THE UNIVERSITY OF CALGARY

Thyroid Hormone Regulation of Hepatic S14 Gene Expression

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Thyroid Hormone Regulation of Hepatic Gene Expression" submitted by Benoit J. Deschamps in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

This thesis describes the adaptation of a cell-free in vitro transcription assay used in determining the role of nuclear proteins that bind to the S14 gene DNase I hypersensitive site (HS-1). Previous studies demonstrated the binding of 2 nuclear proteins P-1 and PS-1 to the S14 DNA sequences -310 to -288 and -63 to -48, respectively. We show that PS-1 binding activity plays an important role in augmenting transcription of the 'G'-free cassette (GFC) reporter DNA sequence in nuclear extracts prepared from livers of hyper- but not hypothyroid rats. Deletional studies suggest that P-1 is a repressor of S14 gene transcription. Additional deletion studies revealed the presence of at least two other cis-regulatory elements within -441 to -296 and -278 to -87 sequences. The transcription activity of various S14-GFC constructs was measured in hepatonuclear extracts of the three thyroid states. In extracts obtained from hyperthyroid animals, the transcription activity of the templates was 2.7 fold higher than in extracts from hypothyroid animals. However, direct addition of L-triiodothyronine (T_3) to the extracts did not alter the transcriptional activity of the different constructs. Together these results suggest a novel mechanism of T_3 action that may involve the modification of factors in vivo which remain active in the extracts. We also report that the tissue-specific expression of S14 is due to the absence of nuclear activators in tissues where the gene is not expressed. In conclusion, our results are consistent with a model in which the regulation of S14 gene transcription by T_3 is mediated in part by hormone-induced increases in transcriptional activation of trans-acting factors.

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ABBREVIATIONS

ANOVA	one way analysis of variance
ATP	adenosine 5'-triphosphate
bp	base pairs
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic adenosine-monophosphate
CBF	CCAAT-binding factor
cDNA	complementary DNA
CP1	CCAAT protein 1
CRF	CCAAT-recognition factor
CTF	CCAAT-transcription factor
CTP	cytosine 5'-triphosphate
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylenediaminetetracetate
FAS	fatty acid synthetase
g	gram
GFC	'G'-free cassette
GH	growth hormone
GPDH	α-glucose-6-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
HRE	hormone-responsive element
HS	hypersensitive site
HSP	heat shock protein
kbp	kilobase pairs
kDa	kiloDalton
M	molar
ME	malic enzyme
mg	milligram
MHC	myosin heavy chain
ml	millilitre
mM	millimolar
Mr	relative molecular weight
mRNA	messenger ribonucleic acid
NF-1	nuclear factor I
NFIII	nuclear factor III
OTF	Octamer-binding transcription factor
PCR	polymerase chain reaction
pI	isoelectric focusing point
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
S14	Spot 14
SDM	standard deviation to the mean
SDS	sodium dodecyl sulfate
t-RNA	transfer ribonucleic acid
T_3	L-triiodothyronine

T_4	L-thyroxine
TBE	tris-borate-EDTA
TFIID	transcription factor IID
TK-CAT	thymidine kinase-chloramphenicol acetyl-transferase
TRE	thyroid hormone-responsive element
UTP	uridine 5'-triphosphate
μCi	microcurie
μg	microgram
μl	microlitre

INTRODUCTION

The Thyroid Hormones

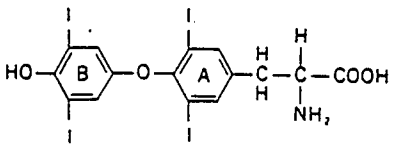
In poikilothermic vertebrate animals the thyroid hormones (L-thyroxine, L-T_4, and L-triiodothyronine, L-T_3) serve critical functions in maintaining body homeostasis. These hormones are synthesized and released by the thyroid gland located in the anterior portion of the neck. Through a series of enzymatic reactions beginning with the iodination of L-tyrosine residues in thyroglobulin, the follicular cells of the thyroid gland synthesize both hormones at a ratio of about 10:1, T_4:T_3, respectively (Reviewed by Di Liegro et al. 1987 and Köhrle et al. 1987). Because of their hydrophobic nature at physiological pH, the thyroid hormones must be bound to carrier proteins following their release from the gland and during transport to the target organs. The 2 major thyroid hormone carriers in blood are thyroxine-binding globulin, which has a 10-fold higher affinity for T_4 than for T_3, and thyroxine-binding prealbumin (Cheung 1985).

The interaction of the thyroid hormones with the target cells involves several events, each of which are believed to be regulated independently. The steps leading to this interaction include: uptake and entry of the hormones in the cells, intracellular deiodination of T_4 to T_3, entry of T_3 into the nucleus, binding of T_3 to specific nuclear receptors, and post-receptor effects of the hormone.

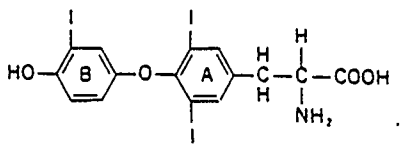
Despite the lipophilic character of the thyroid hormones, evidence indicates that their uptake into the cell is facilitated by a saturable, energy dependent, carrier-mediated transport system in the cell membrane

(Krenning and Docter 1986, Blondeau et al. 1988). It has been suggested that this transport system triggers the entry of amino acids and sugars into the cell via a cAMP-dependent transport mechanism (Reviewed by De Nayer 1987 and Oppenheimer et al. 1987). The interaction of thyroid hormone with the membrane receptors could therefore play an important role in the initial response of the target cells.

T_4 has virtually no intrinsic biological function under normal physiologic conditions. In the target tissues, such as liver and kidneys of both humans and rats, approximately 85% of T_4 is deiodinated at the 5' position of its outer ring by actions of the enzyme 5'-deiodinase.



Thyroxine (3,5,3'5'-tetraiodo-L-thyronine) T_4



Triiodothyronine (3,5,3'-triiodo-L-thyronine) T_3

Actions of this enzyme on L-T_4 yields the active form of the hormone, L-T_3. On a molar basis T_3 is 10 times more potent in terms of biological activity than its precursor (Reviewed by Di Liegro et al. 1987 and Köhrle et al. 1987).

Deiodination of T_4 is localized in the rough endoplasmic reticulum (Reviewed by Köhrle et al. 1987). Following deiodination in the cytosol, T_3 must enter into the nucleus prior to interacting with the chromatin. A stereospecific transport system localized in the nuclear membrane selectively permits the entry of L-T_3 from the cytosol into the nucleus

(Reviewed by De Nayer 1987 and Oppenheimer et al. 1987). For the sake of simplicity and since T_3 has a much higher biological activity than T_4, only T_3 will be used in the remainder of the text.

Although numerous studies have attempted to define the mechanisms by which T_3 modulates gene activity, the precise molecular events remain unknown. Very strong evidence indicates that the binding of T_3 to a nuclear receptor initiates the chain of events leading to the characteristic biological effects on growth, differentiation, and metabolism (Reviewed by Di Liegro 1987, Oppenheimer et al. 1987, Samuels et al. 1988, and De Groot et al. 1989). As early as the mid 1960's, Tata and Widnell (1966) discovered that T_3 administration changes the rate of RNA and protein synthesis in rat liver, thus suggesting that the hormone might somehow control gene expression. Using intact animal and cell culture models, numerous studies have shown that changes in the abundance of proteins by T_3 correlated with similar variations in the corresponding messenger RNAs (mRNAs) (see Reviews for specific references).

The currently accepted model for the nuclear actions of T_3 involves the binding of the hormone to a nuclear receptor. This is followed by the interaction of the hormone-receptor complex with a specific DNA sequence as the first step in modulating expression of T_3-responsive genes. The most compelling evidence supporting this hypothesis comes from studies of the nuclear receptor for T_3. Known characteristics of the receptor include: the nuclear sites have characteristics expected of receptors in general (i.e. high affinity for $T_3[10^{-11}M]$ and low capacity [1 pmole/mg DNA]), the receptor binding characteristics are identical in all tissues and in all species responsive to T_3, there is a close correlation between

nuclear receptor occupancy and the metabolic response (ie., lipogenesis, protein and RNA synthesis), there is a short interval between occupation of the receptor by T_3 and response (ie., increases in specific mRNAs following T_3 administration), analogue studies using compounds that mimick the structure of T_3 (ie., Triac) have T_3-like action, and gene transfer studies demonstrating that the T_3-receptor complex interacts directly with the DNA which flank the 5' end of thyroid hormone responsive genes.

The precise set of events following the binding of the T_3-receptor complex to its cognate DNA sequence, the T_3 responsive element (TRE), and the subsequent effect on RNA polymerase II activity remains unknown. Ichikawa et al. (1988) observed that the binding of T_3 to its receptor altered the chromatographic mobility suggesting a change in the conformation of the complex. They propose that the T_3-induced changes may lead to activation of the receptor. This idea is supported further by results in which occupancy of the receptor by T_3 is not required for binding to DNA (Lavin et al. 1988, Glass et al. 1988). Together these findings suggest that T_3 probably activates the transcriptional role of the receptor rather than its DNA-binding function. The activated receptor-protein complex would alter transcription either by producing an information transfer along the DNA molecule or by allowing the T_3-receptor complex to communicate, through protein-protein interaction, with other transcriptional factors leading to changes in transcriptional activity. Adding to the complexity of the process, Damm et al. (1989) and Sap et al. (1989) demonstrated that in the absence of its ligand, the T_3-receptor (c-erbA oncogene product) can bind the TRE and act as a repressor of transcription. Addition of T_3 abolished the suppression and stimulated

expression of their reporter genes.

The thyroid hormone receptors belong to the family of transcription factors

The differential regulation of gene expression in both eukaryotes and prokaryotes is dependent on complex arrangements of cis-regulatory DNA sequences that are generally located upstream and in close proximity to the transcription initiation site. The cis-regulatory DNA sequences are bound by specific trans-acting (transcription) factors. These transcription factors must interact with each other and RNA polymerase II enzyme to form the active transcription complex. The multitude of factors that form the transcription complex may interact with each other in a variety of combinations, thus giving rise to multiple levels of regulation (ie. if 4 proteins were responsible for regulating a specific gene, each possible combination may result in a different level of expression). The multitude of DNA-protein and protein-protein interactions modulates the activity of transcription initiation thereby providing a reference point in directing RNA polymerase II to the initiation site (Reviewed by Maniatis et al. 1987, Wingender 1989, and Wolffe 1990). In the case of the T_3-receptor complex, it has recently been shown to interact with specific cis-regulatory DNA sequences (Koenig et al. 1987). Therefore, the T_3-receptor belongs to the transcription factor family of proteins. At this stage, a brief review of eukaryotic transcription factors that regulate genes transcribed by RNA polymerase II will bring into perspective the milieu in which T_3 interacts to regulate the expression of specific genes. Due to the space limitation and relevance to the study

of one of the proteins (PS-1) in this project I will concentrate the review in the area of promoter binding proteins.

Many nuclear proteins that bind to specific DNA sequences within the promoter elements of different genes have been well characterized. For example, the "TATA box" with a consensus sequence TATAAA is a well known promoter element in eukaryotic genes (Reviewed by Wingender, 1989 and Breathnach and Chambon, 1981). This motif is present in most eukaryotic promoters and is located approximately 30 base pairs (bp) upstream from the transcription start site. The transcription factor TFII D is the only protein that binds to the "TATA box" (Fire et al. 1984, Sawadogo and Roeder, 1985b). TFII D has recently been purified and characterized (Horikoshi et al. 1990, Hoey et al. 1990). Studies of numerous genes indicate that the "TATA box"-TFII D complex functions to align the RNA polymerase II such that the initiation of transcription is always at the same site (fidelity of transcription). However, the function of the "TATA box-transcription factor complex does not affect significantly the basal level of RNA synthesis (Reviewed by Dynan, 1986).

A second element, the "CCAAT-motif", is present in most eukaryotic promoters (Graves et al. 1986, Myers et al. 1986, Sive et al. 1986). It is generally found in either orientation 70 to 80 bps upstream from the transcription initiation site. Several CCAAT-binding factors have been purified from cultured cells and various tissues. They show different sequence preferences for variants of the CCAAT-motif with each having unique effects on gene transcription. The first two CCAAT-motif binding proteins detected were the C/EBP (CCAAT-binding protein) isolated from rat liver (Graves et al. 1986), and the CTF/NF-1 (CCAAT-transcription factor)

from HeLa cells (Jones et al. 1985, Leegwater et al. 1985). These were soon followed by studies demonstrating the presence of several other members of the same family. For example, the CBF (CCAAT-binding factor) and CP1 (CCAAT protein 1) were isolated from rat liver and HeLa cells, respectively (Chodosh et al. 1988, Maity et al. 1988). CBF and CP1 consist of two subunits and both are required for interaction with the DNA and transcription activity. The CRF (CCAAT-recognition factor) isolated from HeLa cells, although different from CBP and CTF/NF-1, binds to the 3 CCAAT-motifs found in the Adenovirus-2 EIIa-late promoter. The TGGCA binding proteins, isolated from chicken liver, bind to a CCAAT-motif in the 3'-5' orientation and are similar to the CTF/NF1 factors. In view of such a large family of factors that bind to the CCAAT-motif, it raises the possibility that these proteins may provide another level of complexity in the regulation of gene expression. Similar to TF11D, their overall function is essential for maintaining basal promoter activity.

A third group, the so called "octamer binding transcription factors" (OTF), binds to the consensus sequence ATGCAAAT. This sequence has been found in either orientation in the promoter and/or enhancer regions of many genes. The OTFs detected and characterized so far appear to be very closely related to each other in their function as well as in their structure. They activate the expression of specific genes and control subtle processes such as cell-specific and stage-dependent gene expression. Three members of this group have been characterized extensively. OTF-1 (Octamer transcription factor 1) present in most cells is believed to be involved in cell-specific gene expression and functions as a general transcription factor. Nuclear factor III (NFIII) is most

likely identical to OTF-1 and interacts with variants of the octamer motifs present in 5′ region of genes such as histone H2B, immunoglobulin light and heavy chain, U2 snRNA, SV40 enhancer and the Ad_4 origin of replication (Pruijn et al. 1987). The OTF-2 is also probably involved in cell-specific gene expression (Miwa and Strominger, 1987). Upon cloning of its cDNA, the human OTF-2 was shown to contain 4 leucine residues spaced in a fashion identical to that seen with "leucine zippers". The Leucine zipper is believed to be involved with protein-protein interactions (Müller et al. 1988, Scheidereit et al. 1988).

Another cis-acting element often detected in the promoter region of eukaryotic genes is the GC-rich box. The hexanucleotide consensus sequence GGGCGG appears functional in either orientation, and usually present in multiple copies. Unlike the CCAAT-motif, where the binding of different factors is involved for regulation, the Sp1 protein appears to be the only factor that interacts directly with the GC rich sequence. Because the binding affinity of Sp1 to variants of the GC-box sequence may differ by a factor of 10- to 20-fold, the regulation provided by this factor seems to depend solely on its ability to bind DNA.

The mRNA encoding Sp1 has been cloned and sequenced. The deduced amino acid sequence of the Sp1 factor revealed that it belongs to the "Zinc-finger" family of DNA binding proteins (Rhodes and Klug 1986). This was later substantiated by the finding that Sp1 requires Zn^{2+} for specific DNA sequence recognition (Kadonaga et al. 1987).

The "finger motif" model for the DNA binding domain was first identified in the eukaryotic transcription factor TFIIIA (Miller et al. 1985). This nuclear protein is required for efficient transcription of

the gene coding 5S ribosomal RNA. The amino acid sequence of TFIIIA contains a conserved region of cysteine and histidine residues in fixed positions. The combination of the cysteine-histidine repetition pattern, presence of zinc in the protein and x-ray crystallographic studies, led to a model where the structure of TFIIIA forms 9 "finger"-like projections (Miller et al. 1985). Each projection, also termed C-H fingers for cysteine-histidine combination, contains two cysteine and two histidine residues forming a tetrad centered around a zinc ion. The tip of the finger contains a large proportion of basic amino acids responsible for protein-DNA interaction within the major groove of its recognition site. A similar finger structure has been detected in members of the steroid and thyroid hormone receptor superfamily. In this group, however, the two histidine residues are replaced by two cysteine residues in the finger motifs. This type of zinc-finger is therefore referred to as the "C-C-finger" type.

The receptors for steroid hormones (Reviewed by Evans 1988, Beato 1989, and Sluyser 1990) are located in the cytoplasm, complexed to a cytosolic protein, HSP-90. Upon binding with its specific ligand, the receptor forms an active complex with enhanced affinity for chromatin. The receptor dissociates from HSP-90, then translocates into the nucleus and binds to a specific DNA sequence, referred to as the hormone-responsive element (HRE). The interaction of the hormone-receptor complex with the HRE changes transcription of specific genes thereby increasing or decreasing mRNA and protein levels. These modulations ultimately lead to the phenotypic changes characteristic of the hormone.

The steroid HREs display a dyad of symmetry. The significance being

that the receptor is believed to form a dimer prior to interaction with the DNA (Tsai et al. 1988, Reviewed by Beato, 1989). HREs show enhancer-like characteristics in that they are relatively position and orientation independent. In this regard, they can be qualified as hormone-dependent enhancers (Martinez et al. 1987).

The cDNAs encoding the glucocorticoid, progesterone, aldosterone, androgen, estrogen hormone, vitamin D, retinoic acid (vitamin A), and dioxin receptors have been isolated (see Reviews for specific references). The analysis and comparison of their structure, supported by a series of deletion mutant studies (Kumar et al. 1986), allowed for the localization of different domains in the receptor proteins (A to E). The most conserved domain, the DNA-binding region (C), contains 2 putative Zinc-fingers of the C-C type and is located closer to the N-terminal of the protein (Krust et al. 1986, Kumar et al. 1986). A second more hydrophobic region is believed to be the hormone-binding domain (E). It is less homologous than the DNA-binding domain and is located at the carboxy terminus of the receptor protein. A third domain A/B, located within the amino-terminal, displays a variable degree of homology amongst the various receptors and appears to be essential for efficient receptor mediated transcriptional activation of several promoters by the receptor. It was also suggested that this region partially controls specificity of gene activation (Tora et al. 1988). Lastly, a hydrophobic and variable region, the D-region, is thought to act as a hinge between the DNA-binding and hormone-binding domains. Evidence shows that the ligand-binding domain, in absence of hormone, inhibits interaction of the receptor with DNA. Upon ligand binding, the activated hormone receptor complex interacts with

the hormone responsive element and probably facilitates binding of other transcriptional factors leading to productive initiation of transcription (De Groot et al. 1989 and references therein).

The thyroid hormone receptor

The native T_3-receptor has not, as yet, been purified to an extent that would allow direct protein analysis and functional studies. The best attempt at purifying yielded a protein mixture containing at best 5% to 7% pure T_3-receptors (Ichikawa and De Groot 1987). The difficulty in purifying the receptor is due to its low abundance (5000 T_3-receptor/cell compared to 60,000 glucocorticoid receptors/cell in rat liver [see Apriletti et al. 1988 for specific references]) and its instability, particularly after partial purification and in dilute protein solutions (Apriletti et al. 1988). Recent studies using photoaffinity labelling or derivatives of T_3 and T_4 raised the possibility of two nuclear T_3-receptors having relative molecular weights (Mr) of approximately 47 and 57 kDa. The relationship between these 2 species of receptors is unclear and could represent either 2 different receptors or 2 variations of the same protein (see Openheimer et al. 1987 for specific references).

More recently, the T_3-receptor was shown to be related to the avian erythroblastosis virus, v-erb A gene product (Reviewed by Sluyser 1990 and De Groot et al. 1989). Following the cloning of steroid hormone receptor cDNAs, the deducted amino acid sequence of their putative DNA-binding domain had a high homology (45%) with the cysteine-rich region of v-erb A. The protein products obtained from the in vitro translation of the cellular homologue of the v-erb A cDNA, c-erb A, were localized in the

nucleus and bound T_4, T_3 and T_3 analogs (both in vitro and in vivo). The relative affinity of hormone binding to c-erb A was the same as that observed for the native T_3 nuclear receptor. Subsequently, c-erbA cDNAs have been cloned from different human and animal tissues and relative molecular weight of corresponding protein products ranges from 45 to 55 kDa. These weights matched closely with those of the native T_3-receptors. Together, these characteristics suggest strongly that the c-erbA gene products act as T_3 receptors.

Multiple forms of T_3-receptors adds to the complexity of the mechanism by which T_3 regulates gene expression. There is evidence to suggest that different forms of the receptor are expressed in a tissue-specific fashion and compete for the TRE and/or T_3. These multiple forms of the receptor may also be involved in controlling different classes of genes. Since the receptors form a dimer prior to interaction with TRE, it is possible that the formation of heterodimers may be involved in controlling gene transcription. Studies in various rat tissues provide limited data supporting the tissue-specific expression of the individual T_3-receptors. In this regard, Hodin et al. (1989) showed that one of the c-erb-A protooncogenes, c-erbA β_2 is expressed only in the pituitary and the c-erb A α_1 form is dominant in the liver. The same group of investigators (Hodin et al. 1990) reported a marked tissue-specific expression and differential regulation of 4 c-erbA mRNAs by T_3.

Gene models of thyroid hormone action

Attempts to define the mechanisms by which T_3 regulates gene expression have focused mainly on the studies of four model genes.

Included in this list are the growth hormone (GH) gene in pituitary, the α- and β-myosin heavy chain genes in the heart, and the malic enzyme and Spot 14 (S14) gene in rat liver. Obviously, these genes are not influenced solely by T_3 but are used as models because their protein and mRNA levels show marked changes in response to the thyroid states.

The Growth Hormone Gene

The induction of GH by T_3 has been studied extensively in the rat anterior-pituitary in vivo and in vitro using cultured rat pituitary cell lines (Reviewed by Samuels et al. 1988 and De Groot et al. 1989). T_3 increases GH protein and its mRNA in both animal and cultured cell lines (Hervas et al. 1975, Yaffe and Samuels 1984). The increase in GH mRNA was detected within 2 to 3 hours following the addition of T_3 to cultured cells. The maximum level of increase reached 10- to 20-fold above baseline after 48 to 72 hours of exposure to the hormone. The rate of GH gene transcription increased within minutes after the administration of T_3 to hypothyroid rats, or to cultured cells. The increase in the rate of transcription correlated with the number of nuclear receptors bound by T_3 and the increase preceded the rise in GH mRNA. Together, these observations support the idea that the T_3-receptor complex directly increases transcription of the GH gene with little, if any, alteration in the GH mRNA stability (Yaffe and Samuels, 1984).

In attempts to locate the precise site of T_3-receptor complex interaction with the GH gene, different groups of investigators have conducted stable and transient transfection studies in cultured cell lines. Studies examined chimeric plasmids in which sequences flanking the

5′ region of the GH gene were linked to a reporter gene. Although some ambiguity still exists on its exact location, the TRE is believed to be situated between -209 and -146 relative to the transcription initiation site. The strongest evidence for the TRE being located within the -209 to -146 DNA sequence comes from studies by Koenig et al. (1987). They reported that partially purified T_3-receptors bind specifically within this sequence. In addition, the results of Glass et al. (1987) demonstrated that an oligonucleotide containing the -209 to -146 sequence conferred T_3 regulation to an heterologous promoter. More recently Brent et al. (1989) reported that the sequence -190 to -161 was essential for enhancing T_3-responsiveness of the wild type rat GH gene when co-transfected with a c-erb-A expression vector. The direct interaction of the T_3-receptor with a putative TRE located within the -209 to -146 DNA sequence strengthens the notion that GH gene expression is directly modulated by the T_3-receptor complex rather than by secondary gene products responsive to T_3.

The α- and β-Myosin Heavy Chain genes

The cardiac myosin heavy chain (MHC) isoforms are products of 2 highly homologous genes α and β which are linked in tandem and separated by 4.0 kbps of intergenic sequences (Mahdavi et al. 1984, Saez et al. 1987). The inverse manner with which these 2 genes are regulated by T_3 provides a particularly interesting system for studying the regulation of gene expression. In the heart ventricle and in cardiac myocytes, T_3 induces an increase in the level of α-MHC mRNA by about 5-fold but decreases the level of β-MHC mRNA approximately 65%. Both changes occur within 48 hours following the administration of T_3 to hypothyroid rats or

untreated cultured cells (Lompré et al. 1984, Gustafson et al. 1986, and Green et al. 1989). Gustafson et al. (1987) observed that the addition of T_3 to fetal rat heart myocyte cultures caused a 10- to 15-fold increase in α-MHC mRNA relative to the untreated cells within 3 to 4 hours. Lompré et al. (1984) reported that the half-life of both α- and β-MHC mRNAs was not altered by T_3. In addition nuclear run-off studies demonstrated that changes in α- and β-MHC mRNAs correlated with differences in transcriptional activity. These results confirm that the changes in α- and β-MHC mRNAs were due to a direct effect of T_3 on gene transcription. Furthermore, two groups of researchers (Izumo and Mahdavi 1988, Tsika et al. 1990) using either binding and deletional studies or transient transfection studies located a stimulatory TRE sequence highly homologous to the rat GH TRE between -153 and -140 of the rat α-MHC gene. Similar to the rat growth hormone gene, T_3 modulates α- and β-MHC genes expression through binding of the T_3-receptor complex directly to the TRE(s) located in DNA flanking the 5′ end of both genes.

The Malic Enzyme gene

Malic enzyme (ME) is another model used commonly in studying the mechanisms of thyroid hormone action. ME plays a critical role in supplying the bulk of NADPH required for de novo synthesis of fatty acids. T_3-regulation of ME gene expression is limited to the liver, heart and kidney (Dozin et al. 1985, 1986b). Northern blot analysis of hepatic RNA (Dozin et al. 1985) revealed 2 mRNAs of different sizes encoding the ME protein. Strait et al. (1989) reported that the two mRNAs were due to alternate sites of polyadenylation. Kinetic studies demonstrated a lag

time of approximately 2 hours following T_3 administration to hypothyroid rats preceding a detectable rise in ME mRNA (Towle et al. 1980, 1981). The level of hepatic ME mRNA reaches its maximum, 14-fold higher than in the hypothyroid rat between 36 and 48 hours after T_3 administration (Dozin et al. 1985, Strait et al. 1989). In vitro run-off assays revealed only a 3- to 5- fold increase in the rate of transcription of the hepatic ME gene in the transition from hypothyroid to hyperthyroid state (Dozin et al. 1986b). The possibility that post-transcriptional events contribute to the increase in ME mRNA was supported by the demonstration that T_3 stabilizes both the nuclear precursor (Song et al. 1988) and the cytoplasmic ME mRNA (Dozin et al. 1986b).

The ME gene responds synergistically to carbohydrates and T3 both in vivo and in vitro with corresponding increases in the cellular concentration of ME mRNA (Reviewed by Mariash and Oppenheimer, 1983). However, Dozin et al. (1986a) demonstrated that the dietary carbohydrates caused an increase in the ME mRNA level without affecting the transcriptional rate or stability of the nuclear precursor RNA. These findings suggest that carbohydrates prevent the degradation of the cytoplasmic ME mRNA. Taken together, these results suggest that both T_3 and carbohydrates act on different mechanisms in their synergistic effect on hepatic ME mRNA level. T_3-stimulates both ME gene transcription and stabilization of its nuclear precursor RNA. Carbohydrates act predominantly by stabilizing the cytoplasmic ME mRNA. Therefore, the effect of T_3 on ME gene activity appears to be regulated in 3 steps: gene transcription, stabilization of nuclear RNA, and stabilization cytoplasmic RNA.

The Spot 14 gene

In the past several years, we have focused our studies on a T_3 responsive mRNA encoding a protein, referred to as Spot 14 (S14). This protein was first detected by 2-dimensional gel electrophoresis of in vitro translation products directed by total hepatic RNA from rats of the 3 thyroid states: hypothyroid, euthyroid, and hyperthyroid. Using this technique it was possible to resolve approximately 250 different ^{35}S-radiolabelled spots, each of which represented a specific mRNA. Of the 11 sequences stimulated by T_3, the translational product designated S14 increased by 10- to 20-fold within 4 hours after administration of T_3 to thyroidectomized rats (Seelig et al. 1981). By virtue of its rapid response and magnitude of induction by T_3, S14 mRNA appeared to be an ideal model for studying the mechanisms of thyroid hormone action.

S14 gene, mRNA, and protein

S14 is a simple gene measuring 4.5 kb in length, consisting of 2 exons (494 and 820 bps) with an intervening sequence of 3.2 kbps (Liaw and Towle 1984). The 3' end of the gene contains 2 polyadenylation sites which give rise to 2 mRNA species of different size (1350 and 1550 bases). The region encoding the protein is contained entirely within the first exon. Both mRNA species are coordinately regulated in response to the following physiological conditions: thyroid state, tissue specificity, diurnal variation, development, and dietary manipulations (Liaw and Towle 1984, Jump et al. 1984). S14 mRNA codes for a cytosolic (Kinlaw et al. 1989) protein containing 150 amino acids with a Mr of 17,010 and an

isoelectric focusing point (pI) of 4.9 (Liaw and Towle 1984). Comparison of the mRNA nucleotide sequence and the deduced amino acid sequence of the protein with nucleic acid and peptide sequences stored in the national and international data banks have failed to reveal any significant homologies to S14 (Liaw and Towle 1984).

The S14 protein is involved in some aspect of lipogenesis

Despite the detailed information gathered about its structure and regulation, the function of the S14 gene protein still remains to be defined. Several lines of evidence, however, suggest that the function of S14 is associated with some aspect of lipogenesis. The S14 gene is expressed only in lipogenic tissues (Jump and Oppenheimer, 1985). As for other lipogenic enzymes' mRNAs, S14 mRNA is synergistically regulated both by T_3 and carbohydrates (Jump et al. 1984 and references therein). The appearance of the S14 mRNA parallels the increase in activity of lipogenic enzymes such as GPDH, ME, and FAS in neonatal rats (Perez-Castillo 1987). The levels of S14 mRNA and S14 protein exhibit a diurnal variation identical to that of hepatic lipogenesis (Jump et al. 1984, Kinlaw et al. 1989). Finally, Freake and Oppenheimer (1987) observed that the level of S14 mRNA correlated with increased lipogenesis in brown adipose tissues of the rat in response to diverse stimuli such as hypothyroidism, chronic cold exposure and feeding of a "cafeteria" diet.

S14 gene regulation

S14 gene expression is controlled by a variety of mechanisms. Compared to the hypothyroid values, the steady state hepatic S14 mRNA

level is 4- to 5- and 15- to 20-fold higher in euthyroid and hyperthyroid animals respectively. Therefore, the S14 mRNA shows a non-linear (amplified) response to T_3-receptor occupancy by its ligand (Oppenheimer et al. 1977, Jump et al. 1984). This amplified response is defined as a greater fold-increase(s) in the level of S14 mRNA level when receptor occupancy rises from 50% to >95% (euthyroid to hyperthyroid state) as compared to the fold-increase(s) observed during the 5 - 50% receptor occupancy with transition from hypothyroid to euthyroid state.

The expression and regulation of S14 mRNA are tissue specific. S14 mRNA is highly expressed and responsive to T_3 in lipogenic tissues such as: liver, epididymal fat, brown fat and lactating mammary gland. S14 mRNA is almost undetectable in brain, heart, spleen, kidney, lung, testis, and pituitary with measured levels of 6.3%, 0.5%, 0.3%, 0.9%, 2.0% 0.07%, and <0.07% relative to euthyroid liver, respectively. In extra-lipogenic tissues S14 mRNA does not respond to T_3 (Jump and Oppenheimer 1985). This is remarkable, especially in the kidney where the nuclear T_3 receptor level is comparable to that of the liver (Oppenheimer et al. 1974). These findings indicate that tissue-specific factors are important in modulating both basal and T_3 regulated expression of S14 mRNA.

Rat hepatic S14 mRNA undergoes a circadian variation, fluctuating by approximately 3-fold between the low value at 8:00h and the maximal value at 20:00h in unmanipulated animals (Jump et al. 1984, Kinlaw et al. 1987). The diurnal variation was also observed at the protein level using antibodies to peptides in the S14 protein (Kinlaw et al. 1989). The rat hepatic mRNA S14 is regulated by developmental factors as indicated by the 186-fold increase from 15 to 30 days of age. During this time period

weaning occurs (Jump et al. 1986). The level of hepatic S14 is also increased by carbohydrates (Liaw et al. 1983). Mariash et al. (1986) reported that T_3 and carbohydrates interact synergistically to increase the hepatic levels of S14 mRNA. Sucrose administration to euthyroid rats increased the level of hepatic S14 mRNA 2-fold within 30 minutes and 25-fold by 4 hours post-treatment.

T_3 modulates S14 gene expression at the transcriptional level

Administration of a dose of T_3 sufficient to saturate the receptors in thyroidectomized rats led to an increase in the abundance of the hepatonuclear precursor and mature S14 mRNA within 10 minutes (Narayan et al. 1984) and 20 minutes (Jump et al. 1984), respectively. Such a rapid response suggested that the induction of the S14 mRNA was a direct effect of T_3 and not mediated by a secondary T_3-stimulated gene product. Furthermore, the fact that increases in S14 nuclear precursor preceded the rise in mature mRNA suggested that T_3 acts, at least in part, at the nuclear level to modulate production of S14 mRNA (Narayan et al. 1984).

Using a nuclear run-off transcriptional assay, both Narayan and Towle (1985) and Jump et al. (1986) examined S14 gene transcription in various thyroid states and in response to developmental factors, respectively. In both cases the in vitro transcription rate of the hepatic S14 gene did not account completely for observed increases in the level of S14 mRNA. Since the rise in the S14 nuclear precursor RNA occurred prior to increases in the mature S14 RNA, this suggested that T_3 regulates S14 gene expression by stabilization of the nuclear precursor.

However, subsequent experiments using a modified version of the in

vitro run off assay demonstrated that increases or decreases in S14 mRNA correlated closely with changes in the rate of S14 gene transcription. Kinlaw et al. (1986 and 1988) reported that glucagon injected into euthyroid animals during the evening plateau in S14 mRNA caused a sharp diminution in the rate of S14 transcription, a decrease that was sufficient to account for the observed 90% decline in S14 mRNA. T_3 administration reversed within 5 minutes the effects of glucagon on S14 transcription without affecting the apparent nuclear precursor RNA stability. Moreover, Jump et al. (1988) measured a 40-fold increase in transcriptional activity associated with a 100-fold accummulation in S14 mRNA during the weaning period of neonatal rats. Thus the current belief is that the abundance of hepatic S14 mRNA in response to glucagon and developmental factors is regulated at the level of gene transcription.

More recently the induction of S14 mRNA by carbohydrate feeding (Shane Hamblin et al. 1989, Jump et al. 1990) was examined using the modified in vitro run-off assay. Results now demonstrate that parallel changes in S14 mRNA and transcription of the gene probably account for the increase in S14 gene expression. Similar studies were also conducted in animals of various thyroid states (Jump 1989, Jump et al. 1990). A rapid induction in S14 gene transcription was observed within 5 minutes following injection of hypothyroid rats with a receptor saturating dose of T_3. The S14 transcription was augmented 3.8-fold within 15 minutes and reached 70% of the maximum 9-fold induction within 60 minutes, paralleling the increase in hepatic S14 mRNA levels (S14 mRNA increased 2.4, 19, and 24 fold relative to the hypothyroid level within 15 min, 4 hours, and 24 hours, respectively). Thus, the current thinking is that the variations

in hepatic S14 mRNA are due, predominantly, to a rapid effect of T_3 on S14 gene transcription.

A rapidly turning over protein(s) is essential for S14 gene expression

Even if the S14 transcriptional activity increases very rapidly after T_3 administration, it has been shown that ongoing protein synthesis is essential during the initial induction of S14 mRNA by T_3. Studies by Seelig et al. (1982) and Jacoby et al. (1987) have shown that the T_3-induction of S14 mRNA can be prevented by the administration of the protein synthesis inhibitors cycloheximide or emetine 30 minutes prior to the hormone stimulated increase in S14 mRNA. The injection of either one of the inhibitors also caused a rapid disappearance of S14 mRNA when S14 nuclear precursor RNA was highly induced by T_3 (Jacoby et al. 1987). Therefore, this suggests that a very rapidly turning over protein or proteins may be necessary for both the induction and maintenance of the S14 mRNA expression.

The S14 chromatin structure

Jump et al. (1987 and 1988) examined the tissue specific expression of S14 mRNA by comparing the structure of active versus inactive S14 chromatin in different tissues. At least 6 DNase I hypersensitive sites, HS-1, 2, 3', 3, 4, and 5 located at positions -65 to -265, -1.3, -2.1, -2.7, -5.3, and -6.2, respectively relative to the transcription initiation site were identified. The HS-1 site was present only in tissues which expressed S14 mRNA at high levels and where S14 mRNA levels were regulated by T_3. Although not affected by T_3, the DNase I hypersensitivity around

the HS-1 site abruptly increased during the weaning interval correlating with increases in S14 gene activity. Together with its close proximity to the transcription initiation site, these results suggest that nuclear proteins associated with the HS-1 region of S14 chromatin must play an important role in regulating S14 gene expression.

Another DNase I hypersensitive site, HS-3 also showed interesting changes with development (Jump et al. 1988). In contrast to the abrupt changes of HS-1, sensitivity of HS-3 to DNase I increased progressively with age during the weaning period. HS-3 was the only site affected significantly by changes in thyroid status (Jump 1989). The HS-3 site appeared in S14 chromatin within 5 minutes following administration to hypothyroid rats. This event preceded the induction of S14 gene transcription. A comparison between sequences in the vicinity of the HS-3 site with nucleotides -200 to -160 of the rat GH gene showed a region with significant homology (77%) to the GH TRE. The region of highest homology corresponded with the site of binding for the T_3 receptor in the GH gene.

```
S14:-2647  AATTCACATGATGTTCTGGCCAGGCCCTTGACCCCAGTTCC -2607
                     : : :  :  ::::  : ::::: :::
rGH:-200   CGGCGGTGGAAAGGTAAGATCAGGGACGTGACCGCAGGAGA -160
```

These findings suggest strongly that the T_3 receptor may bind to the putative TRE in the HS-3 and not the HS-1 site.

The P-1 and PS-1 proteins

The crucial position of the HS-1 site within the S14 chromatin and its unique presence in tissues where S14 mRNA in abundantly expressed and regulatd by T_3 prompted us to identify the proteins that bind to this site.

The first protein extracted from rat hepatic nuclei, P-1 binds to and protects nucleotides -310 to -288 (AAAAGAGCTATTGATTGCCTGCA) upstream from the S14 gene transcription initiation site (Wong et al. 1989). The P-1 protein appears to be tissue specific and is found only in rat liver. P-1 binding activity correlated with the thyroidal state of the animal and increased approximately 8-fold during the transition from hypothyroid to hyperthyroid state. Its binding activity also increased 3- to 4-fold in the evening correlating with the circadian variation of hepatic S14 mRNA. Since both P-1 binding activity and circadian variation was present only in the liver, it was suggested that P-1 may modulate the circadian rhythm observed in hepatic S14 gene expression. Cycloheximide failed to abolish the binding activity of this protein and a time course study indicated a slow induction of P-1 binding activity by T_3 that followed the rise in S14 mRNA. These results argue against P-1 being the rapidly turning over protein involved in the induction of the S14 gene, and the precise function of the protein remained unknown.

PS-1, a second protein extracted from rat hepatic nuclei was reported to bind and protect the DNA sequence -63 to -48 (TTGGCGTCCTGTCAAT) relative to the S14 transcription initiation site (Wong et al. 1990). The similarity of the sequence of PS-1 and other CCAAT-motifs suggests that PS-1 binding protein(s) may belong to the family of CCAAT-transcription factors. Examination of the PS-1 binding activity indicated the formation of at least 3 protein-DNA complexes. The binding activity of the PS-1 binding protein or proteins increased 4-fold during the transition state from hypothyroid to hyperthyroid transition, again correlating with T_3-regulated expression of the S14 mRNA.

The aims of this study

The rapid effects of T_3 on hepatic S14 gene activity provides an excellent model for studying nuclear actions of T_3. Recent evidence suggests the gene is an ideal candidate for studying the molecular mechanisms by which T_3 modulates gene transcription. Furthermore, the hormonal regulation of the S14 gene expression is a convenient model because one can turn the gene on or off by simply adding or removing T_3, respectively.

The precise role of the P-1 and PS-1 proteins in regulating S14 gene expression remains to be defined. Accordingly, I have established a cell-free in vitro transcription assay to examine the role of P-1 and PS-1 in S14 gene expression. In addition I have used these findings to gain further insights into the mechanisms by which T_3 regulates gene activity.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats weighing 150 to 175 grams were obtained from the Charles-River Breeding Company, St-Constance, Quebec. Animals were maintained on a 12 hour light, 12 hour dark cycle. They were fed ad libitum either a standard rat chow (Purina) or, where indicated, a high carbohydrate fat-free diet (ICN Biochemicals) was given for 7 days. Euthyroid animals were rendered hypothyroid by addition of 0.025% (weight/volume) methimazole (Aldrich) to the drinking water for a minimum period of 3 weeks (Cooper et al. 1984). The presence of hypothyroidism was confirmed by RIA measurement of the total T_3 in serum (Foothills Hospital RIA Laboratory). Euthyroid rats were rendered acutely hyperthyroid by 2 intraperitoneal injections of a receptor saturating dose (200 μg/100 g of body weight) of T_3 (Sigma) dissolved in an alkaline solution (0.1N NaOH) at 24 hours intervals. Doses of T_3 administered were designated to maintain ≥95% receptor saturation for the duration of the experiment (Oppenheimer et al. 1977).

Plasmid constructions

The DNA fragment containing the HS-1 site extending from -441 to -2 position of the S14 gene was synthesized using the polymerase chain reaction (PCR) (Saiki et al. 1988). This DNA fragment was ligated into the Sac I cut blunt-ended $p(C_2AT)_{19}$ vector developed by Sawadogo and Roeder (1985) and referred to as pS14-GFC(1). The $p(C_2AT)_{19}$ plasmid contains a 377-bp DNA fragment 3′ to the Sac I site referred to as the "G-free

cassette" (GFC) and contains no G residues in the sense strand. Both orientations [pS14-GFC(1) and pS14-GFC(1R)] of the S14 fragment relative to the GFC were isolated. The pS14-GFC(6) construct containing the nucleotides -86 to -2 was obtained by subcloning a Hae III restriction fragment of pS14-GFC(1) into the Sma I site of pTZ 19R vector (Pharmacia). The pS14-GFC(2) construct containing the nucleotide -293 to -2 was obtained by subcloning the Pst I restriction fragment of pS14-GFC(1) into the Pst I site of the pTZ 19R vector. The pS14-GFC(2)-Rsa I construct containing the nucleotides -279 to -2 was obtained by digesting pS14-GFC(2) with the restriction enzyme Rsa I and inserting this fragment into the Sma I site of the pTZ 18R vector (Pharmacia). The pS14-GFC(3A) construct containing the nucleotides -1100 to -2 was obtained by ligating the Pst I fragment of the S14 gene extending from -1100 to -293 into the Pst I site of the pS14-GFC(2) construct. The pS14-GFC(5A) construct containing the internal deletion from -1100 to -293 of the S14 gene was obtained by inserting a Pst I fragment of the S14 gene extending from -1635 to -1100 into the Pst I site 5′ to the pS14-GFC(2) construct. The pS14-GFC(316) construct containing the nucleotides -316 to -2 was obtained by using a 30 bp oligomer extending from -316 to -287 (Regional Synthesis Laboratory of the University of Calgary) of the S14 gene and the universal primer (Regional Synthesis Laboratory, University of Calgary) as primers for the PCR with the pS14-GFC(1) construct as the template. The DNA fragment containing the -316 to -2 DNA sequence attached to the 5′ end of the GFC was subcloned into the Sma I site of the pTZ 18R vector. The pS14-GFC(288) construct containing the nucleotides -288 to -2 was detected as a product of the PCR reaction that yielded the construct pS14-GFC(316).

The pP1B#3 construct was obtained by ligating a trimer of the synthetic oligonucleotide -71 to -42 (Regional Synthesis Laboratory, University of Calgary) of the S14 gene into the Bam HI site of the pTZ 18R vector. The construct pP1B#3-GFC(2) was obtained by inserting the pS14-GFC(2) construct into the Pst I site of pP1B#3. The pS14-GFC(1)-TRE was obtained by ligating two copies of a dimer of the TRE, TCAGGTCATGACCTGATCAGGTCATGACCTGA reported by Glass et al. (1988) (synthesized by the Regional Synthesis Laboratory, University of Calgary) into the EcoRI site of the pS14-GFC(1) construct. The construct pS14-GFC(1)-TRE mutant was detected during sequencing. The mutant contains one copy of the T_3 response element dimer missing the underlined nucleotides.

All other basic molecular biology manipulations were performed according to the procedures outlined by Maniatis et al. (1982). The plasmid constructs were confirmed by DNA sequence using the dideoxynucleotide chain termination method described by Sanger et al. (1977), [α-^{35}S]-dATP (Amersham) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals) were used according to the manufacturer's suggested protocol.

Nuclear extract preparation

Nuclear extracts were prepared essentially as described by Gorski et al. (1986). Animals were killed under light anesthesia between 09:00 h and 11:00 h, or at 19:00 h where indicated, to minimize the diurnal effect on S14 gene expression. Tissues of interest were quickly removed, chilled in ice cold saline (0.9% NaCl), and weighed prior to extraction of the nuclei. All of the following manipulations were performed in the

cold or on ice.

All solutions were cooled to ≤4°C. Freshly isolated tissues (7 to 10 g) were minced with scissors in approximately 10 ml of homogenization buffer [10 mM Hepes (pH 7.6), 15 mM KCl, 0.15 mM Spermine (Sigma), 0.5 mM Spermidine (Sigma), 1 mM EDTA, 2.2 M sucrose, and 5% glycerol]. The homogenization buffer also contained dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and Trasylol (Miles) at final concentrations of 0.5 mM, 0.5 mM, and 0.01%, respectively added just prior to use.

For hand homogenization, tissues (7 to 10 g of liver) were homogenized in 85 ml of homogenization buffer in a motor driven teflon-glass homogenizer (Caframo, stirrer type RZR50) with one stroke at the #1 speed and 2 strokes at the #2 position. For blender homogenization, tissues (10 to 30 g) were homogenized in 180 ml of homogenization buffer in a Sunbeam Osterizer #10 (adapted to cell-fractionation procedures by the Technical Services Department of the Health Sciences Centre in Calgary), according to specifications reported by Lichtsteiner et al. (1987) at the "stir" speed for 10 seconds and twice at the "mix" speed for 10 seconds. The homogenate was layered on top of 10 ml cushions, (2 M sucrose, 11.25% glycerol in the same cocktail as the homogenization buffer) in 37 ml Beckman ultra-clear centrifuge tubes and centrifuged at 24,000 rpm for 60 minutes at 0°C using a precooled SW 28 rotor and a Beckman L8-80 ultracentrifuge to sediment the nuclei.

7 to 10 grams of liver, approximately 2 grams of spleen, and 5 grams of testis were homogenized per 60 ml of homogenization buffer. For spleen tissues the nuclear extracts were prepared as described except that 0.5%

non-fat dry milk (Carnation, an aqueous stock solution containing 1 g/10 ml was centrifuged at 4,200 rpm using a TY ds4.2 rotor and Beckman J-6B centrifuge and 1 volume of the supernatant was added to 19 volumes of buffer), along with 100 μM benzamidine, leupeptin (1 μg/ml) and pepstatin A (1 μg/ml) were added to the homogenization buffer (Maire et al. 1989).

After the centrifugation, the solid disc on top of the tubes was removed with a spatula. The supernatant was aspirated carefully and the tubes inverted, rinsed with double distilled H_20 (ddH_20) being careful not to touch the nuclei pellet and excess liquid on the walls of the tube were wiped with gauze. At this stage, if the center of the nuclei pellet contained bits of tissue as commonly seen in preparations from hypothyroid rat liver, these impurities were removed manually by aspiration. The activity of protein extract was greatly enhanced by removing all the red material from the pellet and assuming 50% loss of material for further calculations. The pelleted nuclei was resuspended in nuclear lysis buffer using 2 ml/gram of initial or corrected tissue weight. Nuclear lysis buffer [10 mM Hepes (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 M EDTA and 10% glycerol] required the addition of DTT, PMSF, and Trasylol to final concentrations of 0.5 mM, 0.5 mM, and 0.01%, respectively just prior to use. In the case of spleen, benzamidine, leupeptin and pepstatin A at the same concentrations as in the homogenization buffer were added. The nuclei were resuspended by 7 to 10 strokes using a glass hand held Dounce homogenizer fitted with an A pestle. The nuclei were lysed by the addition of 1/10 volume of 4 M $(NH_4)_2SO_4$ (pH 7.9). The solution was quickly mixed and left on ice for 30 minutes with occasional mixing. The viscous lysate was centrifuged at 26,000 rpm for 60 minutes using Beckman

30 ml polycarbonate tubes. The same rotor and centrifuge were used to pellet the chromatin. Immediately after the centrifugation, the supernatant was rapidly transferred to another Beckman 30 ml polycarbonate tube, avoiding any chromatin contamination, 0.3 g of solid $(NH_4)SO_4$/ml of supernatant was added followed by gentle agitation to dissolve the $(NH_4)SO_4$. The suspension was left to sit on ice for 20 to 60 minutes. The precipitated proteins were sedimented by a 25 minute centrifugation at 26,000 rpm. The supernatant was discarded and the walls of the tubes wiped with gauze to remove the remaining liquid. The tube containing this protein pellet was sealed and stored in an inverted position on ice overnight or resuspended immediately for dialysis.

The protein pellet was resuspended in 0.075 ml of fresh dialysis buffer [25 mM Hepes (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 10% glycerol and 1 mM DTT] per gram of initial weight of tissue. For spleen tissue the pellet was resuspended in 0.3 ml of dialysis buffer/gram of initial weight of tissue. The suspension was transferred to pre-boiled dialysis tubing (Spectra/Por #3, Spectrum Medical Industries, Inc., prepared by boiling for 30 minutes in 2% Na_2HCO_3, 1 mM EDTA followed by 30 minutes in ddH_2O and, stored in ddH_2O at 4°C) and dialysed twice for 2 hours against 250 ml of the same dialysis buffer. The dialyzed extract was centrifuged for 5 minutes at 12,000 rpm in a counter top centrifuge (Sorvall Microspin 245) cooled to 4°C. This step was necessary to sediment the white precipitate that formed during dialysis. The supernatant was quickly frozen at -80°C in 40 μl aliquots. The protein extracts were thawed on ice just prior to use. The protein concentration was determined with the Bradford reagent (Bio-Rad) according to the manufacturer's instructions using Bovine serum

albumin as a standard.

In vitro transcription assays

The in vitro transcription assay was performed by a modification of the procedures described by Gorski et al. (1986). The amount of templates added to the transcription reaction was different depending on the method utilized to homogenize (hand or blender) the tissues. The in vitro transcription assay contained 0.48 pmole of circular S14 DNA templates, 0.48 pmole of pAlb-320 template and 0.04 pmole of the control pAd_2MLP-200 template (gifts from Dr. U. Schibler) when protein extracts obtained from hand homogenized tissues were used. The assay contained 0.6 pmole of S14 DNA template and 0.04 pmole of pAd_2MLP-200 when only one internal control was added and protein extracts were obtained using hand homogenized tissues. The amount of S14 DNA template remained the same (0.6 pmole) but only 0.05 pmole of pAd_2MLP-200 were added to the transcription assay when protein extracts were obtained from modified blender homogenized tissues.

The DNA templates were pre-incubated on ice for 10 minutes in the presence of 80 to 120 μg of protein in a 10 μl volume. A reaction mix was added to bring the final volume to 20 μl and the final concentrations to 14 mM Hepes (pH 7.6), 100 mM KCl, 17 mM $MgCl_2$, 0.15 mM DTT, 15 μM EDTA, 0.65 mM each of ATP and CTP, 0.35 mM UTP (Pharmacia), 7.25 μCi (α-^{32}P)-UTP (Amersham), 0.2 mM 3'-Methoxy GTP, 5% glycerol and 27 units of RNasin (Promega). The transcription reaction was incubated at 30°C for 45 minutes followed by the addition of a stop buffer (280 μl) containing; 0.25 M NaCl, 1% SDS, 20 mM Tris (pH 7.5), 5 mM EDTA, 22 μg carrier yeast

t-RNA and 45 μg Proteinase K (Boerhinger Mannheim). The solution was incubated at 37°C for 30 minutes and extracted with an equal volume of freshly prepared water-equilibrated phenol/chloroform/isoamyl alcohol (25:24:1). The RNA transcripts were precipitated by adding 3 volumes of cold 95% ethanol and placed at -80°C for a minimum of 10 minutes. The RNA was sedimented by centrifugation at 12,000 rpm for 10 minutes in a counter top (Canlab Biofuge A) centrifuge. The supernatant was aspirated and the RNA pellet dried under vacuum. The pellet was resuspended in 5.5 μl of Maxam and Gilbert loading dye (Maniatis et al. 1982), heated at 90°C for 2 minutes and applied to a pre-equilibrated denaturing 8% polyacrylamide gel containing 8 M urea buffered with 1XTBE. Electrophoretic resolution was performed at 1,500 volts, 25-30 milliamperes for 2 hours. The bands were detected by autoradiography with Kodak XAR-5 films using two Kodak X-Omatic regular intensifying screens for approximately 18 hours at -80°C.

Quantification and statistics

The radioactivity incorporated into the individual RNA products was quantitated by cutting and counting the bands directly in Ecolite (+) (ICN Biomedicals) using a scintillation counter (Beckman LS 5000 CE) and/or by computer-assisted videodensitometry of autoradiographs. The relative rate of S14 template transcription was calculated by dividing the radioactivity or optical density in the band arising from transcription of the S14 DNA by the adenovirus product. The data was analysed using one way analysis of variance (ANOVA) and the differences were considered significant if $p \leq 0.05$.

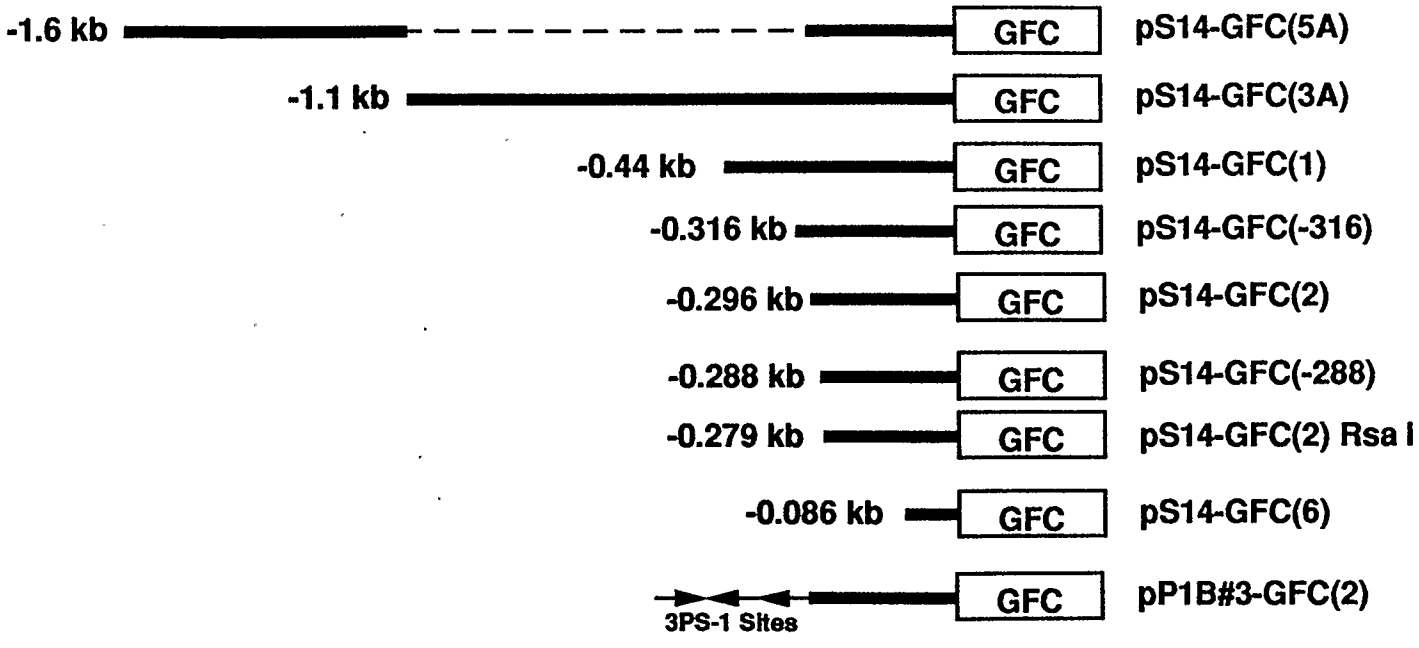
RESULTS

Cell-Free in vitro Transcription Assay

The cell-free in vitro transcription assay described initially by Gorski et al. (1986) was used in combination with the "G"-free cassette (GFC) vector developed by Sawadogo and Roeder (1985). Figure 1 illustrates the constructs containing the S14 gene promoter sequences attached 5′ to a GFC measuring 377 bp in length. Transcription of the template in vitro yielded radiolabelled RNA products 377 bases in size. The RNA transcripts are of a uniform size because transcription initiation is determined by the S14 promoter sequences and termination is fixed at the first G residue 3′ to the GFC. Transcription from other portions of the plasmid is prevented by the addition of 3′-Methoxy-GTP to the reaction mixture because it inhibits RNA chain elongation when the polymerase encounters a G residue. Most of the S14-GFC constructs utilized for this research were generated by the PCR and exploited convenient restriction endonuclease sites.

The nuclear protein extracts were prepared from fresh tissues as described by Gorski et al. (1986) with the modifications mentioned under experimental procedures. The combination of these procedures offered marked advantages. The sensitivity in detecting the transcripts is greatly enhanced because the products of transcription were uniform in size, thus concentrating the signal in discrete bands. The preparation of transcriptionally active nuclear extracts is relatively brief (5-6 h) and easy. The assay from start to finish is usually 2-3 h and up to 20 samples can be processed easily in one day with results the following day.

Figure 1. Map of the S14 DNA constructs and binding sites for P-1, PS-1, and TRE. The limits of the 5′-flanking sequences of the S14 gene inserted 5′ to the GFC are indicated. The head of the arrow indicates the 3′ end of the fragment. See experimental procedures for details about their construction. The nucleotide sequences of the binding sites for the P-1, PS-1, and TRE are shown below.



PS-1 binding site -63 to -48; sequence was (TTGGCGTCCTGTCAAT)

P-1 binding site of S14 DNA, nucleotides -310 to -288 (AAAAGAGCTATTGATTGCCTGCA).

TRE consensus sequence (TCAGGTCATGACCTGA)

The extracts prepared according to methods of Gorski et al. (1986) have been shown to initiate accurately transcription of genes such as Albumin, Adenovirus Major Late (Gorski et al. 1986) and vitellogenin (Corthesy et al. 1988).

Over the last 2 years of using this assay I have uncovered several helpful hints to maximize signal intensity. (1) Fresh tissue is prefered over frozen tissues because extracts from the latter were far less active than from fresh tissues. (2) Freshly equilibrated phenol is essential. It appeared that aged (>30-45 days) phenol was somehow degrading the RNA transcripts. To avoid this problem fresh phenol was prepared every 3 to 4 weeks. The phenol is equilibrated in double distilled water and the aqueous phase was changed at least twice over a 3 day period. RNA transcripts were sensitive to phenol when used before 3 days and after 21-28 days following equilibration with water. The phenol was mixed with chloroform: isoamyl alcohol (24:1) in a 1 to 1 ratio just prior to use, and centrifuged to remove the excess water before being added to the incubation mix.

Optimizing parameters for the cell-free in vitro transcription assay

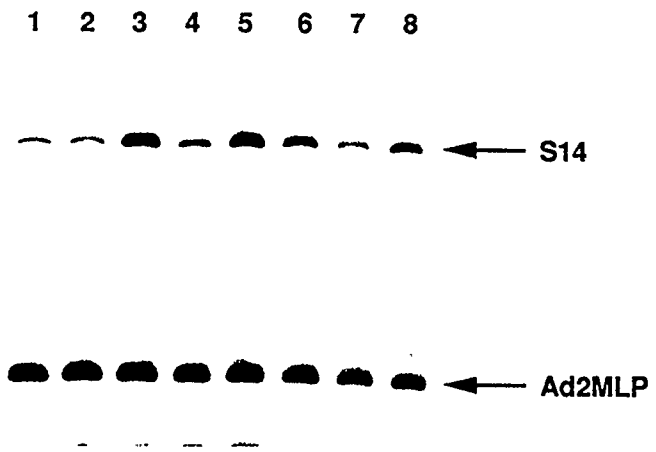
Gorski et al. (1986) and Sawadogo and Roeder (1985) reported extensive variations in transcription efficiencies when different concentrations of DNA, protein and salts were present during incubation. Therefore, it was essential to establish the incubation parameters necessary for optimizing transcriptional activity of the S14-GFC templates in vitro.

The construct pS14-GFC(1) containing -441 to -2 of upstream S14 gene

DNA sequence was prepared (see experimental procedures) and used throughout the standardization assays. Two internal controls pAd_2MLP-200 and pAlb-320 (gifts from Dr. U. Schibler) were used either individually or together in all the incubation reactions. The pAd_2MLP-200 control template contains the -404 to +10 DNA sequence of the Adenovirus 2 major late gene inserted 5′ to a GFC of 200 bps in length (Sawadogo and Roeder 1985). The pAlb-320 control template contains the -650 to +22 DNA sequence of the mouse albumin gene inserted 5′ to a GFC of 320 bps in length (Gorski et al. 1986).

To determine the concentration of nuclear protein required for optimum transcription efficiency, the assay was performed by adding varying amounts of protein to a fixed concentration of template. Figure 2, shows the results obtained with modified blender generated extract added to the incubation in amounts varying from 88 to 286 μg. The quantities of templates pS14-GFC(1) and pAd_2MLP-200 added to the reactions were kept constant at 1.5 μg and 0.05 μg respectively. The pS14-GFC(1) and Ad_2MLP derived transcripts migrated as the upper and lower bands, respectively. In reactions containing less than 44 μg of proteins no transcriptional activity was detected (data not shown). The transcription efficiency of the pAd_2MLP-200 construct remained relatively constant during a wide range of protein concentrations (88 to 286 μg/20 μl, lanes 1 to 8). In contrast, pS14-GFC(1) transcription efficiency was variable with the optimum signal appearing in the reaction containing 154 μg of proteins (lane 3). Although the optimum quantity of protein was different for each extract preparation it was usually between 80 to 160 μg of proteins. The optimum protein concentration was within the same range for both hand and

Figure 2. Representative autoradiogram showing the optimum of nuclear extract concentration for the assay. Each reaction contained 1.5 μg of pS14-GFC(1) and 0.05 μg of the control template, pAd_2MLP-200. pS14-GFC(1) gave rise to the radiolabeled RNA product migrating as the upper band and the product of the pAd_2MLP-200 the lower band in each lane. In lanes 1-8 the protein concentrations were; 88, 132, 154, 176, 198, 220, 242, and 286 μg of protein, respectively.

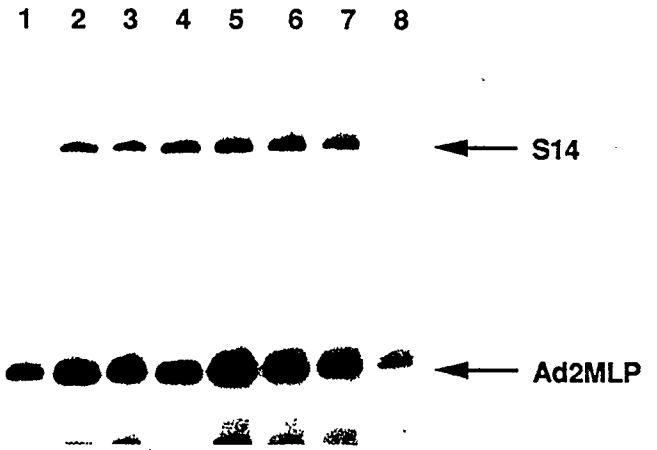

1 2 3 4 5 6 7 8
S14
Ad2MLP

modified blender prepared extracts.

The quantity of template added to each incubation reaction was also optimized. Results in figure 3 represent a typical experiment showing increasing amounts of template added to a fixed aliquot of an extract. Varying amounts of the pS14-GFC(1) construct was added together with a constant quantity of the pAd_2MLP-200 (0.05 μg) to the reactions. The same hepatonuclear extract examined in the previous section was used for these studies with a final protein concentration of 154 μg per reaction. The pAd_2MLP-200 transcription level remained relatively unaffected when pS14-GFC(1) varied from 0.5 to 2.5 μg. The pS14-GFC(1) transcription efficiency was more sensitive to the level of DNA present in the incubation. The pS14-GFC(1) transcripts were barely detectable in the presence of low and high DNA concentrations reaching an optimum between 1.5 and 1.75 μg of pS14-GFC(1) construct. Notice the disappearance of both transcripts at high template concentration, this effect is probably due to a distribution of commonly used transcription factors amongst the templates.

When nuclear proteins were extracted by hand homogenization, the optimum pS14-GFC(1) remained at approximately 1.5 μg per reaction whereas the quantity of pAd_2MLP-200 template had to be augmented to 0.1 μg per reaction. The discrepancy between the 2 methods in transcription efficiency of the pAd_2MLP-200 promoter remains unexplained. It was also determined that the optimum quantity of templates to be used when the 3 templates pS14-GFC(1), pA1b-320 and pAd_2MLP-200 were incubated together are 1.2 μg, 1.2 μg, and 0.1 μg respectively using hand homogenized extracts. Gorski et al. (1986) and Vaulont et al. (1989) previously reported that

Figure 3. Representative autoradiogram illustrating the optimum DNA concentration for the transcription assay. The same quantity of protein (1.54 μg) and control template pAd_2MLP-200 (0.05 μg) was added to each reaction. The nuclear extract utilized was the same as in figure 2. The DNA concentration in lanes 1-8 were: 0.50, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, and 3.00 μg of pS14-GFC(1), respectively.


1 2 3 4 5 6 7 8
S14
Ad2MLP

the efficiency in transcription using this assay was not different using either circular or linear templates. For the sake of simplicity, circular DNA templates were used in all assays.

Next, the optimum salt concentration for maximum transcriptional efficiency of the templates was examined. Figure 4 shows typical results obtained using 1.5 μg of pS14-GFC(1) and 0.05 μg of pAd_2MLP-200 in incubations containing 100 mM KCl and 154 μg of the euthyroid nuclear extract. The parameter being investigated in these studies was the final concentration of $MgCl_2$. Note the sharp variation in the transcriptional efficiency of both templates with changes in $MgCl_2$ concentration. The pS14-GFC(1) transcription was more sensitive to low $MgCl_2$ concentrations whereas both templates were similarly affected at high concentrations. The optimum $MgCl_2$ concentration for this particular assay was between 16 and 21 mM (Figure 4, compare lanes 3 and 4). After repeated trials with different protein extracts, the optimum $MgCl_2$ was standardized at 17 mM. This value was the same for incubations using nuclear extracts prepared by hand or modified blender homogenization of the tissues.

Figure 5 illustrates the results obtained using the optimal $MgCl_2$ (17 mM) concentration but the KCl was variable ranging from 50 to 125 mM. In contrast to the above studies, transcription of the pS14-GFC(1) construct was not affected drastically by changes in the concentration of KCl. Transcription from both templates tolerated a wide range of KCl concentration with the optimum being 100 mM and 58 mM KCl with either modified blender or hand homogenized protein extracts, respectively. The difference in KCl concentration between the 2 extracts remains unexplained since it could not be linked to methodological differences.

Figure 4. Representative autoradiogram showing the optimum $MgCl_2$ concentration for the assay. The quantity of protein, pS14-GFC(1) template and control pAd_2MLP-200 template added to each reaction was 154 μg, 1.5 μg, and 0.05 μg, respectively. The nuclear extract utilized was the same as for the protein study in figure 2. In vitro transcription was performed in reactions containing 6, 11, 16, 21, 26, 31, and 36 mM $MgCl_2$ final concentration for lanes 1 to 7, respectively.

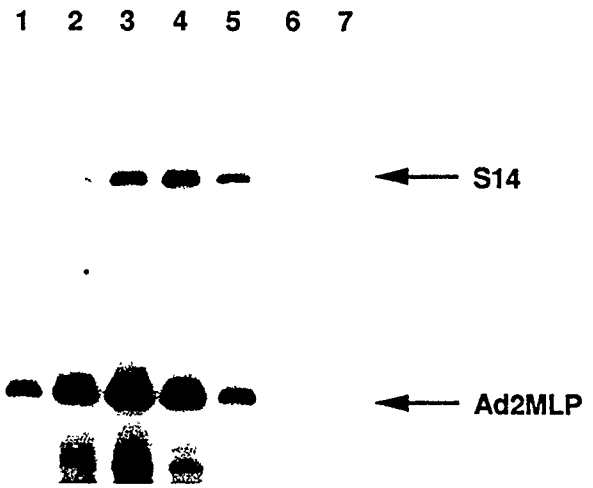
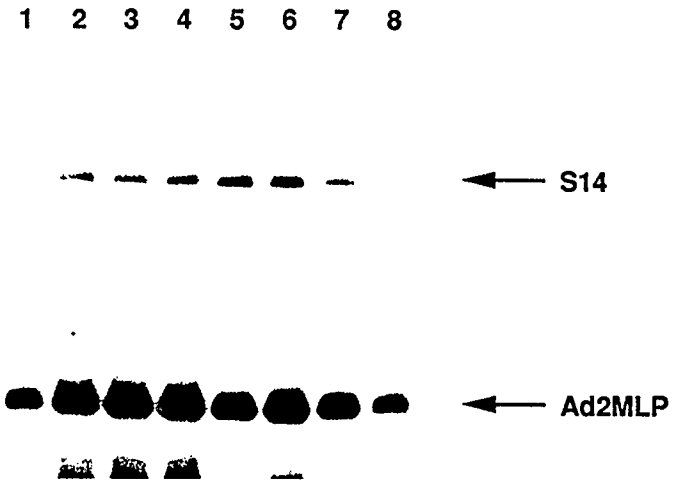

1 2 3 4 5 6 7
S14
Ad2MLP

Figure 5. Representative autoradiogram illustrating the optimum KCl concentration for the assay. The conditions were the same as for the $MgCl_2$ study in figure 4. The $MgCl_2$ concentration was constant at 17 mM. The final KCl concentration was 50, 65, 75, 85, 95, 105, 115, and 125 mM for lanes 1 to 8, respectively.


1 2 3 4 5 6 7 8
S14
Ad2MLP

Next the effect of Zn^{+2} and Mn^{+2} ions on the efficiency of transcription was examined. The addition of as little as 10 mM $ZnCl_2$ to the incubation reaction was sufficient to block completely transcription from both templates (data not shown). The same was true when $MnCl_2$ was used in place of $MgCl_2$ (data not shown). The importance of divalent ions for the transcriptional machinery was confirmed by the addition of 1 μl of 100 mM EDTA to the incubation reaction. This manipulation was sufficient to completely block transcription from either template (data not shown).

The effect of temperature on transcriptional efficiency was also examined. At temperatures <25°C or >42°C transcription from both templates were either nonexistent or barely detectable. The templates appeared to be transcribed with similar efficiency at 30° and 37°C (data not shown). Therefore, the 30°C incubation temperature was selected because it is less likely to activate proteases and RNase at the lower temperature. A 30°C incubation is supported by the findings of Weil et al. (1979) who found that the components required for transcription are stable at this temperature for up to 4 hours.

The preincubation and incubation periods were also studied (data not shown). No significant differences were observed in transcription efficiency of either template by pre-incubating the templates and protein extract on ice for 30 seconds or up to 10 minutes. The 10 minutes pre-incubation period on ice was retained for both convenience and consistency in the assay. The amount of measureable transcripts showed a linear increase between 5 and 30 minutes of incubation at 30°C and reached a plateau at approximately 40 minutes. A 45 minutes incubation period at

30°C was therefore chosen as a standard for the assay.

The radiolabeled bands disappeared when α-amanitin (0.5 μg/ml, an RNA polymerase II inhibitor) or RNase A (0.5 μg/ml) were added to the incubation reaction (data not shown). These results confirmed that the radiolabeled products consisted of RNA synthesized by RNA polymerase II.

Transcription of the GFC is promoter dependent

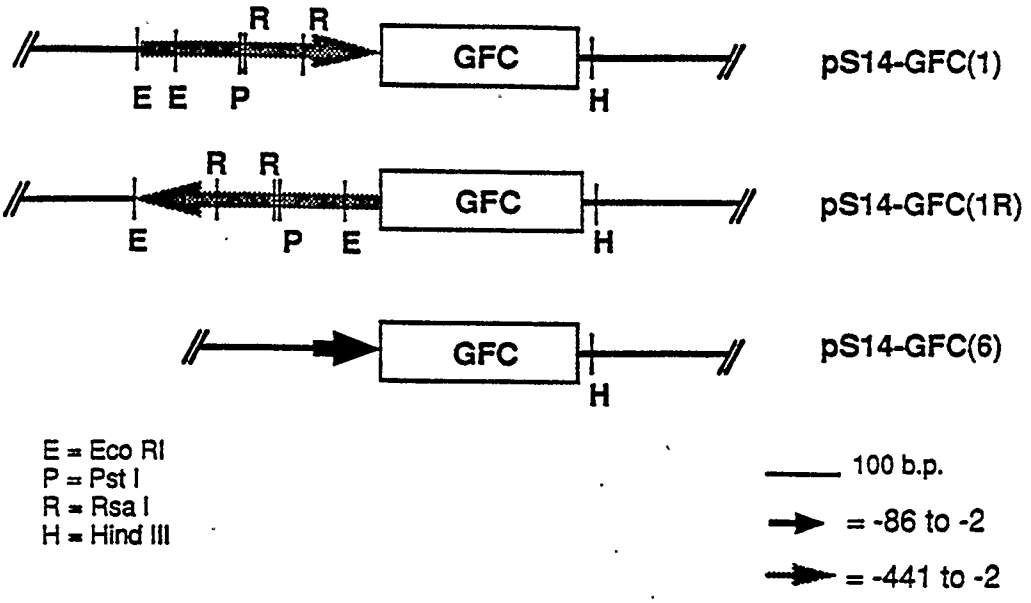
The construct pS14-GFC(1) containing the sequence -441 to -2 of the S14 gene promoter was incubated together with the 2 control templates pAlb-320 and pAd_2MLP-200 in the presence of hepatonuclear extract from euthyroid rats. 3 bands of radioactivity of 377, 320, and 200 bases in length were from pS14-GFC(1), pAlb-320 and pAd_2MLP-200 templates, respectively (figure 6, panel B, first lane). No detectable transcripts were visible at the 377 base length position in assays containing the GFC template without a promoter, $p(C_2AT)_{19}$ (second lane) or the -441 to -2 S14 DNA sequence inserted in the 3′ to 5′ orientation relative to the GFC, pS14-GFC(1R) (third lane). The 2 control templates were transcribed as expected. The last lane shows results using the pS14-GFC(6) template, a construct containing nucleotides -86 to -2 of the gene. Together these observations demonstrate that the -441 to -2 and -86 to -2 sequences of the S14 gene contain promoter activity in vitro and that transcription of the GFC was dependent on orientation of the promoter DNA.

T_3 affects the transcription of pS14-GFC(1) and pS14-GFC(6)

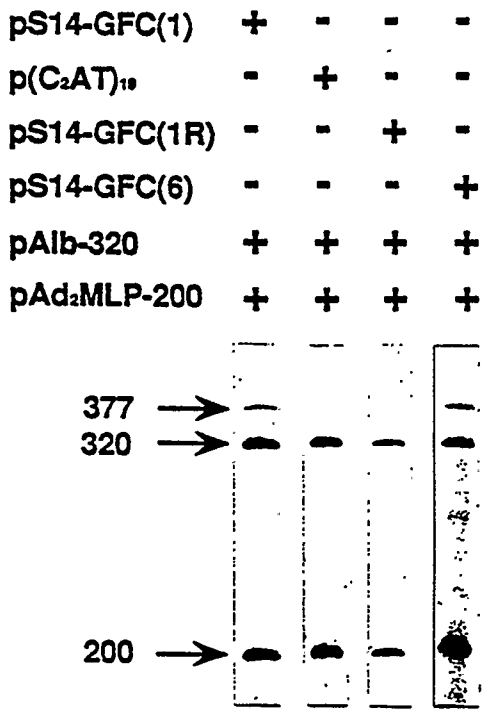
To examine the effect of T_3 on S14 gene transcription in vitro, the pS14-GFC(1) (1.5 μg) and GFC(6) (1.41 μg) templates were incubated in hand

Figure 6. In vitro transcriptional activity of S14 DNA containing the HS-1 site. (Panel A) Map of S14 DNA (-441 to -86 to -2) constructs inserted in front of the 'G' free cassette (GFC), the head of the arrow indicates the 3' end of the fragment. (Panel B) The transcriptional activity of each construct in hepatonuclear extracts from euthyroid animals obtained from hand homogenized tissues. All reactions contained the internal standard plasmids, Alb-320 (1.2 μg) and Ad_2MLP-200 (0.1 μg), the radiolabeled products from these templates are indicated by the arrows positioned at 320 and 200 bases, respectively. Test DNA templates were: lane 1, pS14-GFC(1) (1.2 μg); lane 2, $p(C_2AT)_{19}$ (1.2 μg); lane 3, pS14-GFC(1R) (1.2 μg); and lane 4, pS14-GFC(6) (1.2 μg). The position of the pS14-GFC(1) or pS14-GFC(6) transcribed products are indicated by the arrow at 377 bases.

A


R R
GFC
E E P
H
pS14-GFC(1)
R R
GFC
E P E
H
pS14-GFC(1R)
GFC
H
pS14-GFC(6)
E = Eco RI
P = Pst I
R = Rsa I
H = Hind III
100 b.p.
= -86 to -2
= -441 to -2

B

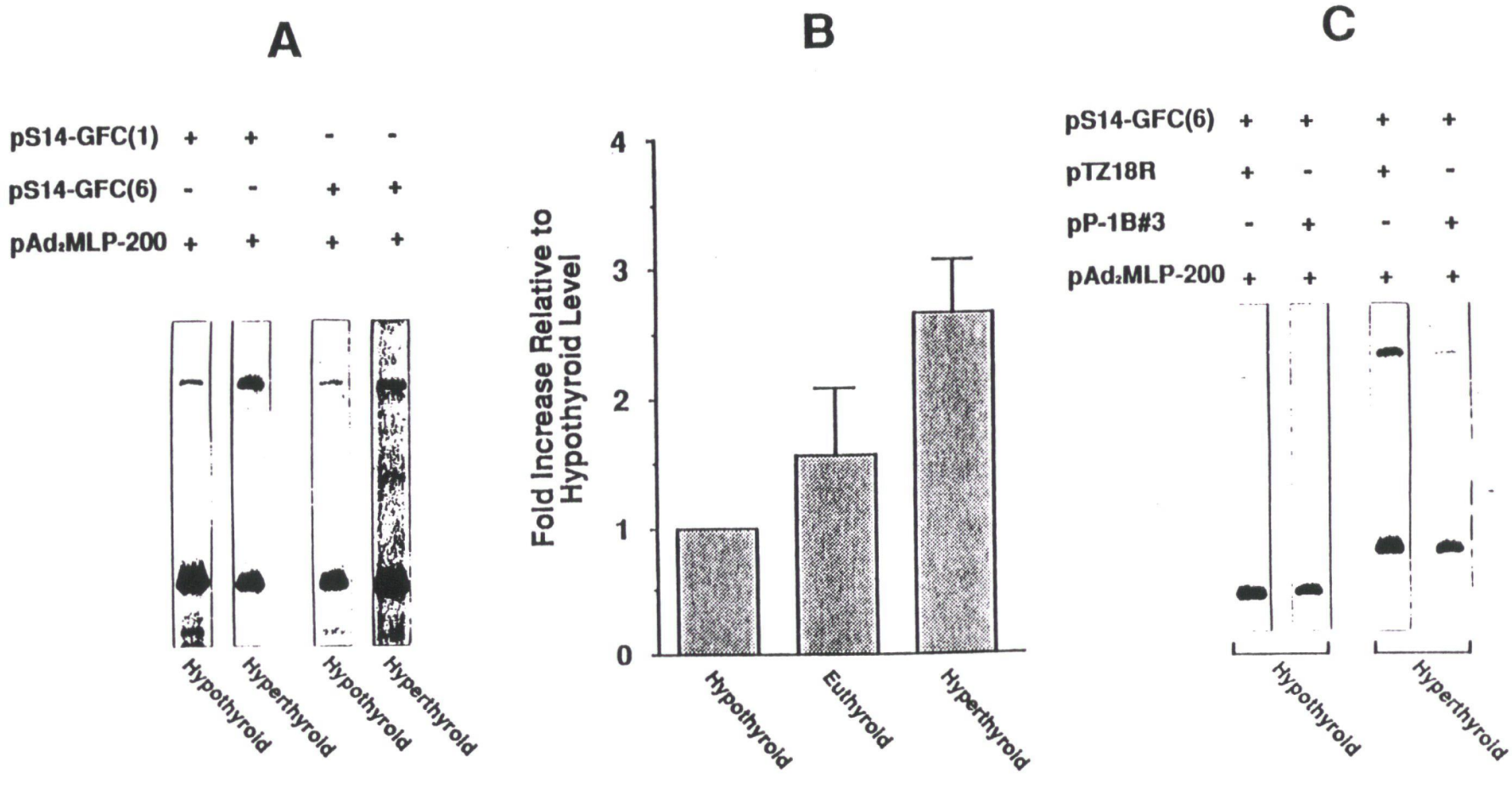

pS14-GFC(1) + - - -
p(C2AT)10 - + - -
pS14-GFC(1R) - - + -
pS14-GFC(6) - - - +
pAlb-320 + + + +
pAd2MLP-200 + + + +
377
320
200

homogenized hepatonuclear extracts from hypo-, eu-, (not shown) and hyperthyroid rats (figure 7, panel A). The internal control pAd_2MLP-200 (0.1 μg) was included to ensure that variations in transcription were not due to a generalized effect of T_3. The amount of radioactivity in each band was quantitated by scintillation counting and the results obtained from pS14-GFC(1) and GFC(6) were expressed as a ratio relative to the pAd_2MLP-200 template RNA product. The results indicate that T_3 increases the level of transcription from the pS14-GFC(6) template by 1-:1.6-:2.7-fold in hypo-, eu-, and hyperthyroid hepatonuclear extracts, respectively, relative to the hypothyroid state (figure 7, panel B). Identical results were observed using pS14-GFC(1) as a template (data not shown) suggesting that the effect of T_3 on transcription of the two templates are probably dependent on the activity of the -86 to -2 sequence. The addition of 10^{-4} to 10^{-9} M T_3 (data not shown) directly to the extracts from the 3 thyroidal states prior to incubation with the templates, failed to affect the transcription rate. These results suggest a novel mechanism of T_3 action which involves the modification of nuclear proteins in vivo that remain active in the nuclear extracts. The increase in transcription during the hypothyroid to hyperthyroid transition did not match the increase in the level of S14 mRNA (15- to 20-fold) reported by Jump et al. (1984). These differences may be due to the limited amount of upstream S14 DNA sequences contained in the constructs.

Deletional studies of the S14 gene upstream DNA sequences

To determine the regions of HS-1 DNA that play important roles in regulating S14 gene transcription, we constructed a series of templates

Figure 7. In vitro transcriptional activity of the S14 DNA and the effect of T_3 and competition for PS-1 binding proteins. Panel A, radiolabeled RNA products from incubations containing S14 templates and hypothyroid or hyperthyroid extracts obtained from hand homogenized tissues. All reactions contained the internal standard plasmid pAd_2MLP-200 (0.1 μg), and the radiolabeled product from this template gave rise to the lower band in each lane. Test DNA templates were: lane 1, pS14-GFC(1) (1.5 μg) with hypothyroid extract; lane 2, pS14-GFC(1) (1.5 μg) with hyperthyroid extract; lane 3, pS14-GFC(6) (1.5 μg) with hypothyroid extract; and lane 4, pS14-GFC(6) (1.5 μg) with hyperthyroid extract. The radiolabeled RNA product from both the pS14-GF(1) and pS14-GFC(6) templates migrated near the top of the gel. Panel B, relative rate of pS14-GFC(6) transcription in hepatonuclear extracts from animals (three per group) of various thyroid states. The ratios of the pS14-GFC(6) to the pAd_2MLP-200 product were quantitated by scintillation counting and video-assisted densitometry. Both techniques revealed the same results. These values were expressed relative to the hypothyroid level and are averages ± S.D. Panel C, competition studies using the PS-1 DNA. All reactions contained the internal standard plasmid, pAd_2MLP-200 (0.1 μg). The rate of pS14-GFC(6) (1.5 μg/reaction) transcription was measured in the presence of control DNA (pTZ18R, 1.5 μg) or competitor DNA, pP-1B#3 (a pTZ18R vector containing three PS-1 binding sites inserted into the Bam HI site, 1.5 μg). Competition studies using hypothyroid extracts appear in lanes 1 and 2 and in hyperthyroid extracts, lanes 3 and 4.


A
pS14-GFC(1) + + - -
pS14-GFC(6) - - + +
pAd2MLP-200 + + + +
Hypothyroid
Hyperthyroid
Hypothyroid
Hyperthyroid
B
Fold Increase Relative to Hypothyroid Level
0
1
2
3
4
Hypothyroid
Euthyroid
Hyperthyroid
C
pS14-GFC(6) + + + +
pTZ18R + - + -
pP-1B#3 - + - +
pAd2MLP-200 + + + +
Hypothyroid
Hyperthyroid

containing various portions of the S14 promoter. This DNA was inserted 5′ to the 377 bp GFC used in the cell-free in vitro transcription assay (figure 1, see experimental procedures for details). Figure 8 illustrates the results obtained when equimolar amounts of the various constructs (0.6 pmole per assay) were incubated with 123 μg of modified blender homogenized nuclear extract obtained from liver of hyperthyroid rat. In addition, we measured the ability of the various constructs to promote transcription of the GFC in nuclear extracts from animals of 3 thyroidal states (figure 9). The internal control pAd_2MLP-200 (0.05 μg) was included in all the reactions. The quantity of radioactivity in each band resulting from the various templates was quantitated by β-scintillation counting. The data were normalized by subtracting the background counts and expressed as a ratio of the transcription value from the S14 promoter over the value from the pAd_2MLP-200 control. The bar graph values represent the mean ± S.D.M. (standard deviation of the mean) of at least 6 assays. Results with a p-value of ≤ 0.05 are marked by an asterisk indicating a statistically significant difference from the results of the same thyroid state with the pS14-GFC(6) template.

The construct pS14-GFC(6) was transcribed approximately 2.5-fold less efficiently than the pS14-GFC(2)-Rsa I template containing the S14 sequence -279 to -2. Similar results were obtained when hepatonuclear extracts obtained from rats of the 3 thyroid states were used. These results suggested that sequences between -279 to -86 enhanced transcription of the S14 gene in vitro. The 2.5-fold increase between the efficiency of transcription of pS14-GFC(6) and pS14-GFC(2)-Rsa I was independent of thyroid state, suggesting that the enhancing function of

Figure 8. Representative autoradiogram of in vitro transcriptional activity of the S14 DNA constructs with hyperthyroid nuclear extracts. Each reaction contained the internal control pAd_2MLP-200 (0.05 μg) and 123 μg of nuclear extract obtained from blender-homogenized hyperthyroid livers. The test DNA templates were: lane 1, pS14-GFC(1) (1.41 μg); lane 2, pS14-GFC(2)-Rsa I (1.43 μg); lane 3, pS14-GFC(288) (1.44 μg); lane 4, pS14-GFC(2) (1.44 μg); lane 5, pS14-GFC(316) (1.45 μg); lane 6, pS14-GFC(1) (1.5 μg); lane 7, pS14-GFC(3A) (1.77 μg); lane 8, pS14-GFC(5A) (1.66 μg); and lane 9, pP1B#3-GFC(2) (1.45 μg).

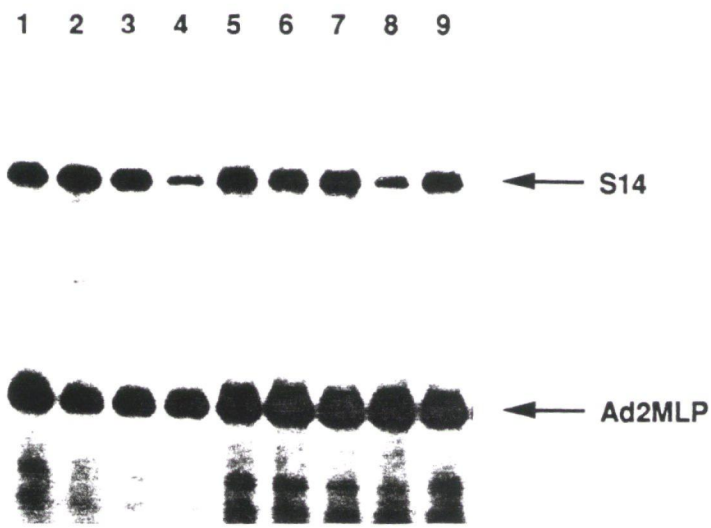
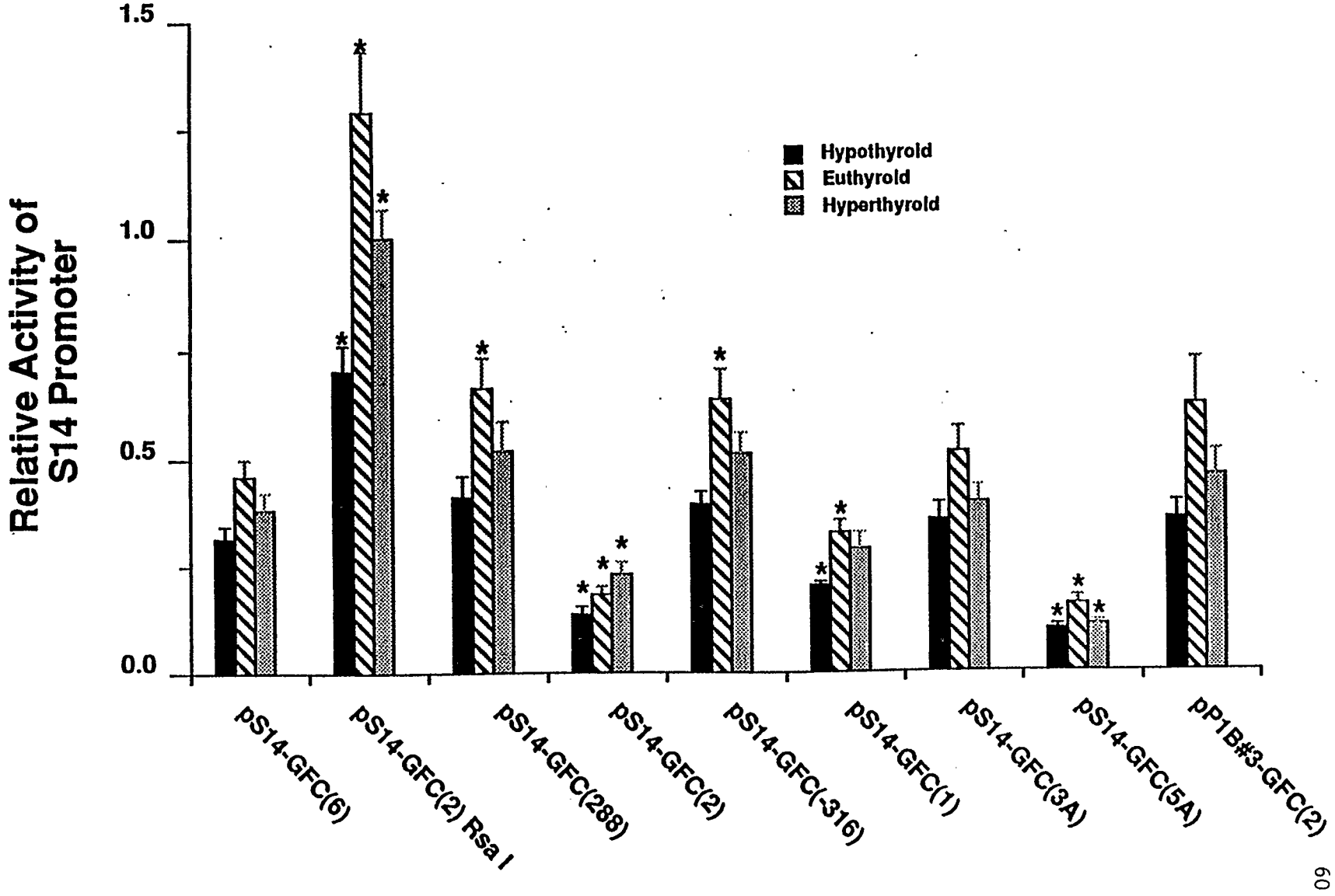

1 2 3 4 5 6 7 8 9
S14
Ad2MLP

Figure 9. Relative rate of in vitro transcription of the S14 DNA constructs in hypo-, eu-, and hyperthyroid hepatonuclear extracts. Values represent ratios of the S14 templates to the pAd_2MLP-200 products quantitated by β-scintillation counting. The bar graph values represent the mean ± S.D.M. of at least 6 assays. Results marked with an asterisk are significantly different ($p \leq 0.05$) from the results of the same thyroid state obtained with the pS14-GFC(6) template. The mean values obtained with the S14 constructs pS14-GFC(6), pS14-GFC(2)-Rsa I, pS14-GFC(288), pS14-GFC(316), pS14-GFC(1) and pP1B#3-GFC(2) in hypothyroid nuclear extracts were significantly lower ($p \leq 0.05$) than the mean values obtained with the same constructs in euthyroid. The differences between the mean values obtained with all the S14 constructs in eu- and hyperthyroid did not reach significance.


Relative Activity of S14 Promoter
1.5
1.0
0.5
0.0
Hypothyroid
Euthyroid
Hyperthyroid
pS14-GFC(6)
pS14-GFC(2) Rsa I
pS14-GFC(288)
pS14-GFC(2)
pS14-GFC(-316)
pS14-GFC(1)
pS14-GFC(3A)
pS14-GFC(5A)
pP1B#3-GFC(2)

the factor(s) is not affected by T_3.

The pS14-GFC(288) template contains the S14 DNA from -288 to -2. The activity of this construct is roughly comparable to that of pS14-GFC(6). Since pS14-GFC(288) contains an S14 promoter sequence only 9 bp longer than the one subcloned in pS14-GFC(2)-Rsa I, this suggests that the additional sequence negatively modulates the transcription of the S14 gene in vitro.

A very interesting sharp decrease in transcription efficiency was obtained with the pS14-GFC(2) construct. This template contained the -296 to -2 sequence of the S14 gene. The transcription efficiency of the pS14-GFC(2) was decreased by almost 6- and 3-fold when compared to pS14-GFC(2)-Rsa I and the construct pS14-GFC(316) containing the -316 to -2 DNA sequence, respectively. These findings suggest that a factor binding within the -316 to -279 of the S14 DNA sequence acts as a strong negative modulator of transcription in vitro. This sequence contains the recognition site protected by the P-1 protein (-288 to -310) previously described by Wong et al. (1989). They reported that the binding activity of P-1 protein correlated closely with the S14 mRNA expression, increasing 8-fold during the hypo- to hyperthyroid transition. As will be discussed later the apparent inverse correlation between P-1 binding and transcriptional inhibition is consistent with a repressor role for P-1.

To be certain that the pS14-GFC(2) was not an artifact of the construct, the sequence -1635 to -1100 of upstream S14 DNA was ligated 5' to the pS14-GFC(2) construct. The reason for designing pS14-GFC(5A) was to eliminate the possibility that the junction between pS14-GFC(2) and pTZ 19R did not create a binding site for a repressor that is not part of

the wild type promoter. The activity of the new construct, pS14-GFC(5A) was also at a very low level in all liver extracts similar to that of pS14-GFC(2). These results support a repressor function for the P-1 protein and the site was not an artifact of DNA manipulation. Since the constructs pS14-GFC(2) and pS14-GFC(5A) do not contain the sequence -297 to -310, part of the DNA protected by P-1 protein, this suggests that the sequence -288 to -296 may be sufficient for a strong interaction between P-1 and its DNA sequence.

A similar scenario was observed with activity of pS14-GFC(1) compared to both pS14-GFC(316) and pS14-GFC(3A). The pS14-GFC(3A) contained the -1100 to -2 DNA sequence of the S14 gene. Activity of pS14-GFC(1) was roughly 2-fold lower than either of the 2 other constructs. This finding suggests the presence of a cis-regulatory region between -441 and -288 interacts with a putative factor that negatively regulates S14 gene transcription.

The efficiency of transcription of the construct pS14-GFC(316) is almost 3-fold and 2-fold higher than the efficiency of transcription of the constructs pS14-GFC(2) and pS14-GFC(1) respectively. These results suggest the presence of a cis-regulatory region 3′ to the position -316 interacting with a positive regulatory factor. The P-1 binding protein has already been identified as protecting the nucleotides -310 to -288 but from the above results it was shown that -288 to -296 appeared sufficient for the repressor protein binding and activity.

The effect of T_3 on the transcription activity of the different S14 DNA constructs using modified blender homogenized hepatonuclear extracts

The transcriptional efficiency of all constructs were tested in blender extracted hepatonuclear proteins from rats of the 3 thyroid states (Figure 9). An increase in activity of approximately 1.7-fold was observed with all the constructs during the transition from hypo- to euthyroid state. This value is in close agreement with the 1.6-fold increase during the same transition measured when hand homogenized extracts were utilized. In contrast to the 50% increase observed during the eu- to hyperthyroid transition when hand homogenized extracts were added to the incubation (figure 7, panel B), the use of blender extracts failed to demonstrate any increase in transcriptional activity during the same transition. The discrepancy between the two methods of preparation for nuclear extracts remains unexplained. It could be due to factors specifically showing increased sensitivity to a variation between the two methods of extraction.

The PS-1 binding protein(s) plays an important role in transcription of the S14 gene in rat liver

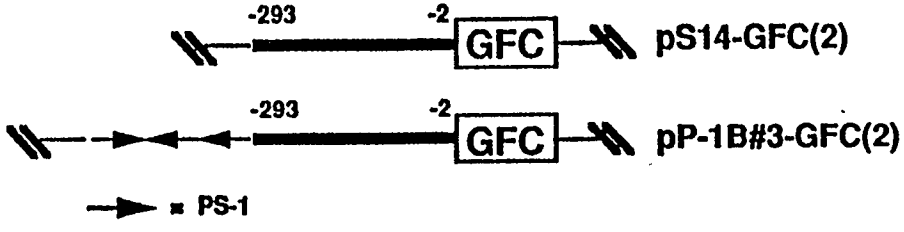
The pS14-GFC(6) construct was shown to contain promoter activity (figure 6, panel B) and to be responsive to thyroidal state (figure 7, panel A). The -86 to -2 S14 DNA sequence contains the binding site (-63 to -48) for the protein(s) PS-1. PS-1 is belived to be a putative CCAAT-motif binding factor, as shown previously by DNase I footprinting studies (Wong et al. 1990). Recently, we have showed that PS-1 binding activity varies with T_3 status; increasing by 1-, 1.8-, and 4-fold in hypo-, eu-,

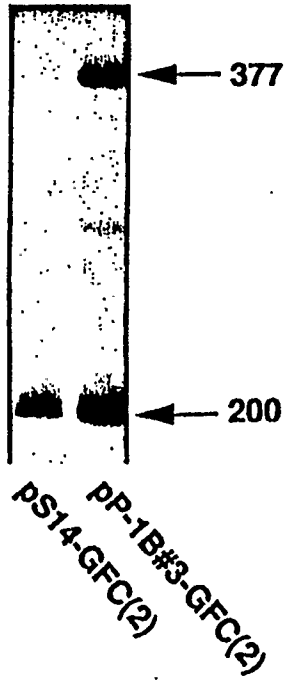
and hyperthyroid states, respectively. As shown in Figure 7 (panel B), binding activity of PS-1 protein(s) correlated with the transcriptional activity of the construct pS14-GFC(6).

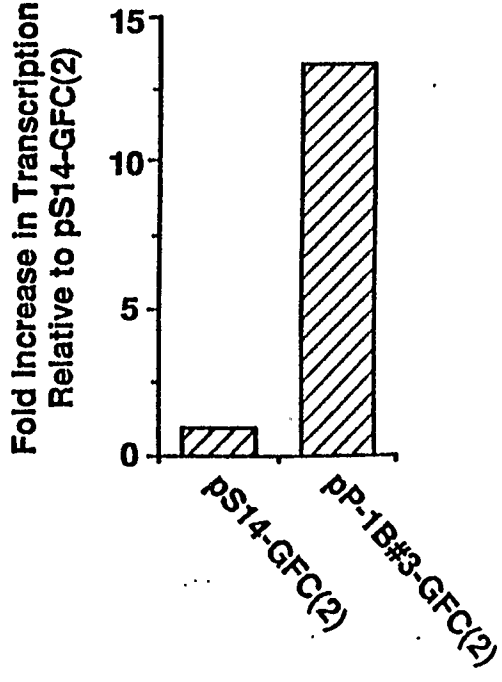
Competition studies were performed to determine the importance of PS-1 binding protein in S14 gene transcription. pS14-GFC(6) (1.5 μg) was incubated in the presence of 0.1 μg of pAd_2MLP-200 control template and 1.5 μg of pP1B#3, a construct containing 3 PS-1 binding sites inserted in the pTZ 18R vector. The level of pS14-GFC(6) transcription was strongly inhibited (93%) in the presence of pP1B#3 in hyper- but not in hypothyroid extracts (figure 7, panel C). As expected, competition with a control plasmid lacking the PS-1 binding sites, pTZ 18R, had no effect on the efficiency of transcription of the pS14-GFC(6) template. Since transcription of the pAd_2MLP-200 template was not affected by the presence of competition DNA, the observed diminution in transcription activity of the pS14-GFC(6) construct appears to be specific. These findings showed that in hyperthyroid extracts, displacement of PS-1 binding from its recognition sequence diminished transcription activity. This suggests that the higher rate of pS14-GFC(6) transcription in hyperthyroid extract may be dependent on PS-1 binding to its recognition sequence.

To further define the function of the PS-1 binding protein in S14 gene transcription, 3 PS-1 binding sites were subcloned 5′ to the -296 to -2 sequence, pP1B#3-GFC(2). The pP1B#3-GFC(2) was incubated (1.5 μg) with the internal control pAd_2MLP-200 (0.1 μg) in hepatonuclear extracts from hypo-, eu-, and hyperthyroid rats. The transcription activity of this construct was 14-fold higher than that of the pS14-GFC(2) template when the nuclear extracts were prepared from hand homogenized tissues (Figure

Figure 10. Multiple PS-1 binding sites increase pS14-GFC(2) transcription in vitro. (Panel A) Map of S14 DNA (-293 to -2) template, pS14-GFC(2) and the same construct containing 3 PS-1 sites, pP-1B#3-GFC(2). (Panel B) Transcriptional activity of each construct in nuclear extract obtained from hand homogenized hyperthyroid liver. All reactions contained the internal standard plasmid pAd_2MLP-200 (0.1 μg), the radiolabeled product from this template appears as the lowest band in each lane. Test templates were: lane 1, pS14-GFC(2) (1.44 μg); and lane 2, pP-1B#3-GFC(2) (1.45 μg). The product from the test templates migrates as the top band in each lane. (Panel C) Relative rates of pS14-GFC(2) and pP-1B#3-GFC(2) transcription in hepatonuclear extracts from hyperthyroid animals. The ratios of the test templates to the pAd_2MLP-200 products were quantitated by β-scintillation counting.


-293
-2
GFC
pS14-GFC(2)
-293
-2
GFC
pP-1B#3-GFC(2)
= PS-1


377
200
pS14-GFC(2)
pP-1B#3-GFC(2)

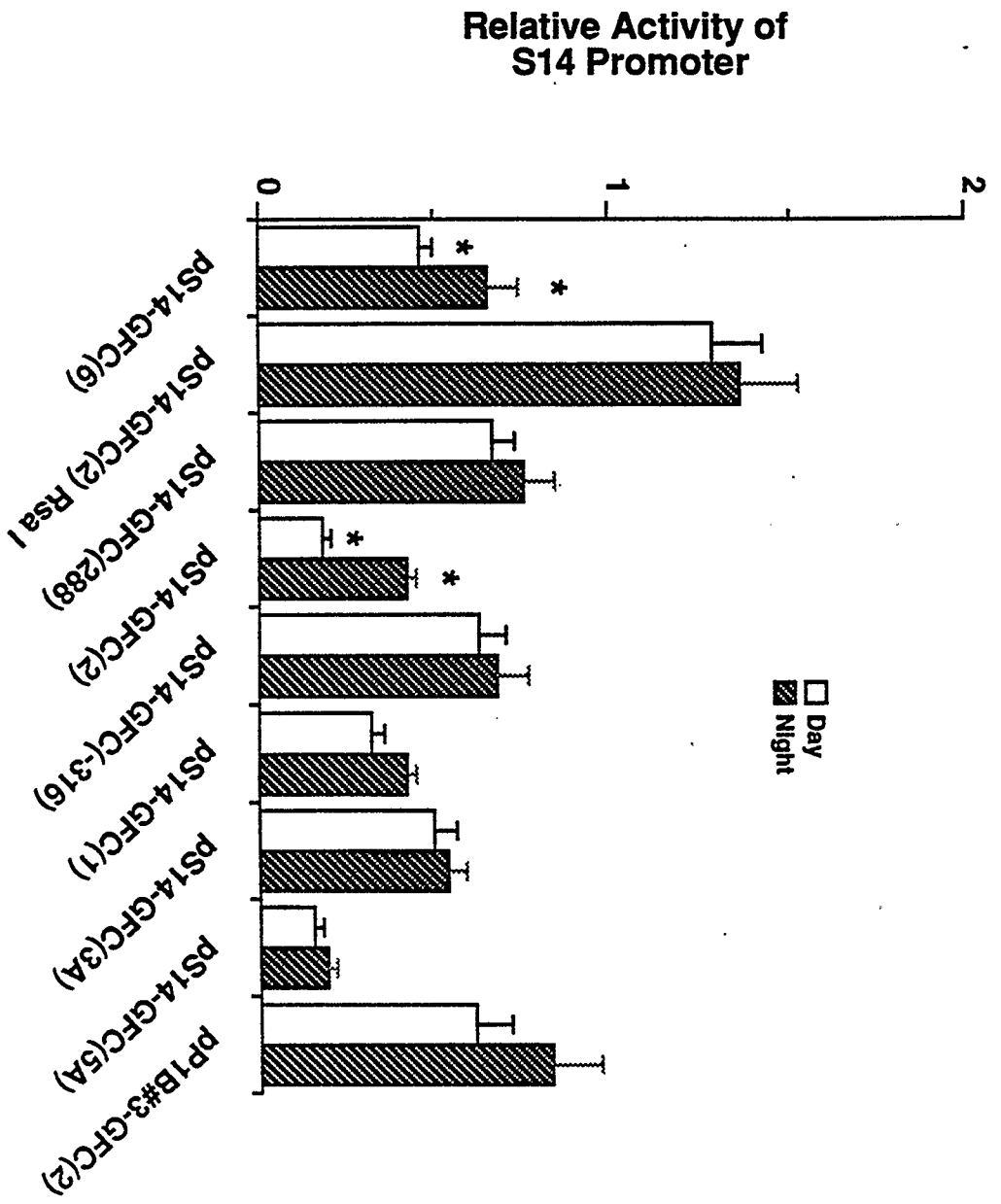

Fold Increase in Transcription
Relative to pS14-GFC(2)
15
10
5
0
pS14-GFC(2)
pP-1B#3-GFC(2)

10). The same construct had a transcription activity approximately 7.5 fold higher relative to pS14-GFC(2) when nuclear extracts were prepared from modified blender homogenization (figure 9). Together these results strongly suggest that PS-1 binding protein(s) plays an important role in the induction of the S14 gene transcription in vitro and that its action is mostly important in the hyperthyroid state.

The P-1 binding protein is associated with circadian regulation of the S14 gene

The S14 mRNA and protein levels are known to undergo a circadian variation. The level varies 3-fold between a maximum at 20:00 h and a minimum at 08:00 h (Jump et al. 1984, Kinlaw et al. 1987, 1989). To investigate the possibility that a regulatory region of the S14 gene may be involved in the circadian response of the S14 gene, unmanipulated rats were killed at 19:00 h. During this time S14 mRNA level is high and P-1 binding protein activity is at its maximum. The constructs containing different lengths of upstream region of the S14 gene and the internal control pAd_2MLP-200 were incubated in modified blender homogenization hepatonuclear extracts of rats killed at 19:00 h. Figure 11 illustrates the transcriptional activity of each construct in normal euthyroid hepatonuclear extracts from rats killed at 09:00 or 19:00 h. Each column represents the mean ± S.D.M. of at least 7 assays. Results marked with an asterisk are significantly different (p-value $\alpha \leq 0.05$) for that construct. Although most of the constructs had a higher transcription efficiency in the presence of "night" extracts than in the presence of the "day" extracts, the results were not significantly different.

Figure 11. Relative rate of in vitro transcription of the S14 constructs with day and night hepatonuclear extracts. Values represent ratios of the S14 templates to the pAd_2MLP-200 products quantitated by β-scintillation counting. The bar graph values represent the mean ± S.D.M. of at least 7 assays. Day and night results marked with an asterisk are significantly different ($p \leq 0.05$) for that construct. The nuclear extracts were obtained from blender homogenized livers.

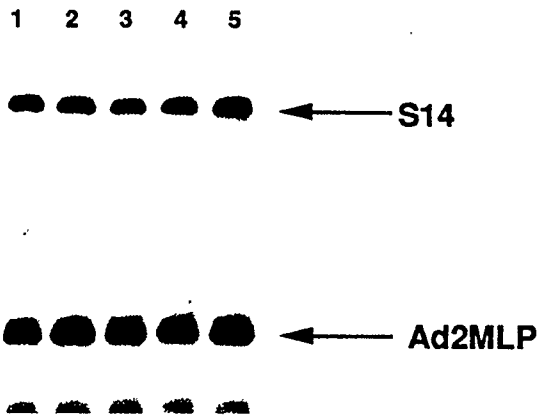

Relative Activity of
S14 Promoter
0
1
2
Day
Night
pS14-GFC(6)
pS14-GFC(2) Rsa I
pS14-GFC(288)
pS14-GFC(2)
pS14-GFC(-316)
pS14-GFC(1)
pS14-GFC(3A)
pS14-GFC(5A)
pP1B#3-GFC(2)

Interestingly, the transcriptional activity of the pS14-GFC(2) construct was 2.4-fold higher in the presence of "night" relative to "day" extracts. As mentioned previously, the pS14-GFC(2) construct contains part of the P-1 binding site. Previous findings showed that DNA binding activity of P-1 binding protein was increased 3- to 4-fold in the evening (Wong et al. 1989). This observation paralleled the circadian variation of hepatic S14 mRNA and protein. The P-1 binding protein could be detected only in the liver, a tissue in which S14 mRNA and protein fluctuate in a circadian pattern. This synchrony raised speculations that the circadian pattern of the hepatic S14 gene expression may be related to the differences in P-1 binding activity between day and night. The 2.4-fold increase in transcription activity of the pS14-GFC(2) construct in "night" extracts over its activity in "day" extracts correlates with the increase in S14 mRNA and binding activity of the P-1 protein. Therefore, the in vitro transcription results strongly support the idea that P-1 binding protein participates in the circadian variation in S14 gene expression.

Thyroid hormone responsive element

To examine the role of T_3-receptor complex in S14 gene transcription, a template containing 4 copies of a synthetic TRE was inserted 5′ to the -441 to -2 sequence of pS14-GFC(1). This new construct was called pS14-GFC(1)-TRE. The chosen TRE was the synthetic dimer of a 16 bp DNA sequence (TCAGGTCATGACCTGA) described by Glass et al. (1988). In cotransfection studies, the 16 bp TRE induced up to 200-fold transcription of the thymidine kinase-chloramphenicol acetyltransferase (TK-CAT) fusion

Figure 12. Representative autoradiogram of in vitro transcriptional activity of S14 construct containing multiple TREs. Each reaction contained the internal control pAd_2MLP-200 (0.05 μg) and 84 μg of nuclear proteins obtained from blender homogenized euthyroid livers. The test DNA templates were: lane, pS14-GFC(1) (1.5 μg); lane 2, pS14-GFC(1)-TRE (1.5 μg); lane 3, pS14-GFC(1)-TRE + 1 μl of 10^{-6} M T_3; lane 4, pS14-GFC(1)-TRE mutant (1.5 μg); and lane 5, pS14-GFC(1)-TRE mutant (1.5 μg) + 1 μl of 10^{-6} M T_3. See experimental procedures for the details on the construction of the different templates.


1 2 3 4 5
S14
Ad2MLP

gene (Damm et al. 1989). Results in Figure 12 illustrates activity of the pS14-GFC(1)-TRE template in hepatonuclear extracts from animals of 3 thyroid states. The addition of T_3 to a final concentration of $5x10^{-8}M$ failed to increase pS14-GFC(1) transcription with or without the TRE. Lanes 4 and 5 represent the results obtained when a construct containing 3 bp deletions in one half of the 16 bp palindromic TRE (see Experimental procedures) was incubated in absence (lane 4) or presence (lane 5) of T_3. Analysis of the results did not reveal any significant difference in template activity using hepatonuclear extracts from animals of 3 thyroid states and in presence or absence of T_3. To be certain that the T_3 receptor was not inactivated due to freezing of the nuclear extracts, similar experiments were performed using freshly prepared extracts. Again, the addition of T_3 failed to increase the transcriptional activity of our templates. These findings suggest that the T_3-receptor is either absent from the protein extracts or, more likely, present but in an irreversibly inactive form (Apriletti et al. 1988).

The -1635 to -2 S14 DNA sequence is insufficient to show carbohydrate regulation of the S14 gene

S14 gene expression is also increased by feeding rats a high carbohydrate, fat-free (lipogenic) diet (Mariash et al. 1986). In an attempt to define a putative carbohydrate responsive element in the S14 DNA 5′ flanking region, the effect of a lipogenic diet on the S14 gene transcription was examined. Activity of 5 S14 constructs was measured, pS14-GFC(6), (2), (1), (3A), and (5A). Rats were fed a high carbohydrate diet for 7 days and killed between 09:00 and 11:00 h on the day of extract

preparation. Each template and the pAd_2MLP-200 internal control were incubated in carbohydrate-fed rat hepatonuclear extracts. No significant difference was observed in the transcriptional efficiency of any of the constructs between hepatonuclear extracts obtained from rats fed the standard rat chow diet or the lipogenic diet (data not shown). These results suggest that the putative cis-regulatory element for carbohydrate modulation of S14 gene expression may be located upstream of the -1635 position. Another possible explanation is that the factor leading to an increased expression of the S14 gene may be absent from the protein extract, or irreversibly inactivated.

The preinitiation complex formation appears unaffected by the thyroid status

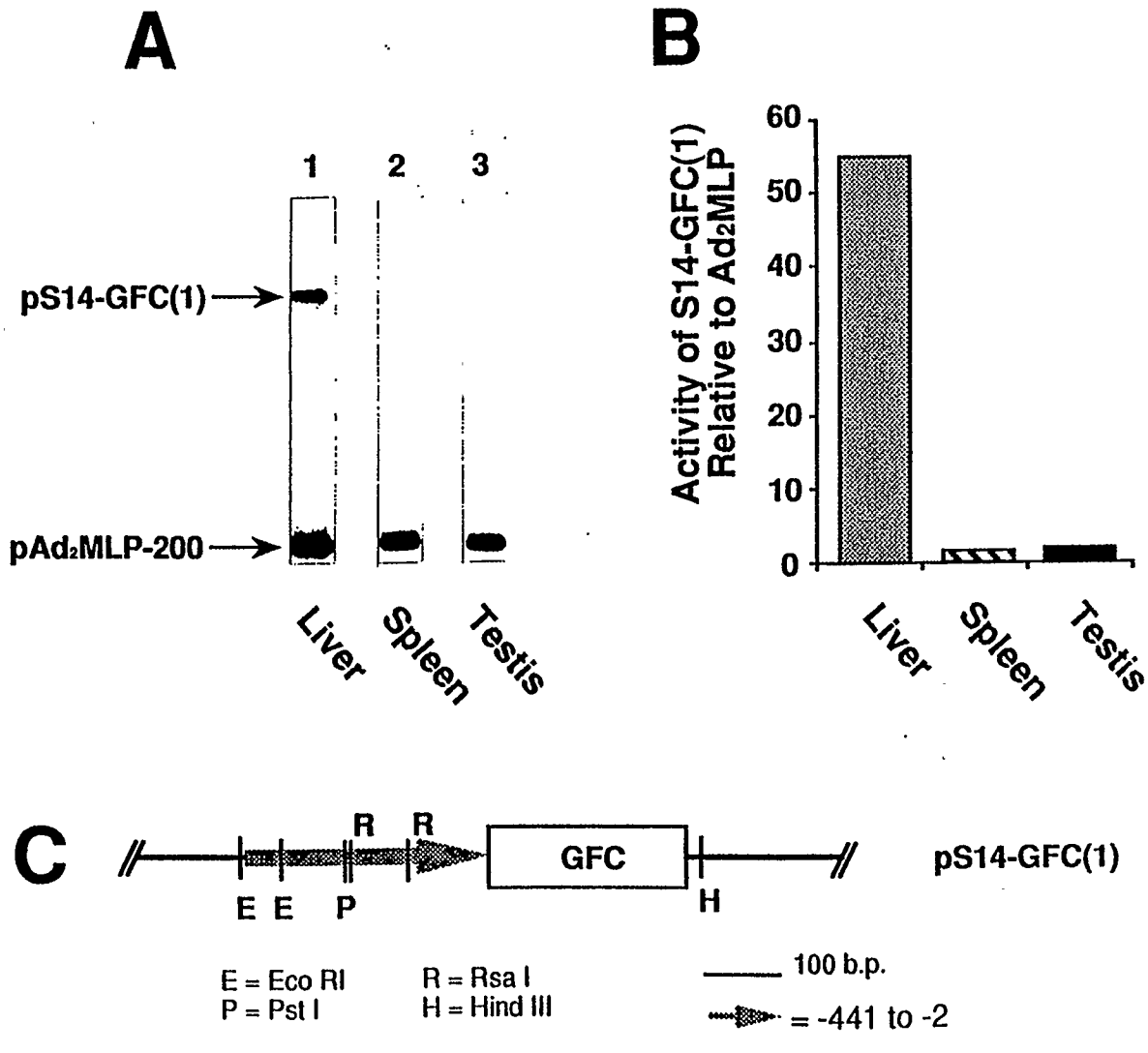
One important aspect of transcription that may be influenced by T_3 is the process of preinitiation complex formation. To study this process, Buratowski et al. (1988) used excess poly (dIdC) to inhibit preinitiation complex formation. The quantity of poly (dIdC) (200 μg) added to the incubation mix stopped any further preinitiation complex formation without inhibiting transcription by the complexes already assembled. Accordingly, the internal control pAd_2MLP-200 was preincubated in hepatonuclear extracts from animals of 3 thyroid states for 10 minutes on ice to allow optimal complex formation on the control template. The pS14-GFC(1) construct was then added followed by the addition of 200 μg of poly (dIdC) at different times from 0 to 10 minutes during the preincubation time on ice. The incubation with α-^{32}P-UTP was the standard 45 minutes at 30°C. The addition of 200 μg of poly (dIdC) to the incubation mix before the pS14-

GFC(1) template completely blocked the transcription of the GFC by the pS14-GFC(1) template but not by the internal control. This result shows that the conditions were favourable for the poly (dIdC) to block preinitiation complex formation (data not shown). Within the sensitivity of our in vitro transcriptional assay, no significant variation in the rapidity of complex formation could be measured in the different thyroid states (data not shown). These results suggest that T_3 had no effect on formation of the preinitiation complex.

The -86 to -2 S14 DNA sequence is sufficient for tissue specific expression of the GFC

The S14 gene is highly expressed in lipogenic tissues such as liver, lactating mammary gland, and brown and white adipose tissue (Jump and Oppenheimer 1985). Therefore, this prompted studies to determine whether tissue specific transcription of the S14 DNA was evident using the pS14-GFC(1) and pS14-GFC(6) templates in the presence of liver, testis, and spleen extracts. Testis and spleen tissues were chosen because S14 mRNA is expressed at very low level in these extra-hepatic tissues: 0.07 and 0.3% in testis and spleen, respectively. Figure 13, panel A illustrates the relative transcriptional activity in euthyroid nuclear extracts from liver, spleen, and testis. The transcription of the pAd_2MLP-200 internal control was not different between the tissues showing that the extracts were equally active. Panel B shows that the transcription activity of pS14-GFC(1) in liver nuclear extracts was 40- and 55-fold higher than in the testis and spleen extracts, respectively. Similar results were obtained with pS14-GFC(6) (data not shown). This result is in agreement

Figure 13. Transcription of the S14 promoter in nuclear extracts from extrahepatic tissues. Panel A. Radiolabeled RNA products from incubations containing pS14-GFC(1) (1.5 μg) and the control pAd_2MLP-200 (0.05 μg) in euthyroid liver, spleen, and testis extracts obtained from blender homogenized tissues. The radiolabeled RNA products from the pS14-GFC(1) and pAd_2MLP-200 templates migrated as the upper and lower band, respectively. Panel B. Transcription activity of pS14-GFC(1) template relative to the activity of the pAd_2MLP-200 control in euthyroid liver, spleen, and testis extracts. The ratios of pS14-GFC(1) to the pAd_2-MLP-200 products were quantitated by β-scintillation counting. Panel C. Map of the pS14-GFC(1) construct.


A
1
2
3
pS14-GFC(1)
pAd2MLP-200
Liver
Spleen
Testis
B
Activity of S14-GFC(1) Relative to Ad2MLP
60
50
40
30
20
10
0
Liver
Spleen
Testis
C
R
R
GFC
E
E
P
H
pS14-GFC(1)
E = Eco RI
P = Pst I
R = Rsa I
H = Hind III
100 b.p.
= -441 to -2

with the tissue distribution of S14 mRNA.

Mixing experiments were performed to determine if the diminished S14 transcriptional activity in testis and spleen was due to the absence of an activator or the presence of an inhibitor. In these studies extracts from spleen and liver or testis and liver were mixed together prior to incubation with DNA template. Figure 14 shows the results obtained when the pS14-GFC(1) construct (1.5 μg) and the internal control pAd_2MLP-200 (0.05 μg) were incubated with decreasing amounts of spleen nuclear extracts and increasing amounts of liver nuclear extracts. pAd_2MLP-200 was equally transcribed in all the incubations showing that lower transcription of pS14-GFC(1) in spleen extracts was not due to decreased activity of the spleen extract to transcribe the template. A linear dose-dependent increase in activity of the pS14-GFC(1) template was observed when increased amounts of hepatic extract was added to the mixture (figure 15). These results indicate that the inability of spleen nuclear extracts to promote efficient S14 gene transcription in vitro is due to the absence of one or more essential activating factors rather than the presence of an inhibitor of transcription. Similar results were obtained when testis nuclear extracts were mixed with increasing quantities of liver nuclear extracts (data not shown). These results demonstrate that the transcriptional activity of -86 to -2 S14 DNA mimicks closely the tissue specific expression of the gene.

Figure 14. Representative autoradiogram of in vitro transcription activity of pS14-GFC(1) template in mixtures of liver and spleen nuclear extracts. The reactions contained 1.5 μg of pS14-GFC(1) template and 0.05 μg of the internal control pAd_2MLP-200. The radiolabeled RNA product from the pS14-GFC(1) construct migrated as the upper band, the product from pAd_2MLP-200 as the lower band. The proportions of each extract added to the reactions were in lanes 1 to 7: 100, 82, 67, 53, 31, 14, and 0% spleen extract, respectively and 0, 18, 33, 47, 69, 86, and 100% liver extract, respectively. The nuclear extracts were obtained from blender homogenized euthyroid tissues.

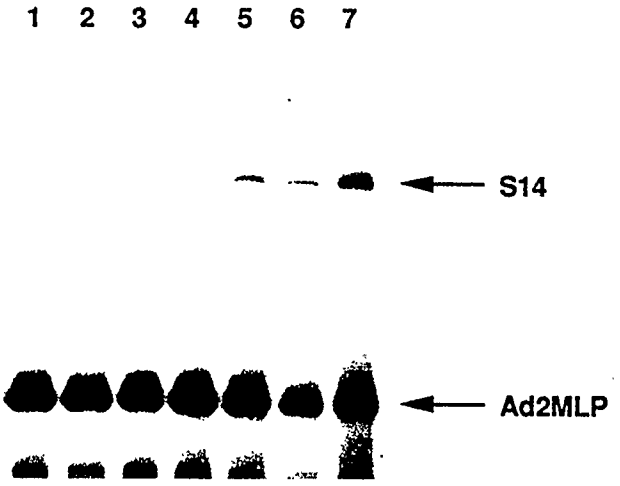
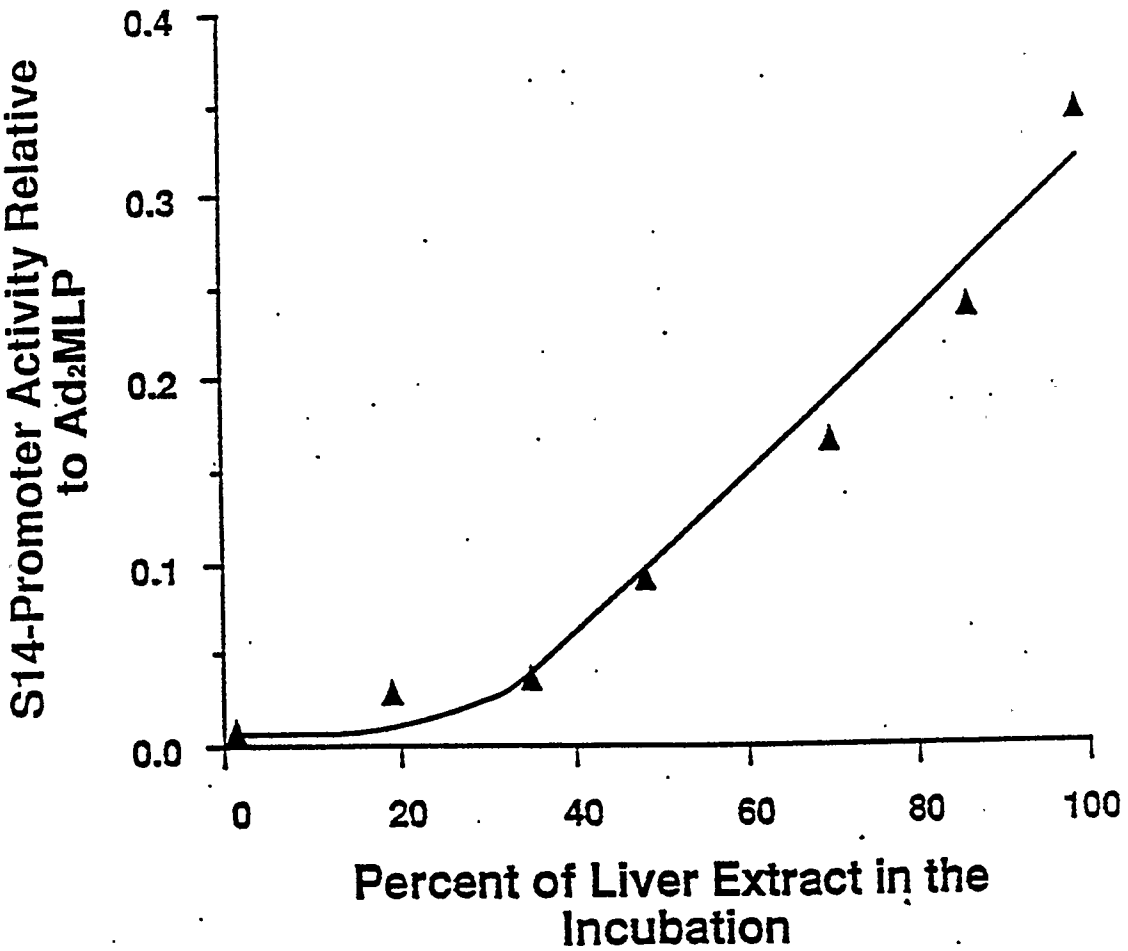

1 2 3 4 5 6 7
S14
Ad2MLP

Figure 15. Relative activity of the S14 promoter in mixtures of nuclear extracts from spleen and liver extracts. The transcription activity of pS14-GFC(1) template relative to the activity of the control pAd_2MLP-200 during the mixing experiment were quantitated by β-scintillation counting of the bands in figure 14. Data points corresponding to lanes 1 to 7, show the effect of increasing amounts of liver extract in the reaction.


S14-Promoter Activity Relative to Ad2MLP
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
Percent of Liver Extract in the Incubation

DISCUSSION

The rapid induction of rat hepatic S14 gene mRNA by T_3 makes it an ideal model for studying the hormonal regulation of gene expression. A significant rise in the levels of hepatic S14 heterogenous nuclear RNA is detectable within 10 minutes following the injection of T_3 into hypothyroid rats (Narayan et al. 1984). Such a rapid response suggests that T_3 has a direct effect on S14 gene transcription. Recent studies attempting to understand the tissue specific expression of the S14 gene demonstrated the presence of at least 6 DNase I hypersensitive sites (HS) in chromatin flanking the 5' end of the gene (Jump et al. 1987, 1988, Jump 1989). The enhanced endonuclease sensitivity of these sites indicate a local opening in the nucleosomal packaging of the chromatin. Such alterations in nucleosomal structure permit the interaction of DNA with transcription factors which regulate the expression of specific genes (Reviewed by Gross and Garrard, 1988).

The HS-1 site of S14 chromatin is found only in lipogenic tissues where the S14 gene is abundantly expressed and regulated by T_3 (Jump 1989). The close proximity of the HS-1 site to the transcription initiation site suggests that the proteins associated with it must play important roles in regulating S14 gene expression. Using the gel-shift assay to examine crude nuclear extracts, previous studies demonstrated the binding of at least two nuclear proteins, P-1 and PS-1, to DNA sequences contained in the S14 HS-1 site. P-1 and PS-1 bind to nucleotides -310 to -288 and -63 to -48 of S14 DNA, respectively (Wong et al. 1989, 1990). In this thesis, I report the adaptation of a cell-free in vitro transcriptional assay used

to examine the potential roles of P-1 and PS-1 in S14 gene transcription. This assay provided the opportunity to examine the function of both P-1 and PS-1 binding proteins. In addition, the use of this assay made possible the investigation of other 5' cis-regulatory sequences in close proximity to the P-1 and PS-1 binding sites.

Cell-free in vitro transcription assay conditions

The initial step was to optimize the various parameters of the cell-free in vitro transcriptional assay. Results showed that the optimums of protein, DNA and salt concentrations were higher than those reported previously by Gorski et al. (1986). They reported optimums of 60 to 100 μg of proteins, 0.8 μg of template, 50 mM KCl and 6 mM $MgCl_2$. In the current study 80 to 160 μg of proteins, 1.5 μg of template, 100 mM KCl and 17 mM $MgCl_2$ were the optimums for the assay. One possible explanation for the apparent discrepancies could be due to the fact that the promoter used in this study was different from the ones examined by Gorski et al. (1986). The optimum protein concentration used in the assay varied somewhat in different preparations of extract. Regardless of the differences in assay conditions, the results show that relative S14 promoter activity (depending on construct tested) was consistent from one extract to the next. Since most extracts were prepared with tissues taken from at least two rats, this rules out the possibility that animal to animal variations was the basis for the differences. Therefore, it was important that the optimum protein concentration be determined for each extract preparation to maximize the transcription signals.

Role of the PS-1 protein(s)

We have previously shown that a nuclear protein PS-1 binds to the -63 to -48 sequence of S14 DNA. Using the cell-free assay, I showed that the S14 DNA -441 to -2, pS14-GFC(1) in the 5′ to 3′ orientation, promotes transcription of the GFC. Deletion of the DNA such that the promoter element contained only the -86 to -2 sequence, pS14-GFC(6) showed that it was equally active in transcription of the GFC. This observation seems to suggest that the -441 to -87 sequence is probably not essential for promoting transcription of the GFC. The transcription rate of both pS14-GFC(1) and (6) increased 1.6- and 2.7-fold in eu- and hyperthyroid, respectively relative to the hypothyroid state (figure 7, panel A). The increased transcription correlated with the 2- and 4-fold rise in PS-1 binding activity in eu- and hyperthyroid nuclear extracts, respectively (Wong et al. 1990). Since the PS-1 site resembles closely the CAAT-motif in both DNA sequence and positioning, this suggests that T_3 may enhance S14 promoter activity via increases in binding of a CAAT-transcription factor. Competition studies demonstrated that displacement of PS-1 binding inhibited by 93% transcription of the pS14-GFC(6) template in hyperthyroid but not in hypothyroid extracts (Figure 7, panel C). In addition, the 7.5-fold increase in transcription of pP-1B#3-GFC(2) containing 3 copies of the PS-1 attached to pS14-GFC(2) (Figure 10 and Figure 9, last lane), reinforces the idea that the site acts to enhance S14 transcription in hyperthyroid animals. Since competition studies with PS-1 site oligomers did not affect activity of the pS14-GFC(6) construct in hypothyroid extracts (Figure 7, panel C), this suggests that PS-1 probably plays a minor role, if any, in basal transcription of the S14 gene.

Role of P-1 protein

The P-1 protein binds to the -310 to -288 sequence of the S14 gene. Its binding activity increases 3- to 4-fold in the evening (Wong et al. 1989) paralleling the circadian 3-fold variation in hepatic S14 mRNA (Jump et al. 1984). P-1 binding activity was present only in the liver, a tissue in which S14 mRNA undergoes circadian variation (Wong et al. 1989). These results suggest that P-1 may be involved in controlling some aspect of circadian variation in S14 gene expression. The construct pS14-GFC(2) containing a portion of the P-1 binding site was the only one to show a large increase (2.4-fold) in transcription activity in extracts obtained from rats killed at night (Figure 11, lane 4). This result supports strongly the role of P-1 in the circadian regulation of the S14 gene.

The results dealing with the function of P-1 is puzzling and represents conflicting roles in S14 gene transcription. The deletion studies using GFC(2)-Rsa I, GFC(2), GFC(316) indicate a strong negative element is present in the region between -279 to -296 with a moderate enhancement between -296 to -316. The P-1 protein binds to -288 to -310 thus spanning both negative and positive regions. It is conceivable that P-1 may serve two functions or P-1 may consist of two proteins. For example, if P-1 is a single protein with two DNA binding fingers (ie. -288 to -310 = 22 bp or 2 turns of the DNA) if one finger binds to the 3' major groove then it is a strong repressor but the binding of both fingers moderates its suppressive activity. This model fits nicely with the observation that transcription of the GFC(2) construct is less than that of GFC(-316). However, it can not explain the 2.4-fold increase in GFC(2)

transcription during the night. One possible explanation to account for this observation may involve enhancer(s) located 3' to the -279 position which overcomes the effects of P-1 during the evening.

It is equally possible that P-1 may consist of 2 proteins each with contrasting functions. The close proximity of these two factors would suggest that direct protein-protein interaction could determine the overall effect of the two factors in determining activity of the templates. However, this protein does not account for all the increase in transcription activity at night since constructs containing S14 DNA sequences without P-1 binding site (Figure 11, lanes 1-3) also show a slight increase in transcription activity. P-1 could therefore act as a moderator of S14 gene transcription. It could control or limit the induction brought about by strong positive transcription factors binding to S14 promoter sequences.

The carbohydrate-responsive sequences are located upstream of the -1635 position relative to the S14 cap site

The S14 gene expression is also modulated by dietary carbohydrates (Mariash et al. 1986). Attempts to define a cis-regulatory sequence responsive to carbohydrates indicated that it was not located within the -1635 to -2 S14 DNA sequence. One possible explanation for these findings is that the DNA element(s) responsible for the response to dietary carbohydrates is not present in the promoter region analyzed. This is in agreement with the transfection studies performed by Jacoby et al. (1989). They reported the presence of multiple carbohydrate responsive elements within the -4316 to -1601 sequences relative to the S14 gene transcription

initiation sites with the major responsive elements located within sequences spanning nucleotides -4316 to -2603.

Tissue specificity is conferred by a short S14 promoter sequence

The tissue specificity in S14 gene transcription was localized to the first 86 bp of upstream sequence. The transcription activity obtained with the constructs pS14-GFC(6) (data not shown) and pS14-GFC(1) using spleen and testis nuclear extracts (Figure 13) correlated closely with the level of S14 mRNA in the extra-hepatic tissues (0.3% and 0.07% in spleen and testis, respectively. Jump and Oppenheimer, 1985). These results suggest that factor(s) responsible for tissue specificity interact with the first 86 bp of the S14 upstream region. The ability of such short sequences to impart tissue specific gene expression was also demonstrated for other genes using a similar in vitro transcription system. For example, Ryffel et al. (1989) reported that the tissue specificity of the Xenopus 68 Kd albumin gene could be conferred by as little as the TATA box and the HP-1, a hepatocyte-specific promoter element. They showed that the TATA box together with the HP-1 site located within 70 bp of each other were sufficient and necessary to generate a minimal promoter with liver-specific activity. Gorski et al. (1986) showed that the mouse albumin gene promoter sequences located between -170 and -55 were required for liver-specific in vitro transcription. Furthermore, this same promoter sequence, when inserted upstream of the parotid-specific Amy-1 promoter, increased the activity of this promoter in liver nuclear extracts to a level comparable to that observed for the albumin promoter. Döbbelin et al. (1988) detected an activator located between -121 and -87

of the A_2 vitellogenin gene that functioned in a liver-specific manner. Monaci et al. (1988) reported that the -137 to -2 promoter sequence of the human α1-antitrypsin gene directs liver specific transcription in vitro. Therefore, it appears that in those cases the first 200 bp upstream of the transcriptional start site plays a crucial role in the tissue-specific expression of the gene.

The results of the mixing experiments suggest that the lack of S14 gene transcription in both spleen (figure 14 and 15) and testis (data not shown) extracts may be due to the absence of tissue-specific transcription activator(s). The requirements of tissue-specific activators as opposed to the presence of inhibitors to confer tissue-specific gene expression is supported by other studies. Using the same assay, Lichtsteiner and Schibler (1989) restored the transcription activity of the liver-specific albumin promoter in spleen extracts by adding purified liver-specific transcription factor, HNF-1. The same group of investigators (Lichtsteiner et al. 1987) demonstrated that 4 of the 6 cis-regulatory elements present within the -164 to -32 sequence of the albumin promoter were occupied by nuclear factors that are several fold more abundant in liver than in spleen or brain. They suggested that the binding of these proteins to the albumin 5′ flanking sequence may account for the activity of the albumin promoter in vitro. In agreement with my results, tissue-specific S14 gene expression appears to be dependent on the presence of tissue-specific transcription activators interacting with proximal promoter sequences of the gene.

Transcription activity was not enhanced by presence of the TRE

The construct pS14-GFC(1)-TRE, containing 4 synthetic thyroid hormone responsive elements (TRE), failed to increase the transcriptional activity of the construct (Figure 12). Incubation of pS14-GFC(1)-TRE in extracts from the 3 thyroid states and addition of T_3 directly to the incubation showed no significant difference in activity of the template. Previous studies reported that the same TRE induced up to 200-fold the transcription of a reporter gene in cotransfection studies following the addition of T_3 to in the culture medium (Damm et al. 1989). Corthesy et al. (1988), using the same assay, succeeded in obtaining oestrogen-responsiveness of heterologous constructs that mimicked the hormonally modulated expression of vitellogenin in vivo. Following addition of 17β-estradiol, they measured a 20-fold induction in transcription activity of a construct containing a native oestrogen-responsive element located within the -596 to +8 sequences of the vitellogenin promoter. These results indicate that the cell-free in vitro transcription assay that we utilized can reproduce hormone responsiveness. Therefore, an explanation for our negative results could be that the T_3-receptor or nuclear factors important for T_3-receptor binding to the TRE may be absent or irreversibly inactivated during the nuclear protein extraction procedure. This is in agreement with the known instability of T_3-receptor (Apriletti et al. 1989 and references therein). Furthermore, recent studies indicate the presence of a nuclear protein that enhances binding of the T_3-receptor to TRE (Burnside et al. 1990). Therefore, it is possible that a lack of response to T_3 in this assay may be due to an inactivity of this ancillary factor. Other studies also suggest that the TRE may have failed to

increase the transcription because our assay utilizes purified, naked DNA rather than reassembled chromatin. Recent reports suggest that histone proteins may play important roles in the organization of T_3-receptor in chromatin and thus preventing the loss of receptor-ligand binding activities (Ichikawa et al. 1987, Sakurai et al. 1989 and references therein).

T_3 modifies nuclear factors in vivo that remain active in the nuclear extracts

T_3 had a modest affect on transcriptional activity of the various S14 promoter constructs. A 2.7-fold increase in activity of the constructs pS14-GFC(6) and pS14-GFC(1) during the transition from hypo- to hyperthyroid state was measured when nuclear extracts obtained from hand homogenized tissues were used. The rise in activity of both templates did not match the 9-fold increase in the transcriptional run-off rate reported by Jump (1989). This could be explained partly by the limited amount of upstream S14 DNA present in these constructs or the possibility that the very rapidly turning over factor(s) necessary for both induction and maintenance of the S14 mRNA (Seelig et al. 1982, Jacoby et al. 1987) may be lost and/or degraded during the process of nuclear protein extraction.

The observed changes in transcription of S14 templates using hepatonuclear proteins from animals of the 3 thyroid states indicate that factors which mediate these changes must be present in the extracts. Furthermore, T_3 added prior to incubation with the S14 templates failed to alter transcriptional activity. Together these results suggest a novel

mechanism of T_3 action that may involve the modification of factor(s) in vivo which remain active in the extracts. The nature of the nuclear factor modification resulting from the actions of T_3 remain unknown. I speculate that the T_3 induction of S14 gene expression is, in part, regulated by pre-existing transcription factors the activity of which is modified by the hormone. Two possible processes involved in the post-translational modification of transcription factors have recently been described: phosphorylation and glycosylation. Phosphorylation has been shown to increase activity of a pre-existing eukaryotic transcription factor, NF-κB. NF-κB is a stage-specific factor that interacts with a specific site in the immunoglobulin κ gene enhancer. Sen and Baltimore (1986) suggested that the activation event of NF-κB depends on phosphorylation of a pre-NF-κB. Angel et al. (1987) and Lee et al. (1987) reported that post-translational phosphorylation directly or indirectly modulated the activity of the human metallothionein IIA gene enhancer binding protein AP-1. Yamamoto et al. (1988) showed that phosphorylation induces binding, dimerization, and transcriptional efficiency of the nuclear factor CREB, a phosphoprotein that binds the cAMP-responsive element of the rat somatostatin gene.

Glycosylation of proteins has been reported to play important roles in protein stability and function (Reviewed by Hart et al. 1988). Sp1 is a factor that activates transcription of various genes through binding to GC-boxes. Jackson and Tjian (1988) showed that glycosylation increases the transcriptional activation properties of the human Sp1 transcription factor without affecting its DNA binding activity. They also investigated the glycosylation of 7 other RNA polymerase II transcription factors

isolated from human HeLa cells and Drosophila embryos (AP-1, AP-2, AP-4, CTF, Zeste, GAGA, and Adf-1). These factors were all glycosylated albeit approximately 3- to 5-fold less than Sp1. Lichtsteiner and Schibler (1989) reported that the liver specific HNF-1 mentioned earlier is also glycosylated. Thus, post-translational modification appears to be a prevalent mechanism by which the activity of transcription factors is regulated.

Conclusions

The size of the S14 chromatin DNase I hypersensitive site I (200-300 bp) together with the results from our deletional studies suggests the presence of at least four cis-regulatory elements within the proximal promoter region of the S14 gene. We have shown previously that the binding activity of both P-1 and PS-1 is modulated by the thyroid state (Wong et al. 1989, 1990). The putative S14 CAAT-transcription factor PS-1 was shown to bind to the -63 to -48 sequence (Wong et al. 1990). PS-1 is important in augmenting transcription of the S14-GFC constructs in hyper- but not in hypothyroid extracts. P-1 was shown to bind to the -310 to -288 sequence (Wong et al. 1989). The P-1 factor appears to act as a negative modulator of transcription and to be involved in some aspect of the circadian regulating of the S14 gene. In addition, our deletional studies suggests the presence of a strong positive and a negative cis-regulatory element within the sequences -279 to -87 and -441 to -288, respectively.

The cell-free in vitro transcription assay that I have adapted for studies of the S14 gene combined with transcription factor purification

techniques will open up a new field in trying to understand the mechanisms involved in T_3 regulation of the S14 gene expression. Data on the abundance, modification and interactions between the transacting factors that bind to the cis-regulatory elements of the S14 gene will help to reveal the overall regulatory machinery of the S14 gene.

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