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Identification of Normoxia-Responsive Elements in the Promoters of the Periportally Expressed Tyrosine Aminotransferase (TAT) And Serine Dehydratase (SerDH) Genes

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ABBREVIATIONS

AA	Amino acid
ACT	β-Actin
AHR	Arylhydrocarbon receptor
Akt	Corresponds to PKB (homolog of v-Akt)
ALD-A	Aldolase A
ANP-CR	Atrial natriuretic peptide clearance receptor
AP	Alkaline phosphatase
AP-1	Activator protein-1
Apo	Apolipoprotein
APS	Ammonium persulfate
ARNT	Arylhydrocarbon receptor-nuclear translocator protein
AT	Amino triazole
BMAL-1	Brain and muscle ARNT-like protein-1
BSA	Bovine serum albumin
bHLH	Basic helix-loop-helix
bp	Base pare
CAD	C-terminal activation domain
cAMP	Cyclic Adenosine-3´,5´-monophosphate
CAT	Chloramphenicol acetyltransferase
CBF	CCAAT-box binding factor
CBP	CREB-binding protein
C/EBP	CAAT/enhancer binding protein
cDNA	Copy desoxyribonucleic acid
Ci	Curie
CMV	Cytomegalie virus
CPS	Carbamoylphosphate synthetase
CRE	cAMP responsive element
CREB	CRE-binding protein
CSPD	Dinatrium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5´-chloro)-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-phenylphosphate
CYP2D	Cytochrome P450-2D
ddNTP	Didesoxy ribonucleoside triphosphate
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMSO	Dimethylsulfoxide
DMTU	Dimethylthiourea
DR0-TF	Transcription factor interacting with DR0 site
ds	Double strand
DSF	Desferrioxamine

DTE	Dithioerythritol
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
E. coli	Escherichia coli
EDTA	Ethylendinitrilo-N, N, N´, N´-tetra-acetate
EGTA	Ethylenglycol-bis-(2-aminoethylether)-N, N´-tetra-acetate
EMSA	Electrophoretic mobility shift assay
ENO	Enolase
EPAS	Endothelial PAS domain protein
EPO	Erythropoetin
Ets	Erythroblastosis factor
FBPase	Fructose-1,6-Bisphosphatase
FCS	Fetal calf serum
g	Gravity
GAPDH	Glycerinaldehyde-3-phosphate dehydrogenase
GK	Glucokinase
G-6-Pase	Glucose-6-phosphatase
GME	Glucocorticoid modulatory element
GNS	Glutamine synthetase
GPX	Glutathione peroxidase
GR	Glucocorticoid receptor
GRBS	Glucocorticoid receptor binding site
GRE	Glucocorticoid responsive element
GRU	Glucocorticoid responsive unit
HC	Hepatocytes
HeLa	Cell line HeLa
Hepes	2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate
HepG2	Hepatoma cell line HepG2
HIF1	Hypoxia inducible faktor-1
HLF	HIF1α-like factor
HNF	Hepatic nuclear factor
HO	Heme oxygenase
HPLC	High Performance Liquid Chromatography
HRE	Hypoxia responsive element
HRF	HIF-related factor
HRP	Horseradish peroxidase
Hx	Hemopexin
ID	Inhibitory domain
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

IκB	NFκB inhibitor
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
Ins	Insulin
kb	Kilo base
kDa	Kilo dalton
LB	Luria Bertani
LDH	Lactate dehydrogenase
LMW	Low molecular weight
LUC	Luciferase
MeS-TF	Methylation-sensitive transcription factor
MOP	Member of PAS superfamiliy
MOPS	3-(N-Morpholino)-propanesulfonic acid
MW	Molecular weight
NaAc	Sodium acetate
NAD	N-terminal activation domain
NaOH	Sodium hydroxide
NB	Northern Blot
NC	Nitrocellulose
NEFA	DNA binding/EF-hand/acidic
NIF	Normoxia inducible factor
NF	Nuclear factor
NLS	Nuclear localization signal
NRE	Normoxia responsive element
OD	Optical density
ODD	Oxygen-dependent degradation domain
ORE	Oxygen responsive element
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PAS	Per-AHR-Sim
pBS	Plasmid Bluescript
PCK	Phosphoenolyruvate carboxykinase
PCR	Polymerase Chain Reaction
PCV	Packed cell volumes
PEG	Polyethylen glykol
Per	Periodic (Drosophila protein)
PFK	Phosphofructokinase
PGK	Phosphoglycerat kinase
PH	Pleckstrin homology-domain
PI3K	Phosphatidylinositol-3-kinase

$PI(4,5)P_2$	Phosphatidyl-inositol-4,5-bisphosphat
PK_L	Pyruvatkinase type L
PK_M	Pyruvatkinase type M
PKB	Proteinkinase B
PLGF	Placental growth factor
PMSF	Phenylmethyl sulfonylfluoride
PTEN	Phosphatase tensin homolog
PVP	Polyvinylpyrrolidon
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
SerDH	Serine dehydratase
Sim	Single minded protein
SP-1	Stimulatory protein-1
ss	Single strand
SSC	Standard saline citrate
TAD	Transactivation domain
TAE	Tris acetate EDTA buffer
TAT	Tyrosin aminotransferase
TBE	Tris borate EDTA buffer
Tceb1	Transcription elongation factor elongin C (SIII) polypeptide 1-like protein
TEMED	N´, N´, N´, N´-Tetramethyldiamine
T4-PNK	T4 polynucleotide kinase
TNF	Tumor necrosis factor
TPA	Tissue type plasminogen activator
Tris	Tris-(hydroxymethyl)-aminomethan
Tse1	Tissue-specific extinguisher-1
uPA	Urokinase type plasminogen activator
USF	Upstream stimulating factor
UV	Ultraviolett
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
Vol.	Volume
X-Phosphate	5-Brom-4-Chlor-3-Indolyl phosphate
ZIP	Zipper

SUMMARY

In the liver, due to the unidirectional bloodflow from the portal vein and hepatic artery to the central vein and due to the oxygen-consuming metabolic processes of the cells along the sinusoid, an oxygen gradient is formed. This gradient in O_2 tension appears to play a key role in the zonated expression of genes such as phosphoenolpyruvate carboxykinase-1 (PCK-1). The PCK-1 mRNA and enzyme activity is maximally expressed in the more aerobic periportal zone. It was then found that a normoxia responsive element (NRE) in the PCK-1 promoter was responsible for the oxygen-modulated PCK-1 expression.

Similarly, NRE homologous sequences exist in the promoters of the periportal tyrosine aminotransferase (TAT) and serine dehydratase (SerDH). While regulation of SerDH expression by oxygen was not investigated yet, it was already demonstrated that the enzyme levels and activity of TAT in hepatocytes was higher under normoxia then under hypoxia. Thus, it was the aim of this study to characterize the oxygen-dependent expression of TAT and SerDH, to identify NRE's of the TAT and SerDH promoter as well as to identify putative candidate NRE binding factors.

Primary rat hepatocytes were cultured under normoxia (16% O_2) or mild hypoxia (8% O_2). TAT mRNA and SerDH mRNA were measured by Northern blot. The cells were transfected with TAT- and SerDH-promoter luciferase (Luc) gene constructs and cultured under normoxia (16% O_2) and hypoxia (8% O_2). Northern blot and transfections with –10095 TAT and –2303 SerDH promoter Luc constructs demonstrated that the TAT and SerDH expression was maximal under normoxia and about half-maximal under hypoxia. It was also shown that normoxia-dependent regulation of TAT and SerDH expression was independent from dexamethasone and glucagon.

Three putative NRE sequences homologous to the PCK-NRE1 were identified in the TAT promoter and designated TAT-NRE1 (-9531/-9524), TAT-NRE2 (-2651/-2644) and TAT-NRE3 (-112/-105). Four putative NRE's, SerDH-NRE1 (-2169/-2162), SerDH-NRE2 (-1904/-1897), SerDH-NRE3 (-578/-571) and SerDH-NRE4 (+35/+45), were found in the SerDH promoter. To determine which putative TAT and SerDH NRE's could be involved in the regulation of gene expression by normoxia, primary hepatocytes were transfected with the Luc gene constructs driven by the first 6100 bp (pGl3TAT-6100), 2556 bp (pGl3TAT-2556), 790 bp (pGl3TAT-790) and 356 bp (pGl3TAT-356) of the TAT promoter as well as by the first 2128 bp (pGl3SerDH-2128), 937 bp (pGl3SerDH-937) and 471 bp (pGl3SerDH-471) of the SerDH promoter. Deletions of TAT-NRE1 and TAT-NRE2 in the construct pGl3TAT-356 Luc as well as deletions of SerDH-NRE1 in the construct pGl3SerDH-2128 Luc completely abolished the modulatory effect of O_2 in transfected primary hepatocytes.

The O_2-response was observed, when primary hepatocytes were transfected with constructs containing 6 copies of putative TAT NRE's in front of the SV40-promoter and the Luc gene TAT-$[NRE1]_6$-Luc and TAT-$[NRE2]_6$-Luc. Furthermore, TAT-$[NRE1]_6$-Luc but not TAT-$[NRE2]_6$-Luc and not TAT-$[NRE3]_6$-Luc expression was also modulated by O_2 in HepG2 and HeLa cells. With the SerDH NRE constructs an O_2-response was only observed with the SerDH-$[NRE2]_6$-Luc construct in both HeLa and HepG2 cells and with the SerDH-$[NRE1]_6$-Luc in HeLa cells. Though the TAT-NRE1 sequence, which functioned best, showes some sequence similarity with a CRE (cAMP responsive element) transfection experiments with TAT-NRE1 Luc constructs indicated that TAT-NRE1 did not function as a cAMP responsive element.

It was further demonstrated in electrophoretic mobility shift assays (EMSA's) with ^{32}P-labelled NRE oligonucleotides and nuclear extracts from cells cultured under 16% O_2 and 8% O_2 that the TAT-NRE1 oligonucleotide was able to bind a complex which was formed much stronger with nuclear extracts from cells cultured under normoxia than with nuclear extracts from cells cultured under hypoxia. Competition experiments, mutation of the NRE-sequences as well as supershift analysis indicated that the TAT-NRE1 was bound by a so far unknown factor. To identify a factor binding to the NRE a yeast one hybrid assay was performed using a yeast strain with genomicly integrated NRE-containing construct and a rat liver cDNA library allowing expression of fusion proteins from different library proteins and Gal4 transactivation domain. As a result 78 clones which could grow on the medium lacking histidine and containing 3 amino triazole (3-AT) were analysed. It was found that 25 K nuclear protein, transcription elongation factor elongin C (SIII) polypeptide 1-like protein (Tceb 1L), DNA binding/EF-hand/acidic protein (NEFA) and ferritin may have the functional capacity to bind to the NRE and to function as putative normoxia responsive element binding proteins.

These results demonstrated that normoxia may not only have a role for the zonation of TAT and SerDH gene expression in liver but also for a wider set of genes in different cells or tissues.

1. INTRODUCTION

1.1 Regulation of gene expression by oxygen

The ability of an organism to adapt to changes in the oxygen concentration is essential for its survival. Accordingly, the expression of numerous genes, products of which are involved in various processes such as hematopoesis, fibrinolysis or carbohydrate metabolism is regulated by oxygen (Tab. 1).

Under physiological conditions the mean oxygen tension in arterial blood is maintained at levels of 74-104 mm Hg (normoxia) and in venous blood at 34-46 mm Hg (mild hypoxia). Oxygen tensions higher than physiological (hyperoxia) could lead to cellular damage due to production of reactive oxygen species (ROS) while oxygen tensions lower than physiological (hypoxia) is dangerous because oxygen is necessary for the functioning of the mitochondrial electron transfer chain and many other metabolic pathways.

It is believed that the general scheme of the oxygen-dependent gene regulation in the case of either hypoxia or normoxia in mammals is similar (Fig. 1). The first stage in this process is oxygen sensing by a sensor molecule that could alter its conformation and/or enzymatic activity under different oxygen concentrations. The next step is signal transduction due to the production of mediators or second messengers which modify regulatory molecules such as DNA-binding transcription factors. These oxygen-modulated transcription factors, which may be different for hypoxic and normoxic conditions, bind in the final step specific response elements in the promoters of oxygen-regulated genes.

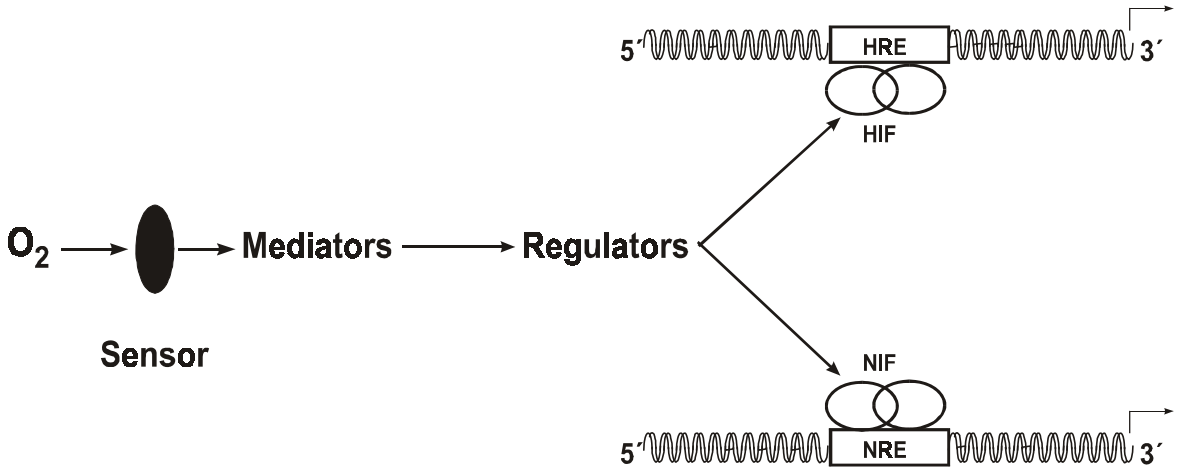


Fig. 1: **Model of the oxygen signalling pathway regulating gene expression.** See text for explanation. HRE, hypoxia response element; NRE, normoxia response element; HIF, hypoxia-inducible factor; NIF, normoxia-inducible factor

Tab. 1: **O_2-modulated processes and genes in mammals.**

Processes	Hypoxia-induced gene expression	Normoxia-induced gene expression
Hematopoesis	Erythropoietin (EPO)[1]	
Angiogenesis	Vascular endothelial growth factor (VEGF)[2]	Placental growth factor (PLGF)[3]
Fibrinolysis	Plasminogen activator inhibitor-1 (PAI-1)[4]	Plasminogen activators [5]
Wound healing/ Inflammation	Inducible nitric oxide synthase (iNOS)[6]	Tumor necrosis factor-α (TNF-α)[7]
Development/ Differentiation	p53 Tumor supressor protein[8]	
	bcl-2 Apoptosis preventing protein[9]	
	Insulin like growth factor binding protein-1 (IGFBP-1)[10]	
Antioxidant defense		Glutathione peroxidase[11]
		Catalase[12]
		Superoxid dismutases[13]
Amino acid metabolism		Tyrosine aminotransferase[14]
Glykolysis/ Gluconeogenesis	Glucokinase (GK)[15]	Cytosolic phosphoenol- pyruvate carboxykinase-1 (PCK-1)[16]
	Aldolase A (ALD-A)[17]	
	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[18]	
	Phosphoglycerate kinase-1 (PGK-1)[19]	
	Pyruvate kinase L (PK_L)[20]	
	Pyruvate kinase M (PK_M)[21]	
	Lactate dehydrogenase A (LDH-A)[22]	

(1) (Jiang et al., 1996); (2) (Forsythe et al., 1996; Levy et al., 1995; Liu et al., 1995); (3) (Gleadle et al., 1995); (4) (Kietzmann et al., 1999; Samoylenko et al., 2001; Kietzmann et al., 2003b); (5) (Pinsky et al., 1998); (6) (Melillo et al., 1995); (7) (Wibbenmeyer et al., 1995); (8) (Koumenis et al., 2001); (9) (Tamatani et al., 1998); (10) (Tazuke et al., 1998); (11); (12) (Li et al., 1989); (13) (Jackson et al., 1996); (14) (Nauck et al., 1981); (15) (Kietzmann et al., 1997); (16) (Hellkamp et al., 1991); (17); (18) (Graven et al., 1999); (19) (Firth et al., 1994); (Semenza et al., 1996); (20) (Krones et al., 2001); (21) (Iyer et al., 1998); (22) (Firth et al., 1995).

1.1.1 Oxygen gradients and metabolic zonation of the liver

Oxygen concentration gradients are formed due to differences in the blood supply within some organs and tissues. Such gradients exist physiologically in organs such as liver and to some extend in kidney, during fetal development and pathophysiologically during tumor formation.

In the liver, the smallest functional unit is represented by the acinus. Within the acinus the blood flows from the region around the hepatic artery and terminal portal vein (periportal zone) into the sinusoids towards the cells located in the region around the central vein (perivenous zone) (Jungermann et al., 1996b; Jungermann et al., 2000). Due to the metabolism of the cells within the sinusoid an oxygen gradient is formed reaching from about 60-65 mm Hg in the periportal area to about 30-35 mm Hg in the perivenous zone. Furthermore, the expression of most genes for enzymes, translocators and receptors is different between the cells of the two zones (Fig. 2). Many genes are expressed predominantly in the first, periportal, or in the second, perivenous, half of the acinus while some genes are expressed or inhibited within only the first or the last quarter of the acinus. The expression of the genes for the rate-controlling enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase-1 (PCK-1) and fructose-1,6-bisphosphatase

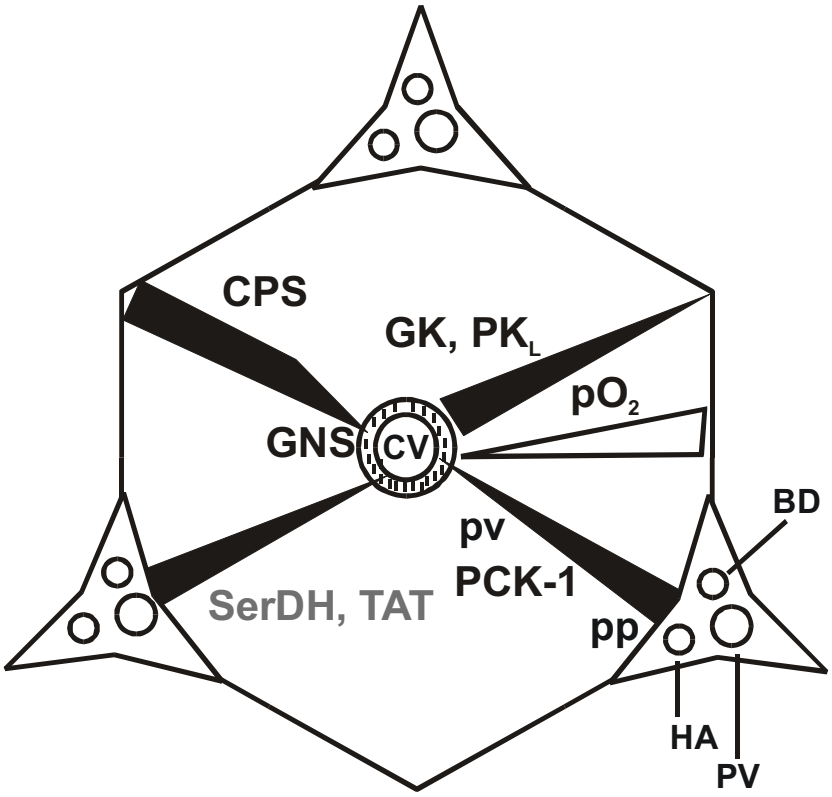


Fig. 2: **Zonation of gene expression in liver.** Scheme of the liver acinus and existing gradients in gene expression as well as of the oxygen tension (pO_2). BD, bile duct; CPS, carbamoylphosphate synthetase; CV, central vein; GK, glucokinase; GNS, glutamine synthetase; HA, hepatic arteriole; PK_L, pyruvate kinase liver type; pp, periportal; PV, portal venule; pv, perivenous; SerDH, serine dehydratase; TAT, tyrosine aminotransferase. Arrows give the gradients in expression.

(FBPase), as well as for the amino acid metabolizing enzymes serine dehydratase (SerDH) and tyrosine aminotransferase (TAT), which are related to gluconeogenesis, are expressed predominantly in the periportal zone, as has been shown for the mRNA, proteins and enzyme activities (Jungermann et al., 1989; Bartels et al., 1990; Ogawa et al., 1994; Ogawa et al., 1995). Furthermore, the key ureagenic enzyme carbamoylphosphate synthetase (CPS) is also expressed periportally (Moorman et al., 1988). In contrast, the genes for the rate-controlling glycolytic enzymes, glucokinase (GK) and pyruvate kinase type L (PK_L), are expressed mostly in the perivenous zone (Jungermann et al., 1989). The mRNA and protein for glutamine synthetase (GNS) were detected exclusively in parenchymal cells of the distal perivenous area (Fig. 2) (Gebhardt et al., 1988; Moorman et al., 1988). These zonal differences in the gene expression pattern constitute the basis for the zonal differerent metabolic capacities. Thus, the capacity for oxidative energy metabolism, glucose output, protective metabolism, urea, cholesterol and bile formation is greater in the periportal area, whereas the capacity for glucose uptake, glutamine formation and xenobiotic metabolism is higher in the perivenous area. Due to the fact that all hepatocytes possess the same genome it was considered that concentration gradients of hormones, metabolic substrates, products and oxygen which are formed during the passage of blood through the sinusoid may be determinants for the zonated gene expression. In line with this, it was shown that the periportal to perivenous gradient in oxygen tension appears to be a key regulator for expression of the periportal PCK and perivenous GK in liver.

1.1.2 Regulation of zonated gene expression by normoxia

The genes such as PCK-1 which are mainly expressed under periportal or normoxic conditions could be either specifically inhibited by low oxygen tensions or induced by a normoxia-induced transcription factor or factors. In addition to PCK-1, other genes induced by normoxia have been identified (Tab. 1), among these are placental growth factor (PLGF) (Gleadle et al., 1995), tissue-type and urokinase-type plasminogen activators (tPA and uPA) (Pinsky et al., 1998), atrial natriuretic peptide clearance receptor (ANP-CR) (Sun et al., 2000), and antioxidant enzymes such as glutathione peroxidase (Li et al., 1989), catalase (Li et al., 1989), manganese-containing and copper/zinc-containing superoxide dismutase (Jackson et al., 1996). In particular, normoxia-induced expression was demonstrated for a number of genes which are predominantly expressed in the periportal region of the liver acinus, such as the heme-binding protein hemopexin (Kietzmann et al., 1995), gluconeogenic enzymes phosphoenolpyruvate carboxykinase-1 (PCK-1) (Hellkamp et al., 1991) and tyrosine aminotransferase (TAT) (Nauck et al., 1981). However, the mechanisms, regulatory transcription factors and the DNA

responsive elements required for normoxia-dependent gene regulation are not completely known and a general view has not been reached.

Many models for oxygen-dependent gene regulation involve signal transduction via oxygen-binding hemproteins and generation of reactive oxygen species (ROS) as mediators. The role of a heme-containing protein as oxygen sensor is based on experiments in which carbon monoxide (CO) which stabilizes the oxy conformation of heme as well as heme synthesis inhibitors such as succinylacetone prevented the response to hypoxia. By contrast, cobalt and nickel, which are incorporated into heme, locked its deoxy state thus mimicking hypoxia (Goldberg et al., 1988; Kietzmann et al., 1997; Jungermann et al., 1997). Spectrophotometric experiments with carotid body preparations and HepG2 cells showed that heme-containing b-type cytochromes such as the NAD(P)H oxidase (Gorlach et al., 1993; Fandrey et al., 1994a) may have a role in the oxygen signalling pathway. By contrast, other experiments with cardiomyocytes pointed also to a role of cytochrome c oxidase in oxygen sensing (Wilson et al., 1994).

It was shown that production of intracellular ROS such as H_2O_2 was increased by high and decreased by low oxygen tension (Kietzmann et al., 1997). Chelators of iron such as desferrioxamine can mimic hypoxia (Wang et al., 1993; Gleadle et al., 1995) which could be explained by the assumption that hydroxyl anions and hydroxyl radicals, produced in the presence of Fe^{2+} from H_2O_2 in a Fenton reaction, were also involved in the transduction of the oxygen signal. This was corroborated for the PCK-1 gene since H_2O_2 mimicked the action of periportal pO_2 (Kietzmann et al., 1996) and two Fenton reaction inhibitors, the iron chelator desferrioxamine (DSF) and the hydroxyl radical scavenger dimethylthiourea (DMTU) (Kietzmann et al., 1998) mimicked perivenous pO_2.

Until now, a common normoxia responsive DNA element in the promoters of normoxia-regulated genes has not been identified. Two oxygen-responsive elements (ORE-1 and ORE-2), which shared the consensus sequence 5'-AYCCTCYRAGAAA-3' (Y=A or T; R=A or G), have been identified in the 5'-flanking region of the human glutathione peroxidase (GPX) gene (Cowan et al., 1993).

In experiments with primary rat hepatocytes, a normoxia response element (NRE) 5'-TTAGGTCAG-3' was located in the PCK-1 promoter at –146/-138 (Bratke et al., 1999) and shown to be involved in the positive modulation by oxygen of the glucagon-dependent PCK-1 expression (Bratke et al., 1999). Although the respective transcription factor binding to the NRE is unknown yet, it might be possible that similar elements and factors may also account for the periportal expression of other genes.

1.1.3 Regulation of zonated gene expression by hypoxia

Many physiological and pathophysiological conditions, such as wound healing, coronary heart disease, chronic lung disease and cancer are associated with hypoxia. The common response to the hypoxic conditions includes activation of genes whose products either increase availability of oxygen, such as erythropoietin (EPO) (Goldberg et al., 1988) and vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), promote metabolic adaptation to decreased oxygen availability, such as glucose transporters (Loike et al., 1992) and glycolytic enzymes (Semenza et al., 1996), or facilitate cell survival, such as insulin-like growth factor 2 (IGF-2) (Feldser et al., 1999) and IGF binding proteins (IGFBP-1, -2, -3) (Tazuke et al., 1998; Feldser et al., 1999).

The mechanisms by which hypoxia stimulates gene activation are not understood to the last detail. Although the nature of an cellular oxygen sensor is not clear yet, the mechanisms of the hypoxia-dependent gene regulation by an appropriate transcription factor, hypoxia inducible factor (HIF), are much better understood (Fig. 3). It was shown that promoters of most genes induced by hypoxia contain a specific O_2 responsive sequence, named the hypoxia response element (HRE) with the consensus sequence 5'-RCGTG-3' (Semenza et al., 1996). This sequence is bound by a family of transcription factors known as the hypoxia-inducible factors (HIF) from which HIF-1 is the best characterized. HIF-1 is a dimer of HIF-1α and HIF-1β (ARNT, arylhdrocarbon receptor nuclear translocator) both belonging to the basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) transcription factor family (Wang et al., 1995a; Wang et al., 1995b). Two other HIF α -subunits, HIF-2α (also known as endothelial PAS domain protein-1 (EPAS-1), member of the PAS (Per-ARNT-Sim) superfamily-2 (MOP-2), HIF-related factor (HRF), HIF-like

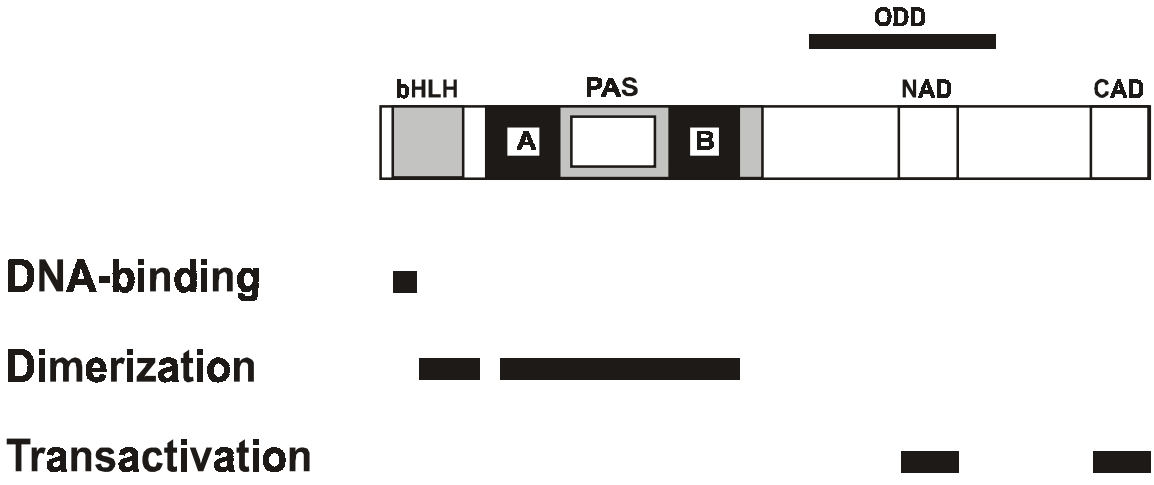


Fig. 3: **Scheme of HIF-1α.** The HIF-1α subunit is shown with basic helix-loop-helix (bHLH) (aa 17-70) and PAS (Per-ARNT-Sim) (aa 106-350) (with A (aa 106-156) and B (aa 249-299) repeats) DNA-binding and dimerization domains, the N-terminal (NAD) (aa 531-575) and C-terminal (CAD) (aa 786-826) transactivation domains. The O_2-dependent degradation domain (ODD) (aa 401-603) is also depicted. HIF-1α has 826 amino acids (Jiang et al., 1996; Semenza, 1999).

factor (HLF)) and HIF-3α, as well as two another ARNT isoforms, ARNT2 and ARNT3 (also known as brain and muscle ARNT-like protein-1 (BMAL-1) or MOP-3), have been identified and the existence of several HIF-dimers composed of different HIF α-subunits and ARNT isoforms was shown (Hirose et al., 1996).

While ARNT was found to be a constitutional protein, HIF-1α protein levels were dependent on the level of hypoxia. Although hypoxia may have small effects on the HIFα mRNA expression, the major regulation appears to occur posttranslationally on the level of protein stabilization (Semenza, 1999). The protein stabilization is conferred by the so called O_2-dependent degradation domain (ODD) which overlaps in part with the N-terminal activation domain (NAD) (Maxwell et al., 1999). Under normoxia two proline residues (P402 and P564) within the ODD are subject to hydroxylation by a new family of prolyl hydroxylases (Epstein et al., 2001; Bruick et al., 2001). The hydroxylation enables the binding of the von Hippel-Lindau (VHL) tumor supressor protein, a component of an E3 ubiquitin ligase complex that targets the HIF α-subunits for degradation by the ubiquitin-proteasome pathway (Jaakkola et al., 2001; Ivan et al., 2001).

Since the prolyl hydroxylases use beside iron and 2-oxoglutarate oxygen as cofactor they were proposed to act also as cellular oxygen sensors (Epstein et al., 2001; Bruick et al., 2001; Hewitson et al., 2002; Lando et al., 2002).

Furthermore, an asparaginyl hydroxylase was shown to be critical for the function of HIF since the oxygen-dependent hydroxylation of an aspargine residue (N803 in HIF-1α and N852 in HIF-2α) within the C-terminal transactivation domain (CAD) prevents the recruitment of the coactivator CBP/p300 and thus the function of HIF (Hewitson et al., 2002; Lando et al., 2002). For the liver it was shown that all three rat HIF α-subunits (HIF-1α, HIF-2α, HIF-3α) were expressed predominantly in the perivenous zone of rat liver (Kietzmann et al., 2001) and may be involved in the zonated expression of perivenously expressed enzymes such as GK and PK_L.

1.2 Regulation of tyrosine aminotransferase (TAT) and serine dehydratase (SerDH) gene expression

1.2.1 Tyrosine aminotransferase

The periportally localized L-tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) is a pyridoxal phosphate-dependent enzyme catalyzing the first step in tyrosine degradation, a transamination reaction resulting in the formation of para-hydroxyphenylpyruvate and L-glutamate (Dietrich, 1992). It is a 454 amino acid protein with the molecular mass of 50 kDa. An about 2.4 kb-long rat TAT mRNA is transcribed from a gene of 11 kb interrupted by 11 introns. TAT deficiency in humans

results in tyrosinemia type II (Richner-Hanhart syndrome) characterized by keratitis, palmoplantar hyperkeratosis, mental retardation and elevated blood tyrosine levels (Huhn et al., 1998).

TAT gene transcription is restricted to the parenchymal cells of liver. TAT expression, like expression of many other gluconeogenic enzymes, is regulated developmentally, since onset of gluconeogenesis occurs around the time of birth (Mcgrane et al., 1992). TAT enzyme activity is virtually absent in fetal rat liver, starts to increase within the first hours after birth and is already stabilized at adult levels by day 2. Glucagon and glucocorticoids positively and insulin negatively affects TAT expression, respectively (Dietrich, 1992).

Many transcription factors binding to the 5' region of the rat TAT gene were identified (Fig. 4). Inside the TAT proximal promoter the sequences spanning -288 to -283 bp, -281 to -268 bp, -175 to -156 bp and -85 to -57 bp were shown to interact with the liver-enriched or ubiquitous transcription factors, namely CCAAT-box binding factor (CBF), hepatocyte nuclear factor-1 (HNF-1), nuclear factor-1 (NF-1) and nuclear factor-Y (NF-Y) (Schweizergroyer et al., 1994) (Fig. 4).

The glucocorticoid-dependent activation of the TAT gene is mediated via cooperative interaction of two glucocorticoid-responsive units (GRU) located at –2.5 and –5.4 kb, (Jantzen et al., 1987; Grange et al., 1989). The TAT GRU at –2.5 kb (ca. -2620/-2320, the precise length of the GRU is not clear since new sites for the binding of regulatory proteins are still being found (Thomassin et al., 2001)) consists of numerous, often overlapping, binding sites for transcription factors such as glucocorticoid receptors (GR), methylation-sensitive transcription factor (MeS-TF), transcription factor interacting with the DR0 site (DR0-TF) and members of the CCAAT/enhancer-binding protein (C/EBP), hepatocyte nuclear factor-3 (HNF-3), stimulatory protein-1 (SP-1) and erythroblastosis factor (Ets) families (Thomassin et al., 2001; Grange et al., 2001). The TAT GRU at –5.4 (-5857/-5362) is much less investigated. It was shown that it contained one functional glucocorticoid receptor binding site (GRBS) (-5450/-5436) that was necessary to achieve the physiological levels of glucocorticoid stimulation (Grange et al., 1989). Also a 21-bp sequence –3.6 kb upstream from the TAT transcription start (-3647/-3627) was reported to be involved in the modulation of TAT gene expression by glucocorticoids and was termed glucocorticoid modulatory element (GME) (Oshima et al., 1992) (Fig. 4).

A cAMP-responsive element (CRE) localized at –3644/-3636 and bound by the CRE binding factor (CREB) is responsible for the glucagon-dependent TAT activation. The CRE also mediates negative regulation of the TAT expression by the tissue-specific extinguisher-1 (Tse-1) (Boshart et al., 1990). The CRE is coupled to the element bound by HNF-4 (-3588/-3579) which confers a strong liver specificity to the TAT promoter (Nitsch et al., 1993a).

Both CRE and the HNF-3-binding site located in the –2.5 kb GRU are targets for the inhibition of the glucagon and glucocorticoid-dependent TAT induction by insulin (Ganss et al., 1994). There is also evidence for not exactly mapped insulin responsive sites within the –111 to -52 bp promoter region of the TAT gene (Cheatham et al., 1992; Carmichael et al., 1992). In addition to the elements already mentioned, the liver-specific enhancer (LSE) activating TAT constitutively and necessary for the developmentally regulated gene expression is located at –10558/-10482 (Nitsch et al., 1990). This enhancer is bound by the proteins of the HNF-3 family (Nitsch et al., 1993b). So far, the oxygen-responsive sequences in the TAT promoter were not identified.

1.2.2 Serine dehydratase

L-serine dehydratase (L-serine ammonia-lyase, EC 4.2.1.13) is an enzyme that catalyzes the pyridoxal phosphate-dependent deamination of serine to produce pyruvate. This enzyme also catalyzes the conversion of L-threonine to alpha-ketobutyrate by the same mechanism and is identical to L-threonine dehydratase (EC 4.2.1.16). It is a homodimeric protein with a 327 amino acid subunit with a molecular mass of 34 kDa (Ogawa et al., 1988; Ogawa et al., 2002). The rat SerDH gene consists of 11 exons and generates a

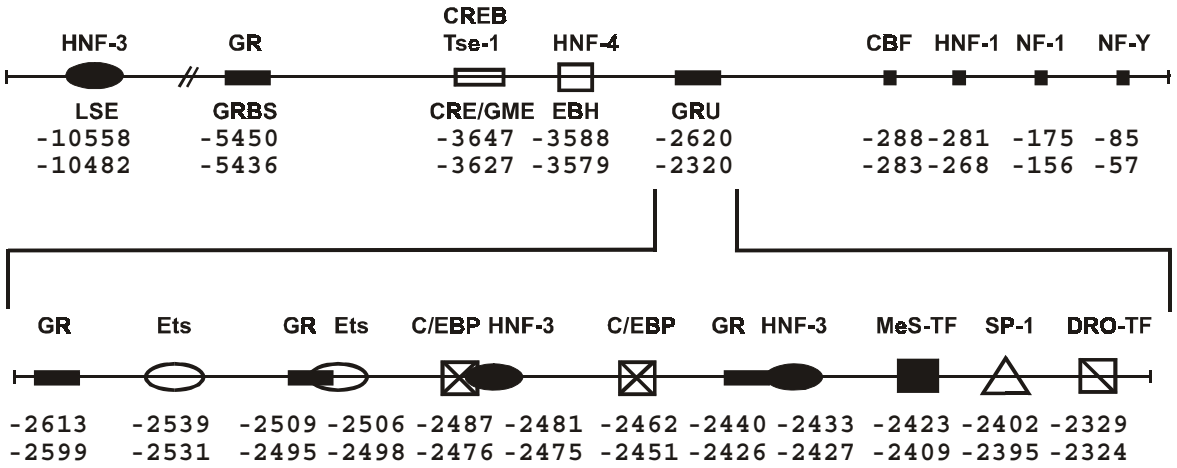


Fig. 4: **Scheme of the TAT gene promoter and its responsive elements.** CBF, CCAAT-box binding factor; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP-responsive element (-3644/-3636); CREB, CRE binding factor; DR0-TF, transcription factor interacting with the DR0 site; EBH, element bound by HNF (-3588/-3579); Ets, erythroblastosis factor; GME, glucocorticoid modulatory element (-3647/-3627); GR, glucocorticoid receptor; GRBS, glucocorticoid receptor binding site (-5450/-5436); GRE, glucocorticoid responsive element; GRU, glucocorticoid-responsive unit (ca. -2620/-2320); HNF, hepatocyte nuclear factor; LSE, liver-specific enhancer (-10558/-10482); MeS-TF, methylation-sensitive transcription factor; NF, nuclear factor; SP-1, stimulatory protein-1; Tse-1, tissue-specific extinguisher-1.
Sequences inside GRU: GRE1 (-2613/-2599) (Jantzen et al., 1987), Ets (-2539/-2531) (Espinas et al., 1994), GRE2 (-2509/-2495) (Jantzen et al., 1987), Ets (-2506/-2498) (Espinas et al., 1994), C/EBP (-2487/-2476) (Grange et al., 1991), HNF-3 (-2481/-2475) (Rigaud et al., 1991), C/EBP (-2462/-2451) (Grange et al., 1991), GRE3 (-2440/-2426) (Jantzen et al., 1987), HNF-3 (-2433/-2427) (Rigaud et al., 1991), MeS-TF (-2423/-2409) (Thomassin et al., 2001), SP1 (-2402/-2395) (Devack et al., 1993), DR0-TF (-2329/-2324) (Thomassin et al., 2001).

mRNA of about 1.5 kb (Ogawa et al., 1990). In humans a case of hereditary deficiency of serine/threonine dehydratase resulting in non-ketonic hyperglycinemia was reported (Krieger et al., 1984).

SerDH is expressed predominantely in the liver and to a lesser extent in the kidney. The SerDH gene becomes transcriptionally activated after birth to reach maximal expression levels after 14 days. It is involved in gluconeogenesis. Accordingly, SerDH expression is enhanced during starvation, diabetes mellitus and by a high-protein diet while it is repressed by a carbohydrate-rich diet (Ogawa et al., 1991). SerDH expression is activated by glucagon and glucocorticoids and repressed by insulin. Glucocorticoids alone could induce maximal expression of the SerDH gene in rat kidney *in vivo* but to achieve maximal induction of the SerDH in rat liver combined treatment with glucocorticoids and glucagon is necessary (Su et al., 1990).

The molecular mechanisms of the SerDH induction by glucagon and glucocorticoids are much less investigated than the mechanisms of TAT regulation. A CRE-1 located at -3528/-3521 of the SerDH gene is not directly involved in the gene activation by hormones (Haas et al., 1999) (Fig. 5) whereas CRE-2 (-3538/-3531), adjacent to CRE-1 and bound by CREB,appears to be critical for cAMP and glucagon-dependent induction of SerDH expression (Su et al., 1992a; Haas et al., 1999).

Three long adjacent sequences (-5356/-5316; -5315/-5228; -5227/-5166), though containing no regions with high homology to the consensus GRE sequence, were shown to be functioning as glucocorticoid-responsive enhancers (Su et al., 1992b). The involvement of the GRE's at –411/-397 and –388/-374 as well as of the CRE's at –954/-950 and -574/-570 in the hormone-dependent regulation of SerDH expression was also suggested (Matsuda et al., 1991).

The sequences between -62 and +10 are essential for liver-specific SerDH transcription, whereas the region between -133 and -63 is essential for maximal transcription not only in hepatocytes but also in nonhepatic cells (Su et al., 1990). The SerDH promoter also contains negative regulatory sequences, bound by an yet unidentified transcription factor, at –555/-531 and +20/+36, and both are functional only in fetal but not in adult liver (Noda et al., 1994).

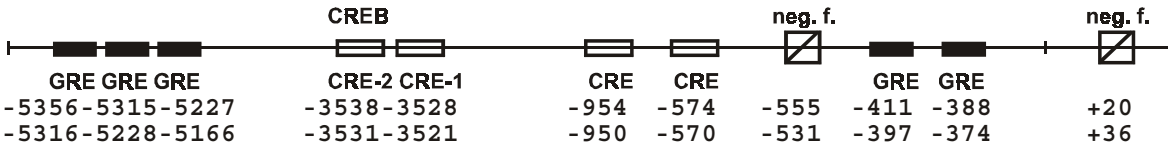


Fig. 5: **Scheme of the SerDH gene promoter and its responsive elements.** CRE, cAMP-responsive element (-3538/-3531; -3528/-3521; -954/-950; -574/-570); CREB, CRE binding factor; GRE, glucocorticoid-responsive element (-5356/-5316; -5315/-5228; -5227/-5166; -411/-397; -388/-374); neg. f.; an unidentified negative factor (-555/-531; +20/+36).

As with the TAT promoter the putative oxygen responsive DNA sequences have not been identified yet.

1.3 Aim of the study

Since TAT and SerDH are expressed in the periportal area of the liver acinus and appear to be modulated by the oxygen gradient it was the aim of the present study to investigate the oxygen-dependent expression of the TAT and SerDH genes and to identify as well as to characterize the possible normoxia responsive elements (NRE) within these genes. In an additional attempt the transcription factor binding to these NRE sequences should be identified.

2. MATERIALS

2.1 Animals

Hepatocytes were isolated from male Wistar rats (180-280 g body weight) (Winkelmann, Borchen/Westfalen). Animals were used for the preparation of hepatocytes not earlier than 6 d after arrival. Rats were kept on a 12 hour day-night rhythm (dark phase: 19 h - 7 h) with free access to water and food (rat diet "ssniff", Fa. Spezialitäten GmbH, Soest/Westfalen) at room temperature of 19-23°C. The rats consumed 12-15 g food and 12-25 ml water per day and had a 30-40 g gain of weight per week. The preparation of hepatocytes was performed during the first 3 h of the light phase. Rats were anesthetized by intraperitoneal injection of nembutal (80 mg/kg body weight).

2.2 Bacterial and yeast strains, vectors and plasmid constructs

2.2.1 Bacterial and yeast strains

Bacterial strains

Two bacterial *E. coli* K 12 strains DH5α and XL1-blue (Stratagene) were used for plasmid transformation.

Yeast strains

The *Saccharomyces cerevisiae* YM4271 strain was used for reporter vector integration. Its genotype is MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-δ512, gal80-δ538, ade5::hisG (Wilson et al., 1991).

2.2.2 Vectors

pBR322 vector

For the cloning and sequencing of DNA fragments the plasmid vector pBR322 (MBI Fermentas) was used. pBR322 is 4361 bp in length and contains the replicon *rep* responsible for the replication of plasmid (source – plasmid pMB1); *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I – RNA II complex to a stable complex and serves to decrease copy number (source – plasmid pMB1); *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source – transposon Tn3) and *tet* gene, encoding tetracycline resistance protein (source – plasmid pSC101) (Fig. 6).

pBS-KSII vector

For the cloning and sequencing of DNA fragments as well as for the *in vitro* transcription of RNA the plasmid vector pBluescript (pBS-KS II) (Stratagene, Heidelberg) was used. The vector, derived from the vector pUC19, consists of 2961 bp. It contains T3 and T7 RNA polymerase promoters, necessary for the *in vitro* transcription, a multiple cloning site (polylinker), containing sites for different restriction endonucleases, and primer sequences (universal and reverse primers), necessary for DNA sequencing (Fig. 7).

pTC10 vector

The TAT promoter DNA sequence was obtained in the vector pTC10 (Grange et al., 1989). The plasmid pTC10 was derived from the promoterless CAT plasmid pSB1 as follows (Grange et al., 1989): the Hind III site upstream from the CAT gene was converted to an Sst I site using a synthetic linker, the TAT 5‘ flanking region was inserted in this new site as a 10 kb-long Sst I fragment originating from the lambda phage TAT3. The basic CAT plasmid pSB1 was constructed as follows (Herbomel et al., 1984): a Hind III – Bam HI fragment containing the coding region of the CAT gene followed by two SV40 fragments, respectively, containig the small t-antigen intron and the early polyadenylation site, was taken out from the plasmid pSV2-CAT and ligated to the Sal I – Eco RI fragment of the plasmid pML2, where a Bam HI and a Hind III linker have been respectively added to the Eco RI and Sal I ends. The pSV2-CAT plasmid contains 5099 bp and can be used for cloning of promoter sequences before the *E. coli cat* gene encoding chloramphenicol acetyltransferase. The pML2 plasmid is derived from pBR322 after deletion of poison sequences (1092 bp to 2484 bp).

pCRII-TOPO vector

For the cloning and sequencing of PCR products as well as for *in vitro* transcription of RNA the plasmid vector pCRII-TOPO (Invitrogen) was used (Fig. 8). The vector consists of 3950 bp. It contains Sp6 and T7 RNA polymerase promoters, necessary for *in vitro* transcription, a multiple cloning site (polylinker), containing sites for different restriction endonucleases, and primer sequences (universal and reverse primers), necessary for DNA sequencing. The PCR product can be inserted in the linearized vector which has single, overlapping deoxythymidine (T) residues.

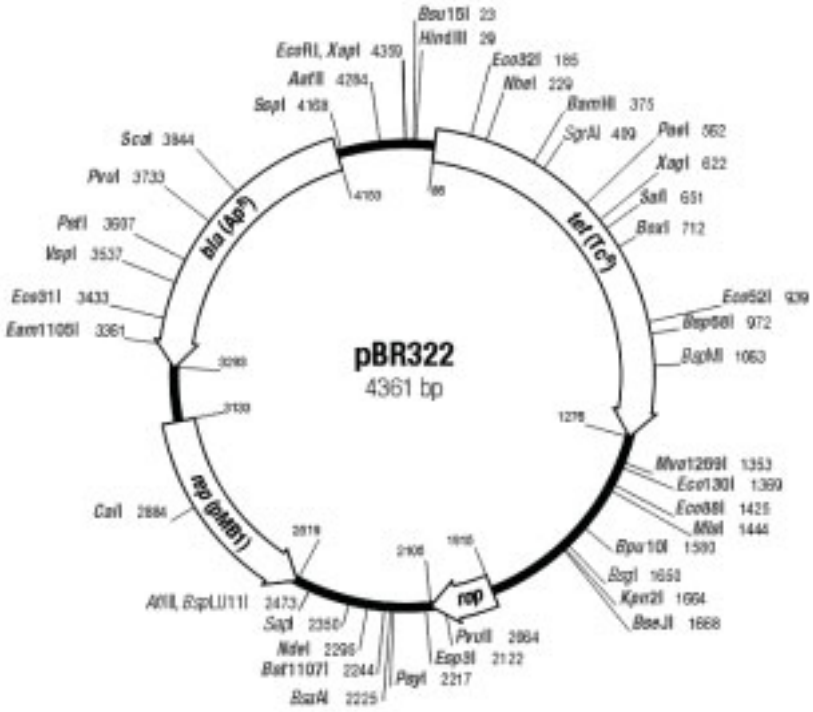


Fig. 6: **Structure of the pBR322.** The vector contains the plasmid origin of replication pMB1, the genes of ampicillin and tetracycline resistance allowing for antibiotic selection.

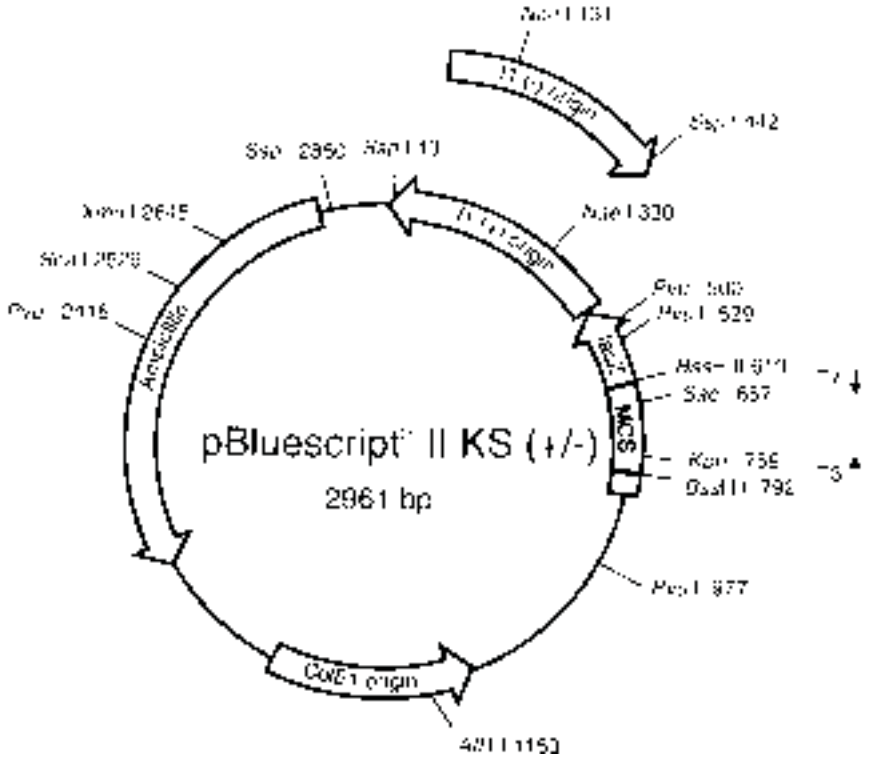


Fig. 7: **Structure of the pBluescript vector (pBS-KSII).** The vector contains the origin of replication ColE1 ori, the ampicillin resistance gene for antibiotic selection, and the LacZ gene coding for β-galactosidase which provides the possibility for blue/white color selection of recombinant clones. The multiple cloning site is flanked by T3 and T7 promoters and by the sequences for T3 and T7 primers, KS and SK primers, universal (M13) and reverse sequencing primers.

pME18S-FL3 vector

The SerDH cDNA sequence was obtained from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin), clone IMAGp998L074634.2, in the cloning vector pME18S-FL3 (Fig. 9). The expression vector pME18S-FL3 is 3392 bp in length. It contains multiple cloning sites in which the SerDH cDNA was cloned into the Xho I site.

2.2.3 The pGL3 basic and pGL3 promoter plasmid constructs

pGL3 basic constructs: pGL3TAT-10095 LUC, pGl3TAT-6100 LUC, pGl3TAT-2556 LUC, pGl3TAT-790 LUC, pGL3TAT-356 LUC, pGL3SerDH-2303 LUC, pGl3SerDH-2128 LUC, pGl3SerDH-937 LUC, pGl3SerDH-471 LUC and pGl3PCK-493 LUC

For the construction of the plasmids pGL3TAT-10095 LUC, pGL3TAT-6100 LUC, pGL3TAT-2556 LUC, pGL3TAT-790 LUC, pGL3TAT-356 LUC, pGL3SerDH-2303 LUC,

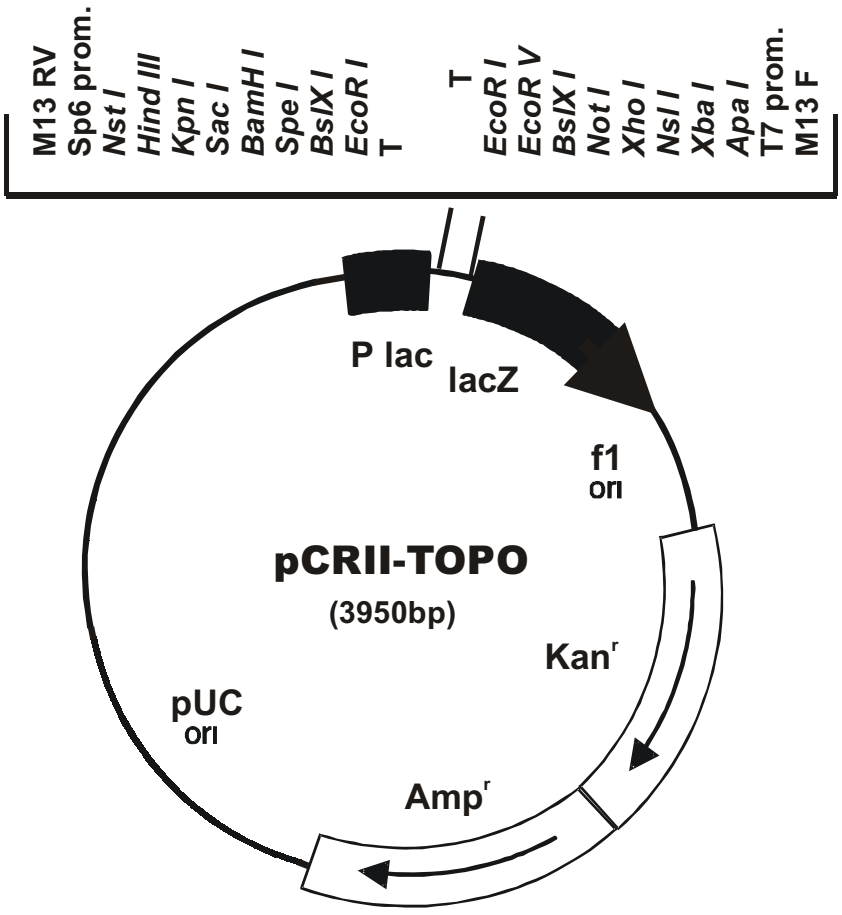


Fig. 8: **Structure of the plasmid pCRII-TOPO.** The vector contains two origins of replication, ColE1 ori and f1, the ampicillin and kanamycin resistance genes for antibiotic selection, and the LacZ gene encoding β-galactosidase which provides the possibility for blue/white color selection of recombinant colonies. The multiple cloning site is flanked by Sp6 and T7 promoters and by the sequences for universal and reverse sequencing primers.

pGL3SerDH-2128 LUC, pGl3SerDH-937 LUC, pGl3SerDH-471 LUC and pGl3PCK-493 the vector pGL3 basic (4818 bp) (Promega) was used. The corresponding regions of either TAT or SerDH promoter were cloned in the polylinker of pGl3 basic. The polylinker is flanked by the primer sequences GL2, RV4 and RV3, which are necessary for DNA sequencing. The vector contains the firefly luciferase gene (luc +) as a reporter gene to estimate the promoter activity, and two polyadenylation signals. The vector pGL3 basic also contains the gene responsible for ampicillin resistance and two origins of replication ColE1 ori and f1 ori, the second of which is necessary for the production of single-stranded DNA (ssDNA) (Fig. 10).

The plasmid pGL3TAT-10095 contains the TAT 5'-flanking sequence (gene bank accession no. X16379) from –10095 to +3 (transcription start is +1) (Fig. 11). It was constructed from plasmid pTC10, in which 10095 bp of the TAT promoter were cloned in front of the CAT gene (Grange et al., 1989). The TAT fragment was excised with Sst I and subsequently ligated into the Sac I digested and dephosphorylated vector pGl3 basic. The Sst I and Sac I restriction enzymes are isoschizomers.

The plasmid pGL3TAT-6100 was constructed from the plasmid pGL3TAT-10095 by excising a 6100 bp promoter fragment with the restriction endonuclease Nco I and

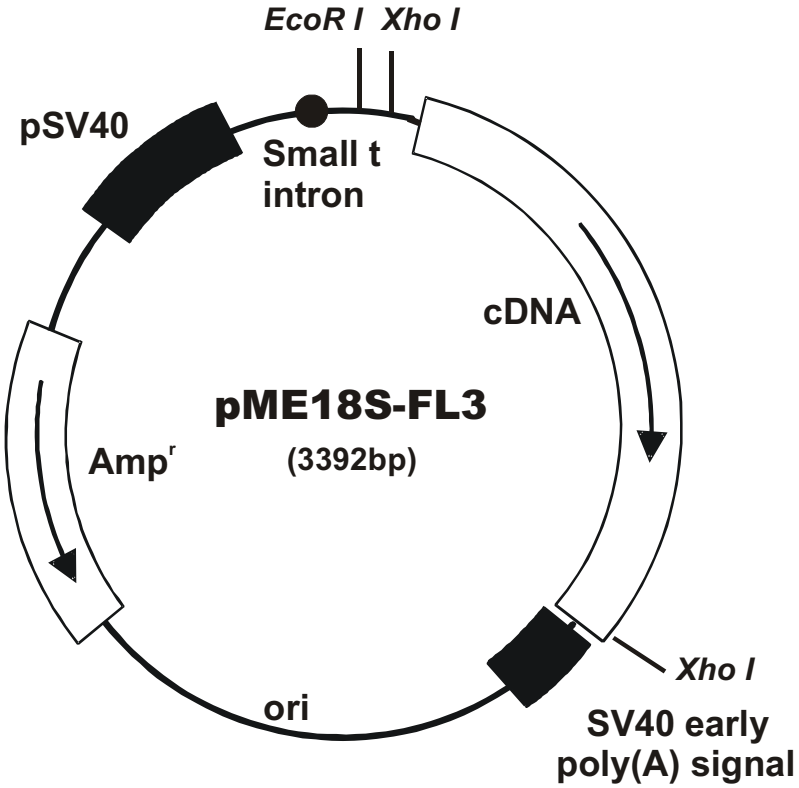


Fig. 9: **Structure of the pME18S-FL3.** The vector contains an origin of replication, ColE1 ori, the ampicillin resistance gene, SV 40 promoter and SV 40 early polyadenylation site.

subsequent ligation of this fragment into the pGl3 basic LUC vector digested by Nco I. The plasmid pGL3TAT-2556 was constructed from the plasmid pGL3TAT-10095 by excising a 2556 bp promoter fragment with the restriction endonucleases Xba I and Nhe I. The resulting fragment was ligated into the pGl3 basic LUC vector digested by Nhe I. The plasmid pGL3TAT-790 was constructed from the plasmid pGL3TAT-10095 by excising a 9125 bp promoter fragment with the restriction endonuclease Pst I and subsequent ligation of the remaining LUC vector. The plasmid pGL3TAT-356 was constructed from the plasmid pGL3TAT-10095 by excising a 9740 bp promoter fragment with the restriction endonuclease Kpn I and subsequent ligation of the remaining LUC vector.

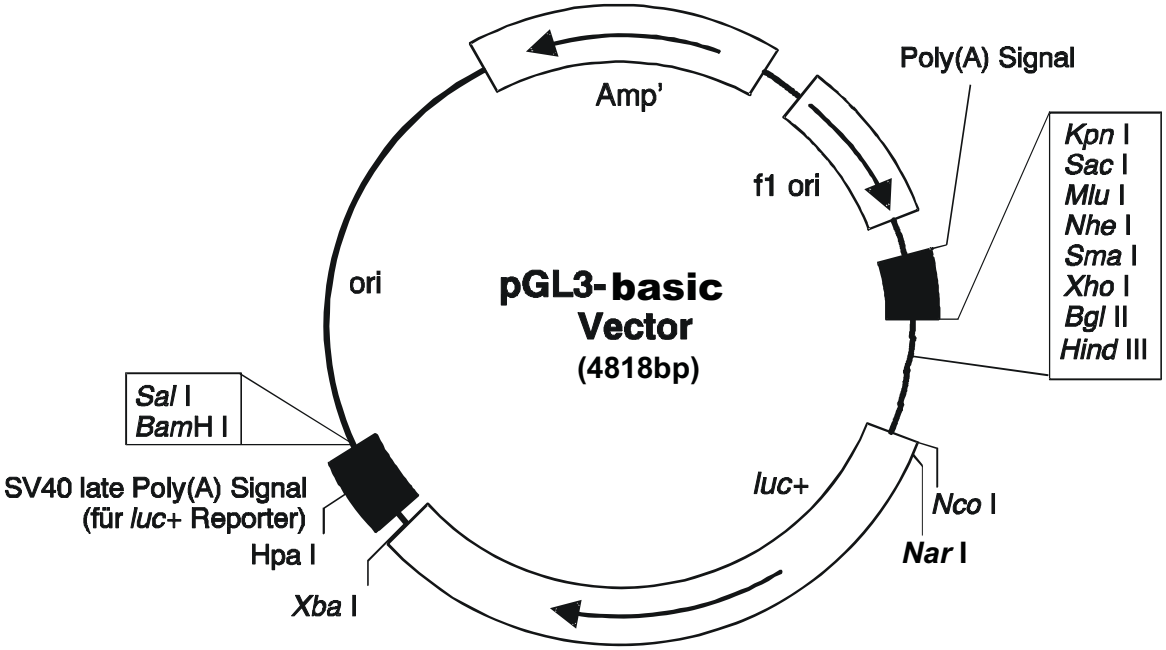


Fig. 10: **Structure of the pGL3 basic vector.** The multiple cloning site (41 bp) is followed by the firefly luciferase gene (luc +, 1649 bp) and the SV40 late polyA signal (221 bp). Another, upstream polyA signal (153 bp), is located directly before the multiple cloning site. The plasmid also contains the ampicillin resistance gene (ß-lactamase; Amp´; 857 bp) and two origins of replication, ColE1 ori and f1 ori (454 bp). The Luc + gene is transcribed clockwise whereas Amp´ is transcribed counterclockwise.

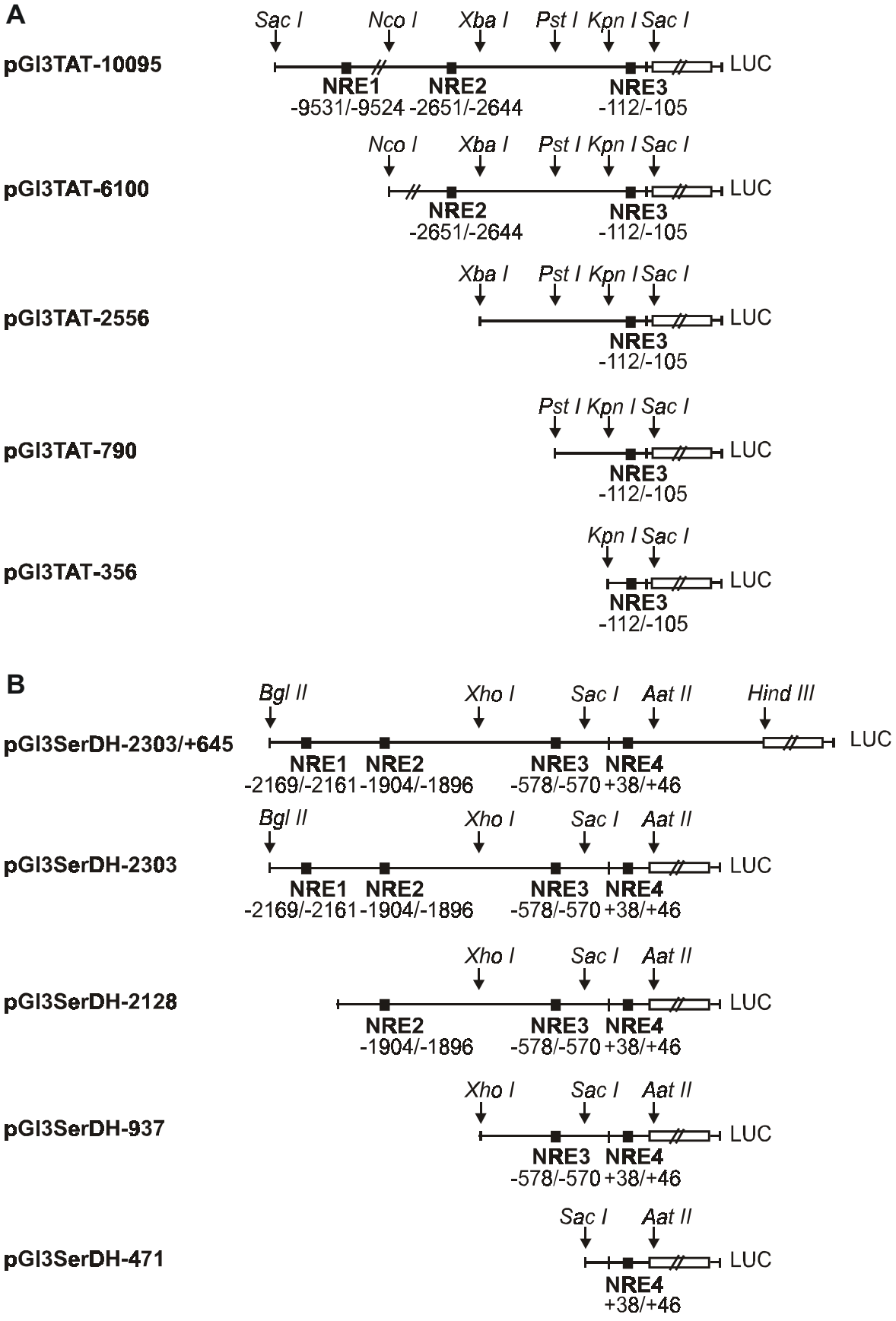


Fig. 11: **Luciferase gene constructs with the regions of TAT and SerDH promoters.** A) The pGL3TAT LUC constructs with the restriction endonuclease sites. B) The pGL3SerDH LUC constructs with the restriction endonuclease sites.

The plasmid pGL3SerDH-2303 contains the SerDH 5‘-flanking sequence (gene bank accession no. X13119) from –2303 to +55 (Fig. 11). It was constructed from the plasmid pBR322, in which the SerDH promoter sequence from –2303 to +645 was cloned into the Eco RI site (Ogawa et al., 1988). The Eco RI fragment from pBR322-SerDH was first ligated into the EcoR I digested pBS vector to give pBS-SerDH. Then a Hind III – Bam HI fragment from pBS-SerDH containing the entire SerDH sequence was ligated into the pGl3 basic vector digested with Hind III and Bgl II. The nucleotides from +55 untill +645 were removed from the pGl3SerDH-2303/+645 as follows: the plasmid was digested with the restriction endonucleases Aat II and Hind III, the resulting vector was treated with the Klenow fragment of DNA polymerase I to produce blunt ends and after religation of the blunt ends pGl3-2303 was obtained.

The plasmid pGl3SerDH-2128 was constructed by performing PCR (3.2.2) using pGl3SerDH-2303 as template and a primer complementary to the –2128/-2106 region of SerDH promoter (2.4.3) together with GL2 primer (2.4.1). The plasmid pGl3SerDH-937 was constructed from the plasmid pGL3SerDH-2303, which was digested with the restriction endonuclease Xho I and the remaining LUC vector was ligated. The plasmid pGL3SerDH-471 was also constructed from the pGL3SerDH-2303, which after excision of a Sac I fragment was subsequently religated.

The plasmid pGl3-PCK-493 was constructed, as described (Bratke et al., 1999), by performing PCR with wild type PCK-CAT construct as template and ligation of the product into the Kpn I and Bgl II sites of the vector pGl3 basic.

<u>pGl3 promoter constructs: pGl3-TAT-NRE1, pGl3-TAT-NRE2, pGl3-TAT-NRE3, pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3, pGl3-SerDH-NRE4 and pGl3-Epo-HRE</u>

The plasmids pGl3-TAT-NRE1, pGl3-TAT-NRE2, pGl3-TAT-NRE3, pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3, pGl3-SerDH-NRE4 and pGl3-Epo-HRE were constructed using the vector pGL3 promoter (5.01 kb) (Promega), in the polylinker of which the corresponding oligonucleotides were cloned in front of the SV40 promoter (Fig. 12).

The oligonucleotides containing six copies of the corresponding NRE elements (Fig. 13) were obtained from the NAPS company (Göttingen) and were all HPLC purified. Each repeating unit (at Fig. 13 shown in brackets) contained 9 bp of the corresponding NRE flanked by 3 bp at the 5´- and by 2 bp at the 3´-ends. The oligonucleotides also contained additional sequences which were necessary for technical reasons, namely either the Spe I or Sma I restriction site in the middle of the sequence for the identification of the positive

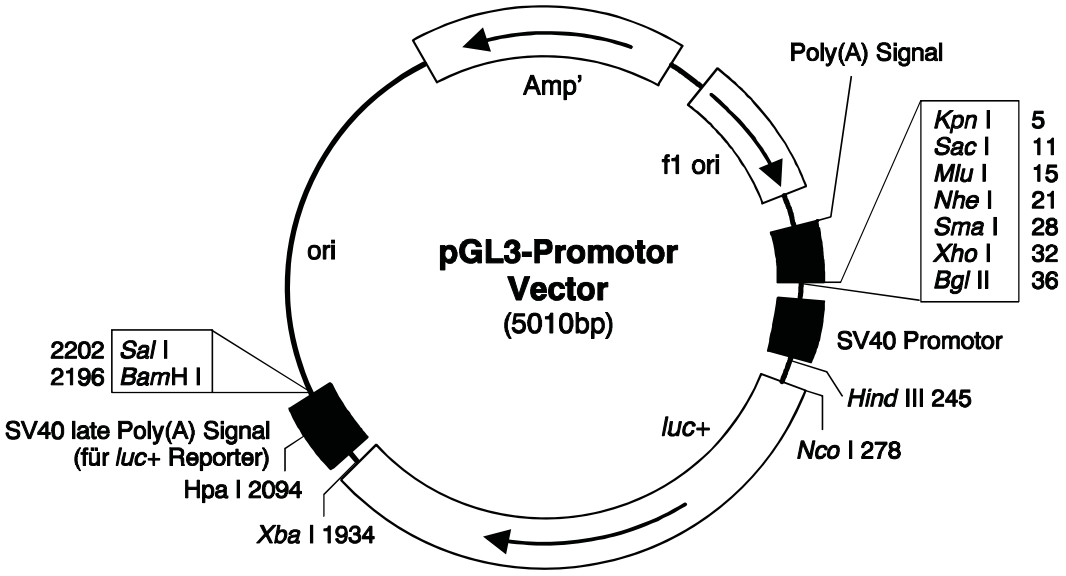
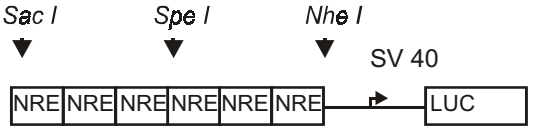


Fig. 12: **Structure of the pGL3 promoter vector.** The multiple cloning site (41 bp) is followed by the SV 40 promoter (195 bp), firefly luciferase gene (luc +, 1649 bp) and a SV40 late polyA signal (221 bp). Another, upstream polyA signal (153 bp), is located directly before the multiple cloning site. The plasmid also contains the ampicillin resistance gene (ß-lactamase; Amp´; 857 bp) and two origins of replication, ColE1 ori and f1 ori (454 bp). The Luc + gene is transcribed clockwise and the Amp´ gene is transcribed counterclockwise.



PCK-NRE 5'GAGCTC(GTGTTAGGTCAGTT)$_6$GCTAGC 3'

TAT-NRE1 5'GAGCTC(GatTTAGcTCAGTg)$_6$CTAGC 3'

TAT-NRE2 5'GAGCTC(GcGTTgGGTCAGag)$_6$CTAGC 3'

TAT-NRE3 5'GAGCTC(GgGgTAGGTCcGgg)$_6$CTAGC 3'

SerDH-NRE1 5'GAGCT(cctTgAGGaCAGcT)$_6$GCTAGC 3'

SerDH-NRE2 5'GAGCTC(GgGTTAtGTgAGac)$_6$GCTAGC 3'

SerDH-NRE3 5'GAGCTC(tccTTAGtcCAGgc)$_6$GCTAGC 3'

SerDH-NRE4 5'GAGCT(cctcTAGaTCAGGa)$_6$GCTAGC 3'

Fig. 13: **Luciferase gene constructs with TAT-NRE´s and SerDH NRE´s as enhancers.** The double stranded oligonucleotides containing the TAT-NRE´s and SerDH-NRE's were cloned in the Sac I (GAGCTC) and Nhe I (GCTAGC) restriction sites of the pGL3 promoter vector. Sequences of the NRE are underlined. Nucleotides different from the PCK-NRE are in lower case letters. The number under brackets correspond to the number of repeats (multimerisation). The oligonucleotides without brackets correspond to the 5´ and 3´ flanking sequences. In the middle of each oligonucleotide one additional Spe I or (in NRE-TAT3) Sma I site was introduced for easier identification of the correct plasmids.

clones and both Sac I and Nhe I sites at the ends for the cloning into the pGl3 promoter plasmid.

The double stranded, phosphorylated oligonucleotides, which contained the sequences complementary to the Sac I restriction site at the 5´- end and to the Nhe I restriction site at the 3´ ends, and the Sac I/ Nhe I digested and dephosphorylated vector pGl3prom were ligated using T4 ligase. The resulting constructs are shown (Fig. 13). The construct pGl3-Epo-HRE contains six copies of the HRE from the erythropoietin gene (Semenza et al., 1992) in front of the pGl3 prom and was already used in the laboratory.

2.2.4 MATCHMAKER one hybrid system vector

pHISi vector

pHISi is a yeast integration and reporter vector for use with the MATCHMAKER one hybrid system (Fig. 14). The pHISi contains the yeast HIS3 gene needed for histidine biosynthesis downstream of the MCS and the minimal promoter of the HIS3 locus (P_{minHIS}). Cis-acting sequences of interest (i.e., target elements) can be inserted into the MCS. Without activation by a target element, constitutive HIS3 expression from P_{minHIS} is very low, but allows enough growth of yeast on selective media when integrated into the host yeast genome. This low level expression can be inhibited by 3-amino triazole (3-AT) which is used during library screening.

The yeast URA3 gene responsible for the uracil biosynthesis and the HIS3 gene of pHISi can be used as selectable markers for integration into the nonfunctional ura3 and his3 loci, respectivly, of the YM4271 host strain. Before integrating, the vector is linearized at the Xho I or Afl II sites (his3 locus) or at the Apa I site (ura3 locus). The Kpn I site cannot be used for integration because it cuts within the coding region of the HIS3 gene, and that region is deleted in YM4271. pHISi cannot replicate autonomously in yeast.

2.3 Rat liver MATCHMAKER cDNA library

A rat liver MATCHMAKER cDNA library RL4004AH was screened in the one hybrid system experiments. MATCHMAKER cDNA libraries are constructed in vectors designed to express fusions of the yeast GAL4 activation domain (AD) with cDNA library inserts. The GAL4 AD vectors carry the LEU2 gene which is necessary for growth on leucine-lacking medium and can be used with the MATCHMAKER one hybrid system to identify proteins that bind to a short DNA regulatory element.

The mRNA sources for the library were normal, whole livers pooled from 100 Sprague-Dawley male rats aged 10-12 weeks. The number of independent clones was about 3.5 X 10^6 with the cDNA size range of 0.4-4 kb (average cDNA size 2 kb). pACT2 vector digested with the restriction endonucleases Xho I and Eco R I was used for the cDNA cloning.

pACT2 vector

pACT2 generates a fusion of the GAL4 activation domain (amino acids 768–881) with a protein encoded by a cDNA in a fusion library cloned into the MCS in the correct orientation and reading frame (Fig. 15). pACT2, which is derived from pACT, contains a unique *Eco*R I site in the MCS. The hybrid protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter (*P*); transcription is terminated at the ADH1 transcription termination signal (*T*). The protein is targeted to the yeast nucleus by the nuclear localization sequence from SV40 T-antigen which has been cloned into the 5'end of the GAL4 AD sequence. pACT2 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the gene which confers ampicillin resistance in *E. coli*. pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow on limiting synthetic media. Transformants with AD/library plasmids can be selected by complementation by the *LEU2* gene by using an *E. coli* strain that carries a *leuB* mutation (e.g., HB101).

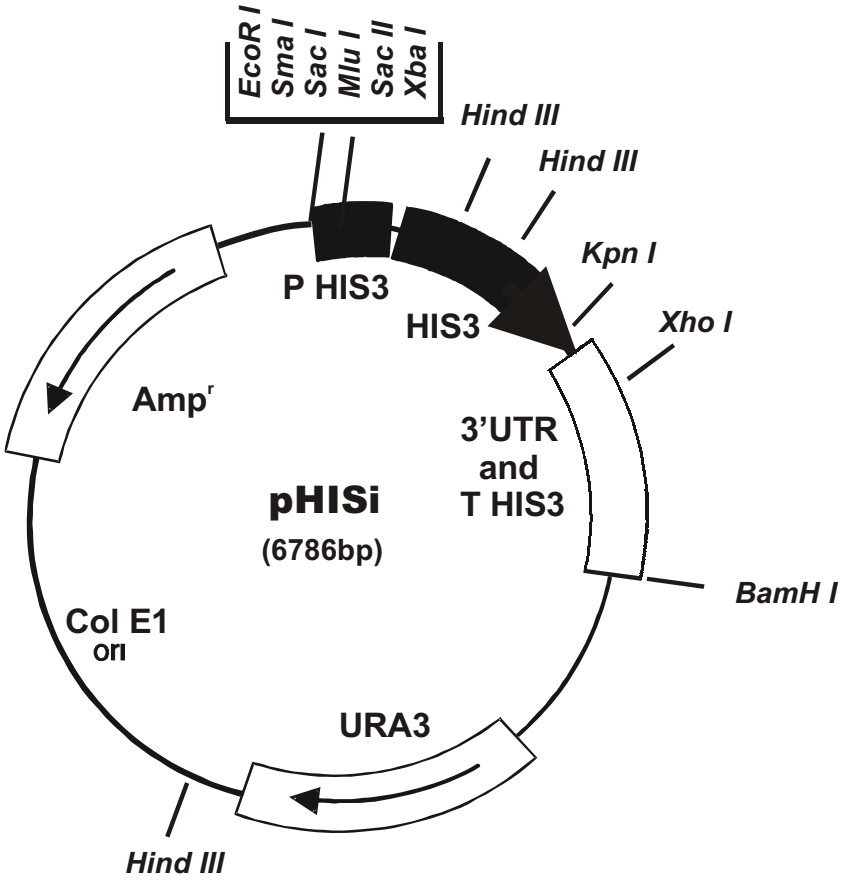


Fig. 14: **Structure of the pHISi.** The vector contains a multiple cloning site, the minimal promoter, 3'-untranslated region and minimal terminator of the HIS3 locus, the yeast HIS3 and URA3 genes. For propagation and selection in E. coli the plasmid also contains a bacterial Col E1 origin (ori) and the ampicillin resistance gene (Amp^r).

2.4 Oligonucleotides

The oligonucleotides were obtained from NAPS (Göttingen) and were HPLC-purified.

2.4.1. Oligonucleotides for sequencing of the plasmides

pBS vector

UNI primer (universal primer or M13 forward):

5´-3´: TGT AAA ACG ACG GCC AG

REV primer (reverse primer or M13 reverse):

5´-3´: ACA GCT ATG ACC ATG ATT

pGL3 basic vector, pGL3 promoter vector

GL2 primer (forward primer):

5´-3´: CTT TAT GTT TTT GGC GTC TTC C

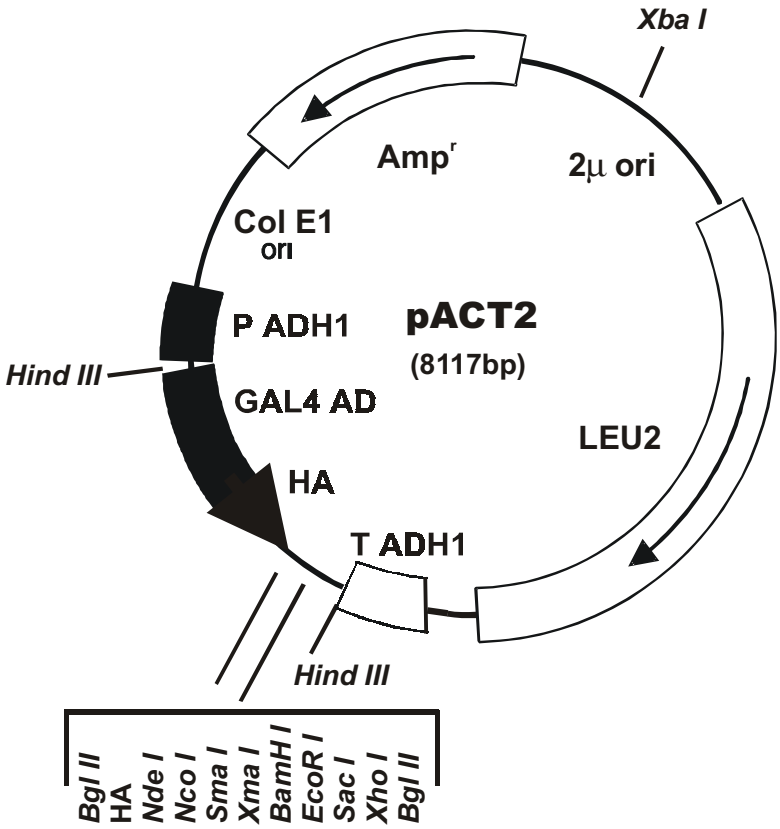


Fig. 15: **Structure of the pACT2 vector.** The vector contains 2μ origin of replication (1–2055), the LEU2 coding sequence (2474–3568), two Lox sites (Lox 1: 4268–4327; Lox 2: 4367–4412), yeast transcription termination signal ADH1 terminator (4415–4742), translation stop codon (4970–4972) in front of the multiple cloning site (4927–5079), hemagglutinin (HA) epitope (5042–5068), GAL4 activation domain coding sequence (5486–5419), SV40 T-antigen nuclear localization signal (5424–5478), promoter fragment carrying the *S. cerevisiae* ADH1 promoter (5504–5901), pBR322 plasmid replication origin (6336–6979) and ampicillin resistance gene (7995-8052).

RV primer (reporter vector primer or reverse primer):
5´-3´: CTA GCA AAA TAG GCT GTC CC

pACT2 vector
GADF primer (forward primer)
5´-3´: CGT TTG GAA TCA CTA CAG G
GADR primer (reverse primer)
5´-3´: ACT TGC GGG GTT TTT C

2.4.2 Oligonucleotides for Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotide from the promoter of the TAT gene (-9536/-9519) containing NRE-1 sequence:
5´-3´: GGG ATT TAG CTC AGT GGT

Oligonucleotide from the promoter of the SerDH gene (+33/+50) containing NRE-4 sequence:
5´-3´: GCC CTC TAG ATC AGG ACG

Oligonucleotide from the promoter of the HO-1 gene (-668/-654) containing CRE/AP-1 sequence:
5´-3´: TGT GTC AGA GCC ATG TGT CCT GAC TTC AGT CT

2.4.3 Oligonucleotides for PCR reaction

Forward primer for the cloning of TAT cDNA fragment (nucleotides 449 to 472)
5´-3´: AGT CGG GAG GAG GTC GCT TCT TAC
Reverse primer for the cloning of TAT cDNA fragment (nucleotides 1478 to 1455)
5´-3´: GAG GAC GGG TGA GGG CTT ATT TGT
SerDH promoter-specific primer (-2128/-2106)
5´-3´: TGT GAG AAT GAT CGG CAA AGC T

2.4.4 Oligonucleotides for pGl3-NRE construction

The double stranded oligonucleotides containing the TAT-NRE´s and SerDH-NRE's, which were cloned in the Sac I (GAGCTC) and Nhe I (GCTAGC) restriction sites of the pGL3 promoter vector, have the following sequences:
TAT-NRE1
5'-GATTTAGCTCAGTGGATTTAGCTCAGTGGATTTAGCTCACTAGTTTTAGCTCAGTG GATTTAGCTCAGTGGATTTAGCTCAGTG-3'

TAT-NRE2
5‘-CGCGTTGGGTCAGAGGCGTTGGGTCAGAGGCGTTGGGTCAGACTAGTTGGGTCAGAGGCGTTGGGTCAGAGGCGTTGGGTCAGAGG-3‘
TAT-NRE3
5‘-CGGGGTAGGTCCGGGGGGGTAGGTCCGGGGGGGTAGGTCCCCCGGGGTAGGTCCGGGGGGGGTAGGTCCGGGGGGGGTAGGTCCGGG-3‘
SerDH-NRE1
5‘-CCTTGAGGACAGCTCCTTGAGGACAGCTCCTTGAGGACAACTAGTTGAGGACAGCTCCTTGAGGACAGCT CCTTGAGGACAGCTG-3‘
SerDH-NRE2
5‘-CGGGTTATGTGAGACGGGTTATGTGAGACGGGTTATGTGAGACTAGTTATGTGAGACGGGTTATGTGAGACGGGTTATGTGAGACG-3‘
SerDH-NRE3
5‘-CTCCTTAGTCCAGGCTCCTTAGTCCAGGCTCCTTAGTCCAGACTAGTTAGTCCAGGCTCCTTAGTCCAGGCTCCTTAGTCCAGGCG-3‘
SerDH-NRE4
5‘-CCTCTAGATCAGGACCTCTAGATCAGGACCTCTAGATCACTAGTTCTAGATCAGGACCTCTAGATCAGGACCTCTAGATCAGGAG-3‘

2.5 Digoxigenin-labeled RNA probes

Digoxigenin-labeled antisense-ß-Actin-(ACT)-RNA as hybridization probe was already used in the laboratory. The ACT probe was synthesized by *in vitro* transcription of pBS-ACT with the T3 RNA polymerase. A 550 bp fragment from nt positions 69 - 618 (EMBL. HSA 1007) of the human β-actin cDNA was transcribed. The transcript was labeled by the incorporation of digoxigenin-labeled uridinemonophosphate (DIG-UMP). In Northern blots the actin specific band of 1.8 kb was detected.

The SerDH cDNA fragment (nt 220-1440) cloned in the Xho I site of the plasmid pME18S-FL3 (Fig. 9) was obtained from the RZPD Deutsches Ressourcenzentrum für Genforschung GmbH (Berlin) as the clone IMAGp998L074634.2. pME18S-FL3-SerDH plasmid was digested with the restriction endonuclease Xho I and the resulting SerDH cDNA fragment of about 1220 bp was ligated into the Xho I site of pBluescript II KS. The pBS-SerDH plasmid was used for the preparation of the digoxigenin-labeled SerDH RNA probe using T7 polymerase and DIG-UMP as described in 3.2.10.

The PCR II-TOPO-TAT plasmid containing a TAT cDNA fragment (nt 449-1478) was constructed with the TOPO TA Cloning of Taq polymerase amplified TAT cDNA products (described in 3.2.3) and was used for the preparation of the digoxigenin-labeled TAT RNA probe using T7 polymerase and DIG-UMP as described in 3.2.10.

2.6 Antibodies

For the detection of c-Jun in the EMSA a purified, rabbit polyclonal antibody against the N-terminus of mouse c-Jun was used (Santa Cruz Biotechnology). The antibody is specific for c-Jun of mouse, rat, chicken and human origin.
For the detection of c-Fos in the EMSA a purified, rabbit polyclonal antibody against c-Fos (K-25) of human origin was used (Santa Cruz Biotechnology). The epitope is mapping within a highly conserved domain of c-Fos which is identical to corresponding mouse, rat and chicken sequences. The antibody is broadly reactive with c-Fos, Fos B, Fra-1 and Fra-2.
For the detection of CREB-1 in the EMSA a purified, mouse monoclonal antibody against human CREB-1 (24H4B) was used (Santa Cruz Biotechnology). The epitope corresponds to amino acids 254-327 within the DNA binding and dimerization domain of human CREB-1.
For the detection of SP-1 in the EMSA a purified, rabbit polyclonal antibody against rat SP-1 (PEP2) was used (Santa Cruz Biotechnology). The epitope is mapping within an internal domain of SP-1 which is identical for SP-1 proteins of rat and human origin. It is mouse, rat and human reactive, recognizes both p95 and p106 SP-1 proteins and is not cross-reactive with SP-2, SP-3 or SP-4.

2.7 Enzymes

Restriction endonucleases:

All restriction endonucleases were obtained from Takara (Taufkirchen). BSA and Triton were added when necessary to the restriction reaction mixture at the final concentration of 0.1%.
Restriction endonucleases can be active only under optimized buffer conditions. For the restriction reactions commercially available buffers (Takara), in which corresponding enzymes had maximal activities, were used.

Enzyme	Specificity	Buffer
Aat II	GACGT^C	T+BSA
Bam HI	G^GATCC	K
Bgl II	A^GATCT	H
EcoR I	G^AATTC	H
Hind III	A^AGCTT	M

Kpn I	GGTAC^C	L
Nhe I	G^CTAGC	M
Not I	GC^GGCCGC	H+BSA+TritonX-100
Sac I	GAGCT^C	L
Sma I	CCC^GGG	T+BSA
Spe I	A^CTAGT	M
Xho I	C^TCGAG	H

Incubation buffers for enzymes of the Takara company

	L	M	H	K	T
Tris HCl	100 mM	100 mM	500 mM	200 mM	-
Tris acetate	-	-	-	-	330 mM
Mg acetate	-	-	-	-	100 mM
K acetate	-	-	-	-	660 mM
$MgCl_2$	100 mM	100 mM	100 mM	100 mM	-
NaCl	-	500 mM	1000 mM	-	-
KCl	-	-	-	1000 mM	-
DTT	10 mM	10 mM	10 mM	10 mM	5 mM
pH at 37°C	7.5	7.5	7.5	8.5	7.9

DNA and RNA modifying enzymes:

Taq DNA-Polymerase

The *Taq* DNA polymerase is isolated from the thermophilic eubacterium Thermus aquaticus BM. This strain produces no *Taq I* restriction endonuclease. The enzyme is a monomer of 95 kDa; it is a 5'-3'-DNA-polymerase, which has no 3'-5'- and 5'-3'-exonuclease activity. The *Taq* polymerase posseses the highest activity at pH 8-9 and a temperature of 70-75°C. It is temperature resistant, could use also modified dNTPs as substrates and can be used for radioactive labeling of DNA fragments as well as for the labeling of DNA fragments with digoxigenin or biotin. The high process capability of *Taq* DNA Polymerase, the lack of exonuclease activity and the high temperature optimum make it possible to use *Taq* DNA polymerase in PCR and DNA sequencing (product information).

Proof*Sprinter*™ DNA polymerase is a mixture of *Taq* and *Pwo* polymerases. The mixture combines the proofreading capacity of *Pwo* with the high process capability of *Taq*, making it a good choice for cloning or sequencing of PCR products. ProofSprinter has robust performance, owing to its high DNA polymerase activity. Thus, in contrast to many

other proof-reading enzymes, ProofSprinter increases PCR yields for both long and short templates. It could also work efficiently with templates that cause problems with *Taq* and other DNA polymerases.

T4 polynucleotide kinase

The enzyme catalyzes the transfer of gamma-phosphate from ATP to the 5'-OH group of DNA, RNA, oligonucleotides or nucleoside 3'-monophosphates. The reaction is reversible. T4 polynucleotide kinase is also a 3'-phosphatase. It catalyzes the hydrolysis of 3'-phosphoryl groups of deoxynucleoside 3'-monophosphates, deoxynucleoside 3', 5'-diphosphates and of 3'-phosphorylpolynucleotides. The pH optimum of the direct reaction is 7.4 - 8.0. The reaction needs Mg^{2+} and sulfhydryliones.

Alkaline phosphatase

Alkaline phosphatases are enzymes removing 5' phosphate groups from DNA and RNA. They are most active at alkaline pH. Shrimp alkaline phosphatase (SAP) is derived from a cold-water shrimp. Its properties are very similar to that of calf intestinal alkaline phosphatase (CIP) but, in contrast to CIP, SAP is readily destroyed by heat (65°C for 15 minutes). There are two primary uses for alkaline phosphatase in DNA manipulations: removing of 5'-phosphates from plasmid and bacteriophage vectors that have been cut with a restriction enzyme preventing self-ligation of the vector and removing 5'-phosphates from fragments of DNA prior to labeling with radioactive phosphate. The SAP was purified nuclease free (product information).

Klenow fragment of DNA polymerase I

DNA Polymerase I, large (Klenow) fragment, is a proteolytic product of *E. coli* DNA polymerase I which retains polymerization and 3´-5´ exonuclease activity, but has lost 5´-3´ exonuclease activity. Klenow retains the polymerization fidelity of the holoenzyme without degrading 5´ termini. It could be used for fill-in of 5´ overhangs to form blunt ends, for removal of 3´ overhangs to form blunt ends, for second strand cDNA synthesis and second strand synthesis in mutagenesis protocols. The enzyme was purified and free of contaminating endonucleases and exonucleases.

T4 DNA ligase

The T4 DNA ligase is isolated from *E. coli* infected with T4 bacteriophages. T4 DNA ligase in solutions is a monomer with the molecular weight of ca. 68 kDa. DNA ligases catalyze formation of a phosphodiester bond between the 5' phosphate of one strand of

DNA and the 3' hydroxyl group of the another. This enzyme is used to covalently link or ligate fragments of DNA together. Most commonly, the reaction involves ligating a fragment of DNA into a plasmid vector.

T3- and T7 DNA-dependent RNA polymerases

Bacteriophage T3 and T7 RNA polymerases are DNA-dependent RNA polymerases with high sequence specificity for T3 or T7 promoters. Both T3 and T7 RNA polymerases synthesize RNA from 5' to 3' and can incoporate ^{35}S, ^{32}P and ^{33}P ribonucleotides. They are used for the generation of strand-specific RNA sequences that may be used as probes for hybridization. The polymerases are Mg^{2+}-dependent and need the four ribonucleoside triphosphates ATP, CTP, GTP and UTP for RNA synthesis. The product has three phosphate groups at the 5'-end and a 3'-OH group at the 3'-end (Sambrook et al., 2001).

Other enzymes:

Collagenase

The collagenase was used for the preparation of rat primary hepatocytes. It is a protease which degrades collagen fibrils. The collagen is the major fibrous component of animal extracellular connective tissue. Collagenase has no specific activity for any single substrate (single protein) but could recognize specific structural patterns inside protein chains.

Lysozyme

Lysozyme is an enzyme that destroys bacterial cell walls by hydrolyzing the glycoside bond of the bacterial mureine. It is a muramidase.

RNase A

Bovine pancreatic ribonuclease A (RNase A) is a small monomeric enzyme of 124 amino acids and a molecular weight of 13.7 kDa. The function of this enzyme is to hydrolyze single-stranded RNA by cleaving the phosphodiester bond. It results in formation of nucleoside 5’-monophosphates. RNase A has a pH optimum at 7.0 - 7.5 (Sambrook et al., 2001). It is used in the isolation of DNA. To inactivate DNases the RNase A solution should be heated before use for 10 min at 100 °C.

2.8 Detection, purification and synthesis systems ("Kits")

JETstar, Plasmid Purification System, Genomed/ Bad Oeynhausen

QIAEXII Gel Extraction Kit, Qiagen/ Hilden

DIG-Nucleic-Acid Detection Kit, Roche/ Mannheim

Luciferase Assay Kit, Berthold/ Pforzheim

ECL-Kit, Amersham/ Freiburg

Nucleotide Removal Kit, Qiagen/ Hilden

Synthetic Oligonucleotides 5´-end Labelling Kit, MBI Fermentas GmbH/ St.Leon-Rot

2.9 Stock solutions

The stock solutions were, unless mentioned, prepared with steril H_2O at room temperature. All solutions for RNA experiments were treated with DEPC-H_2O.

Ammonium acetate 7.5 M

			Final concentration
NH_4Ac	57.8	g/100 ml	7.5 M

The solution was autoclaved.

APS

			Final concentration
APS	100	mg/ml	10 %

APS was always made fresh.

Blocking reagent 10%

50 mg Blocking reagent was diluted with warming in 500 ml 1x maleic acid buffer. The solution was autoclaved and stored at 4°C.

Lithium chloride 4 M

			Final concentration
LiCl	17	g/100 ml	4 M

The solution was autoclaved.

Glucose 2 M

			Final concentration
Glucose	36.4	g/100ml	2 M

The solution was sterile filtered.

Magnesium chloride 1 M

			Final concentration
$MgCl_2$	20.33	g/100ml	1 M

The solution was sterile filtered.

Sodium acetate 2 M pH 4.1

			Final concentration
3 M NaAc	2	parts	2 M
Acetic acid (conc.)	1	part	

The pH was adjusted with concentrated acetic acid to 4.1. The solution was autoclaved.

Sodium acetate 3 M pH 5.2

			Final concentration
NaAc	24.61	g	300 mM
H_2O	to 100	ml	

The pH was adjusted with concentrated acetic acid to 5.2. It should be mentioned that for the right pH value a large amount of acetic acid was added. The solution was autoclaved.

PBS 10x

			Final concentration
NaCl	81.82	g/l	1.4 M
KCl	2.02	g/l	27 mM
$Na_2 HPO_4$	16.02	g/l	90 mM
KH_2PO_4	2.04	g/l	15 mM

The pH was adjusted to 7.0. The solution was prepared with DEPC-H_2O and autoclaved.

SDS

			Final concentration
SDS	10	g/100 ml	10 %

In the case of precipitation of SDS the solution was warmed.

SSC 20x

			Final concentration
NaCl	175.32	g/l	3,0 M
Sodium citrate	88.25	g/l	0,3 M

The pH was adjusted with HCl to 7.0. The solution was autoclaved.

Tris/HCl 1 M

			Final concentration
Tris	12.11	g/l	1 M

The pH values of different solutions were adjusted with HCl to pH 7,5; 8,0; 9,0. The solutions were autoclaved.

Tris/HCl 0.1 M

			Final concentration
Tris	1.211	g/l	0.1 M

The pH values of different solutions were adjusted with HCl to 7.5; 8.0; 9.0. The solutions were autoclaved.

2.10 Chemicals

All chemicals used were of p.a. quality.

Amersham/Braunschweig:

γ^{32}P-ATP (3000 Ci/mmol)

Bayer/Leverkusen:

Trasylol (aprotinin)

Becton, Dickinson and Company

Difco yeast nitrogen base without amino acids

Biometra/Göttingen:

MOPS

Biomol/Hamburg:

NBT, X-phosphate

Boehringer Ingelheim/ Heidelberg:

Takara *Taq* polymerase

Clontech:

Herring testis carrier DNA, -His and –Leu/-His dropout supplements

Roche/Mannheim:

Anti-DIG antibodies, blocking reagent, collagenase, CSPD, deoxyribonucleotide 5´-triphosphates (dATP, dCTP, dGTP, dTTP), DIG RNA labeling mix, FCS, poly d (I-C), GPT, hexanucleotides, leupeptin, NCS, pepstatin A, RNase A, RNA standarts, T3 polymerase, T7 polymerase

Fluka Chemie/Buchs, Schweiz:

Glycerol, guanidinium thiocyanate

Gibco-BRL, Life Technologies/Eggenstein:

Agarose, medium M199, non essential amino acids for MEM, T4 DNA ligase, T4 polynucleotide kinase

Hybaid/Heidelberg:

Proof*Sprinter*™ DNA polymerase mixture

Kodak/Rochester, USA:

Developer and fixative

MBI Fermentas/St.Leon-Rot:

DNA standarts, RNase inhibitor (RNasin)

Merck/Darmstadt:

All usual laboratory chemicals, formamide, H_2O_2

Messer-Griesheim/Düsseldorf:

Nitrogen, CO_2, oxygen

Oxoid/Basingstoke, GB:

Bacto agar, bacto trypton, yeast extract

PAA Laboratories GmbH, Austria:
MEM medium

Pharmacia Biotech/Freiburg:

LMW protein standarts

Pierce/Rockford, USA:

DAB concentrate, peroxidase buffer

Promega/Mannheim:

Luciferase cell lysis reagent (lysis buffer)

Roth/Karlsruhe:

Rotiphorese® Gel 30 (30% acrylamid stock solution with 0.8% bisacrylamid in proportion 37.5:1), Hepes, phenol (in Tris buffer pH 7.0 – 7.5)

Serva/Heidelberg:

Ampicillin, ammonium persulfate, bacitracin, bisacrylamid, bromphenol blue, coomassie blue, DePeX, ß-mercaptoethanol, paraformaldehyd, ponceau S, penicillin, CSA, SDS, Serva Blue, TEMED, Tween 20

Sigma/München:

Antifoam A, CDTA, dexamethasone, dextran sulfate, DMSO, DTE, E.coli DNA, ethidium bromide, formamide, glucagon, D-glucose, insulin, lysozym, N-lauroylsarcosin, sodium vanadate, nembutal, maleic acid, PEG 4000, PMSF, streptomycin sulfate, Tris, Triton X-100, trypsine, 3-amino-1,2,4-triazole (3-AT)

USB (United States Biochemical)/Cleveland, USA:

Shrimp alkaline phosphatase

2.11 Other materials

3 MM Whatman paper, nitrocellulose, folding filter, Schleicher und Schüll/Melsungen

Hyperfilm MP and Hybond N nylon membrane, Amersham/Braunschweig

Culture dishes, culture flasks, reaction glass cups, pipet tips, polystyrol tubes, Greiner/Nürtlingen

Sterile filter Nalgene 0.2 µm, Sartorius GmbH/Göttingen

Braunules 2G14, Braun/Melsungen

Dialysis bags VISKING, TYP 8/32, Roth/Karlsruhe

2.12 Instruments

Analysis system for microscopic photography, SIS System/Münster

Auto Lumat LB 953, Berthold/ Pforzheim

Automatic pipets, type Varipette 4710, Eppendorf/Hamburg

Automatc pipets, type Pipetman P 20, P 200, P 1000, Abimed Analysen-Technik GmbH/Langenfeld

Automatic DNA Sequencer, modell 373 A, Applied Biosystems/Weiterstadt

Drying cupboard, type U 40, Memmert/Schwalbach

Electric power apparatus, type EPS 500/400, Pharmacia LKB GmbH/Freiburg

Electroblotting instrument, built by institute‘s workshop

Eppendorf table centrifuge, types 5414 and 5415 C, Eppendorf-Netheler GmbH/Hamburg

Eppendorf thermostate, type 5320, Eppendorf-Netheler GmbH/Hamburg

Gas controlled incubators “Cytoperm 8080“ and “B 5060 EK/O_2“, Gasmonitor, Heraeus/Hanau

Gel apparatus for EMSA gels, Sigma/Deisenhofen

Gel dryer, Schütt Labortechnik/Göttingen

Glass dishes, Ochs/Göttingen

Halfmicro osmometer, Knauer/Berlin

Hettich centrifuge, type 3850, Hettich/Tuttlingen

Hybridization apparatus OV 3, Biometra/Göttingen

Ice machine, Inco-Ziegra/Isernhagen

Image analysis system, SIS Computersysteme/Münster

Incubator with shaking, modell 3-25, New Brunswick Scientific Co., Inc./Edison, New Jersey 08818, USA

Labofuge II, Heraeus/Hanau

Liquid szintillation counter, beta V with printer Peacock No. D 1018, Raytest Isotopenmeßgeräte GmbH/Straubenhardt

Magnetic mixer with warming, type RCT B, Ika Labortechnik/Staufen

Microscope Zeiss IM (Video camera Hitachi HV-C20), Zeiss/Göttingen

Microwave oven, type KOR-6105, Daewoo Electronics Deutschland GmbH/Butzbach

Millipore apparatus, “ Milli-Q “, Millipore/Neuisenburg

pH meter, pH 535 Multi Cal, Schütt Labortechnik/Göttingen

Phosphorimager with screen plates and eraser, Amerscham/Freiburg

Photometer, lambda 3 UV/VIS with 8 cell programmer (Weef electronic), Perkin-Elmer GmbH/Langen

Sartorius scales, type 2254, H 120 and 2434, Sartorius GmbH/Göttingen

Seesaw apparatus, built by institut’s workshop

Sigma table centrifuge 3 E-1, Sigma Laborzentrifugen GmbH/Osterode/Harz

Sorvall high speed centrifuges, RC 5 and RC 5 B, Du Pont Instruments/ Bad Nauheim

Sonicator, modell W-220 F, Schütt Labortechnik/Göttingen

Sterile bench, type Lamin Air, TL 2472, Heraeus/Hanau

Sterile bench, type MRF 06.12 - GS, Prettl Laminarflow und Prozeßtechnik GmbH/Bempflingen

Sterile pump, DBP Nr. P 24333991, Schleicher und Schüll/Dassel

Thermocycler, Bio-Med/Theres

Thermostats, type 450 LE and Typ R 10/2, Meßgeräte-Werk Lauda, Dr. R. Wobser KG/Lauda-Königshofen

Tube pump " Multifix ", Multifix Motoren, Alfred Schwinherr/Schwäbisch Gmünd

Ultraviolet emitter, type N-90 GL, Konrad Benda/Wiesloch

Ultraviolet emitter, modell C 62, Ultraviolett Productions Inc./Californien, USA

Ultraviolet stratalinker, Modell 1800, Stratagene/Heidelberg

Vakuum blot chamber, Biometra/Göttingen

Vakuum pump, Univac DM 04 with cooler Unicryo MC 2L-60∅C and centrifuge Univapo 150 H, Uniequip Laborgerätebau/Martiensried

Videodensitometer, Biotec Fischer/Reiskirchen

Videodensitometer camera Hitachi KP-140, Hitachi AC Adapter Modell AP-130 E, Biotech Fischer/Reiskirchen

Video camera DNA ES 49 with Mitsubishi video copy processor, Herolab/Wiesloch

Videoprinter and transilluminator UVP, Herolab/Wiesloch

Water bath, type K2R and type NB/S8, Meßgeräte-Werk Lauda, Dr. R. Wobser KG/Lauda-Königshofen

X-ray film cassetes, Intas/Göttingen

3. METHODS

3.1 Cell biological methods

3.1.1 Isolation of primary rat hepatocytes

The isolation of primary rat hepatocytes from liver was performed under sterile conditions by the method of collagenase perfusion (Berry et al., 1969).

Liver perfusion

1. Non-recirculative *in situ* preperfusion of the liver: after laparotomy a braunule (1.3 X 45 mm) was inserted in the vena portae, and the vena cava inferior was ligated above the diaphragma to close the whole body blood circulation. The vena cava inferior was then cut beneath the liver, cannulated with the braunule (22 X 50 mm) and perfusion started with 150-200 ml preperfusion medium (Krebs ringer solution with EGTA) at a flow rate of 30 ml/min untill the liver was free from blood.
2. Recirculative perfusion: the perfusion at a flow rate of 30 ml/min with collagenase perfusion medium was performed untill consistency of the liver became soft, due to digestion of the connective tissue (about 7-11 min). During perfusion the medium was recirculated through a plastic tube connected to the braunule inserted in the vena cava inferior.

Both preperfusion and perfusion medium were pumped through an oxygenator, from which they were directed into the vena portae with a pressure of 10-15 cm of water.

Preparation of the hepatocyte suspension

After perfusion the liver was removed and transferred into a glass cup filled with culture medium M 199. The Glisson's capsule, e.g. collagen tissue around the liver, was carefully removed and discarded. The obtained paste-like liver substance was further disrupted. Finally, connecting tissue and remainders of the liver capsule as well as big cell aggregates were removed by filtration of the primary suspension through a nylon net (pore size 79 µm). Non-parenchymal cells and cell debris were removed by numerous selective sedimentations (20 *g*, 2 min). After the last centrifugation hepatocytes were suspended in medium M 199. 50 ml of M 199 were added per 1 g of wet weight of the sedimented cells; the cell suspension had a density of about 10^6/2.5 ml.

Mediums and solutions for hepatocyte preparation and culture

All mediums and solutions for cell culture were prepared in demineralized water, which was further purified by quartz distillation, sterile filtered in autoclaved bottles and stored at 4°C.

Krebs Ringer stock solution:

			Final concentration	
NaCl	7	g/l	120.0	mM
KCl	0.36	g/l	4.8	mM
$MgSO_4$ x 7 H_2O	0.296	g/l	1.2	mM
KH_2PO_4	0.163	g/l	1.2	mM
$NaHCO_3$	2.016	g/l	24.4	mM

The solution was equilibrated with carbogen and adjusted to pH 7.35.

Preperfusion medium:

			Final concentration	
EGTA	0.1	g/l	0.25	mM

dissolved in Krebs Ringer stock solution.

Collagenase perfusion medium:

			Final concentration	
HEPES	3.356	g/l	15	mM
$CaCl_2$ x 2 H_2O	0.588	g/l	4	mM
Collagenase	0.500	g/l		

dissolved in Krebs Ringer stock solution.

Before each preparation of hepatocytes the collagenase was dissolved in perfusion medium, equilibrated with carbogen for 30 min and finally sterile filtered.

Wash medium:

			Final concentration	
HEPES/NaOH pH 7.4	4.77	g/l	20	mM
NaCl	7.00	g/l	120	mM
KCl	0.36	g/l	4.8	mM

$MgSO_4$ x 7 H_2O	0.30	g/l	1.2	mM
KH_2PO_4	0.16	g/l	1.2	mM
Calf serum albumin	4.00	g/l	0.4	%

Medium 199 :

			Final concentration	
Pulver medium M 199 with Earle`s salts without $NaHCO_3$	9.8	g/l		
Glucose x H_2O	1.1	g/l	5.5	mM
HEPES	3.6	g/l	15	mM
$NaHCO_3$	1.5	g/l	18	mM
Calf serum albumin	4.0	g/l	0.4	%

Solution A was prepared by dissolving 1.5 g $NaHCO_3$ in 550 ml H_2O and equilibrated with carbogen for 3-4 hours. The pH was adjusted to 7.35. Solution B was prepared from powder medium, bovine serum albumin and HEPES dissolved in 450 ml H_2O and the pH was adjusted to 7.35. Then solutions A and B were mixed and again equilibrated with carbogen untill a pH value of 7.35 was reached. Finally, the medium was sterile filtered.

3.1.2 Primary rat hepatocytes culture

Immediately after preparation fetal calf serum (4 ml/100 ml suspension) was added to the cell suspension of hepatocytes for better attachment to the bottom of the polystyrol dishes. Furthermore, the antibiotics (1 ml of stock solution per 100 ml cell suspension), 10^{-7} M dexamethasone and 10^{-9} M insulin as permissive hormones were added. The culture of the hepatocytes was performed on dishes of different sizes and at different cell numbers depending on the type of application:

Application	Cell number / vol. suspension plated	∅ of the culture dish
Transfection	$1x10^6$ /1.5 ml	60 mm
Protein isolation	$2x10^6$ /3.0 ml	60 mm
RNA isolation	$3x10^6$ /9.0 ml	100 mm

After the initial 4 h attachment phase (for transfected cells 5 h) the medium was changed, and the hepatocytes were further cultured in medium M 199 with the same

concentrations of hormones and antibiotics as before but without fetal calf serum. A volume of 2.5 ml medium per 60 mm culture dish and 6 ml per 100 mm culture dish were added. After 24 h the medium was changed again. The incubation of the hepatocytes was performed in gas controlled incubators in the water vapour saturated atmosphere with 8% O_2 (v/v) or 16% O_2 (v/v), 5% CO_2 and, accordingly, 87% (v/v) or 79% N_2 at 37°C.

Hormone and antibiotics stock solutions

All solutions were sterile filtered and stored at -20°C.

Antibiotics

Penicillin G, sodium salt	0.64 g/100 ml
Streptomycine sulfate	1.17 g/100 ml

in 0.9% NaCl solution

Dexamethasone (100 µM)

Dexamethasone	3.92 g/100 ml

in 0.9% NaCl solution
Dexamethasone was first dissolved in 0.3 ml of ethanol and then filled with 0.9% NaCl solution to 100 ml.

Insulin (10 µM)

Insulin	6 mg/100 ml
Bovine serum albumin	100 mg/100 ml

in 0.9% NaCl solution
Insulin was dissolved at pH 2.5, neutralized and then bovine serum albumin was added.

3.1.3 Culture of HepG2 and HeLa cells

A cryotube with the cells frozen in DMSO at -70°C (app. 5 x 10^6 cells) was thawed at RT and app. 30 ml of medium (MEM with 10% FCS and 1% antibiotics) was added. The tube was shortly centrifuged, the supernatant was sucked off, the cells were resuspended in 40 ml of medium and plated in a 170 cm^2 tissue culture flask. When

the cells became confluent (app. 25 x 10^6 cells per 170 cm^2 flask), they were trypsinized (3 ml trypsin per 170 cm^2 flask for app. 5 min at 37°C). The reaction was stopped by adding 10 ml of medium with 10% FCS which contained trypsin inhibitors. For the further culture ca. 1/3 of the HepG2 or HeLa cells were transfered to a new flask, so that the cells after about 3 days were again confluent. For transfection, cells were plated in 2.5 ml of medium on culture dishes of 60 mm diameter, for the preparation of nuclear extracts cells were plated in 16 ml of medium on culture dishes of 150 mm diameter (cell number 1.5 X 10^5/ml).

Three h before transfection and 5 h after transfection of HepG2 cells and 24 h after transfection of HeLa cells the medium was changed. A volume of 2.5 ml medium per 60 mm culture dish and 16 ml per 150 mm culture dish were added. The incubation of HepG2 and HeLa cells was performed in gas controlled incubators in the water vapour saturated atmosphere with 8% O_2 (v/v) or 16% O_2 (v/v), 5% CO_2 and, accordingly, 87% (v/v) or 79% N_2 at 37°C.

Medium for HepG2 and HeLa cells: MEM

Earle's minimum essential medium (MEM) with sodium bicarbonate and with L-glutamine, endotoxin tested and sterile filtered, was obtained from PAA Laboratories GmbH (Austria). Before use, 50 ml of fetal calf serum, 5 ml of a non-essential amino acids solution (Gibco) and 5 ml of antibiotic stock solution were added per 500 ml MEM bottle.

3.1.4 Harvesting of hepatocytes, HepG2 and HeLa cells

When the culture was finished, dishes with cells were taken from the incubator and washed twice with 0.9% NaCl. For RNA preparation the cells were frozen for 2 h at -20°C. The cells, transfected with a Luc construct, were shaked for 15 min in 300 µl of 1 x lysis buffer and then scraped in chilled Eppendorf cups. After 15 s of vortexing primary rat hepatocytes, but not HepG2 or HeLa cells, were frozen in liquid nitrogen and thawed at 37°C. The cell lysates were centrifuged for 2 min (15.800 *g*, 14.000 rpm) and supernatants were transfered into new Eppendorf cups. The supernatants were used for bioluminescence measurements as described in 3.2.9.

5x Lysis buffer

			Final concentration	
Tris	25 ml of	1 M	125	mM
CDTA	10 ml of	200 mM	10	mM
DTT	4 ml of	500 mM	10	mM
Glycerol	115 ml of	85%	50	%
TritonX-100	10 ml of	100%	5	%
H_2O	to 200 ml			

After adjustment of the pH to 7.8 with H_3PO_4 the solution was autoclaved.

3.1.5 Induction experiments in hepatocytes

Hepatocytes were transfected as described, and the medium was changed after 5 h and after 24 h. In the experiments without dexamethasone 10^{-7} M dexamethasone was present in the culture medium only for the first 5 h to allow appropriate attachment. The cells were cultured for the next 24 h under normoxic (16% O_2) or hypoxic (8% O_2) pO_2. The cells were stimulated with glucagon or dexamethasone in concentrations specified for each experiment 12 h or 3 h before harvesting without changing the medium.

3.2 Molecular biological methods

3.2.1 cDNA synthesis

For the synthesis of cDNA a „Superscript™ II RT„ (Life Technologies) reverse transcriptase, a DNA oligonucleotide primer (oligo dT_{12-18}) hybridizing to the polyA tails of the mRNA and total RNA as template were used. The „Superscript™ II RT„ reverse transcriptase is a variant of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, in which RNase H activity was eliminated by removing the C-terminal third of the polypeptide (product description).

First, 2 µg of total RNA prepared from primary rat hepatocytes, 1 µl of oligo dT_{12-18} (500 µg/ml) and 1 µl of 10 X hexanucleotide mix were added to 12 µl of water. To denature RNA secondary structures the reaction was incubated for 10 min at 68°C and cooled on ice for 10 min. Then 13 µl of a cDNA synthesis mix was added. The whole mixture was incubated for 90 min at 42°C, for 30 min at 52°C and for 15 min at 95°C and cooled on ice. To prove the synthesis of cDNA a control β-actin PCR according to a standard protocol (3.2.2) was performed.

cDNA synthesis mix

5 µl 5 X Reaction buffer („first strand buffer„)

2.5 µl 0.1 M DTT

1.5 µl 10 mM dNTP mix

1 µl RNasin (40 U/ml)

1 µl „Superscript^{TM} II RT„ (200 U/ml)

2 µl water

5 X reaction buffer („first strand buffer„)

		Final concentration
Tris/HCl	30.28 g/l	250 mM
$MgCl_2$	3.05 g/l	15 mM
KCl	27.96 g/l	375 mM

The pH was adjusted to 8.3.

3.2.2 Polymerase chain reaction (PCR)

The polymerase chain reaction allows to amplify DNA fragments due to repetitive cycles of DNA synthesis (Fig. 16). The reaction uses two specific, synthetic oligonucleotides (primers), which hybridize to sense and antisense DNA strands of the DNA fragment to be amplified, four deoxyribonucleotide triphosphates (dNTP‘s) and a heat-stable DNA polymerase. Each cycle consists of three reactions which take place under different temperatures. First, the double-stranded DNA is converted into its two single strands (denaturation at 94°C). They function as templates for the synthesis of new DNA. After heating the reaction is cooled (50-60°C) to allow the annealing (hybridization) of primers to the complementary DNA strands. Starting from the primers DNA polymerase extends both DNA strands at 72°C (DNA synthesis). Because the DNA molecules synthesized in each cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle. Already after the

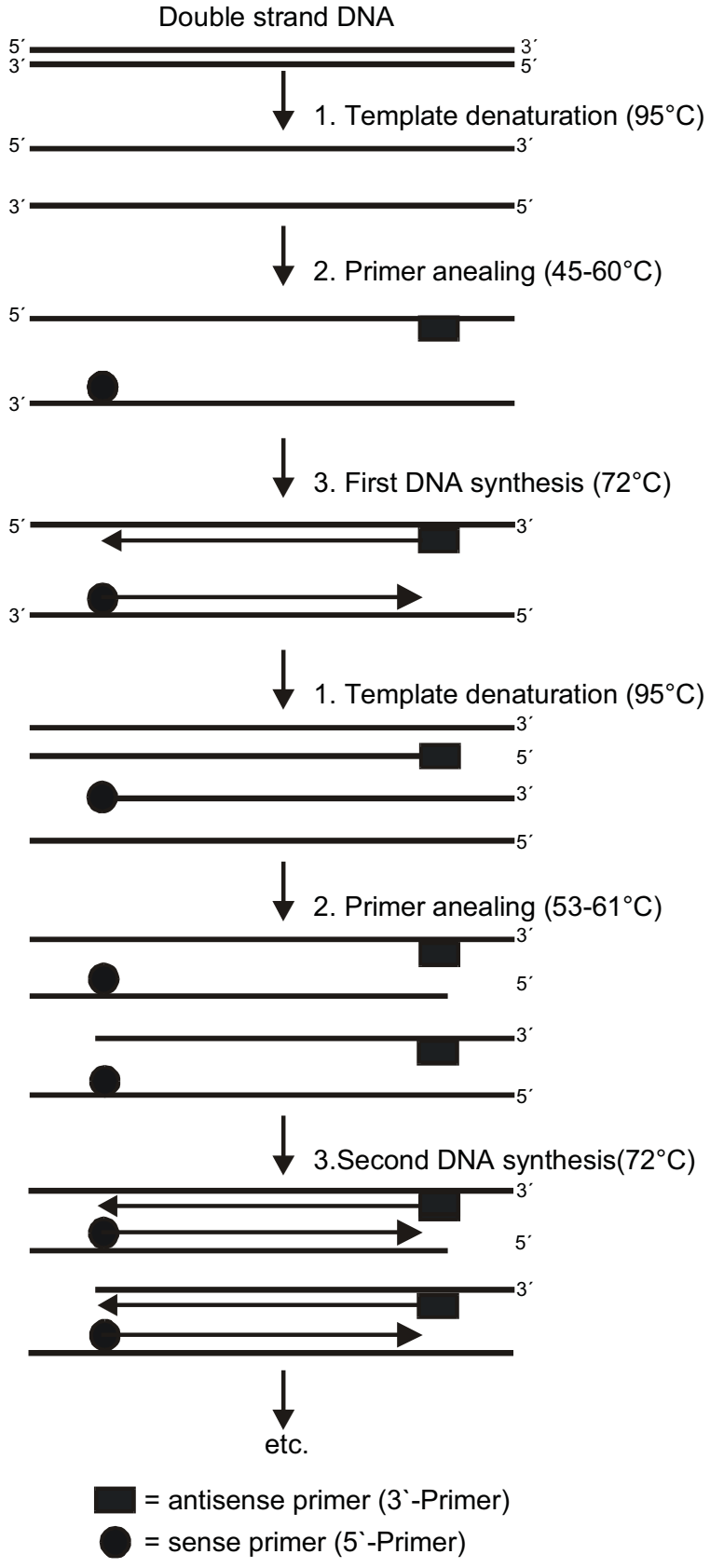


Fig. 16: **Schematic representation of the PCR.** For explanation see text.

third cycle double stranded DNA molecules of the size corresponding to the distance between two primers are synthesized. The repeating cycles of heating and cooling take place in a thermocycler.

Starting from 10 ng of template cDNA the PCR reaction with TAT-specific primers shown in 2.3.3 was performed. The PCR lasted for 30 cycles in a thermocycler under the following conditions:

1. 30 sec denaturation at 96°C
2. 45 sec annealing at 56°C
3. 90 sec (1 kb per min) at 72°C DNA synthesis

The denaturation step at 94°C before the first cycle was extended for 2 min. After the last cycle the synthesis step was prolonged for 7 min to finish synthesis of uncompletely synthesized DNA strands.

The PCR was performed with the Proof*Sprinter*™ DNA polymerase (Hybaid, Heidelberg) which consisted of a mixture of *Taq* and *Pwo* polymerases. The mixture combines the proofreading capacity of *Pwo* with the high process capability of *Taq*, which makes it convenient for cloning PCR products. The specific buffers, solutions and dNTPs were received with the polymerase.

PCR reaction:

10 ng template (cDNA)
30 pmol antisense primer
30 pmol sense primer
0.5 µl 10% tween-20
1 µl DMSO (for the denaturation of secondary structure)
3.5 µl $MgCl_2$ (25 mM)
5 µl 10 x PCR buffer (500 mM Tris/HCl pH 9,1, 140 mM $(NH_4)_2SO_4$)
1 µl 40 mM dNTP mix (10 mM of each dATP, dCTP, dGTP, dTTP)
2,5 U Taq/Pwo polymerase
to 50 µl H_2O

To check the size of the PCR product a 10 µl aliquot of the PCR reaction was electophoretically analyzed in a 1% agarose gel (3.2.12). Then, the PCR product was cloned in the PCR II-TOPO plasmid as described (3.2.3).

3.2.3 TOPO cloning of PCR products

The TOPO TA Cloning System (Invitrogen) is a 5-minute one-step method for cloning of *Taq* polymerase-amplified PCR products into a plasmid vector. A unique aspect of *Taq* DNA polymerase is that it adds a single deoxyadenosine (A) to the 3' ends of PCR products. TOPO Cloning ligates the PCR product into the pCRII-TOPO plasmid vector with topoisomerase I. This can be done because the vector is a linearized plasmid with 3' deoxythymidine (T) overhangs that is activated by being covalently bound to topoisomerase I. The 3' A overhangs of the PCR product complement the 3' T overhangs of the vector and allow fast ligation with the already present topoisomerase I. The plasmid can then be transformed into competent bacterial cells.
About 50 ng of the PCR product (3.2.2) were ligated with 10 ng of pCRII vector.

Ligation of the PCR product with the TOPO kit:

PCR product	50 ng
TOPO vector	1 µl
Salt solution (1.2 M NaCl, 0.06 M $MgCl_2$)	1 µl
H_2O	to 6 µl

The ligation reaction was incubated for 5 minutes at room temperature. Then 2 µl of the reaction mixture was added into a vial of One Shot chemically competent *E. coli* cells and mixed gently. The cells were incubated on ice for 10 minutes, heat-shocked for 30 seconds at 42°C and immediatelly transfered to ice. The tube, to which 250 µl of SOC medium was added, was shaked horizontally (200 rpm) at 37°C for 1 hour. 50 µl of transformation mixture was then spread on a prewarmed selective LB-Amp plate and incubated overnight at 37°C. The next day about 10 white or light blue but not dark blue colonies were picked for analysis as described in (3.2.5).

SOC Medium
2% tryptone
0.5% yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

3.2.4 Cloning of the synthetically synthesized double stranded oligonucleotides into the plasmid pGl3 prom

The synthesized oligonucleotides containing the TAT-NRE´s and SerDH-NRE's were cloned in the Sac I and Nhe I restriction sites of the pGL3 promoter (Fig. 13). The process of cloning had the following steps:

Preparation of oligonucleotides for the ligation in a plasmid

First, the oligodesoxynucleotides (Fig. 13) were phosphorylated at their 5´-ends by T4 polynucleotide kinase. The phosphorylation reaction samples with the sense and antisense oligodesoxynucleotides were combined by transfer to a 1.5 ml tube with a screw cap for annealing. A 1 l Erlenmeyer flask was filled with 800 ml H_2O. The water was warmed up to 90°C. The 1.5 ml tube with the annealing mixture was then transfered into the hot water, which was then allowed to cool to RT for at least 6 h. At this time the annealing of the oligodesoxynucleotides was finished. The ds-oligodesoxynucleotides were precipitated with 0.5 vol. of 7.5 M NH_4Ac and 6 vol. of absolute ethanol for 10 min on ice. The precipitated ds-oligodesoxyribonucleotides were centrifuged for 30 min at 12.500 *g* (12.500 rpm, table centrifuge), washed with 70% ethanol, dried under vakuum and dissolved in 30 µl H_2O. Then the annealing was checked in a 10% native acrylamide gel. For this, 1 µl of the oligodesoxynucleotide solution was loaded.

Reaction mixture:

Oligo (sense or antisense)	2 µg
5 x ligase buffer (5mM ATP) (Gibco)	4 µl
T4 polynucleotid kinase (10U/µl)	1.5 µl
H_2O	to 20 µl

The mixture was incubated at 37°C for 60 min.

Polyacrylamide gels allow much better separation of DNA fragments than agarose gels (3.2.5). They can separate molecules of DNA whose lengths differ only by 1 bp in 1000 bp (Sambrook et al., 2001). Since double stranded oligodesoxyribonucleotides move in the gel much slower than single stranded they are separated. After complete polymerization of the gel, the probes were loaded and the electrophoresis in 1 x TBE buffer for 30 min at 15 mA was performed. Then, the gel was washed for 10 min in ethidium bromide solution (1 µl ethidium bromide in 10 ml 1% glycine) and the DNA was visualized under UV light.

10% Acrylamide gel: (85 mm x 90 mm x 0,8 mm)

		Final concentration
Acrylamide	3.3 ml Rotiphorese®Gel 30 (30% Acrylamide/0.8% Bisacrylamide)	10 %
TBE	0.5 ml 20 x TBE buffer	1 x TBE
H_2O	To 10 ml	
TEMED	2 µl	
APS	70 µl 10% APS solution	

20 x TBE:

			Final concentration	
Tris	108.99	g/l, pH 8,5	900	mM
Borate	55.94	g/l	900	mM
EDTA	7.44	g/l	20	mM

The polymerization of acrylamide monomers via a free radical-mediated reaction in the presence of the cross-linking agents such as bisacrylamide results in the formation of the three-dimensional network of cross-linked acrylamide chains with the pores, sizes of which are dependent mostly on the acrylamide/bisacrylamide concentration. The free radicals are generated in the reaction of ammonium persulfate (APS) with TEMED. The gel was poured immediatelly after the addition of TEMED and APS and left for 45 min at RT for polymerization.

Preparation of the pGL3 promoter vector for ligation

First, the vector pGL3 promoter was linearized with the Sac I and Nhe I restriction endonucleases. To prevent religation of the linearized vector its 5'-ends were dephosphorylated.

The restriction mixture was incubated for 60 min at 37°C. Then SAP was added and the sample was further incubated for 60 min at 37°C and heated for 10 min at 65°C to inactivate alkaline phosphatase.

Restriction of the pGl3 promoter:

Vector	3 µg
Sac I	1.5 µl
Nhe I	1.5 µl
10 x buffer	2 µl
H_2O	to 20 µl

Dephosphorylation of the linearized vector:

Linearized vector	20 µl (ca. 3 µg)
SAP (1 U/µl; Shrimps alkaline phosphatase)	5 µl
10 x Alkaline phosphatase buffer	3 µl
H_2O	to 30 µl

The dephosphorylation mixture was loaded onto a 1% agarose gel, the corresponding DNA bands were cut out and purified with the "QIAEX II Gel Extraction Kit" (Qiagen). The gel slice was transfered into a 1.5 ml microfuge tube and 3 volumes of buffer QX1 were added to 1 volume of the gel, e.g. 300 µl of QX1 to each 100 mg of gel. Then 10 µl of QIAEX II solution, previously vortexed for 30 sec, was added and the mixture was incubated for 10 min at 50°C with shaking. At this step the agarose was solubilized and DNA was bound to QIAEX II particles. After the centrifugation for 30 sec at 13000 rpm the sample was washed with 500 µl of ethanol-containing buffer PE to remove residual agarose and salt contaminants. The pellet was air-dried for 10-15 min, resuspended in 20 µl H_2O and incubated for 5 min at RT for elution. After the centrifugation the supernatant contained purified DNA. To increase the yield by 10-15% the elution step was repeated. The DNA concentration was estimated when 5 µl of the purified DNA was loaded onto a 1% agarose gel in comparison with 10 µl of λ-DNA/*HindIII* of known concentration.

Ligation of the oligodesoxynucleotides into the plasmid pGl3 prom

The 5´-phosphorylated ds-oligodesoxynucleotides were ligated into the linearized and dephosphorylated pGL3 promoter vector by T4 ligase. The enzyme can only catalyze binding of the 5´-phosphorylated ends of the ds-oligodesoxynucleotides with the 3´-dephosphorylated ends of the vector; the 3´-dephosphorylated ends of the ds-oligodesoxynucleotide and the 5´-dephosphorylated ends of the vector could be ligated only by the specific enzymes present in *E. coli*. Therefore *E. coli* cells were

transformed with the ligation mixture immediatelly after finishing of the ligation. The ligation reaction was incubated overnight at 16°C and then transformed in the competent *E. coli* DH 5α cells.

Ligation reaction:

Ds-oligodesoxynucleotide (5´-phosphorylated)	100 ng
Vector (5´-dephosphorylated)	50 ng
5 x Ligase buffer (5 mM ATP) (Gibco)	4 µl
T4 Ligase (1U/µl)	0.5 µl
H_2O	to 20 µl

Components of the ligase buffer:

5 x Ligase buffer	250	mM Tris-HCl (pH 7.6)
	50	mM $MgCl_2$
	5	mM ATP
	5	mM DTT
	25	% (w/v) polyethylenglycol-8000

Stored at -20°C.

Preparation of competent E. coli cells with the one step method

5 ml LB medium was inoculated with one bacterial colony, grown from the *E. coli* DH 5 α or XL1 glycerol culture, and incubated overnight at 37°C. Then 500 ml LB medium was inoculated with 5 ml of this culture. Now the bacterial suspension was incubated for 3-6 h at 37°C untill the OD at 550 nm became 0.3-0.4. The suspension was divided into two 250 ml flasks and centrifuged for 15 min at 4080 *g* (5000 rpm, GSA rotor) at 4°C. The supernatants were removed and the pellets were resuspended each in 5 ml (1/10 vol.) of ice cold TSS buffer, divided in 100 µl aliquots in an ethanol/CO_2 bath and stored at -70°C. The competence of the bacterial cells was checked by the transformation of an ampicillin resistant plasmid.

LB medium (Luria Bertani)

Trypton	10	g/l
Yeast extract	5	g/l
NaCl	10	g/l

The pH was adjusted with NaOH to 7.3. The medium was autoclaved after preparation.

TSS buffer:

			Final concentration	
NaCl	1.0	g/100 ml	1	% (w/v)
Trypton	1.0	g/100 ml	1	% (w/v)
Yeast extract	0.5	g/100 ml	0.5	% (w/v)
$MgCl_2$	0.6	g/100 ml	30	mM
PEG 4000	10	g/100 ml	10	% (v/v)
DMSO	5.0	ml/100 ml	5	% (v/v)

The pH was adjusted with NaOH to 6.5, the buffer was sterile filtered and stored at 4°C.

Transformation of E. coli with the ligation mixture

Bacterial cells which could take up linear or circular (plasmid) double stranded DNA (competent cells) were used for transformation. The ability for DNA uptake could be stimulated in bacteria by incubation with $CaCl_2$ and is maintained in frozen cells at -70°C.

The ligation mixture or the plasmid DNA was carefully mixed with one aliquot of the competent cells and incubated for 30 min at 4°C. After a 2 minute heat shock at 42°C, the bacterial cells grew for 1 h at 37°C in 800 µl LB medium without antibiotics. After 1 h a 300 µl aliquot was spread over an ampicillin-containing agar dish and incubated overnight at 37°C.

Transformation mixture

Competent DH 5α or XL1 cells	100	µl
Ligation mixture	7.5	µl
or plasmid	1	ng

Ampicillin agar dishes

500 ml LB medium containing 6.25 g bactoagar was autoclaved. After cooling to 50°C, 200 µl of an ampicillin stock solution (final concentration 40 µg/ml) was added, mixed and 10 ml were poured into each sterile petri dish. The dishes were turned upside down and left for drying for ca. 6 h at 37°C. The dried dishes were then stored in the dark at 4°C.

Ampicillin stock solution

Ampicillin	100 mg/ml

Dissolved in H_2O; the pH was adjusted with HCl to 7.0; 500 µl aliquots were stored at -20°C.

3.2.5 Isolation and analysis of plasmid DNA (minipreparation)

Colonies of bacteria transformed with a ligation mixture or with a plasmid, which grew on LB-ampicillin media, were inoculated in 5 ml ampicillin-containing (40 µg/ml) LB medium to prove that the bacteria contained the “right“ plasmid. From the 5 ml medium plasmid DNA was isolated by minipreparation.

3 ml of the bacterial culture (2 x 1.5 ml in a Eppendorf cup) were centrifuged in a table centrifuge (30 s, 15800 *g*, 14000 rpm). The medium was removed and the bacterial pellet was resuspended in 200 µl STET buffer. For lysis of cells, 50 µl lysozyme (10 mg/ml) was added. The solution was mixed, heated for 60 s at 95°C and cooled on ice. The resulting bacterial lysate was centrifuged for 10 min (15800 *g*, 14000 rpm) and the sediment containing proteins and genomic DNA was removed with tweezers. The plasmid DNA was precipitated from the supernatant with 150 µl isopropanol (10 min at -20°C). After 5 min of centrifugation the pellet was resuspended in 200 µl Tris/EDTA/NaCl and the plasmid DNA was again precipitated with 200 µl isopropanol (-20°C for 10 min). The pellet was washed once with 70% ethanol, air-dried and dissolved in 40 µl H_2O. The resulting DNA was used for restriction analysis.

STET buffer

			Final concentration	
Tris/HCl, pH 8.0	0.61	g/100 ml	50	mM
EDTA	1.86	g/100 ml	50	mM
Triton-X-100	0.5	g/100 ml	0.5	%
Sucrose	8	g/100 ml	8	%

The buffer was autoclaved and stored at RT.

Tris/EDTA/NaCl

			Final concentration	
Tris/HCl, pH 7.8	0.121	g/100 ml	10	mM
EDTA	37.2	mg/100 ml	1	mM
NaCl	1.75	g/100 ml	300	mM

Restriction analysis

Plasmid DNA	10	µl
10 x buffer (enzyme specific)	2	µl
Restriction enzyme (10 U/µl)	0.5	µl
H_2O	to 20	µl

The restriction reaction mixture was incubated for 1 h at 37°C. Since the minipreparation contained RNA, 1 µl of RNase A (10 µg/µl) was added and incubated for 15 min at 37°C. Then, the sample was mixed with 3 µl of loading buffer and loaded onto a 1% agarose gel. To determine the size of DNA fragments a DNA molecular weight standard was used. The electrophoresis was performed for 45-60 min with 5 Volt/cm. The negatively charged DNA migrated from the catode (-) to anode (+). To visualize DNA in the gel, it was treated with ethidium bromide which intercalated between the bases of DNA double strands forming a complex fluorescent under UV light.

For preparation of the 1% gel, 1 g of agarose was dissolved by boiling in 100 ml of 1 x TAE buffer. After the agarose solution was cooled to 60°C it was poured into a gel chamber (8 cm x 5 cm x 0,4 cm). The electrophoresis chamber was filled with 1 x TAE buffer so that the gel was covered. The restriction samples were loaded to the gel pockets. After electrophoresis was finished, the gel was washed in 500 ml of 1% glycine solution with 10 µl of ethidium bromide and the bands were detected under UV.

After proving that the DNA restriction fragments had the right sizes, a glycerol culture of the transformed bacteria was prepared. It could be stored at -70°C for some years. For preparation of a glycerol culture 500 µl of sterile glycerol was mixed with 500 µl of bacterial suspension.

<u>Ethidium bromide solution</u>

Ethidium bromide	10	mg/ml

Stored at 4°C, protected from light.

<u>10 x Tris/acetate/EDTA (TAE) buffer</u>

			Final concentration	
Tris	6.1	g/100 ml	0.5	M
Sodium acetate	1.6	g/100 ml	0.2	M
EDTA	0.7	g/100 ml	0.02	M

The pH was adjusted with acetic acid to 7.4, the buffer was autoclaved.

Loading buffer

			Final concentration	
Bromphenol blue	0.01	g/100 ml	0.01	%
Glycerol	40	ml/100 ml	40	%
10 x TAE buffer	10	ml/100 ml	1	X

The buffer was autoclaved and stored at 4°C.

3.2.6 Isolation and analysis of plasmid DNA with silicate columns (maxipreparation)

The principle of the plasmid DNA purification with the silicate columns is that after neutralization of the bacterial lysate, obtained with the modified alkaline/SDS method, the plasmid DNA binds to the anion exchange resin inside the column. Washing the resin with the buffers of certain pH and ion strength removes single stranded DNA, RNA and all other impurities such as proteins, metabolites, polysaccharides and dNTPs. Afterwards, the double stranded plasmid DNA can be eluted from the column and finally precipitated by alcohol (product information).

The preparation of plasmid DNA for transfection was performed with JETstar Plasmid Maxiprep Kit (Genomed) according to manufacturer descriptions.

The transformed *E. coli* DH5α cells were cultured in 200 ml LB medium to a density of about 10^9 per ml (OD at 600 nm of 1-1.5). The cells were pelleted by centrifugation for 30 min at –4°C and 5860 *g* (6000 rpm, GSA rotor). The pellet was resuspended in 10 ml of buffer E1, which contained 100 µg/ml of RNase. Then 10 ml of buffer E2 (with NaOH and SDS for bacterial lysis) was added and mixed gently 4 - 6 times (the mixture should not be vortexed to avoid shearing of genomic DNA). After 5 min incubation at RT (longer incubation could lead to irreversible denaturation of plasmid DNA), 10 ml of buffer E3 was added for neutralization of the solution. The solution was centrifuged for 10 min at 20°C and 16300 *g* (10000 rpm, GSA rotor), during which an undense pellet containing bacterial debris, chromosomal DNA etc. was formed, and applied to the column (length 13 cm, ∅ 2,6 cm) equilibrated with 30 ml of buffer E4. When the lysate has been completely run by gravity flow through the column, the column was washed once with 60 ml of buffer E5. Then plasmid DNA was eluted with 15 ml of buffer E6. The eluate was mixed with 10.5 ml of isopropanol to precipitate DNA, stored for 1 h at –20°C and centrifuged for 30 min at 27000 *g* and 4°C (15000 rpm, SS34 rotor). The DNA pellet was washed with 70% ethanol, to remove salts, dried for 30 min and dissolved in 500 µl H_2O.

To determine DNA concentration and the presence of protein in the probes, the OD at 260 nm (DNA) and 280 nm (protein) was measured. 1 OD at 260 nm ≈ 50 µg DNA/ml. In a protein-free solution the ratio OD_{260}/OD_{280} is 2, in the present experiments it was usually 1.6-1.8.

The size of the prepared plasmid and the presence of linearized DNA was proved by restriction analysis.

Buffer E1	50 mM Tris	pH 8.0, adjusted with HCl
	10 mM EDTA	E1 with RNase A stored at 4°C
	100 µg/ ml RNase A	
Buffer E2	200 mM NaOH	
	1% SDS	
Buffer E3	3.1 M potassium acetate	pH 5.5, adjusted with acetic acid
Buffer E4	600 mM NaCl	pH 5.0, adjusted with acetic acid
	100 mM sodium acetate	
	0.15% Triton X-100	
Buffer E5	800 mM NaCl	pH 5.0, adjusted with acetic acid
	100 mM sodium acetate	
Buffer E6	1.25 M NaCl	pH 8.5, adjusted with HCl
	100 mM Tris	

3.2.7 Sequencing of plasmids

To check the sequences of the cloned plasmids sequencing reactions were performed and analyzed with the DNA sequencer (ABI, modell 373 A).

The sequencing was performed by a “dye terminator cycle sequencing“ method according to the manual (Perkin Elmer). In this method a premix solution containing 4 didesoxynucleotides (ddNTP), each labeled with a different fluorescent dye, mixed with unlabeled desoxynucleotides was used. Then the template plasmid and one primer were added to the reaction mixture so that only one DNA strand was synthesized. The cycles of denaturation, annealing and synthesis were repeated 25 times. In this process DNA fragments of different size labeled at their 3´-ends with base specific fluorescent dyes were synthesized.

After finishing of the sequence reaction the solution was cooled at 4°C and purified as follows: 2 µl 3 M NaAc, pH 5.2 and 50 µl 95% ethanol (RT) were added and the mixture was centrifuged for 20 min at 15800 *g* (14000 rpm, table centrifuge). The pellet was washed with 250 µl of 70% ethanol, centrifuged for 10 min, dried and dissolved in 25 µl of water. After denaturation for 2 min at 90°C the probes were loaded on the gel

(4.75% polyacrylamide DNA sequence gel). The DNA fragments were electrophoretically separated in the sequence service laboratory. The fluorescence of fluorescent dye-containing polynucleotides was stimulated by 40 mW argon laser (488 nm and 514 nm). The fluorescent signal was identified by the detector system of the DNA sequencer and quantificated.

Sequence reaction

			Final concentration	
Plasmid	X	µl	800	ng/probe
Primer (2.5 pmol/µl)	2	µl	5	pmol/probe
Premix	8	µl		
H_2O	X	µl	to 20	µl

For the sequencing of the pGL3 promoter vector two primers were used: GL2 primer (forward primer) for the sequencing in 5´ direction from the luciferase gene and RV3 primer (reporter vector primer or reverse primer) for the sequencing in 3´ direction over the polylinker region (2.4.1).
The Ready Reaction Dye Deoxy-Terminator Kit (premix) contains Ampli Taq DNA polymerase FS, fluorescent ddNTPs, dNTPs, dITP and buffer (no information concerning composition was available).
The sequence reaction was performed under following conditions: denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s, polymerization reaction at 60°C for 4 min.

3.2.8 Transfection of hepatocytes, HeLa and HepG2 cells

For transfection of primary hepatocytes, HeLa and HepG2 cells the calcium phosphate precipitation method was used. All the protocols for calcium phosphate precipitation, including the protocol used in the present work, are modifications of the protocol from Graham and van der Eb (Graham et al., 1973). The differences are mostly concerning the type of cells used and the time and duration of transfection (Parker et al., 1979; Chen et al., 1987; Chen et al., 1988; Ginot et al., 1989; Pasco et al., 1989; Rippe et al., 1990). The principle of the method is that the DNA forms together with calcium phosphate small precipitates which are taken up by cells via the process of endocytosis. The precipitate is obtained when the DNA/calcium chloride solution is mixed with a solution containing phosphate.

Transfection mixture for one dish (60 mm)

			Final concentration
Plasmid DNA	2.5	µg	
H_2O	to 67.5	µl	
$CaCl_2$ 2.5 M	7.5	µl	125 mM
2 x Hepes	75	µl	

The transfection mixture was prepared in polystyrol tubes to prevent adhesion of the DNA to the walls of the flask. After pipeting the solutions were mixed and left for 5-10 min at RT. During this time the DNA/calcium phosphate precipitate was formed, which was visible as a light turbidity of the solution. Finally, 150 µl of the mixture was added to 1.5 ml of freshly plated hepatocytes in suspension or to 2.5 ml of precultured HeLa or HepG2 cells in 60 mm ∅ dishes. Alternatively, 940 µl of the suspension was plated in 9.4 ml of precultured HepG2 cells in 150 mm ∅ dishes. Then the cells were incubated at 16% O_2.
The HepG2 and HeLa cells were plated 16-18 h before transfection at a density of 2.8 x 10^6/15.5 ml medium and 3-4 h before the transfection the medium was changed.
With hepatocytes and HepG2 cells the medium was changed 5 h and 24 h after the transfection, with HeLa cells the medium was changed 24 h after transfection.

2 x Hepes

			Final concentration
Hepes	1.192	g/100 ml	50 mM
NaCl	1.636	g/100 ml	280 mM
$Na_2 HPO_4$	0.267	g/100 ml	1.5 mM

The pH was adjusted with 5 N NaOH to 7.05, the buffer was stored in 10 ml aliquots at -20°C.

Calcium chloride 2.5 M

			Final concentration
$CaCl_2$	36.75	g/100 ml	2.5 M

The solution was autoclaved and stored in aliquots at -20°C.

3.2.9 Luciferase detection

The detection of luciferase activity in the cells transfected with reporter vectors containing the luciferase gene from north american firefly (Photinus pyralis) was

performed with the Luciferase Assay Kit (Berthold, Pforzheim). The luciferase assay is based on the enzyme-catalyzed chemiluminescence. Luciferin present in the luciferase assay reagent is oxidized by luciferase in the presence of ATP, air oxygen and magnesium ions. This reaction produces light with a wave length of 562 nm. The luminescence can be measured by a luminometer.

20 µl of the supernatant from the transfected cells were automatically mixed in the luminometer with 100 µl luciferase assay reagent, prepared directly before use by mixing of equal parts of solutions A and B. The reaction was measured 10 times for 2 s. The intensity of luminescence is constant for 20 s and then decreases with a half-life period of 5 min. No information about the composition of solution A and B was given by the supplier.

3.2.10 Preparation of digoxigenin-labeled TAT and SerDH RNA probes

The detection of mRNA on Northern blots was performed after hybridization with digoxigenin labeled TAT and SerDH probes.

The DIG RNA Labeling Kit generates DIG-labeled, single-stranded RNA probes by *in vitro* transcription. RNA probes are labeled with digoxigenin using SP6, T7 or T3 polymerases. A restriction enzyme linearizes the DNA template allowing generation of transcripts of uniform length. One digoxigenin-11-uridininemonophosphate (DIG-UMP) residue is incorporated every 20-25 nucleotides.

For preparation of the digoxigenin-labeled antisense TAT and SerDH RNA‘s the vectors pCRII-TOPO-TAT and pBS-SerDH were linearized by *Kpn I* and used for *in vitro* transcription with T7 RNA polymerase.

Linearization of the plasmids

Restriction reaction:

Plasmid	5	µg
Restriction enzyme *Kpn I*	1.5	µl
10x restriction buffer L	2	µl
H_2O	to 20	µl

The solution was incubated for 2 h at 37°C and then treated for 15 min with RNase. After a phenol/chloroform extraction and ethanol precipitation the pellet was dissolved in 33 µl of DEPC-H_2O.

In vitro transcription

Reaction of *in vitro* transcription:

Linearized plasmid	11	µl
10 x transcription buffer	2	µl
10 x labeling mix	2	µl
RNasin (40 U/µl)	1	µl
T7 RNA polymerase (20 U/µl)	2	µl
DEPC-H_2O	2	µl

Transcription buffer

Tris/HCl pH 7,5	400	mM
$MgCl_2$	60	mM
DTT	10	mM
NaCl	10	mM
Spermidine	10	mM

10 x labeling mix:

ATP	10	mM
CTP	10	mM
GTP	10	mM
UTP	6.5	mM
DIG-UTP	3.5	mM

The solution was incubated for 1.5 h at 37°C. Then 0.5 µl RNA polymerase was again added and the incubation at 37°C was prolonged for another 1 h. Synthesized digoxigenin-labeled RNA was precipitated with 2.5 µl 4 M LiCl and 75 µl absolute ethanol at -20°C for at least 2 h. The solution was centrifuged at 12000 *g* (10000 rpm, SS34 rotor) for 10 min at 4°C, the pellet washed with 80% ethanol and dried in a vacuum centrifuge. The pellet was dissolved in 100 µl DEPC-H_2O.

Estimation of the labeling efficiency

The estimation of probe yield was made by comparison of the DIG-labeled sample RNA with a DIG-labeled control DNA provided in the labeling kit. One µl of the

transcript as well as 0.04-5 ng of a digoxigenin-labeled DNA control were spotted on a Hybond N membrane. The nucleic acids were fixed to the membrane in the UV stratalinker for 2 min with 1200 µJ. The digoxigenin-labeled transcripts were treated as follows: the membrane was washed for 5 min in buffer 1 and incubated with 1% blocking reagent for 20 min (1% blocking reagent = 10% blocking reagent diluted with buffer 1 (maleic acid buffer)). Then the nylon membrane was incubated for 20 min in 20 ml of diluted antibody solution (anti-digoxigenin alkaline phosphatase conjugate in 1% blocking reagent at a concentration of 75 mU/ml = 1:10000 dilution = 2 µl antibody solution in 20 ml 1% blocking reagent). The unbound antibody conjugate was removed by washing of the membrane 2 times in buffer 1 for 15 min. After 2 min equilibration in buffer 3 the color substrate solution containing 45 µl NBT and 35 µl X-phosphate in 10 ml of buffer 3 was added. The color spots started to appear within 3 min. After about 10 min the reaction was stopped by washing the membrane in TE buffer. All steps were performed at RT.

NBT

		Final concentration
NBT	74 mg/ml in 70% DMF	0.41 mM

The solution was stored at -20°C, protected from light.

X-phosphate

		Final concentration
X-phosphate	50 mg/ml in DMF	0.38 mM

The solution was stored at -20°C.
Other solutions are described in 3.2.12.

3.2.11 RNA isolation from primary rat hepatocytes

The isolation of total RNA, used in the Northern blot, was performed by modification of the method described by Chomczynski and Sacchi (Chomczynski et al., 1987). After a combined phenol/chloroform/isoamyl alcohol extraction the RNA was precipitated from the water phase and further purified by washing in ethanol.
3 x 10^6 cells were plated on each culture dish (∅ = 100 mm). After washing (0.9% NaCl) and freezing of the cells (3.1.4) 500 µl of guanidinium thiocyanate buffer was added to each dish. The cells were scraped with a disposable cell scraper and homogenized by pipeting up and down. The homogenate was transfered to a 2 ml

Eppendorf cup. 50 µl 2 M sodium acetate (pH 4.1), 500 µl H_2O-saturated phenol and 100 µl chloroform/isoamyl alcohol (ratio 49:1) were added with gentle mixing one after another. Then the solution was incubated for 15 min on ice and centrifuged for 20 min at 10000 *g* and 4°C in a SS34 rotor. The RNA-containing upper phase was transfered to a new 2 ml Eppendorf cup. One ml isopropanol was added and the RNA was precipitated for 1 h at -20°C. After centrifugation for 10 min at 10000 *g* at 4°C in the SS34 rotor the RNA pellet was washed with 200 µl 80% ethanol. After a new centrifugation for 10 min at 10000 *g* the purified RNA was dried in a vacuum centrifuge at 37°C for 3 min. Finally, the pellet was dissolved in 40 µl 0.1% SDS. After measuring the RNA concentration it was stored at -20°C.

To determine the concentration and purity of the RNA, the extinction at 260 nm and 280 nm was measured. An OD of 1 at 260 nm corresponds to 40 µg RNA/ml. The ratio of the OD at 260 nm and at 280 nm is a measure of RNA purity. In a protein-free solution the ratio OD_{260}/OD_{280} is 2. Due to protein contaminations this coefficient is usually lower. In our experiments it was over 1.7.

Solutions for the RNA isolation

All solutions for RNA experiments were prepared with DEPC-H_2O. By addition of DEPC to millipore water for 12 h RNases in water were inactivated. Then the water was autoclaved.

GTC buffer

			Final concentration	
Guanidinium thiocyanate	47.3	g	4	M
Sodium citrate	2.5	ml 1 M	25	mM
N-lauroyl sarcosine	0.5	g	17	mM
2-mercaptoethanol	0.7	ml	0.1	M
30% antifoam A	0.33	ml	0.1	%
DEPC-H_2O	to 100	ml		

The solution was warmed for 30 min at 65°C. The pH was adjusted with 1 N NaOH to 7.0. 2-mercaptoethanol was added at the end.

Phenol (water saturated)

2/3 volume of phenol was mixed with 1/3 volume of H_2O. After the phase separation the solution was stored protected from light at 4°C.

3.2.12 Northern blot analysis

Northern blot analysis is a method to quantify RNA expression. The RNA is separated in a denaturing agarose gel, transfered by capillary transfer to a nitrocellulose membrane (vacuum blot) and fixed by UV crosslinking. The RNA of interest is identified by hybridization with a specific probe.
All solutions used for the Northern blot were autoclaved, the electrophoresis and blot chambers, gel plates and combs were kept in 3% H_2O_2 for 1 h before use to inactivate RNases.

Denaturation of the RNA

The standard sample to be analyzed in a blot contained 30 µg RNA and 16.5 µl loading buffer. The sample was denaturated at 68°C for 15 min and subsequently cooled on ice for 2 min. After addition of 5 µl sample buffer the RNA was loaded to the gel.

Loading buffer

			Final concentration	
Formamide	15	ml	66.6	%
14.3 x MOPS	2.1	ml	26.7	mM
37% formaldehyde	5.4	ml	8.9	%

The buffer was aliquoted and stored at -20°C.

MOPS 14.3x

			Final concentration	
MOPS	5.93	g	286	mM
NaAc	0.58	g	7.1	mM
EDTA	0.5	g	1.3	mM
DEPC-H_2O	to 100	ml		

The pH was adjusted with NaOH to 7.0 and the solution was autoclaved.

Sample buffer

			Final concentration	
Glycerin	5	ml	50	%
0.5 M EDTA	20	µl	1	mM
Bromphenol blue	10	mg	0.1	%
DEPC-H_2O	to 10	ml		

The buffer was aliquoted and stored at -20°C.

Electrophoresis conditions

The denaturing agarose gel was placed into an electrophoresis chamber filled with 1 x MOPS buffer (900 ml). After the samples were loaded, the electrophoresis was performed at 100 Volt for 2 h.

To denature the RNA, formaldehyde was added to the gel. For preparation of a 1.5% gel 0.9 g agarose was dissolved by heating in 49 ml DEPC-H_2O. Then 6 ml 10 x MOPS and 5 ml formaldehyde were added. After mixing, the gel was poured into the prepared gel plate. The gel plate (10 x 14 cm) and the comb (size of one gel pocket made with the comb was 4 mm; number of bags was 16) were kept for at least 1 h in a chamber filled with 3% H_2O_2 to make them RNase free.

MOPS 10x

			Final concentration	
MOPS	41.9	g	200	mM
NaAc	4.1	g	50	mM
EDTA	3.7	g	10	mM
DEPC-H_2O	to 1	l		

The pH was adjusted with NaOH to 7.0. The solution was autoclaved.

Visualization of the RNA with ethidium bromide

After gel electrophoresis the formaldehyde was washed out by shaking the gel in 150 ml of a 1% glycine solution for 20 min. A 5 µl ethidium bromide solution (3.2.5) was added and the gel was further incubated for 5 min. Ethidium bromide formed a complex with RNA which was fluorescent under UV light (254 nm) and two main bands corresponding to 28S and 18S ribosomal RNA were visible. The gel was photographed with a video camera. Finally, to hydrolyse RNA the gel was washed for 1 h in 50 mM NaOH/10 mM NaCl.

RNA transfer to nylon membranes

RNA separated in the gel was transfered to a nylon membrane by capillary transfer. For this a nylon membrane (10×15 cm) was washed in 2 X SSC for 10 min. The transfer equipment was prepared in the following way: 3 mm thick filter paper, washed in transfer buffer (20 x SSC), on which the nylon membrane was placed, was put on a

vacuum chamber. Also a rubber mat was placed in a way that no free space was left between it and a membrane.

The gel was put on top of the membrane and the rubber mat and the vacuum switched on before the chamber was filled with transfer buffer. The RNA was transfered onto the nylon membrane by capillary force. The transfer was performed for 1-2 h.

After the transfer was finished RNA was fixed on the membrane by UV crosslinking for 2 min. To check if the transfer was complete the gel was again observed under UV light.

Hybridization of the RNA with digoxigenin-labeled RNA probes

The RNA of interest can be identified by a specific probe. As a probe digoxigenin-labeled antisense TAT or SerDH RNA was used.
The nylon membrane was placed for prehybridization into a hybridization tube. The prehybridization, which is necessary to prevent unspecific binding, was performed for 1 h with 10 ml prehybridization solution at 68°C in a hybridization oven. Finally, the prehybridization solution was removed and replaced with 6 ml hybridization solution containing 100 ng of digoxigenin-labeled antisense RNA probe. The hybridization was performed overnight at 68°C. To remove the unbound probe and to decrease background the membrane was washed 2 x 5 min in 2xSSC/0.1% SDS and 2 x 15 min in 0.1x SSC/0.1% SDS at 65°C (posthybridization).

Prehybridization- and hybridization solutions

			Final concentration	
Deionized formamide	12.5	ml	50	%
10% blocking reagent	6	ml	2.5	%
20% SDS	25	µl	0.02	%
10% N-lauroylsarcosine	250	µl	0.1	%
20 x SSC	6.25	ml	5	X

The solution was stored at 4°C.

Detection and quantification

The detection of the hybrides between the RNA of interest and the digoxigenin-labeled asRNA probe was performed using ani-digoxigenin antibodies conjugated to alkaline phosphatase (enzyme immunoassay). During the following dephosphorylation of dinatrium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5´-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-

yl)-phenylphosphat (CSPD) by alkaline phosphatase a chemiluminescent unstable product was formed which produced light of 477 nm. This light signal can be recorded on X-ray films. The quantification was made densitometrically.

The detection was performed with the DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer instructions. First, after hybridization and posthybridization washes of the nylon membrane it was equilibrated in 1 x maleic acid buffer for 1 min. To prevent nonspecific binding of the DIG antibody to the membrane it was washed for 30 min in 100 ml 1% blocking reagent (10% blocking reagent diluted to 1% with 1 x maleic acid buffer). Then the nylon membrane was incubated for 30 min at RT in 20-30 ml diluted antibody solution (anti-digoxigenin, Fab fragments, conjugated to alkaline phosphatase, in 1% blocking reagent at a concentration of 75 mU/ml = 1:10000 dilution = 2-3 µl antibody solution in 20-30 ml 1% blocking reagent).

The unbound antibody conjugate was removed by washing in maleic acid buffer 2 x 15 min. After 2 min equilibration in buffer 3 the nylon membrane was wrapped in film and incubated for 5 min with diluted CSPD solution (CSPD in buffer 3 at a concentration of 25 mM = 1:100 = 10 µl CSPD in 1 ml buffer 3). The liquid was removed and the membrane was incubated for 10 min at 37°C. Then the membrane was exposed to a X-ray film for 30-90 min.

For quantification of the RNA bands the videodensitometer (Biotec-Fischer) was used.

Buffer 1 (maleic acid buffer) 1 x

			Final concentration	
Maleic acid	11.61	g	0.1	M
NaCl	8.78	g	0.15	M
H_2O	to 1	l		

The pH was adjusted to 7.5 with solid NaOH. The solution was autoclaved.

Buffer 3

			Final concentration	
Tris	12.11	g	0.1	M
NaCl	5.84	g	0.1	M
$MgCl_2$	10.17	g	50	mM
H_2O	to 1	l		

The pH was adjusted with HCl to 9.5. Then the buffer was autoclaved and steril filtered, $MgCl_2$ was added to it.

3.2.13 Electrophoretic Mobility Shift Assay (EMSA)

Preparation of probes for analysis of DNA-protein binding

For investigation of DNA-protein interactions with electrophoretic mobility shift assays double-stranded DNA probes of 18 bp were used. These probes were labeled with radioactive ^{32}P ATP.
Before labeling a double-stranded probe was prepared by annealing of two complementary oligodesoxynucleotides.

			Final concentration	
Oligodesoxynucleotide 1 (sense)	50	µg	0.5	µg/µl
Oligodesoxynucleotide 2 (antisense)	50	µg	0.5	µg/µl
5 M NaCl	2	µl	100	µM
1 M Tris, pH 7.8	4	µl	40	µM
H_2O	to 100	µl		

The annealing was performed as described in 3.2.4. To prove that annealing was successful 1 µl of the annealed DNA was loaded together with unannealed oligonucleotides on 10% acrylamide gel.

5'-end labeling of DNA probes by T4 polynucleotide kinase

The radioactive 5'-end labeling of oligodesoxynucleotides was performed by [γ-^{32}P]ATP with the "5'-end labeling kit" (MBI). The T4 polynucleotide kinase in this reaction transfered γ-phosphate of the [γ-^{32}P]ATP to the 5'-hydroxyl group of the oligodesoxynucleotide.

			Final concentration	
Oligodesoxynucleotide (10 pmol/µl)	0.5	µl	5	pmol/sample
10 x kinase buffer	1	µl	1	X
[γ-^{32}P]ATP (10 µCi/µl)	3	µl	30	µCi/sample
T4-PNK (8 Units/µl)	1.5	µl	12	Units/sample
H_2O	to 10	µl		

Composition of the buffer:

10 x kinase buffer	300	µl 0.5 M Tris-HCl, pH 7.6
	0.1	M $MgCl_2$
	50	mM DTT
	1	mM spermidine
	1	mM EDTA, pH 8.0

The solution was incubated for 45 min at 37°C and then for 10 min at 70°C to inactivate the kinase. The labeled probes were purified with the "nucleotide removal kit" (Qiagen). No information about the composition of the buffers was given by the manufacturer. Ten volumes of buffer PN were added to the labeled probe. The mixture was loaded on the column from the kit and centrifuged for 1 min at 6000 rpm in a table centrifuge. At this step all DNA fragments with the size varying from 20 bp to 10 kb were bound to the column. Then the column was washed twice with 500 µl of ethanol-containing buffer PE to remove free nucleotides. Finally, the DNA was eluted with 100 µl H_2O.
To prove the labeling Czerenkov counts from 1 µl of the probe was measured in a LSC (liquid scintillation counter).

Preparation of nuclear extracts from primary hepatocytes and HepG2 cells

The nuclear extracts were prepared according to the method of Dignam et. al. (Dignam et al., 1983). All steps were performed at 4°C to prevent proteolytic degradation of the proteins. The cells were first washed twice with cold 0.9% NaCl and then scraped in 600 µl of 0.9% NaCl. The solution was centrifuged at 2000 rpm for 2 min and then resuspended in 5 PCV ("packed cell volumes") of the hypotonic buffer A. The cells were left on ice for 10 min. Then the swollen cells were homogenized by 10-30 blows of a glas-teflon homogenizer. The cell homogenization was proved by microscopic examination. Too strong homogenization can lead to destruction of cell nuclei and to lower extract yields. Then the homogenate was centrifuged for 10 min at 2300 rpm in a SS34 rotor. The cytoplasmatic supernatant was removed and the pellet was mixed with 3 PCV of buffer A and again centrifuged for 20 min at 14500 rpm in the SS34 rotor. The resulting sediment containing "crude nuclei" was resuspended in 3 PCV of buffer C by 10 blows of the homogenizer. To extract nuclear proteins this solution was stirred in a magnet mixer for 30 min. During the next centrifugation for 30 min at 14500 rpm in the SS34 rotor the destroied nuclei were sedimented. The supernatant containing nuclear proteins was dialysed for max. 4 h against 50 volumes of buffer D to lower the

ionic concentration of the protein-containing buffer. The dialysate was centrifuged at 14500 rpm, the supernatant was aliquoted, frozen in liquid nitrogen and stored at -70°C. The protein concentration of the extracts was measured by the Bradford method. It was usually between 1 and 5 µg/µl.

Buffer A			Final concentration	
Hepes-KOH, pH 7.9	1.19	g/ 500 ml	10	mM
$MgCl_2 \cdot 6\ H_2O$	0.152	g/ 500 ml	1.5	mM
KCl	0.373	g/ 500 ml	10	mM
DTT	500	µl 0.5 M / 500 ml	0.5	mM

Buffer C				
Hepes-KOH, pH 7.9	1.19	g/ 250 ml	20	mM
$MgCl_2 \cdot 6\ H_2O$	76	mg/ 250 ml	1.5	mM
NaCl	21	ml 5 M/ 250 ml	0.42	M
DTT	250	µl 0.5 M/ 250 ml	0.5	mM
EDTA	250	µl 0.2 M/ 250 ml	0.2	mM
Glycerin	50	ml/ 250 ml	20	%

Buffer D				
Hepes-KOH, pH 7.9	4.76	g/ l	20	mM
KCl	7.456	g/ l	100	mM
DTT	1	ml 0.5 M/ l	0.5	mM
EDTA	1	ml 0.2 M/ l	0.2	mM
Glycerin	200	ml/ l	20	%

Protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin and phosphatase inhibitor sodium vanadate were added immediatelly before preparation; DTT was also added from a new tube of stock solution stored at -20°C.

	Stock concentration	Final concentration
PMSF	200 mM in ethanol (stored at 4°C)	0.4 MM
Aprotinin	1.4 mg/ ml (stored at 4°C)	2 µg/ ml
Leupeptin	2 mg/ ml H_2O (stored at -20°C)	2 µg/ ml
Pepstatin A	2 mg/ ml ethanol (stored at -20°C)	2 µg/ ml
Na_3VO_4	1 M in H_2O	1 mM

Product informatin (Roche)

PMSF: inhibits serine proteases like chymotrypsin, trypsin and thrombin as well as the cysteine protease papain (reversible by DTT treatment). Does not inhibit metalloproteases, most cysteine or aspartate proteases. Under some conditions like alkaline pH PMSF has a half life of only 35 min.

Pepstatin: inhibits potently the HIV protease and other aspartate proteases like pepsin, renin, cathepsin D, chymosin and many microbal acid proteases. Pepstatin is not soluble in water.

Leupeptin: inhibits serine and thiol proteases like trypsin, plasmin, proteinase K, kallikrein, papain, thrombin and cathepsin A and B. Not affected are α, β, γ and δ-chymotrypsins, pepsin, cathepsin D, elastase, renin and thermolysin.

Measurement of protein concentration by the Bradford method

For the determination of the protein concentration 1 µl of the nuclear extract was mixed with 99 µl water and then 1 ml Bradford reagent was added. To set the zero value 100 µl H_2O + 1000 µl reagent was used. After 10 min incubation at RT the OD_{595} was measured. A calibration curve was made with 0-6 µg of bovine serum albumin solution in water. The calibration curve up to 6 µg of protein was linear.

Protein concentration measurement:

Nuclear extract	1	µl
or BSA stock solution (1 µg/µl)	1; 2; 4; 6	µl
H_2O	to 100	µl
Bradford reagent	1	ml

Bradford reagent:

Serva blue (no. 35050)	100	mg
Ethanol (95%)	50	ml
Phosphoric acid (85%)	100	ml
To 1 l with H_2O		

Electrophoretic Mobility Shift Assay

The EMSA is a method to detect DNA-protein interactions. Proteins and labeled DNA oligonucleotides were incubated together and then analyzed by electrophoresis in a native acrylamide gel. When DNA is bound by proteins it moves slower in the gel then unbound DNA so that its band is "shifted" compared to free DNA.

Binding reaction of the DNA probes with the nuclear extracts

The binding reactions of the DNA probes with the nuclear extracts were performed in „Ratcliffe“ buffer. The solutions in the absence of labeled DNA were incubated with or without 0.5 µg -1 µg antibody for 2 h at 4°C. Then the DNA probe was added and the binding reaction was further incubated for 45 min at 4°C. After addition of 2 µl loading buffer (1xTBE, 60% glycerol and bromphenol blue) the probes were centrifuged and loaded to the gel. The antibodies were added to identifiy proteins which bound DNA.

Binding mixture

		Final concentration	
Buffer D with nuclear extracts	x µl	10 µg protein/sample	
Poly d(I-C)	1-2.5 µl of the 1:10 dilution of the 1 µg/µl stock solution	100-250	ng/sample
DTT	2 µl of the 50 mM stock solution	5	mM/sample

5x Ratcliffe buffer	4 µl	
DNA probe	0.5 - 2 µl	0.1 – 0.5 ng/sample
H_2O	to 20 µl	

5x Ratcliffe buffer:

			Final concentration	
KCl	1.86	g/100 ml	250	mM
$MgCl_2 \cdot 6\ H_2O$	0.102	g/100 ml	5	mM
EDTA	0.21	g/100 ml	5.5	mM
Glycerol	25	ml/100 ml	25	%

This buffer was autoclaved and stored at RT.

Detection of DNA-protein binding

The gel, on which EMSA samples were loaded, was prepared in a way that between two plates (16 x 21 cm) a rubber joint and a spacer (1 mm) were placed and all together was fixed with clamps. The shift gel solution (50 ml = 10 ml rotiphorese® gel 30 + 5 ml 10 x TBE + 40 ml H_2O) was put under vacuum to exhaust the air for 3 min. Then 20 µl TEMED and 350 µl freshly prepared 10% APS were added, mixed and the gel was poured between the plates. The polymerization took about 1 h at RT.

Before the EMSA reactions were loaded, the gel was electrophoresed in 1 x TBE buffer at 250 V for 1-2 h. To prevent 15-20 bp probes from running out off the gel the electrophoresis was stoped after 3 to 3.5 h. For drying, the gel was taken out from the glas plates, one side of it was covered with film and the other with two sheets of Whatmann paper and put for 2 h at 70°C in a gel dryer. The dried gel was then exposed to a phosphorimager screen.

Phosphorimager

The phosphoimager allows to detect ^{32}P-labeled probes. The dried gel was exposed overnight to the europium-covered phosphoimager screen. Under X-ray irradiation europium electrons are raised to higher energy levels and due to the laser beam of the scanner they return to their original energy levels thereby emitting energy in form of light which is detected by a photomultiplier. The signal is further processed by a special software (ImageQuant) and shown on the computer screen as black spots or bands so that the resulting picture is very similar to the picture obtained with autoradiography film. This method of detection is much more sensitive than the

classical autoradiography and shortens exposure time of the gel app. to 1/10 of the time needed to obtain an autoradiography on film.

3.3 Yeast one hybrid assay

A one hybrid assay is an *in vivo* assay used for isolating genes encoding proteins that bind to a target DNA regulatory element. It is based on the finding that many eucaryotic transcriptional activators are composed of functionally independent DNA-binding and activation domains. This allows construction of gene fusions consisting of the GAL4 activation domain and various DNA-binding domains that, when expressed as fusion proteins in yeast, can bind to a DNA target sequence and activate transcription of a reporter gene (Fig. 17).
The one hybrid assay was performed using the MATCHMAKER One Hybrid System (Clontech). To perform the assay a new yeast reporter strain containing the sequence of a normoxia responsive element upstream of the HIS reporter gene was prepared.

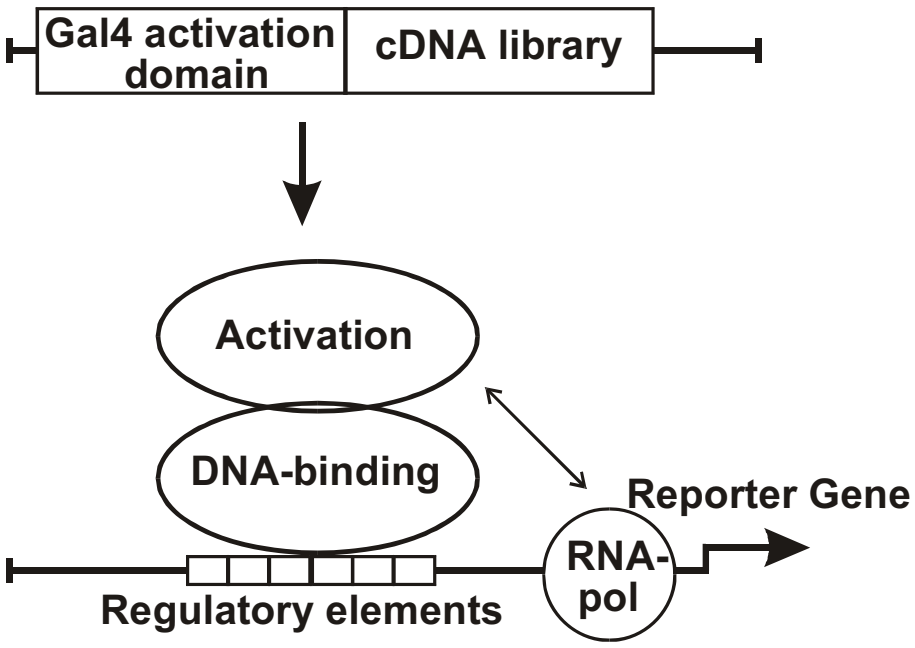


Fig. 17: **Detection of DNA-binding proteins using the one hybrid system.** For explanation see text.

3.3.1 Integration of the target-reporter construct into the yeast genome

Preparation of pHISi-NRE target-reporter construct

The DNA fragment containing 6 copies of the TAT NRE-1 in the pGl3-TAT-NRE1 (Fig. 18) was cut out with Sac I and Bgl II and after purification from agarose gel (3.2.4) cloned into the Sac I and Xba I sites of the MATCHMAKER vector pHISi since restriction enzymes Bgl II and Xba I produce complementary sticky ends.

Next, the target-reporter construct pHISi-NRE was transformed into yeast cells and recombinants with genomically integrated reporters were obtained. The integration of pHISi vector permits yeasts to grow on the selective medium lacking histidine (Fig. 18). The vector pHISi was designed to integrate into the yeast genome. When it is not integrated it would be lost since it could not replicate autonomously from the yeast genome. Linearization in the 3'-untranslated region immediatelly following the HIS3 gene significantly increases the efficiency of homologous recombination at the corresponding locus in the yeast genome.

Linearization of the target-reporter vector

1 μl of pHISi-NRE was digested in a total volume of 20 μl using Xho I. The solution was incubated at 37°C for 2 h. The complete linearization of the plasmid was confirmed by agarose gel electrophoresis.

Small-scale yeast transformation

There are several methods commonly used to introduce DNA into yest, including electroporation and lithium acetate-mediated method (Guthrie et al., 1991). In the present study the yeast transformaton was performed using a modified litium acetate method (Gietz et al., 1992).

Yeast YM4271 cells (Clontech) stored as glycerol stock at –70°C were defrozen and 50 μl of the solution was spread on the YPD agar dish. The dish was incubated at 30°C untill the yeast colonies reached 2 mm in diameter, which took about 5 days. Then 1 ml of yeast YPD medium was inoculated with several yeast colonies and vortexed for 5 min. The inoculated medium was poured into a flask containing 50 ml of YPD and incubated at 30°C for 16-18 h with shaking at 250 rpm to the stationary phase (OD_{600}>1.5). The 30 ml of overnight culture were transfered to a flask containing 300 ml of YPD and incubated at 30°C for 3 h with shaking. At this point the OD_{600}

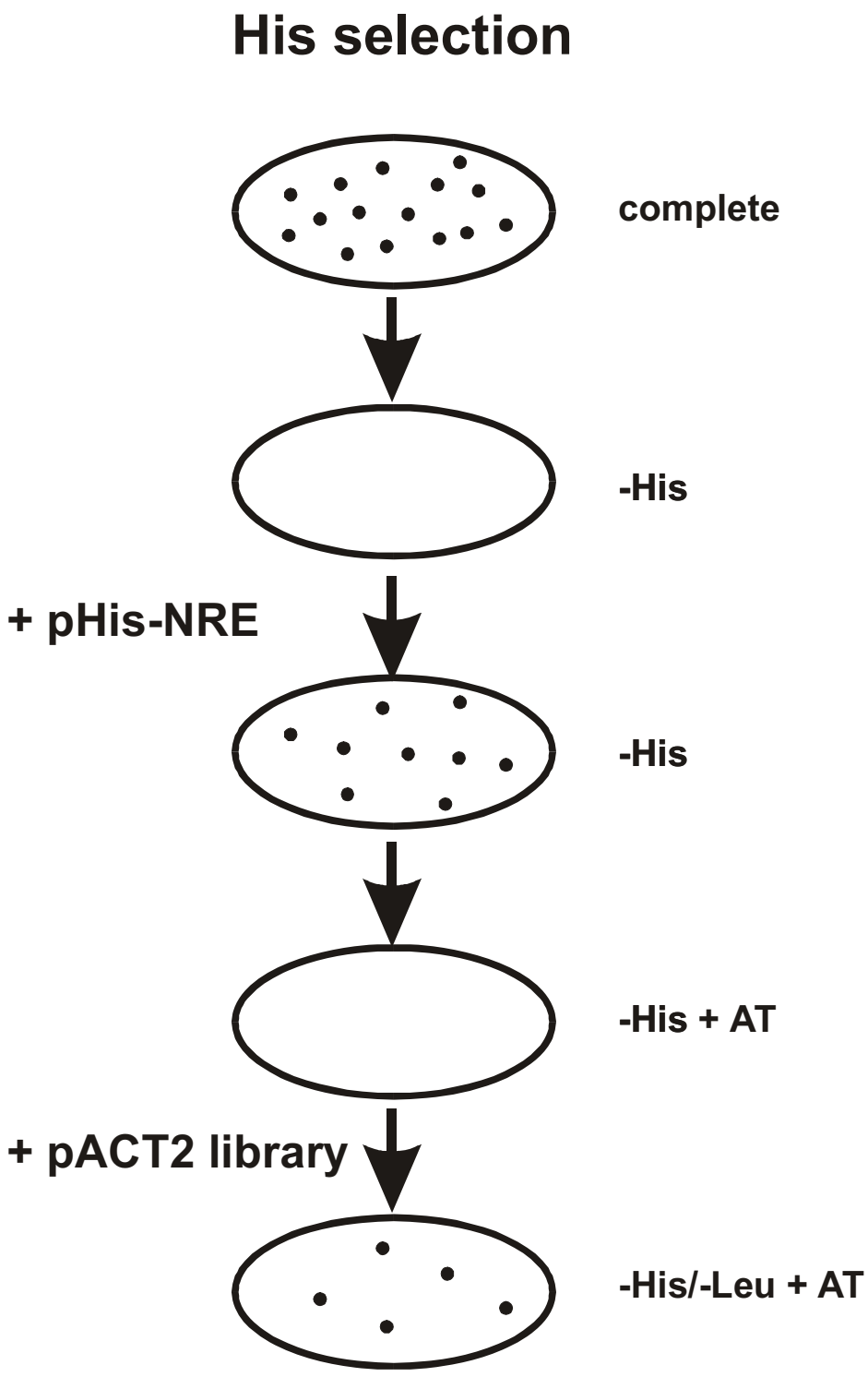


Fig. 18: **Screening of yeast clones on histidine-lacking medium.** When the pHISi-NRE vector is integrated into the host yeast genome a constitutive HIS3 expression allows growth of yeast on -His selective media. This low level expression can be inhibited by 3-AT. When an AD/library fusion protein interacts with the NRE target element, HIS3 reporter gene expression is activated allowing colony growth on minimal medium lacking histidine and containing the concentration of 3-AT needed to inhibit background HIS3 expression.

should be 0.4-0.6. Then the cells were centrifuged at 1000 g (3000 rpm in the rotor SS34) for 5 min at RT. The supernatants were discarded and the pellets were resuspended in sterile TE buffer and pooled together with a final volume of about 50 ml. The cells were again centrifuged at 1000 g (3000 rpm in the rotor SS34) for 5 min at RT. The supernatants were discarded and the cell pellet was resuspended in 1.5 ml of freshly prepared, sterile TE/lithium acetate. At this step preparation of yeast competent cells was finished.

Then 3 μg of linearized pHISi-NRE DNA was mixed with 100 μg of herring testis carrier DNA (Clontech) in a new 1.5 ml Eppendorf tube. The 100 μl of competent yeast cells and 600 μl of sterile PEG/lithium acetate solution were added to it and vortexed. The solution was incubated at 30°C for 30 min with shaking at 200 rpm. Then 70 μl DMSO was added and the solution was mixed by gentle inversion. The cells were heat shocked for 15 min in a 42°C water bath. After chilling of cells on ice they were centrifuged for 5 sec at 14000 rpm at RT in the table centrifuge. The supernatant was discarded and the cells resuspended in 150 μl of TE buffer. Then the entire transformation mixture was plated on one SD/-His plate, which was incubated at 30°C. After 6 days the yeast colonies with integrated pHISi-NRE vector reached the size of 2 mm in diameter and the plates were further stored at 4°C.

YPD medium

Trypton	20	g/l
Yeast extract	10	g/l
Glucose	2	%

The pH was adjusted with NaOH to 6.5. The medium was autoclaved after preparation. Glucose was added only after autoclaving when the temperature of the medium was about 55°C.

Selective (SD) medium

Yeast nitrogen base without amino acids	6.7	g/l
Dropout (-His or –His/-Leu) supplement	0.77	g/l
Glucose	2	%

The pH was adjusted with NaOH to 5.8. The medium was autoclaved after preparation. Glucose and, if indicated, 3-amino triazole (3-AT) were added only after autoclaving when the temperature of the medium was about 55°C.

YPD and SD agar dishes

20 g of agar was added per 1 l of YPD medium or SD medium. The solutions were autoclaved, cooled to 50°C and poured on the dishes.

10 x Tris/EDTA (TE) buffer

			Final concentration	
Tris	1.22	g/100 ml	0.1	M
EDTA	0.35	g/100 ml	0.01	M

The pH was adjusted with acetic acid to 7.5, the buffer was autoclaved.

PEG/lithium acetate solution

		Final concentration	
Polyethylene glycol 3350	8 ml of 50 % PEG	40	%
Lithium acetate	1 ml of 1M stock solution	0.1	M
TE buffer	1 ml of 10 X TE		
H_2O	to 10 ml		

The pH was adjusted with acetic acid to 7.4, the buffer was autoclaved.

Testing new reporter strain for backgroung expression

To determine the optimal 3-amino triazole (3-AT) concentration for inhibiting background HIS3 expression the new reporter strain was plated on the selective dishes with different concentrations of this inhibitor.

A single colony was picked and suspended in 1 ml of TE buffer. 5 μl of the suspension was plated on SD/-His selective plates with 15, 30, 45 and 60 mM 3-AT. After 6 days of incubation at 30°C the colonies appeared only on the plate containing the lowest concentration of 3-AP (15 mM). This concentration of inhibitor was used for the further screening experiments.

3.3.2 Amplification of the AD fusion library and purification of the library DNA

Plasmid library titering

Before use a library should be titered to determine the actual number of clones present in it. For our experiments the rat liver MATCHMAKER cDNA library (Clontech) was used.

First, an aliquot of the library was thawed and placed on ice. Then 1 μl was transfered to 1 ml of LB medium and mixed. It was dilution A ($1:10^3$). Then 1 μl of dilution A was added to 1 ml of LB thus making dilution B ($1:10^6$). 1 μl of dilution A into 50 μl of LB

medium as well as 50 µl and 100 µl aliquots of dilution B were plated on prewarmed LB/Amp plates. After overnight incubation the number of clones on each plate was counted. The library titer was calculated as follows (cfu/ml): for dilution A, number of colonies X 10^3 X 10^3, for dilution B, number of colonies: plating volume X 10^3 X 10^3 X 10^3. The number of clones on the plate with 1 µl of dilution A was about 100, so the titer was 10^8.

Plasmid library amplification

The number of clones which was necessary to get on the plates for the library amplification was determined by the following calculation: number of independent clones in the library (3.5 X 10^6, according to the library certificate) X 3 = 10.5 X 10^6. The exact number of plates required was calculated as follows: number of colonies to screen (10.5 X 10^6): number of colonies per 150 mm plate that they are nearly confluent (20.000) = 525. The amount of the library stock (in ml) to be spread on each plate was determined as follows: number of colonies to screen (10.5 X 10^6) : library titer (10^8) = 105 µl.

After plating 105 µl of original library on each of 525 LB/Amp plates they were incubated at 30°C for 48 h. Then 5 ml of LB containing 25% glycerol was added to each plate and the colonies were scraped into liquid. All the resuspended colonies were pooled in one flask and mixed thoroughly. Then one-third of the library culture was set aside for plasmid preparation and the rest was frozen at –70°C.

Isolation and analysis of the plasmid DNA (gigapreparation)

For preparation of the library plasmid DNA a method that yields a large quantity of highly purified plasmid should be used. For plasmid isolation NucleoBond columns containing AX silica resin from the Giga Plasmid Kit (Clontech) were used. AX is a silica-based anion exchange resin for separation of different classes of nucleic acids. The AX resin consists of hydrophilic, macroporous silica beads coupled to methyl-ethylamine as functional group. The functional group provides a high overall charge density that permits the negatively charged phosphate backbone of RNA and DNA to bind with high specificity to the resin. NucleoBond AX can separate nucleic acids from each other and from proteins, carbohydrates and other cellular components.

A volume of 850 ml of the cells which were scraped from the dishes were pelleted by centrifugation for 30 min at –4°C and 5860 *g* (6000 rpm, GSA rotor). The pellet was resuspended in 85 ml of buffer S1, which contained 100 µg/ml of RNase. Then 85 ml of buffer S2 (with NaOH and SDS for bacterial lysis) was added and mixed gently 4 to

6 times (the mixture should not be vortexed to avoid shearing of the genomic DNA). After 5 min incubation at RT (longer incubation could lead to irreversible denaturation of plasmid DNA), 85 ml of buffer S3 were added to neutralize the solution. The solution was incubated on ice for 5 min and then centrifuged for 60 min at 4°C and 16300 *g* (10000 rpm, GSA rotor) and applied to the NucleoBond column equilibrated with 100 ml of buffer N2. When the gravity-mediated flow of the lysate through the column was complete, the column was washed twice with 100 ml of buffer N3. Then plasmid DNA was eluated with 100 ml of buffer N5. The eluate was mixed with 70 ml of isopropanol to precipitate DNA, kept for 1 h at –20°C and centrifuged for 30 min at 27000 *g* and 4°C (15000 rpm, SS34 rotor). The DNA pellet was washed with 70% ethanol, dried for 30 min and was dissolved in 2 ml H_2O.

To determine the DNA concentration and the presence of protein in the probes, the OD at 260 nm (DNA) and 280 nm (protein) was measured as described in 3.2.6.

Buffer S1	50 mM Tris	pH 8.0, adjusted with HCl
	10 mM EDTA	E1 with RNase A stored at 4°C
	100 µg/ ml RNase A	
Buffer S2	200 mM NaOH	
	1% SDS	
Buffer S3	2.8 M potassium acetate	pH 5.1, adjusted with acetic acid
Buffer N2	900 mM KCl	pH 6.3, adjusted with H_3PO_4
	100 mM Tris	
	0.15% Triton X-100	
	15% ethanol	
Buffer N3	1.15 M KCl	pH 6.3, adjusted with H_3PO_4
	100 mM Tris	
	15% ethanol	
Buffer N5	1 M KCl	pH 8.5, adjusted with H_3PO_4
	100 mM Tris	
	15% ethanol	

3.3.3 Screening of the AD fusion library using the modified yeast reporter strain

First, 1.5 ml of the competent yeast reporter strain containing pHISi-NRE was prepared as described in 3.3.1. Then the yeasts were transformed according to the large-scale transformation protocol (Clontech). 20 µg of AD library plasmid (3.3.2) was mixed with 2 mg of herring testis carrier DNA in a new 50 ml Falcon tube. Then 1 ml of competent yeast cells and 6 ml of sterile PEG/lithium acetate solution were added to it and

vortexed for 10 seconds. The solution was incubated at 30°C for 30 min with shaking at 200 rpm. Then 700 μl DMSO was added and the solution was mixed by gentle inversion. The cells were heat shocked for 15 min in a 42°C water bath. After chilling on ice for 2 min cells were centrifuged at 1000 g (3000 rpm, SS34 rotor) for 5 min at RT. The supernatant was discarded and the cells resuspended in 7 ml of TE buffer. Then 500 μl of the transformation mixture was plated on each 150-mm –His/-Leu selective plate containing 15 mM 3-AT. The medium lacked leucine since the pACT2 vector contained the *LEU2* nutritional gene that allowed transformed yeasts to grow on it. The plates, total number of which was 15, were incubated at 30°C untill the colonies appeared.

3.3.4 Isolation of plasmid DNA from yeast

Each colony growing on the –His/-Leu/+3-AT selective plates was analyzed for its plasmid. For this, the yeast plasmid isolation method described (Ciriacy et al., 1981) was used.

A single yeast colony was inoculated with 5 ml of –His/-Leu selective medium, vortexed and incubated overnight at 30°C untill OD_{600} became 1-2. Then the cells were centrifuged twice for 5 min at 14000 rpm in a table centrifuge, the supernatants discarded, the pellets resuspended in 1 ml of water and again centrifuged. The pellet was suspended in 100 μl of zymolyase mixture and incubated for 30 min at 37°C. Then 100 μl of the lysis mixture was added and heated at 70°C for 20 min. The solution was cooled at RT, then 50 μl of 5 M potassium acetate was added and the mixture was left for 2 h at 4°C. Then it was centrifuged at 14000 rpm in a table centrifuge at 4°C for 12 min. The supernatant (200 μl) was transfered into a new tube and 500 μl of 95% ethanol was added to it. The mixture was incubated for 30 min at –20°C, then centrifuged and the supernatant was removed. The pellet was dried under vacuum pump for 5 min.

The pellet was again dissolved in 100 μl of TE buffer, then 5 μl 5 M sodium acetate and 200 μl 95% ethanol were added and incubated for 30 min at –20°C. The solution was centrifuged for 5 min, the supernatant removed and the dried pellet was dissolved in 40 μl of sterile water.

Finally, 15 μl of the plasmid solution was transformed into *E. coli* using the method described (3.2.4). Then the plasmids were purified using the minipreparation procedure (3.2.5) and sequenced as described (3.2.7). The primers used for sequencing are shown in 2.4.1.

SCE buffer

			Final concentration	
Sorbitol	18.2	g/100 ml	1	M
Sodium citrate	3.57	g/100 ml	0.1	M
EDTA	2.23	g/100 ml	0.06	M

The pH was adjusted with HCl to 7.0, the buffer was autoclaved

Zymolyase mixture

For preparation of the zymolyase mixture 2.5 mg zymolyase 20T and 80 µl β-mercaptoethanol were added to 10 ml of SCE buffer.

Lysis mixture

		Final concentration	
Tris	500 µl of 1 M stock solution	50	mM
EDTA	500 µl of 0.2 M stock solution	10	mM
SDS	2 ml of 10% stock solution	2	%
H_2O	to 10 ml		

The pH was adjusted with HCl to 8.0, the buffer was autoclaved.

3.4 Security measures

All the operations with genetically modified organisms and plasmid DNA were performed according to the „Gentechnikgesetz of 1990“ and to the rules prescribed by the „Gentechnik-Sicherheitsverordnung of 1990“. Materials contaminated with bacterial cells were treated with hydrogen peroxide, soap suds and autoclaved.

The chemicals formaldehyde, DEPC, phenol and ethidium bromide are cancerogenic and were carefully managed and disposed.

All the operations with radiochemicals were performed in a C laboratory, the radiochemicals were disposed according to the instructions.

4. RESULTS

4.1 Normoxia-dependent induction of SerDH and TAT mRNA expression

TAT and SerDH genes are known to be expressed predominantly in the more aerobic periportal region of the liver acinus. To demonstrate the modulation of TAT and SerDH gene expression by normoxia (16% O_2) and mild hypoxia (8% O_2) mRNA levels were measured by Northern blot analysis. Primary rat hepatocytes were used as a model system.

4.1.1 Induction of TAT mRNA expression under normoxia and its modulation by dexamethasone

Primary rat hepatocytes cultured for 24 hours under standard conditions and treated for 24 h with hypoxia (8% O_2) had about 3-fold lower levels of TAT mRNA compared to the controls under normoxia (Fig. 19). This difference was not further modulated by the treatment of the cells with dexamethasone, a putative inducer of TAT expression. When the cells, cultured in the absence of dexamethasone for 30 hours, were stimulated with 100 nM dexamethasone for 12 hours, TAT mRNA was increased by about 8-fold but the difference between normoxic and hypoxic TAT mRNA levels was again about 2-fold. Treatment of the hepatocytes with 100 nM dexamethasone for 48 hours under normoxia and hypoxia also resulted in a 10-fold higher TAT expression under normoxia, whereas the TAT mRNA level under hypoxia was induced by about 4-fold only compared to the normoxic control without dexamethasone. Treatment of cells with glucagon had no influence on TAT mRNA induction (data not shown). Thus, TAT mRNA expression was induced under normoxia and this induction was not further modulated in the presence of dexamethasone.

4.1.2 Induction of SerDH mRNA expression by glucagon and its modulation by oxygen

In the absence of its inducer glucagon SerDH mRNA expression in primary rat hepatocytes was not detectable (Fig. 20). When the cells were treated for 3 hours with 1 nM glucagon, SerDH mRNA expression was induced by about 5-fold under normoxia and by about 2-fold under hypoxia. At a glucagon concentration of 10 nM SerDH mRNA levels were further enhanced by about 12-fold under normoxia and by about 5-fold under hypoxia and thus SerDH mRNA levels under normoxia were again about 2.5-fold higher than SerDH mRNA levels under hypoxia. These results indicate that in the presence of different concentrations of glucagon SerDH mRNA expression under hypoxia is much lower than under normoxia (Fig. 20).

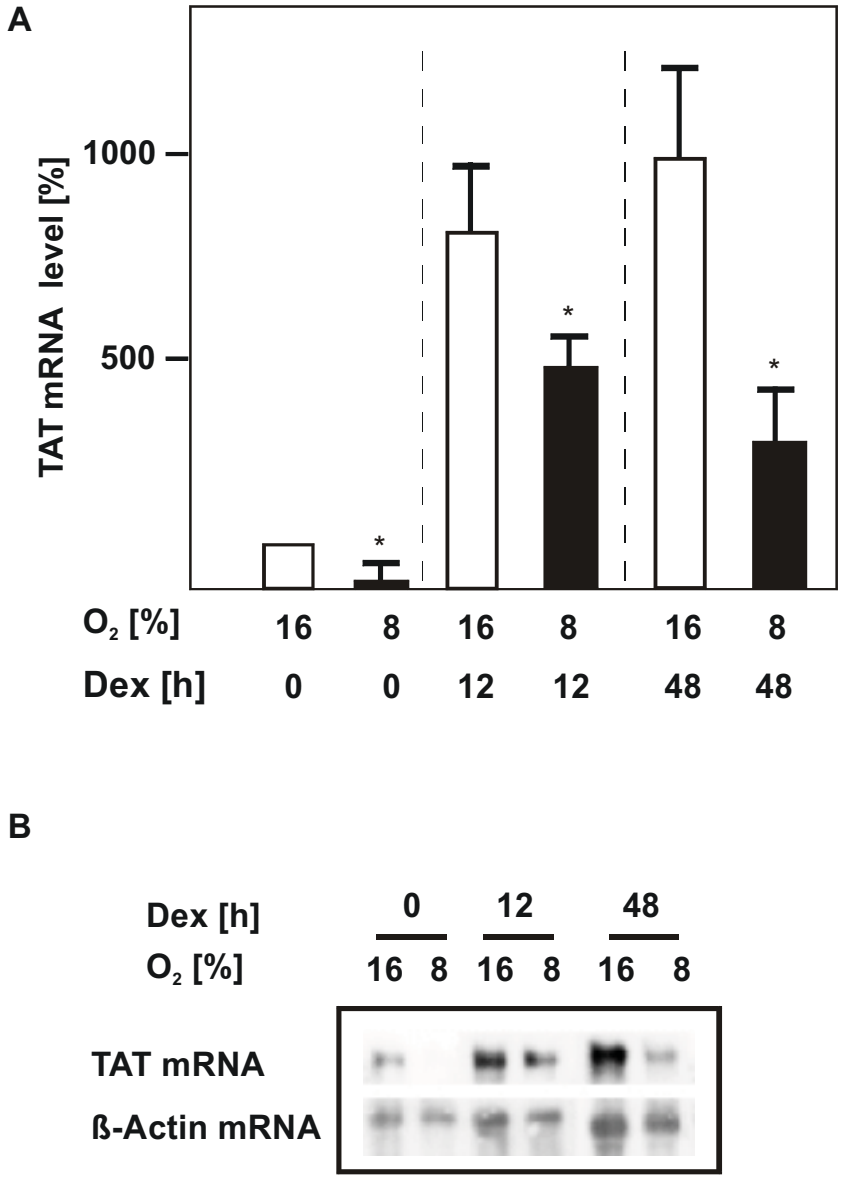


Fig. 19: **Modulation of TAT mRNA expression by oxygen in rat primary hepatocytes.** Hepatocytes were cultured under standard conditions at normoxia (16% O_2) for 24 h. At 24 h the medium was changed and cells were further cultured under normoxic and hypoxic (8% O_2) conditions and treated for the time indicated with 100 nM dexamethasone. (**A**) The TAT mRNA levels were measured by Northern blotting. The mRNA level under normoxia (16% O_2) was set equal to 100%. Values are means ± SEM of three independent culture experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$ (**B**) Representative Northern Blot. For Northern analysis 15 µg total RNA were hybridized to digoxigenin-labelled TAT and β-actin antisense RNA probes (cf. Materials and Methods). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

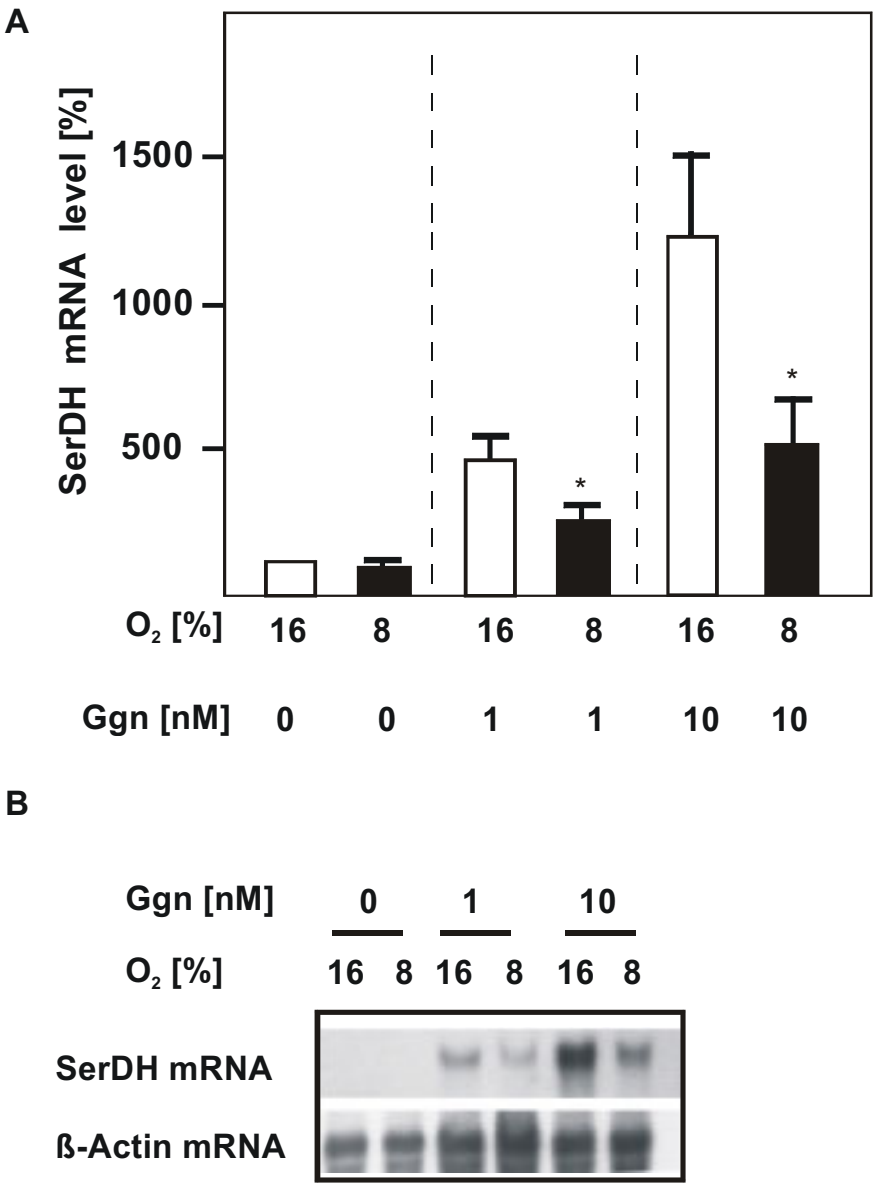


Fig. 20: **Modulation of glucagon-dependent SerDH mRNA expression by oxygen in rat primary hepatocytes.** Hepatocytes were cultured for 24 h under normoxia (16% O_2). At 24 h the medium was changed and cells were further cultured for the next 24 h under normoxic and hypoxic (8% O_2) conditions. At 21 h cells were treated with the indicated concentrations of glucagon. (**A**) The SerDH mRNA levels were measured by Northern blotting. The mRNA level under normoxia (16% O_2) was set equal to 100%. Values are means ± SEM of three independent culture experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$. (**B**) Representative Northern Blot. For Northern analysis 15 µg total RNA were hybridized to digoxigenin-labelled SerDH and β-actin antisense RNA probes (cf. Materials and Methods). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

4.2 Regulation of TAT and SerDH promoter luciferase gene constructs by oxygen

To further substantiate that TAT and SerDH gene expression is transcriptionally regulated by oxygen and to investigate the role of the TAT inducers glucagon and dexamethasone in the modulation of this gene, primary rat hepatocytes were transfected with TAT or SerDH promoter luciferase (Luc) gene constructs and cultured under normoxic or hypoxic conditions.

4.2.1 Sequence analysis of the rat TAT and SerDH promoters.

To find out what sequences could be involved in the regulation of TAT and SerDH gene expression by oxygen, the TAT and SerDH promoter sequences were analyzed for similarities with the normoxia responsive element (NRE) of the phosphoenolpyruvate carboxykinase-1 (PCK-1) gene 5´-TTAGGTCAG-3´ (Bratke et al., 1999).

Sequence analysis of the rat TAT promoter revealed that three putative NRE‘s were present within the 10095 bp of the promoter (Fig. 21). The first potential normoxia response element (NRE-1, -9531/-9523, 5´-TTAGCTCAG-3´) and the second potential normoxia response element (NRE-2, -2651/-2643, 5´-TTGGGTCAG-3´) match the NRE of the PCK-1 gene in 8 out of 9 bp. The third potential normoxia response element (NRE-3, 5´-GTAGGTCCG-3´, -112/-104) which is localized in the proximal region of the TAT promoter matches the PCK-1 NRE in 7 out of 9 bp.

Sequence analysis of the rat SerDH promoter revealed that four putative normoxia response elements (NRE) were present within the first 2303 bp of the promoter (Fig. 22). All four potential normoxia response elements NRE-1, -2169/-2161, 5´-TGAGGACAG-3´, NRE-2, -1904/-1896, 5´-TTATGTGAG-3´, NRE-3, -578/-570, 5´-TTAGTCCAG-3´ and NRE-4, +38/+46, 5´-CTAGATCAG-3´, match the NRE of the PCK-1 gene in 7 out of 9 bp.

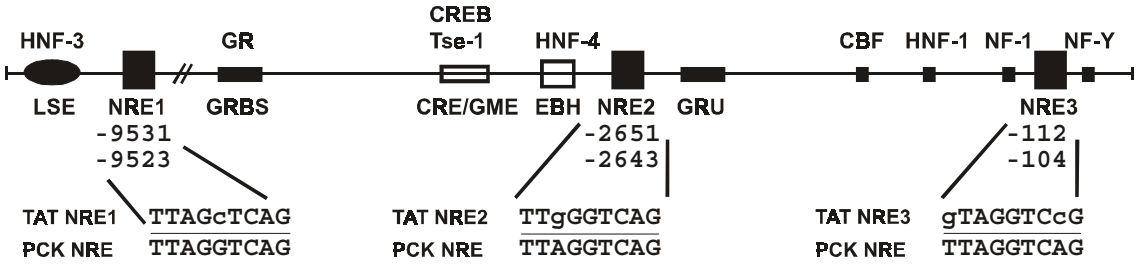


Fig. 21: **Localization of putative normoxia responsive elements within the TAT gene promoter.** The TAT gene promoter with the putative NRE’s and regulatory elements is shown up to –10095 bp. Sequences in the NRE shown in the upper strand correspond to the sequences of the TAT NRE’s, sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between TAT NRE and PCK-1 NRE are shown in lower case letters (see Fig. 4 for details).

4.2.2 Regulation of TAT promoter luciferase gene constructs by oxygen, dexamethasone and glucagon

A 10095 bp fragment of the 5´-flanking region of the rat TAT gene was cloned in front of the luciferase gene in pGl3-basic to generate pGl3TAT-10095. In the cells transfected with pGl3TAT-10095 and cultured under standard conditions in the presence of 100 nM dexamethasone Luc activity was induced maximally under normoxia and to only about 60% under hypoxia (Fig. 23).

To determine which putative TAT NRE could be involved in the regulation of TAT gene expression by normoxia, primary hepatocytes were transfected with the luciferase gene constructs pGl3TAT-6100, pGl3TAT-2556, pGl3TAT-790 and pGl3TAT-356 driven by the first 6100 bp, 2556 bp, 790 bp and 356 bp of the TAT promoter, respectivly (Fig. 23). The construct pGl3TAT-6100 contained both NRE2 and NRE3, but not NRE1, while pGl3TAT-2556, pGl3TAT-790 and pGl3TAT-356 contained only NRE3. The constructs pGl3TAT-6100, pGl3TAT-2556 and pGl3TAT-790 were induced maximally by normoxia and to about 75% by hypoxia. Surprisingly, in the cells transfected with pGl3TAT-356 TAT Luc gene expression was induced to about 200% under hypoxia and not as expected under normoxia. Thus, removal of the TAT NRE-1 sequence significantly reduced oxygen-responsiveness of the TAT promoter.

The Luc activity in cells transfected with pGl3TAT-10095 and treated with glucagon was not significantly induced compared to the control even though the modulatory effect of normoxia was preserved (Fig. 24). In glucagon-treated cells, transfected with pGl3TAT-356, Luc activity under normoxia was not significantly different from the Luc activity under mild hypoxia. Treatment of pGl3TAT-356 transfected cells with glucagon did not further increase Luc activity (Fig. 24).

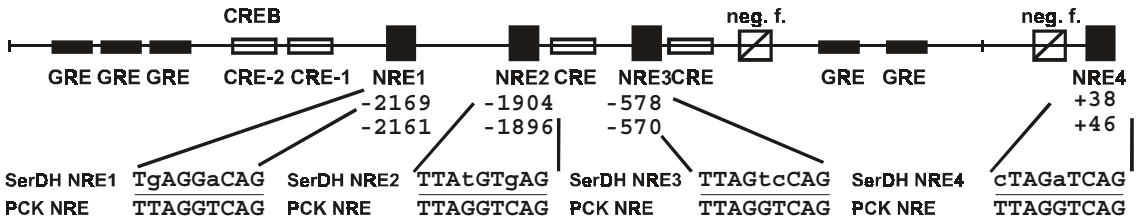


Fig. 22: **Localization of putative normoxia responsive elements within the SerDH gene promoter.** The SerDH gene promoter with the putative NRE's and regulatory elements is shown up to –2303 bp. Sequences in the NRE shown in the upper strand correspond to the sequences of the SerDH NRE's, sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between SerDH NRE and PCK-1 NRE are shown in lower case letters (see Fig. 5 for details).

The lack of induction by glucagon with pGl3TAT-10095 led to the suggestion that the presence of another TAT inducer, dexamethasone, in the culture medium was sufficient to maintain nearly maximal levels of Luc expression. Indeed, when the cells cultured without dexamethasone were treated with 10 nM glucagon for 12 hours Luc activity was stimulated by about 70% under both normoxia and hypoxia (Fig. 25). However, again maximal Luc activity was reached under normoxia compared to hypoxia.

When hepatocytes transfected with the pGl3TAT-356 Luc construct were cultured in the absence of dexamethasone, Luc activity was not induced by glucagon. Again, as in the presence of dexamethasone, an about 60% higher Luc activity under hypoxia was observed in the untreated cells whereas treatment with glucagon abolished modulation by oxygen.

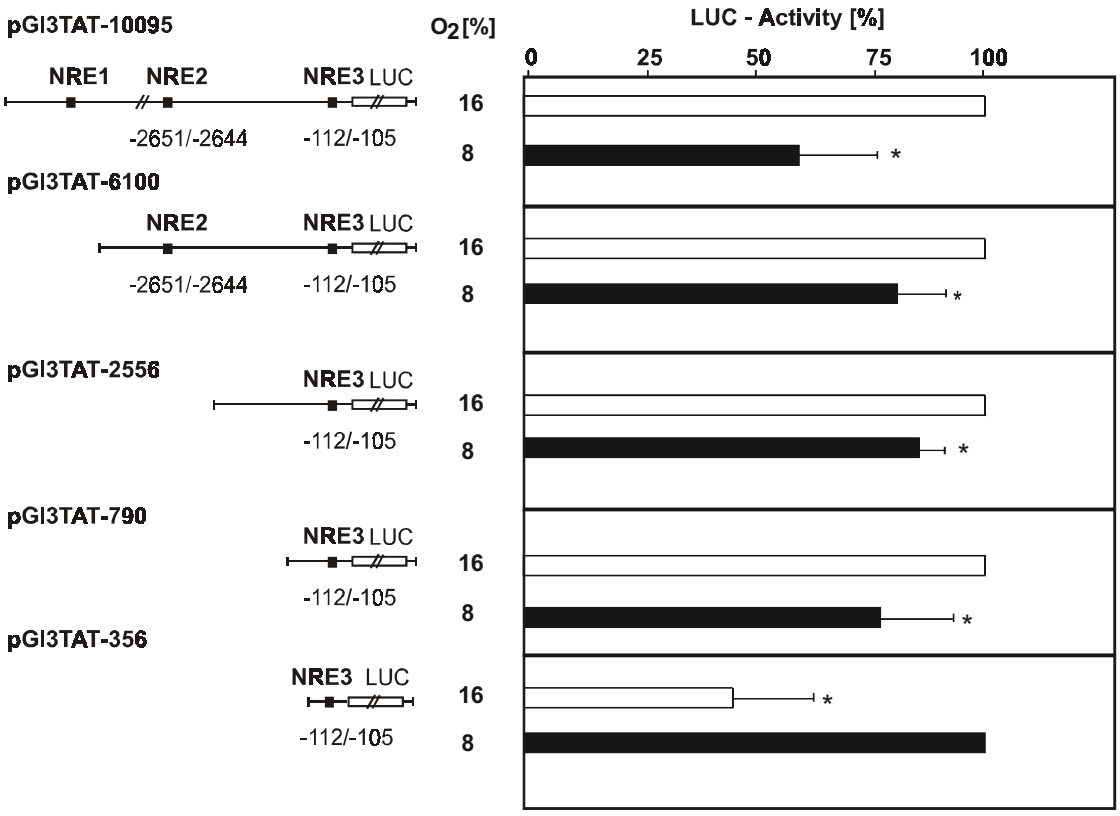


Fig. 23: **Regulation of TAT promoter LUC gene expression by oxygen.** The hepatocytes were transiently transfected with the LUC gene constructs driven by a wild type -10095 bp, -6100 bp, -2556 bp, -790 bp or by a –356 bp rat TAT promoter. Hepatocytes were cultured in the presence of 100 nM dexamethasone for 24 h under normoxia (16% O_2). At 24 h the medium was changed and cells were further cultured in the presence of 100 nM dexamethasone for the next 24 h under normoxic and hypoxic (8% O_2) conditions. In each experiment the percentage of Luc activity was determined relative to the pGl3TAT-10095, pGl3TAT-6100, pGl3TAT-2556 or pGl3TAT-790 16% O_2 controls or to the pGl3TAT-356 8% control which were set equal to 100%. The values represent means ± SEM of six independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$.

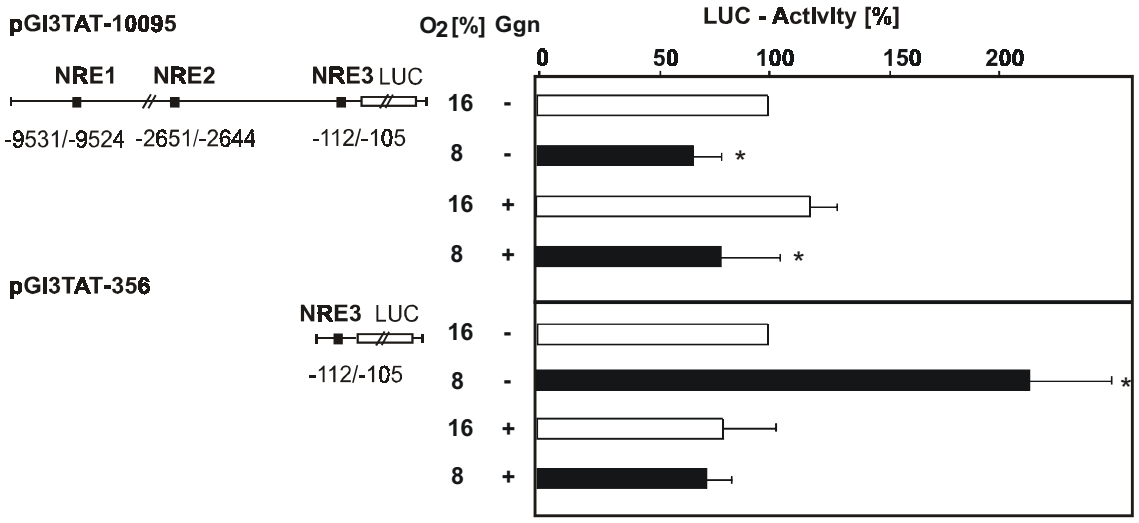


Fig. 24: **Regulation of TAT promoter LUC gene expression by oxygen and glucagon in the presence of dexamethasone.** The hepatocytes were transiently transfected with the LUC gene constructs driven by a wild type -10095 bp or by a wild type –356 bp rat TAT promoter (pGl3TAT-10095 and pGl3TAT-356). Hepatocytes were cultured in the presence of 100 nM dexamethasone for 24 h under normoxia (16% O_2). At 24 h the medium was changed and cells were further cultured in the presence of 100 nM dexamethasone for the next 24 h under normoxic and hypoxic (8% O_2) conditions. The cells were treated with 10 nM glucagon for the last 12 h, as indicated. In each experiment the percentage of Luc activity was determined relative to the pGl3TAT-10095 or pGl3TAT-356 16% O_2 controls which were set equal to 100%. The values represent means ± SEM of six independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$.

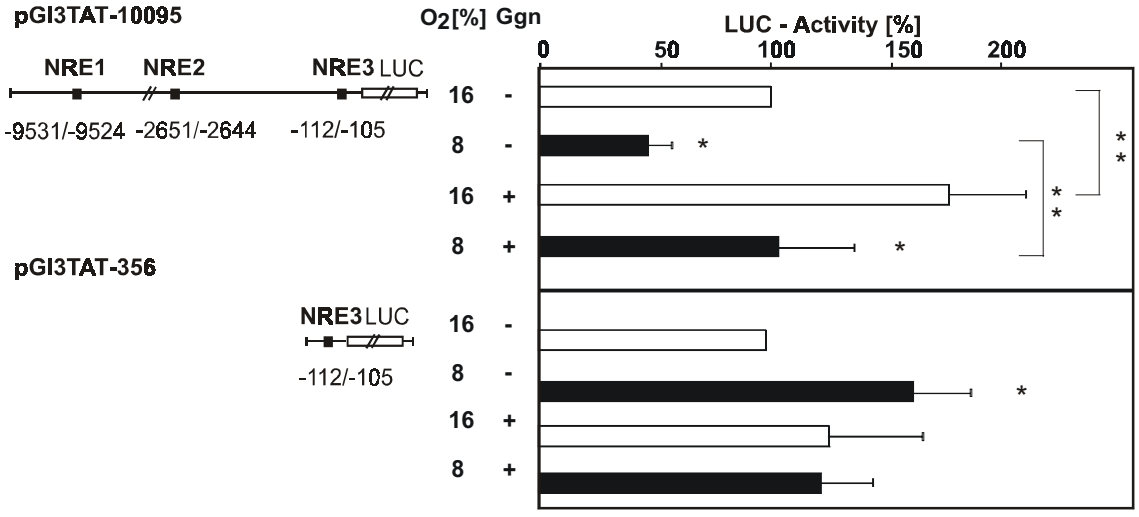


Fig. 25: **Regulation of TAT promoter LUC gene expression by oxygen and glucagon in the absence of dexamethasone.** The hepatocytes were transiently transfected with the LUC gene constructs driven by a wild type -10095 bp or by a wild type –356 bp rat TAT promoter (pGl3TAT-10095 and pGl3TAT-356). Hepatocytes were cultured for 24 h under normoxia (16% O_2). At 24 h the medium was changed and cells were further cultured for the next 24 h under normoxic and hypoxic (8% O_2) conditions. The cells were treated with 10 nM glucagon for 12 h, as indicated. In each experiment the percentage of Luc activity was determined relative to the pGl3TAT-10095 or pGl3TAT-356 16% O_2 controls which were set equal to 100%. The values represent means ± SEM of six independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$.

These results demonstrated that the expression of the –10095 bp TAT promoter Luc gene construct was upregulated under normoxia and the major NRE's involved seem to be localized upstream from –356 bp of the TAT promoter. Normoxia-dependent regulation of TAT expression is independent from dexamethasone and glucagon. Furthermore, a not yet identified putative hypoxia responsive element may be located inside the –356 bp of the TAT promoter.

4.2.3 Regulation of SerDH promoter luciferase gene constructs by oxygen

A 2303 bp fragment of the 5´-flanking region of rat SerDH gene was cloned in front of the luciferase gene in pGl3-basic to generate pGl3SerDH-2303. In hepatocytes transfected with pGl3SerDH-2303 Luc activity was induced maximally under normoxia and to only about 60% under hypoxia (Fig. 26). The Luc activity in cells treated with either dexamethasone or glucagon was not induced compared to the control (data not shown).

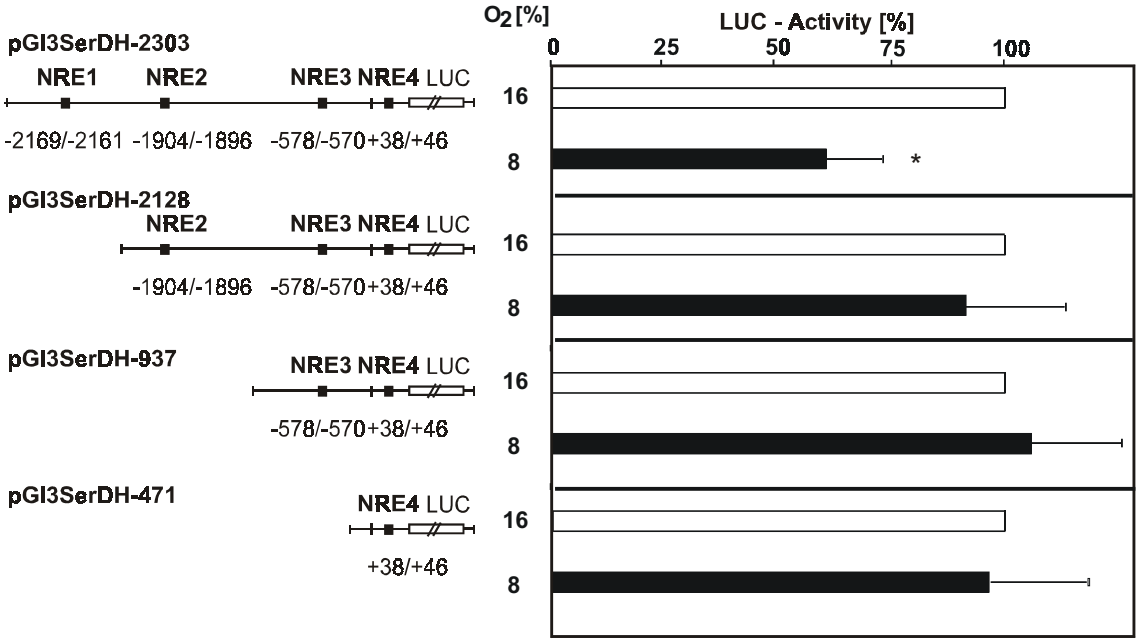


Fig. 26: **Regulation of SerDH promoter LUC gene expression by oxygen.** The hepatocytes were transiently transfected with the LUC gene constructs driven by a wild type –2303 bp, -2128 bp, -937 bp or –471 bp rat SerDH promoter (pGl3SerDH-2303, pGl3SerDH-2128, pGl3SerDH-937 and pGl3TAT-471). Hepatocytes were cultured for 24 h under normoxia (16% O_2). At 24 h the medium was changed and cells were further cultured for the next 24 h under normoxic and hypoxic (8% O_2) conditions. In each experiment the percentage of Luc activity was determined relative to the pGl3SerDH-2303, pGl3SerDH-2128, pGl3SerDH-937 or pGl3TAT-471 16% O_2 controls which were set equal to 100%. The values represent means ± SEM of six independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$.

To determine what region of the SerDH promoter could be responsible for the oxygen-dependent regulation of SerDH gene expression, primary rat hepatocytes were transfected with three serially deleted SerDH promoter luciferase gene constructs pGl3SerDH-2128, pGl3SerDH-937 and pGl3SerDH-471 containing the first 2128 bp, 937 bp and 471 bp of the SerDH promoter, respectively. Normoxia did not induce Luc activity in the cells transfected with either pGl3SerDH-2128, pGl3SerDH-937 or pGl3SerDH-471 indicating that NRE-2, NRE-3 and NRE-4 did not seem to be involved in the regulation of SerDH gene expression by normoxia.

These results demonstrated that the expression of the –2303 SerDH promoter Luc gene construct was induced by normoxia and that the normoxia responsive region of SerDH was localized between –2128 and -2303 bp of the SerDH promoter.

4.3 Induction of the TAT and SerDH NRE enhancer Luc gene constructs by normoxia

Since hypoxia response elements (HRE's) from erythropoietin and other hypoxia-regulated genes could act as transcriptional enhancers when cloned in front of an independent promoter and a reporter gene, the role of the potential NRE's from TAT and SerDH promoters in the normoxia-dependent gene regulation was investigated by using these elements as enhancers regulating expresssion of the reporter luciferase gene. Experiments were performed in primary rat hepatocytes, HepG2 hepatoma cells and HeLa cells.

4.3.1 Induction of the TAT NRE enhancer Luc gene constructs by normoxia

The 90 bp oligonucleotides containing 6 copies of either NRE-1, NRE-2 or NRE-3 enhancers from the TAT promoter were cloned in front of the SV40 promoter and the luciferase gene in pGl3-prom to generate pGl3-TAT-NRE1, pGl3-TAT-NRE2 and pGl3-TAT-NRE3. When primary rat hepatocytes were transfected with pGl3-TAT-NRE1, the Luc activity was by about 70% higher in the cells cultured under normoxia compared to the cells cultured under hypoxia (Fig. 27). The pGl3-TAT-NRE2 construct was less effective since the Luc activity of this construct was only by about 50% higher under normoxia compared to hypoxia. Only a slight difference between the Luc activities under normoxia and hypoxia was observed after transfection of primary rat hepatocytes with pGl3-TAT-NRE3. As a control the pGl3-Epo-HRE construct containing three copies of a hypoxia response element (HRE) from the erythropoietin gene (Kietzmann, Cornesse, 2001) in front of the SV40 promoter and Luc gene was used in the same experiments. Luc activity in the cells transfected with pGl3-Epo-HRE

and cultured under hypoxia was maximal whereas Luc acticvity was reduced to 40% in the cells cultured under normoxia.

To find out whether the observed modulation by oxygen was cell type-specific, HepG2 and HeLa cells were also transfected with the pGl3-TAT-NRE1, pGl3-TAT-NRE2 and pGl3-TAT-NRE3 Luc gene constructs. The Luc activity from pGl3-TAT-NRE1 was induced by normoxia by about 30% in HepG2 cells and by about 50% in HeLa cells. In contrast to pGl3-TAT-NRE1, the expression of the Luc constructs pGl3-TAT-NRE2 and pGl3-TAT-NRE3 was not modulated by oxygen in these cell lines (Fig. 28). Luc activity in the cells transfected with the control hypoxia-inducible pGl3-Epo-HRE was by about 80% higher in the cells cultured under hypoxia. Thus, TAT NRE-1 functioned best in all cell types investigated, whereas NRE-2 and NRE-3 did not.

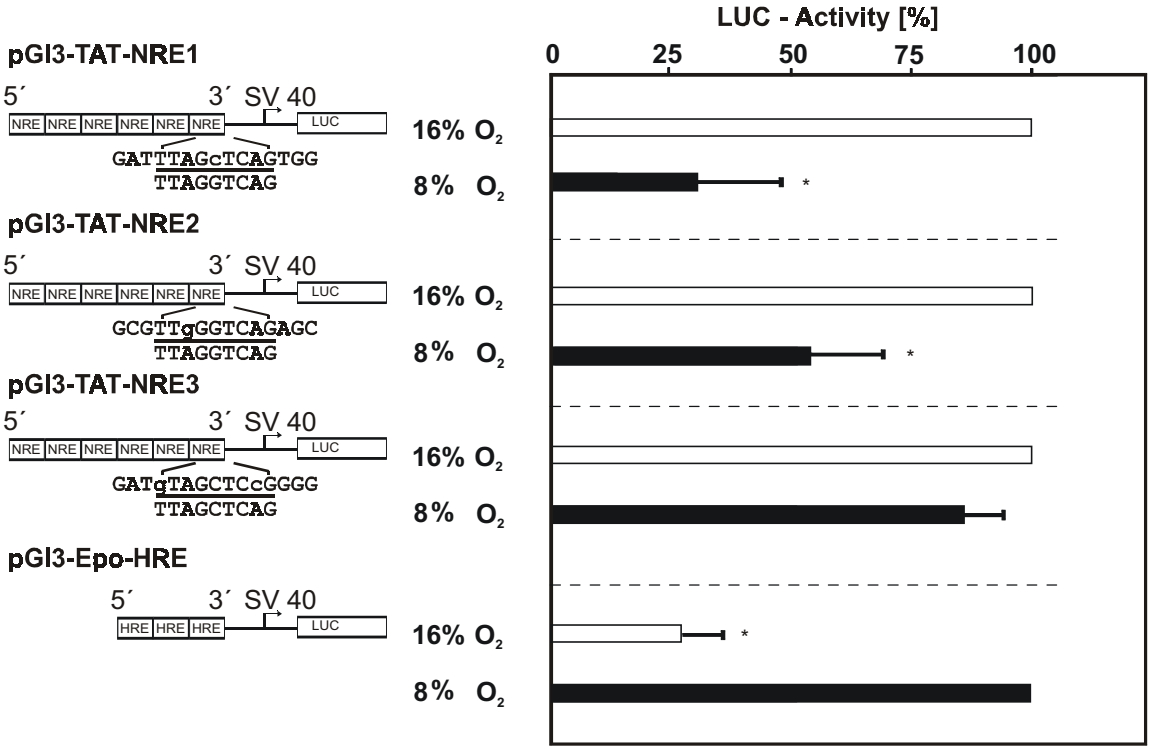


Fig. 27: **Oxygen-dependent expression of pGl3-TAT-NRE Luc constructs in primary rat hepatocytes.** Hepatocytes were transiently transfected with the LUC gene constructs containing six copies of TAT NRE's or three copies of the Epo HRE in front of the SV40 promoter-driven Luc gene (pGl3-TAT-NRE1, pGl3-TAT-NRE2, pGl3-TAT-NRE3 and pGl3-Epo-HRE). After 24 h the cells were cultured under normoxic (16% O_2) and hypoxic (8% O_2) conditions for another 24 h. In each experiment the percentage of Luc activity was determined relative to the pGl3-TAT-NRE1, pGl3-TAT-NRE2 and pGl3-TAT-NRE3 16% O_2 controls which were set equal to 100%. For pGl3-Epo-HRE the 8% O_2 value was set to 100%. The values represent means ± SEM of three independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$. Sequences shown in the upper strand correspond to the sequences of the TAT gene. The NRE sequences are underlined. Sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between TAT NRE and PCK-1 NRE are shown in lower case letters.

Since NRE's are similar to cAMP response elements (CRE) (Bratke et al., 1999) the possibility that glucagon exerts effects on the TAT-NRE1, which was induced by normoxia better then other TAT-NRE's, was investigated. Under standard conditions the Luc activity in cells transfected with pGl3-TAT-NRE1 construct was reduced to 40% under hypoxia compared to normoxia (Fig. 29). In the presence of 10 nM glucagon Luc expression from pGl3-TAT-NRE1 was not induced and the difference between the Luc activity under normoxia and hypoxia was as in the untreated cells about 2-fold. As a control the glucagon-responsive and oxygen-modulated pGl3PCK-493 Luc construct was used. The levels of Luc activity in the cells transfected with pGl3PCK-493 were by about 50% lower under hypoxia compared to normoxia. By contrast, glucagon induced Luc activity in the cells transfected with pGl3-PCK-493 by

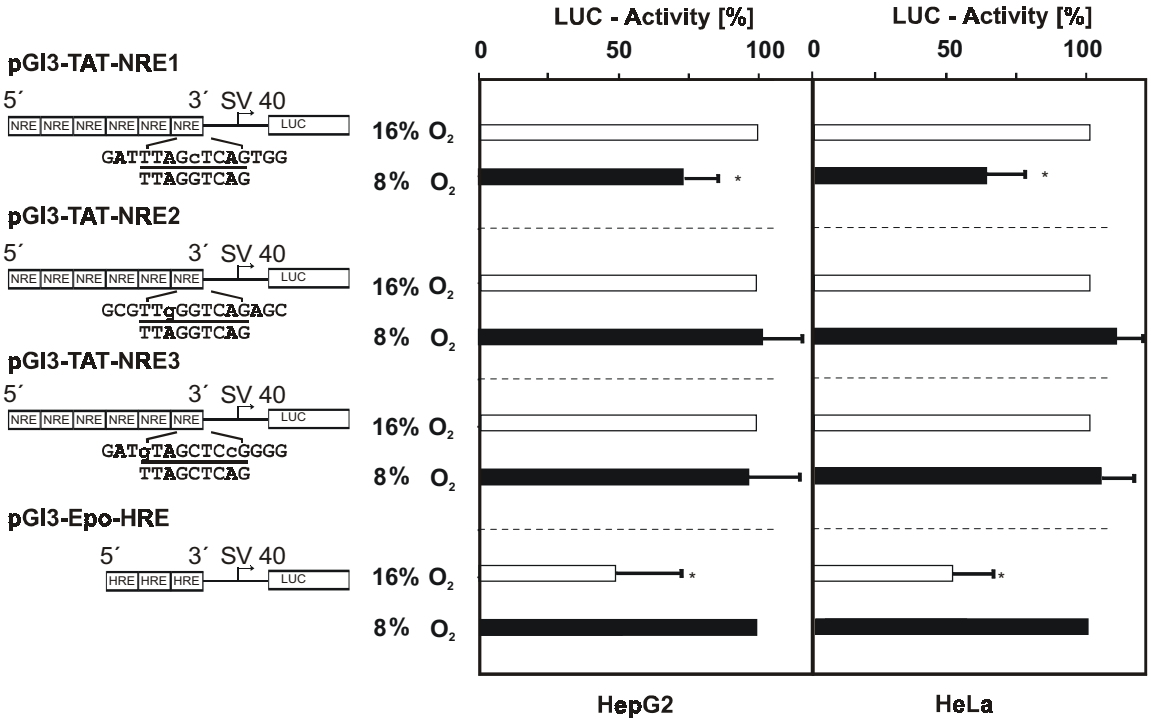


Fig. 28: **Oxygen-dependent expression of pGl3-TAT-NRE Luc constructs in HepG2 and HeLa cells.** HepG2 and HeLa cells were transiently transfected with the LUC gene constructs containing six copies of TAT NRE's or three copies of the Epo HRE in front of the SV40 promoter-driven Luc gene (pGl3-TAT-NRE1, pGl3-TAT-NRE2, pGl3-TAT-NRE3 and pGl3-Epo-HRE) and cultured under standard conditions for 24 h. Then the medium was changed and the cells were were cultured for another 24 h under normoxia (16% O_2) or hypoxia (8% O_2). In each experiment the percentage of Luc activity was determined relative to the pGl3-TAT-NRE1, pGl3-TAT-NRE2 and pGl3-TAT-NRE3 16% O_2 controls or to the pGl3-Epo-HRE 8% control which were set equal to 100%. The values represent means ± SEM of three independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$. Sequences shown in the upper strand correspond to the sequences of the TAT gene. The NRE sequences are underlined. Sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between TAT NRE and PCK NRE are shown in lower case letters.

about 250% under normoxia, whereas the Luc activity was induced by only about 100% under hypoxia. The Luc activity in the cells transfected with the control hypoxia-inducible pGl3-Epo-HRE was by about 200% higher in the cells cultured under hypoxia. Thus, despite some similarity of the TAT-NRE-1 to a CRE sequence the TAT-NRE1 did not function as a cAMP responsive element.

4.3.2 Induction of the SerDH NRE enhancer Luc gene constructs by normoxia

The 90 bp oligonucleotides containing 6 copies of either NRE-1, NRE-2, NRE-3 or NRE-4 from the SerDH promoter were cloned in front of the SV40 promoter and the luciferase gene in pGl3-prom to generate pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3 and pGl3-SerDH-NRE4. When primary rat hepatocytes were

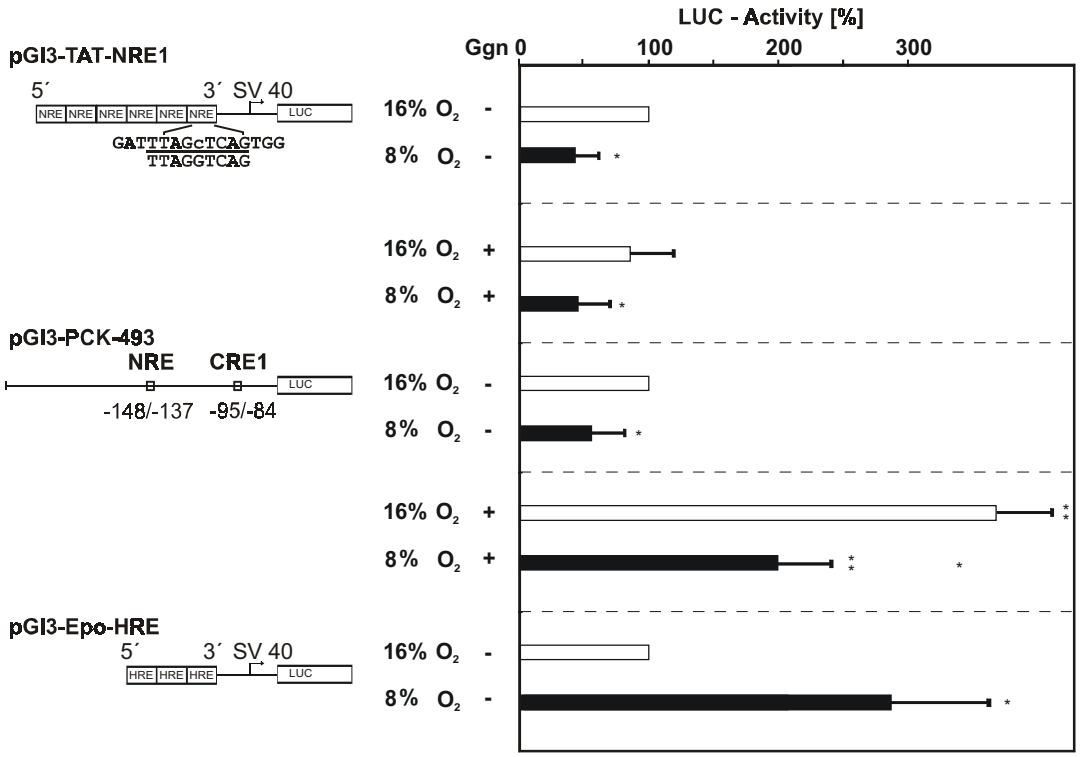


Fig. 29: **Glucagon-dependent expression of pGl3-TAT-NRE1 Luc construct in primary rat hepatocytes.** Hepatocytes were transiently transfected with the LUC gene constructs containing six copies of TAT NRE-1 or three copies of Epo HRE in front of the SV40 promoter-driven Luc gene (pGl3-TAT-NRE1 and pGl3-Epo-HRE) or pGl3-PCK-493 construct containing 493 bp of PCK-1 promoter in front of the Luc gene. After 24 h the cells were cultured under normoxic (16% O_2) and hypoxic (8% O_2) conditions for another 24 h. The cells were treated with 10 nM glucagon for 12 h, as indicated. In each experiment the percentage of Luc activity was determined relative to the pGl3-TAT-NRE1, pGl3-PCK-493 and pGl3-Epo-HRE 16% O_2 controls which were set equal to 100%. The values represent means ± SEM of three independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$; ** significant difference 16% O_2 vs. 16% O_2 + glucagon, 8% O_2 vs. 8% O_2 + glucagon, $p \leq 0.05$. Sequence shown in the upper strand correspond to the sequence of the TAT gene. The NRE sequence is underlined. Sequence shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between TAT NRE and PCK-1 NRE are shown in lower case letters.

transfected with these constructs, neither of them was expressed differentially under normoxia or hypoxia (Fig. 30).

In HepG2 cells only the construct pGl3-SerDH-NRE2 was activated by normoxia by about 40%, while the others displayed the same levels of Luc activity under normoxia and hypoxia. In HeLa cells the expression of two out of four constructs, pGl3-SerDH-NRE1 and pGl3-SerDH-NRE2, was activated by normoxia by about 30% (Fig. 31). Luc activity in the cells transfected with the hypoxia-inducible pGl3-Epo-HRE construct was significantly higher in the cells cultured under hypoxia for all three cell types investigated. These results indicated that NRE-1 and NRE-2 from SerDH promoter

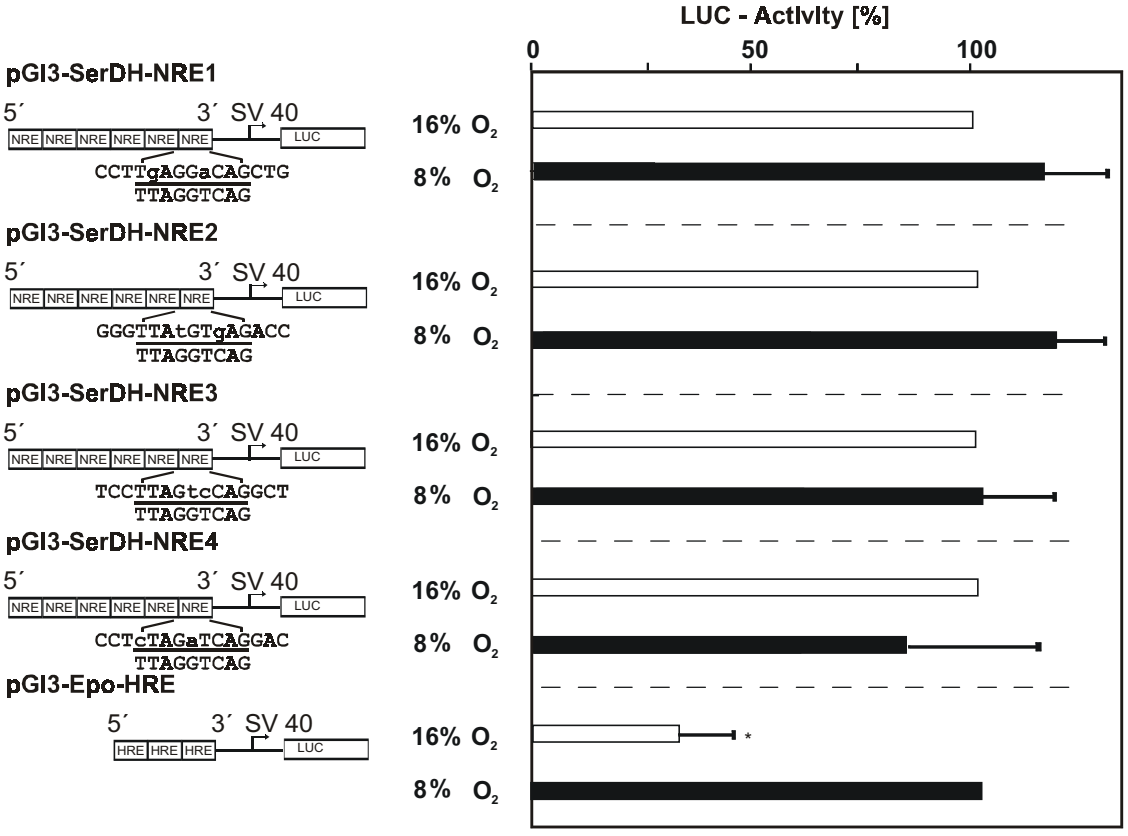


Fig. 30: **Oxygen-dependent expression of pGl3-SerDH-NRE Luc constructs in primary rat hepatocytes.** Hepatocytes were transiently transfected with the LUC gene constructs containing six copies of SerDH NRE's or three copies of the Epo HRE in front of the SV40 promoter-driven Luc gene (pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3, pGl3-SerDH-NRE4 and pGl3-Epo-HRE). After 24 h medium was changed and the cells were cultured for another 24 h under normoxic (16% O_2) and hypoxic (8% O_2) conditions. In each experiment the percentage of Luc activity was determined relative to the pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3 and pGl3-SerDH-NRE4 16% O_2 controls or to the pGl3-Epo-HRE 8% control which were set equal to 100%. The values represent means ± SEM of three independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$. Sequences shown in the upper strand correspond to the sequences of the SerDH gene. The NRE sequences are underlined. Sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between SerDH NRE and PCK-1 NRE are shown in lower case letters.

could act as weak normoxia-responsive elements in HepG2 and HeLa cells, but not in primary rat hepatocytes.

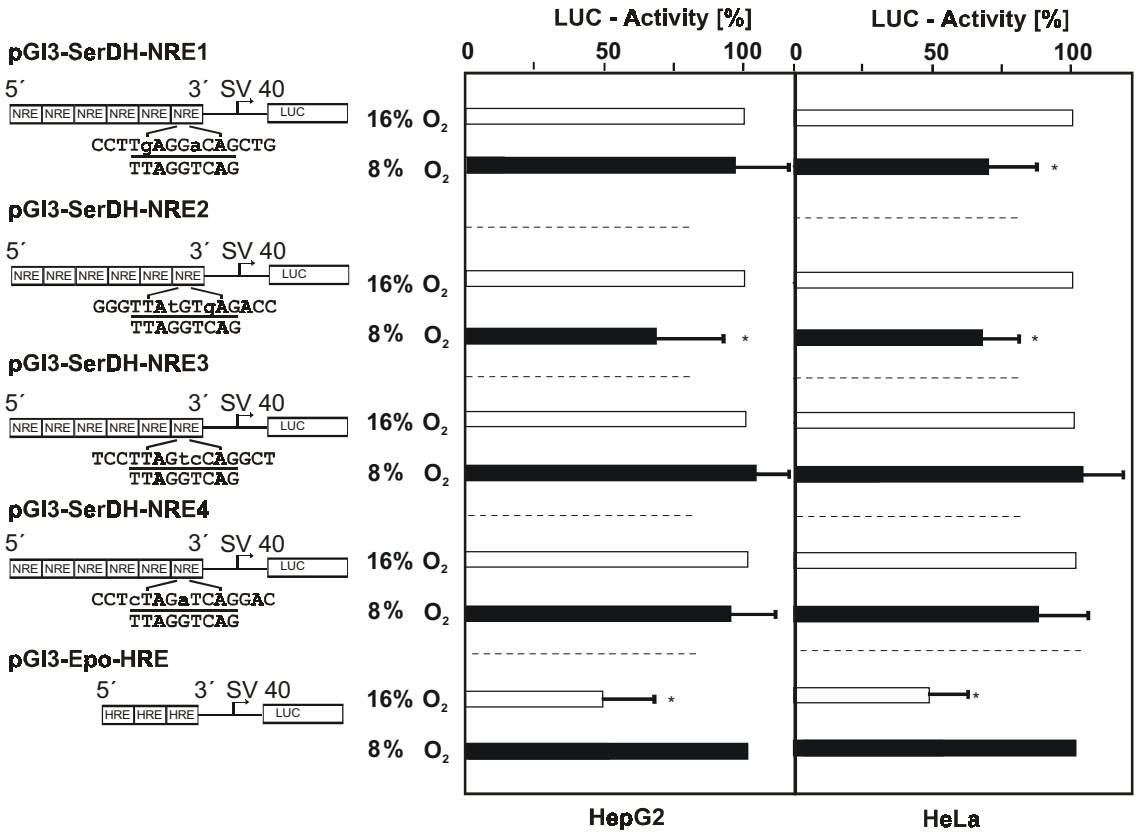


Fig. 31: **Oxygen-dependent expression of pGl3-SerDH-NRE Luc constructs in HepG2 and HeLa cells.** HepG2 and HeLa cells were transiently transfected with the LUC gene constructs containing six copies of SerDH NRE's or three copies of the Epo HRE in front of the SV40 promoter-driven Luc gene (pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3, pGl3-SerDH-NRE4 and pGl3-Epo-HRE). After 24 h medium was changed and the cells were cultured for another 24 h under normoxic (16% O_2) and hypoxic (8% O_2) conditions. In each experiment the percentage of Luc activity was determined relative to the pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3 and pGl3-SerDH-NRE4 16% O_2 or to the pGl3-Epo-HRE 8% controls which were set equal to 100%. The values represent means ± SEM of three independent experiments. Statistics, Student's t-test for paired values: * significant difference 8% O_2 vs. 16% O_2, $p \leq 0.05$. Sequences shown in the upper strand correspond to the sequences of the SerDH gene. The NRE sequences are underlined. Sequences shown in the lower strand correspond to the PCK-1 NRE. The nucleotides different between SerDH NRE and PCK-1 NRE are shown in lower case letters.

4.4 Binding of an oxygen-inducible complex to NRE sequences of the TAT and SerDH promoters

The binding of nuclear proteins to the NRE oligonucleotide probes spanning TAT-NRE-1, which was shown to be involved in the normoxia-dependent regulation of Luc reporter gene expression, or the inactive SerDH NRE-4 was investigated by EMSA. EMSA experiments were performed with nuclear extracts prepared from primary rat hepatocytes and HepG2 cells which were both cultured under normoxia or hypoxia.

The oligonucleotide containing TAT-NRE-1 was able to bind two (for primary rat hepatocytes) (Fig. 32) or one (for HepG2 cells) (Fig. 33) protein complexes formation of which was enhanced with the extracts from cells cultured under normoxia. The SerDH NRE-4 oligonucleotide did not bind any complex when incubated with nuclear extracts from primary hepatocytes but it was able to bind a similar complex as the TAT NRE-1 oligonucleotide when incubated with the nuclear extracts from HepG2 cells. However, in competition experiments a 50-fold excess of unlabelled TAT NRE-1 was sufficient to completely prevent the formation of the SerDH NRE-4 DNA-protein complex with HepG2 nuclear extracts while a 50-fold excess of unlabelled SerDH NRE-4 did not abolish formation of the TAT NRE-1 DNA-protein complex (Fig. 32, Fig. 33). Thus, the binding of the normoxia-induced complex was stronger with the TAT NRE-1.

When the EMSA reaction was additionaly incubated with antibodies against c-Jun, c-Fos, both of which constituting the transcription factor AP-1, CREB/ATF or SP-1, no supershift was detected (Fig. 34). As a control, the supershift with these antibodies and oligonucleotides corresponding to the CRE/AP-1 element of the rat heme oxygenase-1 (HO-1) promoter (-668/-654) were performed (Kietzmann et al., 2003a) (Fig. 34). Thus, the results of the EMSA's, in agreement with the Luc reporter experiments, showed that TAT NRE-1 was able to mediate the oxygen-dependent regulation of gene expression via binding of a normoxia-induced factor whose nature appears to be likely unknown yet.

A

```
TAT NRE1 (TAT1)          5´-GATTTAGCTCAGTGG-3´
                              :  :::  ::::
SerDH NRE4 (SerDH4)      5´-CCTCTAGATCAGGAC-3´
```

B

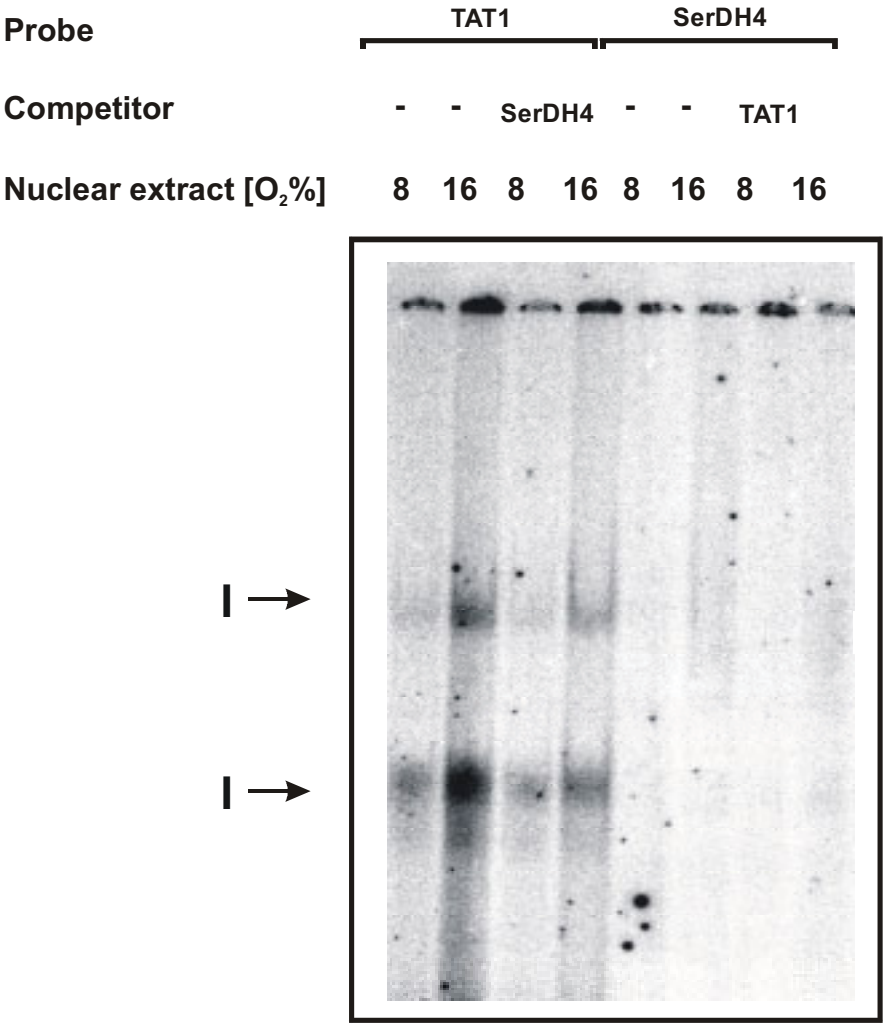


Fig. 32: **EMSA with nuclear extracts from primary hepatocytes.** (**A**) Oligonucleotides: the oligonucleotides containing TAT NRE-1 (-9536/-9519) and SerDH NRE-4 (+33/+50) are shown. The nucleotides of the NRE's are shown in bold, the nucleotides common for TAT NRE-1 and SerDH NRE-4 are indicated by colon. (**B**) Electophoretic mobility shift assay (EMSA): the ^{32}P-labeled TAT NRE-1 and SerDH NRE-4 oligonucleotides were incubated with 10 µg protein of nuclear extracts from either normoxic (cultured under 16% O_2) or hypoxic (cultured under 8% O_2) primary hepatocytes (cf. Materials and Methods). For competition a 50-fold excess of non-labeled NRE oligonucleotides was added. The DNA-protein binding was analyzed by electrophoresis on 5% native polyacrylamide gels. I, normoxia-induced complexes.

A

```
TAT NRE1 (TAT1)          5´-GATTTAGCTCAGTGG-3´
                              : ::: ::::
SerDH NRE4 (SerDH4)      5´-CCTCTAGATCAGGAC-3´
```

B

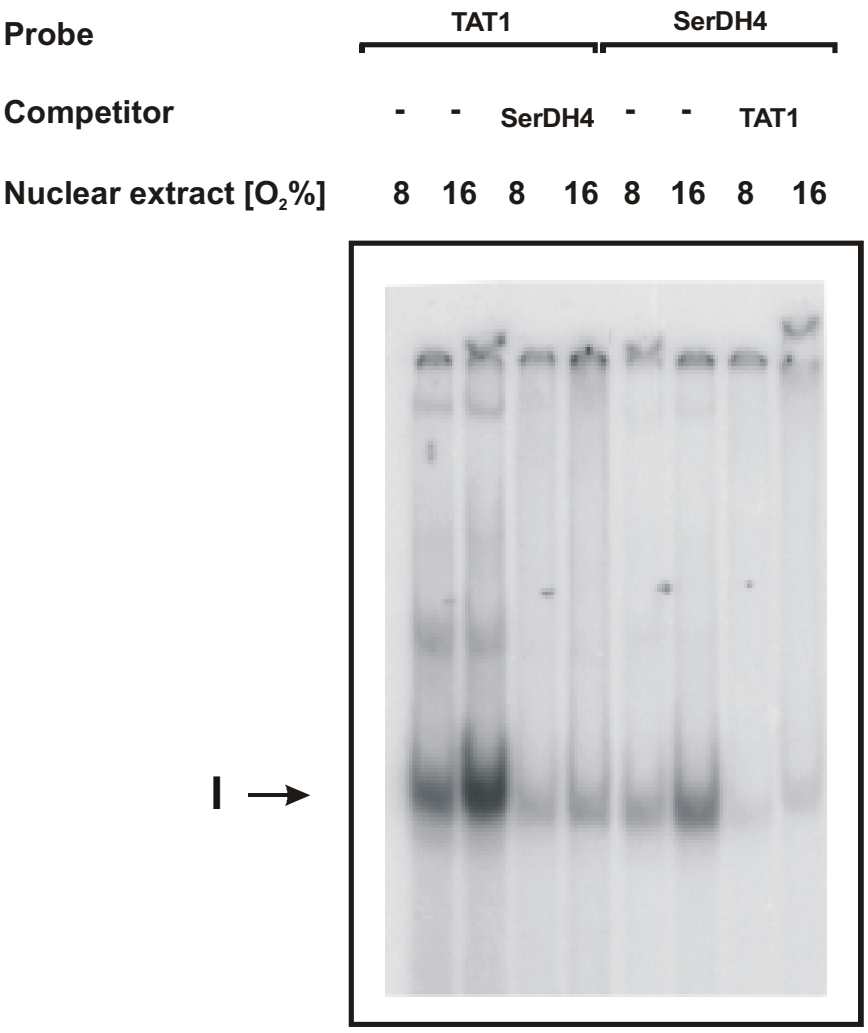


Fig. 33: **EMSA with nuclear extracts from HepG2 cells.** (**A**) Oligonucleotides: the oligonucleotides containing TAT NRE-1 (-9536/-9519) and SerDH NRE-4 (+33/+50) are shown. The nucleotides of the NRE's are shown in bold, the nucleotides common for TAT NRE-1 and SerDH NRE-4 are indicated by colon. (**B**) Electophoretic mobility shift assay (EMSA): the ^{32}P-labeled TAT NRE-1 and SerDH NRE-4 oligonucleotides were incubated with 10 µg protein of nuclear extracts from either normoxic (cultured under 16% O_2) or hypoxic (cultured under 8% O_2) HepG2 cells (cf. Materials and Methods). For competition 50-fold excess of non-labeled NRE oligonucleotides was added. The DNA-protein binding was analyzed by electrophoresis on 5% native polyacrylamide gels. I, normoxia-induced complex.

A

```
AP1 consensus          TGANNTCA
CRE consensus          TGACGTCA
                          :::: :::
HO-1 CRE/AP-1      5´-TCCTGACTTCAGTCT-3´
                          :  :  :::::
TAT NRE1 (TAT1)    5´-GATTTAGCTCAGTGG-3´
```

B

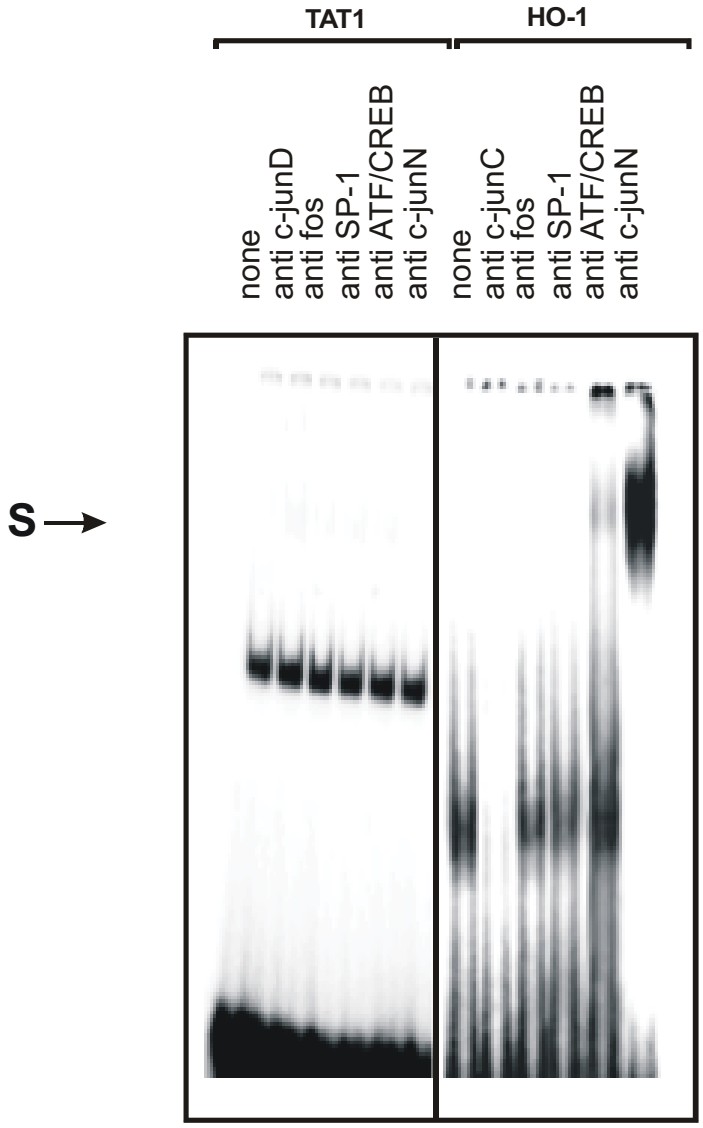


Fig. 34: **EMSA with TAT NRE-1 and HO-1 CRE/AP-1 oligonucleotides.** (**A**) Oligonucleotides: the oligonucleotides containing TAT NRE-1 (-9536/-9519) and HO-1 CRE/AP-1 (-668/-654) as well as AP-1 and CRE consensus sequences are shown. The nucleotides of the NRE and CRE/AP-1 are shown in bold, the nucleotides common for the TAT NRE-1 and HO-1 CRE/AP-1 and for the HO-1 CRE/AP-1 and CRE consensus are indicated by colon. (**B**) Electophoretic mobility shift assay (EMSA): the ^{32}P-labeled TAT NRE-1 and HO-1 CRE/AP-1 oligonucleotides were incubated with 10 µg protein of nuclear extracts from HepG2 cells (cf. Materials and Methods). In EMSA with antibodies the nuclear extracts were preincubated with 1 µl of the JunC, JunN, JunD, Fos, SP-1, ATF/CREB antibodies for 2 h at 4°C before adding the labeled probe. The DNA-protein binding was analyzed by electrophoresis on 5% native polyacrylamide gels. S, supershifted complex.

4.5 Identification of putative NRE-binding proteins by MATCHMAKER one hybrid system experiments

To identify and to clone the NRE binding factor a yeast one hybrid screen was performed. In the one hybrid system experiments a yeast strain containing six copies of TAT-NRE1 integrated into yeast genome was used. This strain was prepared by transfecting yeasts with the linearized plasmid pHISi-NRE, in which six copies of the TAT-NRE-1 were cloned.

The integration of the pHISi-NRE vector resulted in the growth of transformants on the selective medium lacking histidine. To inhibit background HIS3 expression on the selective medium lacking histidine, the new reporter strain was plated on the selective dishes with different concentrations of the HIS3 inhibitor 3-amino triazole (3-AT). A concentration of 15 mM 3-AT was found to be optimal and was used for the further experiments in which the yeast strain was transfected with a rat cDNA library containing plasmids allowing expression of a fusion protein with the GAL4 activation domain (AD). After transfection of the modified yeast reporter strain with the rat liver cDNA/GAL4 AD fusion library the yeasts were plated on selective dishes lacking histidine and containing 15 mM 3-AT.

In total 583 clones were obtained on the dishes. When they were plated on the dishes containing a higher concentration of the HIS3 inhibitor (30 mM 3-AT) only 136 of the clones could grow. The plasmids from these yeast clones were isolated and transformed into E. coli. The plasmids from those clones which could grow after transformation in E. coli were isolated. 78 from 136 clones were thus obtained and sequenced. The sequences were then used to search the entire non-redundant nucleotide gene bank with the BLAST search algorithm (Altschul et al., 1997). The protein found with highest frequency (5 times) in our assay was homologous to a nuclear protein with a molecular weight of 25 K. The protein similar to the rat transcription eliongation factor elongin C (Tceb 1L) was found three times. Among the proteins found twice were NEFA (nucleobindin-2) and ferritin light chain.

To eliminate false positives a dual reporter strain needs to be generated by integrating a lacZ reporter plasmid pLacZi-NRE containing 6 copies of TAT-NRE1 into the genome of the yeast strain already containing HISi-NRE.

Tab. 2: **Putative NRE-binding proteins as identified by the yeast one hybrid experiments**

Protein	Genbank accession number
Nuclear 25 K protein	NM 145474
Transcription elongation factor elongin C (SIII) polypeptide 1-like protein (Tceb 1L)	BC 002115
NEFA (nucleobindin-2)	NM 021663
Ferritin	L 01122

5. DISCUSSION.

In the present study the O_2-dependent expression of TAT and SerDH mRNA and the O_2-dependent regulation of TAT and SerDH promoter activity was demonstrated. Furthermore, the respective normoxia responsive elements in the TAT and SerDH promoter were identified and characterized. Candidate transcription factors which may act as normoxia responsive element binding factors were isolated by using the yeast one-hybrid system. These results are in accordance with previous studies showing the predominant TAT and SerDH expression in the more aerobic periportal zone of the liver acinus and with findings showing that in primary hepatocyte cultures the TAT enzyme levels and activities were positively modulated by periportal pO_2, i.e. normoxia. The data concerning normoxia-induced TAT and SerDH expression confirm the idea that the O_2 gradient within the liver acinus constitutes a major determinant for the zonated gene expression in liver.

5.1 Zonation and regulation of tyrosine aminotransferase (TAT) and serine dehydratase (SerDH) gene expression: control by hormones and oxygen

A number of studies support the metabolic specialization of hepatocytes, the zonation of liver metabolism (Jungermann et al., 1996b; Jungermann et al., 2000). This zonated specialization is based on a heterogenous pattern of gene expression. Although all parenchymal and non-parenchymal cells have the same genome, the zonal differences in the expression pattern of several genes are likely caused due to the heterogeneity in the input and transmission of signals such as substrates including oxygen and products, hormones, mediators and nerves and in cell-to-cell or cell-to-biomatrix interactions.

The present study focussed on the transcriptional regulation of TAT and SerDH gene expression by O_2 and the hormone glucagon and the glucocorticoid dexamethason. In

general, gene expression can be regulated at four levels: transcription, mRNA degradation, translation and protein degradation. Within the liver acinus the same zonal distribution of an mRNA and its encoded protein would indicate control of gene expression at a transcriptional or pretranslational site; an unequal distribution would indicate regulation at a translational or posttranslational site.

Thus the focus on the transcriptional control within this study is based on the observation that the zonal expression of the TAT and SerDH genes as well as a number of other enzymes involved in amino acid and ammonia metabolism appear to be regulated at a pretranslational level. TAT and SerDH had higher mRNA and protein levels in the periportal zone during the daily feeding rhythm and during a starvation-refeeding cycle (Nauck et al., 1981; Ogawa et al., 1991). Furthermore, the mRNA and immunoreactive protein of the ureagenic key enzyme carbamoylphosphate synthetase (CPS) was found to be localized in the periportal and proximal perivenous zone (Moorman et al., 1990). Only a cell layer of 1-3 perivenous hepatocytes did not express CPS mRNA. Conversely, the mRNA and the protein of the key ammonia scavenging enzyme glutamine synthetase (GNS) was found in the layer of 1-3 cells surrounding the central vein (Gebhardt et al., 1988; Moorman et al., 1988). Finally, the mRNA of glutaminase was found in the periportal zone as was the protein (Haussinger et al., 1992).

Similarly, in situ hybridization studies revealed that the mRNAs of the rate-generating enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase-1 (PCK-1) and fructose 1,6-bisphosphatase (FBPase), were predominantly located in the periportal zone during the daily feeding rhythm and during a starvation-refeeding cycle as were the enzyme proteins and activities thus pointing also to a transcriptional control mechanism which regulates the zonated pattern (Jungermann et al., 1996b; Jungermann et al., 2000).

5.1.1 Hormone gradients

The overall daily changes in mRNA levels and activities of TAT, SerDH, PCK and many other carbohydrate metabolizing enzymes in liver are linked to the feeding rhythm and to starvation-refeeding cycles. Since the major hormones during feeding and starvation are insulin and glucagon together with glucocorticoids, respectively, it appears that their gene activity can be expected to be controlled mainly by the insulin/glucagon-glucocorticoid ratio, which is high during the feeding and low during the fasting period (Jungermann et al., 1996a). Thus, in vivo the expression of gluconeogenic TAT, SerDH and PCK decrease with feeding and increase with fasting (Bartels et al., 1990; Ogawa et al., 1991). Conversely, the glucose utilizing glucokinase

and pyruvate kinase (PK_L) decrease with fasting and increase with feeding (Lamas et al., 1987; Eilers et al., 1993).

In the present study Northern blot analyses with RNA prepared from primary rat hepatocytes cultured in the presence of either dexamethasone or glucagon revealed a strong induction of TAT mRNA and promoter activity in the presence of dexamethasone (Fig. 19). This induction was also present when the –10095 bp TAT promoter Luc construct was transfected into primary hepatocytes. However, the glucagon-dependent induction of TAT promoter activity was only observed when the cells transfected with pGl3TAT-10095 Luc were treated in the absence of dexamethasone with 10 nM glucagon for 12 h. This can be explained by the use of dexamethasone as permissive hormone during culture maintenance of primary hepatocytes. Since dexamethasone is a potent inducer of TAT it is likely that the permanent presence of dexamethasone elicited already maximal TAT levels which could not be further induced by glucagon. When the hepatocytes, after the initial attachement phase, were then cultured in the absence of dexamethasone glucagon was able to enhance TAT-10095 promoter activity. The glucagon-dependent induction of Luc activity was not observed with the construct pGl3TAT-356 which did not contain the CRE and both GRU‘s of the TAT gene promoter (Jantzen et al., 1987; Grange et al., 1989).

The effect of glucagon in the presence of dexamethasone on SerDH mRNA expression was only investigated together (Fig. 20) since it was previously shown that the induction of SerDH mRNA in primary cultured rat hepatocytes required both dexamethasone and glucagon or cAMP (Noda et al., 1988). Dot-blot hybridization analysis of mRNA showed that SerDH mRNA was induced by 1 μM dexamethasone and 0.5 μM glucagon together, but not by either alone (Noda et al., 1988). In addition, in our study SerDH promoter-dependent Luc activity was not induced by either dexamethasone or glucagon. The lack of effects of these two inducers was expected since cAMP-regulatory elements (CRE-1 and CRE-2) of SerDH are located approximately 3500 bp upstream from the transcription initiation site, i. e. they are not present in the construct pGl3SerDH-2303 or the shorter constructs used in our experiments (Su et al., 1992a; Haas et al., 1999).

These findings are in line with the observation that physiological glucagon concentrations under the permissive actions of glucocorticoids and thyroid hormones activate the PCK-1 gene in primary hepatocyte cultures and in vivo (Short et al., 1986; Wynshawboris et al., 1986; Runge et al., 1991; Hellkamp et al., 1991), whereas insulin acts as transcription inhibitor (Obrien et al., 1990; Christ et al., 1990). The results are also in accord with the observation that physiological insulin concentrations again

under the permissive action of glucocorticoids induce the GK gene (Probst et al., 1982; Wolfle et al., 1985; Kietzmann et al., 1997). The transcriptional effects of the inducing and permissive hormones are mediated by appropriate hormone response elements in the promoter of the PCK-1 (Short et al., 1986; Wynshawboris et al., 1986; Obrien et al., 1990) and GK gene (Leibiger et al., 1994; Roth et al., 2002). Thus, glucagon appears to dominate the establishment of a high capacity for gluconeogenesis in the periportal area, whereas insulin appears to have a key role in the generation of a high capacity for glucose utilization in the perivenous zone.

In rats with streptozotocin- or alloxan-induced diabetes the periportal to perivenous activity ratio for PCK-1 remained unaltered with elevated levels in the two zones each and the zonal ratio for PK_L was only slightly decreased with lower levels in the two zones (Miethke et al., 1986). This finding indicates that the gradient in the insulin/glucagon-glucocorticoid ratio cannot be the major determinant for the heterogeneous zonal gene expression. Therefore, the oxygen gradient was considered again as a major factor.

5.1.2 Oxygen gradients

In the present study Northern blot analyses with RNA prepared from primary rat hepatocytes cultured under O_2 conditions mimicking periportal and perivenous oxygen tensions revealed a difference between basal normoxic and hypoxic TAT mRNA expression, as expected. The difference between normoxic and hypoxic levels of TAT mRNA and Luc activity is in line with an earlier study (Nauck et al., 1981) which demonstrated the about two-fold difference between hypoxic and normoxic TAT activity in primary rat hepatocyte cultures. In that study, the transcription inhibitor actinomycin and the protein synthesis inhibitor cycloheximide abolished the modulation of TAT activity by O_2 thus pointing to a transcriptional or pretranslational control of gene expression. Indeed, the modulation by O_2 of TAT expression at the transcriptional level was supported by this study with the Luc constructs driven by the -10095 TAT promoter.

Furthermore, in line with the present study the glucagon-dependent induction of TAT enzyme activity in primary cultures of rat hepatocytes under gas atmospheres of normoxia and hypoxia displayed the modulation by O_2 (Nauck et al., 1981). In hepatocyte cultures the activity of TAT was increased by glucagon (100 nM) within 4 h to higher levels under 13% O_2 then under 6% O_2.

The results of the present study are also in agreement with other reports showing that in 24 h primary rat hepatocyte cultures glucagon stimulated PCK-1 gene transcription maximally after 30 min, enhanced PCK-1 mRNA abundance maximally after 2 h and

enzyme activity after 4-6 h under normoxia, whereas glucagon activated the PCK gene only half maximally under hypoxia. Although it appeared that the modulation by O_2 of TAT and PCK expression occurred only in the presence of hormones, it was shown in this and the earlier study, that also small differences at the basal enzyme activities and at the mRNA levels exist (Fig. 19). Thus the presence of the hormones led to an enhancement of mRNA expression and subsequently enzyme activity which could be better detected by the available methods. In our study, both the TAT mRNA expression and TAT promoter-dependent luciferase activity were induced in the presence of dexamethasone, however, the oxygen-dependent modulation did not change indicating an hormone-independent mechanism in the O_2 mediated transcriptional control (Fig. 19, Fig. 25).

The same modulatory role of O_2 was observed with the SerDH gene. Oxygen-regulated expression of SerDH gene as well as of SerDH promoter-dependent Luc constructs in cell cultures was not previously investigated. In our experiments, SerDH mRNA was hardly detectable at basal levels (i.e. no hormones) similar to the in vivo situation as observed in the livers of normal fed rats. Glucagon, as expected - since starvation enhanced SerDH mRNA in vivo - strongly and transiently induced SerDH mRNA expression, with a clear modulation by O_2 (Fig. 20). The increase of the glucagon concentration even enhanced SerDH mRNA but did not affect modulation by O_2. Moreover, the results of this study are in accordance with the zonation data showing predominant SerDH expression in the more aerobic periportal area of the liver acinus.

This modulation of gene expression by oxygen was not due to an impaired energy production at the lower oxygen concentrations, since PAI-1 mRNA (Kietzmann et al., 1999; Samoylenko et al., 2001) and GK mRNA (Kietzmann et al., 1997) as well as EPO-HRE-dependent Luc activity (Fig. 28-31) were induced to higher levels under hypoxia.

5.2 O_2-dependent regulation of gene expression: a common phenomenon

In the present study the O_2–dependent expression of TAT and SerDH was investigated in primary rat hepatocyte cultures and it was found that the culture of hepatocytes under normoxia (16% O_2) and hypoxia (8%O_2) was able to mimic the zonated TAT and SerDH expression pattern observed in the liver acinus. However, O_2-dependent regulation of gene expression is not restricted to the liver although among the genes which were shown to be induced by normoxia many were predominantely expressed in the periportal region of the liver acinus.

In particular, O_2-regulated gene expression was investigated during fetal development.

During the first trimester, a human embryo is located in a low-oxygen environment (3% O_2) (Rodesch et al., 1992). The increase of oxygen tension to the normoxic levels after the first trimester of gestation is supposed to be responsible for a number of developmental processes, among them maturation of human megakaryocytes (Mostafa et al., 2000) and adipogenesis (Yun et al., 2002), spontaneous morphological and biochemical differentiation of human fetal lung cells (Acarregui et al., 1993), proliferation and differentiation of cytotrophoblasts along the highly invasive pathway (Genbacev et al., 1997). Furthermore, severe hypoxia (1% $O_2 \cong$ 7 mm Hg) was found to activate the genes for erythropoietin (EPO) in kidney and fetal liver (Imagawa et al., 1991; Semenza et al., 1991; Eckardt et al., 1994), for vascular endothelial growth factor (Shweiki et al., 1992; Goldberg et al., 1994) and platelet derived growth factor ß (PDGFß) (Kourembanas et al., 1990) in endothelial cells, for endothelin-1 in lung and right atrium (Kourembanas et al., 1991) and for angiotensin converting enzyme (ACE) in pulmonary endothelial cells (King et al., 1989) as well as for tyrosine hydroxylase, a key enzyme of catecholamine synthesis, in the carotid body and in phaeochromocytoma cells (Czyzyk-Krzeska et al., 1994). Hypoxia also induced the genes coding for the glycolytic enzymes lactate dehydrogenase A (LDH A), phosphoglycerate kinase 1 (PGK1), aldolase A (ALD A) and pyruvate kinase M in HepG2, Hep3B or HeLa cells (Firth et al., 1994; Semenza et al., 1994). Thus, the general view has been accepted that O_2 can act as a signaling molecule responsible for the regulation of gene activity in many, if not all, cell types and tissues.

5.2.1 O_2 regulatory elements and transcription factors

So far only a few genes have been found to be positively modulated by normoxia. Little is known about possible normoxia regulatory elements (NRE) and the corresponding transcription factors mediating the modulation. The majority of genes modulated by O_2 has been shown to be induced by hypoxia. The hypoxia regulatory elements (HRE) and the transcription factors involved in the induction by hypoxia have been defined (see below).

Characterization of the normoxia responsive elements

In the present study it was shown that sequences of TAT and SerDH promoters homologous to the normoxia responsive element (NRE) of the PCK-1 gene were involved in the normoxia-dependent gene regulation. Three putative NRE sequences homologous to the PCK-1 NRE were found in the TAT promoter (termed TAT-NRE1, TAT-NRE2 and TAT-NRE3) and four putative NRE sequences were identified in the SerDH promoter (termed SerDH-NRE1, SerDH-NRE2, SerDH-NRE3 and SerDH-

NRE4).

In the experiments, performed with the TAT promoter-dependent Luc constructs, the Luc activity was modulated by O_2 only when the cells were transfected with pGl3TAT-10095. By contrast, after transfection of the hepatocytes with the construct pGl3TAT-356 in which TAT-NRE1 and TAT-NRE2 are deleted Luc activity was induced not by normoxia, as expected, but by hypoxia. This indicates that TAT-NRE1 and TAT-NRE2 are critical for the normoxia-dependent TAT promoter activity and that NRE3 appears to play no role. The induction of the TAT-356 promoter by hypoxia could be explained by the presence of some hypoxia responsive element inside the 356 bp of TAT promoter which is usually not functional within the whole promoter context and therefore might have no physiological importance.

With the SerDH promoter Luc constructs the deletion experiments indicated that SerDH-NRE1 and SerDH-NRE2 present in the construct pGl3SerDH-2303 Luc appeared to be important for the normoxia-dependent promoter activity. The SerDH-NRE3 and NRE4 did not appear to be important since neither normoxia nor hypoxia modulated Luc activity in hepatocytes transfected with the constructs pGl3SerDH-937 and –471 lacking NRE1 and NRE2, respectively.

Under the assumption that the NRE´s may function not only in the whole promoter context of either TAT or SerDH and whether these elements may be able to transmit the response to normoxia to an independent promoter primary hepatocytes, HepG2 hepatoma cells and the epithelial-derived HeLa cells were transfected with reporter gene constructs containing six NRE in front of the SV40 promoter and the Luc gene.

When primary rat hepatocytes were transfected with TAT-$[NRE1]_6$-Luc and TAT-$[NRE2]_6$-Luc but not with TAT-$[NRE3]_6$-Luc constructs the Luc activity was higher under normoxia compared to hypoxia (Fig. 27). These results were in accordance with the observation that cells transfected with pGl3-TAT-356 Luc containing only TAT NRE3 were not induced by normoxia. By contrast, neither SerDH-NRE was able to mediate a normoxia-dependent activation of Luc expression in primary hepatocytes.

In transfections with HepG2 and HeLa cells it was shown that TAT-$[NRE1]_6$-Luc but not TAT-$[NRE2]_6$-Luc and TAT-$[NRE3]_6$-Luc expression was modulated by O_2 in HepG2 and HeLa cells. Contrary to the experiments in primary hepatocytes an O_2-response was observed with the SerDH-$[NRE2]_6$-Luc construct in both HeLa and HepG2 cells and with the SerDH-$[NRE1]_6$-Luc in HeLa cells. This pointed to an eventually more cell type specific regulation of oxygen-dependent transcriptional control.

The TAT-NRE sequences TAT NRE-1 (5‘-TTAG**C**TCAG-3‘) and TAT NRE-2 (5‘-TT**G**GGTCAG-3‘) investigated in this study and functionally active, differ in one base from the first described NRE from the PCK-1 gene (5‘-TTAGGTCAG-3‘). The SerDH

NRE-1 (5‘-T**G**AG**GA**CAG-3‘) and SerDH NRE-2 (5‘-TTA**TG**T**G**AG-3‘) which were active only in HepG2 or HeLa cells were different from PCK-1 NRE in two nucleotides of nine and different from TAT NRE-1 which functioned best, in three nucleotides of nine. Thus, it appeares that the first 5’ bases 5’-TTAG-3’ and the 3’ bases 5’-CAG-3’ at the end of the NRE sequence are most critical for an appropriate function of these elements.

Interestingly, the PCK-1 NRE was previously named cAMP regulatory element 2 (CRE2) on the basis of a 6 out of 8 bp homology with the palindromic CRE consensus sequence 5´-TGACGTCA-3´ and of a footprint with liver nuclear extracts (Roesler et al., 1989), however, functional studies showed that the CRE2 site did not confer inducibility by cAMP but modulation by O_2 of the glucagon-dependent PCK-1 gene activation (Bratke et al., 1999). Thus, the CRE2 site was named normoxia regulatory element (NRE). According to the findings with the PCK-1 NRE, the results obtained within this study also demonstrated that none of the TAT-NRE´s and SerDH-NRE´s which mediated the response to O_2 was sensitive to cAMP (Fig. 29). Thus, the mechanism involving phosphorylation-targeted proteosomal degradation of the cAMP response element binding protein (CREB) may not account for NRE regulated genes such as TAT and SerDH. This type of regulation, proposed from studies investigating the O_2-dependent expression of proinflammatory cytokines such as TNFα and IL-8 in T84 intestinal epithelial cells (Taylor et al., 1999), involves a hypoxia-specific depletion of protein phosphatase-1γ mRNA and protein resulting in the hyperphosphorylation of CREB between amino acids 115 to 121 subsequently initiating ubiquitination and proteasomal degradation of CREB (Taylor et al., 2000). Thus, under hypoxic conditions CREB is very rapidly degraded and by contrast CREB would be stable only under normoxic conditions.

All together this underlined that the NRE´s may display a novel type of regulatory element and based on the experiments in primary rat hepatocytes with NRE enhancer Luc constructs from TAT and SerDH promoters, a first putative consensus sequence 5‘-TTRGSTCAG-3‘(R=A/G, S=G/C) for a common NRE was proposed (Fig. 35). The NRE´s may then be bound by a transcription factor displaying a novel function or by a so far unknown factor.

Normoxia inducible factors

While the role of HIF-1 in the hypoxia-dependent gene regulation is well established, little is known about a transcription factor or factors involved in the regulation of gene expression by normoxia.

In the human glutathione peroxidase (GPX) gene two similar 13 bp oxygen responsive

elements 5´-CCTC**AA**AGAAA**GT**-3´ ORE-1) and 5´-CCTC**TG**AGAAA**AA**-3´ (ORE-2) were found to bind disparate proteins and to confer normoxia-dependent induction of GPX gene expression in human cardiomyocytes (Cowan et al., 1993). It was demonstrated that the transcription factor Ku antigen bound ORE-1 and ORE-2 of the human GPX gene (Merante et al., 2002). Ku antigen is a leucine zipper and HLH domain-containing transcription factor composed of p70 and p80 subunits. This factor was shown to be an ORE-binding protein (OREBP) by sequence-specific DNA affinity chromatography (Merante et al., 2002). The differences in binding affinity of ORE-1 and ORE-2 observed in EMSA experiments could indicate that *in vivo* additional factors were involved in fine tuning of OREBP binding in response to external stimuli (Merante et al., 2002). The sequences of the ORE´s from the human GPX gene are not similar to the NRE sequences identified in our experiments and the possible involvement of Ku factor and ORE's in the regulation of genes other than GPX was not investigated yet.

Hypoxia-inducible factors and hypoxia responsive elements

Activation of gene expression by normoxia and repression of gene expression by hypoxia can not be distinguished without knowing the molecular mechanisms of the processes involved. It might be speculated that higher gene expression under normoxia as shown in this study could be due to an inhibitory effect of HIF-1 under hypoxia. However, HIF-1 acts usually as an activator and the role of HIF-1 as transcriptional inhibitor was demonstrated so far only for the peroxisome proliferator-activated receptor α (PPAR-α) gene (Narravula et al., 2001). In these experiments, performed in the T84 intestinal epithelial cells and *in vivo* with mice subjected to whole-body hypoxia (8% O_2) or normoxia (21% O_2), the down-regulation of PPAR-α by hypoxia correlated with HIF-1α induction and functioned to protect epithelial cells from PPAR-induced amplification of intercellular adhesion molecule-1 (ICAM-1) induction.

The direct interaction of HIF-1α with the consensus HRE inside the PPAR-α promoter was shown using anti-HIF-1α antisense oligonucleotides and with EMSA experiments with antibodies against HIF-1α (Narravula et al., 2001).

This mechanism does not appear to account for TAT and SerDH modulation by oxygen. The TAT and SerDH gene promoters do not possess a complete 8 bp HRE in their 5´-flanking region, but only a 6 out of 8 bp element CGTGCT at positions –9151/-9156, –5889/-5894 and -1248/-1253 in the TAT promoter, which probably can not bind HIF-1 (Semenza, 2000). Thus, it is unlikely that a HRE is involved in the O_2-dependent expression of the TAT and SerDH gene.

An indirect HIF-1-mediated pathway was shown to be involved in the down-regulation

of the peroxisome proliferator-activated receptor γ2 (PPAR-γ2) mRNA expression under hypoxia (Yun et al., 2002). Highly specific for adipose tissues, PPAR-γ2 plays a critical role in the expression of most adipocyte-specific genes and is able to convert nonadipogenic mesenchymal cells, such as fibroblasts and myoblasts, to adipocytes (Tontonoz et al., 1995). In mouse embryonic fibroblasts hypoxia inhibited adipogenesis via the HIF-1-dependent induction of transcription factor DEC1/Stra13 gene expression (Yun et al., 2002). DEC1/Stra13, which contained a basic helix loop helix (bHLH) and an orange domain homologous to those of the HES transcription factors (Boudjelal et al., 1997), inhibited PPAR-□2 expression, at least in part, by interaction with the putative C/EBP sites at -229 and at -200 of PPAR-□2 promoter. The bHLH but not the C-terminal domain of DEC1/Stra13 was responsible for this interaction (Yun et al., 2002). Again, such an indirect mechanism does not appear to be involved in the TAT and SerDH gene expression since no functional C/EBP sites were identified in the

	HC	HepG2	HeLa	
PCK-NRE	-	+	+	GTGTTAGGTCAGTTC
TAT-NRE1	+++	+	++	GATTTAGcTCAGTGG
TAT-NRE2	++	-	-	GCGTTgGGTCAGAGC
TAT-NRE3	-	-	-	GGGgTAGGTCcGGGG
SerDH-NRE1	-	-	+	CCTTgAGGaCAGCTG
SerDH-NRE2	-	+	+	GGGTTAtGTgAGACC
SerDH-NRE3	-	-	-	TCCTTAGtcCAGGCT
SerDH-NRE4	-	-	-	CCTcTAGaTCAGGAC
Consensus				TTRGSTCAG

Fig. 35: **Alignment of normoxia responsive elements. Generation of a first consensus**. The sequences of all putative NRE's from PCK, TAT and SerDH genes with flanking nucleotides and the cells used in experiments are shown. No difference between normoxic and hypoxic levels of Luc activity in a particular cell type is indicated by -, induction below 50% under normoxia indicated by +, strong (ca. 50%) induction under normoxia indicated by ++, very strong (ca. 70%) induction under normoxia indicated by +++.. The NRE sequences are underlined. The nucleotides different between TAT or SerDH NRE and PCK-1 NRE are shown in lower case letters. R=A or G, S=G or C. HC, primary rat hepatocytes.

SerDH promoter and C/EBP sites in the TAT promoter are involvéd in the induction of the TAT gene expression and not in repression (Rigaud et al., 1991).

5.3 Candidate transcription factors functioning as NRE binding proteins

According to the idea of the one hybrid system method, proteins containing DNA-binding domains were expected to be found in this assay. The protein which was found with the highest frequency in our assay was homologous to a nuclear protein with a molecular weight of 25 K, from which the function was not known. The 256 amino acid sequence of this protein was predicted from the mRNA (Strausberg R., 2001, unpublished). The 25 K protein contains a long domain (aa 1 to 228) highly homologous (92%) to the heme-binding proteins of cytochrome P450 CYP2D subfamily, involved in the oxidative degradation of various compounds (Nebert et al., 1991). The presence of a domain which could bind heme makes this protein attractive to be involved as a direct oxygen sensor. It might be speculated that oxygen may directly bind to the heme moiety of the 25 K protein thus modulating its conformation. This conformational change may then affect its DNA binding affinity or its nuclear localization.

Among the other clones found in our assay were proteins similar to the rat transcription elongation factor elongin (SIII) polypeptide-1 (Tceb 1L) and rat NEFA (nucleobindin-2) precursor. Tceb 1L is a 163 amino acid protein of unknown function which showed 31% amino acid identity with elongin C (Sowden et al., 1995). A 112 amino acid protein elongin C was initially identified as a component of the RNA polymerase II elongation factor elongin (SIII), which is a heterotrimer composed of 110 kDa (p110; elongin A), 18 kDa (p18; elongin B) and 15 kDa (p15; elongin C) subunits, involved in stimulation of the overall rate of elongation by RNA polymerase II (Bradsher et al., 1993). Elongin C showes significant sequence similarity with a C-terminal portion of the RNA-binding domain from the termination factor ρ of E. coli (Garrett et al., 1994). Elongin C interacts directly with the ubiquitin-like elongin B subunit as well as with the growing number of proteins containing a conserved BC-box motif and with the Cullin family member protein Cul2 and RING-H2 finger protein Rbx1 (Takagi et al., 1997; Kamura et al., 2001; Hyman et al., 2002). Rbx1 belongs to a zinc RING-containing SAG/ROC/Rbx/Hrt protein family members of which can bind RNA and most likely DNA (Swaroop et al., 2001). However, whether Tceb L1 has a function in the regulation of transcription remains unclear and needs to be investigated.

The NEFA (DNA binding/EF-hand/acidic, also known as nucleobindin-2) is a 420 amino acid protein. Comparison of the amino acid sequence reveals that it contains a basic amino acid rich putative DNA binding domain (b) with a potential nuclear

targeting signal, two calcium-binding helix-loop-helix (HLH) EF-hand motifs, an acidic amino acid region (a) between the EF-hands and a leucine zipper (Z) motif. Thus, NEFA is characterized by a linked motif b-HLH-a-HLH-Z (Barnikolwatanabe et al., 1994). NEFA shares significant sequence identity (61%) and the same domain structure with the DNA/Ca^{2+}-binding protein nucleobindin-1 (CALNUC) (Miura et al., 1992). The DNA binding activity of NEFA, in contrast to nucleobindin-1, is still unclear, the calcium binding capacity of the purified NEFA protein was confirmed (Kroll et al., 1999). Both NEFA and nucleobindin-1 were shown to interact with the nuclear transcriptional repressor necdin (Taniguchi et al., 2000). Since under hypoxia calcium levels in hepatocytes rise (Brecht et al., 1992) it is likely that the calcium bind then to NEFA and modulate its DNA binding activity which was not investigated yet.

Among the candidats found in the one hybrid assay, ferritin light chain has nucleic acid binding properties. Ferritin is the iron storage protein in liver. Three different types of ferritin were described, the L (light) and two variants of H (heavy) chains, of which the L type is prevalent in rat liver. It was shown that ferritin functioned as a RNA-binding protein with the potency to bind a broad range of different RNA species (Heise et al., 1997). In particular, binding of the L type ferritin to the +2611/+1890 region in the rat PCK-1 mRNA was demonstrated (Heise et al., 1997).

5.4 The first steps in the normoxia-dependent gene regulation

Despite the recent years have brought about a number of important investigations concerning O_2 sensing and O_2–dependent gene expression a complete and detailed view has not been reached (Fig. 36).

The first step in the oxygen signalling is the binding of oxygen to a cellular sensor (Fig. 35). While the nature of the O_2 sensor(s) is not known in detail a number of investigations support a model where a heme containing specific “low output” NADPH oxidase isoform or a cytochrome b-type NAD(P)H oxidoreductase (Gorlach et al., 1993; Fandrey et al., 1994b) have been proposed as candidate oxygen sensors (Bunn et al., 1996). This complex leads to the formation of H_2O_2 in dependence of the pO_2 which in the presence of Fe^{2+} is converted in a Fenton reaction. The generated ROS in form of hydroxyl radicals (OH•) may on the one hand prevent activation of HIF-1 and HIF-1-dependent signaling by providing an oxidizing environment. On the other hand NRE binding factors may be stabelized, transported into the nucleus and bound by NRE´s. O_2 which escapes binding by the heme protein may be additionally used by the asparagine and proline hydroxylases which modify HIF-1α and possibly other transcription factors directly to inhibit cofactor recruitment and to mediate proteasomal degradation, respectively.

Further experiments are needed to determine whether one or some of the proteins found in the one hybrid assay are involved in the regulation of oxygen-dependent gene expression but they may constitute interesting new targets.

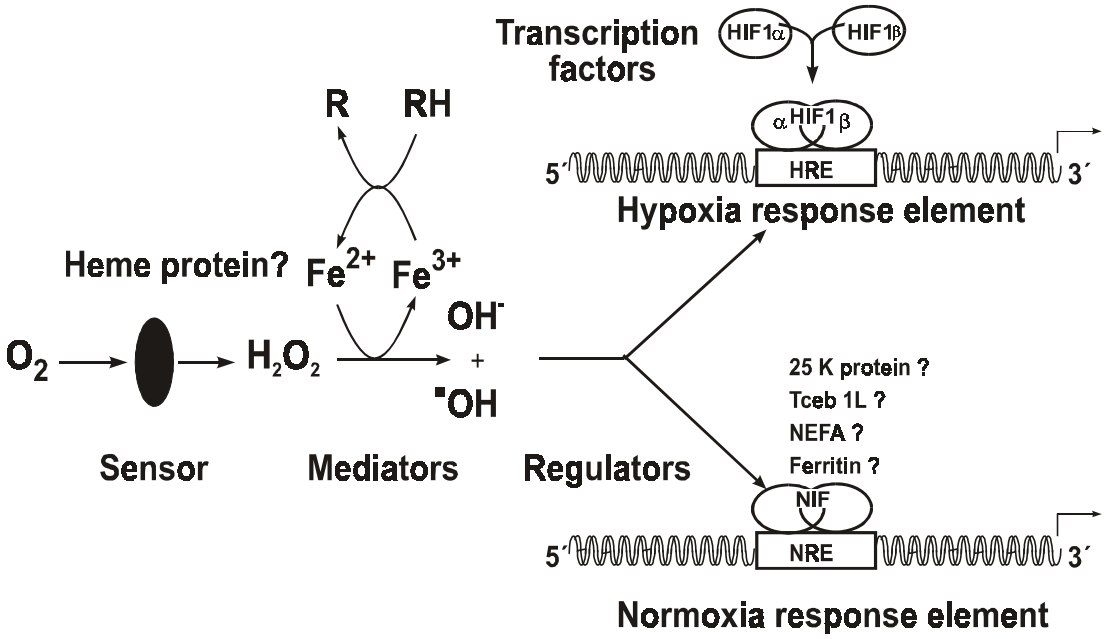


Fig. 36: **Model of the oxygen signalling pathway.** After binding of oxygen to a sensor, production of mediator molecules is enhanced leading to the modification of transcription factors. Oxygen-modulated transcription factors, which may be different for hypoxic and normoxic conditions, bind specific response elements in the promoters of the oxygen-regulated genes. NIF function may be carried out by nuclear protein 25 K, Tceb 1L, NEFA or ferritin. HRE, hypoxia response element; NRE, normoxia response element; HIF, hypoxia inducible factor, NIF, normoxia inducible factor.

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