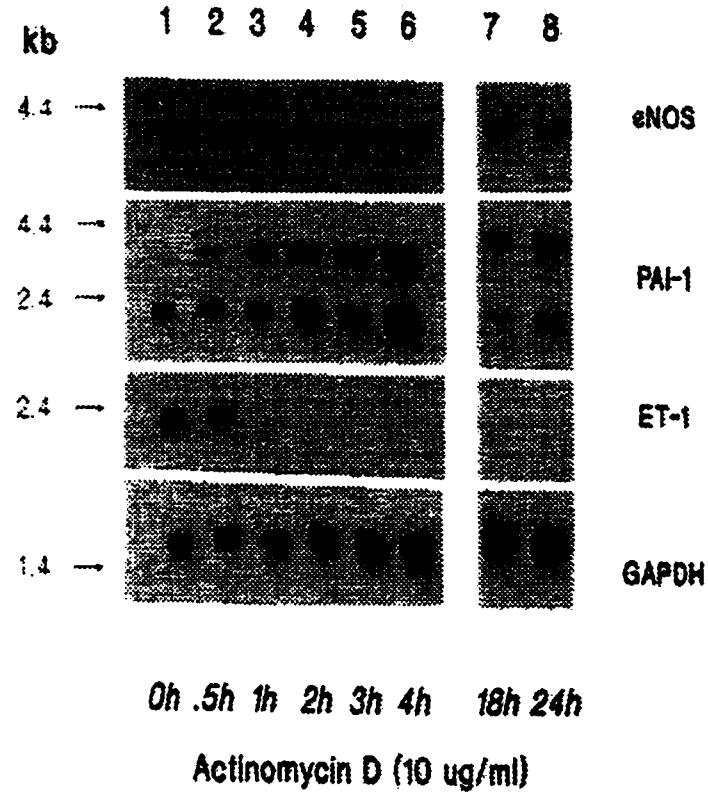


Figure 2.5. Half-life determination by Northern blot analysis for the mRNAs presented in Fig. 2.2. Each lane was loaded with 20 μg total RNA extracted from endothelial cells incubated for varying times after the addition of actinomycin D to inhibit RNA transcription. eNOS indicates endothelial constitutive nitric oxide synthase; PAI-1, plasminogen activator inhibitor-1; and ET-1, endothelin-1.

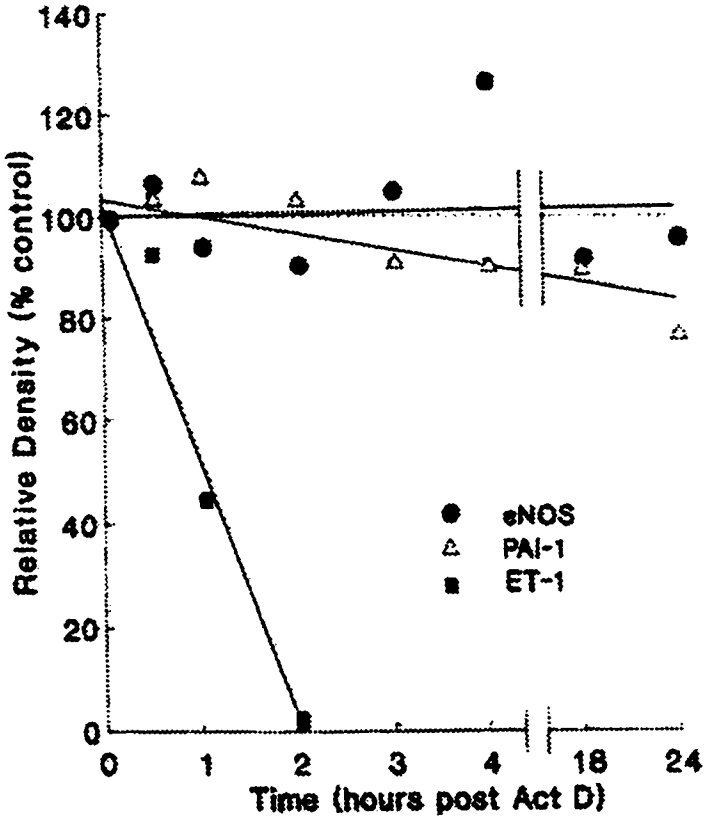


Figure 2.6. Line graph showing levels of mRNA as determined by Northern blot analysis using radiolabeled cDNA probes for endothelial constitutive nitric oxide synthase (eNOS), plasminogen activator inhibitor-1 (PAI-1), and endothelin-1 (ET-1). The density of the hybridization signals relative to GAPDH are presented on the ordinate as a percentage of the control values immediately before the addition of actinomycin D (Act D, 10 μg/mL). Time of incubation following addition of actinomycin D is shown on the abscissa. Each point represents a mean value for two or three experiments.

of PAI-1 remained stable. Cycloheximide had no effect on eNOS mRNA levels prior to the addition of actinomycin D (0 hours) but increased the amount of message at each time point thereafter. In contrast, cycloheximide increased the expression of ET-1 (less so for PAI-1) in the presence of TNFα but had little effect on the half-life, while message for GAPDH was not altered.

The relative density of the eNOS mRNA hybridization signal normalized for GAPDH is presented in Figure 2.8. TNFα produced a shortening of the half-life to less than 12 hours, while co-incubation with TNFα and cycloheximide partially restored eNOS mRNA stability to control levels (i. e. half-life >24 hours). In the presence of SNP, a pharmacological generator of NO, the stability of eNOS mRNA was identical to that under control conditions.

2.5. Discussion:

TNFα produced a marked reduction in steady state mRNA levels for eNOS in human ECs in a time- and concentration-dependent manner, with near maximal down-regulation at concentrations between 10 and 30 U/mL and as rapidly as within 6 hours of its addition to the culture medium. Yet eNOS mRNA was remarkably stable under control conditions, with no detectable loss of signal even 24 hours after treatment with actinomycin D, which effectively prevents further gene transcription (Peltz et al., 1991). Thus, it is unlikely that, without a change in the stability of the message, even the total suppression of transcription could account for the rapid and complete disappearance of steady state eNOS message after exposure to TNFα. Indeed, after a 3-hour pre-incubation with TNFα, the half-life of eNOS message was found to have decreased to less than 12 hours, with nearly no detectable

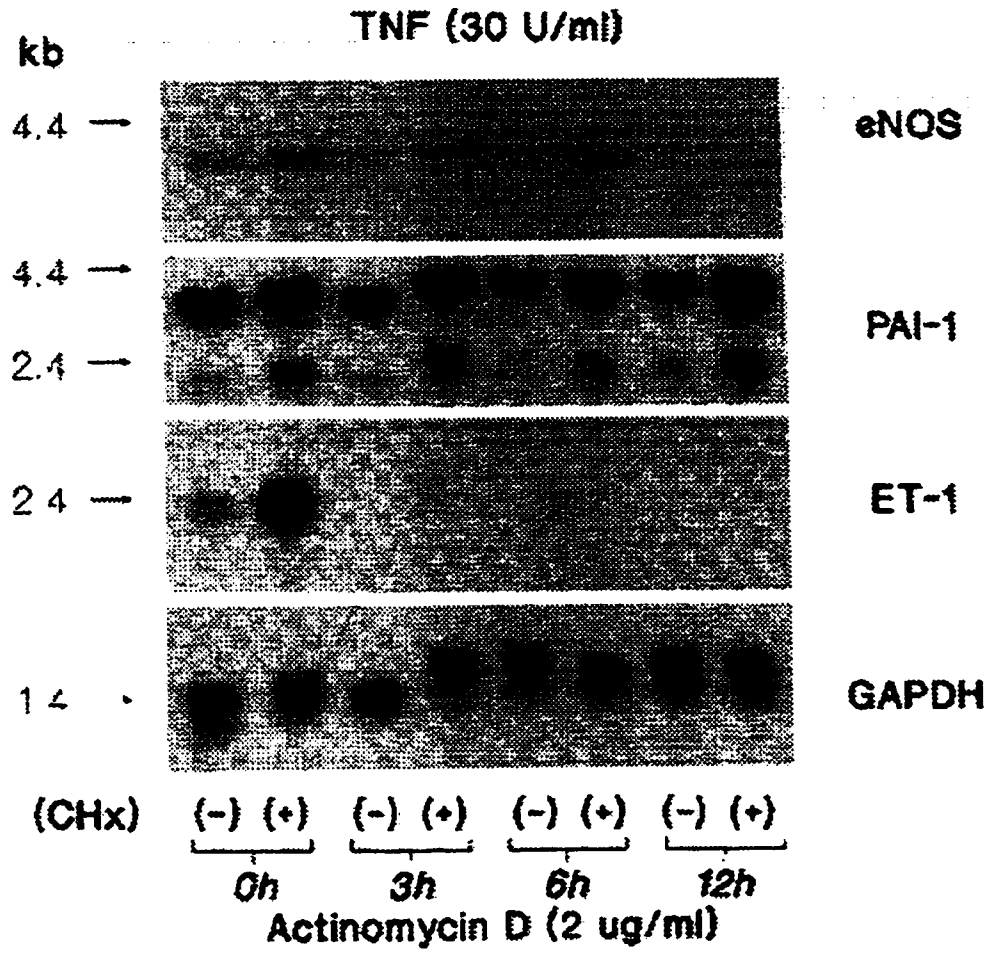


Figure 2.7. Half-life determination by Northern blot analysis for the mRNAs presented in Fig. 2.2 in the presence of tumor necrosis factor (TNF, 30 U/ml). Each lane was loaded with total RNA extracted from endothelial cells incubated for varying time periods following treatment with actinomycin D with (+) or without (-) the addition of cycloheximide (CHX, 1 μmol/L) to inhibit *de novo* protein synthesis. eNOS indicates a constitutive isoform of nitric oxide synthase; PAI-1, plasminogen activator inhibitor-1; and ET-1, prepro-endothelin-1.

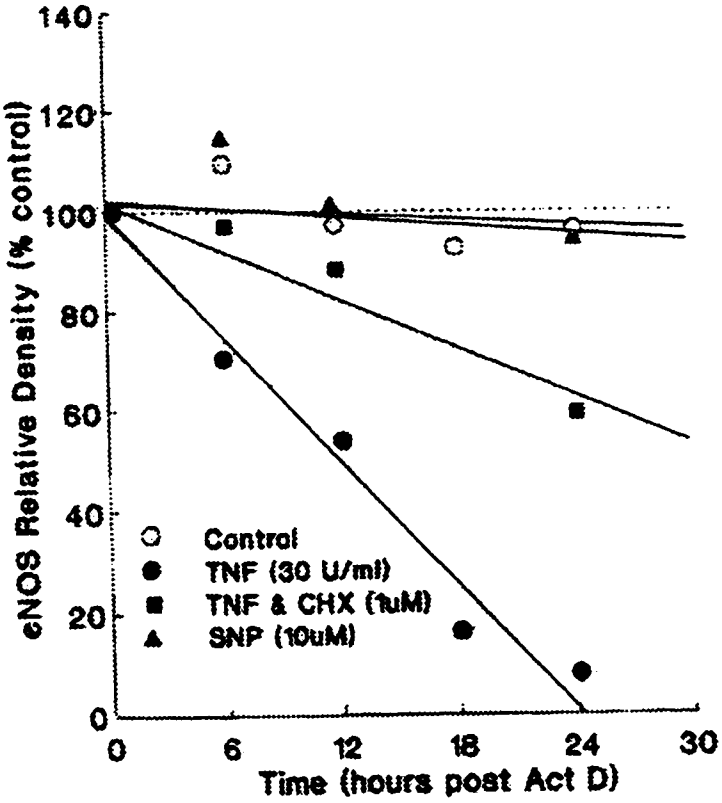


Figure 2.8. Line graph showing levels of endothelial constitutive nitric oxide synthase (eNOS) mRNA as determined by Northern blot analysis of total RNA extracted from endothelial cells incubated in control medium alone (○) or in the presence of tumor necrosis factor (TNF) alone (●), TNF and cycloheximide (CHX,■), or sodium nitroprusiide alone (SNP,▲). The density of the hybridization signal rrelative to GAPDH is presented on the ordinate as a percentage of the control values immediately before the addition of actinomycin D (Act D, 2 μg/mL). Time of incubation following addition of actinomycin D is shown on the abscissa. Each point reprsents a mean value for two or three experiments.

message 24 hours after treatment with actinomycin D. This finding agrees with the earlier report of Yoshizumi et al (Yoshizumi et al., 1993) and strongly suggests that a change in stability of eNOS message is the predominant mechanism for the down-regulation of its steady state expression. Moreover, the destabilization of eNOS message by TNFα could be largely prevented by co-incubation with cycloheximide, indicating that synthesis of new protein(s) was required for this effect.

The degradation of mRNA is a regulated process that is a potentially important contributor to the level of gene expression (Jackson, 1993; Sachs, 1993). Certain motifs can confer stability, such as the stem-loop structures in the 3'UTRs of some bacterial mRNAs. In contrast, UA-rich regions in the 3'UTR of protooncogenes promote the rapid degradation of their messages. Interestingly, the mRNA for both eNOS and ET-1 possesses AUUUA repeats in the 3'UTR, which promote rapid degradation, yet only ET-1 exhibits a short half-life (Inoue et al., 1989). Destabilization of eNOS mRNA in response to TNFα could result from a protein-mRNA interaction, which would allow the rapid degradation elements in the 3'UTR to become active. An example of such a mechanism is the destabilization of transferrin receptor mRNA in the presence of ferrous iron (Harford and Klausner, 1990; Sachs, 1993). Importantly, NO also alters the affinity of the iron regulatory factor for its mRNA binding site (Drapier et al., 1993; Jackson, 1993) and thus regulates the stability of the transferrin receptor message.

The effect of NO on the stability of the transferrin receptor mRNA raises the possibility that, as a product of the NOS pathway, NO could regulate the expression of eNOS mRNA in a "feedback" manner, much as it may regulate the activity of the enzyme (Buga et

al., 1993). However, TNFα-induced down-regulation of steady-state mRNA levels for eNOS was not prevented by inhibition of the synthesis of NO using L-NAME or L-NMMA. As well, pretreatment of ECs with dexamethasone at a concentration that prevents the induction of iNOS in response to cytokine stimulation (Radomski et al., 1990) also failed to modify the effect of TNFα on the expression of eNOS, while the pharmacological generation of exogenous NO by addition of SNP or SNAP had no effect on steady-state levels or stability of eNOS mRNA. Finally, the expression of eNOS protein was down-regulated by TNFα in a manner similar to its message, whereas no induction of iNOS was observed in HUVECs by Western blot analysis (Figure 2.4B) or as shown by the measurement of nitrite, a stable breakdown product of NO. Therefore, the results of the present experiments provide strong arguments against an autocrine role of NO in the cytokine-induced down-regulation of eNOS mRNA in human ECs.

Surprisingly, the message for PAI-1 was stable in human ECs, comparable to those for eNOS and GAPDH. This finding is in disagreement with the short half-life for PAI-1 mRNA reported for bovine ECs (Sawdey et al., 1989) and a human hepatoma cell line (Westerhausen, Jr. et al., 1991). The reason for this discrepancy is not clear, but it may reflect differences in post-transcriptional regulation of PAI-1 mRNA between different cell types. In contrast, the half-life of ET-1 message was very short in our cells (<1 hour), comparable to other reports (Inoue et al., 1989). Of note, the stability of PAI-1 mRNA was not reduced by pretreatment with TNFα, indicating that the destabilization of eNOS message occurred by a selective mechanism rather than by a nonspecific increase in degradation of mRNA.

The present findings may have important implications regarding the role of the

endothelium in the initiation and progression of vascular disorders. The loss of eNOS expression and the resulting decrease in the capacity to produce NO in response to physiological stimuli could compromise the ability of the endothelium to protect against thrombosis, vasoconstriction, and subintimal proliferation. At the same time, TNFα-induced up-regulation of the expression of ET-1 and PAI-1 mRNA would serve to actively promote these pathological processes. Increased levels of cytokines have been demonstrated in a variety of cardiovascular disorders, from atherosclerosis (Barath et al., 1990) to heart failure (Levine et al., 1990). Thus, a better understanding of the mechanisms of TNFα-induced endothelial dysfunction may lead to the development of new strategies in the prevention and treatment of vascular disease.

2.6. Update on eNOS down-regulation by TNFα:

Please refer to Section 3.5.8. entitled "Regulation of eNOS mRNA stability" as well as to the discussion in Chapter 4 (Section 4.5).

3. A REVIEW OF REGULATION OF MESSENGER RNA STABILITY

3.1. Introduction:

Each cell of the body contains the whole genetic makeup of the organism, except for red blood cells, but what renders each cell specialized is the type of proteins it synthesizes to either secrete or to target to various locations within the cell for different purposes and functions, either to act as structural proteins or as enzymes or as receptors, etc. Specific triggers extracellularly or from within the cell cause signal transduction cascade events that lead to the transcription of specific DNA strands, manufacturing an exact replica of the DNA into pre-messenger RNA, which consists of exons and introns. The pre-mRNA is then spliced into mRNA, with the aid of heterogeneous nuclear ribonucleoproteins (hnRNPs) and other splicing factors, in a process where the exons are assembled together and the introns are spliced out (Choi et al., 1986). The mRNA is capped at the 5' end with a 7-methylguanosine (m^7G) cap and the 3' end is polyadenylated with a homopolymer of up to 200 adenosine nucleotides (Wahle, 1995), and is then transported out of the nucleus to be translated into its respective protein at the ribosome. Both modifications act as barriers to the marauding nucleases that would otherwise rapidly degrade mRNAs nucleotide by nucleotide, either from the 5' end by 5' –> 3' exonucleases or the 3' end by 3' –> 5' exonucleases. There is mounting evidence to suggest that splicing of the pre-mRNA occurs after the capping and polyadenylation processes. Konarska et al. have observed that substrate RNAs prepared by transcription with mammalian RNA polymerase II or bacterial RNA polymerase were efficiently spliced when they possessed capped 5' termini (Konarska et al., 1984), suggesting

that cap recognition might be an important step in the formation of a specific RNP complex required for splicing. mRNA 3' end formation in the eukaryotic nucleus involves an endonucleolytic cleavage of the nascent transcript mediated by a protein complex termed CPSF for cleavage and polyadenylation specificity factor, which then activates polyadenylation (Bienroth et al., 1993; MacDonald et al., 1994).

3.2. Role of the poly (A) tail and cap structure:

The poly(A) tail confers a nonspecific stability on mRNAs in eukaryotes, and its removal is a prerequisite for the degradation process. Non-polyadenylated mRNAs are rapidly degraded; addition of a poly(A) tail of 30 adenosines is enough to confer stability to a transcript (Ford et al., 1997). It is possible that the poly(A) tail may block the assembly of a 3' –> 5' exonuclease. Polyadenylation has the opposite effect in prokaryotic (Cohen, 1995) and choroplast (Kudla et al., 1996) mRNAs; it renders the mRNA susceptible to 3' –> 5' exonucleases and therefore promotes efficient degradation of the message. Bacterial mRNA may contain 3' stem-loops that would protect against exonucleases. Polyadenylation by a bacterial poly(A) polymerase would destabilize mRNA with 3' stem-loops by adding a single-stranded tail, facilitating attack by these exonucleases (Raynal et al., 1996).

Polyadenylated mRNAs complex with cytoplasmic proteins to form RNPs, among them the poly(A)-binding protein (PABP), a 70-kDa protein common to most eukaryotes (Munroe and Jacobson, 1990). This complex was thought to play a passive role by functioning as a steric block to 3' –> 5' exoribonucleases. Recent evidence suggests a more active role for the PABP-poly(A) tail complex in RNA metabolism. An mRNA is not

decapped until the 3' poly(A) tail is reduced in length in the deadenylation-dependent pathway (Munroe and Jacobson, 1990). mRNAs in *pab1* mutant cells lacking PABP are decapped even though they possess full-length poly(A) tails (Caponigro and Parker, 1995), indicating that it is the PABP-poly(A) complex at the 3' end that inhibits decapping at the 5' end of the molecule, and not the poly(A) tail *per se*, reinforcing the notion that the mRNA is not a straight linear molecule, but rather that the 5' and 3' ends of the mRNA are physically associated by protein-protein or RNA-protein interactions to form a closed loop.

Current evidence indicates that the poly(A) tail complexed with PABPs is necessary for the formation of the 80S translation initiation complex (Brawerman, 1981). Removal of the poly(A) tail could prevent the joining of the 60S to the 40S ribosomal subunits and mRNA complex, thereby disrupting the formation of the initiation complex and leaving an unstable mRNA vulnerable to degradation. Thus not only does the poly(A) tail help regulate the stability of an mRNA, but it regulates translational efficiency as well (Gallie, 1991). Two eukaryotic translation initiation factors eIF-4F and eIF-4B, known to bind to the cap structure, also bind to poly(A), thus maintaining physical proximity of the termini during translation (Gallie and Tanguay, 1994), a bridge that could allow the synergistic stimulation of translation initiation by the cap and the poly(A) tail..

Once the mRNA is translated into protein, and is no longer needed for numerous translation events, the transcript has to be degraded, so as not to accumulate unwanted RNA or protein. The pathway of degradation would involve an obligate deadenylation step followed by exonucleolytic degradation in the 3' –> 5' direction. mRNAs are not degraded until their tails are shortened below 10 nucleotides, a length that is incapable of binding the

PABP (Sachs, 1993). A deadenylation nuclease (DAN) or poly(A) ribonuclease (PAN) has been found to be the major enzyme responsible for poly(A) tail shortening (Korner and Wahle, 1997). It specifically interacts with the 5' cap structure of RNA substrates, an interaction greatly stimulated by the presence of a poly(A) tail (Gao et al., 2000). Cap-DAN interactions are functionally important for the networking between regulated mRNA stability and translation.

A decapping enzyme has been isolated from yeast (LaGrandeur and Parker, 1998), the activity of which could be triggered by the release of the cap structure by translation initiation factors. Decapping would expose the transcript to 5' –> 3' exonucleases. In bacteria, the decay of many mRNAs is initiated by a primary endonucleolytic cleavage, often by RNase E. This cleavage is followed by exonucleolytic degradation at the new 3' ends by two enzymes: RNase II and polynucleotide phosphorylase (PNPase) since, to date, no 5' –> 3' exonuclease has been found in prokaryotes (Grunberg-Manago, 1999). RNase E is thought to scan the mRNA transcript in a 5' to 3' direction and cleave within adenosine-uridine (AU)-rich segments (Carrier and Keasling, 1997). A magnesium-dependent human endoribonucleolytic activity similar to RNase E, that can cleave AU-rich sequences, has been extracted from polysomes (Wennborg et al., 1995). This finding indicates an evolutionary conservation of the components of the mRNA degradation system. This ribonuclease may derive from the same human gene ard-1 (for activator of RNA decay), first described by Wang et al., that encodes a highly basic 13.3-kDa proline-rich peptide with features in common with RNase E and with other eukaryotic proteins implicated in RNA binding and macromolecular transport (Wang and Cohen, 1994). The human ARD-1 is a site-specific

magnesium-dependent endoribonuclease that cleaves RNA at the same sites as RNase E and generates 5' phosphate termini at sites of cleavage (Claverie-Martin et al., 1997).

3.3. Mechanisms of mRNA regulation:

Post-transcriptional regulation of mRNA expression plays an important role in controlling the level of mRNA. This regulation is particularly important for proteins that must be rapidly produced, function within a narrow time-frame, and thereafter be rapidly cleared, as in the case of oncogenes or growth factors, cytokines, certain enzymes, transcription factors and proteins that control cell cycle progression (Claverie-Martin et al., 1997). Such regulation may take the form of post-translational modification, such as phosphorylation, dephosphorylation or even methylation,, of pre-existing proteins in a direct and rapid manner, or it may involve the stabilization or destabilization of the pool of coding cytosolic mRNAs. Just as rapidly inducible molecules tend to have unstable mRNAs with half-lives in the order of 10-60 minutes, other messages, such as those coding for structural proteins like actin or globin, are very stable with half-lives of 24 hours or more, days as in the case of γ-globin (Lodish and Small, 1976). Since there is such differential regulation in eukaryotic mRNA turnover, mechanisms must exist that predetermine the stability of the transcript and thus regulate the decapping and polyadenylating processes that must precede mRNA degradation. These mechanisms would be influenced by nutritional, hormonal and pharmacological stimuli as well as by the growth rate and requirements of the cell. Viral infection, exposure to toxins and carcinogens and even temperature shifts could also play a role in post-transcriptional regulation (Ross, 1995).

Part of this mechanism is intrinsic to the mRNA itself, where specific nucleotide sequences or *cis*-elements determine the fate of the message. The degradation rates of many eukaryotic mRNAs are determined by the interaction of these *cis*-elements and/or mRNA secondary structures with protein components (*trans*-factors) of cells. Most of the known determinants of mRNA degradation are located in the 3'UTR, although sequences at the beginning and end of the coding regions regulate the degradation of tubulin and *c-fos* mRNAs respectively (Ross, 1995). In some messages, the 5'UTR is known to harbor regulatory *cis*-elements that would play a role in mRNA stability. Most, if not all, mRNAs consist of a 5'UTR and a 3'UTR, so called because they do not get translated into protein.

In a search for elements that targeted mRNAs for rapid turnover, Caput observed that cytokine mRNAs contained AU-rich 3'UTRs (Caput et al., 1986). The 3'UTRs displayed greater evolutionary conservation than either the coding regions or the 5'UTRs, suggesting an important biological role for this region. Since then, AU-rich elements (ARE) have been found in the 3'UTRs of proto-oncogenes like *fos*, *myc*, *myb*, *rel* and *sis*, transcriptional activators like *jun* and *ets*, hematopoietic growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF and ET-1, lymphokines like the interleukins, cytokines like TNFα and IFNγ, neuropeptides like nerve growth factor, neurotensin, met- and leu-enkephalins, dynorphins A and B, cholecystokinin, and many others (Chen and Shyu, 1995). Other motifs or *cis*-elements, that are not AREs, have been discovered to regulate the fate of particular transcripts. These may include C- or CU-rich motifs (Weiss and Liebhaber, 1995), or short U stretches (You et al., 1992), or they may be sequences that are unique to the message(s) that they regulate.

Secondary structures, like stem-loops or hairpins within the mRNA can also affect the stability of the message. The histone mRNA is an example; it lacks a poly(A) tail but it contains a 3' terminal stem-loop that is involved in its regulation (Pandey and Marzluff, 1987). A stem-loop structure makes up the iron-responsive element (IRE), identified as the site of mRNA regulation in iron regulatory genes, where its nucleotide sequence is as important as its secondary structure (Klausner et al., 1993). A remarkable long-range stem-loop structure has been found in the 3'UTR of human, mouse and rat insulin-like growth factor-II (IGF-II) mRNAs to be both an endonucleolytic cleavage site and an mRNA stability determinant (Scheper et al., 1995). IGF-II is expressed primarily in fetal cells, but is also found in adult serum and probably plays a role in cell proliferation and differentiation.

The coding region of some genes also contains information needed to regulate the half-life of an mRNA. Two observations illustrate this: (i) Mutations in the coding region of mRNAs like *c-fos*, *c-myc*, and tubulin result in significant changes in mRNA half-life (Ross, 1995). (ii) The half-lives of *c-myc* and *c-fos* mRNAs lacking their 3'UTR, including the ARE, are only 1-2 hours, which is still relatively short compared with those of many other mRNAs (Bonnieu et al., 1990; Rahmsdorf et al., 1987). The truncated mRNAs must contain instability elements either in their 5'UTR or in the coding region. Sequences in the 5'UTR appear to play a role in the degradation of *c-myc* mRNA in some cells (Rabbitts et al., 1985).

We could classify regulatory mechanisms in terms of class I, where the binding of a factor to an mRNA prevents an initial endonucleolytic cleavage. In class II mechanisms, the binding of one or more factors targets a site on the mRNA for cleavage. In class III mechanisms, the shortening and removal of the poly(A) tail destabilize the mRNA. In class

IV mechanisms, the interaction of a protein with a component of the degradative system alters its activity or ability to recognize the mRNA as a substrate. Examples of all of these regulatory classes exist, as will be outlined below. Another mechanism, which has been termed nonsense-mediated decay (NMD), provides a pathway which monitors premature translation termination, i. e. mRNAs that contain premature STOP codons, or aberrant mRNAs whose 3'UTRs are abnormally extended due to a mutation in the polyadenylation site (Muhlrad and Parker, 1999). It involves a surveillance complex that searches 3' of a nonsense codon for a downstream sequence element (DSE) associated with RNA-binding proteins. Interaction between the complex and the DSE-binding protein triggers NMD (Gonzalez et al., 2000).

The process of translation has been implicated to play a role (sometimes a major one) in the destabilization of mRNA. Cycloheximide is widely used to stabilize labile mRNAs. Because of its ability to inhibit peptidyl transferase, this effect of cycloheximide implies that the destabilization of mRNA is associated with protein synthesis. All mRNAs in yeast are stabilized by cycloheximide (Herrick et al., 1990). Concordantly, the degradation of the mRNAs for histone, ß-tubulin, transferrin receptor, certain proto-oncogenes, and lymphokines is dramatically reduced in the presence of translational inhibitors (Sachs, 1993). These observations suggest that most mRNAs need to be translated to be degraded. The precise mechanism by which labile mRNAs are stabilized by cycloheximide remains to be elucidated. Apoptotic cells have an increased rate of RNA turnover and it is possible that RNA degradation could play a role in the progression to cell death. In the immune system, apoptosis is thought to be involved in the elimination of potentially destructive, self-reactive

immature lymphocytes and excess effector lymphocytes that are generated in primary immune responses (Krammer et al., 1994).

3.4. Regulatory *cis*-elements of mRNA:

3.4.1. The iron-responsive element (IRE):

In a well-defined class I regulatory mechanism, the mRNA encoding transferrin receptor, a protein responsible for importing iron into cells, is destabilized by high iron levels and stabilized when iron is scarce (Klausner et al., 1993). Located within its 3'UTR is a region containing five distinct stem-loops, which consist of a 23- to 27-bp stem and a 6-nucleotide loop, capable of binding the IRE-binding protein (IRE-BP), also called the iron regulatory protein (IRP). The binding of the IRP to the transferrin receptor mRNA stabilizes the message, most likely by preventing association of destabilizing factors to the instability elements, as yet uncharacterized, that must be an intrinsic part of the mRNA. What is known is that the dissociation of the IRE-IRP complex exposes the instability site(s), causing the transferrin receptor mRNA to be cleaved initially by an endonuclease (Binder et al., 1994). As opposed to the 3'UTR of the transferrin receptor mRNA, the 5'UTR of the transferrin mRNA harbors an IRE that is capable of binding the IRP when iron levels are low. That association has been found to promote the translation of the transferrin transcript (Cox et al., 1995).

One copy of the IRE exists near the 5' end of all ferritin mRNAs. Ferritin is a major intracellular iron storage protein. When intracellular stores of iron are low, the IRP binds to this IRE to repress translational activity, without affecting the level of the ferritin transcript

(Klausner et al., 1993). This is an example of how the same regulatory *cis*-acting element can have opposing effects. In low iron concentration, *cis-trans* interactions stabilize the transferrin receptor mRNA and enhance the translation of transferrin so that cells can absorb more iron by endocytosis of transferrin, while at the same time preventing the synthesis of more ferritin that would sequester iron. Obviously structural differences must exist between the IREs that influence their interaction with IRP and that direct their differing translational responses. Iron plays an essential role in many cellular functions, such as oxygen transport, mitochondrial energy metabolism, electron transport, deoxynucleotide synthesis, and detoxification. On the other hand, non-protein bound "free" iron is thought to catalyze the formation of highly reactive radicals that damage membranes and DNA (Hentze and Kuhn, 1996). Therefore the cells have to maintain iron homeostasis to ensure iron supply but to prevent accumulation of excess iron.

3.4.2. The AU-rich element (ARE):

The ARE has been identified as an instability element ever since it was found to be present in the mRNAs (the 3'UTR precisely) of rapidly degraded gene products (Caput et al., 1986) and ever since Shaw and Kamen demonstrated that if the ARE from the 3'UTR of an unstable mRNA, like that of the GM-CSF, was placed within the 3'UTR of a stable transcript, like the ß-globin mRNA with a half-life of over 17 hours, the chimeric transcript decayed within 30 minutes (Shaw and Kamen, 1986). The AUUUA pentamer has been implicated as being the major component of an ARE, although Zubiaga et al. have identified the nonamer UUAUUUAUU as being the key AU-rich sequence motif that mediates mRNA degradation

(Zubiaga et al., 1995). Additional ARE potency is achieved by combining multiple copies of this nonamer in a single mRNA 3' UTR. It is suggested that the UUAUUUAUU motif targets mRNA for rapid deadenylation as an early step in the mRNA decay process. IGF-binding protein (IGFBP-1) contains five AUUUA sequences in its 3'UTR and exhibits a half-life of approximately 2.8 hours. But the half-life of the AUUUA-free mRNA was found to increase to 26.6 hours (Gay and Babajko, 2000). Removal of nucleotides containing the AUUUA sequences from GM-CSF (Shaw and Kamen, 1986) and *c-fos* (Raymond et al., 1989) mRNAs significantly increased their stability.

GM-CSF mRNA is elevated in tumor cells, even though the ARE found in its 3'UTR confers lability to this message in normal cells (Ross et al., 1991). Interleukin-3 (IL-3) mRNA is stabilized in tumor cell lines via a mechanism which does not involve its ARE since no point mutations were found in the ARE (Hirsch et al., 1995), and since that same ARE deletion was able to stabilize IL-3 transcripts in untransformed cells (Stoecklin et al., 1994). There may exist one or more stability elements within those transcripts, in addition to the destabilizing ARE, which may participate in the regulation of RNA stability, depending on the environment within the cell. Obviously when the cell cycle is deregulated, as in cancer cells, different regulatory mechanisms come into play to affect a host of gene products.

Schuler and Cole have described a macrophage derived tumor cell line which selectively stabilizes GM-CSF but not *fos* or *myc* (Schuler and Cole, 1988), demonstrating that AU-rich mRNA turnover signals are recognized differentially in *trans* within the same cell. Lindstein et al. have shown that several AUUUA-containing mRNAs, including IL-2, IL-3, TNFα, IFNγ and GM-CSF, are stabilized in normal T-cells after activation by mitogenic

antibodies (anti-CD3 and anti-CD28), though neither *c-fos* nor *c-myc* were stabilized by the same antibodies (Lindstein et al., 1989). Thus stimuli received at the cell surface can alter gene expression by inducing specific changes in mRNA degradation. GM-CSF mRNA is stabilized in activated eosinophils by a mechanism involving interaction of AU-specific binding proteins (Ruth et al., 1999). There must exist multiple mRNA stabilization or destabilization pathways mediated by AUUUA sequences, each one practically tailored to the molecule it is supposed to regulate, rather than one common mechanism. Regulation of the mRNA via the ARE would then make use of either class I, II, III or even class IV regulatory mechanisms.

A potent ARE from the 3'UTR of *c-jun* proto-oncogene mRNA that does not contain the AUUUA motif has been described to direct rapid shortening of the poly(A) tail as a necessary first step for mRNA degradation (Peng et al., 1996). Polysome profile studies show that the destabilizing function of the *c-jun* non-AUUUA ARE does not require any active transit by ribosomes of the mRNA bearing it, further corroborating that the destabilizing function of AREs is not always tightly coupled to ongoing translation by ribosomes.

3.4.3. Non-ARE-mediated mRNA regulation:

As the post-transcriptional regulation of mRNA became recognized as being an important means of regulating gene expression, this area of research started exploding. As more research opened up this field, it became apparent that the ARE is not the only *cis*-element to play a role in the regulation of mRNA stability. Deletion of the AUUUA motifs from the 3' end of the *c-myc* mRNA failed to stabilize the resultant mRNA (Bonnieu et al., 1990). Kruys et al. failed to detect differences in the turnover of AU-positive and AU-

negative IFN-ε mRNAs in *Xenopus* oocytes (Kruys et al., 1989). Removal of the eleven AUUUA motifs present in the 3'UTR of luteinizing hormone receptor mRNA had no effect on message stability or receptor expression (Nair and Menon, 2000). This would suggest that there are additional *cis*-elements, present within these transcripts in their 3'UTR or elsewhere in their protein-coding region or 5'UTR, that modulate mRNA turnover.

A novel potent destabilizing element in the 3'UTR of the granulocyte colony-stimulating factor (G-CSF), IL-2 and IL-6 mRNAs has been identified as a stem-loop structure that functions independently of the ARE, also present in the 3'UTR of these transcripts (Brown et al., 1996). 261 bases of the m1 muscarinic acetylcholine receptor mRNA, that are important for conferring message destabilization, can form four stable stem-loop structures (Lee et al., 1994). Inverted repeats at the 3' end of plastid mRNAs function to stabilize the mRNAs (Stern et al., 1989).

The prolonged half-life of the α-globin message depends on the assembly of a sequence-specific 3'UTR RNP complex. The *cis*-determinant consists of polypyrimidine tracks, with the human transcript being C-rich, involving CCUCC motifs, while the mouse 3'UTR has both Cs and Us in an equal distribution, as in the CCUUCU motif (Wang and Liebhaber, 1996). The *trans*-factor is a complex composed of several proteins that interact with discontinuous segments of the α-globin 3'UTR. One of these proteins is a 39-kDa poly(C)-binding protein (PCBP); two other proteins (43-kDa and 42-kDa) are non-PCBPs. β-globin is the major protein co-expressed with α-globin in adult erythroid cells, but they do not share the same mechanism for transcript stabilization, as specified by the lack of sequence homology and the lack of C-rich segments in the β-globin 3'UTR (Russell et al., 1998).

Mammalian ribonucleotide reductase is composed of two dissimilar dimeric subunits, R1 and R2, which are encoded by different genes. Both proteins are required for the direct reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides, a rate-limiting step in the synthesis of DNA (Wright et al., 1990). Amara et al. have identified an 83-nucleotide element in the 3'UTR of the R2 mRNA that does not comprise of any known motif, but is responsive to $TGF\beta_1$ (Amara et al., 1993). Phorbol esters, like TPA, cause the elevation of R2 mRNA by inducing a protein that interacts with a 20 nt sequence in the 3'UTR, that is totally independent of the 83 nt segment that confers stability by $TGF\beta_1$ (Amara et al., 1994). TPA further abolishes binding activity of a 45-kDa protein to a 9 nt *cis* element 5'-UCGUGUGCU-3' in the 3'UTR of ribonucleotide reductase R2. Since TPA acts to prolong the half-life of the R2 transcript, the 9 nt sequence must normally function as a destabilizing element (Amara et al., 1996). Likewise TPA can stabilize R1 mRNA via a 49 nt *cis*-element in the R1 3'UTR (Chen et al., 1993). This provides evidence that other sequences, other than the ARE, can act as mRNA destabilizers.

The stability of the heat-stable antigen (HSA) mRNA is regulated by the interplay between two novel *cis*-elements in the 3'UTR. A 160-base element, located in the 3'UTR of HSA mRNA, promotes RNA degradation, and this effect is neutralized by a 43-base fragment located 1 Kb upstream of the negative *cis*-element (Zhou et al., 1998). The presence of positive and negative *cis* elements within the 3UTR of mRNAs suggests that the expression of the proteins they encode for can be regulated by selectively activating or silencing the putative *trans* factors that act on the *cis* elements.

3.4.4. mRNA 3' terminal stem-loop interaction:

Histone mRNA is not polyadenylated, but instead consists of a highly conserved 3' terminal stem-loop structure, with a 6-base stem and a 4-base loop made up of a consensus sequence UYUN. In the presence of DNA synthesis, histone mRNAs are plentiful in the cell, especially during cell division. At the end of the cell cycle and in the presence of DNA synthesis inhibitors, the histone mRNAs get destabilized by a process of autoregulation (Peltz and Ross, 1987). Histone mRNA translation continues after DNA synthesis stops, generating an excess of free histones having no newly synthesized DNA substrate with which to interact. The free histone monomers accumulating in the cytoplasm bind to the 3' terminal stem and loop structure, an interaction which promotes cleavage of the histone mRNA by ribosome-associated nucleases. It is also possible that their interaction with the histone mRNA might affect other proteins that are bound to the stem-loop region. There is evidence that a 45-kDa (Hanson et al., 1996) or a 31-kDa (Whitfield et al., 2000) stem-loop-binding protein (SLBP) binds to the 3' terminus of histone mRNA.

3.4.5. Determinants in the coding region:

Tubulin mRNA also exhibits a form of autoregulation, which involves the beginning of its open reading frame (ORF). Under conditions of tubulin monomer excess, the mRNA becomes destabilized. The first 13 translated nucleotides, that form the N-terminal tetrapeptide, Met-Arg-Glu-Ile, provides the signal to target rapid degradation of that mRNA, while the transcript is still attached to the ribosome and is undergoing translation. This domain is functionally conserved in mouse, human, chicken and yeast .β-tubulin mRNA (Yen et al.,

1988). The lutropin receptor mRNA harbors a regulatory sequence in its ORF, consisting of a bipartite polypyrimidine sequence 5'-UCUC-X_7-UCUCCCU-3', which is sensitive to hormonal regulation (Kash and Menon, 1999). Thymidylate synthase (TS) functions as an RNA-binding protein by interacting with two different sequences on its own mRNA. One site is located in the 5'-upstream region of human TS mRNA while the second site is a 70 nt sequence located within the protein-coding region (Lin et al., 2000). Dihydrofolate reductase protein inhibits its own translation by binding to its mRNA sequences within the coding region. It recognizes a 100-base region of the RNA that contains two putative stem-loop structures (Ercikan-Abali et al., 1997).

Shyu identified the existence of destabilizing sequences within the ORF of *c-fos* mRNA (Shyu et al., 1989) thereby demonstrating that two distinct cellular pathways for rapid *c-fos* mRNA degradation can exist within the same transcript. Each of these pathways recognizes a different, functionally independent instability determinant within the *c-fos* transcript. One instability determinant, located within the 3'UTR, is a 75-nucleotide ARE. Specific deletion of this ARE from *c-fos* mRNA has little effect on the transcript's cytoplasmic half-life due to the presence of the other instability determinant within the protein-coding region. Examination of mRNA decay in cells treated with transcription inhibitors indicates that one *c-fos* mRNA degradation pathway is dependent on RNA synthesis, whereas the other is not (Shyu et al., 1989).

Regulation of the intercellular adhesion molecule (ICAM-1) gene expression involves multiple mRNA stabilization mechanisms (Ohh et al., 1994). ICAM-1 is constitutively expressed at a low level on a subpopulation of hematopoietic cells,on vascular endothelium,

on fibroblasts and on certain epithelial cells, but it is dramatically increased at sites of inflammation. IFNγ and phorbol myristate acetate (PMA) are known to increase the expression of ICAM-1 on many cell types. IFNγ achieves this by stabilizing the otherwise labile ICAM-1 mRNA; the IFNγ-responsive sequence may reside within the protein coding region (Ohh and Takei, 1994). However, the PMA-responsive element is located within the ICAM-1 3'UTR, involving AUUUA multimers (Ohh et al., 1994).

3.5. RNA-binding proteins:

RNA-binding proteins play key roles in the post-transcriptional regulation of gene expression, whereby protein-RNA interactions regulate the stability and translability of the transcripts in the cytoplasm. They partially constitute the ribonucleoprotein or protein machinery required for RNA processing (splicing, polyadenylation and 3' end formation), transport, anchorage and storage. Many RNA-binding proteins have been identified in the past as being structural proteins, as in the case with proteins that make up the ribosomal subunits. As far back as the 70's though, a protein component was isolated from rat liver and Zajdela hepatoma ascites cell nuclei, exhibiting molecular weights of 37-kDa and 40-kDa respectively in SDS gel electrophoresis, which possessed high affinity for hnRNA, polyuridylate (poly(U)), polyadenylate (poly(A)), and double-stranded DNA (Schweiger and Kostka g, 1977). Elongation factors as well as translation initiation factors were being recognized as RNA-binding proteins (Ovchinnikov et al., 1978; Vlasik et al., 1978).

Messenger ribonucleoproteins (mRNPs), first discovered in 1964 as free mRNA-containing particles of fish embryo cytoplasm and designated as informosomes (Spirin, 1979),

proved to have a universal occurrence in eukaryotic cells. It is now widely accepted that the mRNA in eukaryotic cells at different stages of its life time carries on itself the proteins which are required for its own biogenesis, processing and transport (nuclear informosomes), for its existence in a temporarily inactive state (free cytoplasmic informosomes) and for its functioning as a template (polyribosomal informosomes). Transport from the nucleus into the cytoplasm as well as the transition from the free non-translatable state into the polyribosome-bound translatable state must likely be accompanied by essential changes in the protein moiety of the mRNPs (Spirin, 1979). Specific RNA-binding proteins may play a role in determining the subcellular localization of specific mRNPs since both free and polysomal mRNPs may be associated with the cytoskeletal framework (Larson and Sells, 1987).

3.5.1. Nuclear RNA-binding proteins:

Pre-mRNA processing in eukaryotes is thought to take place on a multitude of nuclear RNP complexes, the most abundant of them being the hnRNP complexes. In the eukaryotic nucleus, hnRNPs comprise of 24 polypeptides in the range of 30- to 120-kDa, among them the abundant 30- to 40-kDa proteins, A, B and C (Pinol-Roma et al., 1988). Intron definition and splice site selection occur at an early stage during assembly of the spliceosome, the complex mediating pre-mRNA splicing. Association of small nuclear (sn)RNP with the pre-mRNA is required for these steps (Puig et al., 1999). Five snRNP particles, U1, U2, U4, U5 and U6, play a major role in the spliceosome, though other U snRNPs have been identified. They are each composed of a small nuclear RNA and five common core polypeptides. The production of correctly spliced mRNA requires cleavage at the 5' splice site of the intron

followed by cleavage of the 3' splice site and exon ligation (Pikielny and Rosbash, 1986). A 62-kDa protein binds specifically to the polypyrimidine tract, hence the name polypyrimidine tract binding (PTB) protein, of the 3' splice site region of introns (Garcia-Blanco et al., 1989). The PTB protein has also been identified as the 57-kDa hnRNP I (Perez et al., 1997). Nuclear proteins are subject to post-translational modifications that can affect their binding to RNA. hnRNP C1 undergoes a cycle of phosphorylation-dephosphorylation that modulates its binding to pre-mRNA (Fung et al., 1997). The presence of an RNA-dependent kinase is therefore speculated.

3.5.2. 5'UTR-binding proteins:

Protein binding sites are present on the 5'UTR of many transcripts. It is believed that they represent a general mechanism for the translational regulation of mRNAs in cells. Many ribosomal and viral RNAs contain a polypyrimidine tract in their 5'UTR and this has been shown to interact with cellular proteins, such as the PTB protein, the poly(C) binding protein (PCBP), the La autoantigen that binds most RNA polymerase III transcripts, as well as other uncharacterized proteins of different molecular weights. The binding of these proteins have been linked to the process of translation initiation (Rojas-Eisenring et al., 1995). A mammalian cellular 57-kDa protein binds specifically to the internal translation initiation site in the 5'UTR of foot-and-mouth disease virus RNA, again to a pyrimidine-rich region (Luz and Beck, 1991), as well as to the 5'UTR of other picornaviruses. A high affinity binding site for the PTB protein is located on the 5'UTR of the rat proteinase inhibitor. That same site can function as a positive *cis*-element in a heterologous promoter (Sickinger and Schweizer,

1999).

Other proteins, of molecular masses 25-, 40-, 46-, 58-, 69-, 97-, 110- and 160-kDa, have been found to bind to a region of the 5'UTR of preproinsulin mRNA that is highly conserved between species and that may be associated with a potential stem-loop structure (Knight and Docherty, 1992). Binding of these proteins may result in the stabilization of the transcript. A 66-kDa polysomal protein binds the 5'UTR of the chicken vitellogenin II mRNA. It is liver-specific and believed to be involved in the estrogen-mediated stabilization of this message (Liang and Jost, 1991). A 55-kDa protein present in HeLa and MEL cells, which binds specifically to RNA derived from the first exon of the human *c-myc* gene, i. e. the 5'UTR, has been identified in both cytoplasmic and nuclear fractions. The binding site on the RNA is a purine rich region (Parkin and Sonenberg, 1989).

3.5.3. Stem-loop binding proteins (SLBPs):

The most well-known SLBPs are the ones that bind the 3' end of histone mRNA. Two proteins from calf thymus, of molecular weights 40- and 43-kDa, have been found to bind histone pre-mRNA in a sequence-specific manner (Schaller et al., 1997). The 45-kDa histone SLBP described by Hanson et al. (Section 3.5.4) is found to be distributed mainly in the polysomes (90%) as opposed to the nuclei (10%), whereas the 31-kDa SLBP described by Whitfied et al. (Section 3.5.4) is necessary for pre-mRNA processing; it accompanies the histone mRNA to the cytoplasm, where it is a component of the histone mRNP. When the highly conserved 3' UYUN loop is mutated, so that the first and third bases are changed to purines, binding of the SLBP is abolished as well as histone expression (Pandey et al., 1994).

It is quite likely that binding of the SLBP stabilizes the histone transcript, and that free histones might free this association, causing the mRNA to be degraded. In summary, the SLBP, the histone proteins, the 3' –> 5' exoribonuclease, and perhaps other factors appear to interact so as to autoregulate histone mRNA stability, guarding against untimely and inappropriate histone protein production. The amount of SLBP itself is regulated during the cell cycle at the level of translation as cells progress from G(1) to S phase, although SLBP mRNA levels remain constant during the cell cycle (Whitfield et al., 2000). Regulation of SLBP may account for the post-transcriptional component of the cell cycle regulation of histone mRNA.

Another well characterized SLBP is the IRP that recognizes the IRE as a sequence/structure motif. Alteration of the sequence of the loop or disruption of the base-pairing in the upper stem affects the affinity of the interaction (Bettany et al., 1992). To date, two closely related IRPs (IRP-1 and IRP-2) have been identified as 90- and 100-kDa proteins. Both display IRE-binding under conditions of iron deprivation, but become post-translationally inactivated (IRP-1) or degraded (IRP-2) when the iron supply to cells is increased (Henderson, 1996). Further differences arise in their expression and RNA-binding specificity. The two proteins each recognize a large repertoire of IRE-like sequences, including a small group of exclusive RNA targets (Henderson et al., 1996). IRP-1 is found ubiquitously in all vertebrate tissues and is a cytosolic aconitase, an iron-sulfur protein that interconverts citrate and isocitrate, when it is not bound to RNA (Klausner and Rouault, 1993). Iron itself regulates the function of this enzyme; ligation of the [4Fe-4S] cluster to three cysteine residues will inactivate its RNA-binding function while activating its enzyme

function (Philpott et al., 1994). Not only iron deficiency, but NO can also disassemble the iron sulfur cluster and therefore activate the IRE-binding function of IRP-1 (Weiss et al., 1993).

Chloroplast proteins bind to the 3'UTR of spinach chloroplast petD mRNA, a 33-kDa protein to a hairpin structure in the 3' inverted repeat sequence and a 57-kDa protein to an AU-rich sequence motif that is highly conserved in petD genes of higher plant species (Chen and Stern, 1991). Two *cis*-acting elements in the 3'UTR of IGF-II mRNA interact to form a stable long-range RNA-RNA stem structure, even if they are 2 Kb apart. A cytoplasmic 48- to 50-kDa protein has been identified as the IGF-II cleavage unit binding protein (ICU-BP) since it binds to the stem structure and cleaves the mRNA into an unstable 5' cleavage product containing the IGF-II coding region and a very stable 3' cleavage product of 1.8 kb (Scheper et al., 1996). This endonucleolytic cleavage is most probably the first and rate-limiting step in degradation of IGF-II mRNAs (Scheper et al., 1996). A 69-kDa protein forms redox-sensitive complexes with a 240 nt region of the 3'UTR of catalase mRNA. Two elements are present within this region, a 36-base element that has a computer-predicted stem-loop secondary structure and a CA dinucleotide repeat. Both elements are required for specific binding (Clerch, 1995).

3.5.4. AU-binding proteins:

Many AU-binding proteins have been identified over the past ten years. Malter identified a cytosolic AU-binding factor (AUBF) that binds specifically to synthetic RNA molecules containing four reiterations of the AUUUA motif (Malter, 1989). He observed it to form a 45-kDa RNP complex by RNA mobility shift assay, but the use of reducing agents

separated the complex into 15-, 17- and 19-kDa proteins. AUBF has been shown to bind GM-CSF, IFN-ε, IL-3, *c-fos* and *v-myc* RNAs *in vitro* (Gillis and Malter, 1991). Binding activity can be reversibly blocked by oxidizers like diamide, but irreversibly inhibited by methlymaleimide, suggesting that AUBF contains a redox switch (Malter and Hong, 1991).. AUBF binding activity is increased by TPA and/or calcium ionophore within 30 minutes, a rapid response following treatment, indicating that AUBF is most likely subject to post-translational modification(s) rather than up-regulation of transcription or translation. Indeed AUBF binding activity is abolished by potato acid phosphatase, providing evidence that AUBF binds to RNA as a phosphoprotein (Malter and Hong, 1991). Since cytokine mRNAs are stabilized by TPA and/or calcium, it is likely that modification of AUBF in the cytosol causes it to bind to AU-containing mRNAs to protect them from degradation by nucleases. Thus an AU-binding protein is capable of acting as a stabilizing agent.

Brewer discovered another AU-binding factor which he called AUF, which appears distinct from AUBF in molecular weight and biochemical properties (Brewer, 1991). AUF binds to the AU-rich region of the proto-oncogene *c-myc* mRNA to destabilize the message. Amazingly, proteinase K treatment of the factor can abolish its *c-myc* mRNA degradation activity, but not its RNA-binding activity, suggesting that RNA binding and RNA degradation are separable functions. Many AU-binding factors may bind to the ARE of one transcript to modulate its turnover. Five major proteins have been discovered to bind the 3'UTR of GM-CSF mRNA. They have been identified as being components of hnRNP C and AUF1, both nuclear and cytoplasmic protein complexes, that may be involved in the rapid degradation of that transcript (Nakamaki et al., 1995). AUF1, consisting of a 37-kDa and a 40-kDa isoform,

plays a major role in ARE-directed mRNA decay that is based upon its affinity for different AREs and their potency as mRNA destabilizers (DeMaria and Brewer, 1996). hnRNP D, capable of shuttling between the nucleus and cytoplasm, has been identified as an RNA destabilizing protein *in vivo* in ARE-mediated rapid mRNA decay (Loflin et al., 1999).

Post-translational modification of RNA-binding proteins play a big role in their interaction with nucleic acids. hnRNP A1, an AU-binding protein, shuttles between the cytoplasm and nucleus and plays important roles in RNA metabolism. Nuclear hnRNP A1 binds intronic sequences and modulates splicing, whereas cytoplasmic hnRNP A1 associates with polyadenylated RNA, especially with AREs to modulate mRNA turnover and translation. Its AU-binding activity correlates with serine/threonine dephosphorylation (Hamilton et al., 1997). Thus one protein can serve distinct roles in post-transcriptional regulation of gene expression in both the nucleus and cytoplasm.

Lindsten's group identified three AU-specific mRNA binding proteins, which they termed AU-A, AU-B and AU-C (Bohjanen et al., 1992). AU-A is an abundant, constitutively expressed 34-kDa factor that localizes primarily to the nucleus. It binds to AUUUA multimers with low relative affinity and also binds to other U-rich sequences. AU-A has also been found to be a candidate protein component of RNP complexes that participate in nucleocytoplasmic transport of mRNA and cytoplasmic mRNA metabolism, similar to hnRNP A1. However, monoclonal antibodies to hnRNP A1 and protease digestion patterns show that AU-A and hnRNP A1 are two distinct proteins (Katz et al., 1994). AU-B and AU-C are 30- and 43-kDa cytoplasmic factors that are induced following T cell receptor-mediated stimulation and bind to AUUUA multimers with high affinity, although they require three or more tandem

AUUUA repeats for efficient binding. PMA, known to increase lymphokine mRNA stability, inhibits the induction of AU-B, providing a correlation between the binding of this protein and increased mRNA turnover. Therefore AU-B and AU-C are cytoplasmic regulators of lymphokine mRNA metabolism.

In unstimulated monocyte/macrophage cells, TNFα mRNA is translationally repressed. However, upon stimulation of the cells with various agents (e. g. lipopolysaccharides and viruses), this repression is overcome and translation occurs. The key element in this regulation is the ARE present in the 3'UTR of TNFα mRNA, which is capable of binding two cytosolic proteins. The formation of the LPS-inducible complex, involving a 55-kDa protein, requires a minimal sequence corresponding to the nonamer UUAUUUAUU (Lewis et al., 1998). Whereas a 35-kDa protein binds to a longer segment of TNFα 3'UTR containing seven AUUUA pentamers in tandem (Kim et al., 1996). In unstimulated macrophages, protein binding to the ARE of the TNFα 3'UTR is still observed as three different complexes that form in the cytosol as well as in the nucleus. A fourth complex still forms on the 3'UTR, but is independent of the ARE (Hel et al., 1996). Six macrophage proteins make up these four complexes. It is possible that the binding of those factors may act to modulate the level of the cytokine in the cell, such as to keep it at a low basal level until needed.

Vakalopoulou has shown the existence of a 32-kDa protein which resides principally in the nucleus, whose activity inversely correlates with labile mRNA stability. A single AUUUA sequence in a U-rich context was sufficient for the binding of this protein. Insertion of three copies of this minimal recognition site led to markedly reduced accumulation of β-globin RNA, while the same insert carrying a series of U-to-G changes had little effect on

RNA levels (Vakalopoulou et al., 1991). Parathyroid hormone (PTH) mRNA levels are post-transcriptionally increased by hypocalcemia and decreased by hypophosphatemia, and this is mediated by cytosolic proteins binding to the PTH mRNA 3'UTR. A stabilizing 50-kDa protein that bound the PTH 3'UTR was isolated, sequenced and found to be identical to an AU-rich binding protein, AUF1 (Sela-Brown et al., 2000).

Hypoxia potently induces VEGF expression in several tumor cell lines *in vitro*. This is mediated by both increased transcription and mRNA stability, which likely occurs via a 125-nt ARE in the VEGF 3'UTR. A series of hypoxia-inducible proteins of 90/88-, 72-, 60-, 56-, 46- and 34-kDa bind to this hypoxia stability element to act as mRNA stabilizers (Claffey et al., 1998). The protein associated in the 60-kDa complex is hnRNP L, which binds a 21-base sequence found only in the human and bovine VEGF ARE defined as 5'-CACCCACCCACAUACAUACAU-3' (Shih and Claffey, 1999) that resides at the 5' end of the 125 nt ARE. The 34-kDa protein has been identified as HuR, a member of the Elav family of RNA-binding proteins (described below) that are required for neuronal differentiation. HuR is transcribed in all cells and its total steady-state level is usually unchanged. Therefore a post-translational modification, such as phosphorylation, may regulate its RNA-binding activity (Levy et al., 1998). It is possible that HuR binding alters the structure of the ARE and renders the site either inaccessible to AU-cleaving endonucleases (described earlier) or unable to promote deadenylation.

The embryonic lethal abnormal visual (ELAV) family of AU-rich RNA-binding proteins, first identified in Drososphila (Robinow and White, 1991), plays an important role in neuronal differentiation and maintenance and is highly conserved in vertebrates. In humans,

there are four members; HuR found in all proliferating cells, whereas Hel-N1, HuC and HuD are expressed in terminally differentiated neurons (Akamatsu et al., 1999). Elevation of cytoplasmic HuR levels inhibits *c-fos* ARE-mediated RNA decay but has little effect on rapid decay directed by *c-jun* ARE. HuR can be induced to redistribute from the nucleus to the cytoplasm and this translocation is associated with an altered function. HuR may initially bind to ARE-containing mRNAs in the nucleus and provide protection during and after their export to the cytoplasmic compartment, thus acting as a chaperone. Modulation of the ARE-mediated decay pathway through controlling distribution of the ELAV proteins between nucleus and cytoplasm may be a mechanism by which cell growth and differentiation is regulated (Peng et al., 1998). HuD binds to AREs in 3'UTRs, requiring a minimum of three $(AUUU)_n$ repeats to bind strongly to RNA (Park et al., 2000).

Thus several proteins are possible participants in the regulation of labile mRNA turnover through an interaction with the AU-rich domain. There are as many AU-binding proteins that bind mRNAs to target them for rapid degradation as there are that bind the AREs to stabilize the transcripts. A single AU-binding protein may bind two different mRNAs to give rise to two different outcomes. Recognition might be through primary sequence, tertiary folding, or a combination of both. Their relative affinities for the AU-region, or even protein-protein interactions, may determine the ultimate stability of the mRNA. Differential degradation/stabilization of AUUUA-containing mRNAs may occur through a complex interplay between cytosolic proteins interacting directly with the mRNA and the nucleases that digest it.

3.5.5. U-rich binding proteins (URBPs):

In all eukaryotic nuclei, the La autoantigen binds the U-rich termini of nascent RNA polymerase III transcripts, stabilizing these RNAs against exonucleases. The highly conserved La phosphoprotein is thus a URBP that acts as a chaperone (Xue et al., 2000). Three chloroplast proteins, of 100-, 32-, and 28-kDa, display little sequence or structural binding specificity apart from their preference for U-rich sequences in the 3'UTR of chloroplast mRNAs (Chen and Stern, 1991). Protein kinase A stimulates binding of multiple cytoplasmic proteins to a U-rich domain in the 3'UTR of lactate dehydrogenase (LDH) A mRNA, that is required for the regulation of mRNA stability. The URBPs, that include 50-, 52-, 67- and 96-kDa proteins, lose binding activity when they are dephosphorylated, thus specifying a pathway by which cyclic AMP can induce the stabilization of LDH A (Tian et al., 1998). URBPs of molecular weights 37-, 66-, 71- and 82-kDa, bind a 20 nt U-rich sequence at the 3' end of the *c-fos* 3'UTR to destabilize that mRNA, an association that is distinct and separate from the destabilizing function of the 75 nt ARE rich in AUUUA pentamers found more at the 5' end of the *c-fos* 3'UTR. These four URBPs can form three complexes with the RNA, which consists of three U stretches (5, 4 and 7 in length) separated by single A residues (You et al., 1992). Hel-N1, the human neuron-specific RNA-binding protein, prefers to bind RNAs containing short U stretches similar to those found in the 3'UTRs of oncoprotein and cytokine mRNAs, such as *c-myc*, *c-fos*, and GM-CSF (Levine et al., 1993). HuD, another neuron-specific RNA binding protein, binds a U-rich tract in the 3'UTR of p21, a protein involved in the regulation of the cell cycle (Joseph et al., 1998).

3.5.6. Poly(C) binding proteins (PCBPs):

The most characterized PCBPs are the cytoplasmic proteins that form part of the α-complex that binds the α-globin message, i. e. αCP-1 and αCP-2. The human PCBP cannot bind by itself to the α-globin mRNA, suggesting that protein-protein interactions play a role in the regulation of this mRNA (Wang et al., 1995). The mouse PCBP is expressed ubiquitously, but remains predominantly in the nucleus. It can interact with proteins involved in spliceosomes, for example splicing factor 9G8 and hnRNP L, as well as with cytoskeletal proteins, such as filamin (Funke et al., 1996). αCP can bind to a C-rich sequence in the 3'UTR of collagen α1 to stabilize that mRNA, while a cytoplasmic protein complex can bind to the collagen 5' stem-loop to destabilize the same mRNA (Stefanovic et al., 1999). Since poly(rC) can effectively compete with the lutropin receptor binding protein 1 (LRBP-1) for binding to the lutropin receptor mRNA, then LRBP-1 must be a PCBP. Two PCBPs have been discovered to be acidic hnRNPs, namely a 66-kDa hnRNP K and a 64-kDa hnRNP J. They are located in the nucleoplasm and may play a role in the nuclear metabolism of pre-mRNAs that possess cytidine-rich sequences (Matunis et al., 1992).

PCBP-1 and PCBP- 2 are involved in both stabilization and translational regulation of several cellular and viral RNAs. They interact with the 5' element known as the cloverleaf structure and the large stem-loop IV RNA of the poliovirus 5'UTR (Silvera et al., 1999). They also interact with the hepatitis C virus 5'UTR, once the virus is intracellular. The complete internal ribosome entry site is necessary for efficient binding, suggesting that maintenance of the secondary structure is necessary for recognition of the binding site by the PCBPs (Spangberg and Schwartz, 1999).

3.5.7. RNA-binding proteins and mRNA localization:

mRNA localization plays an important role in directing specific proteins to their correct position within a cell. The molecular mechanisms responsible for these different localization pathways are still largely obscure. mRNA localization is likely to be tightly coupled to translational control. If it is important for a cell to synthesize a protein in a particular place, then the translation of the mRNA must be repressed until it is localized. Indeed, there are already several examples where the direct linkage between translational control and localization has been demonstrated (Curtis et al., 1995).

Annexin II, a 36-kDa protein, binds to mRNAs found in cytoskeleton-associated polysomes (Vedeler and Hollas, 2000). Mammalian staufen, a double-stranded RNA binding protein involved in mRNA transport, can bind tubulin and is localized to the rough endoplasmic reticulum (Wickham et al., 1999), thus implying that staufen can cross-link cytoskeletal and RNA components. These associations may represent pathways by which mRNAs may be targeted to particular subcellular sites. Vimentin mRNA has been shown to be localized to the perinuclear region of the cytoplasm, possibly at sites of filament assembly. The 3'UTR of vimentin mRNA harbors a sequence 61-114 nt downstream of the STOP codon, highly conserved from Xenopus to man, that binds a 46-kDa protein, an interaction that may be important for the localization of this mRNA (Zehner et al., 1997). For most localized mRNAs, the *cis*-acting sequences required for localization reside in their 3'UTRs (St Johnston, 1995). GAPDH, a 36-kDa polypeptide, can bind rather unspecifically different RNAs and can form loose dynamic complexes with polyribosomes. Association of such a kind may be used for compartmentalization of glycolysis near polyribosomes (Ryazanov, 1985).

Not only would mRNA localization direct a transcript to its appropriate location within the cell, but it would prevent the expression of mRNAs in regions where they are not required. For example, myelin basic protein (MBP), a component of the myelin sheath that oligodendrocytes wrap around axons, is an intracellular protein that interacts very strongly with membranes. It would be very difficult to transport MBP from the cell body to the sites of myelin formation, since it would stick to any membrane that it would come into contact with along the way. Therefore the localization of the mRNA would prevent the protein from compacting membranes in the main body of the cell (Trapp et al., 1987). The same principle might be applied to cells which synthesize different isoforms of one protein that multimerizes. For example, β-actin mRNA localizes to the leading lamellae at the cell periphery, while α- and γ-actin transcripts show a perinuclear distribution (Hill and Gunning, 1993). This is important in controlling the composition of actin filaments in differentiating myoblasts.

3.5.8. Regulation of eNOS mRNA stability:

Over the last three years, data has been published concerning the regulation of eNOS mRNA stability by various factors. Some reports have explored potential mechanisms, while others have not. The mechanism of down-regulation of both the bovine and human eNOS mRNA by TNFα has been explored not only by our group but by others. López-Farré's group in Spain have identified a 60-kDa protein that binds to a sequence located in the middle part of the bovine eNOS 3'UTR, comprising a CU-rich element in the 129 end nucleotides of the 5' half of the 3'UTR: GAUUCCCGUACUAUCUCAUCCUCUCAUCUCUAGGUCUG. Binding of the 60-kDa protein was enhanced in the presence of TNFα, which corresponded

to decreased eNOS mRNA levels (Sanchez et al., 1999). The same group has just recently reported that a similar mechanism may be operational in human peritoneal tissue (Arriero et al., 2000). They chose to look at peritoneum as a source of human tissue because the samples were easy to obtain from hernia sacs that were removed during surgical hernia repairs and because the epithelial lining of the peritoneum share similar functions with the endothelium of vessels, in that they provide a nonadhesive surface as well as gating the traffic of molecules and cells between the circulation and body compartments. Moreover they both express eNOS. In this report, the effect of LPS on the half-life of the eNOS message was investigated. They attributed the down-regulation of the eNOS mRNA to the fact that LPS stimulated the production of TNFα by macrophages, and that TNFα then increased binding of the 60-kDa protein to the eNOS 3'UTR. They do not show or explore where the protein might bind on the human eNOS 3'UTR; it is left to the individual to infer and interpret from their previous work with bovine ECs (Arriero et al., 2000).

David Harrison's group at Emory University identified a 99-kDa protein in the nucleus and cytoplasm, a 73-kDa nuclear protein, and a 63-kDa cytoplasmic protein in human aortic endothelial cells that bound to the 3'UTR of eNOS mRNA. They found that binding of the 63-kDa protein was increased in TNFα-treated cells in a dose- and time-dependent manner, whereas binding of the 73- and 99-kDa proteins were not altered (Miwa et al., 1999). Binding of the 63-kDa protein was localized to the middle 146 nt region of the eNOS 3'UTR and was thought to convey mRNA instability. It is possible that the two eNOS-binding proteins described above may be the same protein, identified with slightly different molecular weights, but most likely binding to the same region of the eNOS 3'UTR. In contrast to TNFα,

mevastatin, a 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor, decreased binding of the 63-kDa protein in a dose- and time-dependent manner (Miwa et al., 1999). The same group found a 43 nt sequence at the beginning or the proximal end of the bovine eNOS 3'UTR to be responsible for directing the decay of the eNOS mRNA in confluent growth-arrested bovine aortic endothelial cells. Using UV-cross-linking studies, they identified a 51-kDa protein that bound to this sequence (Searles et al., 1999).

VEGF, secreted by vascular cells and a variety of tumor cells, is a potent angiogenic factor and NO is believed to play a role in the VEGF-induced proliferation of endothelial cells. VEGF was found to increase eNOS mRNA and protein levels, resulting in a maintained increase in NO formation (Bouloumie et al., 1999). It achieved that by stabilizing the eNOS mRNA, which remained elevated in VEGF-treated cells in the presence of actinomycin D. The VEGF-induced increase in eNOS mRNA levels was abolished by tyrosine kinase inhibitors suggesting involvement of a tyrosine kinase-dependent pathway (Bouloumie et al., 1999). Other factors, like oxidized LDL, glucocorticoids and hypoxia, also lead to the down-regulation of eNOS by a post-transcriptional pathway, involving the destabilization of the eNOS message, as mentioned in Section 1.10, but the precise mechanism has not been defined. The antifungal drug, amphotericin B, has been found to alter the stability of the eNOS mRNA by an unknown mechanism (Suschek et al., 2000) but its therapeutic use is limited due to severe side effects, such as the risk of renal failure.

The most recent publication suggests that HUVECs exposed to TNFα can elicit the rapid phosphorylation of Akt/PKB (Murao et al., 2000), but this group was investigating the effect of TNFα stimulation on the chemokine monocyte chemotactic protein-1 (MCP-1)

expression. Chemokines are involved in leukocyte migration, angiogenesis and tumor growth (Salcedo et al., 2000) and therefore may play major roles in atherosclerosis (Shi et al., 2000). Whether the effect of TNFα on eNOS mRNA destabilization is mediated via Akt/PKB is not known. It is possible that Akt activation may in turn phosphorylate eNOS RNA-binding proteins that liaise with the 3'UTR, or it is entirely possible that TNFα does not utilize the Akt-mediated pathway to effect mRNA destabilization. The most plausible explanation may be that, while TNFα causes the destabilization of eNOS mRNA, its activation of Akt/PKB would stimulate the phosphorylation of the eNOS enzyme on serine 1177 (Mitchell et al., 1999) thus activating it, as mentioned in Section 1.10, explaining why there is increased NO production in the face of falling enzyme (Rosenkranz-Weiss et al., 1994).

3.6. Conserved structures of RNA binding proteins:

Some RNA-binding proteins harbor conserved structures that allow the RNA molecule to "dock" onto the protein or vice versa, whilst others adopt other configurations that allow them to bind to specific messages. Some common features that have been identified are listed below.

3.6.1. RNA recognition motif (RRM) or RNP consensus motif:

The RNA recognition motif (RRM) or RNP consensus motif is composed of 90-100 amino acids which form an RNA binding domain. It is most likely an ancient protein structure that has been conserved throughout evolution, since animal, plant, fungal and bacterial cells contain proteins that harbor RRMs in nearly all organelles where RNA is present (Burd and

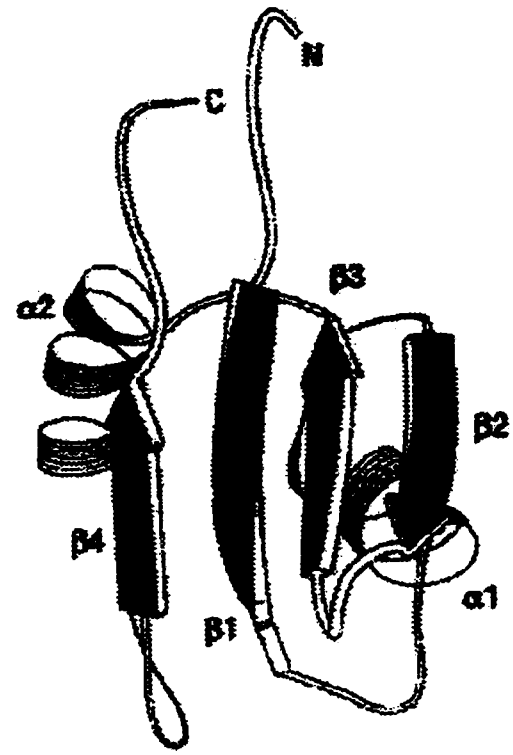


Figure 3.1 Structure of the RNP motif or RNA recognition motif (RRM) of the RNA-binding domain of hnRNP C. Each arrow represents a ß strand and the curled ribbons an α helix. The highly conserved RNP1 and RNP2 consensus sequences, which participate in making contact with the RNA , are juxtaposed on the central ß3 and ß1 strands respectively. N, NH_2-terminus; C, COOH-terminus.

Dreyfuss, 1994). The hnRNPs, PABP, some snRNPs and many RNA-binding proteins share this motif. The RRM is composed of two short sequences, RNP1 and RNP2, and a number of conserved hydrophobic amino acids interspersed throughput the motif, which translate into four antiparallel ß-sheets that form a barrel packed against two perpendicularly oriented α-helices (Figure 3.1). Amino acids of RNP1 and RNP2 are juxtaposed on the two central β strands (β1 and β3) of the folded domain. The charged and aromatic side chains of RNP1 and RNP2 are solvent exposed and make direct contact with bound RNA, probably through hydrogen bonds (Burd and Dreyfuss, 1994). The exposed ß sheet RNA binding surface engages the RNA in an open platform rather than burying it inside a binding crevice. Bound RNA remains potentially accessible for interaction with other RNA sequences or RNA binding proteins. This might be especially relevant when an RNA binding protein may bind to the mRNA to chaperone it and prevent it from degradation, while another RNA binding protein may bind specifically to the mRNA species to effect a totally separate function.

3.6.2. Arginine-rich motif (ARM) and RGG box:

Arginine-rich domains or motifs (ARMs) are used by a variety of RNA-binding proteins to recognize specific RNA hairpins (Tan and Frankel, 1995). The ARM is usually 10-20 amino acids in length and is found in viral, bacteriophage, and ribosomal proteins. The positive charges of the arginine residues may increase the non-specific affinity for RNA, facilitating the search for high-affinity binding sites within the proteins. Serine/arginine-rich proteins are essential for pre-mRNA splicing in metazoans (Graveley and Maniatis, 1998). A 70-kDa protein of U1 snRNP is rich in arginine (20%) and acidic amino acids (18%)

(Theissen et al., 1986). The amino acid sequence for hnRNP G, a glycosylated nuclear RNA-binding protein, shows that it contains one RRM at the amino terminus and a carboxyl domain rich in serines, arginines and glycines (Soulard et al., 1993). Thus a highly conserved structure for the binding of different classes of RNA is utilized by several proteins.

The RGG box, which is defined as closely spaced Arg-Gly-Gly repeats interspersed with other amino acids, is about 20-25 amino acids long and was initially identified in hnRNP U as the only apparent RNA-binding element (Kiledjian and Dreyfuss, 1992). The high density of glycine within this motif suggests that it is not a rigid protein structure; a helical ß-spiral structure is predicted. Many RGG box-containing proteins contain the modified amino acid N^G, N^G-dimethlyarginine (DMA). Methylation would not affect the strong positive charge of the arginine side chain, but it could, by steric constraints, modulate RNA binding (Burd and Dreyfuss, 1994). Three sites of arginine methylation have been located at residues 205, 217, and 224 in the glycine-rich C-terminal third of hnRNP A1 (Kim et al., 1997). This is achieved by a nuclear protein arginine methyltransferase that recognizes the sequence (Phe/Gly)-Gly-Gly-Arg-Gly-Gly-(Gly/Phe). Protein arginine methylation is implicated in signal transduction, nuclear transport, transcription regulation, and most likely RNA metabolism (Gary and Clarke, 1998).

Phenylalanines that are conserved among several RNA-binding proteins form part of a nucleic acid-binding pocket in the 90-amino acid hnRNP A1 (Merrill et al., 1988). A 34-kDa nucleolar protein, highly conserved in plants, B-36, is rich in glycine, phenylalanine and the modified amino acid asymmetrical DMA. The terminus of B-36 contains an interesting nine amino acid sequence, Gly-DMA-Gly-Gly-Phe-Gly-Gly-DMA-Gly, which is precisely

repeated three times in the 110 kD nucleolar protein nucleolin (Christensen and Fuxa, 1988). Nucleolin is an RNA-associated protein implicated in the early stages of ribosome assembly. Similar sequences have also been reported in a yeast nucleolar protein (SSB-1) and several hnRNP proteins (e. g. rat hnRNP A1) (Christensen and Fuxa, 1988). The conserved nature of this unusual sequence is suggestive of an important function which may include RNA-binding since several of these proteins share this feature.

3.6.3. The K homology (KH) domain:

The KH domain, so called because it was first identified in the human hnRNP K protein, which lacks the classical RRM, is defined by a core sequence VIGXXGXXI and is found in one or multiple copies (Siomi et al., 1993a). hnRNP K has 3 KH domains, chicken vigilin has 14, Nova, a neural-specific protein required for the development of the motor nervous system, has 3, and so does PCBP, and the fragile mental retardation (FMR-1) gene product has 2 KH domains, among many (Burd and Dreyfuss, 1994). The Nova-1 protein has recently been shown to regulate alternative splicing of the α2-glycine receptor subunit pre-mRNA by binding to an intronic element containing repeats of the tetranucleotide UCAU. The KH3 domain of Nova recognizes a single UCAY loop element in the context of a 20-base hairpin RNA. This suggest that KH domains in general recognize tetranucleotide motifs and that biological RNA targets of KH domains may use either RNA secondary structure or repeated sequence elements to achieve high affinity and specificity of protein binding (Jensen et al., 2000). Tetranucleotide recognition is supported by an aliphatic α-helix/β-sheet RNA-binding platform.

3.6.4. The double-stranded RNA-binding motif (DSRM):

Some RNA-binding proteins have one or more copies of a 70-amino acid region that binds double-stranded RNA (dsRNA). Conserved residues, including many basic (arginine and lysine) and hydrophobic amino acids are scattered throughout the DSRM. Not many DSRM proteins are currently known and therefore the motif itself may not be well defined, but they do not bind dsRNA in the same manner as dsDNA-binding proteins. Examples of DSRM proteins include staufen (5 DSRMs) in the developing Drosophila oocyte, which is essential for anterior-posterior axis formation, and the interferon-induced protein kinase DAI (2 DSRMs) in mammalian cells (Mathews and Shenk, 1991). DAI shuts off host protein synthesis when activated by ds viral RNA (Mathews and Shenk, 1991).

3.6.5. Other RNA-binding domains:

A small number of RNA-binding proteins contain sequences, such as appropriately spaced cysteine-histidine residues, that relate them to the zinc finger family of DNA-binding proteins. A generalized zinc finger-knuckle motif can be written as $CX_{2-5}C_{4-12}C/HX_{2-4}C/H$, in which X represents any amino acid (Burd and Dreyfuss, 1994). The sequence Trp-Glu-Asp-Glu or Trp-(Gly)n-Glu is conserved between the human and yeast cap binding proteins. The stacking and hydrogen bonding abilities of Trp-Glu-Asp-Glu with m^7G cap structure shows that the fourth Glu residue is important not only for the construction of hydrogen bond pairing with m^7G base but also for strengthening the stacking interaction between the Trp indole ring and m^7G base. This cooperative interaction could be important for the recognition of mRNA cap structure (Ueda et al., 1991).

The three commonly found RNA-binding domains, the RRM domain, the DSRM domain and the KH domain, have now been shown to have an α-helix/β-sheet fold similar to that found in many ribosomal proteins (Nagai, 1996). Many RNP complexes have been studied by atomic resolution. Two main themes emerge: (1) a "groove binder" class of proteins places a protein structure (α-helix, 310-helix, β-ribbon, or irregular loop) in the groove of an RNA helix, recognizing both the specific sequence of bases and the shape or dimensions of the groove and (2) a second class of proteins uses β-sheet surfaces to create pockets that examine single-stranded RNA bases. Some of these proteins recognize completely unstructured RNA, and in others, RNA secondary structure indirectly promotes binding by constraining bases in an appropriate orientation. Binding specificity is generally a function of several factors, including base-specific hydrogen bonds, non-polar contacts, and mutual accommodation of the protein and RNA-binding surfaces (Zhang et al., 1999). Our knowledge of protein interactions with RNA molecules has been, so far, largely restricted to cases in which the RNA itself is folded into a secondary and/or tertiary structure stabilized by intramolecular base pairing and stacking. Until recently, only limited structural information has been available about protein interactions with single-stranded RNA. A breakthrough came in 1999, with the determination of four crystal structures of protein complexes with extended single-stranded RNA molecules. These structures revealed wonderfully satisfying patterns of the ability of proteins to accommodate RNA bases, with the sugar-phosphate backbone often adopting conformations that are different from the classical double helix (Antson, 2000).

3.7. Pathologies associated with messenger RNA deregulation:

The post-transcriptional regulation of gene expression by RNA-binding proteins is important in controlling both normal cell functions and animal or human development. In higher eukaryotes, the expression of about 1 gene in 10 is strongly regulated at the level of mRNA translation into protein. Negative regulatory effects are often mediated by the 5'UTR and rely on the fact that the 40S ribosomal subunit first binds to the cap structure at the 5' end of mRNA and then scans for the first AUG codon. Self-complementary sequences can form stable stem-loop structures that interfere with the assembly of the preinitiation complex and/or ribosomal scanning. These stem loops can be further stabilized by the interaction with RNA-binding proteins, as in the case of ferritin.

Recently, mutations that cause disease through increased or decreased efficiency of mRNA translation have been discovered, defining translational pathophysiology as a novel mechanism of human disease (Cazzola and Skoda, 2000). Hereditary hyperferritinemia or cataract syndrome arises from various point mutations or deletions within a protein-binding sequence in the 5'UTR of the L-ferritin mRNA. Each unique mutation confers a characteristic degree of hyperferritinemia and severity of cataract in affected individuals. Hereditary thrombocythemia (also called familial thrombocytosis) can be caused by mutations in upstream AUG codons in the 5'UTR of the thrombopoietin (TPO) mRNA that normally function as translational repressors. Their inactivation leads to excessive production of TPO and elevated platelet counts. Finally, predisposition to melanoma may originate from mutations that create translational repressors in the 5'UTR of the cyclin-dependent kinase inhibitor-2A gene (Cazzola and Skoda, 2000).

Patients with autoimmune disease may carry antinuclear antibodies in their sera, which

would recognize antigens, like Sm, Ro, and La antigens, that are located on discrete sets of small nuclear or cytoplasmic RNP particles. Others, with systemic lupus erythematosus, myositis and rheumatoid arthritis, carry antibodies that precipitate specific subsets of ribosomal and transfer RNAs (Hardin et al., 1982) The fragile X syndrome, an X-linked disease, is the most frequent cause of inherited mental retardation. The syndrome results from the absence of expression of the FMR1 gene owing to the expansion of a CGG trinucleotide repeat located in the 5'UTR of the gene and the subsequent methylation of its CpG island. The FMR1 gene product (FMRP) is a cytoplasmic protein that contains two KH domains and one RGG box (Siomi et al., 1993b). It is associated with mRNP complexes containing polyadenylated mRNA within actively translating polyribosomes and contains nuclear localization and export signals making it a putative transporter and chaperone of mRNA from the nucleus to the cytoplasm. FMRP is most abundant in neurons and is absent in muscle (Khandjian, 1999).

α-thalassemia is caused by a single base substitution at the translation termination codon (UAA>CAA) of the dominant α2-globin gene (Liebhaber and Kan, 1981). This allows the ribosome to read through into the 3'UTR for an additional 31 codons to terminate at an in-frame UAA located 16 bases 5' to the poly(A) addition site. This readthrough results in the destabilization of the α-globin mRNA and the loss of over 95% of α-globin gene expression from the affected locus, and thus the resultant clinical disease α-thalassemia..

Myotonic dystrophy (DM) is an autosomal dominant neuromuscular disease that is associated with a (CUG)n repeat expansion in the 3'UTR of the myotonin protein kinase (Mt-PK) gene. A CUG repeat pre-mRNA/mRNA-binding protein (CUG-BP) has been identified

that may play an important role in DM pathogenesis (Timchenko et al., 1996). It is an isoform of a novel hnRNP, hNab50, and is localized predominantly in the nucleus, associated with polyadenylated RNAs *in vivo*. Thus triplet repeat expansion would lead to sequestration of this hnRNP on mutant Mt-PK transcripts (Timchenko et al., 1996). CUG-BP binds the human cardiac troponin T pre-mRNA and regulates its alternative splicing, which gets disrupted in DM striated muscle. Thus altered expression of genes regulated post-transcriptionally by CUG-BP may therefore contribute to DM pathogenesis (Philips et al., 1998).

A gibbon lymphoid tumor cell line (ML4 144) exhibits enhanced stability of IL-2 mRNA; decreased AUBP-mRNA association and thus disturbances of mRNA metabolism may contribute to the neoplastic phenotype (Henics et al., 1994). Rapidly growing neoplastic lung epithelial cell lines express a consistently higher abundance of both AUF1 and HuR, two proteins involved in mRNA stability, as opposed to non-tumorigenic cell lines, where both AUF1 and HuR expression decrease with confluence and growth arrest (Blaxall et al., 2000). Hel-NI and HuD, neuronal RNA-binding proteins, have gained recent attention as potential neuroendocrine markers for small-cell lung carcinoma (SCLC) (King, 1997). Members of this conserved family normally appear at different stages of neuronal maturation, raising the possibility that their expression patterns in SCLC reflect the degree of neuroendocrine differentiation (King, 1997). The coding region determinant-binding protein (CRD-BP), that binds to stabilize *c-myc* mRNA, increases c-Myc protein abundance (Doyle et al., 1998). The CRD-BP gene is moderately amplified in 12 of 40 human breast cancers and highly amplified in 2 others (14.4 and 20 copies). Amplification of a gene that might up-regulate c-Myc abundance could accelerate breast cancer (Doyle et al., 2000). Deregulation of RNA-binding

proteins can therefore significantly impact the onset, maintenance, and progression of neoplastic phenotypes.

A highly toxic environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), acts through both transcriptional and post-transcriptional mechanisms to alter gene expression of many genes. TGFα and urokinase-type plasminogen activator (uPA) are examples of genes up-regulated post-transcriptionally by TCDD by mRNA stabilization. A liver cytoplasmic protein of 50-kDa selectively recognizes the 3' UTR of the uPA mRNA in a TCDD-dependent manner, the activation of which is mediated through a protein phosphorylation cascade and not via *de novo* protein synthesis (Shimba et al., 2000). This presents an example of how an external contaminant can have repercussions on RNA stability.

3.8. Therapeutic solutions:

Effective intracellular expression of small RNA therapeutics depends on a number of factors. The RNA, whether antisense, ribozyme, or RNA aptamer, must be efficiently transcribed, stabilized against rapid degradation, folded correctly, and directed to the part of the cell where it can be most effective. One group tested the use of expression cassettes based on the human tRNA(met) and U6 snRNA promoters, in which transcripts encoding small RNA inserts are protected against attack from the 3' and 5' exonucleases (Good et al., 1997).

Antisense (AS) oligonucleotides are designed to bind to and inhibit a target mRNA. Coulis et al. used *in vitro* gel shift assays to determine the best AS that could inhibit RNA binding of the CRD-BP, so as to prevent the stabilization of the *c-myc* transcript by this protein. They identified a set of AS that was able to inhibit RNA binding of the CRD-BP by

75% in K562 cells, and showed a concentration-dependent decrease in both *c-myc* mRNA and protein levels (Coulis et al., 2000). Disrupting RNA-protein interactions might work out as a therapeutic solution in certain instances, such as in deregulated cells like cancer cells. The field of cancer research needs to look into approaches of how to prevent the growth of certain malignant cells by identifying and either turning off the transcription of oncogene(s) involved or by destabilizing their mRNAs. Activation of tumor suppressor genes by increasing their transcription or by preventing the decay of their messages could be another route.

3.9. Future directions:

Nuclear magnetic resonance and X-ray crystallography are being used to unravel the three-dimensional structures and configurations of mRNAs when they are complexed into mRNPs as well as when they exist as free RNAs. It would be of interest to determine whether various regulatory mechanisms involved in mRNA turnover change the transcript configuration and how they might either promote or protect the transcript from attack by nucleases.

Since it is clear that RNA-binding proteins play a major role in the post-transcriptional regulation of messenger RNAs by binding to *cis*-elements in the mRNA, and since they bind to the 3'UTR of mRNA more often than not to effect this process, the post-transcriptional regulation of the eNOS message was investigated by probing for proteins that would bind to the 3'UTR of eNOS mRNA. The 5'UTR of the eNOS mRNA is only 22-25 bases long and therefore may not be of a size to contribute significantly to the regulation of the stability of the eNOS message. Whereas the eNOS 3'UTR is 419 bases long and cntains motifs tht

have been described above, including two AUUUA and three CCUCC motifs. It also contains four CCUCU motifs, although these latter motifs have not been previously defined by any group to harbor any specific function.

4. PROTEIN BINDING TO HIGHLY CONSERVED ELEMENTS WITHIN THE 3' UNTRANSLATED REGION OF THE HUMAN ENDOTHELIAL NITRIC OXIDE SYNTHASE mRNA: MODULATION BY TUMOR NECROSIS FACTOR

4.1. Summary:

TNFα produces a marked decrease in steady-state levels of eNOS mRNA in cultured HUVECs, due to the selective destabilization of this message. Since the 3'UTR is known to play a role in the regulation of mRNA stability, that of the human eNOS mRNA was cloned and sequenced for comparative analysis with other eNOS 3'UTRs. The distal 257 nucleotides exhibited a high degree of homology with the bovine sequence, in which three domains were identified with near identity between species: domain 1 was C-rich and contained a putative CCUCC "stabilizing" motif, domain 2 was CU-rich containing 2 CCUCU motifs, and domain 3 represented an AU-rich element incorporating two AUUUA motifs. Four specific RNP complexes were identified by label-transfer using radiolabeled eNOS 3'UTR incubated with HUVEC cytoplasmic extracts, namely 45-, 53-, 56- and 66-kDa RNP complexes. Deletional mutants of eNOS 3'UTR were created to define the sites of protein binding. In label transfer experiments, the 45- and 66-kDa RNP complexes were formed preferentially with RNA fragments containing domain 3, whereas the 56-kDa protein bound predominantly to the CU-rich elements contained in domain 2. The 53-kDa protein bound unrelated RNA fragments from either the proximal or distal portions of the 3'UTR, suggesting the importance of secondary structure rather than motif specificity. TNFα induced the up-regulation of binding of the 56- and 53-kDa proteins, while also down-regulating that of the 66-kDa protein. We

conclude that the regulation of mRNA stability of human eNOS involves specific binding of cytoplasmic proteins to conserved CU- and AU-rich regulatory elements within the 3'UTR.

4.2. Introduction:

Endothelium-derived NO is now recognized as an important mediator of vasodilation in response to a wide variety of stimuli, both receptor dependent and independent, and likely represents a fundamental physiological mechanism maintaining functional and structural integrity of blood vessels. Release of NO in response to increases in blood flow and intimal shear stress is responsible for the moment-to-moment adjustment of vascular diameter, and maintenance of optimal conductance characteristics of the arterial tree (Furchgott and Vanhoutte, 1989; Griffith et al., 1987; Moncada et al., 1991). Not only is NO a potent vasodilator, but it has important antiplatelet and antiproliferative actions which, over the long-term, may contribute to vascular homeostasis by preventing thrombus formation and abnormal growth of vascular cells within the intima (Moncada et al., 1991). Thus, a defect in the biosynthesis of NO may have far ranging influences in vascular pathobiology.

Three iso-enzymes responsible for NO biosynthesis have been identified (Snyder, 1994). Endothelial cells express a "constitutive" NO-synthase (Marsden et al., 1992; Nishida et al., 1992), the activity of which is tightly regulated by intracellular calcium levels. The expression of this isoform can be profoundly down-regulated in the presence of cytokines, particularly TNFα (Mohamed et al., 1995; Yoshizumi et al., 1993), as well as by pathophysiological stimuli such as hypoxia (McQuillan et al., 1994) and endothelial cell proliferation (Flowers et al., 1995). Down-regulation of eNOS is likely an important

mechanism resulting in endothelial dysfunction, and contributing to a variety of human vascular disorders characterized by reduced endothelium-dependent dilation and impaired ability of the endothelium to generate NO (Vallance and Collier, 1994).

The down-regulation of eNOS induced by TNFα was demonstrated by our group (Mohamed et al., 1995) as well as Yoshizumi et al. (Yoshizumi et al., 1993) to be due to the selective destabilization of its mRNA, by a mechanism likely involving *de novo* protein synthesis (Mohamed et al., 1995), with no alteration in RNA transcriptional activity (Yoshizumi et al., 1993). Similarly, reduced steady-state levels of eNOS expression in hypoxia (McQuillan et al., 1994) and in rapidly proliferating endothelial cells (Flowers et al., 1995) resulted from increased mRNA degradation. Thus, post-transcriptional mechanisms play a predominant role in the down-regulation of eNOS expression in response to a wide variety of pathophysiological influences. The aim of the present study was to define the molecular mechanisms responsible for eNOS mRNA destabilization. Specifically, we examined protein binding to the 3' untranslated region of the eNOS message, since this region is of paramount importance in the regulation of stability of a variety of eukaryote messages (Jackson, 1993).

4.3. Methods:

4.3.1. Cell culture: HUVECs were obtained from the American Type Culture Collection and grown equilibrated in 95% air and 5% CO_2 at 37°C in Ham's F12 medium, supplemented with 15% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml heparin (all from Life Technologies, Burlington, ON), and 20 μg/ml endothelial cell growth factor

(Boehringer Mannheim, FRG). Prior to protein extraction, confluent cells were incubated with or without TNFα (Sigma, St. Louis, MO; 100 U/ml) for 6-24 hours.

4.3.2. Protein extraction: Cytoplasmic proteins were extracted by shaking the cells with hypotonic buffer made of 10 mM HEPES (pH 7.9), 40 mM KCl, 3mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.2% Nonidet P-40, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride (all from Sigma). The nuclei were removed by centrifugation (4500 x g, 15 minutes, 4°C), and the supernatant (cytoplasmic fraction) was aliquoted and immediately frozen at -80°C.

4.3.3. Cloning of eNOS 3'UTR and deletional mutants: A cDNA fragment corresponding to the entire 3'UTR of eNOS was amplified by polymerase chain reaction (PCR) of a 10 μl aliquot of a HUVEC lambda gt-11 cDNA library (generous gift of Dr. Morag Park, McGill University), with sense primers at the 5' end containing a T7 promoter and an EcoR1 cleavage site, and antisense primers at the 3' end containing a BamH1 site. The primers were designed based on the sequence retrieved from Genbank (accession number M93718), and were as follows: Sense primer A 5'- GAGCCGCCTGGCTTTCCCTTC-3'; Antisense primer B 5'-GAGCTGGGGTAGGCACTTTAG-3' (Table 4.1). The PCR product was ligated into the pCRII vector (Invitrogen, San Diego, CA). Its sequence was found to be identical to that previously published for the human eNOS, and, when compared to the published sequence for the bovine eNOS 3'UTR, an overall homology of approximately 68% was found (Figure 4.1). The majority of this sequence homology was confined to the distal 257 nucleotides

Table 4.1

Fragment (length)	sense oligo 5'-3'	antisense oligo 5'-3'	eNOS nucleotide
3'UTR	GAGCCGCCTGGCTTTCCCTTC (primer A)	GAGCTGGGGTAGGCACTTTAG (Primer B)	3659-4077
P305	primer A	GAGAGAGGCAAGAGGAATC	3659-3963
P267	primer A	CAGGAAGCGGGTGGCAGTA	3659-3925
P155	primer A	CTCCTTCCTGGAGGAATAATG	3659-3813
M158	GGAAGGAGCAAAACGCCTCTT	GAGAGAGGCAAGAGGAATC	3805-3963
D285	CATTATTCCTCCAGGAAGGAGC	primer B	3793-4077
D272	GGAAGGAGCAAAACGCCTCTT	primer B	3805-4077
D132	ATTCCTCTTGCCTCTTCTCAGG	primer B	3946-4077
D113	GGAGTATCTTACCTGTAAAG	primer B	3965-4077
ARE (32)	TCAAGTATTTATTATTGAAGATTTACCATAAG	CTTATGGTAAATCTTCAATAATAAATACTTGA	3998-4029

(81%) compared with 56% for the proximal portion of the 3'UTR. Using BLAST (Altschul et al., 1990), three domains of 18 or more nucleotides exhibiting near identity between the human and bovine sequences were identified in the distal region (Figure 4.1). Those domains were also delineated, based on the predicted secondary structure of the eNOS 3'UTR, using PC Gene and DNASIS (Hitachi), since they formed distinct stem loop structures (Figure 4.2).

A variety of deletional mutants of the human eNOS 3'UTR were created by PCR, as shown in Figure 4.5. Deletion mutants lacking progressively more of the 3' homologous sequences were amplified using the complete 3'UTR as a template with sense primer A and antisense primers as shown in Table 4.1. These mutants were named by the amount of bases they carried, preceded by the letter P (to designate that they consisted of the proximal part of the eNOS 3'UTR). Each antisense primer contained a BamH1 cleavage site at the 5'-end. Similarly, the 5'-truncated fragments were amplified with antisense primer B and sense primers as shown in Table 4.1. Again these mutants were named according to the number of bases they consisted of, preceded by the letter D (for the distal sequences of the eNOS 3'UTR). Each sense primer contained an EcoR1 cleavage site at the 5'-end. These PCR products were digested with EcoR1 and BamH1 before being ligated into pGEM3Z vectors. The 3'-truncated mutants P222 and P181 were generated by restriction digestion, namely DraII and StuI respectively.

A middle fragment comprising of 158 nucleotides, encompassing nt 3806 to nt 3963 of the human eNOS message and appropriately named M158, was generated using the primers laid out in Table 4.1. More constructs were generated from this fragment, a proximal part named MP72 spanning nt 3806 to nt 3877, and a distal part named MD86 spanning nt

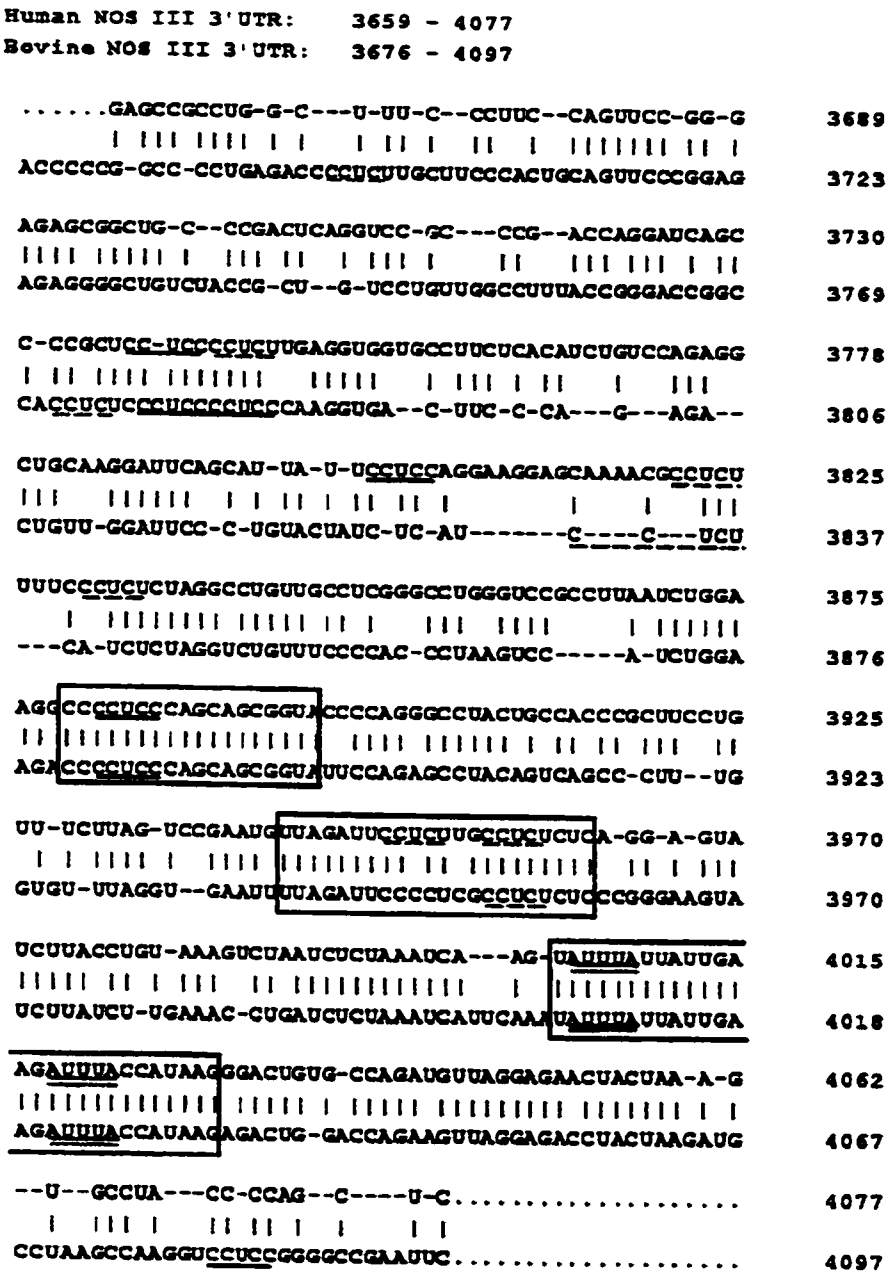


Figure 4.1 Nucleotide sequence for the human (top) and bovine (bottom) eNOS 3' untranslated regions (UTR). Homology between the human and bovine sequences is indicatedby vertical lines. Boxes indicate regions of sequence identity for 18 or more consecutive bases. The underlined sequences indicate previously reported *cis*-acting elements regulating RNA stability: Single underline = CCUCC; double underline = AUUUA and dotted underline = CCUCU.

Figure 4.2 The predicted secondary structure of the 419 bases of the human eNOS 3'UTR, using DNASIS, v 2.5

3878 to nt 3963. The construct MD86 included domains 1 and 2 and the region in between. The construct MD39 was made up of the last 39 bases of MD86, i. e. nt 3925 to nt 3963, that included mostly domain 2, comprising of two CCUCU motifs close to each other (Figure 4.5). As well, a cDNA fragment corresponding to the ARE of domain 3 was constructed by annealing sense and antisense oligonucleotides spanning the region from 3998 to 4029 of the human eNOS 3'UTR (Figure 4.5). The resulting 32 nucleotide fragment (ARE) was then ligated into a pGEM3 vector that had been digested with EcoR1 and BamH1, since the sense oligonucleotide contained an EcoR1 site at its 5' end and the antisense oligonucleotide contained a BamH1 site at its 5' end. A series of concatamers were also generated, namely $(AUUU)_{12}$, $(CCUCU)_{10}$, $(CUUU)_{12}$, $(CUUCU)_{10}$ and $(CU)_{25}$ to further determine the sites of protein binding to the human eNOS 3'UTR.

4.3.4. *In vitro* transcription: The plasmids containing the eNOS 3'UTR or the truncated fragments were linearized with BamH1 and RNA *in vitro* transcribed from 1 μg of the DNA template using T7 RNA polymerase in the presence of 10 mM DTT, 1 U/μl RNAsin (Promega, Madison, WI), 500 μM each of the nonlabeled nucleotides. In order to generate radiolabeled fragments, 25 μM of either cold UTP or CTP and 50 μCi of ^{32}P-UTP or ^{32}P-CTP respectively (specific activity 800 μCi/nmol, Amersham Pharmacia, Hamilton, ON) was substituted in the transcription reaction. Following three hours of transcription at 37°C, the RNA was extracted with phenol-chloroform followed by sodium acetate/ethanol precipitation. The RNA was reconstituted in RNase-free water and the specific activity of the radiolabeled RNA was calculated by counting 1 μl-aliquot in duplicate in a scintillation counter.

4.3.5. Gel mobility shift: 10 fmol of ^{32}P-RNA was incubated for 10 minutes at room temperature with 0.5 μg of cytoplasmic protein in a total volume of 10 μl in 20 mM HEPES (pH 7.6), 60 mM KCl, 10% glycerol, 1 mM $MgCl_2$ and 5U RNAsin (Promega). Heparin (5 μg/μl) was added to suppress nonspecific electrostatic binding. The mixture was also incubated with a 50- to 200-fold molar excess of nonlabeled 3'UTR as a specific competitor in separate reactions. The mixture was then run on a 5% non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide of 60:1). The dried gel was autoradiographed.

4.3.6. Label transfer: 15 μg of cytoplasmic protein from control or TNFα-treated HUVEC was incubated with 15 fmol of ^{32}P-RNA (3'UTR or deletional mutants) with or without a 100-fold molar excess of identical nonlabeled RNA (specific competitor) or 300-500 ng of total yeast RNA (nonspecific competitor) in a total volume of 20 μl of 20 mM HEPES (pH 7.6), 60 mM KCl, 10% glycerol and 1 mM $MgCl_2$ for 10 minutes at 37°C. RNA-protein interactions were stabilized by UV-crosslinking at 250 mJoule/cm^2 followed by digestion of single stranded RNA with RNases A and T1 (Boehringer Mannheim) at 37°C for 15 minutes. 20 μl of 2 x sample buffer containing 0.125 M Tris-HCl, 20% glycerol, 4% SDS, 10% beta-mercaptoethanol and 0.005% bromophenol blue was added to each reaction mixture. The samples were boiled for 4 minutes and loaded onto an 8 to 10% SDS-polyacrylamide gel, along with Kaleidoscope pre-stained markers (Biorad). Electrophoresis was carried out at 125V for 2 hours. The dried gel was either autoradiographed or imaged in a Cyclone phosphorimager (Canberra-Packard).

4.4. Results:

The results of the gel mobility shifts performed with the intact eNOS 3'UTR and the constructs P305, P155, D132, D113 and the ARE are shown in Figure 4.3. Panels A and B show that 3'UTR and P305 form an RNP complex (lane 2), when bound to HUVEC cytoplasmic protein extract, that is larger than the free RNA (lane 1) and thus gets retarded in the gel. A 50 molar excess of specific competitor was not enough to totally prevent the complex formation (lane 3), but a 200 molar excess abolished its formation (lane 4). The shifted band that occurred with either the intact 3'UTR or P305 seemed very insignificant, but these two RNA species were very large to run on a native gel, and most likely exhibited a high degree of secondary structure, such that the mobility of the free RNA through the gel was already highly retarded. P155 in panel C, though, hardly demonstrated any RNP complex formation. The smaller constructs made up of the highly conserved distal domains of the eNOS 3'UTR, D132 in panel D, D113 in panel E and ARE in panel F, exhibited very distinct RNP complexes when bound to HUVEC cytoplasmic proteins. The amount of RNP complexes did not seem to differ whether proteins were extracted under control conditions (lane 2 of panels D, E, F) or under TNFα treatment (lane 4 of panels D, E, F), except for D132 in panel D. The topmost complexes in panel D, which also represent larger protein-RNA complexes, ran as a doublet and exhibited a decreased RNP formation in the presence of TNFα (compare lane 4 to lane 2). In all cases, those RNP complexes were competed out with specific competitor (lanes 3 and 5 of panels D, E, F).

Label transfer experiments were then performed with the intact eNOS 3'UTR as well as with the 3' and 5' truncated 3'UTR mutants, the results of which are presented in Figures

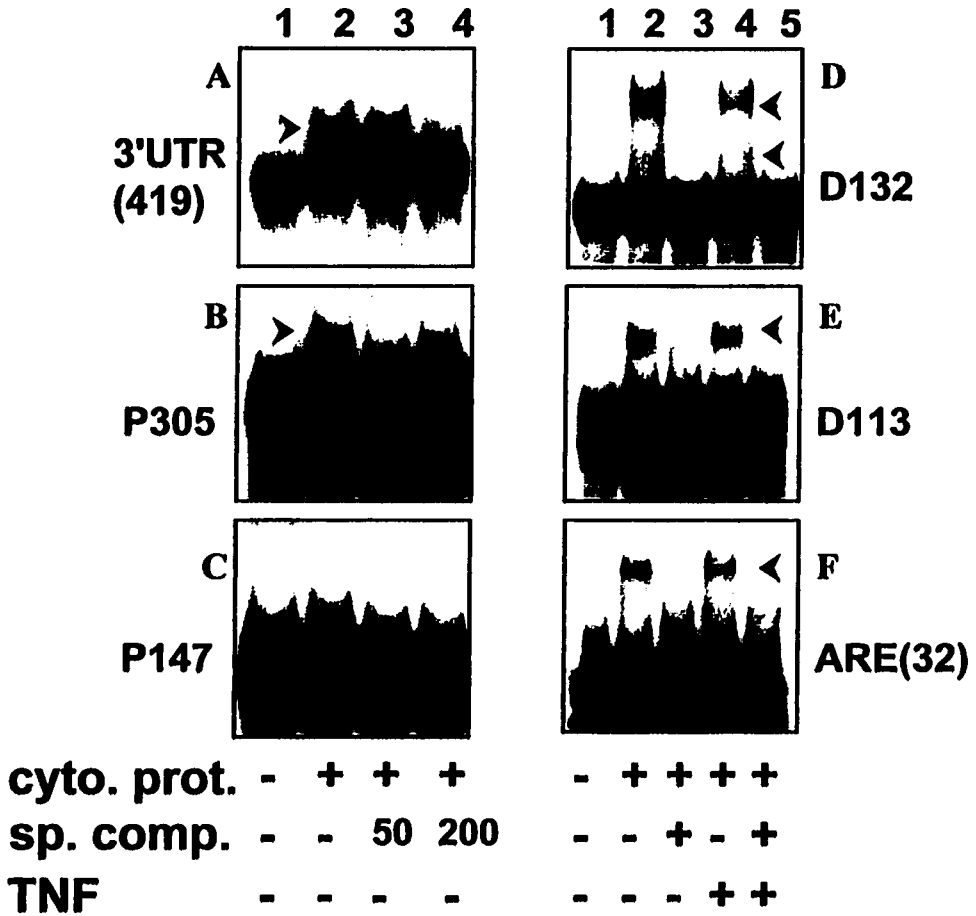


Figure 4.3 Gel mobility shift experiments with eNOS 3'UTR RNA and its various mutants with cytoplasmic proteins (cyto. prot.). Lane 1 of each panel represents free RNA; lane 2, RNA + cyto. prot.; lanes 3 & 4 of panels A, B and C also contain 50 and 200 mM specific competitor (sp. comp.) respectively; lanes 4 & 5 of panels D, E and F contain TNF-treated cytoplasmic proteins; lanes 3 & 5 also contain the sp. comp. Arrows show the position of the shifted RNA-protein complexes.

4.4A and 4.4B. All the label transfers were performed in the presence of total yeast RNA as a non-specific competitor. In the absence of cytoplasmic proteins, no distinct bands could be discerned, indicating complete digestion of free single-stranded RNA (Figure 4.4A, lane 1). However, when radiolabeled 3'UTR was incubated with cytoplasmic extracts from HUVEC, multiple bands were seen, corresponding to RNP complexes of approximate molecular weights of 45-, 53-, 56- and 66-kDa (Figure 4.4A, lane 2). The deletion mutants D285, D271 and D132 (Figure 4.4A, lanes 10, 11 and 12 respectively) displayed the same pattern of binding as the intact 3'UTR. A 100-kDa band or complex was inconsistently observed with the 3'UTR and the mutants D285 and D271, and therefore was not subjected to further investigation. The D113 mutant, which lacked domains 1 and 2, but retained the AU-rich domain3, displayed the formation of the 66- and the 53-kDa RNP complexes.

The proximal mutants in lanes 3-7 (Figure 4.4A) exhibited binding to the 53-kDa RNP complex, and the mutant P305 additionally displayed binding to the 56-kDa RNP complex (lane 3). The mutant M158 showed the formation of the 56-kDa RNP complex alone (lane 8). Thus the 56-kDa RNP complex was formed preferentially with the intact 3'UTR and the deletion mutants P305, M158, D285, D271 and D132 (Figure 4.4A), all having domain 2 in common, which contains two CCUCU motifs in close proximity (Figure 4.5). Further analysis of protein binding to domain 2 was performed using deletional constructs containing different portions of the M158 region: MP72, MD86 and MD39 (Figure 4.4B). The last 39 bases of M158, encompassing nt 3925 to nt 3963, and which included the two CCUCU motifs in domain 2, was sufficient to form the 56-kDa RNP complex (Figure 4.4B, lane 4).

Competition studies were performed with the whole eNOS 3'UTR and its various

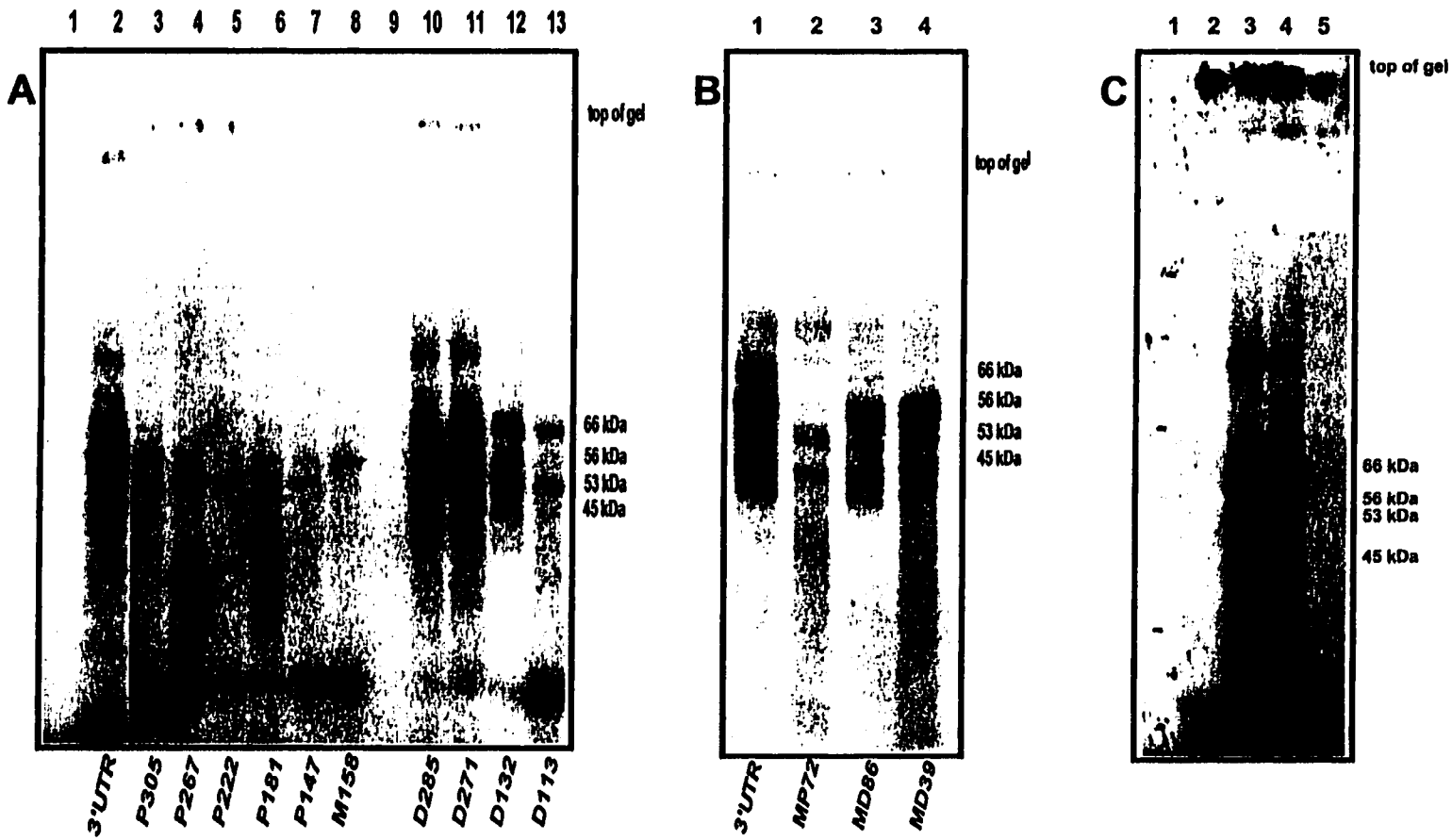


Figure 4.4 (A) Label transfer experiments using radiolabeled eNOS 3'UTR and the truncated 3'UTR fragments illustrated in Figure 4.5 with HUVEC control cytoplasmic proteins. Experiments with each individual RNA fragment were repeated at least 5 times with similar results. **(B)** Label transfer experiment using 100 fmol each of radiolabeled MP72, MD86 and MD39 with HUVEC cytoplasmic proteins. **(C)** A competition study using radiolabeled D132, with lane 1 representing protein markers; lane 2, free RNA; lane 3, label transfer with HUVEC cytoplasmic proteins. 100 molar excess of unlabeled P305 RNA (lane 4) or unlabeled D132 (lane 5) were used for competition. The gel apparatus used for running the competition study was much bigger than the one used for the other label transfers, accounting for the slightly different protein mobilities.

mutants to determine the specificity of protein binding. Figure 4.4C shows a representative label transfer using radiolabeled D132. Formation of the 66-, 56-, 53- and 45-kDa RNP complexes as well as a lower molecular weight RNP complex were clearly visible in lane 3, in which HUVEC cytoplasmic proteins were incubated with the labeled RNA. Upon competition with a 100 molar excess of unlabeled D132 RNA (lane 5), all binding was lost, whereas a 100 molar excess of cold P305 RNA competed mainly for the proteins forming the 56- and 53-kDa RNP complexes and to a certain extent for the 45-kDa-formimg complex, but did not inhibit the formation of the 66-kDa RNP complex (lane 4), reinforcing the observation that the 66-kDa RNP complex formed only with the distal part of the eNOS 3'UTR, which includes domain 3. Figure 4.5 is a schematic representation of the composition and binding data of the eNOS 3'UTR and its mutants. As can be seen, the distal mutant D113 was sufficient to form the 66-kDa RNP complex, MD39 could form the 56-kDa RNP complex, P147 and D113 were sufficient to form the 53-kDa RNP complex, and the 45-kDa complex was preferentially formed with the intact 3'UTR and the distal mutants.

Further label transfer experiments with the synthetic concatamers in Figure 4.6 revealed that the CU-rich RNA motifs formed the 56-kDa RNP complex specifically, but did not exhibit formation of the 53- or the 66-kDa RNP complexes (Figure 4.6A, lanes 4, 7, 8 and 9). A strong band was observed with $(CUUU)_{12}$, $(CCUCU)_{10}$ and $(CUUCU)_{10}$, whereas the CU repeat $(CU)_{25}$ exhibited only weak binding (Figure 4.6A, lane 7). More intense binding of the 56-kDa complex was observed when a higher fmol amount of CU-rich concatamers was used in the label transfer experiments (Figure 4.6B, lanes 3, 4 and 5). The same pattern of binding was obtained when the concatamers were radiolabeled with ^{32}P-CTP (data not

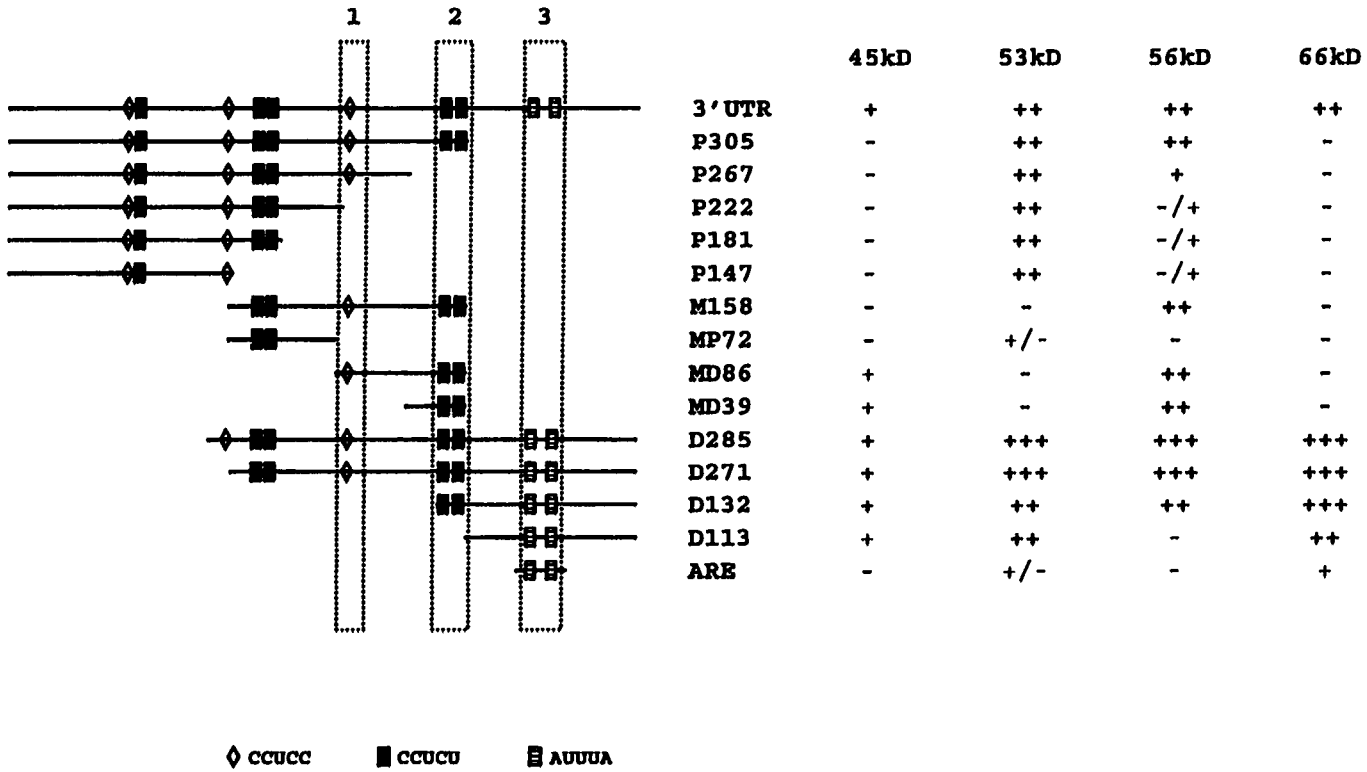


Figure 4.5 Schematic representation of the eNOS 3'UTR, and a series of truncated mutants exhibiting progressively more extensive deletions in the 3' or 5' regions, or both (nomenclature as per Table 1). Little boxes on each construct indicate the position of various motifs (see legend at bottom of figure). The vertical dotted boxes denote the three highly conserved domains between the human and bovine sequences. The binding pattern of the 45, 53, 56 and 66 kDa proteins are illustrated on the right.

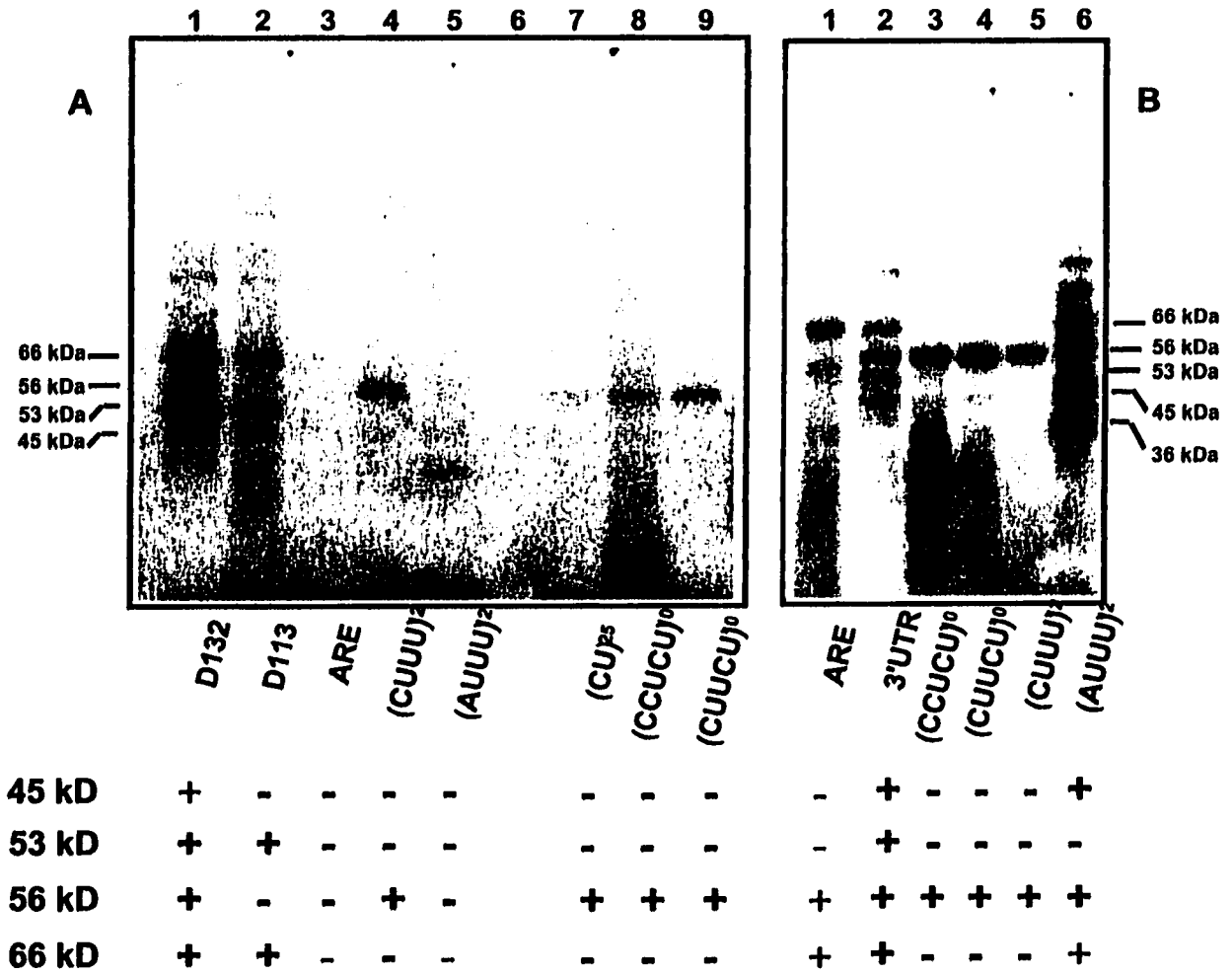


Figure 4.6 (A) Label transfer experiments of someof the distal 3'UTR mutants and various CU- and AU-rich concatamers with cytoplasmic extracts from control HUVEC. Lane 6 is the protein marker lane,whoich is invisible on the phosphorimager. **(B)** lane 1 shows the resultof a label transfer using 40 fmol of radiolabeled ARE,whilst lanes 3-6 used 50-60 fmilog radiolabeled concatamers. Lane 2 used 15 fmol of radiolabeled 3'UTR. The protein binding pattern is depicted at the bottom of the gels.

shown).Of interest, the concatamer $(AUUU)_{12}$ did not exhibit any protein binding activity (Figure 4.6A, lane 5), but when used in a higher molar quantity (40 fmol), it bound to a host of proteins of different molecular weights, including the 66-, 56-, and 45-kDa RNP-forming complexes (Figure 4.6B, lane 6). It is possible that the AUUUA pentamers could recognize other proteins that do not bind the eNOS 3'UTR, but that may be present in cellular extracts to regulate other AU-containing messages. In contrast, the 66- and 45-kDa RNP complexes were not formed with the CU-rich concatamers, whether 15 fmol (Figure 4.6A, lanes 4, 7, 8 and 9) or 50 fmol (Figure 4.6B, lanes 3, 4 and 5) of radiolabeled RNA was used in the label transfer experiments. Although 15 fmol of the ARE transcript displayed almost undetectable binding to any protein (Figure 4.6A, lane 3), increasing the concentration to 30-40 fmol of radiolabeled ARE revealed specific formation of a 66-kDa RNP complex (Figure 4.6B, lane 1). Since the ARE is only a small fragment of the 3'UTR (32 nt), 15 fmol of radiolabeled ARE is likely insufficient to discern RNA-protein interactions.

Figure 4.7A shows a representative label transfer experiment demonstrating the effect of TNFα on RNA-protein interactions, whereas summary data for eight separate experiments is shown in Figure 4.7B. In the presence of TNFα, formation of the 66-kDa RNP complex decreased overall by 42%, whereas that of the 56- and 53-kDa complexes increased by an average of 28% and 24% respectively (Figure 4.7B). In contrast, formation of the 45-kDa complex was not altered by TNFα treatment.

4.5. Discussion:

Post-transcriptional mechanisms are known to play a crucial role in the regulation of

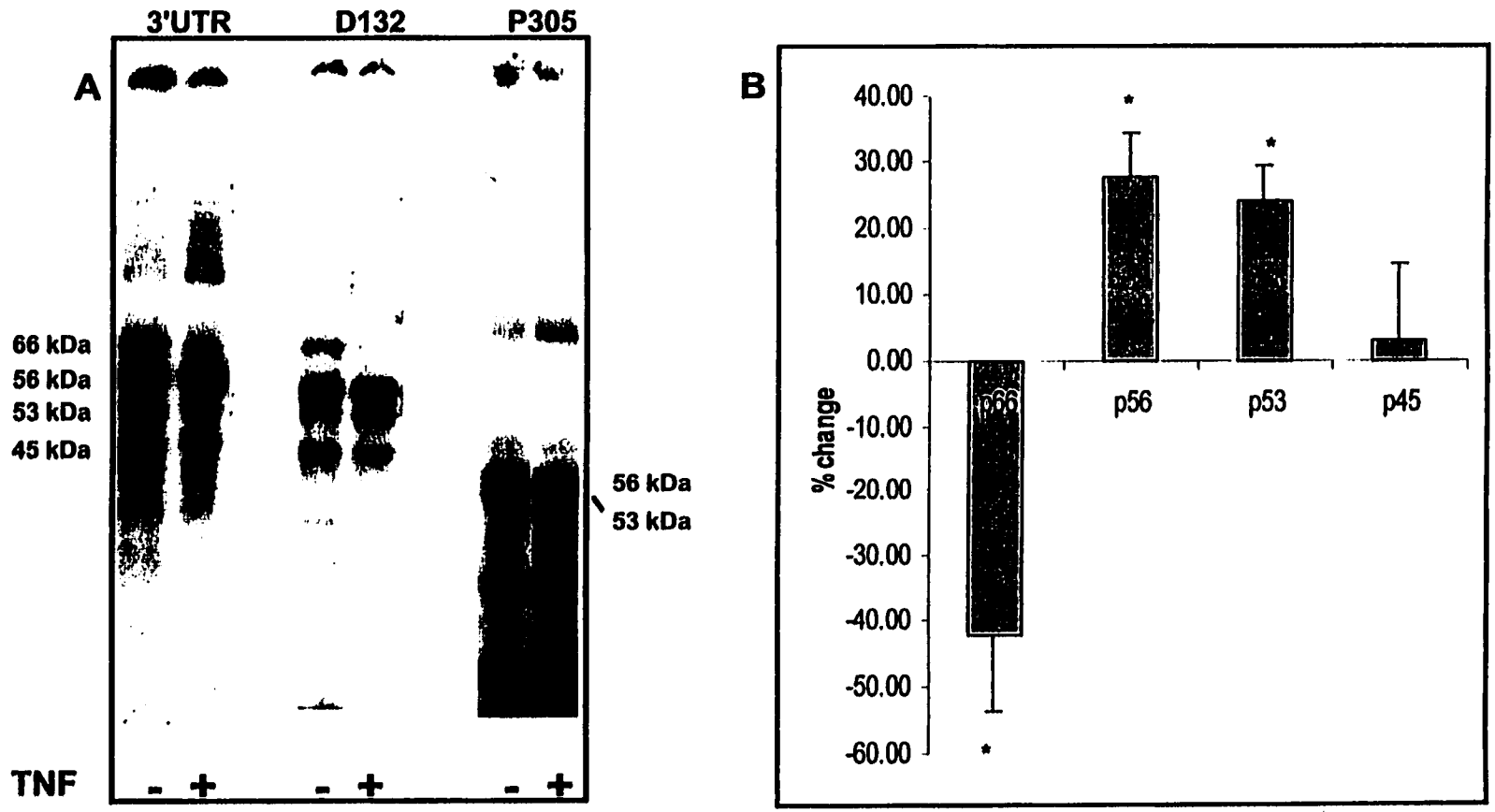


Figure 4.7 (A) Label transfer experiments of the 3'UTR,P305 and D132 with cytoplasmic proteins from HUVEC extracted under control and TNF treatment. Since they are three separate gels, they may have been run slightly differently. **(B)** Summary data of RNA-protein binding,in graphical form, refelcting % change (from control) of binding intensity of the eNOS 3'UTR-binding proteins under TNF -treated conditions; n=4 (p66), n=8 (p56), n=8 (p53), n=4 (p45); *$p < 0.02$ (paired t-test).

expression of an increasing number of eukaryotic genes (Jackson, 1993). The present study examined protein interactions with the 3'UTR of the human eNOS message, since specific elements involved in the regulation of RNA stability have been found in the 3'UTRs of other messages (Jackson, 1993; Ross, 1995). It is anticipated that regulatory elements would be more highly conserved than other non-translated sequences of mRNA. Indeed the distal 257 nucleotides of the human 3'UTR shared a high degree of homology with the bovine sequence, within which were three domains that exhibited near identity between species, 100%, 91% and 100%, respectively (Figure 4.1). Of note, each domain contained nucleotide sequences corresponding to *cis*-acting motifs previously reported to be involved in the regulation of stability of other messages. Domain 1 (3879-3896) was C-rich and contained a CCUCC motif, which has been reported in the human α-globin mRNA to be a *cis*-acting element that contributes to the stabilization of the human α-globin mRNA and increases its expression (Wang et al., 1995). Domain 2 (3942-3963) was a highly CU-rich region that contained two CCUCU motifs. Domain 3 (4003-4029), an AU-rich sequence of 27 nucleotides, contained two AUUUA motifs characteristic of a so-called rapid degradation element (RDE), first identified in the 3'UTR of rapidly degradable messages, as in those of the early messengers, *c-fos*, *c-jun*, and *c-myc* (Jackson, 1993).

In the present study, four highly specific RNP complexes were identified, exhibiting molecular weights of 45-, 53-, 56- and 66-kDa, that bound the human eNOS 3'UTR. Since experiments with either the intact eNOS 3'UTR or any small deletional mutant RNA fragments demonstrated the presence of the same RNP complexes on SDS-PAGE, we thought it highly possible that the RNP complexes reflected the actual size of the proteins

binding to the RNA fragment. In label transfer assays, the RNA was digested following incubation with cytoplasmic proteins and UV cross-linking, such that only the part of the RNA bound to protein would be intact. This most likely corresponded to a very small fraction of the original length of the RNA, and since the protein molecule binding to that small fragment would be of a size that would dwarf the RNA fragment, therefore the mobility of the RNA-binding proteins would not differ significantly from their corresponding RNPs in SDS-PAGE.

The 66- and 45-kDa RNP complexes were formed with the AU-rich distal part of the 3'UTR. Surprisingly though, the 66-kDa complex was not formed with the $(AUUU)_{12}$ concatamer unless a high concentration of radiolabeled concatamer was used in the label transfer, suggesting that other sequences adjacent to the AUUUA pentamers in the distal 3'UTR are most likely involved in the formation of this complex. Whereas the 45- kDa protein displayed strong binding to the $(AUUU)_{12}$ concatamer, raising the likelihood that it specifically recognizes the AUUUA pentamer. In contrast, the CU-rich RNA fragments did not bind either protein (irrespective of whether we used radioactive UTP or CTP in the labels), supporting the hypothesis that these proteins bound solely to AU-rich domains. Although a 36-kDa protein, possibly representing either AUF1 (Zhang et al., 1993) or hnRNP A1 (Wang and Liebhaber, 1996), also bound the $(AUUU)_{12}$ transcript, this would appear to have little relevance for the regulation of the stability of the human eNOS 3'UTR, since it did not bind to this 3'UTR or its various mutants.

The formation of the 56-kDa RNP complex occurred preferentially with specific CU-rich motifs rather than to indiscriminate CU repeats, such as the two CCUCU motifs present

in domain 2 (Figure 4.5). However the presence of CCUCU motifs may be necessary but not sufficient for protein binding, since the fragment MP72 also contained two of those motifs, but could not form the 56-kDa complex (Figure 4.4B). Likewise the mutants P222, P181, and P147, containing 1 CCUCU each (Figure 4.5), either exhibited very faint or no 56-kDa RNP complex formation. Thus it is highly likely that adjacent RNA sequences may be modulating this complex formation or it is also possible that the tertiary structure or the folding of the RNA may mask CU-rich motifs present in other regions of the 3'UTR, such that they are not accessible for binding to any protein.

In contrast, formation of the 53-kDa RNP complex occurred with fragments containing either the proximal or distal portions of the eNOS 3'UTR, but not with the intermediate region, as denoted by the lack of formation of this complex with the mutant M158 consisting of the middle region of the eNOS 3'UTR from nt 3806 to nt 3963. Interestingly, the predicted secondary structure of the eNOS 3'UTR places the proximal and distal regions as two stem loops in close proximity, creating a structural "groove", whereas the medial region is a stem loop, culminating in a series of smaller stem loops, that is removed from the "groove" (Figure 4.2). These results suggest that the protein (s) forming the 53-kDa complex may interact with a "pocket" of the human eNOS 3'UTR, as could result from the tertiary folding of the RNA.

The present experiments have identified highly specific protein binding to well circumscribed segments of the human eNOS 3'UTR, which correspond very closely to *cis*-acting regulatory domains previously shown to enhance or reduce the stability of mRNA. However, there appear to be important differences between the eNOS 3'UTR-binding

proteins identified in the present report and those described previously for other messages containing AU- or CU-rich elements. The role of the ARE in destabilizing a variety of labile mRNAs encoding for a number of transiently expressed proteins including cytokines, oncogenes, and transcriptional activators is well recognized (Ross, 1995). Deletion of these sequences has been shown to markedly stabilize *c-fos* and *c-myc* mRNA, whereas the insertion of the AU-rich motifs into the 3'UTR of highly stable messages results in a pronounced reduction in their stability (Shaw and Kamen, 1986).

A family of proteins which bind with high affinity to the ARE have been identified (Malter, 1989; Shaw and Kamen, 1986; Stephens et al., 1992). A 32-kDa protein, AUH, can interact with AUUUA motifs in *c-fos*, GM-CSF, and interleukin-3 mRNAs to target them for degradation (Nakagawa et al., 1995). AU-binding factors (AUBFs) of 15-, 17- and 19-kDa, that form a trimeric RNP complex, have been identified in cytoplasmic extracts from human mononuclear cells and Jurkat cell lines (Malter, 1989). A 37-kDa protein, AUF-1 (Zhang et al., 1993), found in the nucleus and cytoplasm of human erythroleukemia cells, binds AU-rich sequences to initiate decay (DeMaria and Brewer, 1996). However, the AUUUA motif is not always associated with the destabilization of mRNA. Interestingly, AUF-1 (the 40-kDa isoform) has also been found to be an integral component of the RNP complex implicated in the stabilization of the α-globin mRNA (Kiledjian et al., 1997). Hypoxia-inducible proteins of 17-, 28- and 32-kDa have been reported to bind AU-rich sequences within the 3'UTR of VEGF mRNA, in a manner that promotes stabilization of the message (Levy et al., 1996). Clearly the type of protein(s) binding to the AUUUA pentamers or the neighbouring sequences may play a role in dictating the fate of the mRNA.

hnRNP L has been identified as a 60-kDa protein that interacts with the VEGF mRNA in hypoxic cells to stabilize that mRNA. However the 21-nt hnRNP-binding element is composed of predominanatly C, and is relatively U-poor, clearly not conforming to an AU-rich element (Shih and Claffey, 1999), making it unlikely that hnRNP L is a candidate for our 66-kDa protein. Proteins of molecular masses of 82-, 71-, 66- and 37-kDa, that bind to a 20 nt U-rich sequence (17 U) in the 3'UTR of *c-fos* mRNA, have been identified as U-rich binding proteins (URBPs) (You et al., 1992) since only poly(U) could effectively compete for their binding, and since they were found to be incapable of binding to AUUUA motifs found in the *c-fos* ARE. They are unlikely to be candidates for the eNOS 3'UTR-binding protein described in the present report since the human eNOS 3'UTR does not contain long U stretches. hnRNP A1 and hnRNP C, of approximate molecular masses 34- and 43-kDa, have also been described binding to AUUUA sequences (Hamilton et al., 1993), and thus it is possible that the 45-kDa eNOS 3'UTR binding protein may be analogous to hnRNP C. However, to our knowledge, no "cytoplasmic" protein (or protein complex) of 66-kDa has been reported to bind the ARE, suggesting that this may be a novel AU-binding protein.

Similarly, the 56-kDa RNP complex appears to be distinct from previously identified C-, U- and CU-binding proteins, except for the 60-kDa eNOS 3'UTR-binding protein identified in bovine endothelial cells (Sanchez et al., 1999). The human PCBP is a 39-kDa cytosolic protein that interacts with C-rich elements in the 3'UTR of α-globin mRNA, forming part of the multi-protein complex that stabilizes this message (Funke et al., 1996; Wang and Liebhaber, 1996). This 39-kDa protein binds to poly-C sequences, whereas other components of this complex, the 40-kDa AUF-1 and a 42-kDa protein, may interact either in a sequence

specific manner with the CCUCC stabilizing motif (Wang and Liebhaber, 1996) or with the PCBP itself (Kiledjian et al., 1997). A TGFβ-inducible cytoplasmic factor of 75-kDa, rich in CU-sequences, binds exclusively to an 83-nt fragment of the 3'UTR of ribonucleotide reductase (Amara et al., 1993) to stabilize the mRNA. A 51-kDa protein has been described to bind selectively to a 43-nt fragment of the proximal bovine eNOS 3'UTR (Searles et al., 1999), a G- and C-rich sequence comprising one CCUCU motif, but our 53-kDa protein may be different from it since it is able to also bind the distal conserved part of the human eNOS 3'UTR as well as the proximal half.

The functional consequences of the RNA-protein interactions in the regulation of eNOS mRNA stability were not directly explored in the present study. Evidence of functional importance might be inferred from the similarities in their respective binding domains to those of well characterized *cis*-acting elements important in the regulation of mRNA stability. AUUUA motifs have often been found to promote rapid RNA degradation; however TNFα pretreatment resulted in a decrease in binding of the 66-kDa protein to the distal AU-rich domain of the human eNOS 3'UTR as opposed to an increase. Thus rather than a role in RNA destabilization, the 66-kDa protein may in fact contribute to the stabilization of the human eNOS mRNA. In contrast, the binding of the 56-kDa protein to CU-rich elements was enhanced in the presence of TNFα, suggesting that these motifs, which have been implicated in the stabilization of other messages, might play a role in enhancing human eNOS mRNA degradation. This finding is in accordance with the research from the laboratory of López-Farré. They describe a CU-rich segment in the middle portion of the bovine eNOS 3' UTR that is critical in complex formation with BAEC cytosolic proteins, and they report that

TNFα, which destabilizes eNOS mRNA, increased the binding activity of the cytosolic proteins to that *cis*-element in a time-dependent manner (Alonso et al., 1997). Further work from the same laboratory has revealed the TNFα-responsive *trans* element to be a 60-kDa protein binding to a defined 38 nt sequence within the 5' half of the 129 end nucleotides of the bovine eNOS 3'UTR (Sanchez et al., 1999). Thus it is highly possible that our human 56-kDa RNA-binding protein may be analogous to the bovine 60-kDa eNOS 3'UTR-binding protein.

Alternatively, regulation of mRNA stability may be more complex, and occur not only at the level of binding of these proteins per se, but also involve interactions with other proteins or post-translational modifications (e. g. methylation or phosphorylation) which affect the ability of these proteins to alter mRNA stability. Indeed, this appears to be the case for a growing number of RNA-binding proteins regulating RNA stability, including the PCBP, the binding of which by itself is unable to stabilize the α-globin mRNA (Wang et al., 1995). The novel findings described in the present report provide important new insights into a fundamental mechanism of regulation of the human eNOS mRNA expression. The binding of cytoplasmic proteins to AU- or CU-rich putative regulatory elements within the eNOS 3'UTR, and the modulation of these interactions by TNFα, strongly suggest a role in the post-transcriptional regulation of RNA stability. These results provide a basis for future studies on the mechanisms of regulation of human eNOS mRNA stability, and clearly define potential molecular targets for detailed analyses of RNA-protein interactions.

5. PRELIMINARY CHARACTERIZATION AND PURIFICATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE 3'UTR-BINDING PROTEINS.

5.1. Introduction:

From the literature, it is becoming more evident that a single protein may have several functions, as has been found for the enzymes aconitase, GAPDH, thymidylate synthase and dihydrofolate reductase amongst others, that can also function as RNA-binding proteins to regulate the stability or translation of the mRNA to which they bind. The splicing factors have also been shown to be involved not only in splicing pre-mRNA, but also to participate in regulating the half-life of an RNA species, which can be accomplished either in the nucleus or in the cytoplasm. hnRNPs are now known to shuttle between the nucleus and the cytoplasm, where their RNA-binding functions may vary. A particular RNA-binding protein may regulate the stability of more than one RNA species, as has been found for IRP, the iron regulatory protein, which can bind to ferritin, transferrin and the transferrin receptor mRNAs, and possibly to other mRNA species as well. This might represent a means for the cell to conserve energy, i. e. to make use of its existing repertoire of proteins, rather than having a scenario where each protein expressed would fulfil only one function and where the cell would therefore need a vast number of proteins to be expressed at any time, even for the regulation of each mRNA molecule.

Regulatory proteins bind to DNA, RNA, other proteins, or even lipids to serve many diverse functions. The main interest of this thesis lies with the RNA-binding regulatory proteins, which modulate the stability of different mRNA species. As mentioned above, some

RNA-binding proteins have been discovered to be enzymes, and a number of them seems to regulate the stability of their own mRNA by binding to it to either repress its translation or to target it to compartmental lysosomes, where it can be degraded by endo- and/or exo-nucleases. Thymidylate synthase, required for the conversion of deoxyuridine monophosphate (dUMP) to the DNA precursor deoxyribosylthymine monophosphate (dTMP), and dihydrofolate reductase, which converts dihydrofolate to tetrahydrofolate and is thus involved in the *de novo* synthesis of thymidylate, purines and amino acids, are two examples of enzymes binding to their own mRNA to repress their translation *in vivo*, mentioned in Chapter 3. Another enzyme, isocitrate dehydrogenase, displays binding specificity for the 3'UTRs of mitochondrial mRNAs in yeast (Elzinga et al., 1993). Glutamate dehydrogenase binds to mRNA encoding cytochrome c oxidase (Preiss et al., 1993). A 39-kDa AU-binding protein is found to be a thiolase (Nanbu et al., 1993) and another AU-binding protein, the 32-kDa AUH, possesses hydratase activity (Nakagawa et al., 1995). The eNOS 3'UTR-binding proteins have not been identified yet, but it is unlikely that the eNOS enzyme itself does not bind to the 3'UTR of its own mRNA, otherwise we would have been able to detect an RNP complex of about 135-kDa when the radiolabeled eNOS 3'UTR RNA was mixed with human endothelial cell extracts in the label transfer assays described in Chapter 4.

Interestingly, what the above enzymes have in common are substrates or cofactors that include dinucleotides. GAPDH transforms 3-phosphoglyceraldehyde into diphosphoglycerate, converting NAD^- into NADH in the process. Glutamate and lactate dehydrogenases also utilize NAD^+ or $NADP^+$ in their separate reactions. Dihydrofolate reductase uses NADPH as a cofactor and thymidylate synthase uses dUMP as a substrate.

Aconitase, which also functions as an RNA-binding protein, does not utilize any dinucleotides as cofactors or substrates, but still contains a region of strong amino acid sequence homology with NADH-binding sites, including the βαβ dinucleotide-binding fold of dihydrolipoamide dehydrogenase and other oxidoreductases (Rouault et al., 1990). Catalase, an antioxidant enzyme that protects cells from the toxicity of hydrogen peroxide by converting it into water and oxygen, has been reported to bind to the 3'UTR of its own mRNA, except that it could only do so in the absence of NADPH (Clerch et al., 1996). Yet catalase cocrystallizes with NADPH, even though this enzyme does not require the dinucleotide for enzymatic activity (Fita and Rossmann, 1985; Kirkman and Gaetani, 1984). Hentze has explored the possibility that the dinucleotide-binding and the RNA-binding sites of these enzymes are related, such that either the enzyme has to adopt a different configuration to bind to RNA when it is not bound to any dinucleotide, or the enzyme does not change in configuration, but rather maintains an equilibrium between RNA binding and dinucleotide binding. Therefore the cell conditions would dictate whether it behaves more like an enzyme or an RNA-binding protein. This would also represent a mechanism of metabolic as well as post-transcriptional regulation (Hentze, 1994).

The possibility exists that one or more of the eNOS RNA-binding proteins may turn out to be an enzyme with a known metabolic function, or it is also possible that they may be entirely novel proteins either harboring an unknown enzymatic activity or none at all. Therefore the next step was to try and purify the eNOS 3'UTR-binding proteins from HUVEC cytoplasmic extracts in order to identify them by sequencing or by mass spectrometry. This task was attempted using several methods outlined below. First, the

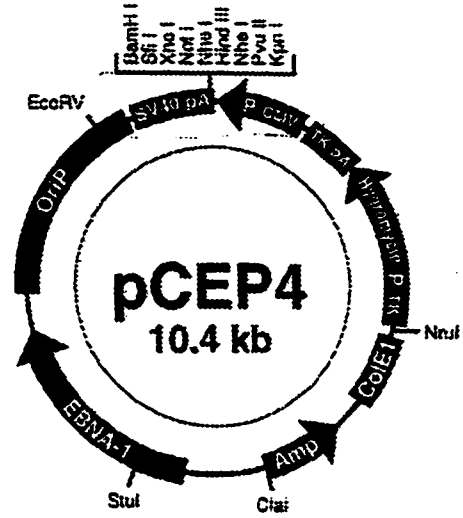
functional importance of these RNA-binding proteins was explored by employing two methods discussed below: (1) attaching the intact eNOS 3'UTR or its mutants to a reporter gene and subsequently investigating the expression and stability of the reporter gene product within the cell and (2) an *in vitro* decay assay that explored rates of decay of the eNOS 3'UTR and various mutants when exposed to cytoplasmic extracts from control and TNFα-treated HUVECs. Other cell types were also tested for expression of the human eNOS 3'UTR-binding proteins.

5.2. Methods:

5.2.1. Reporter gene construct: Luciferase was used as the reporter gene and the constructs were engineered such that the luciferase gene, under the control of a heterologous promoter, had the eNOS 3'UTR cDNA or one of the 3'UTR mutants attached to its 3' end. The vector or plasmid used for this purpose carried a Simian virus (SV40) polyadenylation signal as well as a cytomegalovirus (CMV) promoter to allow for expression in human cells, and an antibiotic-resistant cassette to allow for selection of the clones carrying the construct, as shown in Figure 5.1A. Three constructs were made, one carrying the luciferase gene alone without any 3'UTR (LUC), one carrying the full-length eNOS 3'UTR attached to the luciferase gene (LUC-3'UTR), and one carrying the deletion mutant P155 (LUC-P155), i. e. the proximal part or 5' end of the eNOS 3'UTR, attached to the luciferase gene, as depicted in Figure 5.1B.

5.2.2. Transfection of cells with reporter gene: The cDNA constructs were introduced into

(A)



(B)

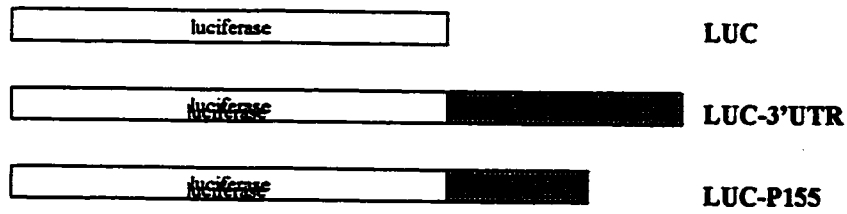


Figure 5.1 (A) Plasmid depicting the multiple cloning sites, promoter, polyadenylation site and antibiotic resistance sites. **(B)** Luciferase constructs: LUC, luciferase alone; LUC-3'UTR, luciferase with the eNOS 3'UTR attached; LUC-P155, luciferase with the deletion mutant P155 attached to its 3' end.

cells by transfection using a liposome-based method, whereby 2 μg DNA was mixed with cationic lipids (lipofectin, Life Technologies, Burlington, ON) in the test tube to form micelles, with the positively charged lipids surrounding the negatively charged DNA. These lipid-DNA complexes were then mixed with Optimem medium (Life Technologies), that was devoid of serum and antibiotics, and placed on top of the cells that were plated onto 100 mm^2 culture dishes at a confluency of 60-70%. Following a 4-6 hour incubation at 37°C, the medium was then replaced with regular cell culture medium containing serum, Ham's F12 (Life Technologies), and the cells left to grow overnight at 37°C.

The transfected cells were either kept under control conditions or were subjected to TNFα at a dose of 30 or 100 U/ml. Since this method represented only a transient transfection of the reporter gene, care had to be taken to harvest cellular RNA or proteins at the right time in order to quantitate the amount of luciferase transcript or protein before dividing cells lost the plasmid altogether. Therefore a time course of luciferase expression was performed, where total protein was extracted from the transfected cells at various time points, namely at 4, 24, 48 and 72 hours following the transfection, and assayed for luciferase activity. The negative controls included cells transfected with vector alone and subjected to either control or TNFα-treated conditions.

Other experiments were performed, where the transfected cells were subjected to actinomycin D (2 μg/mL) which halts the transcription of all genes {Peltz, Brewer, et al. 1991 6689 /id}. Total RNA or total protein were then extracted from the transfected cells at 0, 1, 4 and 24 hours post-actinomycin D for Northern blot analysis and luciferase assay respectively. In this way, we hoped to observe the post-transcriptional and perhaps the post-

translational regulation of the reporter gene product.

5.2.3. Luciferase assay: The luciferase protein, endogenous to the firefly, reacts with luciferin in the presence of ATP and Mg^{2+} to release light. The amount of light generated is directly proportional to the amount of luciferase in the assay. Therefore this simple assay involved the addition of a buffer containing the substrates, luciferin, ATP and Mg^{2+}, to the sample to be tested. The volume of sample was kept constant, and the light emitted, when the substrates and the luciferase in the sample were mixed together, was captured in a luminometer (Berthold), photomultiplied and generated as relative light units.

5.2.4. Northern blot analysis: Total RNA (15-20 μg) extracted from transfected cells were electrophoresed on formaldehyde agarose and transferred onto a nylon membrane. Following UV cross-linking to ensure the attachment of the RNA to the membrane, it was subsequently hybridized with radiolabeled eNOS 3'UTR and luciferase cDNA probes, following the procedure outlined for Northern blotting in Section 2.3.2.

5.2.5. *In vitro* decay assay: A crude assay was developed to explore the differential decay rates between the full-length eNOS 3'UTR and its 5' and 3' deletional mutants. 50 fmol of radiolabeled eNOS 3'UTR RNA or its deletion mutants, that were *in vitro* transcribed using the method outlined in Section 4.3.4., was mixed with 15 μg HUVEC cytoplasmic or polysomal extracts in a reaction buffer containing creatine kinase, creatine phosphate, ATP, GTP, dithiothreitol (DTT), spermine and RNAsin (a ribonuclease inhibitor) prevent the

spontaneous degradation of the RNA in the reaction tube. Following incubation at 37°C, an aliquot from the reaction mixture was taken out at each of the time intervals corresponding to 0, 10, 30, 60 and 90 minutes and quenched with phenol. The RNA was extracted with phenol/chloroform and run on a 10% sequencing gel or a native Tris-Borate-EDTA (TBE) gel to visualize the amount of RNA present at each time point, as well as its degradation products. Controls included RNA alone that underwent the same period of incubation and the same process of extraction.

5.2.6. Use of North-Western blotting to screen a HUVEC expression library: A biotinylated eNOS 3'UTR RNA probe was transcribed *in vitro*, using the procedure described in Section 4.3.4, except that biotinylated UTP was substituted for radioactive UTP, rendering the RNA probe less dangerous to work with. The lambda-phage HUVEC expression library was plated at a density of 1 million colonies on agar containing Y1090 bacteria, which carried ampicillin resistance. Transcription was stimulated with isopropyl-1-thio-β-*D*-galactoside (IPTG) since the HUVEC library was engineered to be under the control of the lac Z promoter. The proteins expressed were lifted onto a nitrocellulose membrane. Each plaque forming unit (pfu) represents an expressed protein, leaving room for the fact that more than one pfu may express the same protein, depending on the number of copies of transcripts present at the time of making the cDNA library. The membrane was subsequently hybridized with the biotinylated eNOS 3'UTR probe, such that if the RNA bound to any protein, binding activity was detected by adding a streptavidin-alkaline phosphatase conjugate that would attach to any biotinylated eNOS RNA bound to one or more proteins attached to the

membrane. Alkaline phosphatase substrates, that turned blue as they got converted into their products, were used for visual detection.

5.2.7. Use of magnetic beads to capture eNOS 3'UTR-binding proteins: The full-length biotinylated eNOS 3'UTR RNA, as well as two biotinylated deletion mutant RNA probes, namely P305 and D113, were also used to isolate eNOS RNA-binding proteins by a method utilizing streptavidin-coated magnetic beads (Dynal, N.Y.). Approximately 30 μg HUVEC protein extracts, isolated under control and TNFα-treated conditions, and 30 fmol of biotinylated RNA were allowed to mix in a sterile Eppendorf tube, after which the streptavidin-coated beads were added. The beads were pulled to the side of the tube with the aid of the magnet applied to the exterior wall of the tube. With the magnet still holding the beads, the rest of the liquid reaction mixture was gently pipetted out, such that the eNOS 3'UTR-binding proteins would be separated from the whole cell extract by being physically bound to the biotinylated RNA bound to the magnetic beads. The RNA-binding proteins were then released from the eNOS 3'UTR RNA by eluting with a high salt buffer and separated by SDS-PAGE, in the light that several proteins are capable of binding to eNOS 3'UTR, discussed in detail in Chapter 4. The gel was then silver stained for visualization of the proteins. Negative controls included biotinylated RNA alone as well as protein extract alone, undergoing the same procedure with the magnetic beads.

5.2.8. Purification of the eNOS mRNA-binding proteins: Since HUVECs were not an abundant source of cells, a vascular organ or tissue, like the human placenta, was used to

purify human eNOS 3'UTR-binding proteins. It was readily available, easy to harvest and a good source of endothelial cells. The placenta was homogenized in a buffer containing Tris-HCl at a pH of 7.4, 5 mM $MgCl_2$, 10% glycerol, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 0.1 mM PMSF with the aid of a polytron and the resultant mixture spun at 3400 rpm for 10 minutes at 4°C to remove a lot of debris. The supernatant was respun for 30 minutes at 11,500 rpm at 4°C, after which it was subjected to a crude ammonium sulfate extraction. Ammonium sulfate powder was added to the supernatant at a concentration of 35% and left to stir for 2 hours. The mixture was centrifuged to pellet out proteins, and the resultant supernatant was further mixed with 65% ammonium sulfate and stirred for another hour, after which it was centrifuged again for 30 minutes at 11,500 rpm. The resulting pellets were dissolved in and dialyzed with 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 5% glycerol, aliquoted and stored at -80°C.

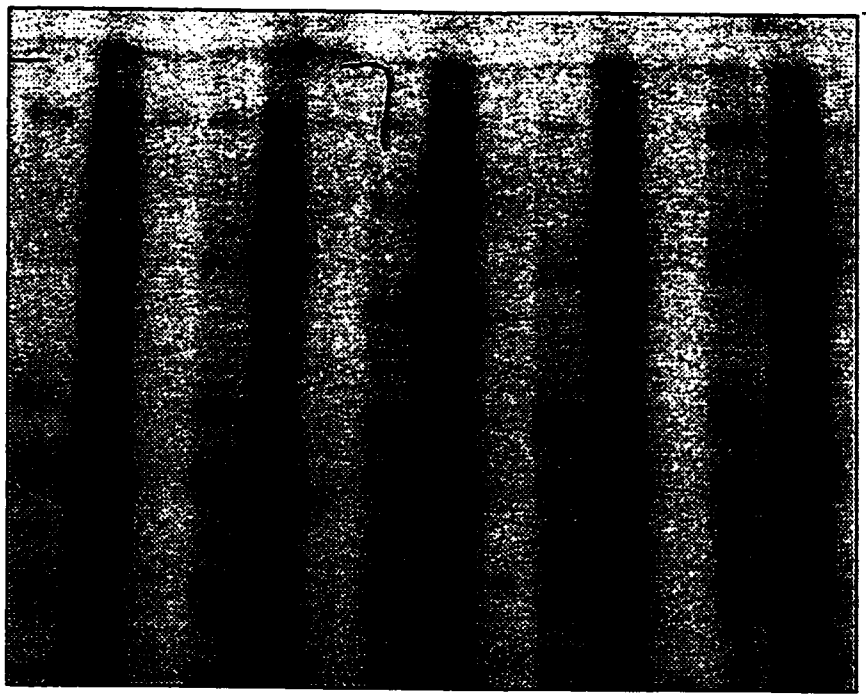
Next, molecular size exclusion was used to try and separate out the proteins present in the 65% ammonium sulfate precipitation. The 65% pellet (dissolved and dialyzed as specified above) was passed through P-100 (Bio-Rad, ON), a cellulose-based matrix which allows the separation of proteins above and below the molecular weight cut-off of 100-kDa, and a total of 60 fractions collected at a rate of 5 mL/hour in 10 mM HEPES pH 7.5, 2.5 mM $MgCl_2$ and 40 mM KCl.

The 35% ammonium sulfate precipitate, containing all the eNOS 3'UTR-binding proteins, was also fractionated through DEAE-cellulose, once it was dissolved and dialyzed as described above. The proteins were eluted with a gradient of up to 0.5 M sodium chloride (NaCl) in 10 mM Tris-HCl pH 7.5. Fractions containing the 56-kDa protein were pooled,

dialyzed against a low salt buffer and subjected to RNA-affinity chromatography. This was achieved by immobilizing polyadenylated eNOS 3'UTR RNA onto an oligo(dT) cellulose matrix in a column to which RNAsin was added for preservation of the RNA. The protein mixture was passed several times through the column to ensure maximal binding of the RNA-binding proteins. Following a wash step, the proteins were then eluted from the column with a high salt buffer containing a progressive concentration of 1 to 4 M KCl. The products of each protein purification step were assayed for RNA binding by label transfer assays utilizing radiolabeled eNOS 3'UTR RNA.

5.3. Results:

5.3.1. Occurrence of eNOS 3'UTR-binding proteins: Other EC types as well as non-ECs were tested for expression of the eNOS 3'UTR RNA-binding proteins. Cells included porcine and bovine pulmonary artery endothelial cells, PPAECs and CPAEs respectively, and non-EC types, like porcine aortic smooth muscle cells, PASMCs, human fibroblasts, human hepatocytes, HepG2, and rat macrophages, IC-21. Cytoplasmic proteins from these cells, extracted using the procedure outlined in Section 4.3.2., were subjected to label transfer assays with radiolabeled eNOS 3'UTR and P305 RNA, following the method outlined in Section 4.3.6. Figure 5.2 show that most of the cells displayed the same pattern of binding as the HUVECs, suggesting that these RNA binding proteins may be ubiquitously expressed and may serve to regulate the half-lives of other messages, especially in cells that do not express eNOS. CPAE and human fibroblasts were not included in the gel in Figure 5.2.



HUVEC PPAEC HepG2 IC-21 PASMC

Figure 5.2 Label transfer assays of radiolabeled eNOS 3'UTR (first lane of each set) and its deletion mutant, P305 (second lane of each set) with cytoplasmic extracts from various cell types. The cell type is named at the bottom of each set. The proteins that bind to the RNA are separated by 10% SDS-PAGE and thereafter exposing to X-ray film or to a phosphorimaging screen (Canberra Packard). The protein markers are not illustrated since the purpose of this gel was to find out whether the eNOS 3'UTR-binding proteins are found ubiquitously.

5.3.2. Reporter gene expression: Four different types of cells were used for the transfection experiments: HUVEC, PPAEC, COS (SV40 transformed monkey kidney cells) and HepG2, a human hepatocelluar carcinoma. The last two were used for their ease of transfection, since transformed cells seem to pick up more DNA than normal cells and since they are known not to produce any eNOS. Cells that produce eNOS, like HUVEC and PPAEC, may potentially not exhibit any regulation of the reporter gene product if the RNA-binding proteins are engaged or involved in binding to endogenous eNOS mRNA, whereas non-EC types would provide no such interference. The possibility exists though that non-EC types may not produce any of the eNOS 3'UTR-binding proteins, but label transfer assays with the cytoplasmic extracts from HepG2 and radiolabeled eNOS 3'UTR RNA seem to indicate that they do (see Figure 5.2 for HepG2).

The results of the luciferase expression during the time course experiments were normalized to the total amount of protein extracted from the cells and are presented in tabular and graphical forms in Figure 5.3. HUVECs expressed the reporter gene maximally 4 hours following transfection (A), whereas PPAECs showed maximal expression 24 hours after transfection (B). The COS cells also showed maximal expression at 24 hours, but the expression of the reporter gene was sustained at the maximal rate for 96 hours (4 days) or could maybe have gone longer (Table 5.1). When the cells were subjected to TNFα (30 U/mL) after the transfection, the results were inconsistent, as outlined in Table 5.2. HUVECs showed an increase in luciferase expression with the LUC-3'UTR construct at one time, a decrease in luciferase at another time with the same construct, and no change yet another time, when exposed to TNFα. The LUC construct seemed to show a decrease in luciferase

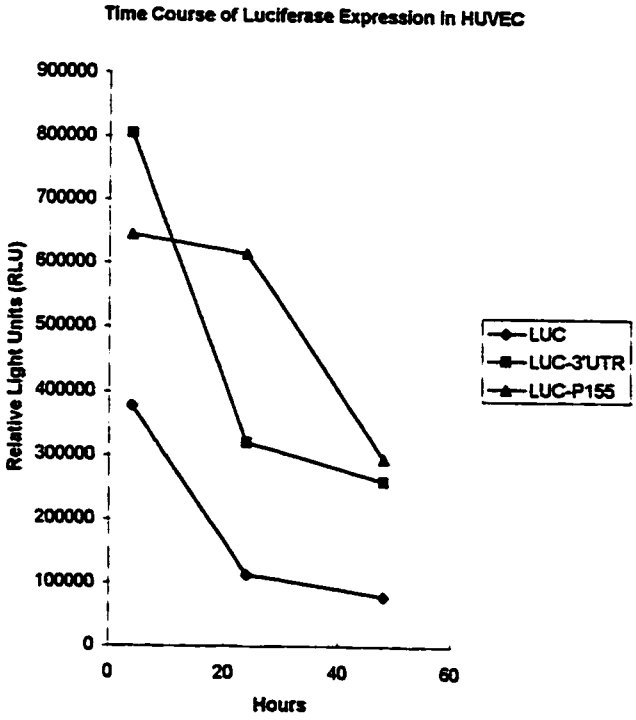
TIME COURSE of LUCIFERASE EXPRESSION
IN TRANSFECTED CELLS

(A) HUVEC

Hours	LUC	LUC-3'UTR	LUC-P155
4	377831	804673	644737
24	113493	320223	614678
48	79374	260051	295805

(B) PPAEC

Hours	LUC	LUC-3'UTR	LUC-P155
4	59003	150137	100152
24	2170659	5855112	4308042
48	35208	42672	109652
72	8803	4788	21670



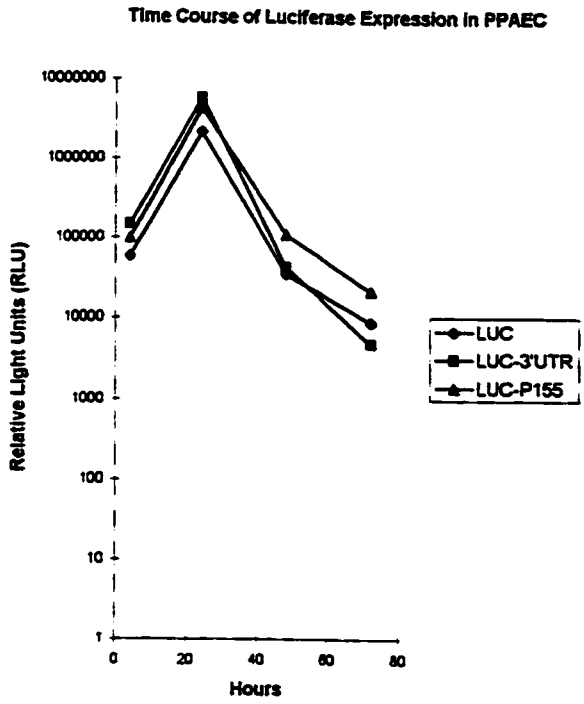


Figure 5.3 Time course expression of luciferase expression following transfection with LUC, LUC-3'UTR and LUC-P155 cDNA constructs in **(A)** HUVEC and **(B)** PPAEC. The relative light units (RLU) are linear scale for HUVEC and log scale for PPAEC.

Table 5.1

Time course of luciferase expression in COS cells following transfection of the luciferase (LUC) cDNA constructs.

	LUC	LUC-3'UTR	LUC-P155
4 hour	540,558	53,668	189,611
24 hour	6,323,244	567,024	4,308,834
48 hour	3,912,594	655,328	4,626,565
72 hour	2,555,160	873,265	2,198,900
96 hour	2,798,146	1,216,863	3,599,938

Luciferase is expressed as relative light units (RLU)/mg total protein.

Each data point is the mean of 2.

Table 5.2

Luciferase expression in HUVEC under control and TNFα-treated conditions 24 hours following transfection with luciferase (LUC) cDNA constructs.

Experiment Date	cDNA construct	CONTROL		TNFα (30 U/mL)	
8 Nov. '95	**LUC**	13452390	n=3	4369482	n=2
8 Nov. '95	**LUC-3'UTR**	31723429	n=3	55896429	n=2
15 Nov. '95	**LUC**	921877	n=6	528769	n=3
15 Nov. '95	**LUC-3'UTR**	620452	n=6	272496	n=3
6 Dec. '95	**LUC**	94172	n=4	232271	n=4
6 Dec. '95	**LUC-3'UTR**	335329	n=4	378784	n=4
6 Dec. '95	**LUC-P155**	674629	n=4	1198286	n=4

Luciferase is expressed as relative light units (RLU)/mg total protein.

Each data point represents a mean of two to six tissue culture dishes.

expression following exposure to TNFα, except for the last experiment in Table 5.2, which shows the opposite. The absolute values, i.e. the relative light units, varied so widely between experiments (Table 5.2) that this line of research was not pursued.

The luminometer was also tested for linearity. Different volumes of cell extracts containing luciferase ranging from 0.1-10 μL were added to the substrates and the light emitted measured in the luminometer. The results, presented in Figure 5.4, demonstrate that both the luciferase assay and the luminometer were linear since the intensity of the light emitted was proportional to the amount of luciferase-containing sample.

Table 5.3 and Figure 5.5 represent the level of luciferase protein in PPAECs following actinomycin D treatment. They illustrate that protein expression will go down once the transcription of a gene is prevented. What is interesting though is that the cells containing the LUC-3'UTR cDNA construct exhibited a greater level of decreased expression of luciferase at 1, 4, and 24 hour post-actinomycin D, compared to the cells transfected with either the LUC or the LUC-P155 construct (Figure 5.5). Yet before the addition of actinomycin D, PPAECs transfected with the LUC-3'UTR construct showed the highest expression of luciferase. This would infer that the products of the three constructs are regulated differently post-transcriptionally; in other words, the eNOS 3'UTR either confers an overall instability to the luciferase transcript, or interferes with its translation into protein. The LUC-P155 construct seemed to confer the highest stability to the luciferase mRNA, as depicted by the higher amount of protein expressed at the 4 hour time point, compared to the other two constructs at the same time point (Figure 5.5).

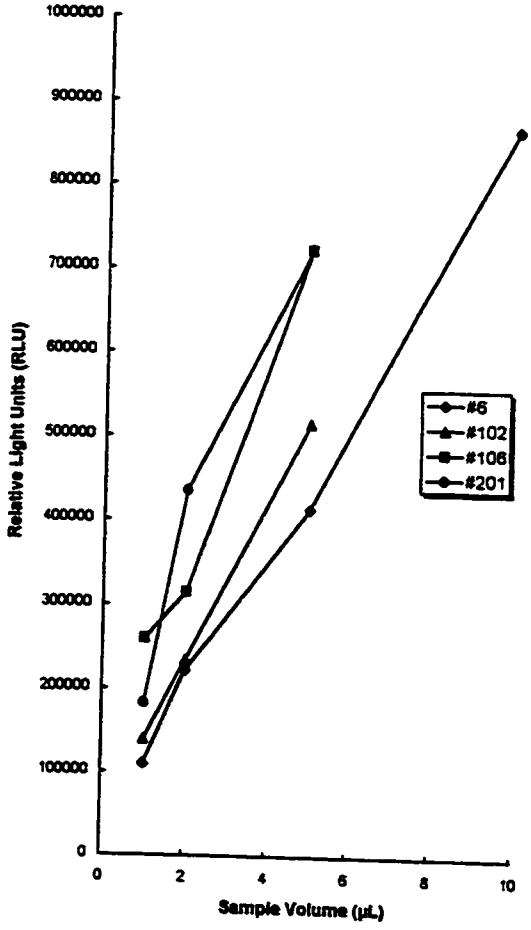
COS cells did not show any of this regulation, as shown in Table 5.4 and illustrated

LUCIFERASE ASSAY

TESTING LINEARITY of LUMINOMETER

	VOLUME of SAMPLE			
	1 µL	2 µL	5 µL	10 µL
SAMPLE #	1	2	5	10
#3	97353	130316	443319	too high
#6	110057	222131	414236	869888
#102	139211	234637	516078	too high
#106	259601	314495	722764	too high
#201	182872	434985	722940	too high

	VOLUME of SAMPLE				
	0.1 µL	0.25 µL	0.5 µL	0.75 µL	1.0 µL
SAMPLE #	0.1	0.25	0.5	0.75	1
#38	15326	34483	65107	108708	146413
#48	19384	51199	100625	130189	165119



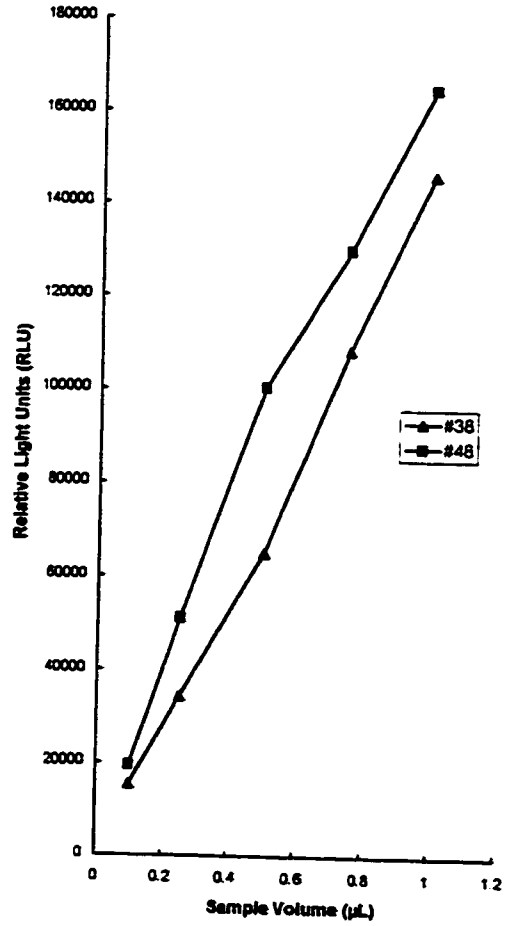


Figure 5.4 Testing the linearity of the luciferase assay and the performance of the luminometer by varying the sample volumes in the assay.

Table 5.3

Time course of luciferase protein expression following actinomycin D (2 μg/mL) treatment of porcine pulmonary artery endothelial cells (PPAEC) 24 hours after transfection with various luciferase (LUC) cDNA constructs.

	LUC	LUC-3'UTR	LUC-P155
0 hour	2164179	5872522	4298678
1 hour	1028044	1212121	2010788
4 hour	258816	302451	1711320
24 hour	43894	16865	41653
48 hour	11109	27174	41244

Luciferase is expressed as relative light units (RLU)/mg total protein.

Each data point is the mean of 2.

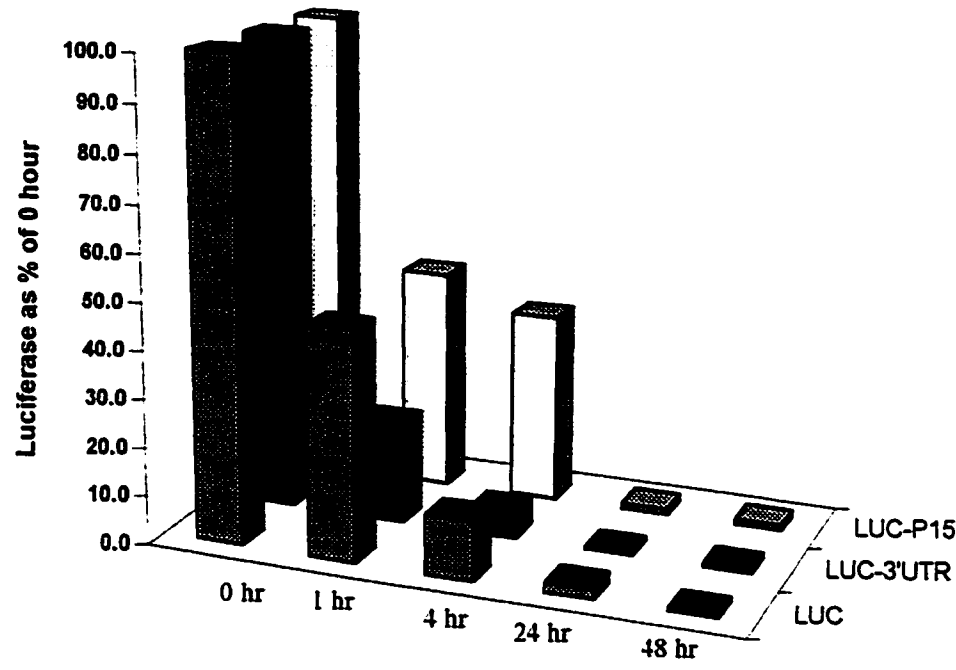


Figure 5.5 Time course of luciferase protein expression in PPAEC following addition of actinomycin D 24 hours after transfection with the three luciferase cDNA constructs. The amount of luciferase is expressed as a % of the 0 hour (set at 100%) of actinomycin D addition.

Table 5.4

Time course of luciferase protein expression following actinomycin D (2 μg/mL) treatment of COS cells 24 hours after transfection with various luciferase (LUC) cDNA constructs.

	LUC	LUC	LUC-3'UTR	LUC-3'UTR	LUC-P155	LUC-P155
	CON	TNFα	CON	TNFα	CON	TNFα
4 hour	268 mil.	390 mil.	309 mil.	344 mil.	524 mil.	451 mil.
24 hour	26 mil.	36 mil.	28 mil.	33 mil.	29 mil.	41 mil.
48 hour	5 mil.	7 mil.	7 mil.	10 mil.	12 mil.	20 mil.
72 hour	3 mil.	5 mil.	6 mil.	12 mil.	22 mil.	31 mil.

Luciferase is expressed as relative light units (RLU)/mg total protein in millions (mil.)

Each data point is the mean of 3.

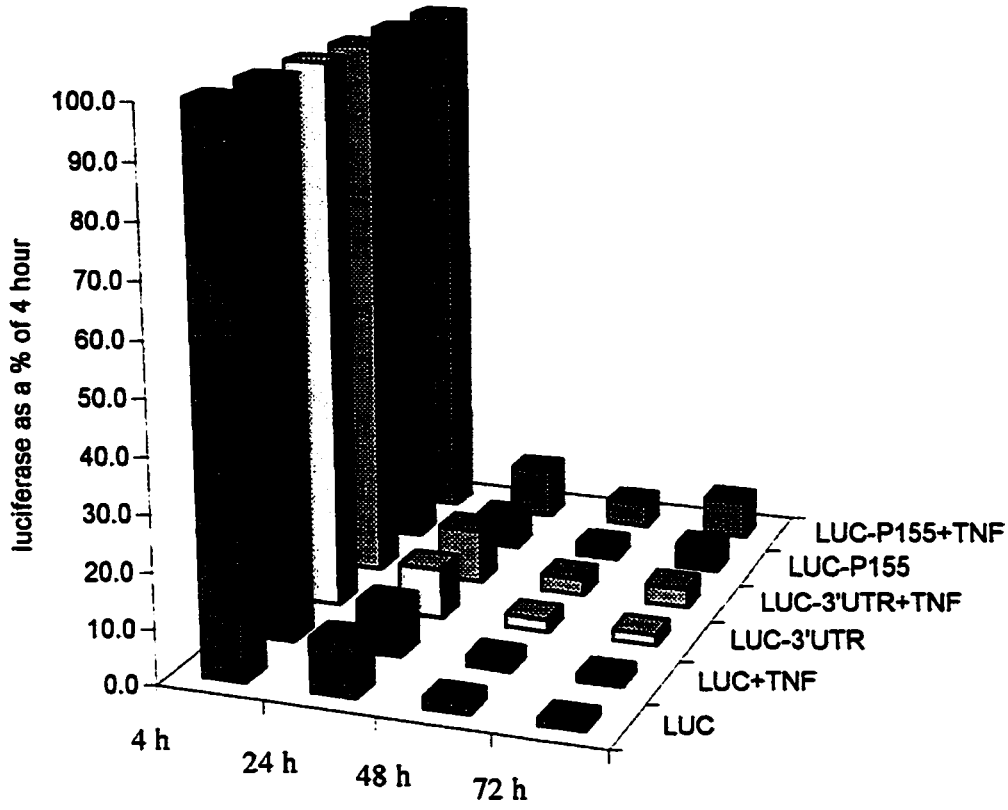


Figure 5.6 Time course of luciferase protein expression following addition of actinomycin D 24 hours after transfection with the three luciferase constructs. Amount of luciferase is expressed as a % of the 4 hour time point, which is set at 100%. The other three time points represent 24, 48 and 72 hours after actinomycin D addition. The transfected cells were exposed to either control or TNF-treated conditions.

in Figure 5.6. It is therefore possible that only endothelial cells, rather than non-endothelial cell types, carry all the factors or machinery needed for the post-transcriptional regulation of the eNOS mRNA via its 3'UTR. Another possible explanation may be that COS, being a transformed cell line, does not express the eNOS 3'UTR-binding proteins seen in other non-endothelial cell types, illustrated in Figure 5.2.

Since we were interested in monitoring the post-transcriptional regulation of the luciferase transcript, Northern blots were performed to observe the stability of each of the three luciferase transcripts following a time course of actinomycin D treatment on transfected cells. Cells used for these experiments included HUVEC and COS. Total RNA from HUVECs was probed using the eNOS cDNA probe, since the endogenous eNOS as well as the luciferase transcripts containing the eNOS 3'UTR or its mutant would show up on a Northern blot. Results of actinomycin D experiments with transfected HUVECs are shown in Figure 5.7. HUVECs displayed expression of the endogenous eNOS mRNA, an expected size of 4.1 Kb, at 0, 1, 4 and 24 hours post-actinomycinD, but the size of the luciferase transcripts in the same HUVECs were far bigger than expected (Figure 5.7). The luciferase gene was supposed to encode a mRNA of 1.7 Kb in length; the mRNA containing the full-length eNOS 3'UTR was supposed to be 2.11 Kb long, and that of luciferase with the P155 mutant at its 3' end was supposed to be 1.85 Kb long (Figure 5.1B). Probing the same Northern blot with a luciferase cDNA probe also revealed that there were several luciferase RNA bands per lane (data not shown), indicating that the STOP codon, engineered in the vector and situated after the SV40 polyadenylation signal, was not being recognized or was inefficient. What was tentatively observed though was that the eNOS mRNA was stable over

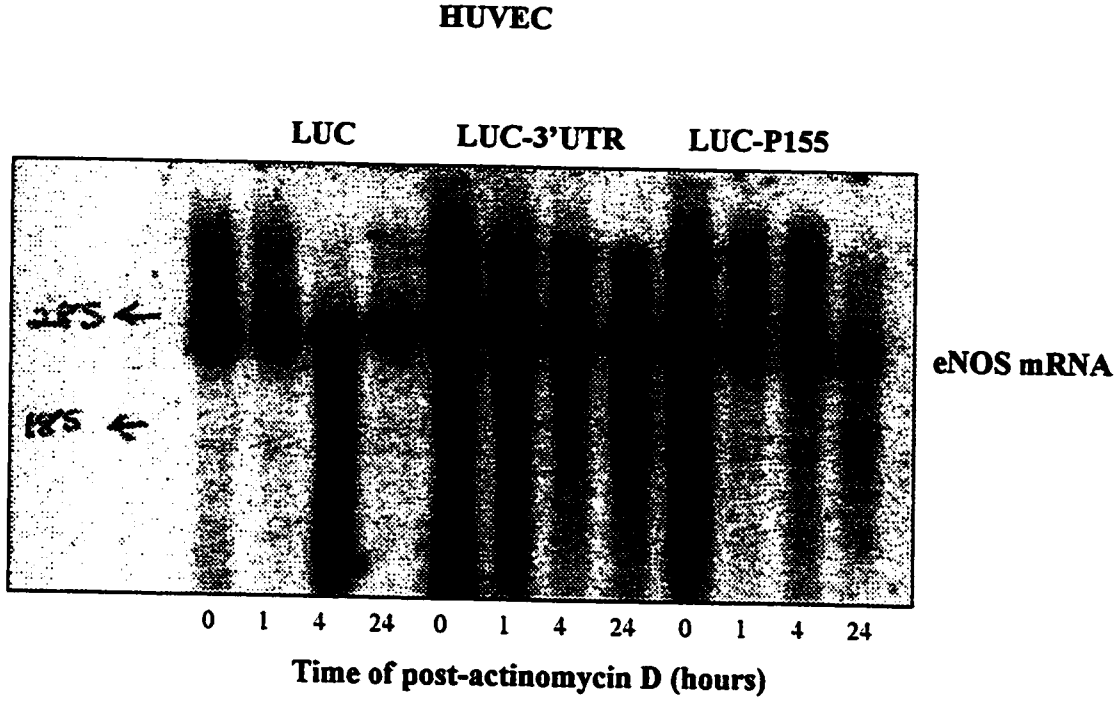


Figure 5.7 Northern blot of total RNA extracted from transfected HUVECs hybridized with the eNOS cDNA probe. The experiments represent a time course of actinomycin D treatment, where RNA was harvested at 0, 1, 4 and 24 hours post-actinomycin D. The first 4 lanes were transfected with LUC, middle 4 lanes with LUC-3'UTR, and the last 4 lanes with LUC-P155. The positions of the 28S and 18S ribosomal RNAs are delineated on the gel.

24 hours, but that the luciferase transcript seemed to degrade fairly rapidly (Figure 5.7).

COS cells do not produce any endogenous eNOS, therefore only the luciferase mRNA that contained the eNOS 3'UTR or its mutant could be detected with the radiolabeled eNOS 3'UTR cDNA probe (last 8 lanes of Figure 5.8A), whereas they were all detectable when hybridized to a luciferase cDNA probe (Figure 5.8B). Lanes 1-4 represent RNA extracted at 0, 1, 4, and 24 post-actinomycin D treatment from COS transfected with LUC; lanes 5-8, RNA from LUC-3'UTR transfected cells and lanes 9-12, RNA from LUC-P155 transfected COS at the same time points mentioned above. What was seen was that there was more than one band of the expected transcript per lane, again indicating that the STOP codon may not be fully functional, although in COS, the luciferase transcripts of the right size seemed to predominate (Figure 5.8). What the Northern blots showed was that the luciferase transcript was stable over the 4 hour time point, but had degraded by 24 hours irrespective of which luciferase cDNA construct was used. Therefore other experiments, where time points, between 4 and 24 hours following actinomycin D treatment, would be utilized to harvest RNA from transfected COS cells, would have had to be performed in order to pinpoint the half-lives of the three different luciferase transcripts.

5.3.3. *In vitro* decay: Figure 5.9 shows a representative gel from experiments utilizing HUVEC cytoplasmic extracts and different radioactive eNOS 3'UTR RNA species. It indicated tentatively that the intact eNOS 3'UTR was more unstable than its deletion mutants P305, P155, D285 and D132, since no intact 3'UTR RNA was detected at the 30 and 60 minute time points, as compared to the mutants. The full-length eNOS 3'UTR already showed

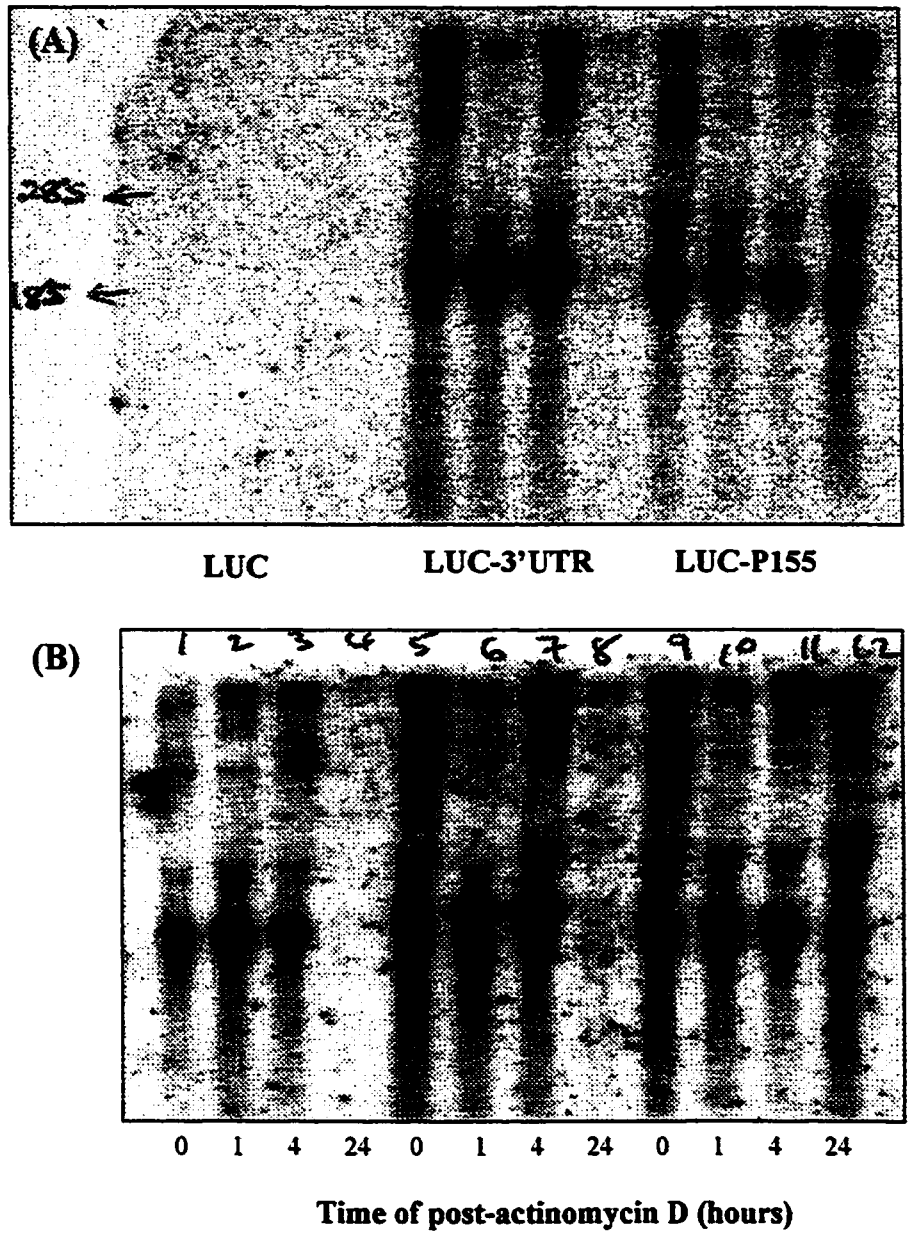


Figure 5.8 Northern blot of total RNA extracted from transfected COS cells hybridized with **(A)** eNOS 3'UTR or **(B)** luciferase cDNA probes. This represents a time course of actinomycin D treatment of COS cells transfected with either LUC (first 4 lanes) or LUC-3'UTR (middle 4 lanes) or LUC-P155 (last 4 lanes). RNA was harvested at 0, 1, 4 and 24 hours following actinomycin D (2 μg/ml). The positions of the 28S and 18S ribosomal RNA bands are shown on the gel in **(A)**.

signs of decay at 10 minutes (Figure 5.9); the mutant D285, which was made up of the last 285 bases of the eNOS 3'UTR and encompassed all three distal homologous domains (Figure 4.5), showed signs of progressive decay as did the mutant P305, consisting of the proximal 305 bases of the eNOS 3'UTR, encompassing domains 1 and 2 (Figure 4.5). Whereas the mutants P155 and D132, both lacking domain 1, seemed to be stable and had not undergone decay by 60 minutes (Figure 5.9). When polysomal extracts were used in these decay assays, as a potential source of RNA-binding proteins, inconsistent results were obtained (data not shown), for example, the stable mutants P155 and D132 exhibited a greater rate of decay with polysomal extracts. Therefore these experiments proved to be difficult to interpret.

5.3.4. Screening a HUVEC expression library: Using North-Western blotting to screen the HUVEC expression library, twelve pfus out of a million pfus were identified that turned blue with the substrates for alkaline phosphatase, and therefore agar plugs were taken at those sites. The phage particles were amplified and replated at a density of 1,000 - 2,000 pfus for easier separation of the different pfus that may have been picked up with the agar plugs. The same procedure was repeated, i. e. the plaques were lifted onto nitrocellulose, hybridized with the biotinylated eNOS RNA probe and subsequently visualized using substrates for alkaline phosphatase. What was baffling and surprising was that all the pfus turned blue and no explanation was forthcoming as to why that would happen. Whether radioactive eNOS RNA probes, rather than biotinylated ones, would have made a difference in the results is unknown.

5.3.5. Isolation of RNA-binding proteins by magnetic beads: Figure 5.10 shows the result

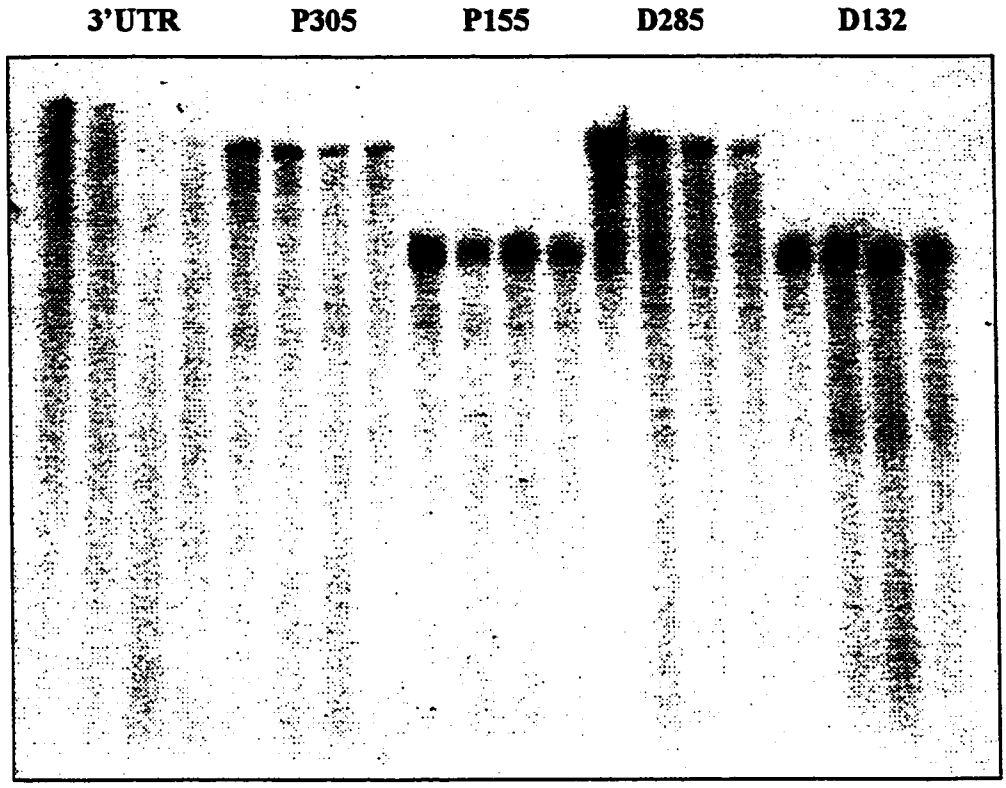


Figure 5.9 *In vitro* RNA decay, showing the decay rates of eNOS full-length 3'UTR and the deletion mutants P305, P155, D285 and D132 at 0, 10, 30 and 60 minutes following addition to HUVEC cytoplasmic cell extracts. The extracted RNA was run on a native gel. The various RNA species are distinguishable from their differences in length, except for P155 and D132.

of a silver stained gel that was run following the separation of cytoplasmic proteins that bound biotinylated eNOS 3'UTR RNA (from the cocktail of HUVEC cytoplasmic extracts) with streptavidin-coated magnetic beads. The lane containing the biotinylated RNA alone exhibited no silver staining (lane 8), as would be expected, since there were no proteins to elute. The protein marker lane exhibited staining all through the lane (lane 7), which could be due to overloading of the markers. Surprisingly, the other negative control, i. e. the lane containing HUVEC cytoplasmic proteins alone (lane 9) stained for the same proteins as the lanes that contained proteins eluted from the magnetic beads, even though there was no biotinylated RNA to bind and capture any eNOS RNA-binding proteins from the mixture. The gel also denoted no differences in the amount or type of proteins that were isolated by either the control or the TNFα-treated extracts as well as by the different eNOS 3'UTR RNA species (compare lanes 1 and 2 for 3'UTR, 3 and 4 for P305, 5 and 6 for D113).

5.3.6. Purification of eNOS 3'UTR-binding proteins: Figure 5.11A shows the result of a label transfer assay using radiolabeled eNOS 3'UTR RNA and the ammonium sulfate fractions (lanes 4 and 5) as well as cytoplasmic extracts from HUVECs (lane 1) and ECV-304 (lane 2), a spontaneously transformed HUVEC cell line that secretes the same factors as HUVECs, except that these cells do not senesce and present a compact cobblestone appearance, as opposed to HUVECs.. Some of the 66-kDa protein appeared in the 35% (lane 4) and 65% (lane 5) ammonium sulfate precipitates, whereas all the 56-, 53- and 45-kDa proteins precipitated out in the 35% ammonium sulfate pellet (lane 4). The 65% pellet also contained an intense band of a higher molecular weight protein, close to 200-kDa (lane 5). What was

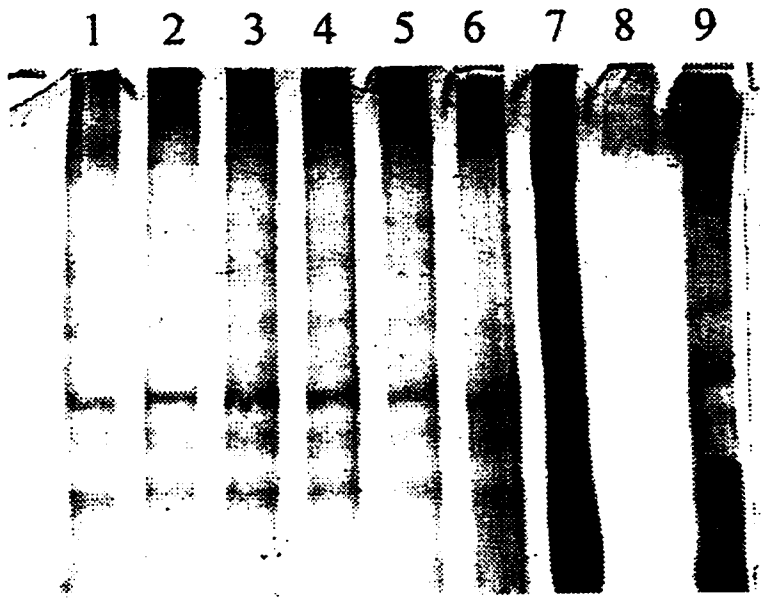


Figure 5.10 Silver stained gel following magnetic bead separation of the eNOS 3'UTR-binding proteins. The protein markers were run in lane 7, RNA alone in lane 8, and CON proteins alone in lane 9. Proteins under control and TNF-treated conditions were either bound to biotinylated 3'UTR (lanes 1 and 2 respectively), P305 (lanes 3 and 4 respectively), or D113 (lanes 5 and 6 respectively).

unknown was whether this 200-kDa band represented a single protein or a multimer of proteins, e. g. it could well be a trimer of the 66-kDa proteins or a heteromer of the 66-, 56-, 53- and 45-kDa proteins. Therefore the proteins that bound to the radiolabeled eNOS 3'UTR in the label transfer assay were subjected to reducing conditions, with the addition of β-mercaptoethanol in the sample buffer, before being separated by gradient SDS-PAGE. The same pattern of binding was seen with the 65% pellet under both reducing and non-reducing conditions (data not shown).

Figure 5.11B is the result of molecular size separation of the 65% ammonium sulfate precipitate using P-100. Fractions 36-39 seemed to be enriched in the 66-kDa protein as seen by label transfer assay, but the assay also revealed the higher molecular weight protein(s) to be present in the same fractions. P-100 most likely does not represent the right material to separate the 66-kDa protein from any higher molecular weight proteins in the mixture, since it is too close to the molecular weight cut-off. It is also quite possible that UV cross-linking of the proteins to the RNA in the assay, prior to digesting the unbound RNA with RNases and prior to running the mixture on SDS-PAGE, may strengthen any protein-protein interactions, such that they cannot be separated by reducing agents. UV cross-linking is necessary to strengthen the interaction between radiolabeled RNA and protein so that hydrogen bonding may turn into a stronger covalent bonding and thus enable the visualization of the RNA-binding proteins by SDS-PAGE followed by exposure to X-ray film or a phosphorimager.

Figures 5.12A and 5.12B show label transfer assays with fractions eluted after passing the reconstituted and dialyzed 35% ammonium sulfate precipitate through DEAE-cellulose. Fractions 9-14 seemed to be enriched in the 56-kDa protein (Figure 5.12A) while Figure

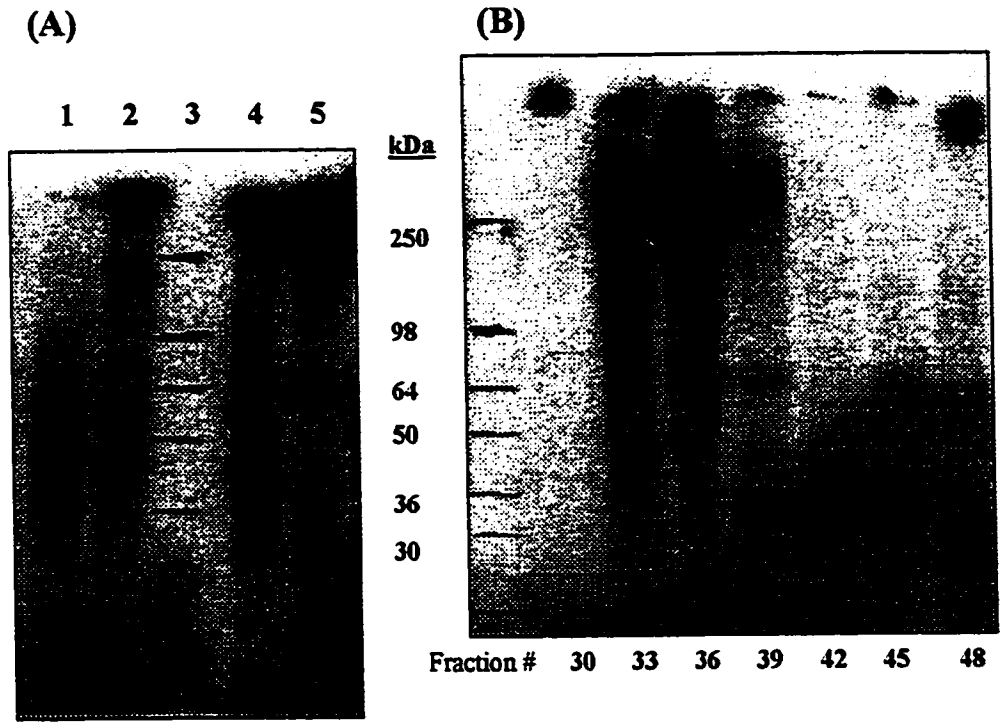


Figure 5.11 (A) Label transfer assay of radiolabeled eNOS 3'UTR with control cytoplasmic extracts from HUVEC (lane 1) and ECV-304 (lane 2) as well as with placental extracts from 35% (lane 4) and 65% (lane 5) ammonium sulfate precipitation. Lane 3 represents the protein markers. **(B)** Molecular size separation of 65% ammonium sulfate placental extract using P-100. Figures at the bottom of (B) represent fraction numbers. The gels have been aligned according to the protein markers denoted in between.

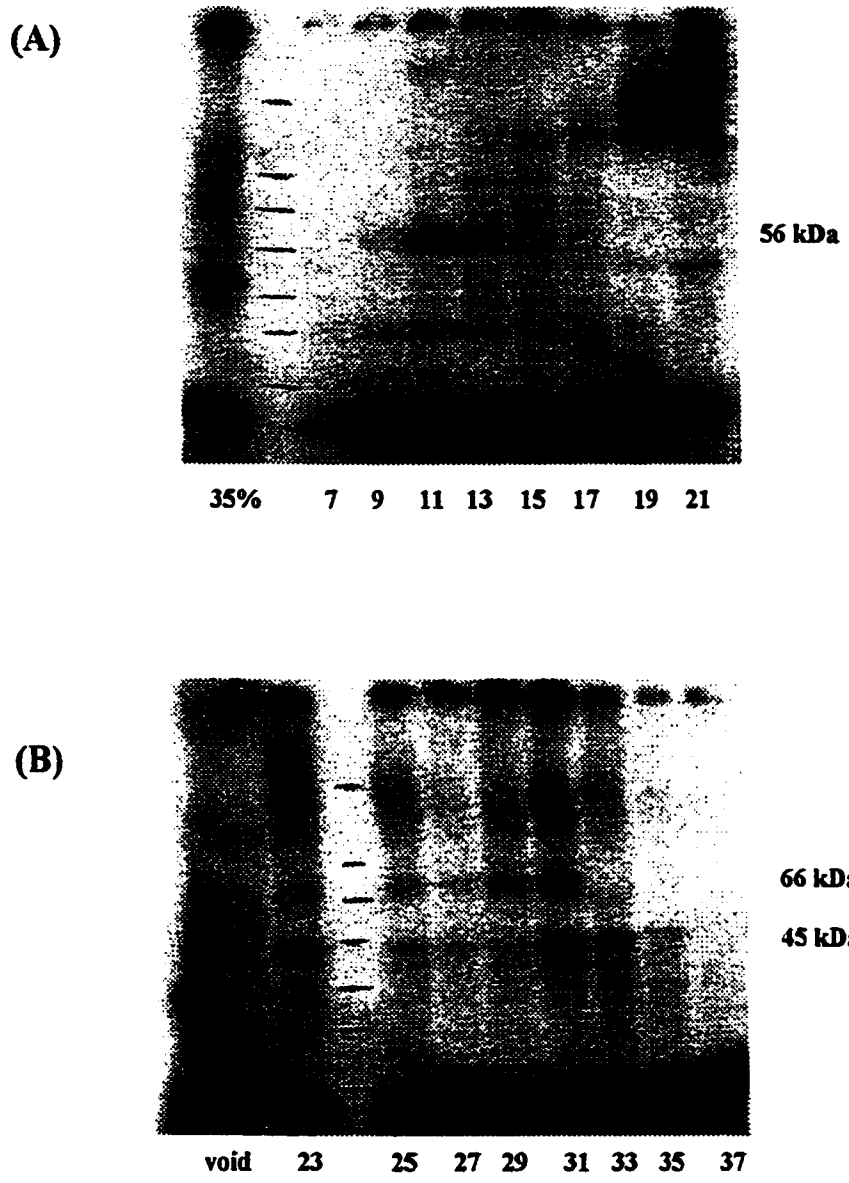


Figure 5.12 The gels **(A)** and **(B)** are the result of a label transfer assay with radiolabeled eNOS 3'UTR and various fractions eluted after passing the 35% ammonium sulfate extract through DEAE-cellulose. The positions of the 45-, 56- and 66-kDa RNP complexes are indicated on the right.

5.12B show up the 66-kDa protein band in fractions 23-31. But again, no clear cut separation of the proteins were achieved, since other molecular weight protein bands were faint but visible in those fractions (Figure 5.12). Fractions 9-14 were therefore subjected to RNA affinity chromatography as described in the methods and the results are presented in Figure 5.13. Label transfer experiments show that, while a small amount of the 56-kDa protein band present in the pooled fractions 9-14 (lane 2) was detectable in the void volume (lane 3), no RNA-binding protein eluted with 2 M KCl (lane 4) or with 4 M KCl (lane 5). The oligo(dT) column was monitored for its efficiency to bind polyadenylated eNOS RNA, as well as for its leakiness, by passing radioactive ^{32}P-labeled 3'UTR RNA through the column and measuring for radioactive ^{32}P in the void volume, the wash and the eluted fractions. The column seemed to bind RNA efficiently; 8-10% of the RNA was recovered in the void volume, another 8-10% recovered in the wash step, but only 1.5% in the elution steps. Thus the problem seemed to lie either in the elution of the RNA-binding proteins from the column or in the fact that very little or no proteins could bind to the immobilized RNA and therefore was not detectable in the elution steps.

5.4. Discussion:

Both the reporter gene experiments and the *in vitro* decay assay described above yielded preliminary data that indicated that the eNOS 3'UTR as a whole could confer instability to a messenger RNA. The instability element is likely present in the middle portion of the eNOS 3'UTR, since the mutants comprising either the very proximal or the very distal parts of the 3'UTR seemed to exhibit lower decay rates, but the results were not conclusive.

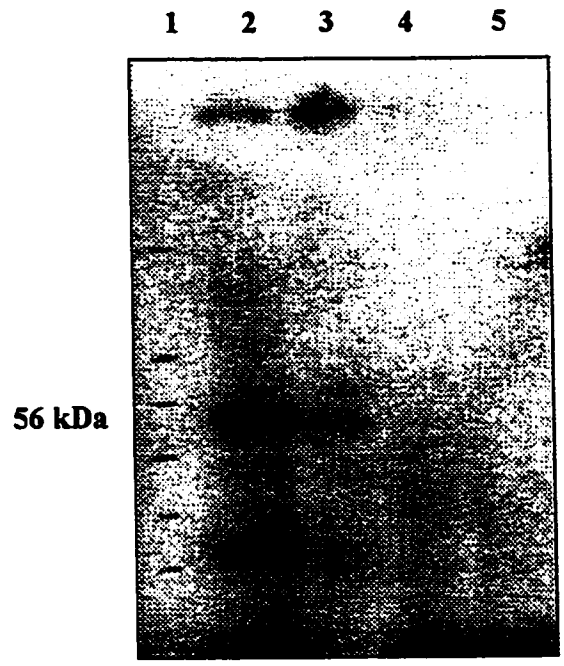


Figure 5.13 RNA affinity chromatography using eNOS 3'UTR RNA immobilized onto an oligo (dT) column. Lane 1 represents the protein markers; lane 2, pooled fractions 8-14 from DEAE-cellulose; lane 3, void volume; lane 4, 2M KCl eluted fraction; lane 5, 4M KCl eluted fraction. The above is a label transfer using radiolabeled eNOS 3'UTR with the extracts mentioned above.

The *in vitro* decay assay proved to be unsatisfactory since the half-life of the RNA species could only be measured in minutes rather than hours because of spontaneous degradation of RNA in the test tube at ambient temperature or at 37°C. *In vitro* decay experiments that explored longer time points revealed that all RNA species had decayed by 90 minutes, and TNFα-treated cytoplasmic extracts exhibited the same decay rates as control extracts (data not shown). One of the drawbacks of this method is that the decay rate of the eNOS 3'UTR is already highly accelerated *in vitro*. Therefore this method could not be utilized to assess the function of TNFα on the stability of the eNOS 3'UTR or its mutants.

The reporter gene experiments exhibited many drawbacks, the major one being the presence of more than one luciferase transcript within the cell, which therefore presented a degree of difficulty in the quantitation of the reporter gene stability. A few enzymes, like β-galactosidase, chloramphenicol acetyl transferase (CAT), and luciferase have been used as reporter genes because they are not produced by mammalian cells and are therefore easier to detect since there is no background interference from endogenous gene products. The luciferase constructs should most likely be re-engineered into other mammalian expression vectors, such as one that would utilize the bovine growth hormone polyadenylation signal for example, that could perhaps function more efficiently. Another drawback with this method is that the transfection efficiency of the reporter gene could not be quantitated with each experiment, and the possibility exists that this may vary between experiments. Vectors are now available that use the green fluorescent protein (GFP), such that the eNOS 3'UTR or any of its deletion mutants could be attached to the 3' end of the GFP cDNA and the expression of GFP within the transfected cells followed by microscopy.

It should also be noted that human ECs are not the easiest to transfect with liposomes, whose lipids fuse with the cell membranes and deliver the DNA of interest into the cells, which then transcribe the DNA and express the reporter protein. At the present time, there are many different types of liposomes available, which, for the purpose of this study, would have taken too long to explore for transfection efficiency. Other transfection agents are endosome-oriented rather than liposome based, in which case may function more efficiently in delivering the DNA of interest into the cells. Proprietory reasons do not allow the end user to gather all the information surrounding the technology that accompanies these transfection agents though. Adenoviruses would most likely be the best vector to introduce DNA into human cells, since these viruses that cause the common cold can easily get into human cells, but the cost of engineering adenoviruses remains high and out of reach for most research laboratories. Our laboratory utilized the agent lipofectin, which yielded a transfection efficiency of only about 5% in ECs.

Protein purification of the eNOS 3'UTR RNA-binding proteins proved to be a daunting task. More time and effort was needed to fine-tune and troubleshoot the techniques employed, as well as trying out new separation procedures. The streptavidin-coated magnetic bead method could have been explored further if there was a sufficient source of endothelial cell extracts. One of the problems may lie with the fact that the eNOS 3'UTR-binding proteins may represent such a minimal amount of the total cell extract that a larger amount of cellular proteins would have to be utilized in this procedure. Since the purpose of this thesis was to explore the mechanism(s) of RNA regulation, the major task of purifying the eNOS 3'UTR RNA-binding proteins was not undertaken.

Other RNA-binding proteins have been successfully purified from cell extracts. The IRP has been extracted from placental tissue by first fractionating homogenized extract through Sephacryl S-300 and then adsorbing the pooled fractions, that show IRP activity, onto heparin-Sepharose beads equilibrated in buffer containing a reducing agent. The bound proteins were then eluted with 250 mM KCl in 10 mM HEPES pH 7.5, 3 mM $MgCl_2$, 5% glycerol, and then passed through an RNA-affinity column containing immobilized RNA, which included the five IREs from the human transferrin receptor 3'UTR. The IRP was eluted with a buffer containing 1 M KCl, with an overall yield of 30% (Neupert et al., 1990).

Another method involves biotinylating RNA by *in vitro* transcription, mixing it with unfractionated cytosol, and then binding to avidin, such that the RNA-binding proteins would bind to the RNA, which is bound to avidin as a complex. The whole mixture is then subjected to biotinylated agarose beads with gentle mixing, after which the beads are allowed to pellet out of the solution so as to separate the RNA binding proteins from the rest of the mixture. Once the beads are washed, the proteins are eluted from the beads with high salt and utilized for gel mobility or label transfer assays to test for RNA binding. In this way, Rouault and his colleagues were able to purify IRP from human liver, but the recovery was only 0.2% (Rouault et al., 1989). Thus it is clear that protein purification of the eNOS 3'UTR RNA-binding proteins is a major undertaking and is outside the scope of this thesis.

6. CONCLUSIONS

This thesis has shown that the constitutively expressed human eNOS messenger RNA is down-regulated by the cytokine TNFα, and that the mechanism of down-regulation is of a post-transcriptional nature, which most likely involves the destabilization of the transcript. This process seems to occur by the differential regulation of binding of proteins that bind to the 3'UTR of the eNOS message. What was found was that many proteins bind to the eNOS 3'UTR under basal conditions, but their RNA-binding activity may be regulated under conditions where TNFα is abundant, such as in inflammation or viral infection. A 66-kDa RNP complex, formed with the distal AU-rich region of the eNOS 3'UTR, was found to be decreased in the presence of TNFα, inferring that this complex may function to stabilize the eNOS mRNA under control or physiological conditions. A 56-kDa RNP complex formed between cytoplasmic extracts and a CU-rich region, namely two CCUCU motifs located between nucleotides 3942 and 3963 of the eNOS mRNA, demonstrated the opposite, i. e. binding was increased in the presence of TNFα, suggesting that it may act to destabilize the eNOS transcript. It is still not entirely clear what the role of the 53-kDa RNP-forming complex is, even though preliminary data suggest that it may also act to destabilize the mRNA. The results presented in this thesis are in contrast to what has been reported previously in the literature about the stable genes exhibiting protein binding to the C- or CU-rich regions in their 3'UTR, but is in accordance with what has been reported for the destabilization of eNOS mRNA. López-Farré's group also reports the binding of a "destabilizing" protein to a C-/CU-rich region of the bovine eNOS 3'UTR, which is 60-kDa

in size (Sanchez et al., 1999). Although the *cis*-element it binds to contains only one CCUCU motif, (refer to Section 3.5.8 and Section 4.5), it seems to closely resemble the 56-kDa human eNOS 3'UTR-binding protein in size and function. It is entirely possible that the "stabilizing" and "destabilizing" proteins bind the eNOS mRNA even under normal conditions to maintain an equilibrium, such as to establish a basal level of eNOS mRNA and protein within the endothelial cell.

Since the human eNOS 3'UTR-binding proteins have not been characterized, it is not entirely clear whether the size of the proteins corresponds to the size of the RNP complexes that they form. It could be argued that no matter which construct was used in the label transfer assays, the size of the RNP complexes did not alter, and therefore it could be assumed that the RNP complexes actually represented or came close to the size of the proteins constituting them. It is also not known whether the 53- and 56-kDa RNP-forming proteins are different proteins or whether they may be different forms of the same protein, such as a phosphorylated or methylated form, since they mostly seemed to run as a doublet on SDS-PAGE and since their binding to the eNOS 3'UTR was up-regulated simultaneously in the presence of TNFα. Experiments would have to be performed, where the endothelial cell cytoplasmic extracts would have to be subjected to a phosphatase before use in label transfer assays with radiolabeled eNOS 3'UTR or any of the mutants. In that way, we would obtain some information as to whether any of the proteins bind as a phosphoprotein or not.

How would proteins binding the eNOS 3'UTR destabilze the mRNA? It can be speculated that binding of the "destabilizing" protein(s) to the 3'UTR of the eNOS mRNA could cause either the recruitment of nucleases to digest the transcript, or could trigger the

PABP, which usually binds the poly(A) tail and protects the mRNA, to dissociate from the mRNA, thus exposing the 3' end of the message to non-discriminating 3' exonucleases. In the same vein, the stabilizing 66-kDa RNP-forming protein might cooperate with the PABP to protect the 3' end of the transcript from marauding nucleases, such that removal of the 66-kDa RNP complex from the 3'UTR would also trigger the removal of the PABP from the poly(A) tail. Since protein molecules are relatively large compared to the area of the RNA where they bind, they would form large complexes that would surround the RNA and this is how they may prevent its degradation. Since the 66-kDa RNP complex is formed at the very distal 3' end of the eNOS 3'UTR, this may represent a plausible mechanism in that it protects the 3' end from marauding exonucleases. The binding of the "destabilizing" proteins could, on the other hand, allow or promote the targeting of the transcript to specific compartments of the cell, such as lysosomes, where it can be degraded. The exact mechanism(s) of degradation remains unknown.

It has to be pointed out that the experiments performed to dissect out the RNA-protein binding data, as outlined in Chapter 4, utilized the eNOS 3'UTR or its 5' and 3' deletional mutants rather than the whole eNOS mRNA itself. Protein binding to the eNOS coding region, or to the eNOS 3'UTR in the context of the entire intact eNOS mRNA, was not explored, the reason being the degree of difficulty associated with working with large transcripts, not knowing whether the entire message gets *in vitro* transcribed and stays intact in the test-tube, as well as with interpreting which region(s) of the entire mRNA would be responsible for binding to which protein. For the prupose of the study, the eNOS 3'UTR, a 419 base fragment, already represented quite a big molecule to handle and this presented

some difficulties with certain techniques, such as gel mobility shift assays and RNA affinity chromatography.

6.1. Suggestions for future study:

The function of the eNOS RNA-binding proteins could be further explored by introducing the eNOS 3'UTR cDNA into cells like HUVECs, such that it would get transcribed into RNA and sequester the eNOS 3'UTR RNA-binding proteins. Thereafter the amount of endogenous eNOS mRNA would be quantitated by Northern blotting to monitor the level of eNOS transcript present in the ECs under control and TNFα-treated conditions. The eNOS 3'UTR mutants, especially those that exhibited specific binding to one particular protein, as for example, M158 which bound the 56-kDa protein, or D113 that formed the 66-kDa RNP complex, would be used in the same manner to try and fine-tune the mechanism of binding. If the 66-kDa RNP complex bound to stabilize the eNOS mRNA, then introducing an RNA species in the ECs that would sequester this protein should result in enhanced degradation of the eNOS message, even under basal conditions. Vice versa, introducing an RNA species, that would sequester the 56- or 53-kDa proteins, should result in the stabilization of the eNOS message even under conditions of TNFα treatment. The greatest degree of difficulty with performing these kinds of experiments lies with the transfection efficiency of the cDNA species, which can vary from one experiment to the next. Therefore adenoviral delivery of the cDNAs would be a more efficient approach, since it can guarantee almost a 100% transfection efficiency.

The experiments from the preliminary characterization of the eNOS 3'UTR RNA-

binding proteins might provide an insight into strategies that would prove useful in the purification of these proteins. Exploring the functions of and purifying these RNA-binding proteins would constitute a whole study on its own and could be the basis for a future thesis.

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