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Transcriptional Regulation Of The Urea Cycle Enzyme Genes In The Liver Of Rana Catesbeiana Tadpoles During Spontaneous And Thyroid Hormone-induced Metamorphosis

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TRANSCRIPTIONAL REGULATION OF THE UREA CYCLE ENZYME GENES IN THE LIVER OF *RANA CATESBEIANA* TADPOLES DURING SPONTANEOUS AND THYROID HORMONE-INDUCED METAMORPHOSIS

by

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ABSTRACT

The ornithine urea cycle enzymes, carbamyl phosphate synthetase (CPS-1), ornithine transcarbamylase (OTC) and arginase, are liver-specific proteins. Their expression is coordinately activated during the metamorphosis of the *Rana catesbeiana* tadpole by thyroid hormone (TH), and their presence is critical for the shift of this amphibian from an aquatic, ammonotelic larva into a terrestrial, ureotelic adult. My studies were focused on analyzing the transcriptional regulation of the genes encoding these urea cycle enzymes and determining whether these genes were upregulated directly or indirectly by TH. With this thought in mind, I isolated and characterized the sequences in the promoter regions of the CPS-1 and OTC genes and found that they lacked thyroid hormone response elements (TREs). This observation implies that TH is not directly regulating the expression of these genes. However, the presence of C/EBP (CAATT/enhancer binding protein) binding elements in the promoter regions of both of these gene prompted the thought that this transcription factor may be TH-inducible and play a role in the TH-induced expression of the CPS-1 and OTC genes. Thus, I isolated and characterized cDNAs encoding two different C/EBP-like proteins. One of them, RcC/EBP-1, encodes a *Rana* homologue of the mammalian C/EBPα, and protein synthesized from it was found to bind specifically to the mammalian C/EBP-like sequences present in the *Rana* CPS-1 and OTC genes. Although no TREs are evident in the promoter region of this RcC/EBP-1 gene, Southern hybridizations

suggest that more than one copy of this gene is present in the *Rana* genome and Northern hybridizations indicate that at least one of them is upregulated by TH. The TH-induced upregulation of an RcC/EBP-1 mRNA is concurrent with the upregulation of mRNAs encoding a thyroid hormone direct-response gene, TRβ, and precedes, by at least 12 hours, the upregulation of mRNAs encoding CPS-1, OTC and arginase. These results imply that the TH-induced expression of urea cycle enzyme genes involves a cascade of molecular events in which a member of the RcC/EBP-1 family plays a role in orchestrating the expression of these genes in the liver of this tadpole during both spontaneous and TH-induced metamorphosis.

To my wife, Ye

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°C	degrees Celsius
AA	amino acid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine 5'-triphosphate
AL	argininosuccinate lyase
Arg	arginase
AS	argininosuccinate synthetase
Blotto	bovine lacto transfer technique optimizer, *i.e.* skim milk
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
CPS	carbamyl phosphate synthetase
cpm	count per minute
d	day
Da	Dalton
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethlypyrocarbonate
ddH_2O	double-distilled water
dGTP	deoxyguanosine 5'-triphosphate
DIG	digoxigenin
dNTP	deoxynucleotide 5'-triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
h	hour
HBD	hormone binding domain
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic

	acid
HRE	hormone response element
HS	heat shock
HSE	heat shock element
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobase
LSTF	liver-specific transcriptional factor
LSRE	liver-specific response element
min	minute
M_r	relative molecular mass
mRNA	messenger RNA
MW	molecular weight
HCO_3^-	bicarbonate ion
NH_4^+	ammonium ion
nt	nucleotide
ORF	open reading frame
OT	ornithine transporter
OTC	ornithine transcarbamylase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
P_i	inorganic phosphate
PMSF	phenylmethylsulfonyl fluoride
poly-A	polyadenylated
PP_i	diphosphate
RAR	retinoic acid receptor
RE	response element
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
T_3	3, 5, 3'-triiodothyronine
T_4	thyroxine
T/B	a ratio of tail length to body length

TCA	trichloroacetic acid
TH	thyroid hormone
TR	thyroid hormone receptor
TRAP	thyroid hormone auxiliary protein
TRE	thyroid hormone response element
Tris	tris (hydroxymethyl) aminomethane
UTP	uridine 5'-triphosphate
UTR	untranslated region
UV	ultraviolet

CHAPTER 1 GENERAL REVIEW OF THE MOLECULAR BIOLOGY OF AMPHIBIAN METAMORPHOSIS

1.1 General Introduction

All vertebrate organisms have the capacity to synthesize thyroid hormones *de novo*, and L-thyroxine (T_4) represents the major circulating form of this hormone (for a review see Eales, 1990). Removal of the 5' iodine moiety by tissue deiodinases (Galton, 1988) results in the production of the more active metabolite, 3,5,3'-L-triiodothyronine (T_3). Thyroid hormone (TH) regulates diverse aspects of cellular development and homeostasis (Ingbar, 1981; Glass and Rosenfeld, 1991). The effect of TH on postembryonic development is most dramatically exemplified in its ability to precociously induce amphibian metamorphosis (Gilbert and Frieden, 1981). During spontaneous metamorphosis, the endogenous levels of TH increase in the tadpole (Galton and St. Germain, 1985) and are maximal at the beginning of metamorphic climax, a time in which the tadpole undergoes a series of rapid morphological, biochemical and behavioral postembryonic changes which remodel it into a juvenile frog (*e.g.* growth and development of its limbs and lungs, resorption of its tail and gills, synthesis of liver-specific urea cycle enzymes, etc.). Similar climatic-type responses in gene expression and gross metamorphic changes can be induced by exogenous TH administration to a premetamorphic tadpole (for reviews see Frieden, 1961; Frieden and Just, 1970; Cohen, 1970; Gilbert and Frieden, 1981; Galton, 1983, Shi, 1994; Atkinson, 1994).

Taylor and Kollros (1946) characterized the postembryonic development of the *Rana catesbeiana*, North American bullfrog into 25 different stages (stages I to XXV). These stage can be divided into three phases (Etkin, 1968): 1) Premetamorphosis (stages I to XII) is characterized by growth of the tadpole with no gross morphological changes; 2) Prometamorphosis (stages XIII to XIX) is characterized by a reduced rate of body growth and an acceleration of hindlimb growth; 3) Metamorphic climax (stages XX to XXV) is characterized by a cessation in body growth and extensive morphological and biochemical remodeling.

1.2 Thyroid Hormone (TH) Is Obligatory for Amphibian Metamorphosis

As early as 1912, Gudernatsch found that an extract of the thyroid gland could induce metamorphosis. The active ingredient was later identified as thyroid hormone (Etkin, 1935; Kollros, 1961; Dodd and Dodd, 1976). In the 1970's, several laboratories measured the T_4 and T_3 levels in pooled plasma from tadpoles at various stages of metamorphosis (Just, 1972; Leloup and Buscaglia, 1977; Miyauchi *et al.*, 1977; Krug *et al.*, 1978; Regard *et al.*, 1978), and demonstrated that TH levels begin rising in the premetamorphic tadpole, increase dramatically during prometamorphosis, and reach peak levels at metamorphic climax. Although the levels of TH decline in the froglet and remain low in the frog, they are still much higher than the levels during premetamorphosis. Immersion in, or a single injection of, an appropriate quantity of T_4 or T_3 can precociously induce the

metamorphic response in pre- and prometamorphic tadpoles in 3 to 5 days (Gilbert and Frieden, 1981). While all of these observations suggest that TH plays a paramount role in anuran metamorphosis, the mechanism by which it affects these metamorphic changes is poorly understood.

The identification of nuclear-localized TH receptors (TRs; Samuels *et al.*, 1973; Samuels and Tsai, 1973; Oppenheimer *et al.*, 1974), which bind TH with much higher affinity than cellular or plasma TH-binding proteins, led to the suggestion that TH controls metamorphosis by regulating gene expression (Galton, 1983). This presumption was supported when the avian and mammalian TRs were cloned and found to act as transcriptional factors (Evans, 1988; Green and Chambon, 1986). Since then, a new, molecular approach has been adopted to study this phenomenon.

1.2.1 Thyroid Hormone Receptors

The thyroid hormone receptors (TRs) belong to the steroid hormone receptor superfamily (Evans, 1988; Green and Chambon, 1986). Members of this family include receptors for glucocorticoids, androgens, estrogens, retinoic acid, etc., and they share three properties, namely, a nuclear site of action, binding to particular DNA elements, and the ability to regulate gene transcription. The most highly conserved region of these proteins is the DNA-binding domain (DBD) containing two "zinc fingers" (Evans and Hollenberg, 1988). The carboxyl-terminal hormone-binding domain, which varies according

to the specific receptor ligand, is absolutely essential for hormonal regulation.

1.2.1.1 Multiple TR Isoforms

The TRs are divided into two major groups, α and β , based on the sequence similarity to the v-*erb*-A and human placenta cDNAs, respectively (for reviews see Chatterjee and Tata, 1992; Lazar, 1993; Oppenheimer *et al.*, 1994; Tata, 1993; Tata, 1994; Yen and Chin, 1994). They are coded by two distinct genes. The human TRα gene resides on chromosome 17 (Dayton, *et al.*, 1984), whereas the human TRβ gene is located on chromosome 3 (Weinberger *et al.*, 1986). The cloning of TRα from human (Nakai *et al.*, 1988; Benbrook and Pfahl, 1987), rat (Thompson *et al.*, 1987), mouse (Prost *et al.*, 1988; Masuda *et al.*, 1990; Moeller *et al*, 1989), chicken (Sap *et al.*, 1986) *Xenopus laevis* (Yaoita *et al.*, 1990) and *Rana catesbeiana* (Schenider and Galton, 1991) and TRβ from human (Weinberger *et al.*, 1986), rat (Koenig *et al.*, 1988; Murray et al., 1988), mouse (Wood *et al.*, 1991), chicken (Forrest *et al.*, 1990; Showers *et al.*, 1991) *Xenopus laevis* (Brooks *et al.*, 1989; Yaoita *et al.*, 1990), and *Rana catesbeiana* (Helbing *et al.*, 1992; Davey *et al.*, 1994) clearly indicates that the existence of multiple TRs extends across a variety of species.

TR diversity is further increased by the generation of additional isoforms through alternative splicing of the primary transcripts from each of the α and β genes. The major products of the α gene are the α receptor isoform designated TRα1, and a second

protein, called TRα2, which does not bind thyroid hormone (Lazar *et al.*, 1988; Koenig *et al.*, 1989). Remarkably, there is evidence that the opposite strand of the α gene is also transcribed to produce another member of this receptor superfamily, called rev-TRα (Lazar *et al.*, 1989), the ligand of which is unknown. The β receptor gene also generates multiple proteins differing in their N-terminal composition (Hodin *et al.*, 1989).

1.2.1.2 Tissues-Specific Expression of TR Isoforms

Several functional differences between these receptor proteins have been noted in mammalian tissues, and the various isoforms are expressed in a tissue-specific manner (for reviews see Lazar, 1993; Oppenheimer *et al.*, 1994; Tata, 1993; Tata, 1994; Yen and Chin, 1994). TRα1 is found predominantly in brain, cardiac and skeletal muscle, and brown fat, whereas TRβ1 is most abundant in kidney and liver, and TRβ2 is found exclusively in the pituitary (Chin, 1991). In both the rat and chicken, the levels of TRα1 remain relatively constant in the brain during embryonic development, whereas TRβ1 expression in the brain is induced at birth or following hatching (Forrest *et al.*, 1991; Mellstrom *et al.*, 1991). Similarly, TRα and TRβ genes are also differentially regulated by thyroid hormone during amphibian metamorphosis (for reviews see Tata, 1993; Tata, 1994).

The specific roles of TRα and TRβ in development are poorly understood and it is not known if the levels of TRα and TRβ mRNAs accurately reflect tissue levels of their proteins. In certain instances, a discrepancy between mRNA and protein levels has been observed (Strait *et al.*, 1990; Lane *et al.*, 1991). However, an attractive hypothesis to explain tissue-specific effects of TH is that differential expression of TR variants determines the individual tissue's response to TH.

1.2.1.3 TRs in Amphibian Metamorphosis

The obligatory requirement of thyroid hormones, and hence TRs, for the development of some amphibian larvae to adults is firmly established (for reviews see Tata, 1993; Atkinson, 1994; Tata, 1994; Atkinson *et al.*, 1995). In *Xenopus laevis*, TRα is expressed at higher levels than TRβ in early development and is present in tissue even prior to the formation of the thyroid gland (Banker *et al.*, 1991; Kawahara *et al*, 1991; Yaoita and Brown, 1991). The appearance of TR mRNAs early in tadpole development correlates well with the ability of these tadpoles to respond to TH (Tata, 1970). As development proceeds, TRβ mRNA accumulation increases substantially, whereas TRα mRNA increases are modest, reaching the maximum levels by metamorphic climax (Baker and Tata, 1990; Yaoita and Brown, 1991). This pattern is compatible with the differential and rapidly increasing sensitivity of tadpole tissues to thyroid hormones as metamorphosis progresses. The presence of TR mRNAs in early developmental stages of *Xenopus* tadpoles was confirmed by *in situ*

hybridization analyses. Strong hybridization signals were recorded in tissues programmed for morphological and biochemical remodeling (brain and liver), *de novo* morphogenesis (limb buds) and cell death (tail, intestine) during T_3 induced metamorphosis (Kawahara *et al.*, 1991). *Rana catesbeiana* TRα and TRβ mRNA levels, like the levels in *Xenopus*, increase during both spontaneous and T_3-induced metamorphosis (Schneider and Galton, 1991; Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Chen *et al.*, 1994; Davey *et al.*, 1994; Helbing and Atkinson, 1994). In the liver of *Rana catesbeiana* tadpoles, the level of TRβ mRNA increases more than the level of TRα mRNA in response to exogenous T_3 (TRα ~3-fold and TRβ >50-fold; Helbing *et al.*, 1992; Atkinson *et al.*, 1994).

1.2.2 The Mechanism of Thyroid Hormone Action

Although TH regulates diverse aspects of cellular development and homeostasis (Ingbar, 1981; Glass and Rosenfeld, 1991), the discovery of TRs implies that some common underlying mechanism of TH action must be in place. Thyroid hormone is thought to enter a cell, move to the nucleus, and bind to a TR which is already bound to a thyroid hormone response element (TRE) in the promoter region of a target gene (Oppenheimer *et al.*, 1987; Evans, 1988). The formation of a ligand-bound TR complex with a thyroid hormone response element is, presumably, a necessary first step for activation or suppression of a target gene (Oppenheimer *et al.*, 1987; Evans, 1988). While the precise mechanisms of TH action is poorly understood, recent advances from a number of laboratories have

shed new light on various aspects of this phenomenon. For example, TRs usually bind to their TREs as dimers which include both homodimers, and dimerization with other, albeit related, nuclear proteins (*i.e.* heterodimers; see Figure 1 and Holloway *et al.*, 1990, Lazar *et al.*, 1991, Williams *et al.*, 1991; Yu *et al.*, 1991; Forman *et al.*, 1992; Kliewer *et al.*, 1992; Marks *et al.*, 1992; Yen *et al.*, 1992a; Yen *et al.*, 1992b; Zhang *et al.*, 1992; Yen and Chin, 1994). The DNA binding of these TR-complexes has been found to be differentially regulated by phosphorylation of the TR (Andersson *et al.*, 1992; Lin *et al.*, 1992; Miyamoto *et al.*, 1993; Yen *et al.*, 1994; 1993; Yen *et al.*, 1992b). Finally, TREs have been shown to consist of two half elements, whose consensus sequence can either be AGGTCA or AGGACA, arranged as a palindrome, a direct repeat with a spacing of 4 bp or as an inverted palindrome with a spacing of 6 bp (See Figure 2 and Glass *et al.*, 1988; Baniahmad *et al.*, 1990; Naar *et al.*, 1991; Umesono *et al.*, 1991; Andersson *et al.*, 1992; Miyamoto *et al.*, 1993; Yen *et al.*, 1993; Desvergne, 1994).

1.2.3 The Mechanism of Thyroid Hormone Action During Amphibian Metamorphosis

TH can induce a cascade of gene regulation in each tissue that undergoes a metamorphic response (for a review see Shi, 1994). The presumption is that TH binds to a TR and the resulting complex activates or represses a set of genes at the transcriptional level. If the products of these direct response genes are transcription factors, they are expected to regulate yet another set of genes which

Figure 1. Multiple thyroid hormone receptor complexes bind to thyroid hormone response elements (TREs). TRAP, thyroid hormone auxiliary protein; RXR, retinoid X receptor; RAR, retinoic acid receptor. This figure is modified from Yen and Chin (1994).

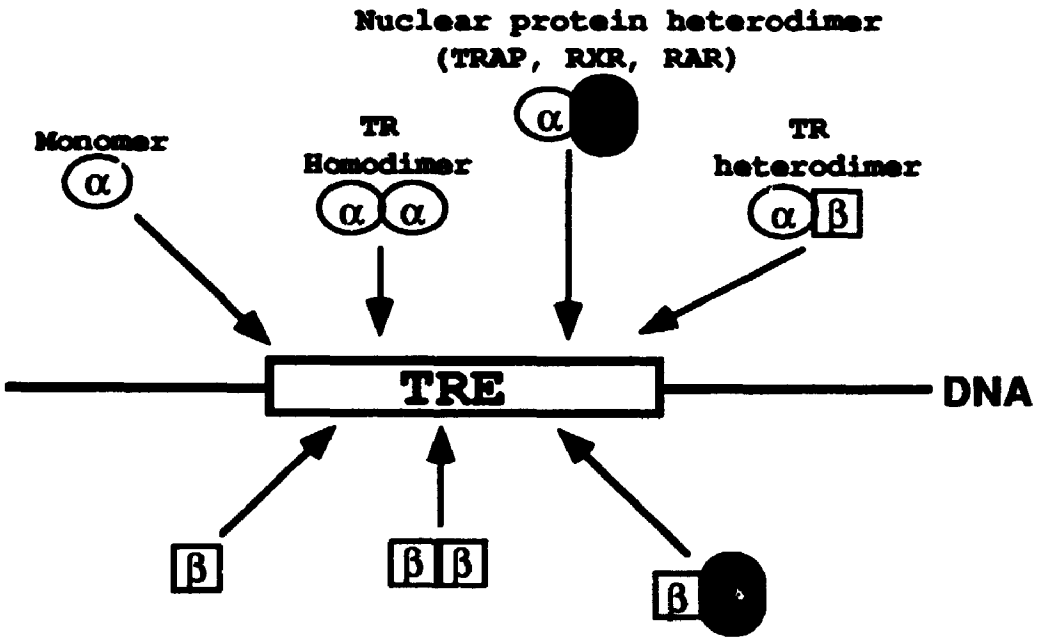

Nuclear protein heterodimer
(TRAP, RXR, RAR)
Monomer
TR
Homodimer
TR
heterodimer
α
α α
α β
TRE
DNA
β
β β
β

Figure 2. Spacing and orientation of thyroid hormone response elements (TREs).

Idealized TREs: a palindrome (TREpal), a direct repeat (DR), and an inverted palindrome (IP) are depicted. Boxes enclose half site sequences and arrows denote orientation of half site sequences. Nucleotide spacers between half-site sequences are designated by N with the optimum spacer number for each type of TRE depicted. The consensus TRE half-site is shown in the lower box. This figure is modified from Yen and Chin (1994).

TREs	Nucleotide Sequence
Palindrome (TREpal)	
Direct Repeat (DR)	
Inverted Palindrome (IP)	

Consensus TRE half-site:	AGGTCA A

characterize the tissue-specific phenotype. If the products of the direct response genes are not transcription factors, they could still be indirectly involved in regulating genes later in the process through other signal-transduction mechanisms. According to this model, tissue-specific changes during metamorphosis are determined by the activation or repression of tissue-specific genes at various steps of the cascade (Lazar, 1993; Oppenheimer *et al.*, 1994; Tata, 1993; Atkinson *et al.*, 1994; Tata, 1994; Yen and Chin; 1994).

Using a polymerase chain reaction (PCR)-based differential screening method (Wang and Brown, 1991), many TH responsive genes from the limb, tail, and intestine of *Xenopus laevis* tadpoles have been isolated. Some of thyroid hormone-induced genes expressed in the limb, tail, and intestine during amphibian metamorphosis are thought to be direct-responsive genes, that is, genes which are activated within a very short time after exposure to TH (4-8 h; Buckbinder and Brown, 1992; Shi and Brown, 1993; Wang and Brown, 1993). Other genes appear to have a delayed response, normally longer than 1 day. The late response of these genes suggests that they are not responding directly to TH but, instead, require the synthesis of some proteins, such as tissue-specific transcription factors, for their upregulation (for a review see Atkinson, 1994). Many of the TH late-response genes have been studied over the years (Morris, 1987; Schultz *et al.*, 1988; Mathisen and Miller, 1989; Shi and Brown, 1990; Helbing *et al.*, 1992; Buckbinder and Brown, 1992; Shi and Hayes, 1993; Xu *et al.*, 1993), and most, if not all, of these genes are tissue-specific. The activation or repression of these genes likely represents a terminal transition

induced by TH in a given tissue. One of the best-studied systems is the activation of the ornithine-urea cycle enzymes in the liver of some amphibians upon their transition from ammonotelism to ureotelism (for reviews see Frieden, 1961; Frieden and Just, 1970; Cohen, 1970; Gilbert and Frieden, 1981; Galton, 1983, Shi, 1994; Atkinson, 1994). The activity of the urea cycle enzymes is coordinately upregulated during both spontaneous and T_3-induced metamorphosis (for a review see Dodd and Dodd, 1976). This activation is associated with a dramatic increase in the mRNA levels for the genes encoding these enzymes (Morris, 1987; Helbing *et al.*, 1992; Xu *et al.*, 1993; Atkinson *et al.*, 1994; Chen *et al.*, 1994). The upregulation of these mRNAs requires about 1 to 2 days of T_3 treatment. Therefore, the activation of the urea cycle enzyme genes is thought to be a late-response to T_3. Similarly, the adult epidermal keratin gene (Mathisen and Miller, 1989) and the myosin heavy-chain gene (Buckbinder and Brown, 1992) in the amphibian also take about 2 days of T_3 treatment for their mRNAs to be upregulated. These results suggest that some other, albeit tissue-specific, transcriptional factors must be involved in the expression of these tissue-specific, TH-induced late-response genes.

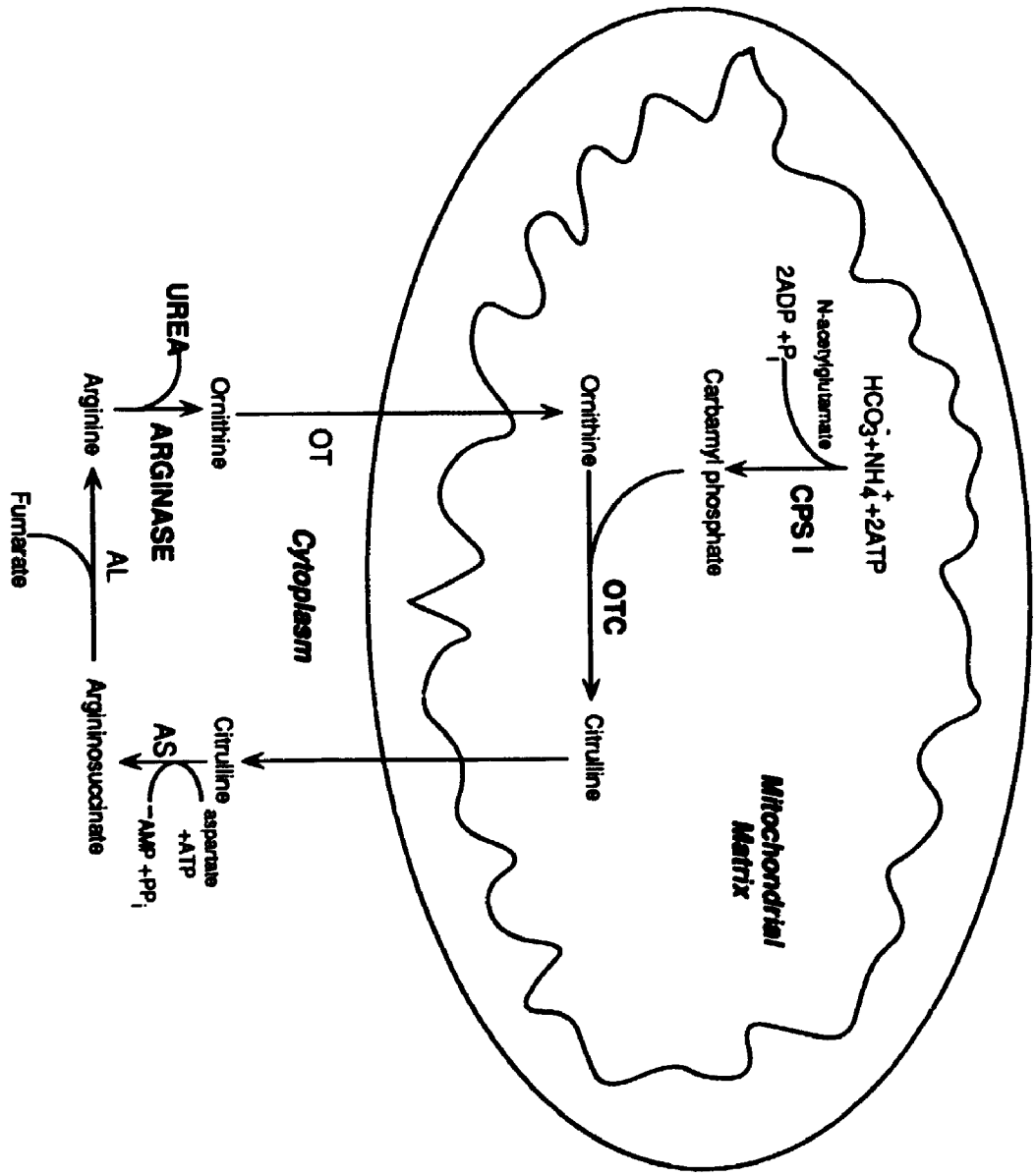
1.2.4 Thyroid Hormone Affects Gene Expression in the Tadpole Liver during Amphibian Metamorphosis

The liver of adult amphibians is the source of serum proteins, including albumin, and, since most adult amphibians are ureotelic, the source and site of the enzymes required for the

biosynthesis of urea (Frieden, 1967; Atkinson, 1995; Atkinson *et al.*, 1995). The liver of most amphibian tadpoles, however, produces little or no serum albumin and, since the fully aquatic tadpoles are ammonotelic, is not normally involved in producing urea (Frieden, 1967; Atkinson, 1995). During the postembryonic development/metamorphosis of most amphibians, the tadpole liver begins to assume the adult liver phenotype by synthesizing new and/or enhanced levels of albumin and other serum proteins (Herner and Frieden, 1960; Chen, 1970), and by marked elevations in the activities and levels of the enzymes involved in urea biosynthesis (Cohen, 1966). Indeed, by metamorphic climax (Stage XX; Taylor and Kollros, 1946) the levels (Feldhoff, 1971; Nagano *et al.*, 1973) and synthesis (Ledford and Frieden, 1973) of serum albumin have increased 6-fold in *Rana catesbeiana*, and the activities of the ornithine-urea cycle enzyme (Figure 3), carbamyl phosphate synthetase-1 (CPS-1), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL) and arginase (Arg), are elevated 30-, 8-, 35-, 20- and 30-fold, respectively (Brown *et al.*, 1959; Paik and Cohen, 1960; Cohen 1966; 1970; 1978). Measurements of the urea cycle enzyme activities in the liver (Cohen *et al.*, 1978) and of albumin concentrations in the serum of spontaneously metamorphosing *Rana catesbeiana* tadpoles (Feldhoff, 1971; Nagano *et al.*, 1973) demonstrate that both the urea cycle enzyme activities and serum albumin levels increase at a time in development when a measurable increase in plasma thyroid hormone levels is detectable (Galton and St. Germain, 1985) and the degradation of the tadpoles' tail is just beginning to be evident. These biochemical changes appear to occur in anticipation of new demands,

Figure 3. The ornithine-urea cycle and the enzymes responsible for the production of urea in the liver cells of *Rana catesbeiana.*

Two urea cycle enzymes, carbamyl phosphate synthetase-1 (CPS-1) and ornithine transcarbamylase (OTC) are localized in the mitochondrial matrix; the other three enzymes, argininosuccinate synthetase (AS), argininosuccinate lyase (AL) and arginase, are cytoplasmic. OT is an ornithine transporter. The enzymes shown in bold represent the products of genes which are emphasized in this thesis.


$HCO_3^- + NH_4^+ + 2ATP$
N-acetylglutamate
2ADP + P_i
CPS I
Carbamyl phosphate
Ornithine
OTC
Citrulline
Mitochondrial Matrix
Ornithine
OT
Cytoplasm
Citrulline
aspartate
+ATP
AS
-AMP +PP_i
Arginosuccinate
AL
Fumarate
Arginine
UREA
ARGINASE
Ornithine

namely, the subsequent transition from a fully aquatic tadpole to a fully or partially terrestrial adult.

Although the obligatory control of amphibian metamorphosis by thyroid hormones was established in the early 1900's, it was not until the 1950's that investigators demonstrated that administration of this hormone to premetamorphic tadpoles results in a precocious increase in the levels of serum proteins (Frieden *et al.*, 1957), in urea excretion (Munro, 1953) and in liver arginase activity (Dolphin and Frieden, 1955; Brown and Cohen, 1959). Subsequent studies have confirmed these results and established that administration of TH to premetamorphic tadpoles also induces the precocious synthesis of albumin (Ledford and Frieden, 1972) and a concerted increase in the levels of activity of all of the ornithine-urea cycle enzymes in the tadpole liver (Cohen, 1966; 1970; Wixom *et al.*, 1972; Cohen *et al.*, 1978). Moreover this last group of studies revealed that each of the ornithine-urea cycle enzymes attained a level of activity (based on a ratio of metamorphic to premetamorphic activity) in the liver of thyroid hormone-induced tadpoles approximating that found in the liver of spontaneously metamorphosing tadpoles (Cohen, 1966; Cohen *et al.*, 1978).

The striking increase in CPS-1 activity prompted Cohen and his colleagues to investigate the means by which thyroid hormone effects the activity of this urea cycle enzyme. Purification of CPS-1 from *Rana catesbeiana*, and preparation of an antibody against it, permitted these investigators to demonstrate that the increase in CPS-1 activity in the liver of tadpoles exposed to thyroid hormone is the result of *de novo* synthesis (Metzenberg *et al.*, 1961). This

observation has been confirmed by others (Kim and Cohen, 1968; Pouchelet and Shore, 1981; Helbing *et al.*, 1992) and, coupled with the studies by Ledford and Frieden (1972) on the thyroid hormone induction of albumin synthesis in this tadpole's liver, clearly implicates a role for thyroid hormone in both albumin and urea biosynthesis and in establishing the adult liver phenotype in this amphibian.

Since the administration of thyroid hormone to premetamorphic *Rana catesbeiana* tadpoles results in the precocious synthesis and accumulation of serum albumin and liver CPS-1 protein, studies were undertaken to establish if the thyroid hormone-induced synthesis of these proteins is coordinated with a rise in the hepatic mRNAs encoding them. Investigations by Schultz *et al.* (1988), using monospecific antibodies for immunoprecipitating albumin synthesized from *in vitro* translations of *Rana catesbeiana* tadpole liver mRNAs, supported the contention that thyroid hormone administration precociously upregulates the level of hepatic mRNAs encoding serum albumin in this organism. Results from Northern and slot-blot hybridization studies, using a cDNA encoding a portion of a *Rana catesbeiana* albumin (Averyhart-Fullard and Jaffe, 1990) and a cDNA encoding domains II and III of *Xenopus laevis* albumin (Moskartis *et al.*, 1989), have confirmed the observations of Schultz and his colleagues. Moreover, studies of albumin mRNA levels in the liver, of spontaneously metamorphosing tadpoles (Schultz *et al.*, 1988; Moskartis *et al.*, 1989) reveal that the amount of albumin mRNAs increases as the amount of endogenous thyroid hormone increases in the plasma, is maximal during metamorphic climax, and decreases after metamorphic climax. Unfortunately, none of the

reported studies has made a direct correlation between the timing of albumin synthesis and the upregulation in the level of albumin mRNAs, nor have they addressed the question of why the level of albumin mRNAs appears to decrease after metamorphic climax. Whatever the case, these reports clearly indicate that thyroid hormone, directly or indirectly, influences mRNAs encoding serum albumin to accumulate in the liver of these tadpoles.

Studies by Mori *et al.* (1979) and Pouchelet and Shore (1981), using antibodies to immunoprecipitate CPS-1 synthesized by *in vitro* translations of tadpole liver mRNAs, have shown that administration of thyroid hormone to premetamorphic tadpoles results in a premature up-regulation in the levels of hepatic mRNAs encoding CPS-1. Results obtained from hybridization analyses, using nucleotide sequences encoding a portion of a rat CPS-1 cDNA (Morris, 1987; Galton *et al.*, 1991; Helbing *et al.*, 1992), have supported the data obtained from the in vitro translations. More recently, Helbing and Atkinson (1994) isolated and characterized cDNAs encoding a complete *Rana catesbeiana* CPS-1 and used specific portions of the nucleotide sequence as probes to reassess the studies reported with the rat CPS-1 probes. Results from both hybridization and reverse transcription-polymerase chain reaction (RT-PCR) analyses (Helbing and Atkinson, 1994; Atkinson *et al.*, 1994; Helbing, 1993) confirmed the earlier studies and demonstrated that by 48 hours after the administration of thyroid hormone the level of CPS-1 mRNA is increased 6-fold and remains elevated.

While these observations support the conjecture that thyroid hormone regulates the expression of the genes encoding the

enzymes involved in urea biosynthesis, they were limited to studies with only CPS-1. However, the recent isolation and characterization of amphibian cDNAs encoding two other enzymes in the ornithine-urea cycle, OTC and arginase (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Xu *et al.*, 1993), have enabled this hypothesis to be more thoroughly investigated. Results from RT-PCR analyses, using PCR primers specific for each of these mRNAs, and from Northern- and dot-blot hybridization analyses using nucleotide sequences specific for OTC and arginase mRNAs as probes (Helbing *et al.*, 1992; Atkinson *et al.*, 1994), substantiate that administration of thyroid hormone to a premetamorphic *Rana catesbeiana* tadpole induces a marked up-regulation in the level of the hepatic mRNAs encoding OTC and arginase. Studies of CPS-1, OTC and arginase mRNA levels in the liver of spontaneously metamorphosing tadpoles (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994) reveal that the amount of each of these mRNAs increases as the level of endogenous thyroid hormone increases in the plasma, is maximal during metamorphic climax, and thereafter remains more or less at the level attained during metamorphic climax.

The major mechanism of thyroid hormone action is thought to be at the transcriptional level and is mediated through the binding of this hormone to a nuclear-localized receptor protein (for reviews: Tata, 1993; Tata, 1994). Since the amount of thyroid hormone receptor proteins (TRs) and the mRNAs encoding them have been shown to increase in tadpole tissues following thyroid hormone treatment (Kistler *et al.*, 1975; Galton and St. Germain, 1985; Baker and Tata, 1990; Kawahara *et al.*, 1991; Schneider and Galton, 1991; Yaoita and Brown, 1991), investigators hypothesized that an

accumulation of the mRNAs encoding the TRs should precede, or be coincidental with, the up-regulation of CPS-1, OTC and arginase mRNAs in the liver of thyroid hormone-treated tadpoles. The recent isolation and characterization of cDNAs encoding the *Rana catesbeiana* TRs, TRα and TRβ (Schneider and Galton, 1991; Helbing *et al.*, 1992; Davey *et al.*, 1994), enabled these studies to be conducted. Hybridization and RT-PCR analyses of RNA isolated from the liver of control and thyroid hormone-treated tadpoles, using nucleotide sequences specific for each of the *Rana catesbeiana* TRs, revealed that the mRNAs encoding these TRs accumulate in the liver of hormone-treated animals (Helbing *et al.*, 1992; Helbing *et al.*, 1994). TRβ mRNA accumulation is detectable by 12 hours after thyroid hormone treatment, is maximal by 48 hours and drops substantially by 72 hours. TRα mRNAs, which are 10 times more abundant than TRβ mRNAs in the liver of control animals, follow an accumulation pattern similar to the TRβ mRNAs except that there is never more than a 3-fold increase in the level of TRα mRNAs. As expected, the up-regulation of the mRNAs encoding the TRs, as well as the ornithine-urea cycle enzymes, occurs well before the enhanced synthesis and excretion of urea and before any gross morphological changes (*e.g.* tail reduction) occur in the tadpole. The most interesting observation, however, is that the up-regulation of the TR mRNAs is detectable within 12 hours after administration of thyroid hormone to the tadpole, whereas little or no accumulation of the CPS-1, OTC or arginase mRNAs is detectable until 24 hours after administration of the hormone. The early accumulation of TR mRNAs in response to thyroid hormone suggests that they are encoded by genes which may

be directly regulated by thyroid hormone, and the more extended time period (~24 hours) between the administration of the hormone and the up-regulation of the CPS-1, OTC and arginase mRNAs - the so called "lag" phase - suggests that these mRNAs are encoded by genes which are not directly regulated by thyroid hormone.

Hybridization and RT-PCR analyses of RNA isolated from the liver of *Rana catesbeiana* tadpoles at various stages of spontaneous metamorphosis disclose that no TRβ mRNA is detectable in the liver of tadpoles until stage XV. After stage XV, however, TRβ mRNA levels increase, attain maximal levels (~7-fold increase) at the beginning of metamorphic climax (stage XX), and thereafter, decrease (Helbing *et al.*, 1992). While TRα mRNAs are detectable in tadpole liver in all of the postembryonic developmental stages studied (VI-XXIV), they also appear to accumulate (2- to 3-fold) at metamorphic climax (Helbing, 1993). It is well known that the mRNAs encoding both the TRs and ornithine-urea cycle enzymes accumulate before urea excretion is elevated and before any reduction in the tadpole tail is evident (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994). Moreover, the correlation between the accumulation of these mRNAs and increasing levels of endogenous thyroid hormone in the plasma of spontaneously metamorphosing *Rana catesbeiana* tadpoles (Galton and St. Germain, 1985) supports the contention that the thyroid hormone-induced precocious accumulation of these mRNAs is a reflection of the natural sequence of events which occur during the postembryonic development/ metamorphosis of this organism.

Early studies on the thyroid hormone induction of albumin synthesis and the enhanced activity and synthesis of CPS-1 in the liver of *Rana* tadpoles were concerned with the amount of time that lapsed between the administration of the hormone and the detection of a change in the synthesis of albumin and in the synthesis and/or activity of CPS-1 (Paik and Cohen, 1960; Metzenberg *et al.*, 1961; Shambaugh et al., 1969; Ledford and Frieden, 1972), the so-called "lag" phase. Investigators, attempting to shorten the response time (i.e. reduce the lag phase), used methods which included administration of thyroid hormone by injection (rather than by immersing the tadpole in a solution of the hormone), use of higher doses of the hormone (T_3 and T_4) and/or maintenance of the tadpoles at a higher environmental temperature. All of these changes in protocol led to a reduction of the lag phase, however a lag phase of ~24-48 hours still appeared to be required. The observation that a lag phase (~24 hours) is also evident in the thyroid hormone-induced upregulation of the mRNAs encoding CPS-1, OTC and arginase in the liver of tadpoles maintained at 25°C has renewed interest in elucidating the molecular events which occur during this time.

The possibility that this lag phase may represent a period of time required for the synthesis and/or interaction of other, perhaps thyroid hormone-inducible, extrahepatic factors necessary for this response, has been addressed in serum-free, tadpole liver culture systems (Shambaugh *et al.*, 1969; Helbing *et al.*, 1992; Atkinson *et al.*, 1994). These culture systems have been used to assess the response of CPS-1 synthesis, and the up-regulation of the mRNAs encoding CPS-1, OTC, and arginase, as well as the TRα and TRβ mRNAs.

Administration of thyroid hormone to cultures of liver from premetamorphic tadpoles results in an early (~12 hours) up-regulation of TRα and TRβ mRNAs, a delayed (~24 hours; similar to that observed *in vivo*) up-regulation of CPS-1, OTC and arginase mRNAs, and the enhanced synthesis of CPS-1 (Shambaugh *et al.*, 1969). These data support the contention that responses observed *in vivo* are affected by thyroid hormone and are not dependent upon other extrahepatic factors. While these results do not clarify whether thyroid hormone directly or indirectly affects the expression of the genes encoding the TRs and/or the ornithine-urea cycle enzymes, they clearly establish that the lag phase does not reflect a period of time required for the synthesis and/or interaction of other extrahepatic factors.

1.3 Summary and Thesis Objectives

Neither the synthesis and/or interaction of extrahepatic factors, other than thyroid hormone, are required for the TH-induced expression of the genes encoding the ornithine-urea cycle enzymes. Consequently these phenomena cannot account for the lag phase. These observations have resulted in the conjecture that the lag phase may represent a period of time in which a cascade of molecular events occur that are required for initiating the terminal differentiation of the resident hepatocytes and, subsequently, orchestrating a reprogramming of gene expression which enables genes characteristic of the adult liver phenotype to be expressed (Chen *et al.*, 1994; Atkinson, 1994). This hypothesis implies that

thyroid hormone does not directly effect the expression of the ornithine-urea cycle enzyme genes but, instead, is responsible for initiating molecular events which lead to the terminal differentiation of the liver cells which, subsequently, results in the expression of the ornithine-urea cycle enzyme genes.

Thus, one objective of my thesis was aimed at elucidating intermediate factors involved with regulating these hormone-induced (metamorphic) changes in gene expression in the tadpole liver of *Rana catesbeiana*. Since the ornithine urea-cycle enzyme genes generally exhibit coordinate expression, I hypothesized that the 5'-flanking regions of these genes should share some DNA regulatory elements in common. Therefore, as a first step, I isolated and characterized the promoter regions of the genes encoding two of this amphibian's ornithine urea-cycle enzymes, CPS-1 and OTC. Characterization of the promoter regions of these genes revealed that they do not contain any recognizable thyroid hormone response elements (TREs), but do contain DNA elements that are recognized by C/EBP (CCAAT/enhancer-binding protein), or C/EBP-related transcriptional factors (Howell *et al.*, 1989; Murakami *et al.*, 1990; Takiguchi and Mori, 1991). Since the C/EBPα has been implicated in liver development (for reviews see Lai and Darnell, 1991; Sladek and Darnell, 1992; McKnight, 1992), CPS-1, OTC and arginase gene expression (Lagace *et al.* 1987; Takiguchi *et al.*, 1988; Murakami *et al.*, 1990; Takiguchi and Mori, 1991; Lagace *et al.*, 1992; Nishiyori, *et al.*, 1994), and terminal cell differentiation (Samuelsson *et al.*, 1991; Lin and Lane, 1992), I focused my studies on characterizing the *Rana* homologue of C/EBPα and establishing whether this particular

transcriptional factor might be involved in regulating the expression of some of the ornithine urea-cycle enzyme genes in the liver of this metamorphosing amphibian.

CHAPTER 2 CHARACTERIZATION AND EXPRESSION OF THE ARGINASE GENE IN THE LIVER OF *RANA CATESBEIANA* TADPOLES DURING SPONTANEOUS AND THYROID HORMONE-INDUCED METAMORPHOSIS

2.1 Introduction

This chapter describes the isolation and characterization of a novel cDNA isolated from our *Rana catesbeiana* adult liver cDNA library. This cDNA encodes the complete ORF of arginase, the last enzyme in the ornithine-urea cycle pathway (Figure 3). The arginase cDNA has been used as a probe to examine the relative, temporal expression of this gene in tadpole liver during spontaneous and T_3-induced metamorphosis.

2.2 Materials and Methods

2.2.1 Animal Experimentation

Rana catesbeiana tadpoles and adults were obtained from W. A. Lemberger Co. (Oshkosh, WI) and maintained in dechlorinated, aged tap water. Tadpoles were fed tadpole food (Boreal Laboratories) and adults were fed chopped beef liver. For spontaneous metamorphic studies, *Rana catesbeiana* developmental stages were assessed based on morphological criteria (Taylor and Kollros, 1946) and five animals for each developmental stage were used in each experiment (n=5). For each TH-induction experiment, five

premetamorphic tadpoles, stages VI-VIII, were selected for each time point and were injected intraperitoneally with 3,5,3'-triiodothyronine (T_3; Sigma Chemical Co., St. Louis, MO) at a dose of 0.3 nmol/g body weight (Atkinson *et al.*, 1972; Atkinson, 1981). Control animals were injected with a vehicle solution of 200 μM NaOH. T_3 immersion studies were performed by immersion of stage VI-VIII tadpoles in 25°C water containing 10^{-7} M T_3 or lacking it (Ashley *et al.*, 1968). The water was changed daily. Tadpoles were anesthetized in 0.1% tricaine methane sulfonate (Syndel Laboratories Ltd., Vancouver, B.C., Canada) and sacrificed by severing the truncus arteriosus.

2.2.2 Cloning and Sequencing of *Rana catesbeiana* cDNAs Corresponding to Arginase

A *Rana catesbeiana* cDNA library, made from poly-A mRNAs isolated from the liver of adult frogs, consisting of 1 x 10^6 independent recombinant phage, was constructed in the *EcoR* I/*Xho* I sites of a UniZap vector (Stratagene Inc., La Jolla, CA). The cDNA library was screened with a 1.28 kb *EcoR* I [α-^{32}P]-dCTP-labelled (NEN Dupont Canada; specific activity >3,000 Ci/mmol) cDNA fragment containing a *Xenopus laevis* arginase ORF (Xu *et al.*, 1993). The cDNA fragment used as a probe was radiolabelled using a T_7 QuickPrime Kit (Pharmacia Inc.). Hybridizations were done in a solution containing 50% formamide (BRL, Gaithersburg, MD), 5X SSPE (1X = 0.18 M NaCl, 0.01 M Na_2HPO_4, 1 mM EDTA), 5X Denhardt's solution [1X = 1% (w/v) of each of Ficoll Type 400, polyvinyl

pyrrolidone (MW 360,000) and bovine serum albumin (BSA; Fraction V, Sigma)], 0.1% SDS and 0.1 mg/ml denatured herring sperm DNA at 42°C for 24h. Two washes were done at room temperature with 2X SSC (1X = 0.15 M NaCl, 0.015 M Na citrate) and 0.1% SDS for 1h each followed by two washes with 1X SSC and 0.1% SDS at 48°C for 1h each. Six positive clones were isolated and *in vivo* excised into pBluescript II SK$^-$ (Stratagene Inc., La Jolla, CA). The cloned inserts were sequenced by the Exo III-based DNA sequencing method (Li and Tucker, 1993) using [^{35}S]-dATP (Dupont Canada, Mississauga, Ontario, Canada; specific activity 600 Ci/mmol) and a Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH) for direct dideoxynucleotide sequencing. In some cases, particular sequences were confirmed by subcloning DNA fragments into pBluescript II SK$^-$ and using T_3, T_7, SK, KS primers for dideoxynucleotide sequencing (Chen and Seeburg, 1985). Two of six cDNA clones (Arg-1 and Arg-7) were found to contain a complete ORF.

2.2.3 Computer Analyses

Sequence characterization was accomplished using DNA Strider version 1.0 (Marck, C., Service de Biochimie-Department de biologie, Institute de Recherche Fondamentale, Commissariat á l'Energie Atomique-France), and DNA Inspector II$^+$ (Textco, West Lebanon, NH) software. The EMBL and GenBank databases were searched using the Wisconsin Genetics Computer Group sequence analysis software package Version 6.1 (Devereux *et al.*, 1984).

2.2.4 Southern Hybridization Analysis

DNA was isolated from *Rana catesbeiana* liver tissue using the proteinase K method of Ausubel *et al.*, (1990), and digested with various restriction enzymes. The resultant fragments were electrophoresed on 0.7% agarose gel, vacuum-blotted, using a LKB 2016 Vacugene vacuum blotting unit (LKB Instruments, Rockville, MD), onto a Zetaprobe membrane (BioRad, Richmond, CA), and UV-fixed using a UV Stratalinker (Stratagene). The membrane was prehybridized in a solution containing 10% formamide, 3X SSPE, 1% SDS, 0.5% Blotto and 0.5 mg/ml denatured herring sperm DNA for 4 h at 55°C. Hybridization was performed in a similar solution containing 10% dextran sulfate and a 700 bp *EcoR* I/*Hind* III [α-^{32}P]-dCTP-labelled (NEN Dupont Canada; specific activity >3,000 Ci/mmol) fragment from the *Rana catesbeiana* Arg-1 cDNA (nt 1 to 700 in Figure 5) for 20 h at 55°C. Hybridizations were performed in a hybridization incubator (Model 400, Robbins Scientific, Sunnyvale, CA). DNA fragment sizes were estimated using *Hind* III digested λ DNA-*Hae* III digested ϕX174 RF DNA markers (Pharmacia Inc.).

2.2.5 RNA Extraction, and Northern and Dot-Blot Hybridization Analyses

Liver was excised from tadpoles, perfused with cold amphibian phosphate-buffered saline (PBS), minced on ice, rinsed with cold PBS and homogenized. In each case, the tissues were

homogenized in 10 volumes of 4 M guanidinium isothiocyanate, 25 mM Na citrate, pH 7.0, 0.5% Sarkosyl and 0.1 M β-mercaptoethanol using a motor-driven teflon pestle and glass homogenizer. Total RNA was isolated from the homogenate using a modification of the guanidinium isothiocyanate single step isolation method (Puissant and Houdebine, 1990). In some cases, poly-A mRNA was isolated using the PolyATract mRNA isolation system (Promega Corporation, Madison, WI). The RNA samples were run on 1.0% agarose gels containing 2.6 M formaldehyde (Khandjian, 1986) and vacuum-blotted to a Zetaprobe membrane. Dot-blots were prepared (Sambrook *et al.*, 1989) with a dot-blotting apparatus (BioRad).

Northern and dot-blot hybridizations were performed on companion and/or stripped membranes with [α-^{32}P]-labelled DNA probes containing a 700 bp *EcoR* I/*Hind* III) fragment from Arg-1 cDNA (Atkinson *et al.*, 1994), a 1224 bp *Sac* I/*Hind* III fragment from TRα cDNA (Schneider and Galton, 1991), a 531 bp *Taq* I/*BamH* I TRβ genomic fragment containing its exon sequence (Helbing *et al.*, 1992), a 441 bp *EcoR* I fragment from *Rana catesbeiana* CPS-I cDNA (Helbing and Atkinson, 1994), and a 518 bp *EcoR* I/*Xho* I fragment from OTC cDNA (Helbing and Atkinson, 1994) in a solution similar to the Southern hybridizations except that the hybridization solutions contained 50% formamide and 3X SSPE and the final wash was done at 60°C. Dot-blot hybridizations with a *Hind* III/*Pvu* II 960 bp fragment from *Xenopus* cytoskeletal actin cDNA (Mohun *et al.*, 1984) were done in 22% formamide and 5X SSPE at 50°C. The final washes were done in 0.1X SSC and 1% SDS at 37°C. The molecular sizes of detected mRNA species were determined from the mobility of a

coelectrophoresed 0.24-9.49 Kb RNA ladder (BRL). The membranes were exposed to preflashed Kodak XR-Omat RP film (Eastman Kodak Co.; Laskey and Mills, 1975) with an intensifying screen. The X-ray films were processed using Kodak GBX developer and fixer. Autoradiograms of the dot blots were quantitated with a Ultroscan XL Laser Densitometer (LKB).

2.2.6 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Due to the sensitivity of the RT-PCR procedure (Innis *et al*, 1990), several precautions were taken to ensure comparability between samples. All reactions of a given experiment were performed at the same time using the same reaction premixes. Specific 20 nt primers for RT-PCR analyses were designed with the aid of OLIGO software (Rychlik and Rhoads, 1989; search parameters: 760 pM nucleic acid and 50 mM salt). All primers were synthesized by Vetrogen (London, Ontario, Canada). The sizes of the amplified fragments, diagnostic restriction enzyme sites and expected digestion products, and the results of PCR amplification of genomic DNA (a potential contaminant) are summarized in Table 1.

For cDNA synthesis, 2.5 μg of total *Rana catesbeiana* liver RNA was solublized in a 10 μl water, denatured for 10 min at 65°C and plunged into ice. A 6.5 μl aliquot of reaction premix [Pharmacia First-Strand cDNA Synthesis Kit (#27-9261-01)] was added (premix for 50 reactions: 275 μl bulk first strand reaction mix, 25 μl d(N6)

Ta el 1. Summary of RT-PCR primers used and the expected product sizes.

The primer designations are italicized and their locations in the pubilished sequences are indicated in brackets underneath the primers. The genomic results were obtained from using 10 ng of *Rana catesbeiana* genomic DNA. Since the ferritin primers gave a larger band from the genomic DNA template (asterisk; presumably due to an intron), the presence or absence of this band was used to assess the level of genomic DNA contamination in all cDNA template samples. The references were shown on the right side of this tabel.

Specificity	Primer pair (location in sequence)	RT-PCR Product length (bp)	Genomic DNA Product lengt[b] (bp)	Diagnostic RE Sites (product size; bp)	Reference
TRα	*pα-3* (nt 869-888) *pα-4* (nt 1046-1027)	179	179	*Sac* I (131, 48)	Schneider and Galton, 1991
TRβ	*pβ-1* (nt736-757) *pβ-2* (nt 984-965)	248	248	*Sac* I (no cut sites)	Helbing *et al.*, 1992
Arginase	*Arg-1* (nt 145-164) *Arg-2* (nt 690-671)	545	no product	*EcoR* V (300, 245)	Atkinson *et al.*, 1994
Ferritin	*pFERR-1* (nt 161-180) *pFERR-2* (nt 383-364)	223	400 bp*	*Pst* I (79, 78, 66)	Didsbury *et al.*, 1986

primers and 25 µl DTT solution) to the RNA tubes, and the mixture was incubated at 37°C for 1 h. The resultant first-strand cDNA samples were stored at -20°C and used as reaction template for all PCR reactions for a given experimental set.

In order to ensure comparability between samples, all of the PCR reactions of a given set (for example: arginase mRNA accumulation during various time periods after T_3-induced metamorphosis) were performed at the same time, using the same reaction master mixes and same Taq DNA polymerase. A reaction master mix for 20 reactions consisted of 100 µl 10X Taq DNA Polymerase buffer (1X = 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0 at room temperature), 80 µl dNTP stock solution (2.5 mM stock of each dNTP; final concentration = 200 µM) and 30 µg each of the appropriate specific primers (see Table 1). The final volume was brought up to 980 µl by DEPC-treated double-distilled water. One µl of cDNA or genomic DNA control solution (10 ng/µl) was added to 49 µl of the appropriate master mix in a sterile, 0.5 ml thin-walled polypropylene tube. The mixture was overlaid with 30 µl paraffin oil and the tube placed into a Perkin Elmer Cetus Thermal cycler preheated to 97°C. The samples were incubated for 5 min at 97°C and immediately cooled to 75°C. The polymerase chain reaction was "hot-started" by the addition of 0.5 µl (2.5 U) *Taq* DNA polymerase (Pharmacia or BRL) and a 30-cycle step programme was initiated as follows: 1 min at 94°C, 1 min at 53°C, and 3 min at 72°C. Following completion of the thirtieth cycle, a final extension was performed at 72°C for 10 min. The aqueous layer was collected following the

addition of 100 µl chloroform and a 2 min centrifugation at 14,000 rpm in an Eppendorf microfuge. Equal volumes of reaction products were coelectrophoresed with *Hind* III digested λ DNA-*Hae* III digested ϕX174 RF DNA markers (Pharmacia Inc.) on a 2.5% agarose gel. The identity of the bands was confirmed by electrophoretic mobilities of the uncut band and of those cut with diagnostic restriction enzymes (see Table 1 and Figure 4).

2.3 Results

2.3.1 Isolation and Characterization of cDNAs Encoding the *Rana catesbeiana* Urea Cycle Enzyme, Arginase

Two of the six arginase cDNA clones (Arg-1, 1520 nt; Arg-7, 1516 nt) contained an entire ORF of 969 nt (Figure 5) which encodes a 323 AA protein with an M_r of 35,253. Although the 3' ends of all of the clones contained polyadenylated tails, the polyadenines were found at different positions in each clone (nt 1372, 1516, 1518 and 1520). None of them were preceded by a complete polyadenylation consensus sequence. Comparison of the ORF nucleotide sequence with the nucleotide sequences from cDNAs encoding arginase in *Xenopus laevis* (Xu *et al.*, 1993), human (Haraguchi *et al.*, 1987), and rat (Kawamoto *et al.*, 1987) showed that they share 70%, 66% and 65% identity, respectively. Comparison of their derived amino acid sequences reveals 71%, 68% and 65% identity, respectively (Figure 6). Southern hybridization analyses suggest that the arginase probe recognizes a single copy gene (Figure 7). Since RT-PCR analyses of genomic DNA for this region of Arg-1 (Figure 4) does not yield a

Figure 4. Characterization of RT-PCR products.

RT-PCR primers (see Table 1), specific for the nucleotide sequences encoding *Rana catesbeiana* TRα (**A**; Schneider and Galton, 1991), TRβ (**B**; Helbing *et al.*, 1992), arginase (**C**; Atkinson *et al.*, 1994) and ferritin (**D**; Didsbury *et al.*, 1986), were used to analyze the relative level of these mRNA transcripts. The RT-PCR products (Lane 2) are shown beside *Hin*d III-digested λ DNA-ϕX174 *Hae* III digested DNA markers (Lane 1) on 2.5% agarose gels stained with ethidium bromide. The RT-PCR products were digested with internal diagnostic restriction enzymes (Lane 3) listed in Table 1. PCR amplification products from 10 ng *Rana catesbeiana* genomic DNA template (Lane 4) are also shown. Relative sizes are indicated on the left.

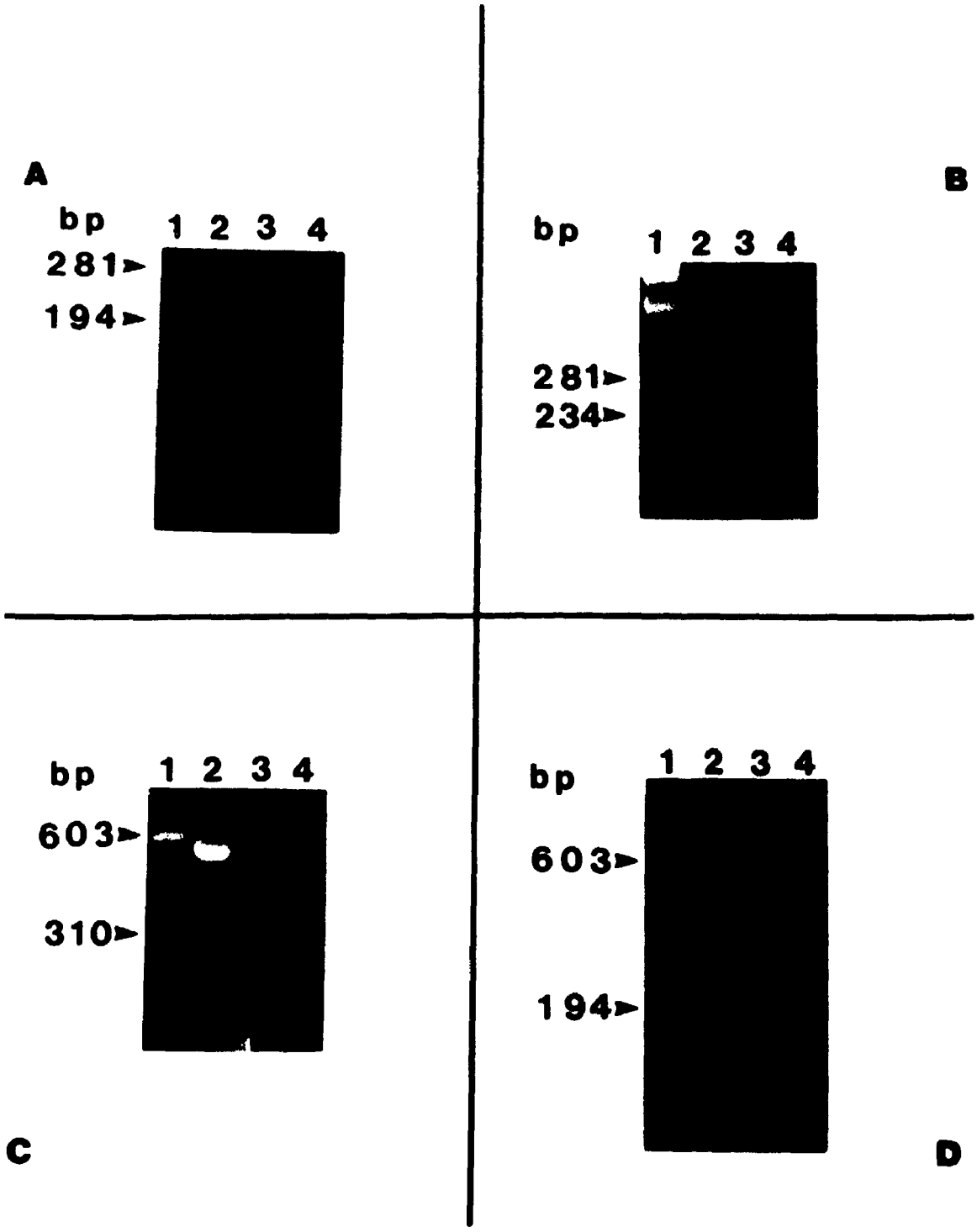

A
bp
1 2 3 4
281
194
B
bp
1 2 3 4
281
234
C
bp
1 2 3 4
603
310
D
bp
1 2 3 4
603
194

Figure 5. Nucleotide sequence of *Rana catesbeiana* arginase cDNA.

Numbers to the left refer to the nucleotide locations with respect to the 5'-most end of the sequence. The incomplete 5'-UTR (nt 1-5) is indicated in lowercase letters. The open reading frame (ORF) is shown in uppercase letters, and the ATG translation start site and TGA stop codon are highlighted in bold letters. The 3'-UTR is indicated in lowercase letters. No putative polyadenylation signals were found, however, the clones did have poly-A tails at different positions. The nucleotide positions at which poly-A tails were located in inserts having exactly the same sequence are double underlined. The *Hin*d III site used for preparing a 0.7 kb hybridization probe containing a large proportion of the ORF and the 5 nt of the 5'-UTR is underlined. The arrows indicate the primer sequences used for the RT-PCR studies.


1 gcaca
ATGAGCGAAAGAACTAAGAGATCAGTAGGAGTTCTTGGAGCACCCTTTTCTAAAG
61 GGCAGGCCAGAGGTGGAGTGGAAGAAGGACCAATTTACATAAGGAGAGCAGGTTTAATTG
121 AAAAACTGGAAGAACTCGAATATGAGGTGAGGGATTACGGTGATCTGCATTTTCCTGAGC
181 TGCCCTGTGATGAACCCTTCCAGAATGTGAAGAATCCACGCACGGTTGGTCAAGCTGCTG
241 AGAAAGTTGCTAATGCTGTTTCAGAAGTTAAGAGAAGTGGAAGAGTTTGCTTAACCCTCG
301 GTGGAGATCACAGCTTGGCAGTTGGGACCATCACAGGACATGCTAAGGTTCACCCTGATC
361 TGTGTGTTGTTTGGGTGGATGCCCATGCAGATATCAACACTCCAATAACATCACCCAGTG
421 GCAATCTACATGGACAACCTGTTTCTTTCCTAATCAGAGAGCTACAAACCAAGGTGCCAG
481 CCATCCCAGGATTCTCTTGGGTGCAGCCAAGTCTGTCTGCCAAAGATATAGTATACATTG
541 GACTGAGAGATGTAGACCCTGGAGAGCATTATATTCTGAAGACTCTTGGGATTAAGAGCT
601 ACTCAATGTCAGACGTGGACAGGCTTACAATAAATAAAGTGATGGAAGAAACTATTGAAT
661 TTTTGGTTGGAAAGAAGAAAAGACCCATCCACTTAAGCTTTGACATAGATGGTTTGGACC
721 CTAGTGTTGCGCCTGCTACTGGCACACCCGTCCCTGGAGGTCTCACCTACAGAGAGGGCA
781 TGTACATCACAGAGCAGCTTTACAATACAGGCTTACTTTCTGCAGTGGATATGATGGAGG
841 TCAACCCATCGCGTGGAGAAACAGAGCGAGAATCCAAGCTGACTGTAAACACCTCCCTCA
901 ATATGATCCTGTCCTGCTTCGGGAAGGCACGAGAAGGCTTTCATGCATCCTCACTGCGTG
961 TTCCTGATCTGATCTGA
atgccaagtcctctgctatgtcaaccttgcatacttcattttt
1021 aataattagaaaccgtatattttaatggacagaatactcatatcaaaatgcataaattgc
1081 catttctcagcaactttgttacattatacagtagagttgagaatttctttcattctcatg
1141 ttgaaaaaatataactgcacaagataaggaatcatagcacaaataatatttaagttatat
1201 taacttcttttcttttgaaagtaagcatttcgaatttgaggtctttgatctctgccactt
1261 gtttttgtatgcttgttaatgtagtttatcttatatatatttttagagagaagaatagctt
1321 gcggttttgatgttaccagtcttaatttattgatataaatgaaaagttttacaatgcctc
1381 catttctgtctgatgggggggttacatacttctgctgcagtgcagttaatgtagaacaca
1441 tcctttgccagttgactgctttcaaattgcatctgttaaatgtctaagcaatattaaatt
1501 tggaaccatgttttggacataaaaaaaaaaaaaa

Figure 6. Comparison of the derived amino acid sequence from the ORF of *Rana catesbeiana* arginase with published sequences for arginase.

The derived *Rana* arginase amino acid sequence is compared with the amino acid sequences from *Xenopus laevis* (Xu *et al.*, 1993), human (Haraguchi *et al.*, 1987) and rat (Kawamoto *et al.*, 1987) liver arginases. Dashes represent amino acid identity, dots represent deletions or insertions and asterisks represent stop codons. The percent identity of each of the compared sequences to the derived *Rana* amino acid sequence is shown.

```
          1                                                  50
Rana      MSERTKRSVG VLGAPFSKGQ ARGGVEEGPI YIRRAGLIEK LEELEYEVRD
Xenopus   .MAKERH--- ---------- P-R------K -L-E------ -R-FGND---
Human     --AKS.-TI- II-------- P--------T VL-K---L-- -K-Q-CD-K-
Rat       --SKP-.PIE II-------- P-----K--A AL-K---V-- -K-T--N---

          51                                                 100
          YGDLHFPELP CDEPFQNVKN PRTVGQAAEK VANAVSEVKR SGRVCLTLGG
          C---D--DV- N-T--N---- -----K-T-I L----TA--K ADKT-QSI--
          ----P-ADI- N-S---I--- --S--K-S-Q L-GK-AQ--K N--IS-V---
          H---A-VDV- N-S---I--- --S--K-N-Q L-AV-A-TQK N-TISVV---

          101                                                150
          DHSLAVGTIT GHAKVHPDLC VVWVDAHADI NTPITSPSGN LHGQPVSFLI
          ---------A ---A---N-- ---------- ---S---C-- -----L---M
          -----I-S-S ---R-----G -I-----T-- ---L-TT--- ---------L
          ---M-I-S-S ---R------ -I-----T-- ---L-TS--- ------A--L

          151                                                200
          RELQTKVPAI PGFSWVQPSL SAKDIVYIGL RDVDPGEHYI LKTLGIKSYS
          K--KA-M--V ---E--K-C- RS-------- ---------- -------YL-
          K--KG-I-DV ------T-CI ---------- ---------- -------YF-
          K--KG-F-DV ------T-CI ---------- ---------- I------YF-

          201                                                250
          MSDVDRLTIN KVMEETIEFL VGKKKRPIHL SFDIDGLDPS VAPATGTPVP
          -IE--Y-KDD ------L-Y- ---------- ---------- I-------C-
          -TE----G-G ------LSY- L-R------- ---V------ FT-------V
          -TE--K-G-G ------FSY- L-R------- ---V-----V FT-------V

          251                                                300
          GGLTYREGMY ITEQLYNTGL LSAVDMMEVN PSRGETERES KLTVNTSLNM
          --R-----RI LH---HK--- --G--TIWME STSRGETKRD VEVTVKTALD
          --------L- ---EI-K--- --GL-I---- --L-K-PE-V TR----AVAI
          ---S----L- ---EI-K--- --GL-I---- -TL-K-PE-V TR----AVPL

          301                        324   Identity
          ILSCFGKARE GFHASS...L RVPDLI*      -
          MTLSCFGKAR EGFHA-T--* ......      71%
          T-A---L--- -N-KPIDYLN PPK*..      68%
          T-----TK-- -N-KPETDY- KP-K*.      65%
```

product under the conditions used, this suggests that there is at least one large intron in this region.

2.3.2 Expression of the Gene Encoding Arginase in the Liver of *Rana catesbeiana* Tadpoles

A DNA fragment (*EcoR* I/*Hind* III; nucleotides 1-700 in Figure 5) was excised from one of our arginase cDNA clones (Arg-1), labelled with [α-^{32}P]-dCTP, and used as a probe to hybridize Northern blots of RNA extracted from the liver of premetamorphic (stages VI-VIII) control, and 3-day T_3-treated *Rana catesbeiana* tadpoles, as well as from adult liver. The autoradiographic results of the hybridizations (Figure 8) clearly demonstrate that this probe recognizes a 1.8-Kb mRNA transcript that accumulates in the liver of both T_3-treated tadpoles and adults.

Autoradiograms from dot-blot hybridization analyses of RNA isolated from the liver of stage VI-VIII tadpoles at various times after a single intraperitoneal injection of T_3, and from the liver of spontaneously metamorphosing tadpoles are shown in Figures 9A and 9B, respectively. Quantitative laser densitometric analyses of these autoradiograms reveal that the amount of arginase mRNA transcripts appears to increase (approximately 2.5-fold) in the liver of a stage VI tadpole 12 h after T_3 treatment. By 72 h after T_3 treatment, the amount of arginase mRNA transcripts in the tadpole liver has increased 10-fold (Figure 9A), an increase similar to that found between stage VI and stage XXIV tadpoles during spontaneous

Figure 7. Southern blot hybridization analysis of *Rana catesbeiana* genomic DNA with a *Rana* arginase-specific probe.

Rana catesbeiana genomic DNA (20 μg) was digested with *Bam*H I (Lane 1) , *Eco*R I (Lane 2) or *Hin*d III (Lane 3). The digested DNAs were separated on a 0.7% agarose gel, the fragments transferred to Zetaprobe membrane and hybridized to a [α-^{32}P]-labelled 700 bp *Eco*R I /*Hin*d III Arg-1 cDNA fragment as outlined in the "Materials and Methods". The relative sizes of the DNA fragments were determined from a coelectrophoresed DNA ladder and are shown on the right in kilobases (kb).

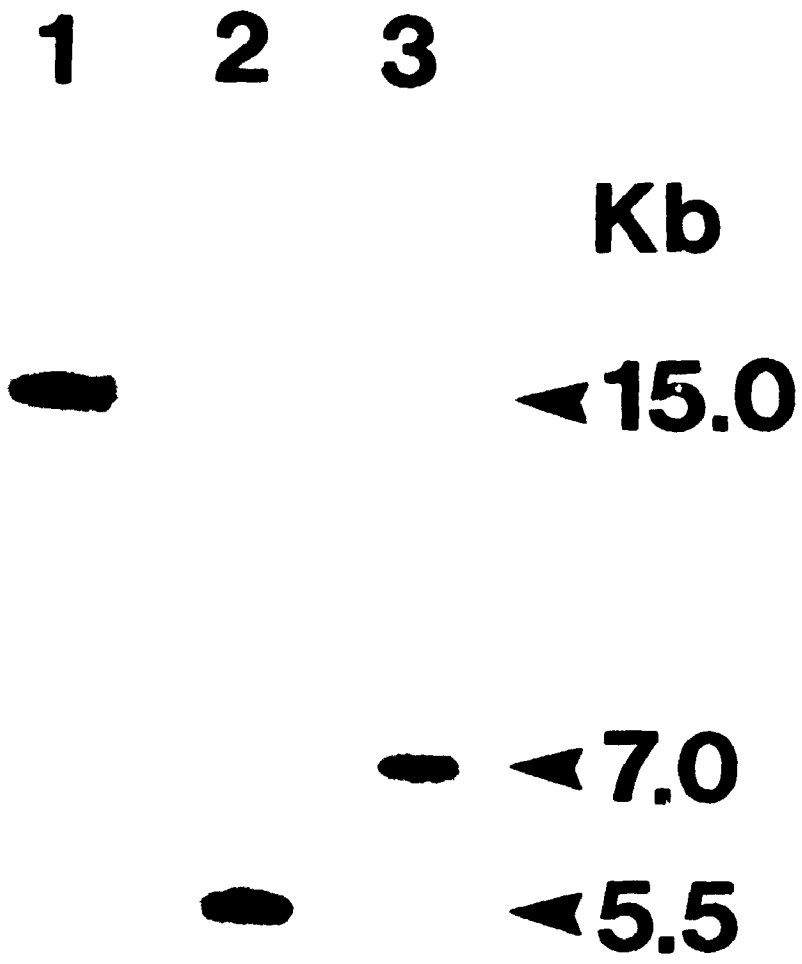
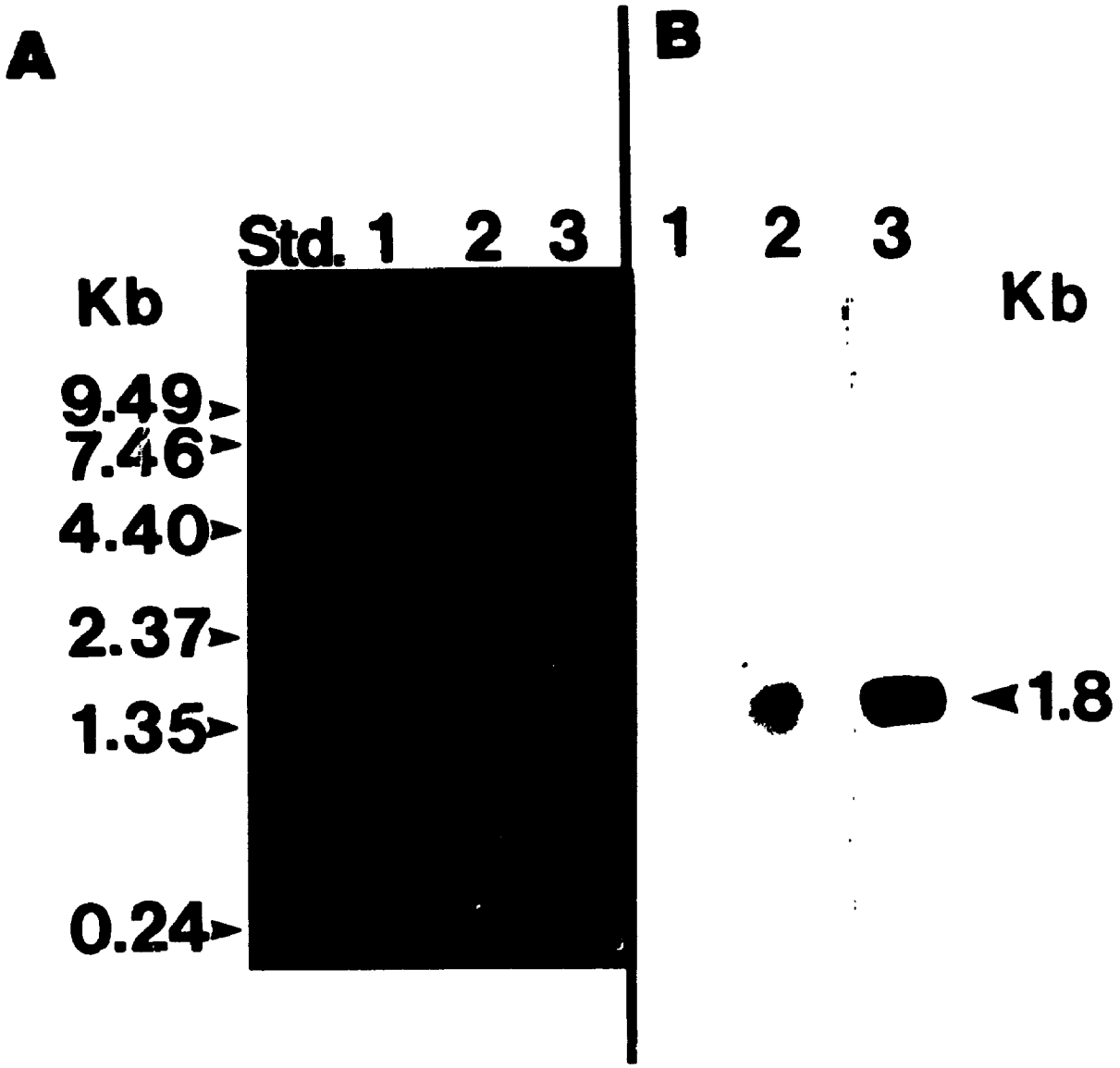

1
2
3
Kb
15.0
7.0
5.5

Figure 8. Northern hybridizations demonstrating the accumulation of arginase mRNA transcripts in the liver of T_3-treated *Rana catesbeiana* tadpoles and in the liver of adult *Rana catesbeiana.*

A: An Ethidium bromide-stained 1.0% formaldehyde-agarose gel shows the total RNA (10 μg), isolated from the liver of stage VI-VIII (Taylor and Kollros, 1946) control (Lane 1) and 3-day T_3-treated tadpoles (Lane 2), from liver of adults (Lane 3). The RNA standards (Std.) are shown on the left side of panel A.

B: The gel from panel A was transferred to a Zetaprobe membrane and probed with a [α-^{32}P]-labelled 700 bp I/*Hin*d III nucleotide fragment from Arg-1. That relative molecular size (kb) of the mRNA transcripts recognizing this probe is given on the right side of panel B.


A
B
Std.
1
2
3
1
2
3
Kb
Kb
9.49
7.46
4.40
2.37
1.35
0.24
1.8

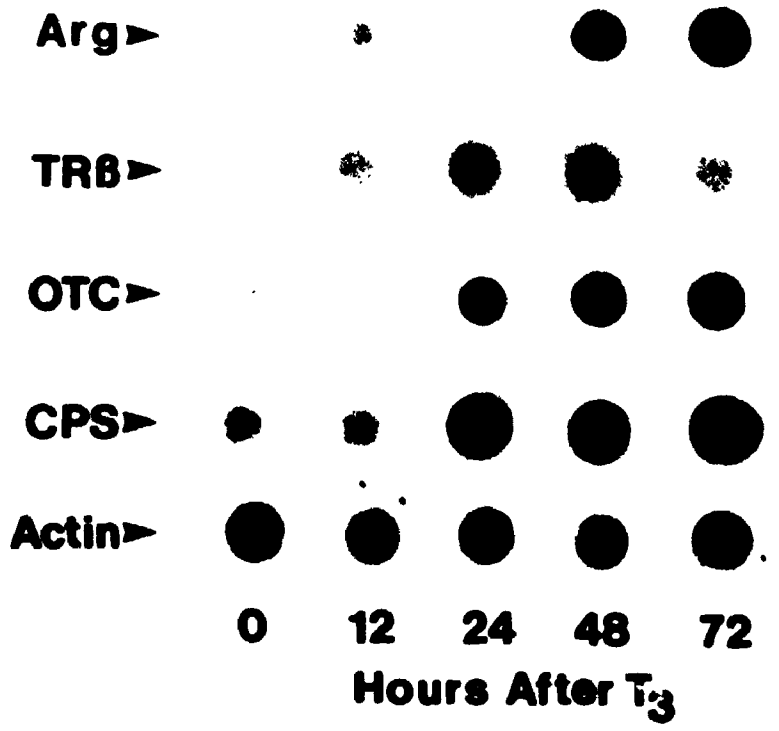
metamorphosis (Figure 9B). Dot-blot hybridization results of TRβ, OTC and CPS-I mRNA accumulation in tadpole liver during T_3-induced metamorphosis and spontaneous metamorphosis is compared to liver arginase mRNA accumulation in Figure 9A and 9B. These results confirm previous work reported by Helbing (1993). In T_3-induced metamorphosis (Figure 9A), TRβ mRNAs are detectable by 12 h, show a >11-fold increase by 48 h and drop substantially by 72 h. Both CPS-1 and OTC mRNAs show increased accumulation at 24 h and, by 48 h, maintain 6- and 9- fold increases, respectively. In spontaneous metamorphosis (Figure 9B), the TRβ mRNAs are not detectable in the liver of the tadpoles until stage XV, reach maximal levels (7-fold increase) at the beginning of metamorphic climax (stage XX), and decrease in the froglet (stage XXIV). Both CPS-1 and OTC mRNA accumulate at approximately stage XV and reach maximal levels at metamorphic climax (7- and 6-fold increases respectively). The relative level of liver cytoskeletal actin mRNAs remain relatively constant during both T_3-induced metamorphosis (Figure 9A) and spontaneous metamorphosis (Figure 9B).

RT-PCR analyses of RNA isolated from the liver of stage VI-VIII tadpoles at various times after a single intraperitoneal injection of T_3 or after the immersion of the tadpoles in water containing T_3, using PCR primers specific for *Rana catesbeiana* arginase (see Table 1, and Figures 4 and 5) also demonstrate (Figure 10A and 10B) that an enhanced accumulation of arginase mRNA transcripts occurs after thyroid-hormone treatment. RT-PCR analyses of these same RNA samples, using primers (see Table 1 and Figure 4) specific for the

Figure 9. Autoradiograms showing the accumulation of arginase (Arg), TRβ, OTC, and CPS mRNA transcripts in tadpole liver during T_3-induced (**A**) and spontaneous (**B**) metamorphosis.

Total RNA (10 μg), isolated from the liver of stage VI-VIII tadpoles at various times after T_3 injection (**A**) and from the liver of tadpoles at specific stages (Taylor and Kollros, 1946) of spontaneous metamorphosis (**B**), was dot-blotted to Zetaprobe membranes and hybridized with [α-^{32}P]-labelled DNA fragments specific for sequences in the genes encoding *Rana catesbeiana* arginase (Arg), TRβ, OTC, and CPS (see "Materials and Methods" for details). A [α-^{32}P]-labelled *Xenopus laevis* cytoskeletal actin cDNA fragment (Mohun *et al.*, 1984) was also hybridized to the same blots.

A


Arg►
TRB►
OTC►
CPS►
Actin►
0
12
24
48
72
Hours After T_3

B

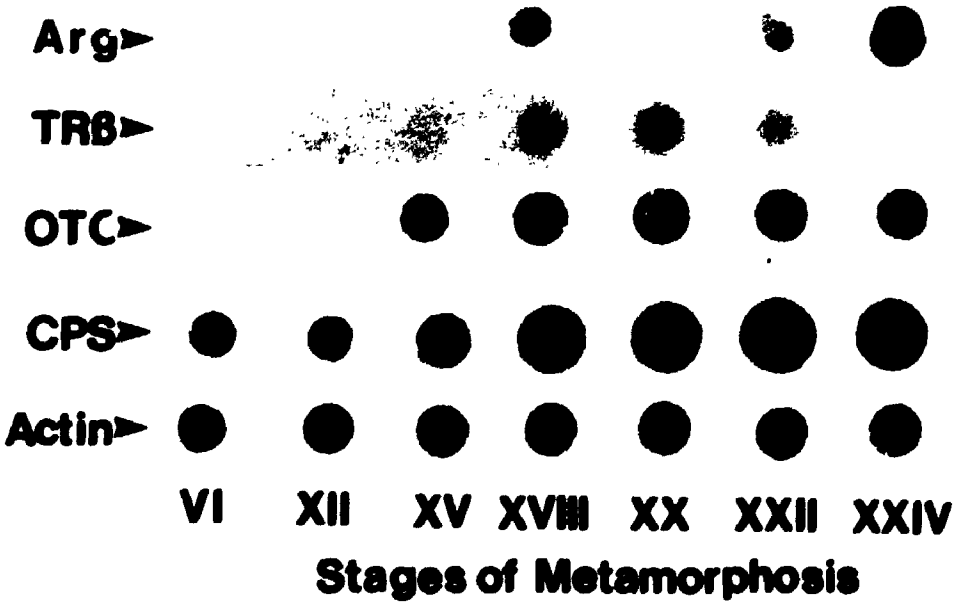
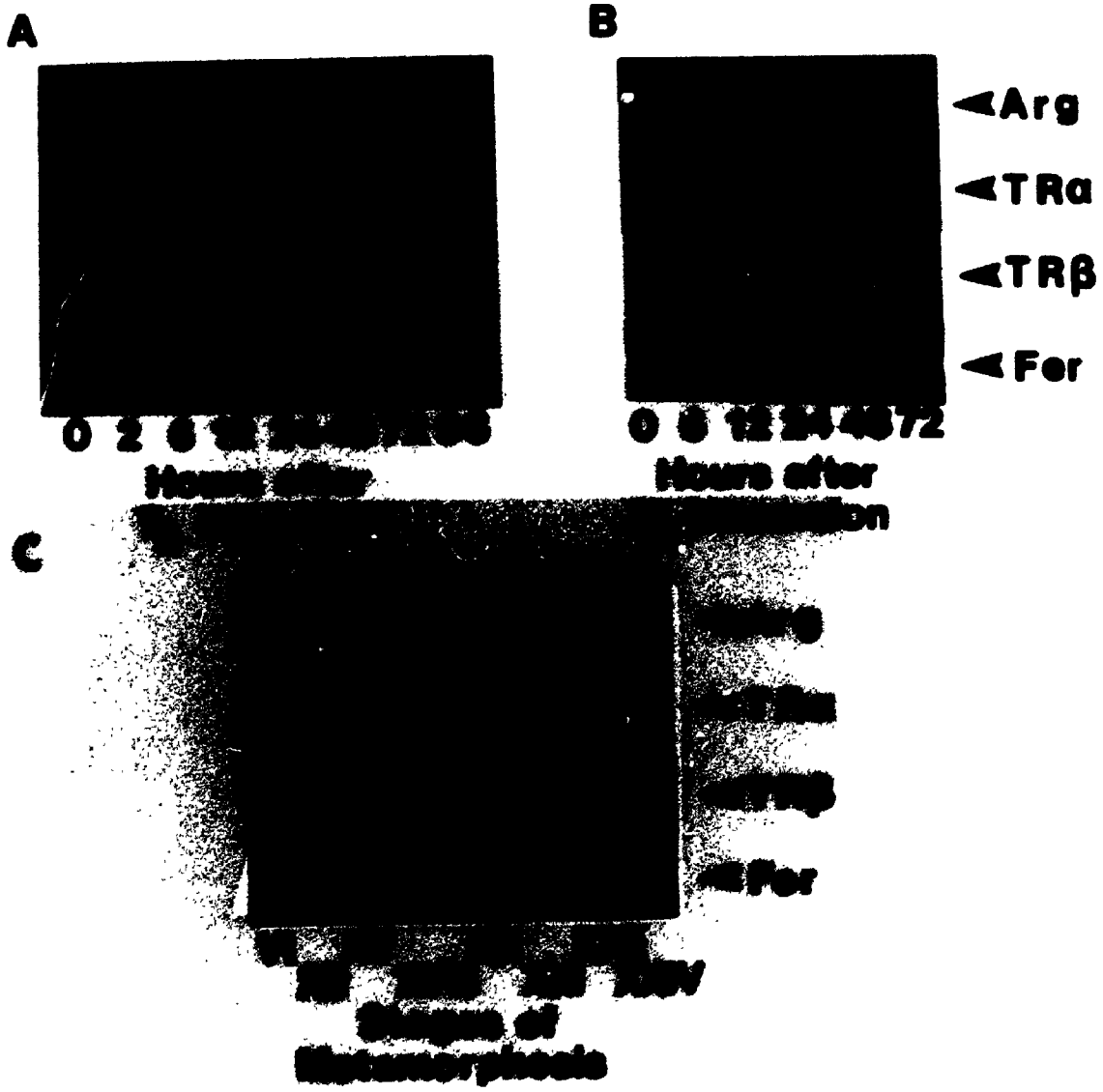

Arg►
TRB►
OTC►
CPS►
Actin►
VI
XII
XV
XVIII
XX
XXII
XXIV
Stages of Metamorphosis

Figure 10. RT-PCR analyses of RNA isolated from the liver of (**A** and **B**) T_3-induced and (**C**) spontaneously metamorphosing *Rana catesbeiana* tadpoles.

RT-PCR primers, specific for the nucleotide sequences encoding *Rana catesbeiana* arginase, TRα, TRβ and ferritin (see Table 1 for details), were used to analyze the relative level of mRNA transcripts encoding these proteins in the liver of stage VI tadpoles at various times after a single injection of T_3 **(A)** or following immersion of the tadpoles in T_3-containing water **(B)**. The same RT-PCR primers were used to assess the relative levels of mRNAs encoding these proteins in the liver of spontaneously metamorphosing tadpoles **(C)**. In each case RT-PCR primers were prepared for a sequence in an *Rana catesbeiana* ferritin cDNA clone encoding an mRNA reported to remain constant throughout T_3-induced and spontaneous metamorphosis (Dickey *et al.*, 1987).


A
B
Arg
TRα
TRβ
For
Hours after
C

Rana catesbeiana TRα and TRβ sequences suggest that the T_3-induced up-regulation of arginase mRNAs is coincidental with, or occurs slightly after, the TH-induced up-regulation of the mRNAs encoding these TRs (see Figures 10A and 10B). RT-PCR analyses of RNA isolated from the liver of tadpoles at various stages of spontaneous metamorphosis (Figure 10C), using the same arginase and TRα and TRβ primers as in the T_3 studies, reveal that arginase mRNAs begin to accumulate in the liver of these tadpoles, just prior to metamorphic climax (stage XX). Results from these latter studies also demonstrate that the accumulation of arginase mRNAs coincides, in a stage-specific manner, with the accumulation of TRα and TRβ mRNAs in the liver of spontaneously metamorphosing tadpoles. RT-PCR analyses of these same RNA samples, using primers (see Table 1 and Figure 4) specific for the *Rana catesbeiana* ferritin sequence show that the level of liver ferritin mRNAs remain relatively constant at all times after T_3 treatment (Figure 10A and 10B) and at different stages of spontaneous metamorphosis (Figure 10C). The results from both dot-blot hybridization and RT-PCR analyses, lend credence to the suggestion that T_3 may play a direct role in up-regulating the mRNAs encoding the urea cycle enzyme, arginase.

2.4 Discussion

The ornithine urea cycle enzymes have evolved quite early from the arginine metabolic pathway, and their primary structure is highly conserved in eukaryotic and prokaryotic organisms (Takiguchi *et al.*, 1989). It is therefore not surprising that the DNA sequence of

Rana arginase reveals a high degree of homology to those in other species.

It is apparent, from the studies reported herein, that we are not able to unequivocally establish that the T_3-induced up-regulation of arginase mRNA in the liver of this tadpole represents an "early gene response" (Tata, 1993) to this hormone. Indeed, it seems likely that the T_3-induction of TRα and TRβ mRNAs precedes the up-regulation of arginase mRNAs. However, quantitation and summation of the data from these investigations and other studies (Helbing *et al.*, 1992), as shown in Figures 11 and 12, suggest that the T_3-induced up-regulation of arginase mRNAs may precede the up-regulation of the mRNAs encoding the two mitochondrial-localized ornithine-urea cycle enzymes CPS-1 and OTC. Thus, our current model of T_3 action on the gene encoding the ornithine-urea cycle enzyme, arginase, in the *Rana catesbeiana* tadpole liver proposes (i) that the up-regulation of arginase mRNAs is a direct effect of T_3 (see scheme I in Figure 13) but, as suggested by Tata (1993), subject to activation by differential thresholds of receptor concentration or that the up-regulation of arginase mRNAs is an indirect effect of T_3, requiring an intermediate step involving liver-specific transcription factors (LSTFs), such as proposed in scheme II of our model (Figure 13). On the other hand, as shown in scheme III of Figure 13, the up-regulation of arginase mRNAs may require both the formation of a T_3-TR complex and liver-specific transcription factors (LSTFs).

A comparison of the data summarized in Figures 11 and 12 also emphasizes the similarities in the expression of the *Rana*

Figure 11. Relationship of TRα, TRβ, CPS-1, OTC and arginase mRNA levels in the liver of thyroid hormone-induced metamorphosing *Rana catesbeiana* tadpoles to some of the morphological and physiological changes occurring in these tadpoles.

A: Summary bar graphs depicting the accumulation of TRα, TRβ, CPS-1, OTC, and arginase (Arg) mRNAs in the liver of stage VI (Taylor and Kollros, 1946) tadpoles which had been injected (time 0) with 3, 5, 3'-triiodothyronine (T_3) at a dose of 3×10^{-10} moles/g body weight. The amount of urea-nitrogen excreted by similarly-treated tadpoles is superimposed on the bar graphs. The average relative amounts of the mRNA transcripts were determined by laser densitometric quantitation of dot blot hybridizations and RT-PCR products, and the plotted values were corrected based on the abundance of an mRNA (either actin or ferritin) which had been shown not to change during this treatment (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994).

B: The amount of urea-nitrogen excreted by T_3-induced metaorphosing tadpoles at various times after the hormone treatment is compared with a morphological change in the tadpoles (tail loss; a ratio of tail length to body length or TB) and with decreases in the relative amount of the T_3 originally injected in each tadpole [the last comparison is plotted from data obtained from tadpoles maintained at 25°C as reported by Yamamoto *et al.*, (1966)].

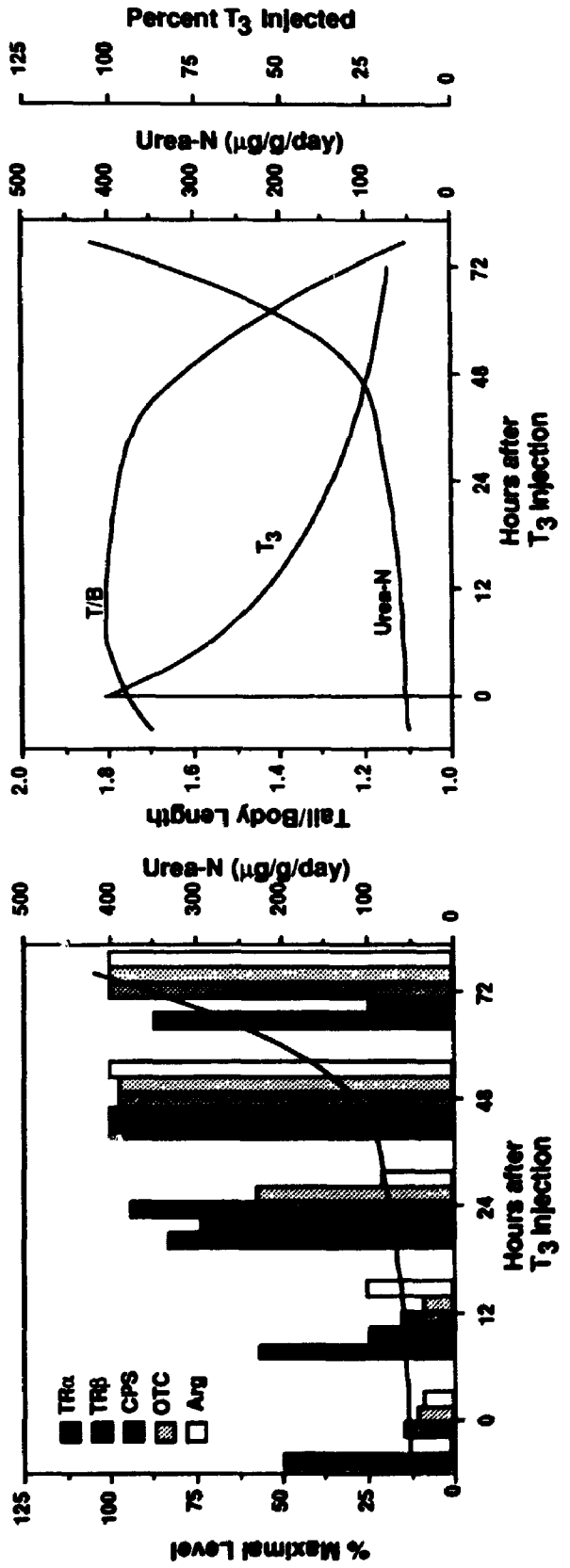
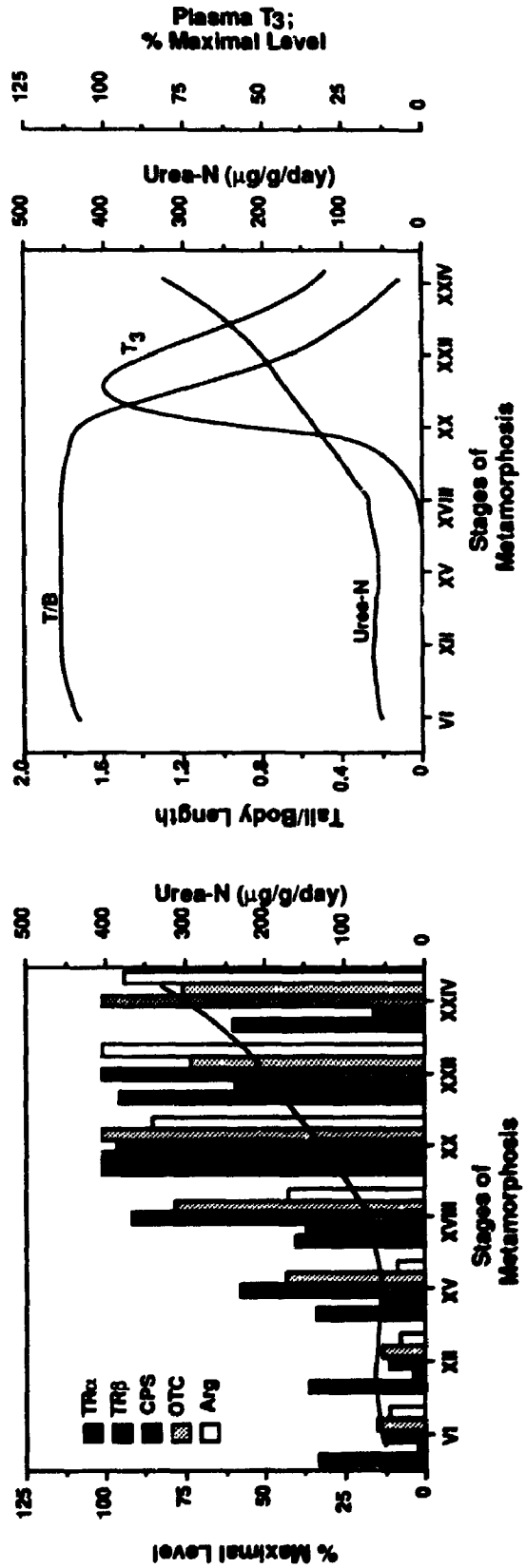

Percent T_3 Injected
125
100
75
50
25
0
Urea-N (μg/g/day)
500
400
300
200
100
0
72
48
24
12
0
Hours after T_3 Injection
T/B
T_3
Urea-N
2.0
1.8
1.6
1.4
1.2
1.0
Tail/Body Length
Urea-N (μg/g/day)
TRα
TRβ
CPS
OTC
Arg
% Maximal Level

Figure 12. Relationship of TRα, TRβ, CPS-1, OTC and arginase mRNA levels in the liver of spontaneously metamorphosing *Rana catesbeiana* tadpoles to some of the morphological and physiological changes occurring in the tadpoles.

A: Summary bar graphs depicting the accumulation of TRα, TRβ, CPS-1, OTC and arginase (Arg) mRNAs in the liver of tadpoles undergoing spontaneous metamorphosis. The amount of urea-nitrogen excreted by these tadpoles at various stages (Taylor and Kollros, 1946) of their postembryonic development is superimposed on the bar graphs. The average relative amounts of the mRNAs were determined as in Figure 11, and the plotted values were corrected based on the abundance of actin mRNA (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994).

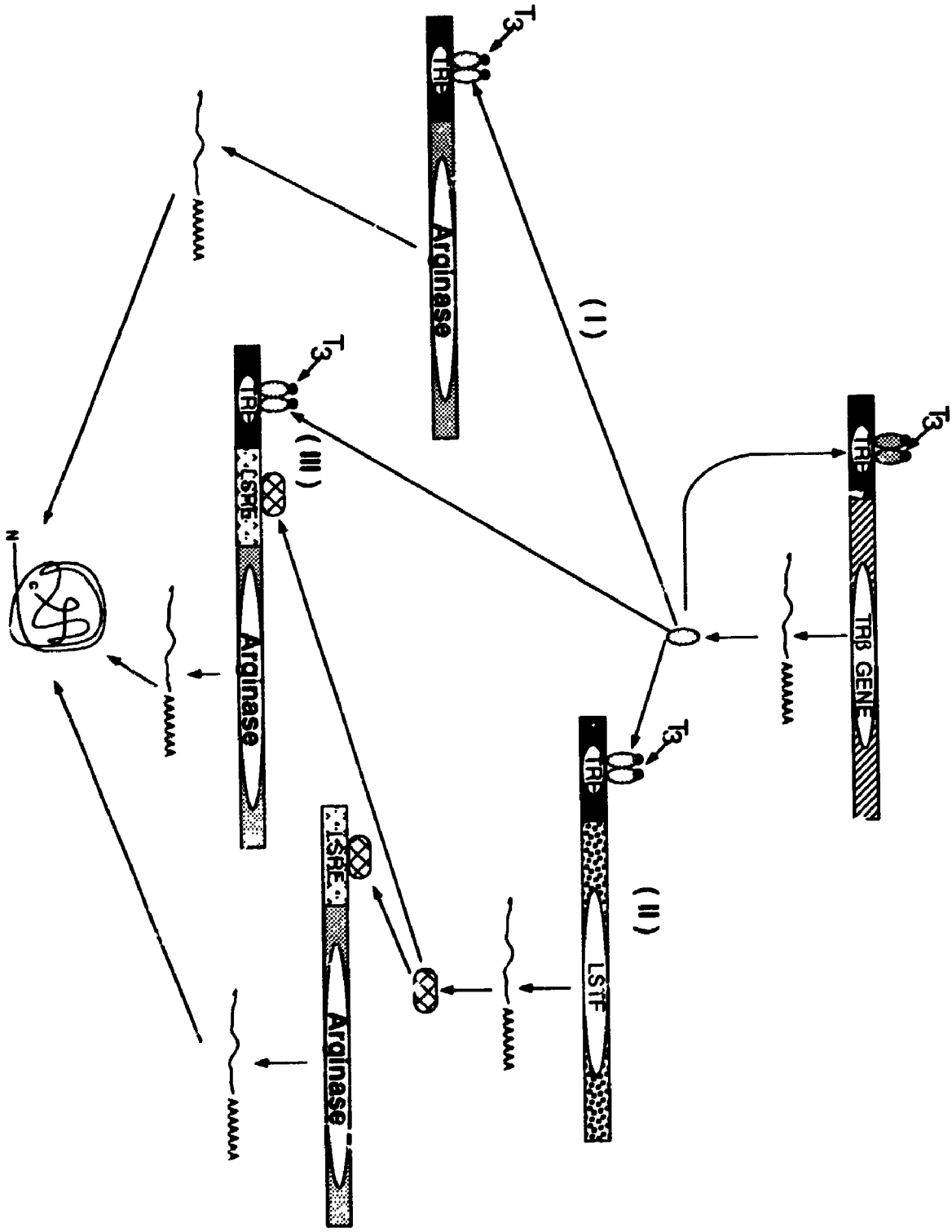
B: Comparison of the amount of urea-nitrogen excreted by spontaneously metamorphosing tadpoles with a morphological change in the tadpoles (tail loss; a ratio of tail length to body length or T/B) and with the relative amount of endogenous thyroid hormone reported in the plasma of staged-tadpoles [the last comparison is plotted from data reported by Galton and St. Germain(1985)].


Plasma T3;
% Maximal Level
125
100
75
50
25
0
Urea-N (μg/g/day)
500
400
300
200
100
0
T3
T/B
Urea-N
VI
XII
XV
XVIII
XX
XXII
XXIV
Stages of Metamorphosis
Tail/Body Length
2.0
1.6
1.2
0.8
0.4
0
Urea-N (μg/g/day)
TRα
TRβ
CPS
OTC
Arg
% Maximal Level

catesbeiana TRα, TRβ, CPS, OTC, and arginase genes in the liver of T_3-induced and spontaneously metamorphosing tadpoles. It is evident that T_3 induces an up-regulation of the mRNA transcripts from these genes that, 48 h after T_3 treatment of the tadpole, is comparable, in each case, to the level found at metamorphic climax (stage XX) in the liver of spontaneously metamorphosing tadpoles. Finally, the fact that the expression of TRβ mRNAs in this tadpole's liver appears to coincide with changes in the endogenous level of T_3 in the tadpole during spontaneous metamorphosis [T_3 increases after stage XVIII, is maximal at approximately stage XX, and returns to premetamorphic levels by stage XXIV (see Figures 11 and 12)] implicates it, rather than TRα, in the subsequent up-regulation of mRNAs encoding the liver urea cycle enzymes responsible for this organism's transition from ammonotelism to ureotelism. However, the demonstration that both TRα and TRβ mRNAs are coincidentally up-regulated in the liver of this tadpole by administration of T_3, albeit to quite different extents (Trα ~3-fold and TRβ >11-fold; see Figure 11), raises the possibility that both of these TRs may have specific roles in up-regulating the mRNAs encoding CPS, OTC, and arginase in the *Rana catesbeiana* tadpole liver.

Figure 13. Putative mechanism(s) for the T_3-induced up-regulation of the mRNAs encoding the urea cycle enzyme, arginase, in the liver of *Rana catesbeiana* tadpoles.

The schemes proposed depend upon an early autoinduction of mRNAs encoding the thyroid hormone β receptor protein (TRβ), and the enhanced synthesis and accumulation of this receptor protein. The proposed schemes also suggest that thyroid hormone subsequently interacts with newly-synthesized TRβs which have accumulated on putative thyroid hormone response elements (TREs) located upstream from genes thought to be regulated by this hormone. These schemes suggest three possible, albeit simplistic, means by which T_3 might cause an up-regulation in arginase mRNAs. The first scheme (I) presumes that T_3 has a direct effect on the gene encoding arginase (*i.e.* T_3 forms a complex with a TR(s) bound to TRE(s) on the promoter region of the gene encoding arginase). The second scheme (II) predicts that the up-regulation of arginase mRNAs is an indirect effect of T_3, requiring an intermediate step, such as the activation of gene(s) encoding liver-specific transcriptional factor(s) (LSTFs), which, in turn, regulate(s) the expression of the arginase gene. The third scheme (III) suggests that up-regulation of arginase mRNAs may require an interaction of T_3 with TR(s) bound to TRE(s) on the arginase gene promoter as well as with LSTF(s).


T3
TR
Arginase
(I)
T3
TR
(III)
LSRE
Arginase
N
T3
TR
TRβ GENE
T3
TR
LSTF
(II)
LSRE
Arginase

CHAPTER 3 ISOLATION AND CHARACTERIZATION OF THE PROMOTER AND UPSTREAM REGULATORY REGIONS OF THE CARBAMYL PHOSPHATE SYNTHETASE-1 (CPS-1) AND ORNITHINE TRANSCARBAMYLASE (OTC) GENES FROM *RANA CATESBEIANA*

3.1 Introduction

The results from my studies, and the report from Helbing *et al.* (1992), suggest that the expression of CPS-1, OTC and arginase mRNAs may be either directly or indirectly influenced by T_3. In order to obtain a better understanding of the relationship between the T_3-induced gene expression of these urea cycle enzymes and thyroid hormone receptors (TRs), I isolated and characterized the promoter and the upstream regulatory regions of the CPS-1 and OTC genes from *Rana catesbeiana*. These studies were aimed at defining possible regulatory elements upstream from the promoter regions of these genes and, as such, serve as a first step towards elucidating the transcriptional regulatory mechanisms governing the expression of these genes.

3.2 Materials and Methods

3.2.1 Southern-Blot Hybridization Analyses

Southern blots of *Rana catesbeiana* genomic DNA were prepared and hybridized with a 561 bp *Apa* I/*Eco*R I fragment or a

80 bp *Apa* I/*Nci* I fragment from a *Rana* CPS-1 cDNA (clone 5'-9; Helbing and Atkinson, 1994), and with three different DNA fragments from *Rana* OTC cDNA clone 1 (Helbing *et al.*, 1992), namely, a 516 bp *Eco*R I/*Xho* I fragment, a 316 bp *Sau*3A I/*Xho* I fragment and a 220 bp *Eco*R I/*Sau3A* I fragment. Similar or the same blots were subsequently hybridized with DNA fragments encoding the first exon in CPS-1 and the first exon in OTC. In each case, the membrane was prehybridized in a solution containing 50% formamide, 3X SSPE, 1% SDS, 0.5% Blotto and 0.5 mg/ml denatured herring sperm DNA for 4 h at 50°C. Hybridization was performed in a similar solution containing 10% dextran sulfate and a [α-^{32}P]-dCTP (NEN Dupont Canada; specific activity >3,000 Ci/mmol) random primer-labelled (Pharmacia Inc.) DNA fragment for 20 h at 50°C. The final stringency washes were done in 1X SSC and 1% SDS for 30 min at 55°C. Kodak XR-Omat RP film was preflashed and exposed to the membrane with a Dupont Cronext Lightening Plus intensifier screen at -80°C.

3.2.2 Genomic Cloning, Isolation and Sequencing of the Promoter Region of the OTC Gene

A *Rana catesbeiana* genomic library was constructed in the *Xho* I site of a λFIXII vector (Stratagene Inc.) and consisted of 2.8 x 10^6 independent recombinant phage. For isolating clones containing the OTC gene promoter, the genomic library was screened with an [α-^{32}P]-dCTP-labelled (NEN Dupont Canada; specific activity >3,000 Ci/mmol) 220 bp *Eco*R I/*Sau*3A I OTC cDNA fragment. Two clones,

screened from 2 X 10^6 plaques, survived tertiary screening. These two positive clones, designated RcOTC-1 and RcOTC-2, contained inserts of approximately 15 kb and 14 kb, respectively.

A *Not* I cassette, containing the insert from RcOTC-1 and the flanking T3 and T7 promoter vector sequences, was prepared and digested with various restriction enzymes. The resultant fragments were separated on a 1.0% agarose gel and vacuum blotted onto a Zetaprobe membrane. To identify potential OTC gene promoter-containing fragments, the blot was hybridized with the 220 bp *Eco*R I/*Sau*3A I OTC cDNA fragment. A ~5 kb *Sac* I positive fragment from RcOTC-1 was cloned into pBluescript II SK$^-$ (Stratagene Inc.) at the *Sac* I site and named as pGOTC-1. A ~1.1 kb *Sac* I/*Hin*d III fragment from pGOTC-1, subsequently found to contain the OTC gene promoter region, was subcloned into pBluescript II SK$^-$ at the *Sac* I/*Hin*d III sites and designated as pGOTCpro.

The nucleotides in the relevant portion of pGOTC-1 and in all of pGOTCpro were sequenced from both strands by the Exo III-based DNA sequencing method (Li and Tucker, 1993) using [^{35}S]-dATP (Dupont Canada, Mississauga, Ontario, Canada; specific activity 600 Ci/mmol) and a Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH) for direct dideoxynucleotide sequencing. In some cases, particular sequences were confirmed by subcloning DNA fragments into pBluescript II SK$^-$ and using T_3, T_7, SK, KS primers for the dideoxynucleotide sequencing (Chen and Seeburg, 1985).

3.2.3 Genomic Cloning, Isolation and Sequencing of the Promoter Region of the CPS-1 Gene

The same *Rana catesbeiana* genomic library was screened with an [α-^{32}P]-dCTP-labelled 80 bp *Apa* I/*Nci* I fragment from *Rana* CPS-1 cDNA (clone 5'-9; Helbing and Atkinson, 1994). One clone, screened from 2 x 10^6 plaques, survived tertiary screening. This positive clone, designated RcCPS-1, contained an insert of approximately 15 kb.

RcCPS-1 was digested with restriction enzymes which have no cut sites in the λFIXII vector and delineate the unique sites flanking the RNA promoters. The resultant fragments were separated on a 1.0% agarose gel and vacuum blotted onto a Zetaprobe membrane. To identify potential CPS-1 gene promoter-containing fragments, the blot was hybridized with the 80 bp *Apa* I/*Nci* I fragment from the *Rana* CPS-1 cDNA clone 5'-9. A ~7 kb *Sac* I positive fragment from RcCPS-1 was cloned into pBluescript II SK$^-$ (Stratagene Inc.) at the *Sac* I site and named pGCPS-1. A ~1.4 kb *Pst* I/*Sac* I fragment from pGCPS-1, subsequently found to contain the CPS-1 gene promoter region, was subcloned into pBluescript II SK$^-$ at *Pst* I/*Sac* I sites. It was designated as pGCPS-1pro and was completely sequenced from both strands by the same method as used for determining the sequences of pGOTCpro.

3.2.4 Computer Analyses

Sequence characterization was accomplished using DNA Strider version 1.0 (Marck, C., Service de Biochimie-Department de biologie, Institute de Recherche Fondamentale, Commissariat á l'Energie Atomique-France), and DNA Inspector II$^+$ (Textco, West Lebanon, NH) software. The EMBL and GenBank databases were searched using the Wisconsin Genetics Computer Group sequence analysis software package Version 6.1 (Devereux *et al.*, 1984).

3.2.5 Generation of the First Exons of CPS-1 and OTC Genes by the Polymerase Chain Reaction (PCR)

Specific 17 nt primers, pCPSexon1.1 (5'-$^{+1}$CAGAAAGAGC-TGAACAG^{+17}-3'), pCPSexcn1.2 (5'-$^{+151}$CTTGACGCTCATAAGCC^{+135}-3'), pOTCexon1.1 (5'-$^{+1}$CATACACATGTGAAGAG^{+17}-3') and pOTCexon1.2 (5'-$^{+185}$CCAAACTGACGAACAAT^{+169}-3') for generation of CPS-1 and OTC first exons were designed with the aid of OLIGO software (Rychlik and Rhoads, 1989; search parameters: 760 pM nucleic acid and 50 mM salt). All primers were synthesized by Vetrogen (London, Ontario, Canada).

A reaction consisted of 1 ng DNA template (pGCPS-1pro plasmid for generation of the CPS-1 first exon, or pGOTC-1 plasmid for generation of the OTC first exon), 10 µl 10X Taq DNA Polymerase buffer (1X = 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0 at room temperature), 8 µl dNTPs stock solution (2.5 mM stock of each

dNTP; final concentration = 200 μm) and 0.6 μg of each of specific primers (pCPSexon1.1 and pCPSexon1.2 for generation of the CPS-1 first exon, pOTCexon1.1 and pOTCexon1.2 for generation of the OTC first exon). The final volume was brought up to 100 μl with aut. claved double-distilled water (ddH_2O). The PCR conditions used were similar to those previously described for RT-PCR (see section 2.2.6) except that the annealing temperature was set at 58°C. After the PCR reaction, 2 μl of the klenow fragment (Pharmacia Inc.) was added to each sample for 30 min at 37°C to blunt-end the amplified exons. The exon fragments were isolated from 2% agarose gels and cloned into pBluescript II SK$^-$ at the *Sma* I site and their sequences were confirmed by dideoxynucleotide sequencing (Chen and Seeburg, 1985).

3.2.6 Primer Extension

A 28 nt primer, complementary to CPS-I mRNA at position +35 to +62, and a 29 nt primer, complementary to OTC mRNA at position +29 to +57 were ^{32}P end-labelled using T4 polynucleotide kinase (Pharmacia Inc.). Briefly, 2 μl (100-200 ng) of primer, 1.5 μl of 1 M Tris-HCl (pH7.5), 3 μl of 0.1 M $MgCl_2$, 1.5 μl of dithiothreitol (DTT; Sigma), 10 μl of [γ-^{32}P]-ATP (NEN Dupont Canada; specific activity >3,000 Ci/mmol), 10 μl of ddH_2O and 2 μl (10-20 Units) of T4 polynucleotide kinase were combined in an eppendorf tube. The mixture was incubated at 37°C for 1 h. The end-labelled primers were separated from the free [γ-^{32}P]-ATP by a NICK Column

(Pharmacia Inc.) and the radioactivity of the primers was determined in a Beckman LS 5801 scintillation counter.

Approximately 5 X 10^5 cpm of primer was annealed to 20 µg of total *Rana catesbeiana* liver RNA, or to 20 µg of yeast tRNA (Pharmacia Inc.), or added to ddH_2O lacking RNA. In each case, hybridization was performed at 55°C for 4 h in a securely sealed tube in which the total volume was 15 µl containing 0.15 M KCl, 10 mM Tris-HCl (pH8.3) and 1 mM EDTA. After the hybridization period was completed, the reaction mixture was diluted to 100 µl with a solution containing 50 mM Tris-HCl (pH8.3), 10 mM DTT, 6 mM $MgCl_2$, 0.2 mg/ml actinomycin D (Sigma), 0.25 mM dNTPs and 15 units avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia Inc.) and incubated at 42°C for 1 h. After the incubation at 42°C, 100 µl of RNase mix [100 µg/ml salmon sperm DNA and 20 µg/ml RNase A (DNase-free; Pharmacia Inc.)] was added to each primer extension reaction tube and the tubes were incubated for 15 min at 37°C. The samples were extracted with phenol, precipitated with ethanol, and, following electrophoresis in a 6% polyacrylamide sequencing gel containing 8 M urea, the products were visualized by autoradiography. Also included in the same gel were sequencing reactions in which the same primers were used for sequencing the 5'-flanking regions of these genes and delineating the precise transcription start sites.

3.3 Results

3.3.1 Generation of 5'-specific Nucleotide Probes from the OTC and CPS-1 cDNAs to Screen a *Rana catesbeiana* Genomic Library

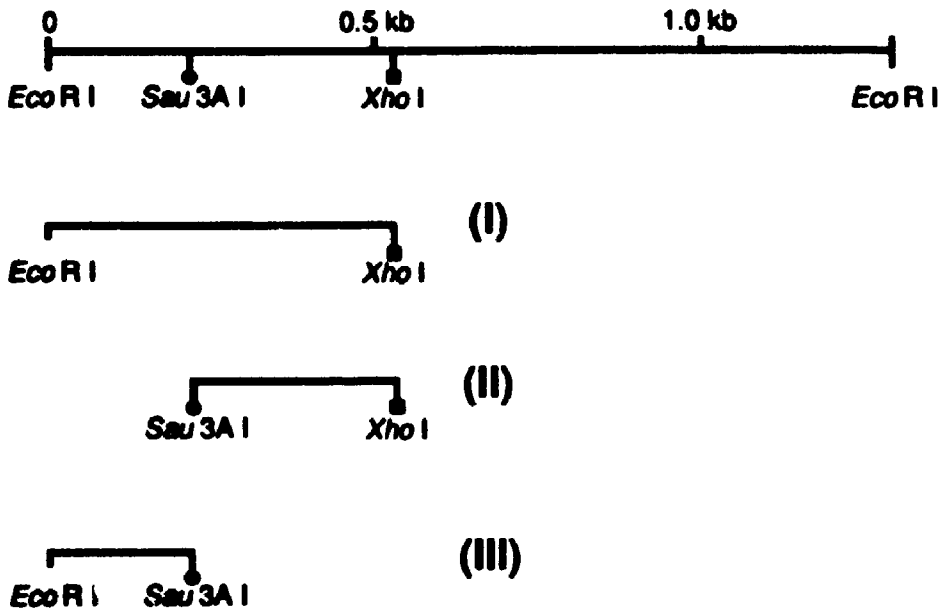
The size of OTC mRNA in *Rana catesbeiana* is approximately 1.6 kb (Helbing *et al.*, 1992). Since the size of the OTC gene in other organisms is approximately 75 to 85 kb in size (Takiguchi, *et al.*, 1987; Hata *et al.*, 1988; Scherer *et al.*, 1988), I assumed that the *Rana catesbeiana* OTC gene was also of similar size. Thus, in order to isolate the promoter region for the OTC gene, I generated 5'-nucleotide probes from the OTC cDNA to screen our *Rana* genomic library (Figure 14). The DNA fragments prepared from the 5' portion of the OTC cDNA, shown in Figure 14A, were initially used as probes, on Southern blots of *Rana* genomic DNA, to assess the possibility of their use for screening our *Rana* genomic library. Southern blot hybridization analyses with these DNA fragments disclosed that two of these DNA fragments [a 516 bp *Eco*R I/*Xho* I fragment (I) and a 316 bp *Sau3A* I/*Xho* I fragment (II)] hybridized with a number of different-sized pieces of DNA in all of the restriction enzyme digestions (see Figure 14B I and II). However, a third DNA fragment [a 220 bp *Eco*R I/*Sau3A* I fragment (III)] hybridized with only a single piece of DNA in each of the restriction enzyme digests (see Figure 14B III), and this fragment was used as a 5'-specific probe to screen the *Rana* genomic library for the promoter region of the OTC gene.

Figure 14. Generation of 5'-specific probes for OTC to screen a *Rana catesbeiana* genomic library.

A: Enzyme restriction map of a *Rana catesbeiana* OTC cDNA (clone 1; Helbing *et al.*, 1992) showing the origin of the DNA fragments considered for use as probes to screen a genomic library for the OTC gene.

B: Southern blot hybridization analyses of *Rana catesbeiana* genomic DNA using the probes defined in A. Genomic DNA (20 μg) was digested with *Bam*H I (Lanes 1, 4 and 7), *Pst* I (Lanes 2, 5 and 8) and *Eco*R I (Lanes 3, 6 and 9). The digests were electrophoresed through a 0.7% agarose gel, transferred onto a Zetaprobe membrane and hybridized with the [α-^{32}P]-labelled DNA fragments described in A and denoted as I (Lanes 1, 2 and 3), II (Lanes 4, 5 and 6) and III (Lanes 7, 8 and 9). The relative sizes of the DNA fragments from the enzyme digests were determined from a coelectrophoresed DNA ladder shown on the left in kilobases (kb).

A


0
0.5 kb
1.0 kb
EcoR I
Sau 3A I
Xho I
EcoR I
(I)
EcoR I
Xho I
(II)
Sau 3A I
Xho I
(III)
EcoR I
Sau 3A I

B

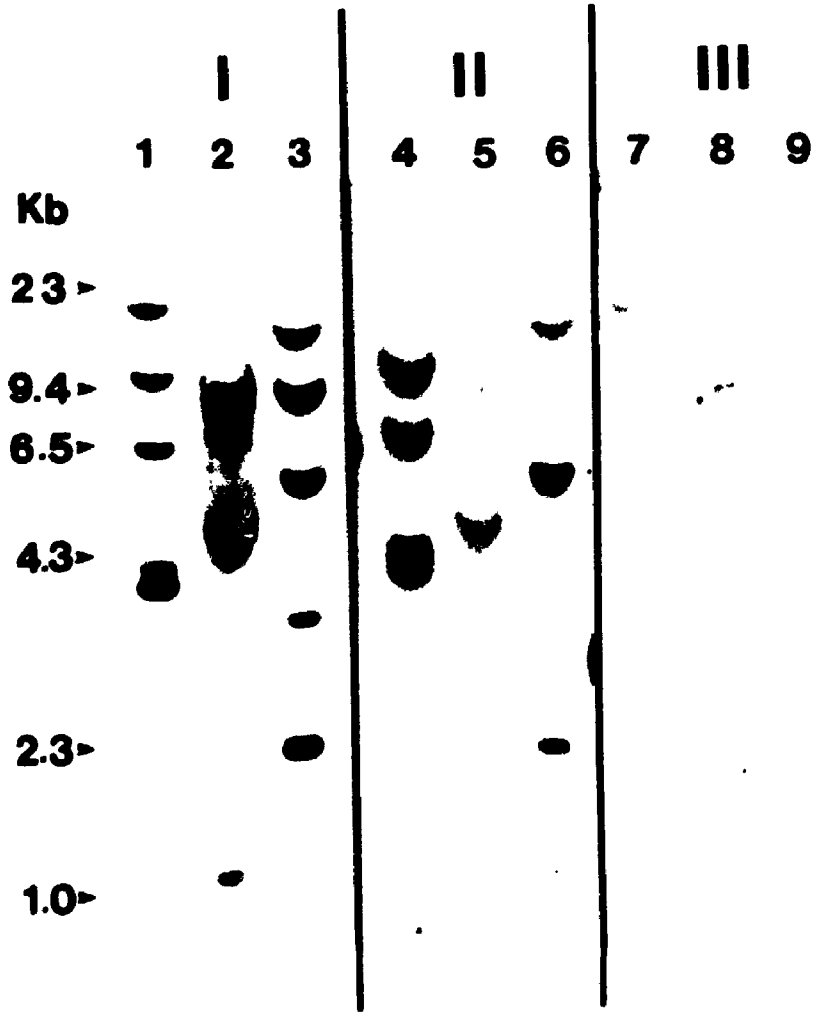
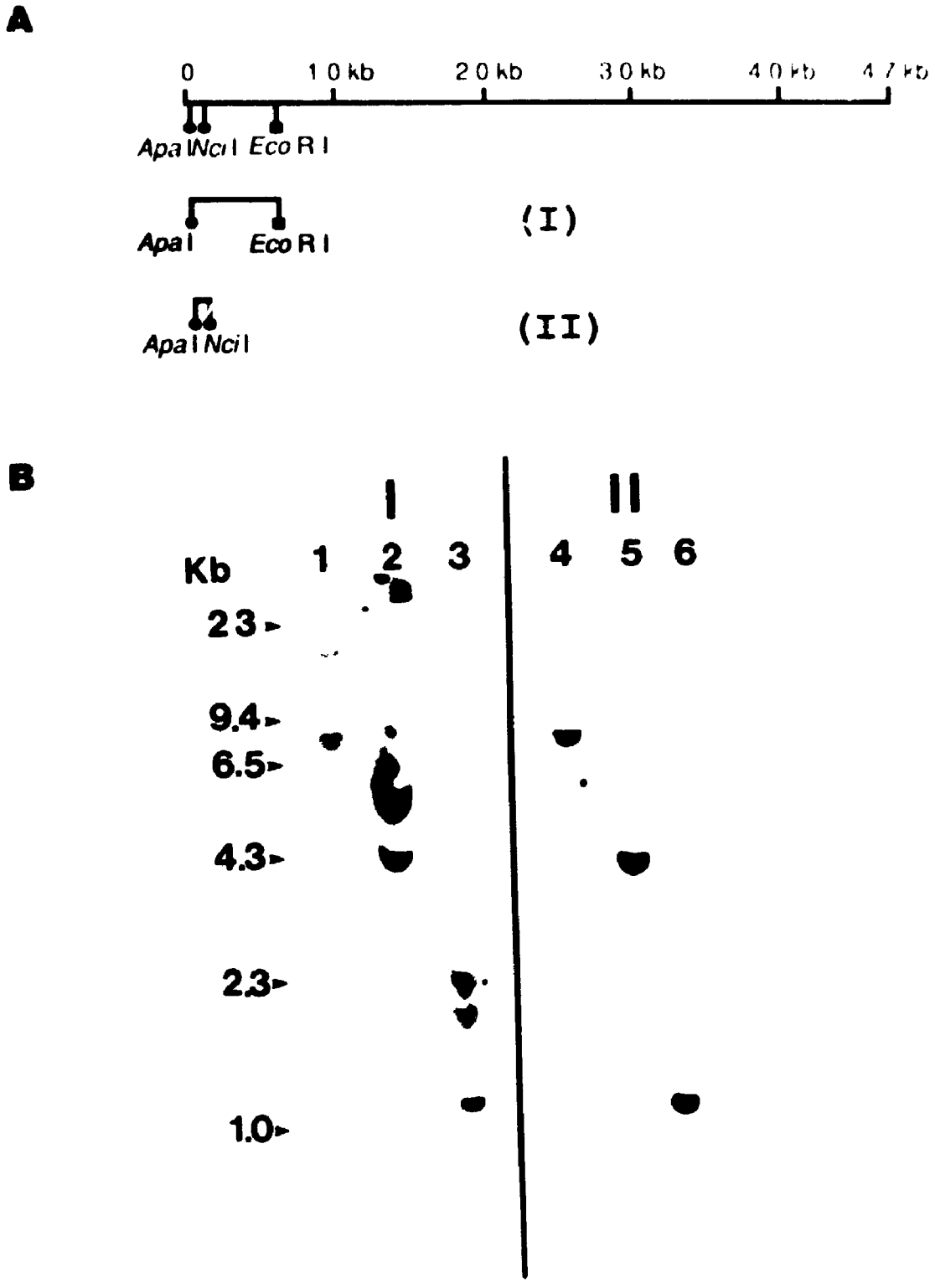

I
II
III
1 2 3 4 5 6 7 8 9
Kb
23
9.4
6.5
4.3
2.3
1.0

Figure 15. Generation of 5'-specific probes for CPS-1 to screen a *Rana catesbeiana* genomic library.

A: Enzyme restriction map of a *Rana catesbeiana* CPS-1 cDNA (Helbing and Atkinson, 1994) showing the origin of the DNA fragments considered for use as probes to screen a genomic library for the CPS-1 gene.

B: Southern blot hybridization analyses of *Rana catesbeiana* genomic DNA using the probes defined in A. Genomic DNA (20 μg) was digested with *Bam*H I (Lanes 1 and 4), *Hin*d III (Lanes 2 and 5) and *Pst* I (Lanes 3 and 6). The digests were electrophoresed through a 0.7% agarose gel, transferred onto a Zetaprobe membrane and hybridized with the [α-^{32}P]-labelled DNA fragments described in A and denoted as I (Lanes 1, 2 and 3) and II (Lanes 4, 5 and 6). The relative sizes of the DNA fragments from the enzyme digests were determined from a coelectrophoresed DNA ladder shown on the left in kilobases (kb).


A
0
10 kb
20 kb
30 kb
40 kb
47 kb
ApaI NciI EcoRI
ApaI EcoRI
(I)
ApaI NciI
(II)
B
I
II
Kb
1
2
3
4
5
6
23
9.4
6.5
4.3
2.3
1.0

The size of CPS-1 mRNA in *Rana catesbeiana* is approximately 6.5 kb (Helbing *et al.*, 1992) and is similar in size to the rat CPS-1 mRNA (Adcock and O'Brien, 1984; Nyunoya *et al.*, 1985). Since the rat CPS-1 gene was partially cloned and found to contain numerous large introns (Lagace *et al.*, 1987), I presumed that *Rana* CPS-1 gene also contained introns. Thus, in order to isolate the promoter region for the CPS-1 gene, I generated 5'-nucleotide probes from the CPS-1 cDNA (clone 5'-9; Helbing and Atkinson, 1994) to screen our *Rana* genomic library (Figure 15). The DNA fragments prepared from 5' portion of the CPS-1 cDNA, shown in Figure 15A, were initially used as probes, on Southern blots of *Rana* genomic DNA, to assess the possibility of their use for screening our *Rana* genomic library. Southern blot hybridization analysis with a 561 bp *Apa* I/*Eco*R I fragment (I) disclosed that this DNA fragment hybridized with a number of different-sized pieces of DNA in all of the restriction enzyme digestions (see Figure 15B I). However, a DNA fragment [a 80 bp *Apa* I/*Nci* I fragment (II)] hybridized with only a single piece of DNA in each of the restriction enzyme digests (see Figure 15B II), and this fragment was used as a 5'-specific probe to screen the *Rana* genomic library for the promoter region of the CPS-1 gene.

3.3.2 Isolation and Characterization of the Promoter and Upstream Regulatory Regions in a *Rana catesbeiana* OTC Gene

As a first step towards elucidating the transcriptional regulatory mechanisms governing the T_3-induced, tissue-specific expression of the urea cycle enzyme genes in the liver of *Rana catesbeiana*, I isolated the OTC gene promoter region by using a 220 bp 5'-specific OTC gene probe (see Figure 14) to screen the *Rana* genomic library. Two positive clones, containing inserts of approximately 15 kb and 14 kb, survived tertiary screening. The nucleotide sequence of the OTC gene 5'-flanking region was determined from these clones and is shown in the Figure 16.

Inspection of the nucleotide sequence in the upstream portion of the *Rana* OTC gene reveals a putative TATA motif located at -10 and another TATA motif at position -154. Two putative C/EBP (CAATT/enhancer core binding protein) binding sites, (5'-GCAAT-3'; Graves *et al.*, 1986; Vinson *et al.*, 1989; Cao *et al.*, 1991), exist at -121 and -305. Motifs, [5'-ATTACACAAG-3', (Ryden and Beemon, 1989; Xu and Tata, 1992) and 5'-ATTGGGAAAT-3' (Ryden *et al.*, 1993)] also believed to serve as C/EBP binding sites, are located at positions -91 to -82 and -23 to -14, respectively. A well-conserved heat shock element, consisting of tandem arrays of inverted 5 bp units with the consensus sequence nGAAn (Amin *et al.*, 1988; Xiao and Lis, 1988), is located between -172 and -163.

Computer-assisted analyses of the OTC gene promoter region revealed the presence of two sets of direct 23 nucleotide repeats (positions -918 to -896 and -523 to 501; positions -891 to -869 and -496 to -474) and three sets of inverted nucleotide repeats, two sets, consisting of 14 nt each, share the same sequence (5'-AATGAACGGACTTT-3') and are located at positions -918 to -905 and

-751 to -738; positions -751 to -738 and -523 to -510). The other set of inverted repeats is made up of 12 nt and is located at positions -275 to -264 and -119 to -108. In addition, one set of 4 nt inverted repeats was found to be present at the 5'-end of OTC transcript (+5 to +8 and +9 to +12).

The transcriptional start sites within the OTC gene were identified by primer extension (Figure 17). I defined cytosine as the +1 nt of OTC gene transcript, although the next two nucleotides downstream from it (adenine and thymine) may also be cap addition sites. However, the fact that the OTC cDNA (clone 1) from which the 220 bp 5'-specific probe was derived also extends upstream to this cytosine (Helbing *et al.*, 1992) supports my contention that this cytosine is the transcription start site.

The first exon in the OTC gene is 185 nt in size and encodes most (24 out of the 32 amino acid residues including all of the basic residues) of the amino terminal presequence that functions as a mitochondrial targeting signal (Takiguchi *et al.*, 1987; Helbing *et al.*, 1992; Lathrop and Timko, 1993). The first intron is larger than 3 kb (Figure 16), and a consensus sequence, 5'-GTAAGT-3', for exon-intron splicing is located at the 5' end of the first intron of the OTC gene (Padgett *et al.*, 1986).

Using the nucleotide sequence in the first exon, generated by PCR methodology (see "Materials and Methods"), as a probe, I performed Southern blot hybridization analyses with enzyme-restricted genomic DNA from *Rana catesbeiana* (Figure 18). The results from this hybridization show that a single DNA fragment is

Figure 16. Enzyme restriction map and partial sequence of pGOTC-1.

A: Enzyme restriction map of pGOTC-1. H, and S represent the relative location of restriction enzyme sites for *Hind* III, and *Sac* I, respectively. The hatched-boxed area indicates the area sequenced (shown in B) in both directions. An intron start site is indicated by an arrowhead. The relative 1 kb size is shown on the top right side of this panel.

B: Nucleotide sequence of the promoter region, exon 1 and part of intron 1 in the *Rana catesbeiana* OTC gene. The transcription start site is designated +1. Two TATA motifs (underlined) are present in this promoter. The boxed areas delineate C/EBP (CAATT/enhancer binding protein) binding consensus sequences. A well-conserved heat shock element is shown in double underlined. Two direct repeats (paired arrows 1 and 2) and four inverted repeats (paired arrows a, b, c and d) are in the 5'-flanking region. The first exon is 185 nt in size. The translation start codon, ATG, is located at +112. The first intron begins at +186, and a consensus sequence (bold) for exon-intron splicing is present at the 5'-end of intron 1. One letter code words for the amino acids corresponding with the codons in the first exon are shown.

A

B

```
-1029                                                        GAGCTC
                                                             Sac I
-1023 GCAAGGTGTCAGTCTCACCGATAAAAGCGGCAAGATTGACTTCCTTTTCT
 -973 AGTCCTGTCATACCTCATCATGTTCAAAATGAACAGACTTTAGTTCATGT
                (1)                                  (2)
 -923 GTGGGAAAGTCCGTTCATTCGGAAGTCCATCGTAACTCCGCCAAAAGTCA
           (a)
 -873 GTCGGGAAAAACGCAGGGCACCCCCCTTCCCCAAAACACCCACCCCCCCT
 -823 TCTTGAGAGCATGTGGCCTGGTACAGTTCAGGAGGGGGGGCACTCGCTTG
 -773 TCCCCACCCCCTTTCCCGACTGGCCGGGCTGCATGCTCGGATAAGGGTCT
 -723 GGTGTGGATTTGGGGGGACCCCCACGCCGTTTTTTCGGCATAGGGGGTTC
 -673 CCCTTAAAATCCATACCAGACCTAAGCCGTCCCGCGACAGGGCTCGCAAG
 -623 GTGTCAATCTCGCTAATAAAAGCGGCAAGATTGACTTCCTTTTCTAGTCC
                                 (a) & (b)
 -573 TGTCGTACCTCATCACGTTCAAAATGAACGGACTTTAGTTAGTGTGTGGG
      (1)                                  (2)
 -523 AAAGTCCGTTCATTCGGAAGTCCGCCATAACTCCGCCAAAAGTCAGTCGG
           (b)
 -473 CAAAAACGTTGGGCCTAGTCTGCCGGAAAGTCCGGTCGTGTGTGCGCAAG
 -423 TCCGATCATTCGAAAAGTCCATTGGAAGTCCGTCGAAAGTCCGTCTGACT
 -373 TTTGATGCTGAAAACTCCGCTCGTGTGTACGCGGCATAATACCAGACATC
 -323 CAGTGGTATCTGCTCTTAGCAATGCTTTCTCTTTTGAAAAATTAAACTGA
                                                       (c)
 -273 AATACAGATTTACTTTTAAAAGACACTGTCATTGTGACATTGCCTCCCTA
 -223 CAATCCTAATGGGCAGTTGATGTTATCTCTTAAAGAATTTAAAAAGGAGG
 -173 CTGAATGTTCTGTTATTTTTATAAGACAAAAATGTATCAGTTAGCTTTAA
 -123 CAGCAATCTGTATTTCCACAGATAAGGTAATTATTACACAAGACAACTGA
                    (c)
  -73 CTGTTCAAAGAGATTTTCGCTTGTCCAGTACAGAGCTGGCACTTTGATCA
  -23 ATTGGGAAATCACTATAATTGTT
                                                  HindIII
                                  +1 CATACACATGTGAAGAGCTAAGCTTTA
                                  (d)  (d)
  +28 AGTGATTCTGAATAGAACTGAAAAACTTCTACAATAAACTACAAACGTGT
                                      *
  +78 GCTTACTAGAGGAACAATCTGCAAGCAGCTCAAAATGTTACATCATATGA
                                                M  L  H  H  M
 +128 GAACTATTATTAATGCTTCATGGAGATATGGTAATAAGTGCATTGTTCGT
      R  T  I  I  N  A  S  W  R  Y  G  N  K  C  I  V  R
 +178 CAGTTTGG +188 gtaagttgtatttttaatcttctcaattatatatatttt
       Q  F          Intron 1
 +205 tttttctatcattgctgacttatttttaaaaa---> Sac I
```

Figure 17. Determination of the *Rana catesbeiana* OTC gene transcription start site by primer extension.

A 29 nt primer, complementary to OTC mRNA at position +29 to +57 (see Figure 16) was ^{32}P end-labelled using T4 polynucleotide kinase. Approximately 5 x 10^5 cpm of this primer was annealed to 20 μg of total *Rana catesbeiana* liver RNA (Lane 3), 20 μg of yeast tRNA (Lane 2) or ddH_2O (Lane 1). The extended products were analyzed on a 6% polyacrylamide sequencing gel containing 8 M urea. Included in the gel were sequencing reactions in which the same primer was used for sequencing the 5'-flanking region of the OTC gene (T=Thymine, G=Guanine, C=Cytosine and A=Adenine).

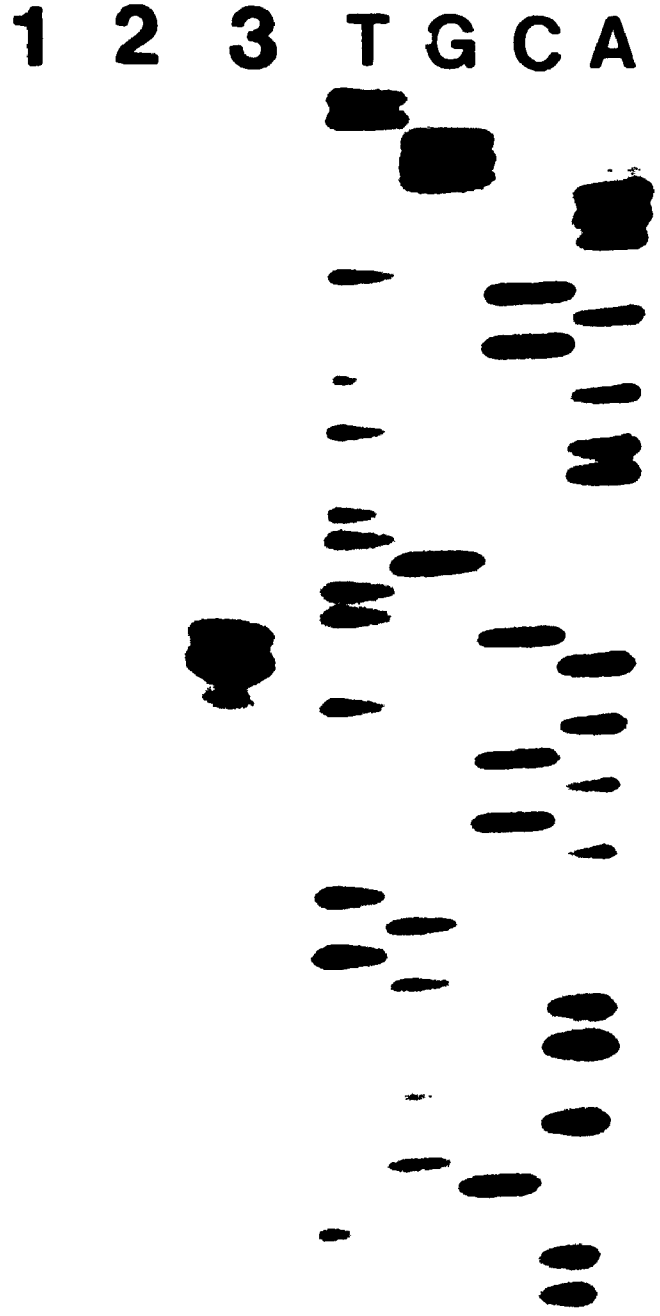
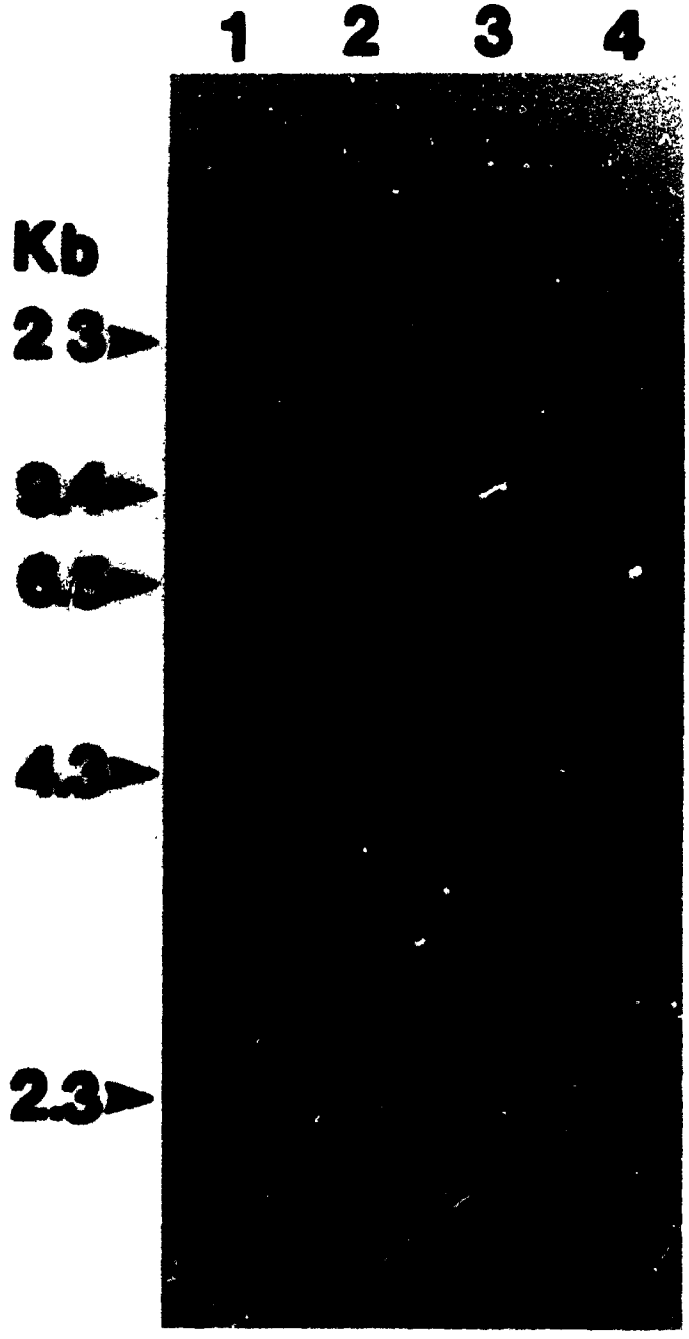

1 2 3 T G C A

Figure 18. Southern blot hybridization analysis of *Rana catesbeiana* genomic DNA with the first exon in the OTC gene.

Rana catesbeiana genomic DNA (20 μg) was digested with *Bam*H I (Lane 1), *Eco*R I (Lane 2), *Hin*d III (Lane 3) or *Pst* I (Lane 4). The digested DNAs were separated on a 0.7% agarose gel, and the fragments were transferred onto a Zetaprobe membrane and hybridized to the first exon in the OTC gene as outlined in "Materials and Methods". The relative sizes of the DNA fragments from the enzyme digests were determined from a coelectrophoresed DNA ladder shown on the left in kilobases (kb).


1
2
3
4
Kb
23
9.4
6.5
4.3
2.3

recognized by digestion of genomic DNA with *Bam*H I, *Eco*R I or *Pst* I. Whereas, *Hin*d III digestion, as predicted (the first exon of OTC gene has an internal *Hin*d III site at +20), results in two DNA fragments recognizing this probe. Therefore, these results suggest that the OTC gene is a single copy gene in the *Rana catesbeiana* genome.

3.3.3 Isolation and Characterization of the Promoter and Upstream Regulatory Regions in a *Rana catesbeiana* CPS-1 Gene

Using a 80 bp 5'-specific probe to screen our *Rana* genomic library, and using the same strategy as I used for isolating the OTC gene promoter region, I cloned the CPS-1 gene promoter region. One positive clone, containing an insert of approximately 15 kb, survived tertiary screening. The nucleotide sequence of the CPS-1 gene 5'-flanking region is showed in the Figure 19. The sequence includes 1180 nucleotides upstream and 209 nucleotides downstream from the transcription start site. Comparison with the cDNA and protein sequence of the CPS-1 precursor (Helbing and Atkinson, 1994) reveals that the region I sequenced contains the first exon and extends downstream into intron 1. Exon 1 comprises the 5'-untranslated region (40 nucleotides) of the CPS-1 precursor plus the 5'-translated sequence encoding the entire signal peptide of the pre-enzyme (33 amino acids), the signal cleavage site (amino acids 33 and 34), and the first 4 amino acids (34-37) of the amino terminus of the mature enzyme. The first two nucleotides at the 5'-end of the

first intron of the CPS-1 gene are in agreement with the nucleotide consensus for exon-intron splicing (Padgett *et al.*, 1986).

Seven putative TATA motifs (-80, -105, -176, -433, -608, -616 and -689), one CCAAT box (-550) and one Sp1 box (-742; GGGCGG) are located in the CPS-1 gene promoter region. Two putative C/EBP binding sites, (5'-GCAAT-3'; Vinson *et al.*, 1989; Cao *et al.*, 1991), exist at -330 and -780. Two 10 nt motifs, 5'-ATGTTGCAAC-3' and 5'-TTGTTGAAAG-3', also suggested to be a C/EBP binding sites (Howell *et al.*, 1989; Lagace *et al.*, 1992; Goping *et al.*, 1992; Ryden *et al.*, 1993; Goping and Shore, 1994), are located at -201 to -194 and -1075 and -1066, respectively.

Computer analyses of the CPS-1 gene promoter region reveals the presence of four inverted repeats: 1) a 22 nt inverted repeat (between -593 to -572 and -482 to -461); 2) a 14 nt inverted repeat (between -566 to -553 and -501 to -488); 3) a 13 nt inverted repeat (between -794 to -782 and -775 to -763); and 4) a 6 nt inverted repeat (between -7 to -2 and -1 to +5). Moreover, an 8 nt sequence, 5'-ATACCACC-3,' between nucleotides -456 and -449, is a direct repeat of a sequence located 15 nt upstream from the transcription start site of the CPS-1 gene.

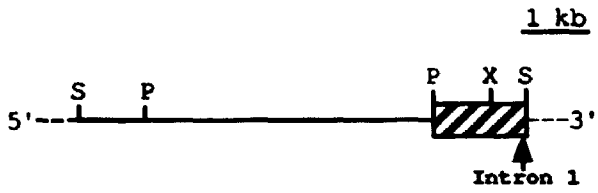
The putative transcription start site within the CPS-1 gene was identified by primer extension (Figure 20). The results suggest that either cytosine or adenine may be employed as the cap addition site.

Figure 19. Enzyme restriction map and partial sequence of pGCPS-1.

A: Enzyme restriction map of pGCPS-1. P, S, and X represent the relative location of restriction enzyme sites for *Pst* I, *Sac* I and *Xba* I, respectively. The hatched-boxed area indicates the portion sequenced (shown in B) in both directions. An intron start site is indicated by an arrowhead. The relative 1 kb size is shown on the top right side of this panel.

B: Nucleotide sequence of the promoter region, exon 1 and part of intron 1 in the *Rana catesbeiana* CPS-1 gene. The transcription start site is designated +1. Seven putative TATA motifs (underlined) are present in this promoter. The boxed areas delineate C/EBP (CAATT/enhancer binding protein) binding consensus sequences. One CAAT motif (bold and underlined) and one GC motif (bold and double underlined) are present in this promoter. A direct repeat (double underlined) and four inverted repeats (paired arrows a, b. c and d) are found at the 5'-flanking region of the CPS-1 gene. The first exon is 151 nt in size. The translation start codon, ATG (asterisk), is located at +41. The coding region in exon1 encodes the entire signal peptide (33 amino acids) and the first 4 amino acids from the amino terminus of the mature enzyme. The signal cleavage site is showed by a arrowhead. The first intron begins at +152, and the first two nucleotides (bold) are in agreement with the nucleotide consensus for exon-intron splicing.

A



B

-1180 CTGCAGAGATCAACAGCTCAGTTGGAAGAATCTGTCCACAGGATAACTAT
Pst I

-1130 TACTCGTGCACTCCACAAATCTGGCCTTTATGGAAGAGTGGCAAGAAGAA

-1080 AGCCATTGTTGAAAGAAAGCCATAAGAAGTCCCGTTTGCAGTTTGGGAGA

-1030 AGCCATGTGGGGGACACAGCAAACATGTGGAAGAAGGTGCTCTGGTCAGA

-980 TGAGACCAAAATTGAACTTTTTGGCCTGAAAGCAAAATGCTATGTGTGGC

-930 AGAAAACTAACACTGTACATCACCCTGAACACACCACCCCAACCATGAAA

-880 CATGGTGATGGCAGCATCATGTTGTGGGGATGCTTTTCTTCAACAGGGAC

-830 AGAGACGCTGGTCAGAGTGAGATGGGAAGATGGATGGAGCCAAATACAGG
(1) →

-780 GCAATCTGTATTTGGCTCAGGGCACAGACTTGAGACCGGGGCGGAGGTTC
(1) ←

-730 ACCTTCCAGCAGGACAACGACCCTAAACATACAGCCAGAGCTATAATGGA

-680 ATGGTTTAGATCAAAGCATATCATGTGTTAGAATGGCCCAGTCAAAGTCC

-630 AGACCTAAATCACATATAAATCTATAGGAAGGTTTAATAAAACTGGTGCA
(2) →

-580 CACAGAATCTGTTACAGCTGTGCATAGTAGCCAATCAGTTTCTAGATGTG
(3) → (3) ←

-530 AACAAGCTGAAATTAAAAACTGATTGGTTACTATGCACAGCTGCACCTGA
(2) ←

-480 TTCTGTGTGCACCAGTTTTAGTAAATACCACCCTATGTCTTTTAAAATAT

-430 AACTTGGTACATTTACTTATGTTCATAGTGCATAGTGTCAAAACTAAAGA

-380 ACTCTTGAGTTTGTTGTACTTTTACGTTAAAAAGGCTGTTTCAGGACAGT

-330 GCAATAATTTATTTTCGCCTATTCTATTGATTGCCTACCATAGGCTAGTA

-280 TGGCTTTACTGATAAAAAAGCAGAGGTCTTATTGAGTTGTCTGTTTGAAC

-230 ATCACTTCAAAAGATAATGTTTAATCTATGTTGCAACCAAGTTACACAGA

-180 CACGTATAATATCAAGGATTGACTTCAGTGTAAAAGAGGGTGGGGATACA

-130 GGCAAGGCGGAGTTTGGCTTCAATTTATTTAATGCCCACATAGGTGCAAA

-80 TATTAGCAAATGTGACTCGGGCACAAGTCTCTTTCTCGCCAGCGGCTCTG

-30 AGGAAGAAGCATCTCATACCACCTTCTGAT
(4) → (4) ←

+1 CAGAAAGAGCTGAACAGAAC

+21 AGCACCTCAGGGCCCCAAACATGACTCGGATTTTGTCTGTGTTTAAGACA
M T R I L S V F K T

+71 GCAAAGTCAGGGGTTCTCAATGCAGCAGCCCATCGTTACCGGGGCTTCTC
A K T G V L N A A A H R Y R G F S

+121 CAAAGCCGGCGTTCGGCTTATGAGCGTCAAG +151 gtatcaatctatgttt
K A G V R L M S V K Intron 1

+168 tacttgtttcaaaatgactatatttgttaatacttttagcgagctc+213
Sac I

Figure 20. Determination of the *Rana catesbeiana* CPS-1 gene transcription start site by primer extension.

A 28 nt primer, complementary to CPS-1 mRNA at position +35 to +62 (see Figure 19) was ^{32}P end-labelled using T4 polynucleotide kinase. Approximately 5 x 10^5 cpm of this primer was annealed to 20 µg of total *Rana catesbeiana* liver RNA (Lane 3), 20 µg of yeast tRNA (Lane 2) or ddH_2O (Lane 1). The extended products were analyzed on a 6% polyacrylamide sequencing gel containing 8 M urea. Included in the gel were sequencing reactions in which the same primer was used for sequencing the 5'-flanking region of the CPS-1 gene (T=Thymine, G=Guanine, C=Cytosine and A=Adenine).

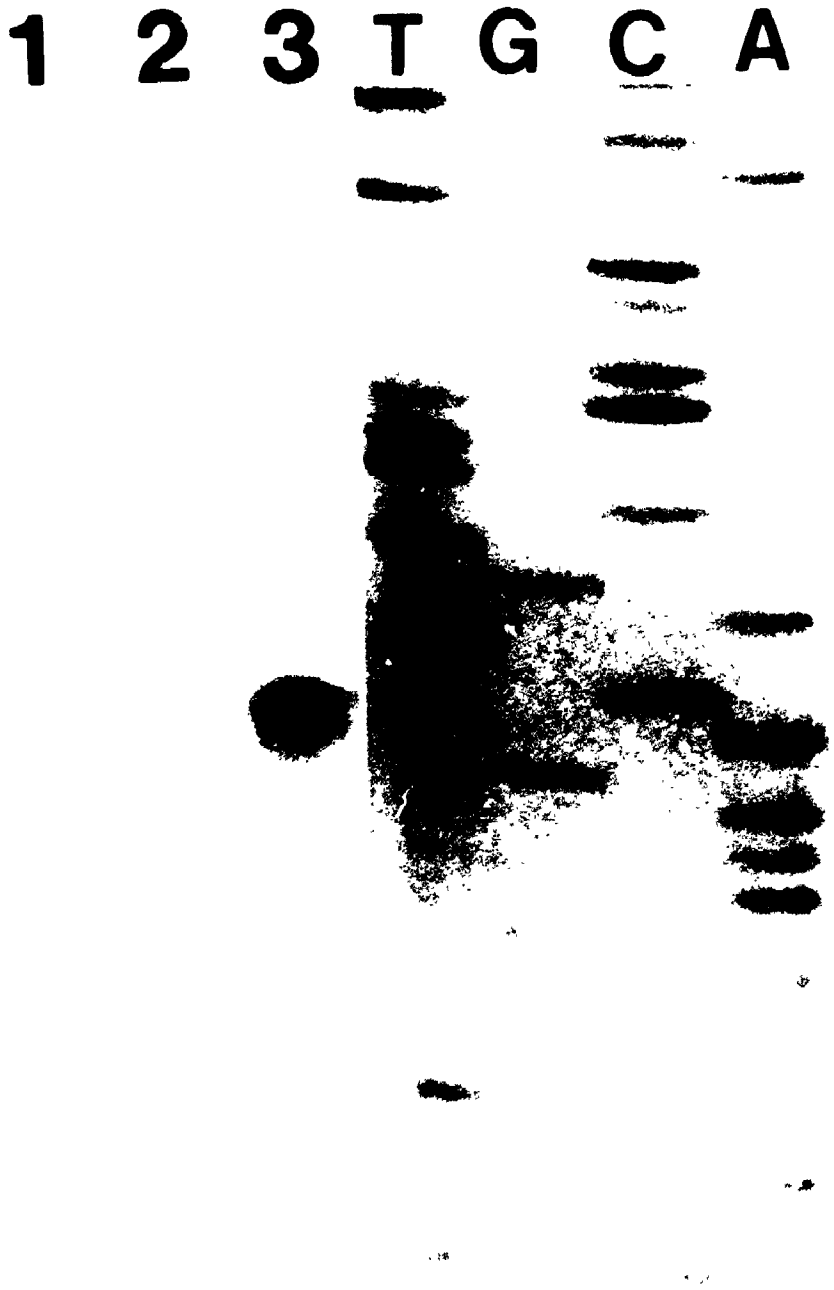

1 2 3 T G C A

Figure 21. Southern blot hybridization analysis of *Rana catesbeiana* genomic DNA with a sequence from the first exon in the CPS-1 gene.

Rana catesbeiana genomic DNA (20 μg) was digested with *Bam*H I (Lane 1), *Hin*d III (Lane 2) or *Pst* I (Lane 3). The digested DNAs were separated on a 0.7% agarose gel, and the fragments were transferred onto a Zetaprobe membrane and hybridized to a sequence from the first exon in the CPS-1 gene as outlined in the "Materials and Methods". The relative sizes of the DNA fragments from the enzyme digests were determined from a coelectrophoresed DNA ladder shown on the left in kilobases (kb).


Kb
1
2
3
9.4
6.5
4.3
2.3
1.0

Using the first exon, generated by PCR methodology (see "Material and Methods"), as a probe, I performed a Southern blot hybridization and the results demonstrate (see Figure 21) that a single DNA fragment is recognized by digestion of genomic DNA with *Bam*H I, *Hin*d III or *Pst* I. These results suggest that the CPS-1 gene also appears to be a single copy gene in the *Rana catesbeiana* genome.

3.4 Discussion

Using nucleotide probes specific for the 5' region of cDNAs encoding *Rana catesbeiana* OTC and CPS-1 mRNAs enabled me to isolate genomic sequences containing the promoter and upstream, putative regulatory regions (Veres *et al.*, 1986; Hata *et al.*, 1986; Takiguchi *et al*, 1987; Howell *et al.*, 1989; Lagace *et al.*, 1992; Goping *et al.*, 1992) of these genes. Moreover, isolation of these genomic sequences permitted me to establish, by primer extension, the transcription start site for each of these genes (Figures 17 and 20). Comparison of the OTC and CPS-1 cDNA sequences with the corresponding genomic sequences reveals the location of the first exon-intron boundary in each of these genes, and use of the exon-1-specific probes for Southern blot hybridization analyses revealed that these genes are single-copy genes in the *Rana catesbeiana* genome (Figures 18 and 21). Interestingly, much like the first exon in the human (Hata *et al.*, 1986) and rat (Takiguchi *et al*, 1987) OTC genes, the first exon in the *Rana catesbeiana* OTC gene contains most (24 out of the 32 amino acid residues) of the amino terminal

presequence that functions as a mitochondrial targeting signal (Takiguchi *et al.*, 1987; Helbing *et al.*, 1992; Lathrop and Timko, 1993). Moreover, the 3'-terminal end of the first exon in the *Rana* CPS-1 gene occurs at exactly the same position as the first exon found in the rat CPS-1 gene (after the nucleotide sequence encoding the fourth amino acid from the amino terminal end of the mature protein; Lagace *et al.*, 1987).

The *Rana* OTC gene promoter, like the promoters found in rat (Takiguchi *et al*, 1987), mouse (Veres *et al.*, 1986) and human (Hata *et al.*, 1986) OTC genes, is atypical in that it appears to lack consensus TATA and CCATT motifs at the positions they are usually found in other eukaryotic genes. While a number of direct and inverted repeats of unknown function are present in the presumed 5'-regulatory region of this gene, it was surprising to discover a well-conserved heat shock element (Amin *et al.*, 1988; Xiao and Lis, 1988) located between -172 and -163. Although four well-conserved C/EBP (CAATT/enhancer binding protein) binding sites (Graves *et al.*, 1986; Howell *et al.*, 1989; Ryden and Beemon, 1989; Vinson *et al.*, 1989; Lagace *et al.*, 1992; Goping *et al.*, 1992; Ryden *et al.*, 1993; Chen *et al.*, 1994) were identified in the 5'-regulatory regions of this gene, no consensus sequences for thyroid hormone response elements (TREs; See Figure 2; Glass *et al.*, 1988; Baniahmad *et al.*, 1990; Naar *et al.*, 1991; Umesono *et al.*, 1991; Andersson *et al.*, 1992; Miyamoto *et al.*, 1993; Yen *et al.*, 1993; Desvergne, 1994) are present in ~1 kb of the 5' region upstream from the transcription start site of this gene.

The *Rana* CPS-1 gene promoter contains TATA, CCAAT and Sp1 boxes far upstream from the usual locations. This observation

questions whether these elements play a role in the expression of the *Rana* CPS-1 gene. However, the fact that an 8 bp nt sequence (5'-ATACCACC-3'; Figure 19) in the 3'-end of the untranscribed promoter of this gene (nt -7 to -15) is directly repeated (nt -449 to -456) 20 bp upstream from one of the TATA boxes raises the possibility (see Ptashne, 1986; Takiguchi *et al.*, 1987) that an interaction of these direct repeats with their binding proteins might place this putative TATA box in the vicinity of the transcription start site. In this manner, putative TATA box (-80 to -84, -105 to -99, -176 to -172, -433 to -429, -608 to -605, -616 to -612, and -689 to -685), CCAAT box (-550 to -546) and Sp1 box (-742 to -737) elements (see Figure 19) may become functional. Whatever the case, the 5'-regulatory region of the *Rana* CPS-1 gene, like the *Rana* OTC gene (Figure 16) and the rat CPS-1 gene (Howell *et al.*, 1989; Lagace *et al.*, 1992; Goping *et al.*, 1992) contains several C/EBP elements, but no TREs.

Since these urea cycle enzyme genes generally exhibit coordinated expression in the liver of *Rana catesbeiana* during spontaneous and T_3-induced metamorphosis (Helbing *et al.*, 1992; Helbing and Atkinson, 1994), it seemed reasonable to believe that the 5'-flanking regions of these genes should share some regulatory elements in common. The absence of any TRE consensus sequences in the 5'-regulatory regions of the *Rana* OTC and CPS-1 genes, at least within the ~1 kb upstream sequences analyzed in my study, suggests that the thyroid hormones must exert their effects on the expression of the OTC and CPS-1 genes in an indirect fashion. The observation that the 5'-regulatory regions of both of these genes contain several C/EBP elements, coupled with the fact that a number of reports have

implicated these transcription factors (*i.e.* the C/EBPs) in the terminal differentiation of mammalian hepatocytes and in the expression of live-specific genes (Friedman *et al.*, 1989; Umek *et al.*, 1991; Sladek and Darnell, 1992), as well as current data indicating that C/EBP binding elements are present in the mammalian CPS-1, OTC, and arginase gene promoter regions (Howell *et al.*, 1989; Murakami *et al.*, 1990; Takiguchi and Mori, 1991), raised the possibility that thyroid hormone may be upregulating the expression of the liver C/EBPs which, in turn and in a coordinated fashion, upregulate the expression of the OTC and CPS-1 genes.

CHAPTER 4 CHARACTERIZATION AND EXPRESSION OF C/EBP-LIKE GENES IN THE LIVER OF *RANA CATESBEIANA* TADPOLES DURING SPONTANEOUS AND THYROID HORMONE-INDUCED METAMORPHOSIS

4.1 Introduction

The absence of consensus thyroid hormone receptor binding elements (TREs) and the presence of CAATT/enhancer binding protein (C/EBP) elements in the promoter regions of both CPS-1 and OTC genes (see Chapter 3) raised the possibility that *Rana* homologues of the mammalian C/EBPs, might play a role in the TH-induced terminal differentiation and reprogramming of gene expression in the tadpole liver. Thus, I isolated and characterized cDNAs from adult *Rana catesbeiana* liver mRNAs which encoded two different C/EBP-like proteins, and, subsequently, evaluated the expression of these genes in the liver of tadpoles undergoing spontaneous and TH-induced metamorphosis. The marked, TH-induced up-regulation of mRNAs encoding one of these *Rana* C/EBP-like proteins (RcC/EBP-1) prompted me to isolate and characterize the promoter and 5'-regulatory regions of the gene encoding this C/EBP-like protein. The results reported in this chapter implicate a role for a *Rana* homologue of the mammalian C/EBPα in the TH-induced transition occurring in the liver of this amphibian.

4.2 Materials and Methods

4.2.1 Animal Experimentation

As described in Chapter 2, *Rana catesbeiana* tadpoles and adults were obtained from W. A. Lemberger Co. (Oshkosh, WI) and maintained in dechlorinated, aged tap water. For spontaneous metamorphic studies, *Rana catesbeiana* developmental stages were assessed based on morphological criteria (Taylor and Kollros, 1946) and five animals for each developmental stage were used in each experiment (n=5). For each TH-induction experiment, five premetamorphic tadpoles, stages VI-VIII, were selected for each time point and were injected intraperitoneally with 3,5,3'-triiodothyronine (T_3; Sigma Chemical Co., St. Louis, MO) at a dose of 0.3 nmol/g body weight (Atkinson *et al.*, 1972; Atkinson, 1981). Control animals were injected with a vehicle solution of 200 mM NaOH. Tadpoles were anesthetized in 0.1% tricaine methane sulfonate (Syndel Laboratories Ltd., Vancouver, B. C., Canada) and sacrificed by severing the truncus arteriosus.

4.2.2 Cloning and Sequencing of *Rana catesbeiana* RcC/EBP-1 cDNA

A cDNA library, made from poly(A)$^+$mRNAs isolated from the liver of adult *Rana catesbeiana* frogs and consisting of 1 x 10^6 independent recombinant phage, was constructed in the *Eco*R I/*Xho* I sites of a UniZap lambda vector (Stratagene Inc., La Jolla, CA). The cDNA library was screened with a 841 bp *Eco*R I/*BamH* I cDNA fragment containing most of the sequence coding for a *Xenopus* C/EBP (Xu and Tata, 1992) under conditions similar to those

described previously (see Chapter 2). Two positive clones, clone 1 (subsequently designated as RcC/EBP-1) and clone 2, were isolated and *in vivo* excised into pBluescript II SK$^-$ (Stratagene Inc.) The cloned inserts were sequenced by the *Exo* III-based DNA sequencing method of Li and Tucker (1993) using [^{35}S]-dATP (Dupont Canada, Mississauga, Ontario, Canada; specific activity 600 Ci/mmol) and a Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH) for direct dideoxynucleotide sequencing. In some cases, particular sequences were confirmed by subcloning DNA fragments into pBluescript II SK$^-$ and using T3, T7, SK, or KS primers for dideoxynucleotide sequencing (Chen and Seeberg, 1985).

4.2.3 Cloning and Sequencing of *Rana catesbeiana* RcC/EBP-2 cDNA

The adult liver cDNA library was screened at a reduced hybridization stringency (Lee, 1990) with a 385 bp DNA fragment (nucleotides 642-1,027 in Figure 22) containing the bZIP domain of RcC/EBP-1. Briefly, hybridizations were carried out in a solution containing 10% formamide (BRL, Gaithersburg, MD), 5X SSPE (1X = 0.18 M NaCl, 0.01 M Na_2HPO_4, 1 mM EDTA), 5X Denhardt's solution [1X = 1% (w/v) of each of Ficoll Type 400, polyvinyl pyrrolidone (MW 360,000) and bovine serum albumin (BSA; Fraction V, Sigma)], 0.1% SDS and 0.1 mg/ml denatured herring sperm DNA at 37°C for 24h. Two washes were done at room temperature with 2X SSC (1X = 0.15 M NaCl, 0.015 M Na citrate) and 0.1% SDS for 1h each followed by two washed with 1X SSC and 0.1% SDS at 42°C for 1 h each. Eleven

clones, screened from 5 x 10^5 plaques, survived tertiary screening. Nine of these clones tested positive when probed with a 1,668 bp *Pvu* I/*Xba* I DNA fragment corresponding to the 3'-untranslated region of RcC/EBP-1 cDNA. These clones were found to contain the RcC/EBP-1 sequence and were discarded. The remaining two clones, clone 1 and 5, were isolated and *in vivo* excised into pBluescript II SK^- (Stratagene Inc.). Clones 1 and 5 were completely sequenced in both directions by the method of Li and Tucker (1993). Inspection of their sequences revealed that clone 1 was a truncated version of clone 5 and, since the sequence in clone 5 was different from the sequence in RcC/EBP-1, I designated it as RcC/EBP-2.

4.2.4 Cloning and Sequencing of a *Rana catesbeiana* RcC/EBP-1 Gene

A *Rana catesbeiana* genomic library, as described in Chapter 3, was screened with a 1027 bp *Eco*R I/*Bam*H I cDNA fragment from RcC/CEBP-1 (nt 1 to 1027; see Figure 22). One clone, screened from 1 x 10^6 plaques, survived tertiary screening. This positive clone, designated as RcGC/EBP-1, contained an insert of approximately 18 kb.

RcGC/EBP-1 was digested with *Xho* I to release the 18 kb insert from the λFIXII vector, and the digest was separated on a 0.7% agarose gel and vacuum blotted onto a Zetaprobe membrane. The membrane was sequentially hybridized with four different DNA fragments from RcC/EBP-1 (see Figure 22): 1) a 90 bp *Eco*R I/*Nco* I fragment (used as a 5'-UTR probe; nt 1-90), 2) a 318 bp *Nco* I/*Hind*

III fragment (used as a 5'-ORF probe; nt 324-642), 3) a 385 bp *Hin*d III/*Bam*H I fragment (used as a 3'-ORF probe; nt 642-1,027), and 4) a 1668 bp *Xba* I/*Pvu* I fragment (used as a 3'-UTR probe; nt 1127-2795). Restriction enzyme analyses of RcGC/EBP-1 revealed a ~7 kb *Hin*d III fragment which hybridized with probes from both the 5'-UTR and 5'-ORF fragments, and, subsequently, was cloned into pBluescript II SK$^-$ (Stratagene Inc.) at the *Hin*d III site. The clone containing this *Hin*d III DNA fragment was named pGC/EBP-1.

pGC/EBP-1 was partially sequenced by the Exo III-based DNA sequencing method (Li and Tucker, 1993) using [^{35}S]-dATP (Dupont Canada, Mississauga, Ontario, Canada; specific activity 600 Ci/mmol) and a Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH) for direct dideoxynucleotide sequencing. In some cases, particular sequences were confirmed by subcloning DNA fragments into pBluescript II SK$^-$ and using T_3, T_7, SK, KS primers for the dideoxynucleotide sequencing (Chen and Seeburg, 1985).

4.2.5 Computer Analyses

Characterization of the RcC/EBP-1 and RcC/EBP-2 cDNA sequences and the RcC/EBP-1 gene sequence was accomplished using DNA Strider version 1.0 (Marck, C., Service de Biochimie-Department de Biologie, Institut de Recherche Fondamentale, Commissariat a l'Energie Atomique-France), and DNA Inspector II+ (Textco, West Lebanon, NH) software. The EMBO and GenBank databases were

searched using the Wisconsin Genetics Computer Group sequence analysis software package Version 6.1(Devereux *et al.*, 1984).

4.2.6 Primer Extension

A 28 nt primer, complementary to RcC/EBP-1 mRNA at position +28 to +55 (see Figure 32B) was ^{32}P end-labelled using T4 polynucleotide kinase (Pharmacia Inc.) as described in Chapter 3. Approximately 5 X 10^5 cpm of the primer was annealed to 20 μg of total *Rana catesbeiana* liver RNA, 20 μg of yeast tRNA (Pharmacia Inc.), or ddH_2O. Hybridization and primer extension reactions were performed at the same condition as described for OTC mRNA primer extension (see Chapter 3). The samples were extracted with phenol, precipitated with ethanol, and visualized following electrophoresis in a 6% polyacrylamide sequencing gel containing 8 M urea. Also included in the gel were sequencing reactions in which the same primer was used for sequencing the 5'-flanking region of pGC/EBP-1 and delineating the transcription start site.

4.2.7 Southern Hybridization Analyses

Southern blots were prepared as described previously (see Chapter 2). The blots were sequentially hybridized with a 234 bp *Nco* I/*Nco* I fragment from RcC/EBP-1 cDNA (nt 96-330 in Figure 32), and two different DNA fragments from the RcC/EBP-2 cDNA [a 847 bp *Eco*R I/*Sma* I fragment (nt 1-847 in Figure 24), and a 482 bp *Sac*

I/*Sma* I fragment (nt 365-847 in Figure 24)]. Prehybridizations and hybridizations were performed under the same conditions as the Southern hybridizations used with the OTC and CPS-1 first exons (see Section 3.2.1). The final stringency washes were done in 1X SSC and 1% SDS for 30 min at 60°C. Kodak XR-Omat RP film was preflashed and exposed to the membrane with a Dupont Cronex Lightening Plus intensifier screen at -80°C.

4.2.8 RNA Extraction, and Northern- and Dot-Blot Hybridization Analyses

Liver was excised from TH-induced and spontaneously metamorphosing tadpoles, perfused with cold amphibian PBS, minced on ice, and homogenized. Total RNA was isolated from the homogenate using a modification of the guanidinium isothiocyanate single step isolation method (Puissant and Houdebine, 1990). Samples of the RNA were run on 1.0% agarose gels containing 2.6M formaldehyde (Khandjian, 1986) and transferred onto a Zetaprobe membrane as described previously. Dot blots were prepared (Sambrook *et al.*, 1989) with a dot blotting apparatus (Bio-Rad).

Northern- and dot-blots were hybridized with a 318 bp *Nco* I/*Hin*d III fragment (nt 324-642 in Figure 22) from RcC/EBP-1, a 482 bp *Sac* I/*Sma* I fragment (nt 365-847 in Figure 24) from RcC/EBP-2, a 441 bp *Eco*R I fragment from *Rana catesbeiana* CPS-1 (Helbing and Atkinson, 1994), and a 960 bp fragment from *Xenopus* cytoskeletal actin (Mohun *et al.*, 1984). In each case, the membranes were prehybridized in a solution containing 50% formamide (BRL,

Gaithersburg, MD), 3X SSPE, 1% SDS, 0.5% Blotto, and 0.5 mg/ml herring sperm DNA for 4 h at 55°C. Hybridization was performed in a similar solution containing 10% dextran sulfate and [α-^{32}P] dCTP (Dupont Canada; specific activity 3,000 Ci/mmol) random primer-labelled probes (BRL; Pharmacia, Piscataway, NJ) for 20 h at 55°C. The membranes were rinsed with 2X SSC followed by two 15 min washes in 2X SSC and 0.1% SDS, and one 15 min wash in 1X SSC and 0.1% SDS at room temperature. The stringency wash was done in 1X SSC and 1% SDS for 30 min at 60°C. Kodak XR-Omat RP film (Eastman Kodak Co., Rochester, NY) was preflashed and exposed to membranes with a Dupont Cronex Lightening Plus intensifier screen at -80°C.

4.2.9 *In situ* Hybridization

Freshly dissected tadpole and frog tissues were fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin. Tissues were cut into 5 µm sections and mounted on Superfrost/Plus glass slides (Fisher Scientific, Toronto, Canada). The sections were deparaffinated, rehydrated, treated sequentially with 0.2 N HCl, 0.3% Triton X-100, and Proteinase K (20 µg/ml), fixed in paraformaldehyde, and acetylated in 0.25% acetic anhydride containing 0.1 M triethanolamine. The sections were prehybridized in 50% formamide containing 2X SSC for 2 h at 45°C, and hybridized overnight at 50°C in a hybridized buffer containing 0.01 M Tris-HCl (pH 7.5), 12.5% Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 2X SSC, 50% formamide, 0.5% sodium dodecyl sulfate, 250 µg/ml salmon sperm DNA, 5 µg/ml

sodium pyrophosphate, 50% dextran sulfate, and approximately 150 ng/slide of a digoxigenin (DIG)-labelled RNA probe (Boehringer Mannheim Canada, Laval, Quebec). Sense and anti-sense DIG-labelled RNA probes were prepared, using a DIG RNA Labeling Kit (Boehringer Mannheim), from cDNAs subcloned into pBluescript II SK$^-$, containing either a 441 bp *Eco*R I fragment from *Rana catesbeiana* CPS-1 (Helbing and Atkinson, 1994) or a 318 bp *Nco* I/*Hin*d III fragment from RcC/EBP-1. Following hybridization, the sections were rinsed in 2X SSC and incubated in 2X SSC for 30 min at 37°C with RNase A (100 µg/ml in 2X SSC) to digest any unbound probe. The RNase A-treated sections were washed three times for 30 min in 2X SSC at 55°C, twice for 30 min in 0.1X SSC at 55°C, once in 0.1X SSC at room temperature, and once in digoxigenin buffer (0.1 M Tris-HCl, pH 7.5, 0.15M sodium chloride) for 5 min at room temperature. The washed sections were incubated for 2 h with alkaline phosphatase conjugated anti-digoxigenin sera (Boehringer Mannheim) diluted 1:1,000 with digoxigenin buffer containing 10% normal rat serum and 2 mM levamisole. Bound antibody was detected by incubating the sections overnight in a staining solution consisting of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate. In some cases, the sections were counterstained with haemotoxylin prior to being mounted in permount. The tissue sections were photographed on a Zeiss photomicroscope III using Kodak technical pan film.

4.3 Results

4.3.1 Isolation and Characterization of cDNAs Encoding *Rana catesbeiana* Homologues of C/EBP mRNAs

A number of C/EBP-related clones were isolated from a cDNA library prepared from the mRNAs in the liver of an adult *Rana catesbeiana* frog. Sequence analyses of the cloned DNA inserts revealed that they contained sequences from two different C/EBP-like genes. One clone, RcC/EBP-1, was completely sequenced from both strands (see Figure 22) and found to contain 3233 base pairs (bp) which includes 91 bp of 5'-untranslated region (UTR), an open reading frame (ORF) of 879 bp, and a 3'-UTR consisting of 2263 bp. A complete polyadenylation consensus sequence signal begins 19 bp upstream from the polyadenylated tail. The nucleotide sequence in the ORF of RcC/EBP-1 shares 68 to 79% identity (Figure 23A) with the ORFs encoding C/EBPα in rats (Lincoln *et al.*, 1994; Landschulz *et al.*, 1988a), mice (Christy *et al.*, 1991; Cao *et al.*, 1991), chickens (Calkhoven *et al.*, 1992) and *Xenopus* (Xu and Tata, 1992), and less than 49% identity (Figure 23B) with ORFs encoding other C/EBPs from the same organisms (Cao *et al.*, 1991; Descombes *et al.*, 1990, Williams, Cantwell and Johnson, 1991; Burk *et al.*, 1993).

The other clone, RcC/EBP-2, was completely sequenced from both strands (see Figure 24) and consists of 2,271 bp which includes 84 bp of 5'-UTR, an ORF of 855 bp, and a 3'-UTR consisting of 1,332 bp. A complete polyadenylation consensus sequence signal begins 9 bp upstream from the polyadenylated tail. The nucleotide sequence in the ORF of RcC/EBP-2 shares 62-66% identity (Figure 25A) with

Figure 22. Nucleotide sequence of a cDNA, RcC/EBP-1, encoding a *Rana catesbeiana* homologue of a mammalian C/EBP.

Numbers to the left refer to the nucleotide locations with respect to the 5'-end of the cDNA insert. The 5'-UTR (nucleotides 1-91) is indicated in lowercase letters. The ATG translation start site is shown in bold letters. The ORF is shown in uppercase letters, and ends with a stop codon (TGA) in bold letters. The 3'-UTR is indicated in lowercase letters and three putative polyadenylation signals are underlined.

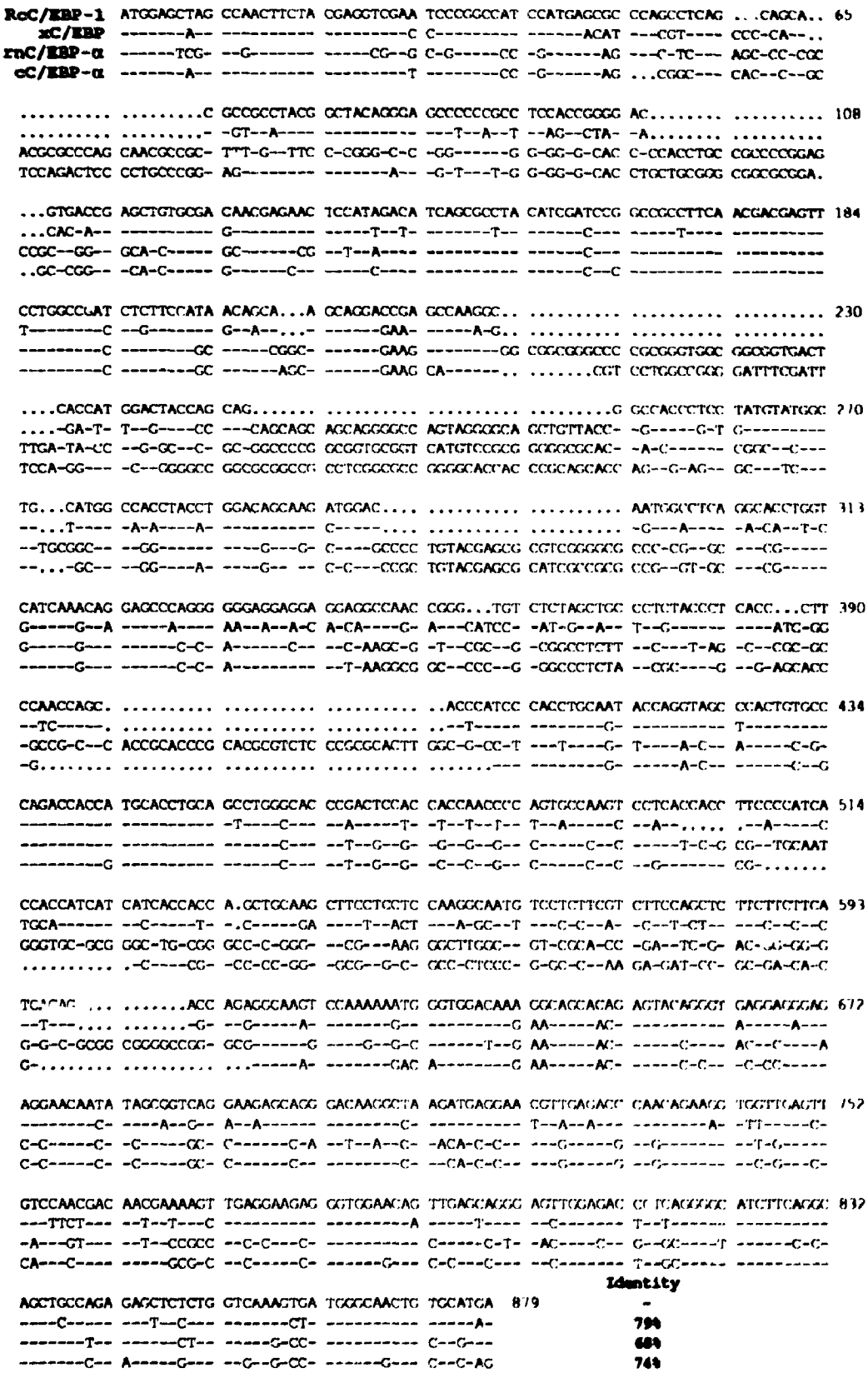
```
   1 gagagtccgccgattgatcgcgcagagtcagcccgagctcatcctggatt
  51 cccggagctcgtcaccatgcttggctggctataggagctcc
                                        ATGGAGCTA
 101 GCCAACTTCTACGAGGTCGAATCCCGGCCATCCATGAGCGCCCAGCCTCA
 151 GCAGCACGCCGCCTACGGCTACAGGGAGCCCCCCGCCTCCACCGGGGACG
 201 TGACCGAGCTGTGCGACAACGAGAACTCCATAGACATCAGCGCCTACATC
 251 GATCCGGCCGCCTTCAACGACGAGTTCCTGGCCGATCTCTTCCATAACAG
 301 CAAGCAGGACCGAGCCAAGGCCACCATGGACTACCAGCAGGGCCACCCTC
 351 CTATGTATGGCTGCATGGCCACCTACCTGGACAGCAAGATGGACAATGGC
 401 CTCAGGCACCTGGTCATCAAACAGGAGCCCAGGGGGGAGGAGGAGGAGGC
 451 CAACCGGGTGTCTCTAGCTGCCCTCTACCCTCACCCTTCCAACCAGCACC
 501 CATCCCACCTGCAATACCAGGTAGCCCACTGTGCCCAGACCACCATGCAC
 551 CTGCAGCCTGGGCACCCGACTCCACCACCAACCCCAGTGCCAAGTCCTCA
 601 CCACCTTCCCCATCACCACCATCATCATCACCACCAGCTGCAAGCTTCCT
 661 CCTCCAAGGCAATGTCCTCTTCGTCTTCCAGCTCTTCTTCTTCATCAGAG
 701 ACCAGAGGCAAGTCCAAAAAATGGGTGGACAAAGGCAGCACAGAGTACAG
 751 GGTGAGGAGGGAGAGGAACAATATAGCGGTCAGGAAGAGCAGGGACAAGG
 801 CTAAGATGAGGAACGTTGAGACCCAACAGAAGGTGGTTGAGTTGTCCAAC
 851 GACAACGAAAAGTTGAGGAAGAGGGTGGAACAGTTGAGCAGGGAGTTGGA
 901 GACCCTCAGGGGCATCTTCAGGCAGCTGCCAGAGAGCTCTCTGGTCAAAG
 951 TGATGGGCAACTGTGCATGA
                                    gatcgaacccaacctcctcgttcttgaccc
1001 ccccccccccccaatccatctcttgtggatcctaaccaagtgccaagagt
1051 gataaggacacttccatgaacttggatcccagcctttgactcttttttt
1101 tctttgcatgaggtccatctccaaattctagactttctttctccatctcc
1151 tgtggatcctaaccaagcaccaagagtggtaaggacacttccatgaactt
1201 ggatcccaacctttgacacttttttctttctctctatgttgcatgaggt
1251 ccaactcaacctccaatttctttacttcccctctccatccatccaccatc
1301 tcctgtggatcctaaccaagcaccaagagtgataaggacacatccatgaa
1351 cttggatcccagcctttgacccccttctctctttctacagttttgccaaa
1401 acattggatatttgaatgattgattgtggtgaccattttgcaagacagtt
1451 atggctaagttctcacaacctggtgctaatgcagttgtgaggagcctcta
1501 ttagtcaaccacagtctaccaaatactgtacttttttatggttcttattt
1551 tttatactctttgtaagaaaaaaaaaaagtttgaataacttctatgcaac
1601 ctgtggctggtcaaactattttattatgtttttttaaggggcagtaaagt
1651 ttggtaattgttgaacagtttgctgcaactcccaatggcatttggtgggg
1701 taaataacaagatccttttaagacatgcccagtaggtcacaggccagttc
1751 gggcaacttttggcttcggttgttttggcatcacagactccagtgtgccc
1801 tgtcaacacaggaaatattggaagttttggtgccaacccagctggtggct
1851 ttaagttgttgaattggtttctgacataatgggtttggttatgaataggg
1901 gcagtaaagattggtaaacattgaagaacagtttgctgcaactctcaatg
1951 gcatttagtggagtaataaataggatgataggattctatagcacaagcc
2001 caatgggtcacagtacatgttcaggcaacttttagcttctggttgtattg
2051 gcaccacaaactccagagtgccctttcaacacagaaaacatttgaggttt
2101 tggtgccagcccaaccgatggcttcagattgttgaagtatcttgtgatat
2151 gatgggttttgctatgaacagggtataattatgcacctttttttaaaagtg
2201 ccttctcctcatcatcatttagtgtgtctgttactttaaaatgctaccta
2251 cagtccagtgcagctcattaacgcaccagcagattatctatcaggagttt
2301 atgatatgaagcagatatggacttctacaaatatttatatatatttcttc
2351 aattcatagacaagctttattcaggataaacccttgccttcactatcaga
2401 gacgatcaatgtgccaagagacttttttttatttttttattttatcgaacgc
2451 acaaaacttatttaatgatttcaatgacaagctgcattgtggagtaaagg
```

2501 gaatggtgtctttgtgtgacacaggccatcacattatcatactgtattac
2551 agaagaaaaaaaaaacacattaaggcatttttataaaaaataaataaatc
2601 ttgactacctttaacaagctgctattcgctgattgcattgcgccttttttg
2651 tctcttgtatatattggctttaaactttgtcagatttaagtgaacccggt
2701 tgaatgtgaatcagaaaattgcagttttaagctttcgaacatttctttt
2751 tcatcatattcttgccatttttttaaaattcttattatcatcagcgatcg
2801 ctctagcatgttgcaggtctacgtacctggcaaccttgagtagctagtat
2851 tgatgcacaaacacccccctcccaattgtgtacaatgcaccctaaccaac
2901 ccagcaccccactaacccatgactttcataactgtgccttaattattgta
2951 cgcatagcaccgcttaattaacccatcctatcacttttttttttttaaccc
3001 ctggagcaaatttacgtcagaagatttttttctaccaatttatcatccc
3051cttttctgaaatcccaagatgtttcaagagtttaatattattttaaagga
3101 tttatgtctgaaaatttttatttttaaaaaaagaaatggttctattcattt
3151 tatttgttattaattataatttgaaacacaatggtatatgttttggttt
3201 ttattttcattataaataaagaatatacactgtaaaaaaaaaaaaaaaaa
3251 aaaaa

Figure 23. Comparison of the nucleotide sequence in the ORF of RcC/EBP-1 with the ORF sequences reported in the C/EBP isoforms of other organisms.

The nucleotide sequence in the ORF of RcC/EBP-1 is compared to the ORF sequences of *Xenopus* (xC/EBP; Xu and Tata, 1992), rat (rnC/EBPα; Lincoln *et al.*, 1994; Landschulz *et al.*, 1988), and chicken (cC/EBPα; Calkhoven *et al.*, 1992) C/EBPα isoforms (**A**), and to the ORF sequences of the C/EBPδ and C/EBPβ isoforms of the rat (**B**). Dashes represent nucleotide sequence identity, dots represent insertions or deletions. Numbers to the right refer to the nucleotide locations of RcC/EBP-1 cDNA with respect to the translation start site (ATG). The percent identity of the compared sequences to the ORF of RcC/EBP-1 is shown.

A



B

```
RcC/EBP-1  .......... .......... .......... .......... ..ATGGAGCT AGCCAACTTC TACGAGGTCG 28
rnC/EBP-δ  .......... .......... .......... .......... .......... .......... ..........
rnC/EBP-β  ATGCACCGCC TGCTGGCCTG GGACGCAGCA TGCCTCCCGC CGCCGCCCGC C---TTTAGA CC-AT--AA-

AATCCCGGCC ATCCATGAGC GCCCAGCCTC AGCAGCACGC CGCCTACGGC TACAGGGAGC CCCCCGCCTC CACCGGGGAC 108
.......... ........-TG AG-GCCG--- TTTTCAG-CT A-A-AG-CCA GCAC-C-GCG -A---TGGC- -- A-A-CC-
TGG--AACTT C-A-TACGAG C--G-CTGC- T-GCCT---G G---A-C-CG GC-C-C-CCG -G--GCG-G- -C---CC-C-

GTGACCGAGC TGTGCGACAA CGAGAACTCC ATAGACATCA GCGCCTACAT CGATCCGGCC GCCTTCAACG ACGAGTTCCT 188
-...-G-CCT -C-A---GCC A-GC-GGGTG GGCA-GCCAG -ACGAGGGCC G--G--T-GG -ATC-GGGG- -GCC-GG-TC
-A-C-G-CCA -CG----GC- --A-CG-G-- --C---T--- --C-----C- G--G---CT- --GCC-GC-- C--C-GA-T-

GGCCGATCTC TTCCATAACA GCAAGCAGGA CCGAGCCAAG GCCACCATGG ACTACCAGCA GGGCCACCCT CCTATGTATG 268
CA-GACC-CT GC-...-TGT ATG-CG-C-- GA-C----TC -A-TT--GC- C----ATTG- TTC-ATGG-- G-CG--CCCA
C----CG-C- GCG...CC-G CGC-C--C-- -TTCCTTTCC -A-CT-T-C- C-G--G-CT- C......GGC G-C-A-CCGA

GCTGCATGGC CACCTACCTG GACAGCAAGA TGGACAATGG CCTCAGGCAC CTGGTCATCA AACAGGAGCC CAGGGGGGAG 348
C-CTAGA-TT GTG-C--GAC --G-T-TTCG CC---CTCTT -AA---CA-T -AC..... --AGC-GC-G GC-C---C--
--AAG-A-C- GT--G--TAC -GTTA-GT-- GCCT-GGCC- -GCGG-CGC- AA-..... GC-GCACCG- -C-CCT-CTT

GAGGAGGAGG CCAACCGGGT GTCTCTAGCT GCCCTCTACC CTCACCCTTC CAACCAGCAC CCATCCCACC TGCAATACCA 428
CCT-GA-CT- -TGCAG--CG -C-CTAC--G A---C-GGGT G-GGGGTCAA TCG----GGG --CG-TG-AG C--G-AC--G
CCC-CC-CC- --TC---CCG CA---A--GC CGAGC-GGG- T--GAA-CCG -GGA-T---A G-GCG-GGA- GA-GCGC--G

GGTAGCCCAC TGTGCCCAGA CCACCATGCA CCTGCAGCCT GGGCACCCGA CTCCACCACC AACCCCAGTG CCAAGTCCTC 508
AC-G-GG-GA C-GCGA-GCG --GGGC..TC -----T---G -C---ACT-G -AGTGTGCG- GCAG.ACAGT GGTGAG-T-G
CCAT-G-GG- C-GCTT-CCG TTCG-CCTGC G-GC-TA--- ----TA--AG GCGACG-CGA GCGG-AGCA- -GGCAG---G

ACCACCTTCC CCATCACCAC CATCATCATC ACCACCAGCT GCAAGCTTCC TCCTCCAAGG CAATGTCCTC TTCGTCTTCC 588
G-GG--GCGG -ACAGC---- A.....-CA- C-ACTTC--C CG-GC--C-T CGAGG--GCC -TGGAC-GAG CCTT......
T----G-CGT -GTCGT---G -.....-CG- C-GGGAC--C -AGCC-CG-- GA-G------ -CGC-C--G- CG-C-GC-T-

AGCTCTTCTT CTTCATCAGA GACCAGAGGC AAGTCCAAAA AATGGGTGGA CAAAGGCAGC ACAGAGTACA GGGTGAGGAG 668
...G-GC--G GCC-CGTCCG AGAG-AG--- GCCGG---G- GGG-TCC--- -CGG------ C-T------C --CA-C-AC-
GCGGGGC-GC -GG-CG-GCC CG---AG-C- ---G----G- -GGC------ ---GCTG--- GAC------- A-A--C--C-

GGAGAGGAAC AATATAGCGG TCAGGAAGAG CAGGGACAAG GCTAAGATGA GGAACGTTGA CACCCAACAG AAGGTGGTTG 748
C---C-C--- --C--C--T- -GC-C----- -C-------- --C---CGCC -----CAG-- --TG--G--- ---C----G-
C---C-C--- --C--C---- -GC-C----- -C-C------ --C------C -----C-G-- ---G--G--C ------C-G-

AGTTGTCCAA CGACAACGAA AAGTTGAGGA AGAGGGTGGA ACAGTTGAGC AGGGAGTTGG AGACCCTCAG GGGCATCTTC 828
--C----GGC ---G-----G ---C--CATC --C-T----- G---C-C-C- C----CC--- CC-G----C- -CAGT-----
--C--A-GGC G--G-----G CG-C--CA-- ---A------ G---C--TCG C-A-A-C-CA GC--G---C- -AA-T-G---
                                                                    Identity
AGGCAGCTGC CAGAGAGCTC TCTGGTCAAA GTGATGGGCA ACTGTGCATG A...... 879      -
-AAG-----= -CAGCCCGC- -T-CC-GCCG CCC-CC---- ...CC-AC-- CCGGTAA          47%
-A-------- -C---CCGCT G----C-TCG -C-GGTCA-T G--AG..... .......          48%
```

the ORFs encoding C/EBPδ in rats (Williams, Cantwell and Johnson, 1991), mice (Cao *et al.*, 1991) and humans (Kinoshita *et al.*, 1992), and less than 52% identity (Figure 25B) with ORFs encoding other isoforms of the C/EBPs (Landschulz *et al.*, 1988a; Descombes *et al.*, 1990; Christy *et al.*, 1991; Cao *et al.*, 1991; Poli *et al.*, 1990).

A comparison of the nucleotide sequences in RcC/EBP-1 and RcC/EBP-2 reveals that they share 50.5% identity in their ORFs (Figure 26B), and 45.6% and 39.4% identity in their 5'- (Figure 26A) and 3'-UTRs (Figure 26C), respectively. Although the nucleotide sequence in RcC/EBP-1 and -2 share little identity, they both contain a repeated sequence, albeit different in each clone, in the 5'-proximal ends of their 3'-UTRs. A 76 nucleotide sequence in RcC/EBP-1 is repeated three times. Although these repeats are not contiguous in RcC/EBP-1 (nucleotides 1015 to 1090, 1142 to 1217, and 1295 to 1370; see Figure 22), they share 91 to 95% identity in their sequences. An 80 nucleotide sequence the 3'-UTR of RcC/EBP-2 is contiguously repeated four times (nucleotides 1025 to 1103, 1104 to 1181, 1182 to 1261, and 1262 to 1341; see Figure 24) and, except for the last 18 nucleotides of the last repeat, each repeat shares 85-90% identity (one- and two-nucleotide deletions are also evident in the first and second repeats, respectively). Whiie the function of the direct repeats is not known, it is of some interest that a number of AU-rich, 10-12 nucleotide inverted repeats are also evident in the 3'-UTRs of both of these *Rana catesbeiana* C/EBP-like mRNAs.

Figure 24. Nucleotide sequence of a cDNA, RcC/EBP-2, encoding a *Rana catesbeiana* homologue of a mammalian C/EBP.

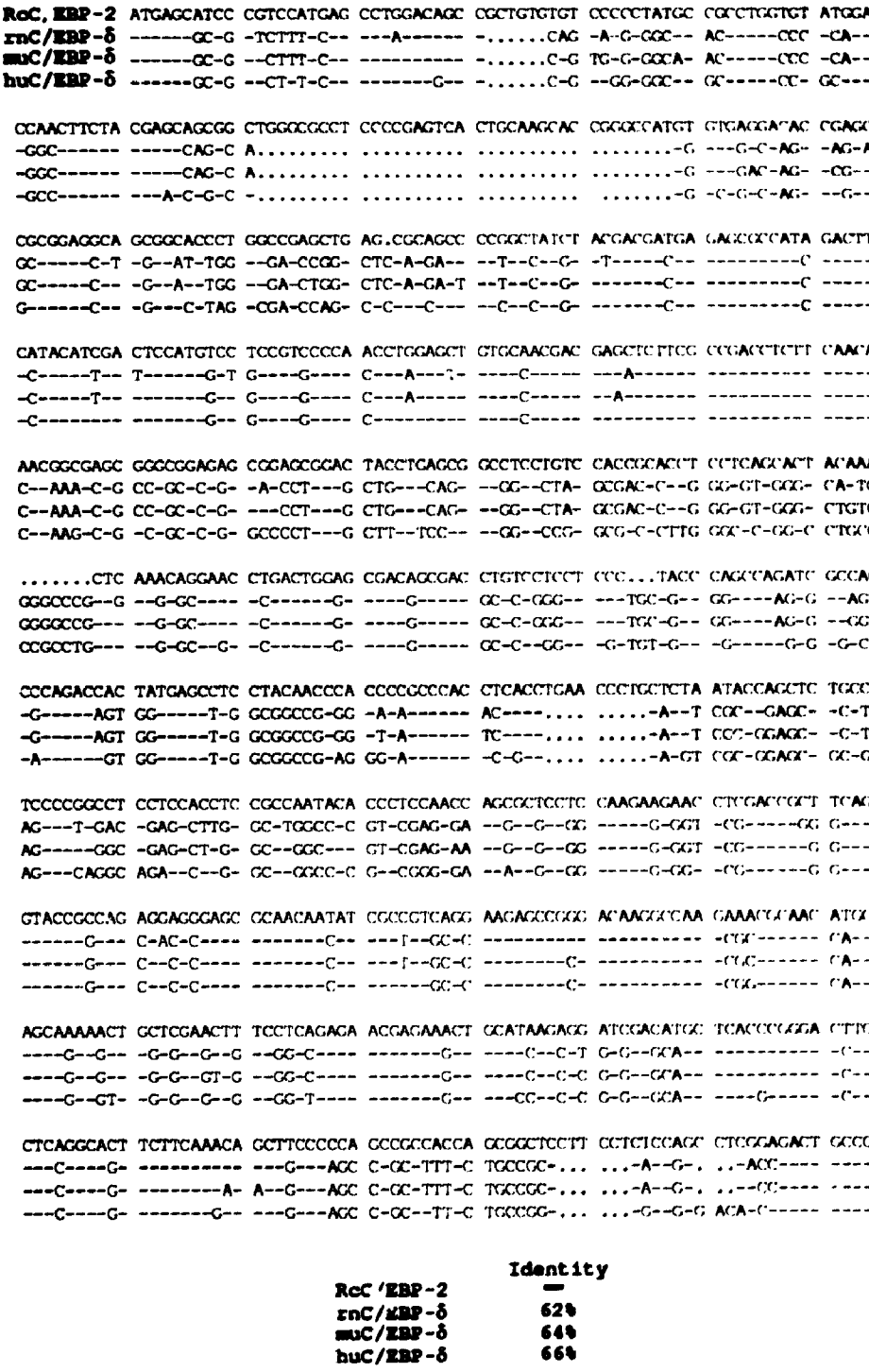
Numbers to the left refer to the nucleotide locations with respect to the 5'-end of the cDNA insert. The 5'-UTR (nucleotides 1-84) is indicated in lowercase letters. The ATG translation start site is shown in bold letters. The ORF is shown in uppercase letters, and ends with a stop codon (TAA) in bold letters. The 3'-UTR is indicated in lowercase letters and a putative polyadenylation signal is underlined.

```
   1 ccgatctatatcgctcctcttcctccagagtgtggggatcccgac-tcgt
  51 catcattgctcagatcttcccttcgatcagtcac
                                        ATGAGCATCCCGTCCA
 101 TGAGCCTGGACAGCCGCTGTGTGTCCCCCTATGCCGCCTGGTGTATGGAG
 151 CCCACCAACTTCTACGAGCAGCGGCTGGGCGCCTCCCCGAGTCACTGCAA
 201 GCACCGGGCCATGTGTGAGGACACCGAGCCCCCGCGCGGAGGCAGCGGCA
 251 CCCTGGCCGAGCTGAGCGCAGCCCCGGCTATCTACGACGATGAGAGCGCC
 301 ATAGACTTCAGTTCATACATCGACTCCATGTCCTCCGTCCCCAACCTGGA
 351 GCTGTGCAACGACGAGCTCTTCGCCGACCTCTTCAACAGCAAGAACGGCG
 401 AGCGGGCGGAGAGCGGAGCGGACTACCTGAGCGGCCTCCTGTCCACCGCA
 451 CCTCCTCAGCACTACAAATCCCTCAAACAGGAACCTGACTGGAGCGACAG
 501 CGACCTGTCCTCCTCCCTACCCAGCCAGATCGCCACCTGTGCCCAGACCA
 551 CTATGAGCCTCCTACAACCCACCCCGCCCACCTCACCTGAACCCTGCTCT
 601 AATACCAGCTCTGCCTGCCCGTCCCCGGCCTCCTCCACCTCCGCCAATAC
 651 ACCCTCCAACCAGCGCTCCTCCAAGAAGAACCTCGACCGCTTCAGTCCGG
 701 AGTACCGCCAGAGGAGGGAGCGCAACAATATCGCCGTCAGGAAGAGCCGG
 751 GACAAGGCCAAGAAACGCAACATGGACATGCAGCAAAAACTGCTCGAACT
 801 TTCCTCAGAGAACGAGAAACTGCATAAGAGGATCGACATGCTCACCCGGG
 851 ACTTGACCAGCCTCAGGCACTTCTTCAAACAGCTTCCCCCAGCCGCCACC
 901 AGCGGCTCCTTCCTCTCCAGCCTCGGAGACTGCCGGTAA
                                        ccccccggtgt
 951 gtgtgtcacacggactatagagaatacctcatccctgagagagagagaga
1001 gggggagacctcccaatcgggcttcacctacctggactgtgtgccctcta
1051 actcggtgtcatcaggtagcacctatctggactgtgtgcatcccaccata
1101 cagcacacgtctgatctgtgtgccctcccaatcagtgtcatcaggcagca
1151 cctacctggactgtgctaccccaccatacagcacctgccttgtctgtgcg
1201 ccctctcacaatccgtgtcttcaggcagcacctacctggactgtgctacc
1251 ccaccatacagcacctgcctggtctgtgcgccctctcacaatcagtgtca
1301 tcaggcagcacctatctggactgtgtactacccccacctcctctccacac
1351 tgagccacaatctcagccttgtccagcatcatcagcctagtgcaataaga
1401 cttctatagcaaagagacattgggggaccccagcaacattgtgccaaag
1451 atttgggtcacctcccactattaaaggggcagtgcacctgacaccctaat
1501 attcatgacctgatgggtgtgaagctgctgcatatgattttgtttatata
1551 tagagtatcaggctacagcctcacatttcatgcaaacaaagcaggaggta
1601 cggactgaacgctaattctgtaaacactttagagctaagtttgatgtaag
1651 cgttttatgactacaaccatgataatgttctgctaaccattgtccgatat
1701 ctacctgccatttaagtgcctgtttgcttttctagtgtctacttgttctt
1751 tttttgtatgtgagatgttggttcttttccttttttttttttttccttgt
1801 ctcatgttgtcctgctagtgcccaagtggagccatttacaccagcagaag
1851 catttgatatgttctgttagtgccttgcaaaagcggcctctgtaagctga
1901 aagtactgtagggaagggaggggggggtgggcacattttgggcagtgca
1951 aacagaaattgtgctggtcttcagtttccttttgttgaatgtgtagcca
2001 gatgaatagtgctacccccctggaattatagaactataaatatatatattt
2051 ttttcttctccacaaaactataagacaaagctaaaacatttccctttcct
2101 taaattatttttgtaatttttggttttctatattctctatggatgcagct
2151 atggtacatttgtaaaatgattacagagaaaaagactttgtattgtagat
2201 attagggaaagaaaatgagcatgctcaagttttatatgattttacagc
2251 atttttaataataataaaatgaaaaaaaaaaaaaaaaa
```

Figure 25. Comparison of the nucleotide sequence in the ORF of RcC/EBP-2 with the ORF sequences reported in the C/EBP isoforms of other organisms.

The nucleotide sequence in the ORF of RcC/EBP-2 is compared to the ORF sequences of rat (rnC/EBPδ; Williams, Cantwell and Johnson, 1991), mice (muC/EBPδ; Cao *et al.*, 1991), and human (huC/EBPδ; Kinoshita *et al.*, 1992) C/EBPδ isoforms **(A)** and to ORF sequences encoding other isoforms of the C/EBPs **(B)**. Dashes represent nucleotide sequence identity, dots represent insertions or deletions. Numbers to the right refer to the nucleotide locations of RcC/EBP-2 cDNA with respect to the translation start site (ATG). The percent identity of the compared sequences to the ORF of RcC/EBP-2 is shown.

A



	Identity
RcC 'EBP-2	—
rnC/EBP-δ	62%
muC/EBP-δ	64%
huC/EBP-δ	66%

B

```
RoC/EBP-2  .......... .......... .......... .......... .......... .......... ..........
rnC/EBP-α  ATGGAGTCGG CCGACTTCTA CGAGGCGGAG CCGCGGCCCC CGATGAGCAG CCACCTCCAG AGCCCCCCGC
   nC/EBP  ATGGAGCAAG CCAACTTCTA CGAGGTCGAC CCCCGGCCAT CCATGAACAT CCACGTTCAG CCCCC.....
rnC/EBP-β  .......... .......... .......... .......... .......... .......... ..........

 .......... .......... .......... .......... .......... .......... .......... .ATGAGCATC 9
 ACGCCCCCAG CAACGCCGCC TTTGGCTTTC CCCGGGGCGC GGGCCCCGCG CCGCCCCCAG CCCCACCTGC CGCCCCGGAG
 .......... .......... .........AC ACGGTGCATA CGGCTACAGG GAGCCTCCAG CTTCAGCCCT AGAACA--A-
 .......... .......... .......... ........AT GCACCGCCTG CTGGGCTGGG ACGCAGCATG CC-CCCGCCG

 CCGTCCATGA GCCTGGACAG CCGCTGTGTG TCCCCCTATG CCGCCTGGTG TATGGAGCCC ACCAACTTCT ACGAGCAGCG 89
 ---CTGGGCG --A-CTG-GA G-A-GAGAC- --TATAG-CA T-AG-GCC-A C--C--C--G G--GC----A ----CG--TT
 GA-C-G.... .....TG-GA GAA-GAGAAC ---ATTG--A T-AG-GCT-A C--C--C--G G--GCT---A ----CG--TT
 ---C--GCCG C-T-TAGACC -ATGGAA--- G--AA--TCT A-TA-GA-CC CGACTGC-TG G--T--GGGG C-A--GC-GC

 GCTGGGCGCC TCCCCGAGTC ACTGCAAGCA CCGGGCCATG TGTGAGGACA CCGAGCCCCC GCGCGGAGGC AGCGGCACCC 169
 C----C--A- CT-TTCCAG- --A--CG--- G-A--AG-A- GCCA---CGG -G-C-GG--- CGCG--T--- G----TGA-T
 T----C--A- CTGTTC...- -TA----CA- G-A--AG-AA GCCA-..... .......... .......... ..A---GA-T
 C-GC-C---G C-G-GCGCC- C-GC-GCCG- G-C-------C G-C---CA-G AG........ .......C-- GC-AT-GA-T

 TGGCCGAGCT GAGCGCAGCC CCGGCTATCT ACGACGATGA GAGCGCCATA GACTTCAGTT CATACATCGA CTCCATGTCC 249
 -T-A-T-C-C -G----CC-G G---GCCC-G G-GGT-CG-T C-TGT--GCG -GGGCGCACG G-CC-CCTCC -GG-TACGG-
 -T-AGT-C.. .....-CC-A G-A--AGCAG CAGGG-CCAG T--G..GGC- -CTG-T-CCG GGC--CCGCT GATGTATGG-
 -CAG-CCCTA CCTG-AGC-G -TC--GCC-G C-GC--CG-- CTT----GCG CC-GCGCCCG -GC--CA--- --T-C-T---

 TCCGTCCCCA ACCTGGAGCT GTGCAACGA. .......... .......... .......... CGAGCTCTTC GCCGACCTCT 298
 -GT-CGG-GG C-GGCT-C-- -GA-GG-AGG CTGGAGCCCC TGTACGAGCG CGTCGGGGCG -CC--G--G- -G-CG-TGG-
 -GT...ATGG CAAACT-CA- -GA--G-A-G CTGGA..... ........ . .........C A-T-GA--CA -A-C--T-GC
 GA-C--TT-G C-GAC--CTA CG--GC-A-G C.......... .......... ...CGAGCAA GA---CG-C- -A-T--GGT-

 TCAACAGCAA GAACGGCGAG CGGGCGGAGA G.CGGAGCGG ACTACCTGAG CGGCCTCCTG TCCACCGCAC CTCCTCAGCA 377
 GATCA----G --G-C---C- A--AG-AC-- -G--A---A- CTGG-GCTG- -C-G-CT--T C--CTAT--G -C--CGCCGC
 GATCA----- --G-C..... ......A--G -AA-A--AA- CAGCAGCC-- -A-GGCATCC --ATTG---G ---TGT-C-C
 A-GTG---CT CGG-C---C- G-C--CA--. -C--C-C--C C-GC-TGCTT -CCG-CG-C- C-TC----CG -A-TCA--GC

 CTACAAATCC CTCAAACAGG AACCTGACTG GAGCGACAGC GACCTGTCCT CCTCCCTACC CAGCCAGATC GCCACCTGTG 457
 -GC-GCCG-- AC-GC--CC- C--GC-T--C CC----..CA- TTGGCCG--C -TCA-T-G-A GTT------- --ACA---C-
 TC--C-TG-G GC-TC..... .......... ..........- C-G-AC--A- --CA---G-A GTA----G-A --TCA-----
 -G-GCCGGG- T--G--.... .........C CC----..GA- TG-AA-CG-G -GGA-GACG- GCC-GCC--G --GG--G-CT

 CCCAGACCAC TATGAGCCTC C......TAC AACCCACCCC GCCCACCTCA CCTGAACCCT GCTCTAATAC CAGCTCTGCC 531
 G---------- C---CA---G -AGCCTGGC- -C--T--G-- ---GC-CA-G --C-TG---A --C--C--C- -GCGC----A
 ---------- C---CA---G -AGTCTGGC- -C--A--T-- T--TC-TA-T ----T---AA --C-AC--CA -CATC----A
 T--C-TT-G- C---C--GC- TACCTGGGCT -C-AGG-GA- ---G..... .......AGC -GCAGC-GCG GCAGC---T-

 .......... .......... .......... .......... ......TGCC CGTCCCCGGC CTCCTCCACC TCCGCCAATA 565
 ATGGGTGCTG CGGCCCTGCC GGGCCCCGGG CGCTCGCTCA AGGGCT--G- T-GT--GCA- -C-GA--T-- G-AC-GGCGG
 .......CAT CATCACCACC ATCACCTGCA GACTTCTTCA CTCAAAG--A TT-----CT- A-----T--T --TT--TCCT
 CACGTCGTCG TCGTCCAGCC CGCCCGGGAC GCCGAGCCCC GCCGACGC-A A-G--G--C- -G--G--TG- -T---GGGGC

 CACC...... .....CTCCA ACCAGCGCTC CTCCAAGAAG AACCTCGACC GCTTCAGTCC GGAGTACCGC CAGAGGAGGG 634
 -GG-GGCGGC GGCGGGG--G G-GC-G--AA GG-------- TCGG-G--TA AGAA---CAA C--------G GTAC--C---
 -CT-TGAG.. .........- G-AG-G--AA A-----A--- TGGG-G---A AGAA---CAA C--------A-G GTA-----A-
 -G--GGC... ..CGCGC--G C-AA-GC-AA GG-------- GCGG-G---A AGC-G--C-A C------AAG AT-C--C-C-

 GCGCAACAA TATCCCCGTC AGGAAGAGCC GGCACAAGGC CAAGAAACGC AACATGGACA TGCAGCAAAA ACTGCTCGAA 714
 A-------- C-----G--G C-C------- -A--T--A-- ---AC-G--- ---G----G- C------G-- GC--T-G--G
 --A-G----- C--A--A--G --A--A---A ---------- -----TGA-G --TG-A--A- CC--A--G--- -G-TT-T--G
 ---------- C-----G--G C-C------- -C-------- -----TG--- ---C----G- C------C-- GG----G--G

 CTTTCCTCAG AGAACGAGAA ACTGCATAAG AGGATCGACA TGCTCACCCG GGACTTGACC AGCCTCAGGC ACTTCTTCAA 794
 T-GA--AGT- -C--T--CCG C----GC--- C--G-G--AC A---G-G--- T--AC--GA- -CG--GC--G GTA-----CG
 --G--T--T- -C--T--T-  G---AGG--- ---G-G--AC AAT-G-GTA- ---GC--GAG -CT--T---G G-A------G
 --GA-GG-G- --------A G G-----G--- -A-G-G--GC A---GT-G-- A--GC-C-G- -CG--GC--A ----G-----
                                                                                 Identity
 ACAGCTTCCC CCAGCCGCCA CCAGCCGCTC CTTCCTCTCC AGCCTCGGAG ACTGCCGGTA A 855          -
 C-----G--T GAGAG-T--T TGGT-AAGG- -A-CGG-AA- T--GCGT--. .......... .             52%
 G-----C--A GAGAGTT--C TGGT-AAAG- TA-CGG-AA- T-TGCATA-. .......... .             46%
 G-----G--- GAGC-GCTGC TGGC-TCGG- GCGT-A--G- TAG..... .... ..... .              51%
```

Figure 26. Comparison of the nucleotide sequence in RcC/EBP-1 with the nucleotide sequence in RcC/EBP-2.

The nucleotide sequence in the 5'-UTR **(A)**, ORF **(B)**, and 3'-UTR **(C)** of RcC/EBP-1 is compared to the nucleotide sequence in the 5'-UTR, ORF, and 3'-UTR of RcC/EBP-2, respectively. Dashes represent nucleotide sequence identity, dots represent insertions or deletions. Numbers to the left and right refer to the nucleotide locations of RcC/EBP-1 or -2 cDNAs with respect to the 5'-end of the cDNAs (see Figures 22 and 24). The percent identities are shown.

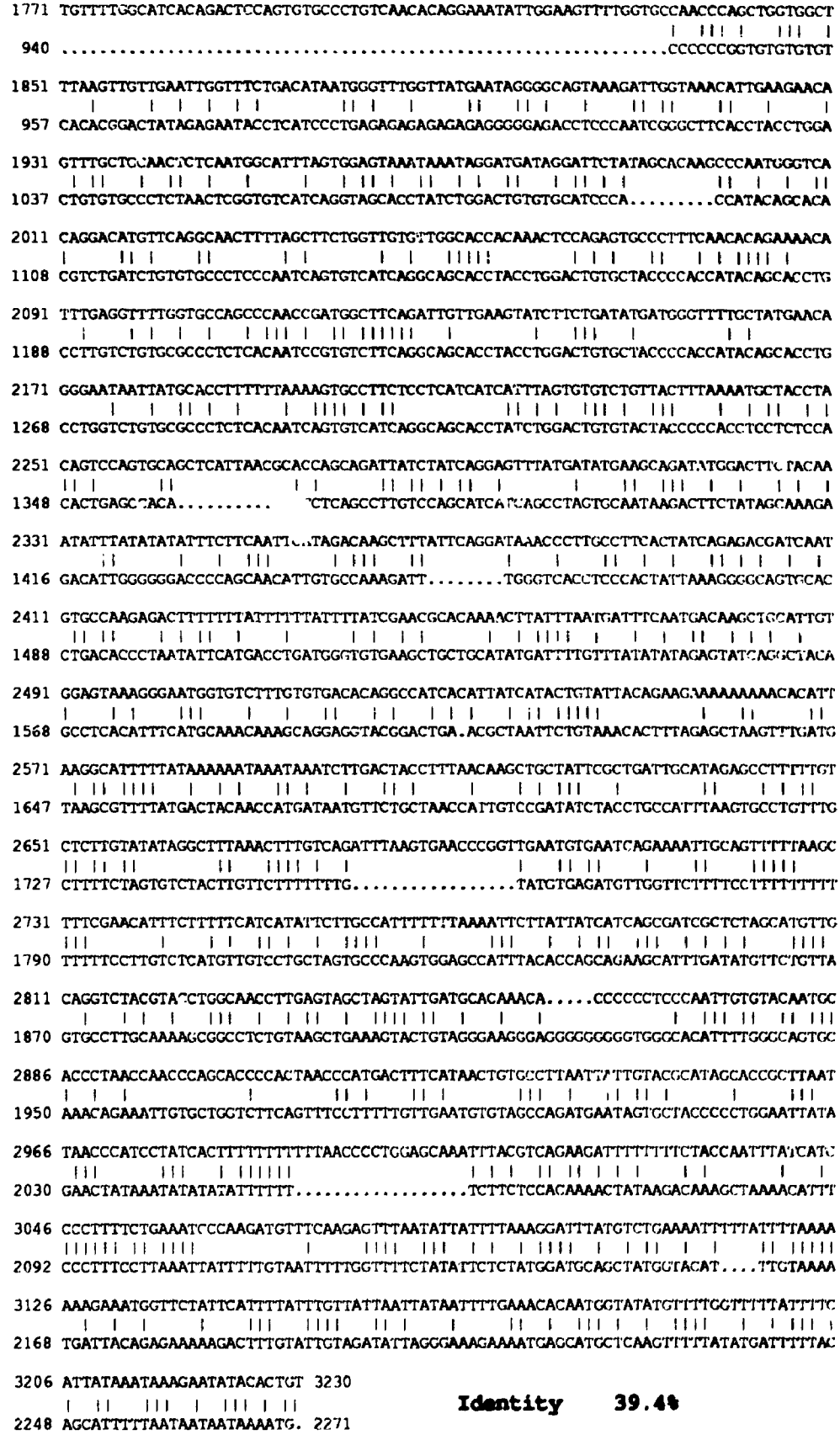
A

```
  1 GAGAGTCCGCCGATTGATCGCGCAGAGTCAGCCCGAGCTCATCCTGGATTCCCGGAGCTCGTCACCATGCTTGGCTGGCT
          |||||  ||  |||         | | ||   |   |||    || ||  |||||| |||   |       |
  1 .........CCGATCTATATCGCTCCTCTTCCTCCAG...AGTGTGGGGATCCCGACATCGTCATCATTGCTCAGATCTT

 81 ATAGGAGCTCC..... 91
        ! ||                          Identity     45.6%
 69 CCCTTCGATCAGTCAC 84
```

B

```
 92 ATGGAGCTAGCCAACTTCTACGAGGTCGAATCCCGGCCAT..CCATGAGCGCCCAGCCTCAGCAGCACGCCGCCTACGGC
                                | |||||| ||||   | ||  |  ||    |  |   | |   |||  | |
 85 ......................ATGAGCATCCCGTCCATGAGCCTGGACAGCCGCTGTGTGTCCCCCTATGCCGCCTGG

170 TACAGGGAGCCCCCCGCCTCCACCGGGGACGTGACCGAGCTGTGCGACA...ACGAGAACTCCATAGACATCAGCGCCTA
    |  | |||||||  ||  || |  || | |   |   |      | |   |   ||   ||   |   | |||  | |   |
142 TGTATGGAGCCCACCAACTTCTACGAGCAGCGGCTGGGCGCCTCCCCGAGTCACTGCAAGCACCGGGCCATGTGTGAGGA

247 CATCGATCCGGCCGCCTTCAACGACGAGTTCCTGGCCGATCTCTTCCATAACAGCAAGCAGGACCGAGCCAAGGCCACCA
    || ||| ||  |   |      '  ||    |||||||||  ||   |     |           ||   ;   ||  | |||
222 CACCGAGCCCCCGCGCGGAGGCAGCGGCACCCTGGCCGAGCTGAGCGCAGCCCCGGCTATCTACGACGATGAGAGCGCCA

327 TGGACTACCAGCAGGGCCACCCTCCTATGTATGGCTGCATGGCCACCTACCTGGACAGCAAGATGGACAATGGCCTCAGG
    | |||| |         |  |    ; |||      | |   | | || |||||||        |  ||| |   | ||
302 TAGACTTCAGTTCATACATCGACTCCATG......TCCTCCGTCCCCAACCTGGAGCTGTGCAACGACGAGCTCTTCGCC

407 CACCTGGTCATCAAACAGGAGCCCAGGGGGGAGGAGGAGGAGGCCAACCGGGTGTCTCTAGCTGCCCTCTACCCTCACCC
     |||| ||| ||   || |   |  | ||| ||||   |  ||  ||    ;|      |    ||| | | |    ||
376 GACCTCTTCAACAGCAAGAACGGCGAGCGGGCGGAGAGCGGAGCGGACTACCTG...AGCGGCCTCCTGTCCACCGCACC

487 TTCCAACCAGCACCCATCCCACCTGCAATACCAGGTAGCCCACTGTGCCCAGACCACCATGCACCTGCAGCCTGGGCACC
    | |  | ||  ||  |||| |        | |||| | |  ||||   | | | |  | ||  ||| |  |||   || |
453 TCCTCAGCACTACAAATCCCTC......AAACAGGAACCTGACTGGAGCGACAGCGACCTG.TCCTCCTCCCTACCCAGC

567 CGACTCCACCACCAACCCCAGTGCCAAGTCCTCACCACCTTCCCCATCACCACCATCATCATCACCACCAGCTGCAAGCT
    |   ||  |  |    |||||  |    |  |  |       |||| | |  |||  |           || |||  ||  |
526 CAGATCGCCACCTGTGCCCAGACCACTATGAGCCTCCTACAACCCACCCCGCCCACCTCACCTGAACCCTGCTCTAA..T

647 TCCTCCTCCAAGGCAATGTCCTCTTCGTCTTCCAGCTCTTCTTCTTCATCAGAGACCAGAGGCAAGTCCAAAAAATGGGT
     ||  |||          |||| |  | || |||| |||  |   | || |    | |    ||   ||'|| ||     |
604 ACCAGCTCTGCCTGCCCGTCCCCGGCCTCCTCCACCTCCGCCAATACACCCTCCAACCAGCGCTCCTCCAAGAAGAACCT

727 GGACAAAGGCAGCACAGAGTACAGGGTGAGGAGGGAGAGGAACAATATAGCGGTCAGGAAGAGCAGGGACAAGGCTAAGA
    |||     |||  | ,|||||  |   |||||||||| | |||||||| || ||||||||||| |||||||||| ||||
684 CGACCGCTTCAGTCCGGAGTACCGCCAGAGGAGGGAGCGCAACAATATCGCCGTCAGGAAGAGCCGGGACAAGGCCAAGA

807 TGAGGAACGTTGAGACCCAACAGAAGGTGGTTGAGTTGTCCAACGACAACGAAAAGTTGAGGAAGAGGGTGGAACAGTTG
      | ||| | || |  || || ||  || | ||  | |||   || ||||| ||  ||   |||||| | ||   | |
764 AACGCAACATGGACATGCAGCAAAAACTGCTCGAACTTTCCTCAGAGAACGAGAAACTGCATAAGAGGATCGACATGCTC

887 AGCAGGAGTTGGAGACCCTCAGGGGCATCTTCAGGCAGCTGCCAGAGAGCTCTCTGGTCAAAGTGATGGGCAACTGTGC
    | | |||| |||   | |||||||  | ||||||  ||||| ||      | |      |      |   |  | |
844 ACCCGGGACTTGACCAGCCTCAGGCACTTCTTCAAACAGCTTCCCCCAGCCGCCACCAGCGGCTCC'"CCTCTCCAGCCT

967 ATGA..... 970
      ||                          Identity     50.5%
924 CGGAGACTGCCCGTAA 939
```

C


1771 TGTTTTGGCATCACAGACTCCAGTGTGCCCTGTCAACACAGGAAATATTGGAAGTTTTGGTGCCAACCCAGCTGGTGGCT
940 .....CCCCCCGGTGTGTGTGT
1851 TTAAGTTGTTGAATTGGTTTCTGACATAATGGGTTTGGTTATGAATAGGGGCAGTAAAGATTGGTAAACATTGAAGAACA
957 CACACGGACTATAGAGAATACCTCATCCCTGAGAGAGAGAGAGAGGGGGAGACCTCCCAATCGGGCTTCACCTACCTGGA
1931 GTTTGCTCCAACTCTCAATGGCATTTAGTGGAGTAAATAAATAGGATGATAGGATTCTATAGCACAAGCCCAATGGGTCA
1037 CTGTGTGCCCTCTAACTCGGTGTCATCAGGTAGCACCTATCTGGACTGTGTGCATCCCA.....CCATACAGCACA
2011 CAGGACATGTTCAGGCAACTTTTAGCTTCTGGTTGTGTTGGCACCACAAACTCCAGAGTGCCCTTTCAACACAGAAAACA
1108 CGTCTGATCTGTGTGCCCTCCCAATCAGTGTCATCAGGCAGCACCTACCTGGACTGTGCTACCCCACCATACAGCACCTG
2091 TTTGAGGTTTTGGTGCCAGCCCAACCGATGGCTTCAGATTGTTGAAGTATCTTCTGATATGATGGGTTTTGCTATGAACA
1188 CCTTGTCTGTGCGCCCTCTCACAATCCGTGTCTTCAGGCAGCACCTACCTGGACTGTGCTACCCCACCATACAGCACCTG
2171 GGGAATAATTATGCACCTTTTTTAAAAGTGCCTTCTCCTCATCATCATTTAGTGTGTCTGTTACTTTAAAATGCTACCTA
1268 CCTGGTCTGTGCGCCCTCTCACAATCAGTGTCATCAGGCAGCACCTATCTGGACTGTGTACTACCCCCACCTCCTCTCCA
2251 CAGTCCAGTGCAGCTCATTAACGCACCAGCAGATTATCTATCAGGAGTTTATGATATGAAGCAGATATGGACTTCTACAA
1348 CACTGAGCCACA..... CTCAGCCTTGTCCAGCATCATCAGCCTAGTGCAATAAGACTTCTATAGCAAAGA
2331 ATATTTATATATATTTCTTCAATTC..TAGACAAGCTTTATTCAGGATAAACCCTTGCCTTCACTATCAGAGACGATCAAT
1416 GACATTGGGGGGACCCCAGCAACATTGTGCCAAAGATT.....TGGGTCACCTCCCACTATTAAAGGGGCAGTGCAC
2411 GTGCCAAGAGACTTTTTTTATTTTTTATTTTATCGAACGCACAAAACTTATTTAATGATTTCAATGACAAGCTGCATTGT
1488 CTGACACCCTAATATTCATGACCTGATGGGTGTGAAGCTGCTGCATATGATTTTGTTTATATATAGAGTATCAGGCTACA
2491 GGAGTAAAGGGAATGGTGTCTTTGTGTGACACAGGCCATCACATTATCATACTGTATTACAGAAGAAAAAAAACACATT
1568 GCCTCACATTTCATGCAAACAAAGCAGGAGGTACGGACTGA.ACGCTAATTCTGTAAACACTTTAGAGCTAAGTTTGATG
2571 AAGGCATTTTTATAAAAAATAAATAAATCTTGACTACCTTTAACAAGCTGCTATTCGCTGATTGCATAGAGCCTTTTTTGT
1647 TAAGCGTTTTATGACTACAACCATGATAATGTTCTGCTAACCATTGTCCGATATCTACCTGCCATTTAAGTGCCTGTTTG
2651 CTCTTGTATATAGGCTTTAAACTTTGTCAGATTTAAGTGAACCCGGTTGAATGTGAATCAGAAAATTGCAGTTTTTAAGC
1727 CTTTTCTAGTGTCTACTTGTTCTTTTTTTG.....TATGTGAGATGTTGGTTCTTTTCCTTTTTTTTT
2731 TTTCGAACATTTCTTTTTCATCATATTCTTGCCATTTTTTTAAAATTCTTATTATCATCAGCGATCGCTCTAGCATGTTG
1790 TTTTTCCTTGTCTCATGTTGTCCTGCTAGTGCCCAAGTGGAGCCATTTACACCAGCAGAAGCATTTGATATGTTCTGTTA
2811 CAGGTCTACGTACCTGGCAACCTTGAGTAGCTAGTATTGATGCACAAACA.....CCCCCCTCCCAATTGTGTACAATGC
1870 GTGCCTTGCAAAAGCGGCCTCTGTAAGCTGAAAGTACTGTAGGGAAGGGAGGGGGGGGGTGGGCACATTTTGGGCAGTGC
2886 ACCCTAACCAACCCAGCACCCCACTAACCCATGACTTTCATAACTGTGCCTTAATTATTGTACGCATAGCACCGCTTAAT
1950 AAACAGAAATTGTGCTGGTCTTCAGTTTCCTTTTTGTTGAATGTGTAGCCAGATGAATAGTGCTACCCCCTGGAATTATA
2966 TAACCCATCCTATCACTTTTTTTTTTTAACCCCTGGAGCAAATTTACGTCAGAAGATTTTTTTTCTACCAATTTATCATC
2030 GAACTATAAATATATATATTTTTT.....TCTTCTCCACAAAACTATAAGACAAAGCTAAAACATTT
3046 CCCTTTTCTGAAATCCCAAGATGTTTCAAGAGTTTAATATTATTTTAAAGGATTTATGTCTGAAAATTTTTATTTTAAAA
2092 CCCTTTCCTTAAATTATTTTTGTAATTTTTGGTTTTCTATATTCTCTATGGATGCAGCTATGGTACAT....TTGTAAAA
3126 AAAGAAATGGTTCTATTCATTTTATTTGTTATTAATTATAATTTTGAAACACAATGGTATATGTTTTGGTTTTTATTTTC
2168 TGATTACAGAGAAAAAGACTTTGTATTGTAGATATTAGGGAAAGAAAATGAGCATGCTCAAGTTTTTATATGATTTTTAC
3206 ATTATAAATAAAGAATATACACTGT 3230
2248 AGCATTTTTAATAATAATAAAATG. 2271
Identity 39.4%

4.3.2 Comparison of the Deduced Amino Acid Sequences in the Proteins Encoded from the ORFs of RcC/EBP-1 and RcC/EBP-2

The ORF of RcC/EBP-1 encodes a protein consisting of 292 amino acids (Mr=33,088) which shares 64% amino acid sequence similarity (44% identity) with the 284 amino acid protein (Mr=31,215) encoded from the ORF of RcC/EBP-2 (Figure 27). Although the archetypical C/EBP, rnC/EBPα in rats (Lincoln *et al.*, 1994; Landschulz *et al.*, 1988a), encodes a protein consisting of 358 amino acids, its amino acid sequence shares 81% similarity and 65% identity with the sequence in the RcC/EBP-1 protein (Figure 28), and only 64% similarity and 44% identity with the sequence in the RcC/EBP-2 protein (not shown). High levels of amino acid sequence identity with the deduced RcC/EBP-1 protein were also found with the amino acid sequences reported for the C/EBPα isoform other organisms [mouse, 63% (Christy *et al.*, 1991; Cao *et al.*, 1991); chicken, 71% (Calkhoven *et al.*, 1992); and *Xenopus*, 80% (Xu and Tata, 1992)], while other reported isoforms of C/EBP from these same organisms (Cao *et al.*, 1991; Descombes *et al.*, 1990; Williams, Cantwell and Johnson, 1991; Burk *et al.*, 1993) and from humans (Poli *et al.*, 1990; Kinoshita *et al.*, 1992) exhibited less than 43% identity to the RcC/EBP-1 sequence. Comparison of the deduced amino acid sequence from the ORF of the RcC/EBP-2 protein, with the amino acid sequences reported for isoforms of C/EBP from other organisms, disclosed that it shares its highest identity (59-60%) with the mammalian C/EBPδ isoform (Figure 29); except for a 50% identity

Figure 27. Comparison of the derived amino acid sequence of RcC/EBP-1 with the derived amino acid sequence of RcC/EBP-2.

Dashes represent amino acid identity, dots between two sequences represent amino acid similarity, dots inside the same sequence represent deletions or insertions, and asterisks represent stop codons. The percent identity and similarity are shown.

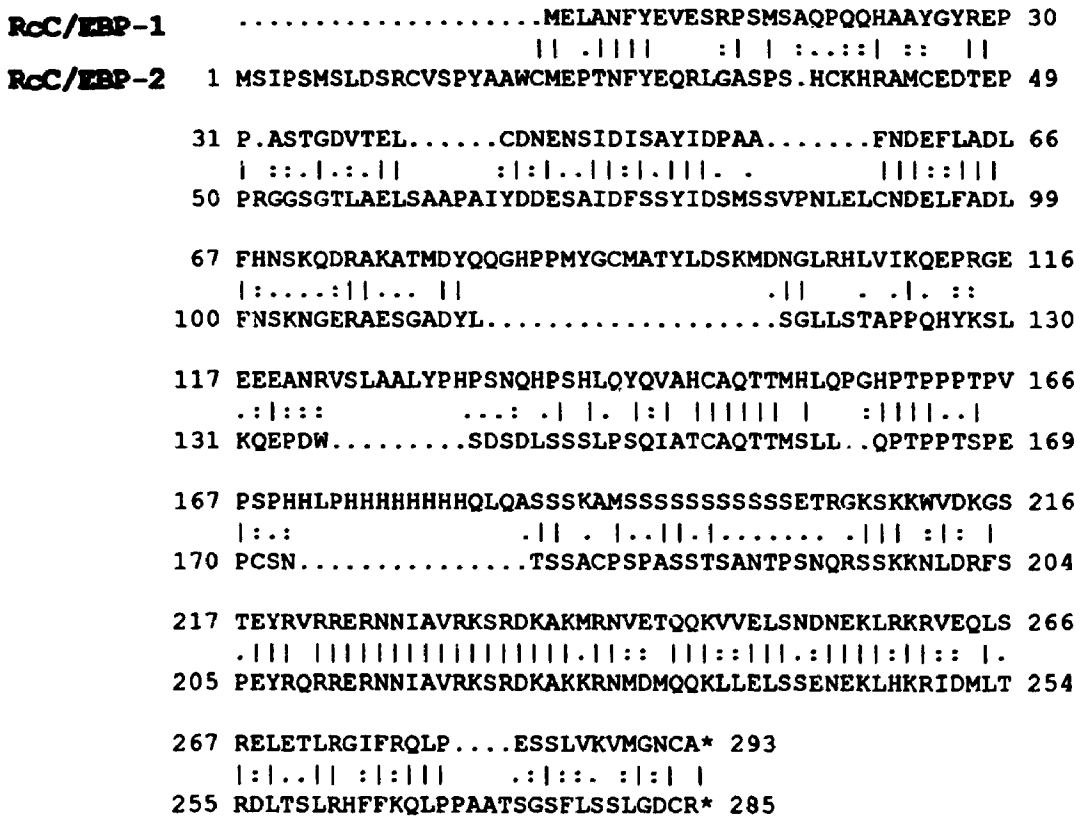
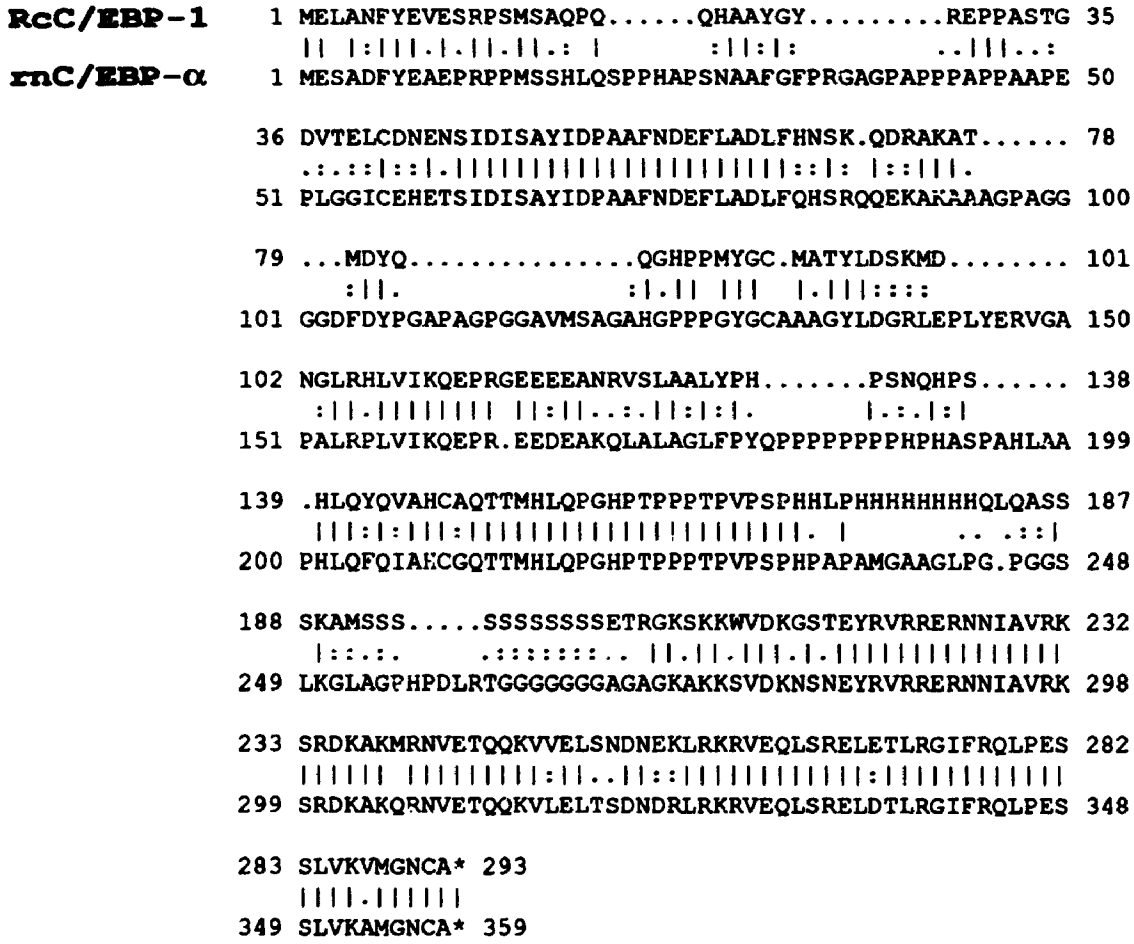

RcC/EBP-1
RcC/EBP-2
Similarity: 64%
Identity: 44%

Figure 28. Comparison of the derived amino acid sequence of RcC/EBP-1 with the amino acid sequence of rat C/EBPα.

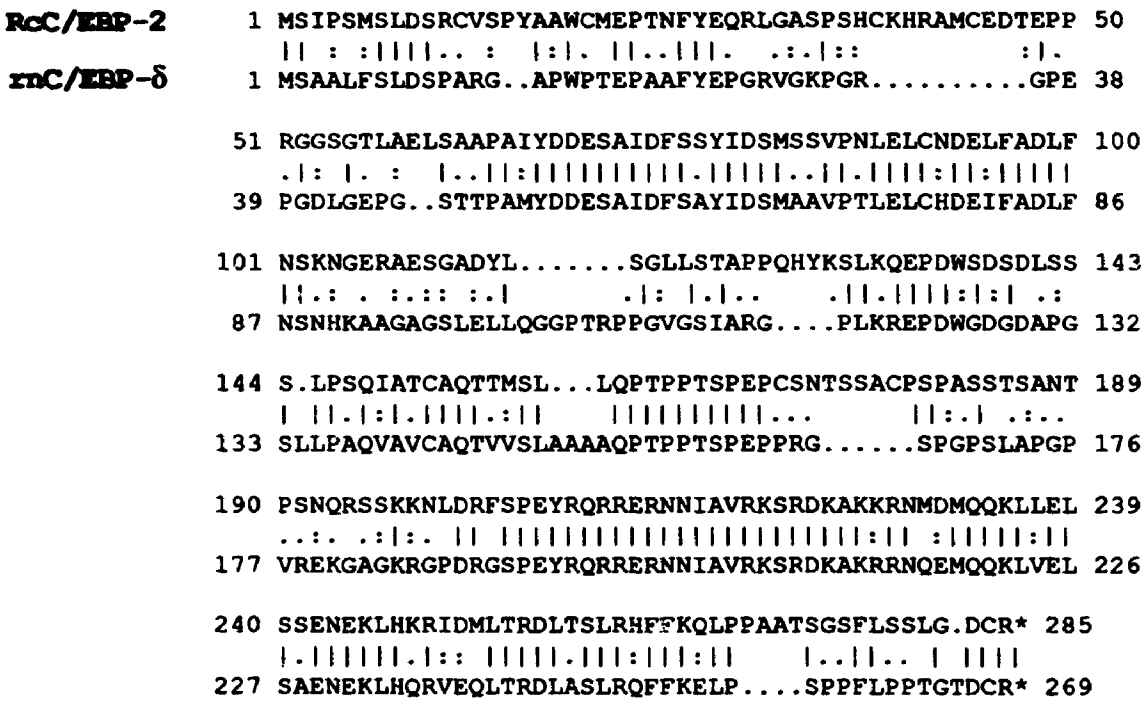
The derived amino acid sequence from the ORF of *Rana catesbeiana* RcC/EBP-1 is compared to the sequence reported for a rat C/EBPα (Lincoln *et al.*, 1994). Dashes represent amino acid identity, dots between two sequences represent amino acid similarity, dots inside the same sequence represent deletions or insertions, and asterisks represent stop codons. The percent identity and similarity are shown.



Similarity: 81%
Identity: 65%

Figure 29. Comparison of the derived amino acid sequence of RcC/EBP-2 with the amino acid sequence of rat C/EBPδ.

The derived amino acid sequence from the ORF of *Rana catesbeiana* RcC/EBP-2 is compared to the sequence reported for a rat C/EBPδ (Williams, Cantwell and Johnson, 1991). Dashes represent amino acid identity, dots between two sequences represent amino acid similarity, dots inside the same sequence represent deletions or insertions, and asterisks represent stop codons. The percent identity and similarity are shown.



Similarity: 72%
Identity: 59%

with the *Xenopus* xC/EBP protein, all other reported C/EBP isoforms from these organisms exhibited only 36-45% identity.

While the amino-terminal two-thirds of the mammalian C/EBPα, C/EBPβ, and C/EBPδ are largely unrelated at the level of primary amino acid sequence (Cao *et al.*, 1991) the carboxy-terminal third, containing the so-called bZIP domain (Vinson *et al.*, 1989), are closely related and, as such, have been used as an index for identifying homologues of the C/EBP isoforms in different organisms. In Figure 30, the amino acid sequence in the carboxy-terminal, putative bZIP domain of the RcC/EBP-1 protein (a region rich in basic amino acids linked to a dimer-forming region called a leucine zipper) is compared with similar domains reported for various isoforms of other C/EBP proteins. The results of these comparisons demonstrate that the putative bZIP domain of RcC/EBP-1 shares more identity, in both its basic amino acid region and its leucine zipper region, with *Xenopus* C/EBP and the C/EBPα isoforms than it shares with the RcC/EBP-2 protein or with any other C/EBP isoform from these different organisms. A comparison of the putative bZIP domain of the RcC/EBP-2 protein, with the bZIP domain of the C/EBP isoforms from these different organisms, indicates that it shares more identity with the mammalian C/EBPδ isoform than it shares with any other isoform.

Figure 30. Comparison of the RcC/EBP-1 and RcC/EBP-2 carboxy-terminal amino acid sequences with published carboxy-terminal sequences of C/EBP isoforms from other organisms.

Alignment of the amino acid sequence in the carboxy-terminal end [nt 711 to 967 for RcC/EBP-1 (see Figure 22) and nt 679 to 736 for RcC/EBP-2 (see Figure 24)], containing both a basic amino DNA-binding region **(A)** and a leucine zipper **(B)**, of the protein derived from RcC/EBP-1 with similar sequences in the carboxy-terminal ends of proteins derived from RcC/EBP-2, a *Xenopus* (x) C/EBP (Xu and Tata, 1992), a chicken (c) C/EBPα (Calkhoven *et al.*, 1992), a chicken (c) C/EBPβ (Burk *et al.*, 1993), a rat (rn) C/EBPα (Landschulz *et al.*, 1988), a rat (rn) C/EBPδ (Cao *et al.*, 1991), a rat (rn) C/EBPδ (Cao *et al.*, 1991). Dashes represent amino acid identity, and the leucine residues making up the leucine zipper domain are denoted by arrowheads over the RcC/EBP-1 sequence. The percent identity of each of the aligned sequences to RcC/EBP-1 or RcC/EBP-2 carboxy-terminal sequences is shown.

A

Basic Region

		% Identity with RcC/EBP-1	% Identity with RcC/EBP-2
RcC/EBP-1	KSKKWVDKGSTEYRVRRERNNIAVRKSRDKAKMRNVET	-	71.1
RcC/EBP-2	S---NL-RF-P---Q----------K--MDM	71.7	-
xC/EBP	--------N-N----------	94.7	71.1
cC/EBPα	----T---N-N----------Q-----	89.5	71.1
cC/EBPβ	-P--C---H-D--KL----------L--	81.6	65.8
rnC/EBPα	-A--S---NN----------Q-----	86.8	68.4
rnC/EBPβ	-A--T---L-D--LM----------	84.2	65.8
rnC/EBPδ	AG-RGP-R--P---Q----------Q-M	73.7	76.3

B

Leucine Zipper

		% Identity with RcC/EBP-1	% Identity with RcC/EBP-2
RcC/EBP-1	QQKVVELSNDNEKLRKRVEQLSRELETLRGIFRQLPESSLVKVMGNCA	-	43.7
RcC/EBP-2	---LL---SE----H--IDM-T-D-TS--HF-K---PAATSGSFLGDC	43.7	-
xC/EBP	----F---S--D----------A-----	91.7	43.7
cC/EBPα	----L--TT---R----------A--S--	87.5	41.7
cC/EBPβ	-H--L--TAE--R-Q-K--------S---NL-K----PL-ASSPRC--	58.4	41.7
rnC/EBPα	----L--TS--DR----------D----------A-----	85.5	41.7
rnC/EBPβ	-H--L--TAE--R-Q-K--------S---NL-K----PL-ASAGMC--	58.4	41.7
rnC/EBPδ	---L----AE----H-Q----T-D-AG--QF-KK--SPPFLPPTGADC	48.0	60.4

4.3.3 Isolation and Characterization of the Promoter and 5'-Regulatory Regions of a *Rana catesbeiana* Gene Encoding RcC/EBP-1

A 1027 bp *Eco*R I/*Bam*H I cDNA fragment (nt 1-1027) from RcC/EBP-1 (Figure 22) was used to screen our *Rana catesbeiana* genomic library. A positive clone of approximately 18 kb was isolated and designated as RcGC/EBP-1. Since this 18 kb genomic fragment hybridized with sequences from the 5'-UTR and most, if not all, of the ORF of RcGC/EBP-1 (Figure 31A), I reasoned that this fragment contained the 5'-flanking region of this gene. Southern blot hybridization analyses of enzyme restriction fragments from this clone identified a *Hin*d III/*Hin*d III fragment of approximately 7 kb which appeared to contain the promoter and 5'-regulatory regions of this gene (Figure 31B). This fragment was subcloned and designated as pGC/EBP-1.

A cartoon depicting a restriction map and delineating the region of pGC/EBP-1 which was sequenced is shown in Figure 32A. The actual nucleotide sequence of the promoter region as well as some of the 5' transcribed region of this gene is shown in Figure 32B. A comparison of this sequence with the sequence established for RcC/EBP-1 (Figure 22) reveals that the gene encoding RcC/EBP-1 contains an intron (greater than 2 kb) which begins 318 nt after the translation start codon (ATG).

In order to characterize the promoter region of the gene encoding RcC/EBP-1, primer extension analyses were conducted to

Figure 31. Characterization of a RcC/EBP-1 genomic clone, RcGC/EBP-1, by Southern blot hybridization analyses.

A: RcGC/EBP-1 (5 μg) was digested with *Xho* I, and the digested DNA was separated on a 0.7% agarose gel and vacuum-blotted onto a Zetaprobe membrane. The membrane was sequentially hybridized with four different ^{32}P-labelled DNA fragments from RcC/EBP-1 (for details see Figure 22): **1)** a 90 bp *Eco*R I/*Nco* I fragment (a 5'-UTR probe; nt 1-90); **2)** a 318 bp *Nco* I/*Hin*d III fragment (a 5'-ORF probe; nt 324-642); **3)** a 385 bp *Hin*d III/*Bam*H I fragment (a 3'-ORF probe; nt 642-1,027); and **4)** a 1668 bp *Xba* I/*Pvu* I fragment (a 3'-UTR probe; nt 1127-2795). **B:** RcGC/EBP-1 (5 μg) was digested with *Xho* I and *Hin*d III, and the digested DNA was separated on a 0.7% agarose gel and vacuum-blotted onto a Zetaprobe membrane. Hybridization of this blot with the most 5' fragment from RcC/EBP-1 (*i.e.* the 90 bp *Eco*R I/*Nco* I fragment) revealed that it recognized a ~7 kb *Hin*d III/*Hin*d III fragment (Designated as pGC/EBP-1). The relative sizes of the DNA fragments were determined from a coelectrophoresed DNA ladder and are shown on the left in kilobases (kb).

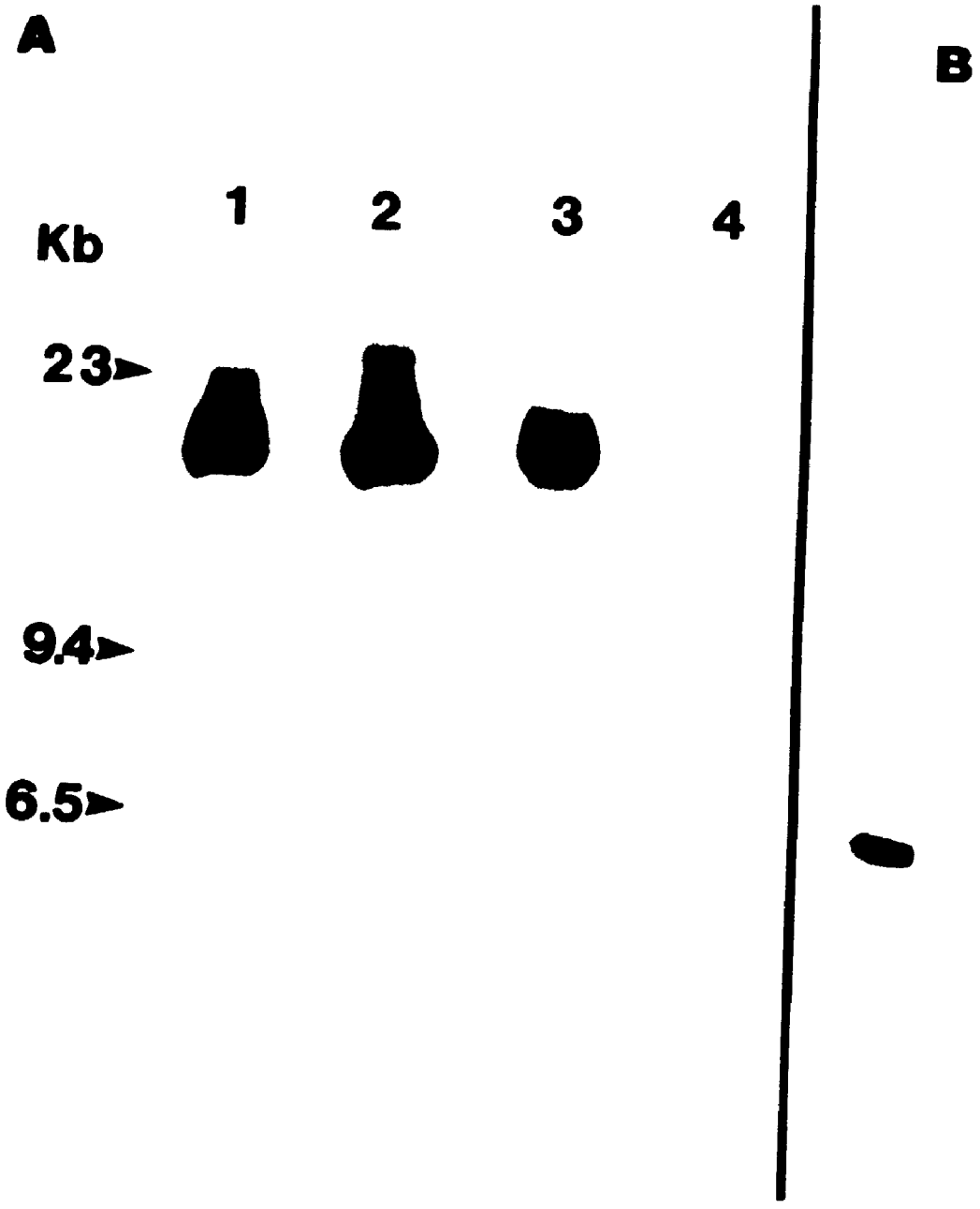
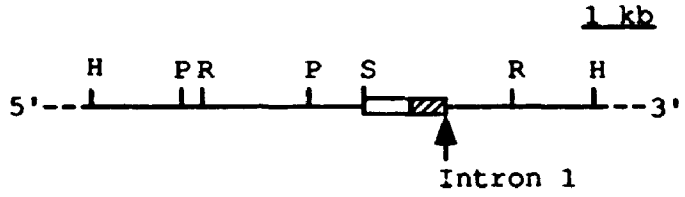

A
B
Kb
1
2
3
4
23
9.4
6.5

Figure 32. Enzyme restriction map and partial sequence of pGC/EBP-1.

A: Enzyme restriction map of pGC/EBP-1. H, P, R, and S represent the relative location of restriction enzyme sites for *Hind* III, *Pst* I, *Eco*R I, and *Sac* I, respectively. The open- and the hatched-boxed areas indicate the areas sequenced (shown in B) in both directions and represent the relative positions of the promoter region (open-box) and first exon (hatched-box), respectively. An intron start site is indicated by an arrowhead. The relative 1 kb size is shown at the top right side of this panel.

B: Nucleotide sequence of the promoter region, exon 1 and part of intron 1 of a *Rana catesbeiana* RcC/EBP-1 gene. The transcription start site is designated +1. A conserved TATA motif at -53 and a possible TATA motif at -18 are double underlined, and a well-conserved heat shock element is single underlined. The boxed area indicates a sequence reported to represent a C/EBP (CAATT/enhancer binding protein) binding consensus sequence (Graves *et al.*, 1986; Vinson *et al.*, 1989; Ryden and Beemon, 1989). Two direct repeats (paired arrows a and b) and two inverted repeats (paired arrows 1 and 2) are present in the promoter region of this RcC/EBP-1 gene. The first exon is 417 nt in size. The ATG (bold) translation start codon is located at +97. The coding region in exon 1 encodes approximately one-third of the RcC/EBP-1 protein. The first intron is larger than 2 kb.

A



B

```
                                                    Sac I
-545                                                GAGCTCAATTAACCCT
                                                        (a)
-529  CACTAAAGGGAGTCGACTCGATCAGGTTGCACGAGAGGTGGGTCCCTTCG
                (1)      (1)                       (b)
-479  TCGTGTGCGGGTTGTGATTGGCTAGCAGGAAAGAAAGGCGGGACCTTGCT
                                        (a)
-429  TGTGGGCACGGGCTGTGATTGATCAGCAGGCACGAGAGGCGGGACCTCGC
                                                (b)
-379  TGTGTGTGCGGACCGTGATTGGTTAGCAGGCACAAGAGGCGGAACCTTGC

-329  TGTGGGTGCGGGCTGCGATTGGGCAGAAATTCAAGGAACTTGACCGTAGA

-279  CATCAGATTACTGCACACTTATCGGCGTTATTGGCAATGTGTGCCCCGAG
          (2)
-229  AACATTAATCTCATCGAGGACGCGGCACAGGACGGCGGACCTCGGAGGGA

-179  GTTGCGGGGAAGGAAGCGCTGAAGGCGGCCGGAGAAGTTCCAGAAGTTTT

-129  CTCGCTGTAGCGCAGCCTTGTGTGCAGTACCTGTAAGCGGCCATGAGGTG
                                (2)
 -79  TCGCTGCCCCGCCCTCGCCTCCCGCCTATATATAGAGTGACAGCGGCGCT

 -29  CCGGCAGTCAGTATTGAGCGGTGTCGCCT
                                  +1
                                  GCTTGGAGAGTCCGCCGATTG
 +22  ATCGCGCAGAGTCAGCCCGAGCTCATCCTGGATTGTCCCGGAGCTCCACC
 +72  ATGCTTGGCTGGCTATAGGAGCTCCATGGAGCTAGCCAACTTCTACGAGG
+122  TCGAATCCCGGCCATCCATGAGCGCCCAGCCTCAGCAGCACGCCGCCTAC
+172  GGCTACAGGGAGCCCCCCGCCTCCACCGGGGACGTGACCGAGCTGTGCGA
+222  CAACGAGAACTCCATAGACATCAGCGCCTACATCGATCCGGCCGCCTTCA
+272  ACGACGAGTTCCTGGCCGATCTCTTCCATAACAGCAAGCAGGACCGAGCC
+322  AAGGCCACCATGGACTACCAGCAGGGCCACCCTCCTATGTATGGCTGCAT
+372  GGCCACCTACCTGGACAGCAAGATGGACAATGGCCTCAGGCACCTG
                                                    +417
+418  gtcaatgctacccaaccagcgcccatcctgcctgcaccccgccaaccaac
     Intron 1
+468  cagcgtgcaccccgccaaccaaccagcgcccatcccgcctgcaccccgcc
+518  aaccaaccagcg ——→ Hind III
```

Figure 33. Primer extension analyses to determine the *Rana catesbeiana* RcC/EBP-1 gene transcription start site.

A 28 nt primer, complementary to RcC/EBP-1 mRNA at position +28 to +55 (see Figure 32B) was ^{32}P end-labelled using T4 polynucleotide kinase. About 5 x 10^5 cpm of this primer was annealed to 20 μg of total *Rana catesbeiana* liver RNA (Lane 3), 20 μg of yeast tRNA (Lane 2) or ddH_2O (Lane 1). The extended products were analyzed on a 6% polyacrylamide sequencing gel containing 8 M urea. Also included in the gel were the sequencing reactions in which the same primer was used for sequencing the 5'-flanking region of the RcC/EBP-1 gene.


1 2 3 T G C A

establish the transcription start site. Results from these analyses (Figure 33) demonstrate that the 5' terminus of RcC/EBP-1 is a guanine and it is denoted in the nucleotide sequence (Figure 32B) as +1. Inspection of the nucleotide sequence upstream from the transcription start site (Figure 32B) reveals the presence of a putative TATA motif (-53), a possible TATA motif (-18), a well-conserved heat shock element (nt -142 to -135; Amin *et al.*, 1988; Xiao and Lis, 1988), and a number of GC-rich areas. Moreover, a DNA element, partially consistent with a putative C/EBP binding motif, 5'-GCAAT-3' (Graves *et al.*, 1986; Vinson *et al.*, 1989; Cao *et al.*, 1991) is located at -246 to -242. Computer analyses of this RcC/EBP-1 gene promoter region discloses the presence of two inverted nucleotide repeats [one consists of 5 nt (between -520 to -516 and -515 to -511) and another of 10 nt (between -271 to -262 and -110 to -101)] and two direct repeats [one consists of 10 nt (between -501 to -492 and -400 to -391) and another of 11 nt (between -343 to -434 and -393 to -383)].

4.3.4 Southern Blot Hybridization Analyses of *Rana catesbeiana* Genomic DNA with DNA Fragments from RcC/EBP-1 and -2

Using a 234 bp *Nco* I/*Nco* I fragment which contains most of the first exon in the RcC/EBP-1 gene (nt 91-325 in Figure 32B) as a probe, I performed Southern blot hybridization analysis (Figure 34). All of the lanes showed multiple bands. Thus, these results suggest

Figure 34. Southern blot hybridization analysis of *Rana catesbeiana* genomic DNA with a cDNA probe specific for the first exon of RcC/EBP-1.

Rana catesbeiana genomic DNA (20 μg) was digested with *Bam*H I (Lane 1) , *Eco*R I (Lane 2), *Hin*d III (Lane 3) or *Pst* I (Lane 4). The digested DNAs were separated on a 0.7% agarose gel, and the fragments were transferred onto a Zetaprobe membrane and hybridized to a 234 bp *Nco* I/*Nco* I fragment from RcC/EBP-1 cDNA (nt 96-330 in Figure 32B). The relative sizes of the DNA fragments were determined from a coelectrophoresed DNA ladder and are shown on the left in kilobases (kb).

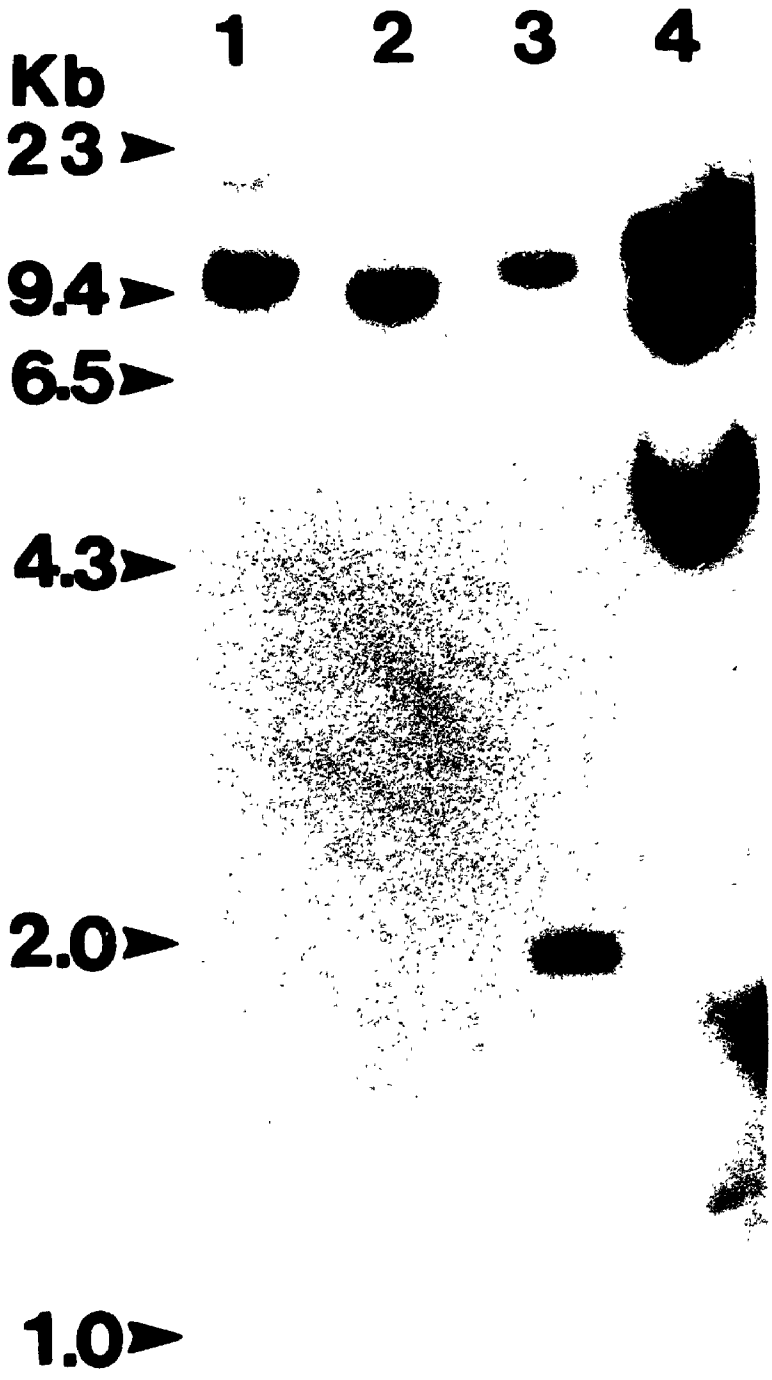
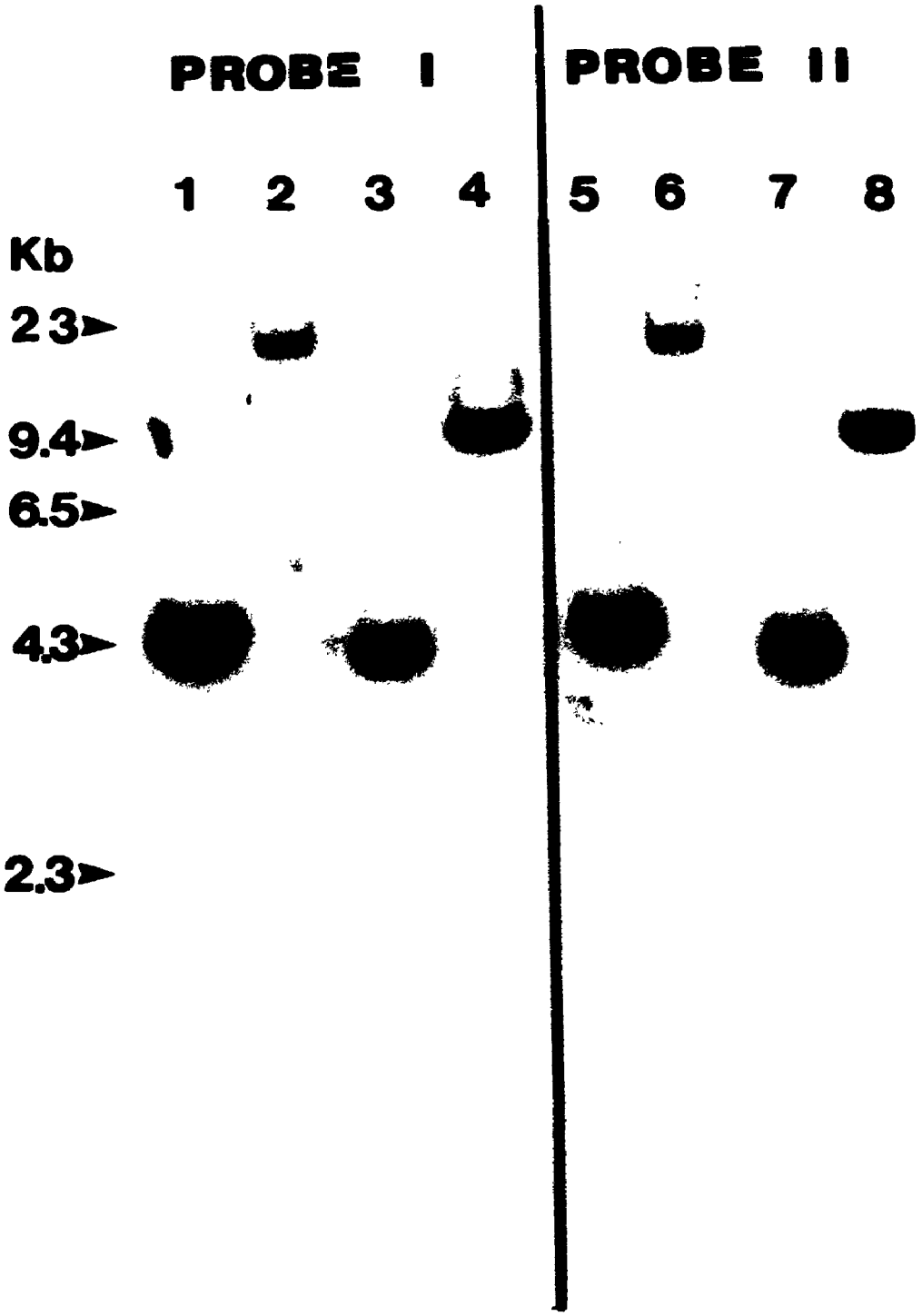

1
2
3
4
Kb
23
9.4
6.5
4.3
2.0
1.0

Figure 35. Southern blot hybridization analyses of *Rana catesbeiana* genomic DNA with cDNA probes specific for RcC/EBP-2.

Rana catesbeiana genomic DNA (20 μg) was digested with *Bam*H I (Lanes 1 and 5) , *Eco*R I (Lanes 2 and 6), *Hin*d III (Lanes 3 and 7) or *Pst.* I (Lanes 4 and 8). The digested DNAs were separated on a 0.7% agarose gel, and the fragments were transferred onto a Zetaprobe membrane and hybridized to Probe I, a 847 bp *Eco*R I/*Sma* I fragment (nt 1-847 in Figure 24), and Probe II, a 482 bp *Sac* I/*Sma* I fragment (nt 365-847 in Figure 24). The relative sizes of the DNA fragments were determined from a coelectrophoresed DNA ladder and are shown on the left in kilobases (kb).


PROBE I
PROBE II
1 2 3 4 5 6 7 8
Kb
23
9.4
6.5
4.3
2.3

that the *Rana catesbeiana* genome has multiple copies of the RcC/EBP-1 gene.

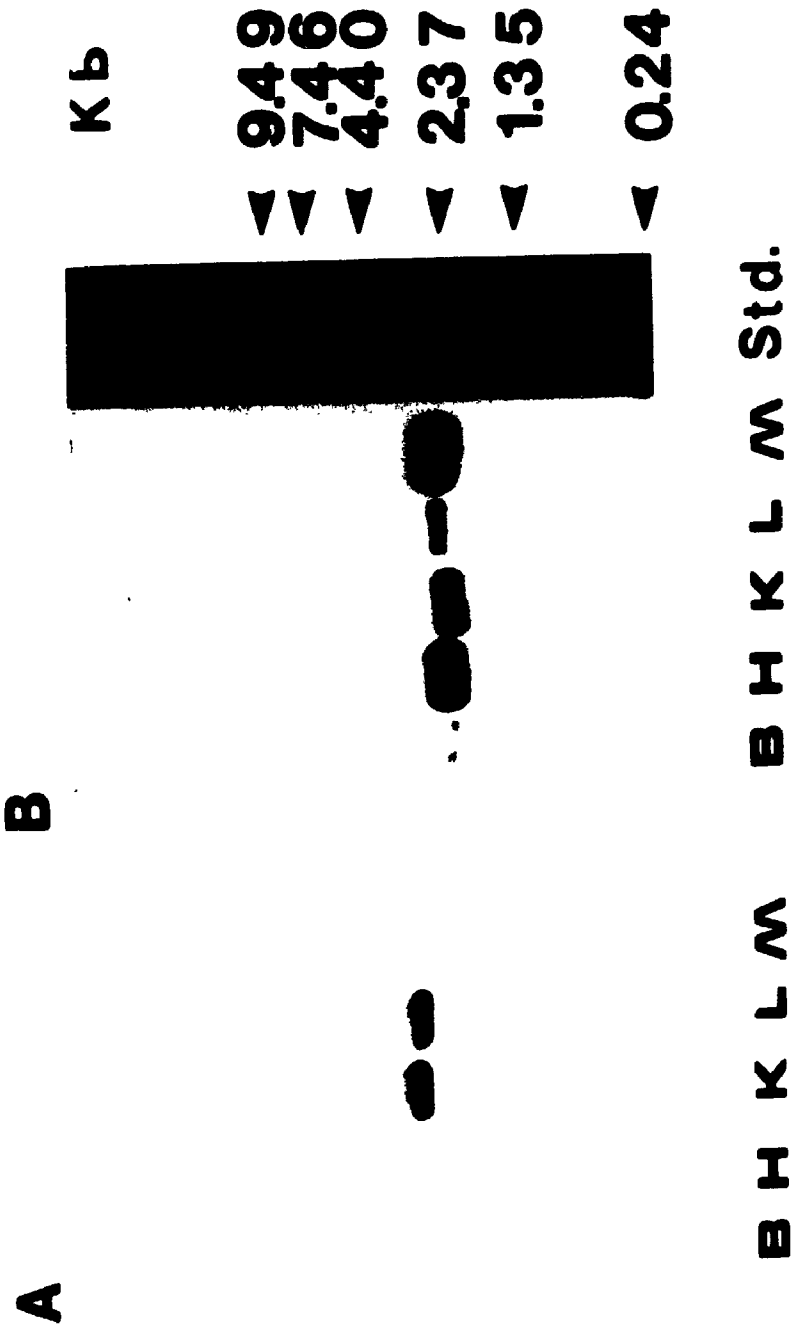
Southern blots of enzyme digested *Rana catesbeiana* genomic DNA which were hybridized with two different RcC/EBP-2 cDNA probes [a 847 bp *Eco*R I/*Sma* I fragment (nt 1-847 in Figure 24) and a 482 bp *Sac* I/*Sma* I fragment (nt 365-847 in Figure 24)] reveal only single-band hybridizations (Figure 35). These results suggest that RcC/EBP-2, unlike RcC/EBP-1, appears to be a single copy gene sequence in the *Rana* genome.

4.3.5 Tissue Distribution of mRNA Transcripts Recognizing *Rana catesbeiana* C/EBP-1 and C/EBP-2 Sequences

The distribution of mRNA transcripts recognizing RcC/EBP-1 and RcC/EBP-2 was determined in various *Rana catesbeiana* tissues by Northern-blot hybridization. Total RNA was isolated from liver, kidney, brain, heart and skeletal muscle of adult frogs, and used for the Northern blot hybridization analyses shown in Figure 36. The results shown in panel A, using a DNA fragment specific for RcC/EBP-1, demonstrate that mRNA transcripts which recognize RcC/EBP-1 can be detected in adult liver and kidney. but not in adult brain, heart, or skeletal muscle. Hybridization of the same RNA blots with a DNA fragment specific for RcC/EBP-2 (Figure 36B), demonstrates that, although each of these adult tissues contain RNA transcripts which recognize RcC/EBP-2, the transcripts are most abundant in heart and skeletal muscle. The mRNA transcripts recognizing RcC/EBP-1 and RcC/EBP-2 are approximately 3.4 and 2.4 kilobases

Figure 36. Northern blot hybridization analyses of the levels of the RcC/EBP-1 and RcC/EBP-2 mRNA transcripts in various tissues from *Rana catesbeiana* frogs.

Total RNA (10μg), isolated from the brain (B), heart (H), kidney (K), liver (L), and skeletal muscle (M), was electrophoretically separated on a 1.0% formaldehyde-agarose gel. The RNA was transferred to a Zetaprobe membrane and hybridized (see Materials and Methods) with [α-^{32}P]-labelled DNA fragments specific for RcC/EBP-1 **(panel A)** and RcC/EBP-2 **(panel B)**. The relative molecular size (kb; kilobases) of mRNA transcripts corresponding to RcC/EBP-1 (~3.4 kb) and RcC/EBP-2 (~2.4 kb) were determined from the relative mobility of the concurrently separated, ethidium bromide-stained RNA standards (Std.) shown on the right side of panel B.


Kb
9.49
7.46
4.40
2.37
1.35
0.24
A
B
B H K L M
B H K L M Std.

(kb) in size, respectively. Although the size of RcCEBP-1 mRNA transcripts is compatible with the size of C/EBPα mRNAs (~2.7kb; Landschulz *et al.*, 1988a), the mRNA transcripts hybridizing with RcC/EBP-2 are approximately twice the size of human and rat C/EBPδ mRNAs (Kinoshita *et al.*, 1992; Cao *et al.*, 1991). Results from the Northern-blot hybridizations were confirmed by *in situ* hybridization (not shown) of sections from some of the same adult tissues used for preparing the RNAs for the Northern blots.

4.3.6 Accumulation of *Rana* C/EBP mRNAs in *Rana catesbeiana* Tadpole Liver During TH-Induced and Spontaneous Metamorphosis

Autoradiograms from dot-blot hybridization analyses of RNA isolated from the liver of tadpoles at various stages of spontaneous metamorphosis (Figure 37A) reveal that the relative level of mRNA transcripts encoding RcC/EBP-2 do not change during spontaneous metamorphosis, while those encoding RcC/EBP-1 begin to accumulate in liver tissue after stage XV, reach maximal levels at the beginning of metamorphic climax (stage XX), and decrease after stage XXII. The observation that the temporal accumulation of RcC/EBP-1 mRNAs is similar to that detected for endogenous TH (Galton and St. Germain, 1985) as well as for mRNAs encoding the thyroid hormone receptor protein, TRβ, and some of the liver-specific urea cycle enzymes (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Chen *et al.*, 1994; Helbing and Atkinson, 1994), including, as shown in

Figure 37A, carbamyl phosphate synthetase-1 (CPS-1), implicates a role for TH in the up-regulation of RcC/EBP-1 mRNAs.

Autoradiograms from dot blot hybridization analyses of RNA isolated from the liver of stage VI tadpoles at various times after TH administration (Figure 37B) demonstrate that the first detectable accumulation of a RcC/EBP-1 mRNA transcripts occurs by 2 h after TH treatment of the tadpole. Although no change in the level of RcC/EBP-2 mRNAs is evident in the liver of tadpoles treated with TH, an accumulation of mRNAs encoding one of the urea cycle enzymes, CPS-1, is detectable, as shown here and elsewhere (Helbing *et al.*, 1992), between 12 and 24 h after TH treatment. The relative levels of liver cytoskeletal actin mRNAs appear to remain constant at all times after TH treatment of the tadpole.

The fact that an accumulation of liver RcC/EBP-1 mRNAs occurs soon after TH-treatment of the tadpole and that its accumulation precedes, by 6 to 12 h, the accumulation of mRNAs encoding proteins associated with an adult liver phenotype, such as CPS-1 mRNAs, caused us to speculate about the role(s) that RcC/EBP-1 might be playing during this 12 h lag period. We questioned whether this 12 h lag period might represent a period of time required for transcription factors, such as RcC/EBP-1 and/or the TRs, to either terminally differentiate and reprogram gene expression in the resident hepatocytes or, as some investigators have suggested (Smith-Gill and Carver, 1981), promote the proliferation of a population of stem cells exclusively responsible for expressing mRNAs encoding adult proteins, such as the CPS-1 mRNAs. We assessed these possibilities by *in situ* hybridization studies using

Figure 37. Dot-blot hybridization analyses of the levels of the RcC/EBP-1 and RcC/EBP-2 mRNA transcripts in liver from *Rana catesbeiana* tadpole undergoing **(A)** spontaneous and **(B)** T_3-induced metamorphosis.

Total RNA was isolated from the liver of tadpoles at various stages of metamorphosis (Taylor and Kollros, 1946), and from the liver of stage VI tadpoles at various times after TH-treatment of the tadpoles. Aliquots (10 μg) of the RNA were dot-blotted to a Zetaprobe membrane and hybridized with [α-^{32}P]-labelled DNA fragments specific for RcC/EBP-1, RcC/EBP-2, CPS-1 and cytoskeletal actin. The hybridization conditions and probes used are described in Materials and Methods.

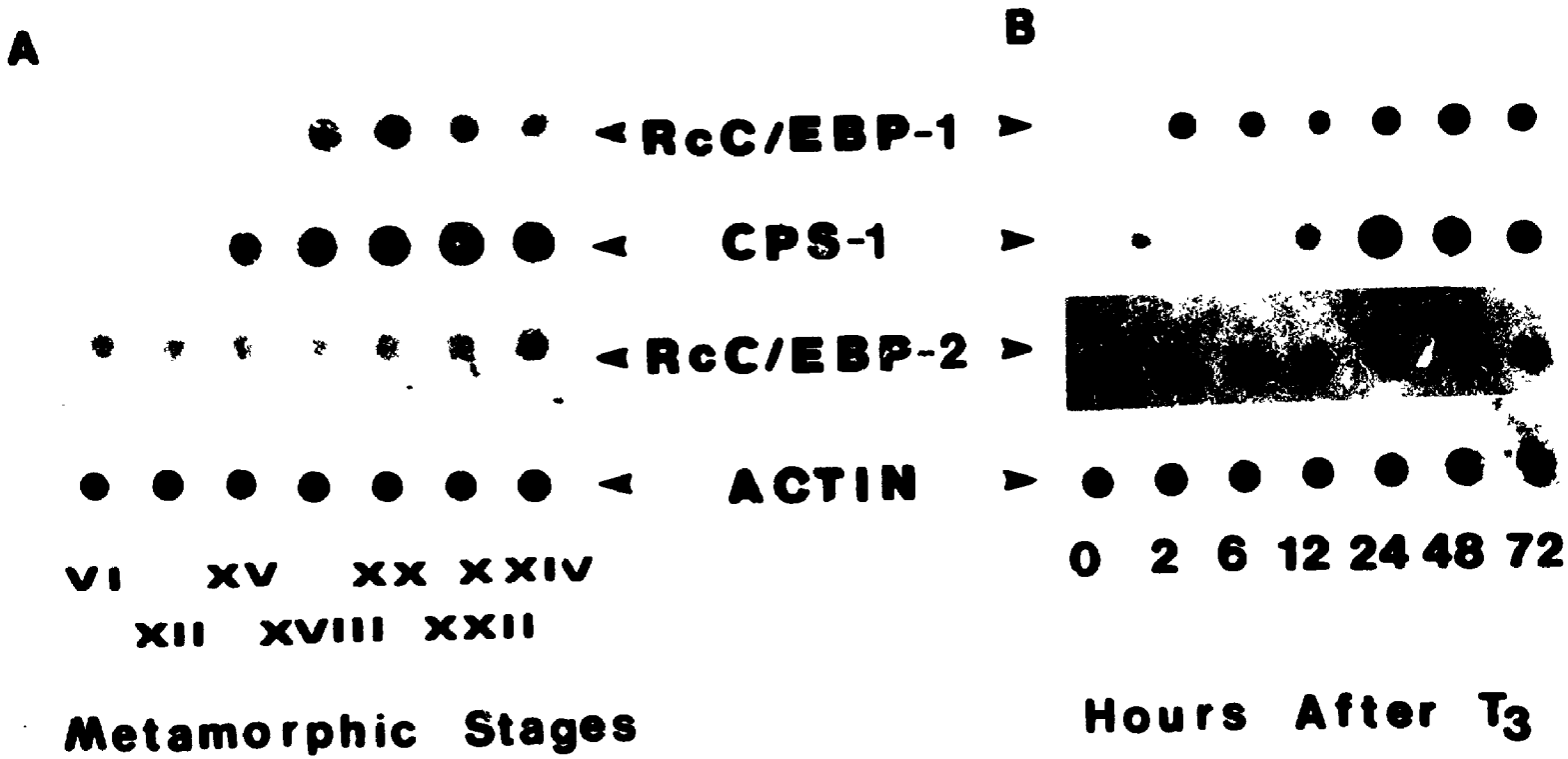

A
B
RcC/EBP-1
CPS-1
RcC/EBP-2
ACTIN
VI XV XX XXIV
XII XVIII XXII
Metamorphic Stages
0 2 6 12 24 48 72
Hours After T3

Figure 38. *In situ* hybridization analyses of CPS-1 **(A)** and RcC/EBP-1 **(B)** mRNA accumulation in the liver of *Rana catesbeiana* tadpoles (stage VI) which had not been exposed to TH (CON), or had been treated with 3,5,3'-triiodothyronine 15 (T_3 -15), 24 (T_3 -24), or 48 (T_3 -48) h earlier.

Serial sections of liver tissue were hybridized with antisense and sense DIG-labelled RNA probes (see Materials and Methods). No hybridization was evident in the sections treated with the sense DIG-labelled RNA probes or in RNase A-treated sections (not shown). Arrows in the first panel of A indicate pigment granules, and arrowheads in subsequent panels denote the central vein in a liver lobule. The clear circles are unstained nuclei and the unstained area between the cords of liver cells are the hepatic sinusoids. Magnification is the same in all photographs and the bar represents 10 μm.

antisense RNAs directed against RcC/EBP-1 and CPS-1 mRNA transcripts (Helbing *et al.*, 1992; Chen *et al.*, 1994 Helbing and Atkinson, 1994). We reasoned that if the stem cell proliferation theory is correct, then *in stiu* hybridization analyses should detect, in some cases, the expression of CPS-1 transcripts in discrete populations of liver cells, particularly in the 12 to 24 h period after TH treatment of the tadpole. If, on the other hand, the reprogramming theory is correct, we presumed that CPS-1 mRNA transcripts should be detectable in all of the liver parenchymal cells (hepatocytes). The results, shown in Figure 38, support the "terminal differentiation and reprogramming" hypothesis by demonstrating that the expression of CPS-1 mRNA transcript is not confined to discrete populations of hepatocytes, but, like the RcC/EBP-1 mRNAs, is discernible in all of the hepatocytes present in the liver of tadpoles treated 15, 24, or 48 h earlier with TH.

4.4 Discussion

In an effort to elucidate the means by which TH influences the liver of *Rana catesbeiana* tadpoles to express tissue-specific genes which are characteristic of an adult liver phenotype, such as the urea cycle enzyme genes, I explored the possibility that TH, in combination with its receptor protein, might initially affect the expression of genes encoding specific transcription factors. Since the CCAAT/enhancer binding proteins (C/EBPs) are transcription factors which have been implicated in both the terminal differentiation of mammalian hepatocytes and liver-specific gene expression (Umek *et*

al., 1991; Friedman *et al.*, 1989; Sladek and Darnell, 1992), and since putative binding elements for them are present in the promoter regions of both the CPS-1 and OTC genes (see Chapter 3), I reasoned that one or more of the regulatory proteins/transcription factors required for the TH-influenced terminal differentiation of this tadpole's hepatocytes might be C/EBP-related. Guided by this notion, I isolated and characterized cDNAs encoding *Rana* homologues of the C/EBPs. The proteins encoded by these *Rana* cDNAs, termed RcC/EBP-1 and RcC/EBP-2, have relative molecular masses of approximately 33,000 and 31,000, respectively. While the amino-terminal two-thirds of these proteins are largely unrelated at the level of primary amino acid sequence, the carboxy-terminal third of these proteins are highly conserved and each protein contains a bZIP DNA-binding domain similar to ones reported for particular isoforms of the C/EBPs (Vinson *et al.*, 1989; Landschulz *et al.*, 1988a; Cao *et al.*, 1991). A comparison of the deduced amino acid sequence in the bZIP domain of the proteins encoded from these *Rana* cDNAs, with the bZIP sequences reported for the various isoforms of c/EBPs from other organisms (Landschulz *et al.*, 1988a; Descombes *et al.*, 1990; Poli *et al.*, 1990; Cao *et al.*, 1991; Christy *et al.*, 1991; Williams, Cantwell and Johnson, 1991; Calkhoven *et al.*, 1992; Xu and Tata, 1992; Kinoshita *et al.*, 1992; Burk *et al.*, 1993; Lincoln *et al.*, 1994), suggests that the protein encoded from RcC/EBP-1 is the *Rana* homologue of C/EBPα and that the protein encoded from RcC/EBP-2 is the *Rana* homologue of C/EBPδ.

The relative distribution of RcC/EBP-1 and -2 mRNAs in adult *Rana catesbeiana* tissues was determined by Northern blot

hybridization analyses and confirmed by *in situ* hybridizations (not shown). Among the tissues assessed (brain, heart, liver, kidney, and skeletal muscle), mRNAs encoding RcC/EBP-1 were present in the liver and kidney but not detectable in brain, heart, or skeletal muscle, whereas mRNAs encoding RcC/EBP-2 were present in all of the tissues examined but, interestingly, most abundant in heart and skeletal muscle. The limited distribution of the RcC/EBP-1 mRNAs in the adult tissues of this frog corresponds well with the tissue-distribution of mRNAs encoding the C/EBPα isoforms in other organisms (Landschulz *et al.*, 1988a; Birkenmeier *et al.*, 1989; Xu and Tata, 1992).

The relative levels of both RcC/EBP-1 and -2 mRNAs were also assessed in the liver of tadpoles at various stages of spontaneous metamorphosis. The results of these studies disclosed that while the level of liver RcC/EBP-2 mRNA transcripts remained relatively constant during metamorphosis, the level of liver RcC/EBP-1 mRNAs changed in a manner corresponding with changes in both the endogenous level of TH (Galton and St. Germain, 1985) and the level of liver TRβ mRNAs (Helbing *et al.*, 1992; Atkinson *et al.*, 1994). These observations, coupled with the fact that mRNAs encoding the liver-specific urea cycle enzymes appear to accumulate in the tadpole liver at approximately the same time as RcC/EBP-1 mRNA levels increase, support the conjecture that TH, directly or indirectly, influences the expression of gene(s) encoding this particular C/EBPα-related transcription factor.

In order to more fully appraise the possibility that TH influences the expression of the gene(s) encoding RcC/EBP-1, I

administered TH to stage VI tadpoles and, subsequently, determined the RcC/EBP-1 and -2 mRNA levels in the liver of these tadpoles. Results from these studies demonstrate that RcC/EBP-1 mRNAs accumulate in the liver of this tadpole within 2 h after the administration of TH. While the relative level of liver RcC/EBP-1 mRNAs continues to increase and remain high for at least 72 h after TH treatment, no changes in the relative level of RcC/EBP-2 mRNAs are detectable. The rapid and sustained up-regulation of RcC/EBP-1 mRNAs in the liver of TH-induced tadpoles coincides with the TH-induced up-regulation of liver TRβ mRNAs, and precedes, by 6 to 12 h, the TH-induced up-regulation of CPS-1 and other liver-specific urea cycle enzyme mRNAs (Helbing *et al.*, 1992; Helbing, 1993; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994).

The 6 to 12 h time difference between the up-regulation of RcC/EBP-1 mRNAs and the urea cycle enzyme mRNAs, such as CPS-1 and OTC, prompted us to evaluate whether this lag period represents time required for the proliferation of stem cells expressing genes characteristic of the adult phenotype (Smith-Gill and Carver, 1981) or for terminally differentiating and reprogramming gene expression in the resident hepatocytes. Results from *in situ* hybridizations, using probes specific for RcC/EBP-1 and CPS-1 mRNAs, support the "terminal differentiation and reprogramming" hypothesis. If, in fact, this is the case, then this *Rana* homologue of C/EBPα might have both the anti-mitotic and transcriptional regulatory properties of the mammalian C/EBPα transcription factor (Umek *et al.*, 1991; Vasseur-Cognet and Lane, 1993). Assuming that the TH-induction of RcC/EBP-1 mRNAs would be reflected in that of its protein product, it seems

reasonable to suggest that the product from the TH-induced expression of RcC/EBP-1 mRNA may play an early role, perhaps in concert with a thyroid hormone receptor protein or other transcription factors, in terminally differentiating and/or coordinating the reprogramming of gene expression in this tadpole's liver cells during both spontaneous and TH-induced metamorphosis.

In an attempt to evaluate the possibility that thyroid hormone directly effects the expression of the gene encoding RcC/EBP-1, I isolated and partially characterized a ~7 kb genomic sequence containing the 5'-region flanking the transcription start site and a portion (417 nt) of the transcribed gene. Primer extension analyses were used to demonstrate the transcription start site of this gene and sequence analyses disclosed that this gene, unlike its presumed mammalian homologues (Cao *et al.*, 1991; Williams, Cantwell and Johnson, 1991), contains at least one intron, beginning 417 nt after the transcription start site. Southern blot hybridization analyses, using a 234 bp *Nco* I/*Nco* I fragment from the first exon of this gene, revealed that this gene, unlike RcC/EBP-2, appears to be present in multiple copies in the *Rana catesbeiana* genome.

A search for transcriptional regulatory elements in the region of this gene upstream from the transcribed portion of it, revealed the presence of a possible TATA box at -18, a well-conserved TATA box at -53, a number of GC-rich areas, a well-conserved HSE at -142 (Amin *et al.*, 1988; Xiao and Lis, 1988), and an element presumed to be a C/EBP binding site (Graves *et al.*, 1986; Ryden and Beemon, 1989; Vinson *et al.*, 1989). While the presence of C/EBP binding elements in the regulatory region of the RcC/EBP-1

gene suggests that this gene, similar to the mouse C/EBPα (Legraverend *et al.*, 1993), is autoregulatory, the absence of conserved consensus sequences for TREs, at least in the first 545 nt flanking the 5' portion of the transcribed region of this gene, suggest that the expression of this gene is not directly influenced by thyroid hormone. However, since both our understanding of DNA binding elements and how well they are conserved in different organisms, specifically TREs, are still in their infancy, the suggestion that thyroid hormone may not directly influence the expression of the RcC/EBP-1 gene is inconclusive. Indeed, the early thyroid hormone-induced upregulation of the mRNAs encoded from this gene certainly give credence to the idea that thyroid hormone is directly influencing the expression of this gene. Moreover, since more than one copy of this gene appears to be present in the *Rana* genome (see Figure 34), another member of the RcC/EBP-1 gene family may contain TREs in its promoter and, in fact, be responsible for the elevated levels of RcC/EBP-1 mRNAs detected in the liver of T_3-treated tadpoles. Whatever the case, the results presented in this chapter implicate the product of the RcC/EBP-1 gene in the hormone-induced transitions occurring in the liver of this amphibian.

CHAPTER 5 THE PROTEIN SYNTHESIZED FROM THE RcC/EBP-1 GENE BINDS TO DNA SEQUENCES FOUND IN THE PROMOTER REGIONS OF THE *RANA* OTC AND CPS-1 GENES

5.1 Introduction

The presence of putative C/EBP binding elements in the promoter regions of *Rana catesbeiana* CPS-1 and OTC genes (see Chapter 3), coupled with the demonstration that the thyroid-hormone induced up-regulation of RcC/EBP-1 mRNAs is apparent well before the up-regulation of the mRNAs encoding the urea cycle enzymes CPS-1 and OTC (see chapter 4; Chen *et al.*, 1994; Atkinson *et al.*, 1994; Helbing *et al.*, 1992), support my contention that the thyroid hormone-induced expression of the RcC/EBP-1 mRNAs may be required for the up-regulation of CPS-1 and OTC mRNAs. In this chapter, I will demonstrate that the protein synthesized from the RcC/EBP-1 gene binds to DNA sequences in the promoter regions of both the *Rana* OTC and CPS-1 genes.

5.2 Materials and Methods

5.2.1 *In vitro* Trar scription and Translation of the RcC/EBP-1 and RcC/EBP-2 cDNAs

In order to translate the information encoded by RcC/EBP-1 in a cell-free system, I subcloned a *Eco*R I/*Hinc* II (nt 1-1,503) fragment of it into the *Eco*R I/*Eco*R V sites of pBluescrip! II SK-

(designated as RcC/EBP-1-ORF). Both the subcloned RcC/EBP-1-ORF and the RcC/EBP-2 plasmid DNAs were linearized by cutting them at the *Xho* I site downstream from the 3' end of their DNA inserts, and RNAs were transcribed from the T_3 promoter of the pBluescript SK$^-$ vector. The transcription reactions were performed at 37°C for 30 minutes using the Stratagene mCAP RNA capping kit. Approximately 0.5 mg of transcribed RNA was mixed with a rabbit reticulocyte lysate translation system (Dupont Canada) containing [^{35}S]-methionine (Dupont Canada) and incubated at 37°C for 60 min. The [^{35}S]-labelled translation products were electrophoretically separated on 7.5-17.5% SDS-polyacrylamide gradient gels (Laemmli, 1970) and fluorograms were prepared from them as described elsewhere (Helbing *et al.*, 1992).

5.2.2 Production and Purification of the Recombinant RcC/EBP-1 Protein

A 1412 nt *Nco* I/ *Xho* I fragment from RcC/EBP-1 ORF (nt 92-1504 in Figure 22) was cloned into the *Bam*H I/*Xho* I sites of a pTrcHis A vector, a pUC-derived expression vector designed for efficient protein expression and purification from genes cloned in *E. coli* (Invitrogen Co., San Diego, CA), and designated as pTrcHisC/EBP-1. In order to have the pTrcHisC/EBP-1 in the open reading frame, the *Nco* I end of RcC/EBP-1 ORF and the *BamH* I end of the digested pTrcHis A vector were end-filled by the Klenow fragment (Pharmacia Inc.). A 21 nt pTrcHis forward sequencing primer, 5'-GAGGTATATATTAATGTATCG-3' (Invitrogen Co.), was used to

sequence the pTrcHisC/EBP-1 to confirm that it was in frame. The final recombinant RcC/EBP-1 protein is a 328 amino acid fusion protein which contains 292 amino acids encoded by RcC/EBP-1 cDNA plus 36 N-terminal amino acids, including six tandem histidine residues from the pTrcHis vector.

The expression and purification of recombinant RcC/EBP-1 protein was done according to the protocol recommended from the company (Invitrogen Co.). Briefly, a 1.0 ml aliquot of an overnight culture of *E. coli* cells, transformed with pTrcHisC/EBP-1, was inoculated into 100 ml of SOB media containing 50 mg/ml ampicillin and incubated at 37°C. When the O. D. value of the growing culture reached 0.3, IPTG was added to a final concentration of 1 mM and the cultures were incubated for an additional 5-6 hours. The cells were lysed by lysozyme and the recombinant RcC/EBP-1 protein was purified from the *E. coli* cell lysates by a one-step procedure using Probond resin columns (Invitrogen Co.; a column which has a high affinity for histidine residues) according to the protocol recommended by the company. The concentration of recombinant RcC/EBP-1 was determined by the TCA-turbidity method of Comings and Tack (1972).

Samples of the purified recombinant RcC/EBP-1 proteins were coelectrophoresed with the *E. coli* cell lysate and with low molecular weight protein standards (Pharmacia Inc., Piscataway, NJ) on a 7.5 - 17.5% SDS-polyacrylamide gradient separating gel overlaid with a 4% polyacrylamide stacking gel. Electrophoresis was performed using a BioRad mini-protein vertical gel apparatus at 50 voltage (V) until the bromophenol blue entered the separating gel

upon which the voltage was turned up to 150 V and the gel was run until the bromophenol blue ran off the gel.

The gel was stained overnight in 0.2% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid. The gel was destained for 2 h with 50% methanol and 10% acetic acid, and subsequently destained in 10% methanol and 10% acetic acid until the gel background was clear. The gel was photographed with transillumination using a green #11 filter (Eastman Kodak Co., Rochester, NY).

5.2.3 Gel Mobility-Shift Analyses

To assess the ability of the proteins generated from the *Rana* RcC/EBP-1 and RcC/EBP-2 to bind to established, mammalian C/EBP-DNA-binding elements, a 23-mer oligonucleotide and its complementary sequence were synthesized (Vetrogen Inc.). This particular oligomer, 5'-TCTCTTAGATTGCGCAATCTGCC-3', contained a consensus nucleotide motif for C/EBP binding described by Ryden and Beemon [1989; namely, 5'-T(T/G)NNG(C/T)AA(T/G)-3'] as well as one described by Graves *et al.* (1986; namely, 5-GCAAT-3'). In addition, 25-mers and their complementary sequences were designed to exactly match sequences in the regulatory regions of the *Rana* OTC and CPS-1 genes which share consensus with the mammalian C/EBP binding elements. One of these oligomers, 5'-GGTAATTATTACACAAGACAACTGA-3' (nt -98 to -74 in Figure 16), contains the C/EBP binding element motif corresponding to 5'-MTTRCNNMA-3' (Xu and Tata, 1992), and the other, 5'-AATCTATGTTGCAACCAAGTTACAC-3' (nt -208 to -184 in Figure 19),

contains motifs corresponding to both the 5'-GTTGCAAC-3' (Howell *et al.*, 1989; Lagace *et al.*, 1992) and 5'-T(T/G)NNG(C/T)AA(T/G)-3' (Ryden and Beemon,1989) C/EBP binding motifs. Finally, a nonspecific oligonucleotide and its complementary sequence, containing two heat shock element consensus sequences (5'-AGAACGTTCTAGAACATTCGC-3'; Perisic *et al.*, 1989), was used in the gel mobility-shift assays as an unrelated competitor for the C/EBP binding-element sequences. To conduct the gel mobility-shift analyses, I annealed the oligonucleotides designed to contain the consensus or predicted C/EBP binding elements to their complementary sequences and used them as means (probes) to assess the ability of the proteins generated from the *Rana* RcC/EBP-1 and the *Rana* RcC/EBP-2 to recognize these sequences. In each case, the 5'-terminal end of these probes were radiolabelled, using a T4 polynucleotide kinase (Pharmacia Inc.), with [γ-^{32}P] ATP (Dupont Canada, specific activity 3,000 Ci/mmol).

The reaction mixture (20 µl) for each gel mobility-shift assay contained 25 mM Hepes (pH 7.8), 10% (v/v) glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) NP40, 0.2 µg bovine serum albumin, and 200 µg of poly(dI-dC)poly(dI-dC) with or without the unlabelled competitor oligonucleotides. Either a 100 ng aliquot of purified recombinant RcC/EBP-1 protein (see section 5.2.2), or an aliquot of the translation products (2 µl from 50 µl of the translation reaction) obtained from cell-free transcriptions/translations of RcC/EBP-1 or RcC/EBP-2, was added to the reaction mixtures. After 20 min at room temperature, 10^4 cpm of the ^{32}P-labelled DNA probe (approximately 1 ng of

probe) was added to each reaction and they were incubated for another 20 min at room temperature. After the last incubation, the reaction mixtures were loaded onto a 6% polyacrylamide gel containing 50 mM Tris base, 380 mM glycine, 2 mM EDTA, and 5% glycerol (pH 8.5). Gels were electrophoresed at a constant rate of 30 mA at 4°C for 2-3 h, dried onto Whatman 3MM filter paper with a BioRad Model 443 Slab Dryer, and exposed to Kodak XR-Omat RP film (Eastman Kodak Co.) without an intensifying screen. The X-ray film was processed using Kodak GBX developer and fixer.

5.3 Results

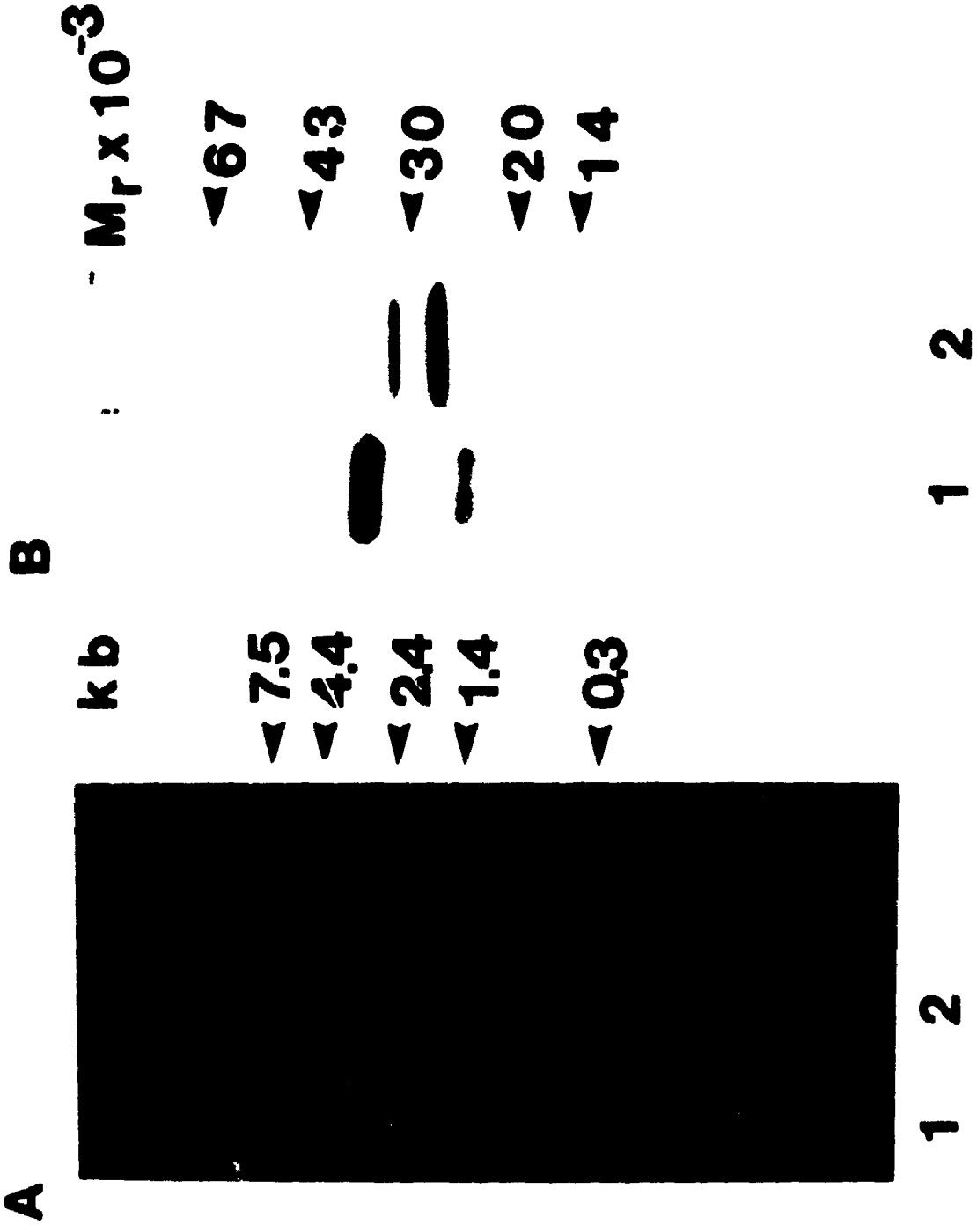
5.3.1 *In vitro* Transcription and Translation Analyses of the Proteins Encoded from RcC/EBP-1 and RcC/EBP-2 cDNAs

The deduced relative masses (M_r) of the proteins encoded from RcC/EBP-1 and RcC/EBP-2 cDNAs were confirmed by *in vitro* transcription and translation analyses. Figure 39A shows an ethidium bromide-stained gel of electrophoretically separated RNAs transcribed from a 1,503 bp subclone of RcC/EBP-1 (see Materials and Methods) and from the RcC/EBP-2 cDNA. Figure 39B shows a fluorogram from a one-dimensional, SDS-polyacrylamide gel electrophoretic separation of the [^{35}S]-methionine-labelled proteins translated from the RNAs transcribed from the RcC/EBP-1 subclone and RcC/EBP-2 cDNAs. The fluorogram of the electrophoretically separated proteins demonstrates that although each cDNA produces a protein with an M_r corresponding in size to one predicted from its cDNA sequence (M_r=33,088 for RcC/EBP-1, and M_r=31,215 for

Figure 39. *In vitro* transcription and translation of RcC/EBP-1 and RcC/EBP-2.

A: Ethidium bromide-stained gels of electrophoretically separated RNAs transcribed from a 1,053 bp subclone of RcC/EBP-1, designated RcC/EBP-1 ORF **(Lane 1)** and RcC/EBP-2 **(Lane 2)**. the relative mobility and size (kb) of concurrently separated standards are shown on the right side of this panel.

B: Fluorograms of the electrophoretically separated, [^{35}S]-methionine-labelled proteins translated from the RNAs transcribed from RcC/EBP-1 ORF **(Lane 1)** and RcC/EBP-2 **(Lane 2)**. The mobility and relative molecular mass (M_r) of standard proteins (Pharmacia Inc., Piscataway, NJ) applied to the gel is shown on the right side of this panel.


A
1 2
kb
7.5
4.4
2.4
1.4
0.3
B
1 2
$M_r \times 10^{-3}$
67
43
30
20
14

RcC/EBP-2) a truncated form of each protein is also present. While truncated forms of C/EBP have been detected in preparations of rat liver (Landschulz *et al.*, 1988a) and adipocytes (Birkenmeier *et al.*, 1989), it is possible that in this cell-free system their synthesis results from the use of in-frame, downstream AUGs as translation start codons (Tahara *et al.*, 1991).

5.3.2 Binding of the Proteins Produced by *in vitro* Transcription and Translation of the *Rana* RcC/EBP-1 and RcC/EBP-2 cDNAs to a Consensus Sequence for C/EBP Binding

Gel electrophoretic mobility shift assays were used to determine if the proteins produced by *in vitro* transcription and translation of the RcC/EBP-1 and RcC/EBP-2 cDNAs would bind to an oligonucleotide consensus sequence shown to be a binding site for C/EBP-like proteins (see Materials and Methods for details). The results, shown in Figure 40, demonstrate that the RcC/EBP-1 and RcC/EBP-2 proteins bind with high affinity to the consensus binding site for C/EBP-like proteins (Vinson *et al.*, 1989; Ryden and Beemon, 1989). The specificity of the binding of these proteins to this sequence was confirmed by competition with increasing amounts of either the same (Figure 40, lanes 5-7) or different (Figure 40, lanes 8-10) unlabelled oligonucleotide sequences.

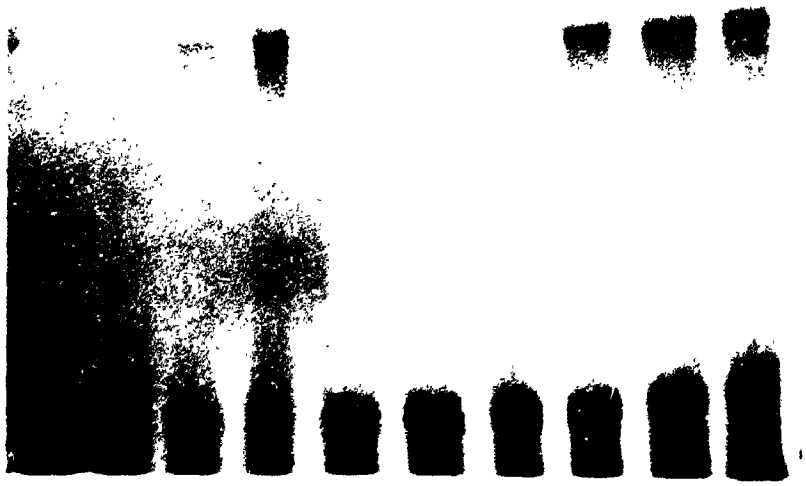
Figure 40. Specific binding of RcC/EBP-1 **(A)** and RcC/EBP-2 **(B)** proteins to the consensus nucleotide binding site for C/EBP-like proteins.

Gel electrophoretic mobility shift analyses (see Materials and Methods) were carried out using 0.1 to 2.0 µl aliquots of the translation mixture from the in vitro transcription and translation of RcC/EBP-1 or -2, and a [^{32}P]-labelled C/EBP consensus binding site oligonucleotide probe (10^4 cpm or ~1.0 ng of oligonucleotide) with or without unlabelled competitor oligonucleotides. **Lane 1**: 2.0 µl of translation mixture to which no mRNA had been added. **Lanes 2-4**: 0.1, 1.0, and 2.0 µl translation mixture containing the synthesized proteins. **Lanes 5-7**: 2.0 µl translation mixture containing the synthesized proteins and 0.5, 5.0, and 50 ng of the unlabelled C/EBP-binding site oligonucleotide. **Lanes 8-10**: 2.0 µl translation mixture containing the synthesized proteins and 0.5, 5.0, and 50 ng of an unlabelled oligonucleotide which contains a consensus sequence for the heat shock element. The [^{32}P]-labelled probe, to which nothing had been added, had the same mobility (not show) as the sample shown in lane 1.

A

1 2 3 4 5 6 7 8 9 10

B



1 2 3 4 5 6 7 8 9 10

5.3.3 Binding of the Recombinant RcC/EBP-1 Protein Produced by *E. coli* to the Putative C/EBP Binding Consensus Sequence in the *Rana* OTC and CPS-1 Gene Promoter Regions

I cloned the RcC/EBP-1 ORF into a pTrcHis expression vector, which allowed the production of large amounts of purified recombinant RcC/EBP-1 protein (~1 mg purified protein per 100 ml culture media). As demonstrated in Figure 41, the purification of this recombinant protein from *E. coli* lysates is only a one-step procedure when using a Probond resin column (Invitrogen Co., San Diego, CA); this column has high affinity binding to the histidine residues incorporated into the amino terminal end of this recombinant protein.

The purified recombinant RcC/EBP-1 protein produced by the *E. coli* cells can bind to the synthesized consensus binding site for C/EBP-like proteins (Figure 42). The specificity of the binding of the purified recombinant RcC/EBP-1 protein to this sequence was confirmed by competition analyses with increasing amounts of either the same (lanes 3-5 in Figure 42) or different (lanes 6-8 in Figure 42) unlabelled oligonucleotide sequences.

I also designed two 25-mers and their complementary sequences to match sequences in the regulatory regions of *Rana* OTC (nt -98 to -74 in Figure 16) and CPS-1 (nt -208 to -184 in Figure 19) genes which share consensus with mammalian C/EBP binding elements (see Materials and Methods). As demonstrated in lane 2 of

Figure 41. One-dimensional SDS-PAGE separation of the recombinant RcC/EBP-1 protein produced by *E. coli* cells.

RcC/EBP-1 was cloned into a pTrcHis vector which was transfected into and expressed by *E. coli* cells (see Materials and Methods for details). The *E. coli* cells were lysed with lysozyme and the recombinant RcC/EBP-1 protein was purified from the lysate using Probond resin columns (Invitrogen Co.). **Lane 2** shows the proteins in the *E. coli* cell lysate before purification of the recombinant RcC/EBP-1 protein. **Lanes 3 and 4** show the recombinant RcC/EBP-1 protein purified either once (lane 3) or twice (lane 4) by a Probond resin column. **Lanes 1 and 5** are coelectrophoretically separated standard proteins (Pharmacia Inc., Piscataway, NJ). The relative molecular mass (M_r) of the standard proteins is shown on the left side of this figure.

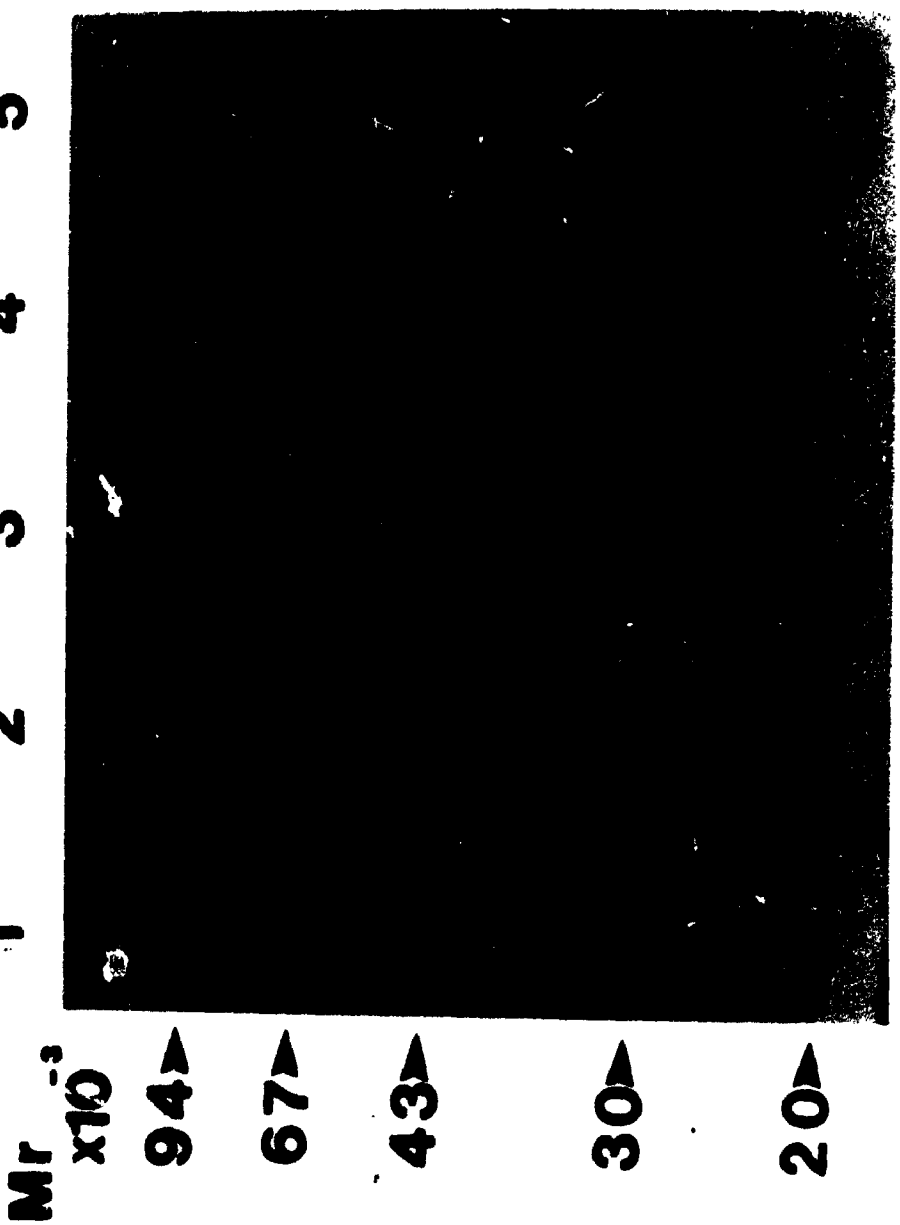

1
2
3
4
5
Mr x10⁻³
94
67
43
30
20

Figure 42. Specific binding of recombinant RcC/EBP-1 protein produced by *E. coli* to the consensus nucleotide binding site for C/EBP-like proteins.

Gel electrophoretic mobility shift analyses (see Materials and Methods) were carried out using 100 ng recombinant RcC/EBP-1 protein, and a [^{32}P]-labelled C/EBP consensus binding site oligonucleotide probe (10^4 cpm or ~1.0 ng of oligonucleotide) with or without unlabelled competitor oligonucleotides. **Lane 1**: 100 ng BSA. **Lane 2**: 100 ng recombinant RcC/EBP-1 protein. **Lanes 3-5**: 100 ng recombinant RcC/EBP-1 protein and 0.5, 5.0, and 50 ng of the unlabelled C/EBP-binding site oligonucleotide. **Lanes 6-8**: 100 ng recombinant RcC/EBP-1 protein and 0.5, 5.0, and 50 ng of an unlabelled oligonucleotide which contains a consensus sequence for the heat shock element.

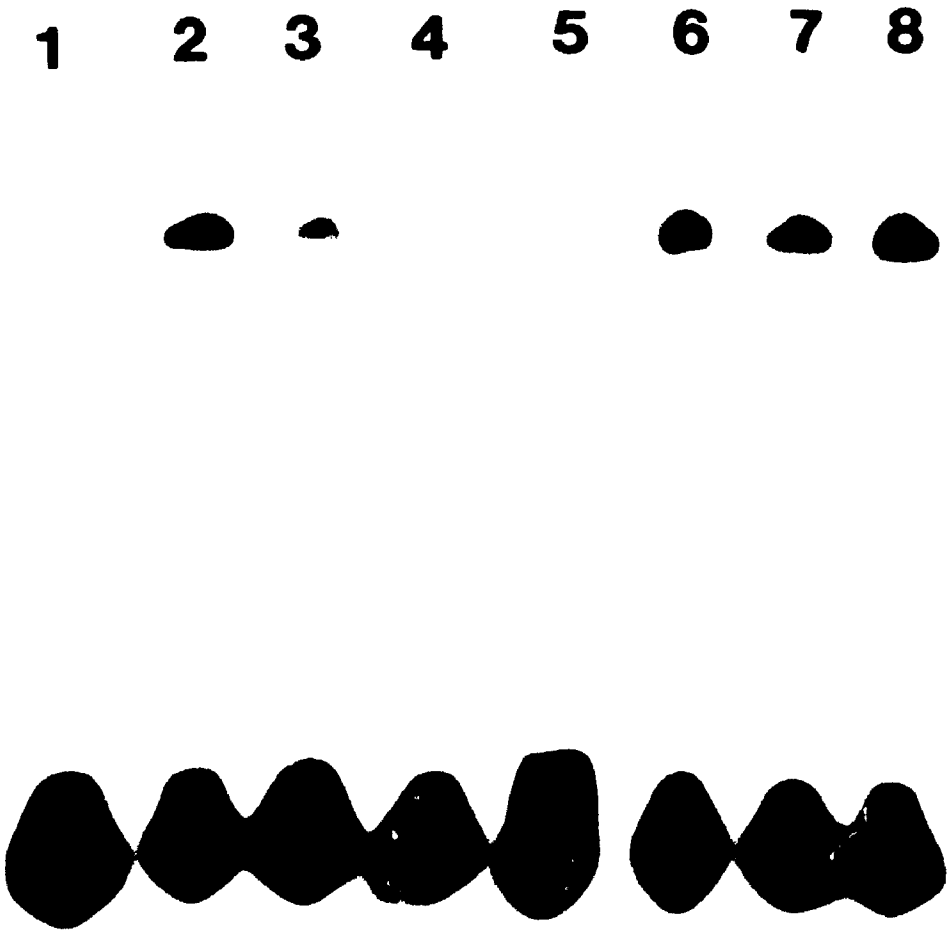
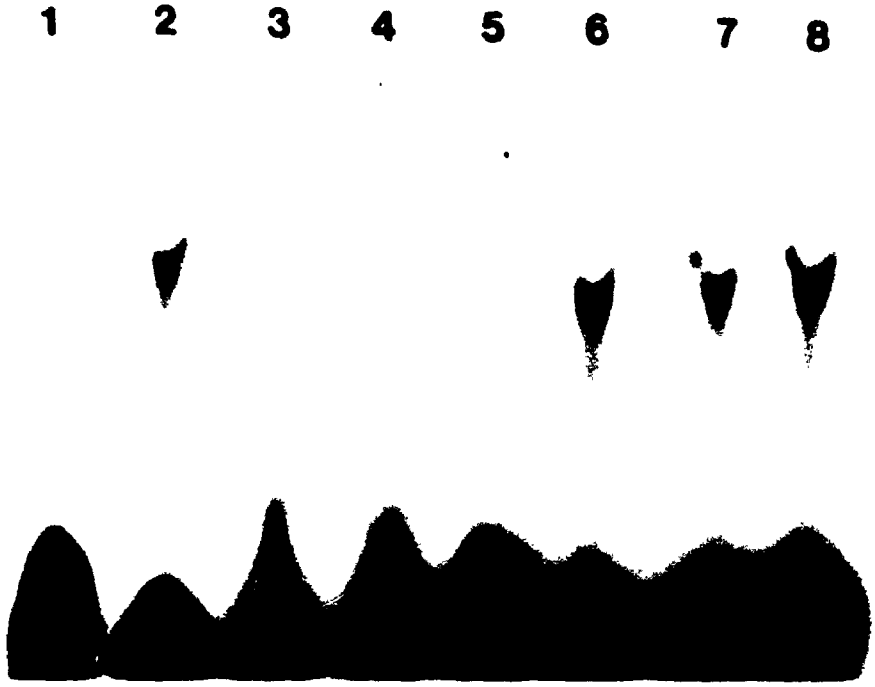

1
2
3
4
5
6
7
8

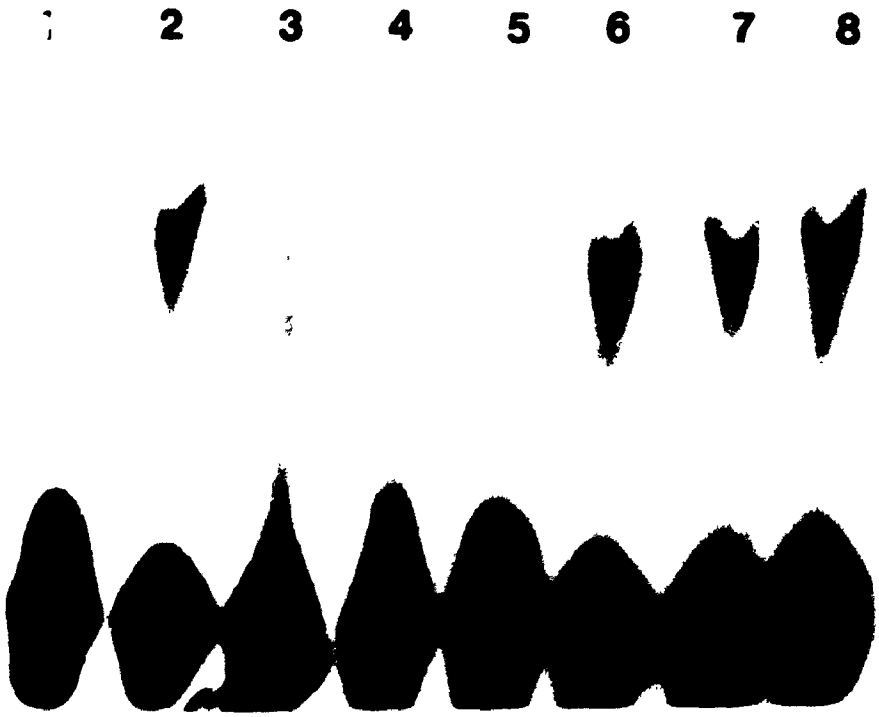
Figure 43. Specific binding of recombinant RcC/EBP-1 protein produced by *E. coli* to a C/EBP binding element from the OTC gene promoter region **(A)** and one from the CPS-1 gene promoter region **(B)**.

Gel electrophoretic mobility shift analyses (see Materials and Methods) were carried out using 100 ng recombinant RcC/EBP-1 protein and [^{32}P]-labelled C/EBP binding elements (10^4 cpm or ~1.0 ng of oligonucleotide), **(A)** 5'-GGTAATTATTACACAAGACAACTGA-3' (nt -98 to -74 in Figure 16) from the OTC gene promoter region or **(B)** 5'-AATCTATGTTGCAACCAAGTTACAC-3' (nt -208 to -184 in Figure 19) from the CPS-1 gene promoter region, with or without unlabelled competitor oligonucleotides. **Lanes 1:** 100 ng BSA. **Lanes 2:** 100 ng recombinant RcC/EBP-1 protein. **Lanes 3-5:** 100 ng recombinant RcC/EBP-1 protein and 0.5, 5.0, and 50 ng of unlabelled C/EBP-binding site oligonucleotide from the OTC gene promoter region **(panel A)** or from the CPS-1 gene promoter region **(panel B)**. **Lanes 6-8:** 100 ng recombinant RcC/EBP-1 protein and 0.5, 5.0, and 50 ng of an unlabelled oligonucleotide which contains a consensus sequence for the heat shock element.

A



B



Figures 43A and B, the purified recombinant RcC/EBP-1 protein can bind to these two different oligonucleotide sequences. The specificity of the binding was confirmed by competition analyses with increasing amounts of either the same (lanes 3-5 in Figure 43) or different (lanes 6-8 in Figure 43) unlabelled oligonucleotide sequences.

5.4 Discussion

The results described in this chapter initially demonstrate that the RcC/EBP-1 and RcC/EBP-2 cDNAs can be transcribed and translated in cell-free systems and can produce proteins which bind specifically to C/EBP consensus binding sites (Figure 40). Moreover, My results also demonstrate that a recombinant RcC/EBP-1 protein, produced in *E coli* cells transfected with a pTrcHis vector containing the RcC/EBP-1 encoding sequence, can also recognize and specifically bind to a C/EBP consensus binding site (Figure 42). These data, coupled with the computer analyses of the derived amino acid sequences of these proteins (see Chapter 4), indicate that RcC/EBP-1 and -2 are members of the CCAAT/enhancer binding protein (C/EBP) family. Since the C/EBPs are transcription factors which have been implicated in both the terminal differentiation of mammalian hepatocytes and liver-specific gene expression (Umek *et al.*, 1991; Friedman *et al.*, 1989; Sladek and Darnell, 1992), I explored the possibility that these *Rana* homologues of the mammalian C/EBPs might play a role in the T_3-induced terminal differentiation and

reprogramming of gene expression in the liver of *Rana catesbeiana* tadpoles.

While it is evident that the ultimate result of T_3 action involves a change in gene expression, the underlying mechanism(s) of T_3 action on tadpole liver cells, or, for that matter, on any other tadpole cell/tissue, is poorly understood. Since the urea cycle enzyme genes generally exhibit coordinate expression *in vivo*, it is reasonable to believe that the 5'-flanking regions of these genes share some DNA regulatory elements in common. Since multiple C/EBP binding elements are located in both the *Rana* CPS-1 and OTC gene promoter regions (see Figures 16 and 19), and since mammalian CPS-1, OTC, and arginase gene promoter regions also contains C/EBP binding elements (Howell *et al.*, 1989; Murakami *et al.*, 1990; Takiguchi and Mori, 1991), I hypothesized that C/EBP and its related proteins might be involved in regulating the expression of the urea cycle enzyme genes. My results revealed that the recombinant RcC/EBP-1 protein could specifically bind to C/EBP-like binding elements located in the promoter regions of both the OTC and CPS-1 genes (Figure 43). This observation, coupled with the demonstration that the thyroid-hormone induced up-regulation of RcC/EBP-1 mRNAs was apparent well before the up-regulation of the mRNAs encoding the urea cycle enzymes, CPS-1 and OTC (see Chapter 4; Chen *et al.*, 1994; Atkinson *et al.*, 1994; Helbing *et al.*, 1992), supports my contention that the thyroid hormone-induced expression of the RcC/EBP-1 mRNAs may be required for the expression of the CPS-1 and OTC genes.

Thus, my current model for the putative molecular mechanism(s) to account for the thyroid hormone-induced

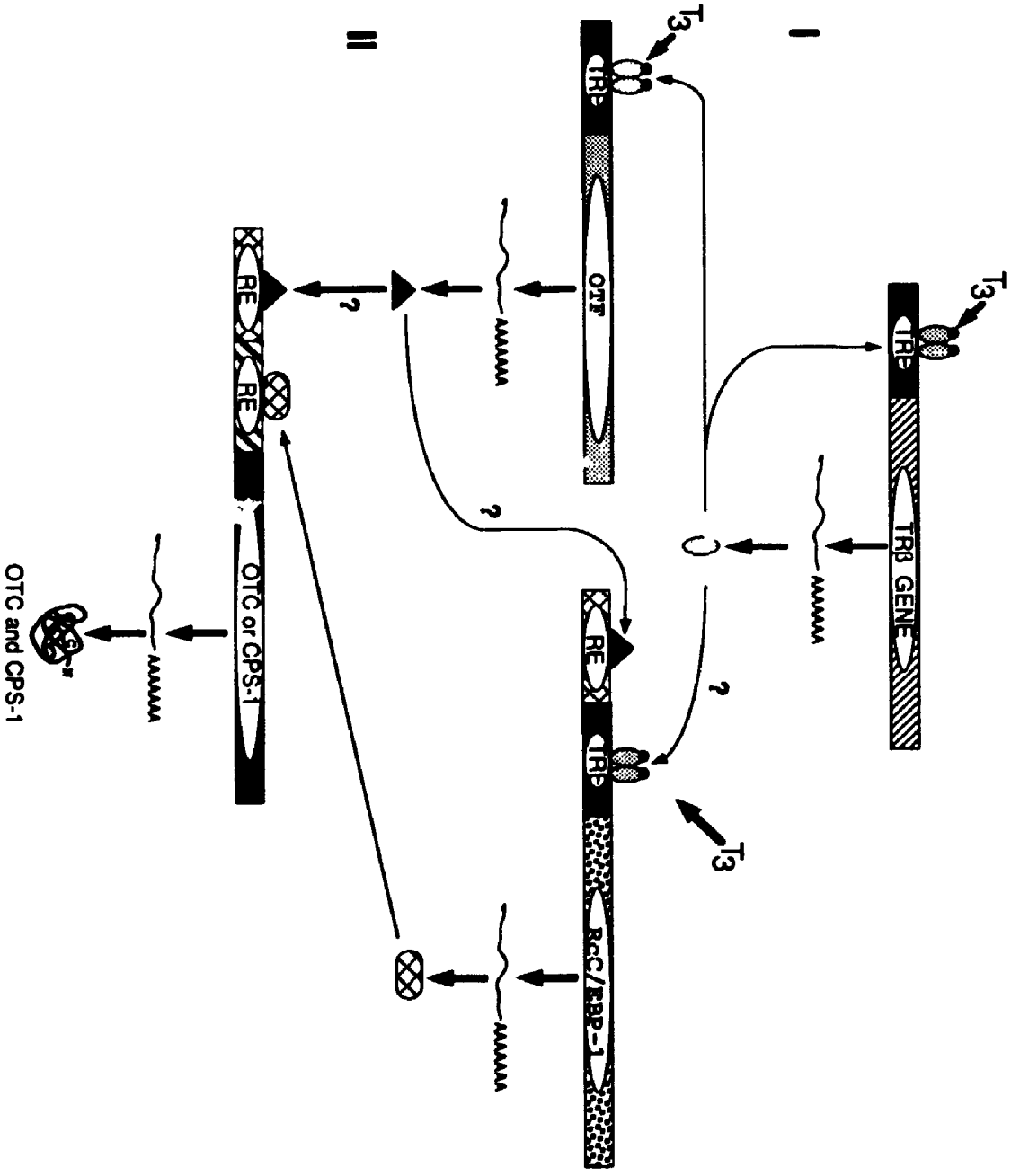
expression of the genes encoding the ornithine-urea cycle enzymes, CPS-1 and OTC, in the liver of *Rana catesbeiana* tadpoles consists of two phases (Figure 44). Phase I depicts molecular events thought to occur during the lag period - the time between the administration of thyroid hormone and the expression of the ornithine-urea cycle enzyme genes, CPS-1 and OTC - and depends upon thyroid hormone (T_3) binding to thyroid hormone receptor proteins (TRβs) which are bound to thyroid hormone response elements (TREs) located in the promoter regions of the genes proposed to be directly regulated by this hormone. However, the molecular events occurring during phase I are poorly understood, and may or may not (see question marks in Figure 44) involve thyroid hormone directly regulating the expression of each of these early responding genes (*i.e.* the TRβ gene and/or a member of the RcC/EBP-1 family of genes). Phase II shows a scheme for initiating the molecular events, the expression of the CPS-1 and OTC genes, occurring after the lag period. Phase II is thought to be initiated by the products of the genes directly regulated by this hormone-TRβ complex. The products, transcription factors [*e.g.* a member of the RcC/EBP-1 family or other transcription factors (OTF)], are envisioned to bind to their appropriate response elements (RE) in the promoter regions of the CPS-1 and OTC genes, and, subsequently, activate and sustain the expression of the these genes.

Figure 44. Putative molecular mechanism(s) to account for the thyroid hormone-induced expression of the genes encoding the ornithine-urea cycle enzymes, CPS-1 and OTC, in the liver of *Rana catesbeiana* tadpoles.

The molecular mechanism(s) proposed involves a cascade of molecular events in which this hormone is though to initially up-regulate the expression of genes encoding transcription factors, including TRβ, which, in turn up-regulate the expression of the genes encoding the ornithine-urea cycle enzymes, CPS-1 and OTC. For simplicity, this cascade has been separated into two phases.

Phase I depicts molecular events thought to occur during the lag period-the time between the administration of thyroid hormone and the expression of the ornithine-urea cycle enzyme genes, CPS-1 and OTC. Phase I depends upon thyroid hormone (T_3) binding to thyroid hormone receptor proteins (TRβs) which are bound to thyroid hormone response elements (TREs) located in the promoter regions of the genes proposed to be directly regulated by this hormone. However, the molecular events occurring during phase I are poorly understood, and may or may not (see question marks) involve thyroid hormone directly regulating the expression of each of these early responding genes.

Phase II shows a scheme for initiating the molecular events, the expression of the CPS-1 and OTC genes, occurring after the lag period. Phase II is thought to be initiated by the products of the genes directly regulated by this hormone-TRβ complex. The products, transcription factors [*e.g.* RcC/EBP-1 and/or other transcription factors(OTF)], are envisioned to bind to their appropriate response elements (RE) in the promoter regions of the CPS-1 and OTC genes and, subsequently, activate and sustain the expression of these ornithine-urea cycle enzyme-encoding genes.


I
II
T3
TRβ
TRβ GENE
AAAAA
OTF
RE
RE
?
?
?
RcC/RBP-1
OTC or CPS-1
OTC and CPS-1

CHAPTER 6 SUMMARY AND DISCUSSION

The liver in a metamorphosing *Rana catesbeiana* tadpole does not show marked morphological changes, such as those seen in the total regression of its tail or in the emergence and growth of its limbs, but the liver does undergo extensive cytological, biochemical and molecular remodeling which is thought to involve both a reprogramming of gene expression and the terminal differentiation of the resident hepatocytes (Cohen *et al.*, 1978; VanDenbos and Frieden, 1976; Chen *et al.*, 1994). While the occurrence of these postembryonic changes in the liver cells of a metamorphosing tadpole is dependent on increasing circulating levels of TH and, in fact, can be induced precociously by administration of T_3 to a premetamorphic tadpole, the means by which this hormone initiates these changes are only now beginning to be understood.

The ornithine urea cycle is an essential metabolic pathway for disposal of the toxic metabolite ammonia in terrestrial vertebrates, and is catalyzed by five hepatic enzymes (see Figure 3), carbamyl phosphate synthetase-1 (CPS-1), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL) and arginase (Arg). In this amphibian, CPS-1 and OTC are located in the mitochondrial matrix, and the remaining three enzymes are cytosolic. In *Rana catesbeiana*, like most amphibians, the tadpoles excrete ammonia directly into the water and are ammonotelic, but the frogs are ureotelic (for reviews see Frieden,, 1961; Frieden and Just, 1970; Cohen, 1970; Gilbert and

Frieden, 1981; Galton, 1983, Shi, 1994; Atkinson, 1994). More than 20 years ago, Cohen and his colleagues demonstrated that both the activities of urea cycle enzymes and urea excretion increase at a time in development (Stages XVIII to XX) when the gross morphological changes in the tadpole (*e.g.* loss of the tadpole's tail) are only just beginning to be evident (for reviews see Cohen, 1966; Cohen, 1970; Cohen, 1978). Indeed, these biochemical changes in the liver of this organism occur in anticipation of new demands for a terrestrial lifestyle, and their initiation and completion are dependent upon and tightly controlled by thyroid hormone (Metzenberg *et al.*, 1961; Paik and Cohen, 1960; Kim and Cohen, 1968; Pouchelet and Shore, 1981; Helbing *et al.*, 1992).

The isolation and characterization of the nucleotide sequences encoding two of the *Rana catesbeiana* mitochondrial-localized enzymes, CPS-1 (Helbing and Atkinson, 1994) and OTC (Helbing *et al.*, 1992), one of the cytosolic-localized enzymes involved in this cycle, arginase (see Chapter 2), and *Rana* TRs, TRα and TRβ (Schneider and Galton, 1991; Helbing *et al.*, 1992; Davey *et al.*, 1994), permitted a reliable means of assessing the intracellular levels of mRNAs encoding these proteins in the liver of *Rana catesbeiana* tadpoles undergoing thyroid hormone-induced or spontaneous metamorphosis. It is apparent, from the studies reported in Chapter 2 and other recent reports (Helbing *et al.*, 1992; Atkinson *et al.*, 1994; Helbing and Atkinson, 1994), that the up-regulation of the TR mRNAs is detectable within 12 hours after administration of thyroid hormone to the tadpole, whereas little or no accumulation of the CPS-1, OTC or arginase mRNAs is detectable until 24 hours after

administration of this hormone. The early accumulation of TR mRNAs in response to thyroid hormone suggests that they are encoded by genes which may be directly regulated by thyroid hormone, and the more extended time period (~24 hours) between the administration of the hormone and the up-regulation of the CPS-1, OTC and arginase mRNAs - the so called "lag" phase - suggests that these mRNAs are encoded by genes which are not directly regulated by thyroid hormone.

The major site of thyroid hormone action is thought to be at the transcriptional level and is mediated through the binding of this hormone to a nuclear-localized receptor protein (for reviews see Tata, 1993; Atkinson, 1994; Tata, 1994). The formation of ligand-bound TR complexes specifically interacting with thyroid hormone response elements (TREs) located in regulatory regions of target genes is, presumably, a necessary first step for activation or suppression of target genes (Oppenheimer *et al.*, 1987; Oppenheimer *et al.*, 1994). In order to obtain a better understand of the relationship between the T_3-induced gene expression of the urea cycle enzymes and thyroid hormone receptors (TRs), I isolated and characterized the promoter and the upstream regulatory regions of the CPS-1 and OTC genes from *Rana catesbeiana* as a first step towards elucidating the transcriptional regulatory mechanisms governing the expression of these genes (see Chapter 3).

The absence of any TRE consensus sequences in the 5'-regulatory regions of the *Rana* OTC and CPS-1 genes, at least within the ~1 kb upstream sequences analyzed in my study (see Chapter 3), suggests that the thyroid hormones must exert their effects on the

expression of the OTC and CPS-1 genes in an indirect fashion. Since these urea cycle enzyme genes generally exhibit coordinated expression in the liver of *Rana catesbeiana* during spontaneous and T_3-induced metamorphosis (Helbing *et al.*, 1992; Chen *et al.*, 1994; Helbing and Atkinson, 1994), it seemed reasonable to believe that the 5'-flanking regions of these genes should share some regulatory elements in common. The observation that the 5'-regulatory regions of both of these genes contain several C/EBP elements raised the possibility that thyroid hormone may be upregulating the expression of the liver C/EBPs which, in turn and in a coordinated fashion, upregulate the expression of the OTC and CPS-1 genes.

C/EBP, like other members of the basic-region leucine-zipper (bZIP) class of transcription factors, possesses a basic-region which dictates DNA-binding specificity (Agre *et al.*, 1989) and a leucine-zipper which dictates dimerization specificity (Smeal *et al.*, 1989). A "scissors grip" model is proposed for explaining the site-specific interaction of C/EBP with its DNA-binding element (Vinson *et al.*, 1989). This model visualizes a Y-shaped dimer of C/EBP monomers held together by a coiled-coil zipper interaction in the carboxyl-terminal stem. The bifurcating arms of the Y are visualized to wrap around the target DNA within the major groove, facilitating the interaction of amino acid side-chains with substituents in the target site. Presumably, the regulatory domain(s) of C/EBP, which reside in the amino-terminal two-thirds of the bifurcating arms, provide the specific contacts necessary for transactivation of the target genes (for reviews see Lamb and McKnight, 1991; Vasseur-Cognet and Lane, 1993). To date, five C/EBP isoforms have been

identified in mammals and/or birds. These include (1) C/EBPα (Landschulz *et al.*, 1988a; Lincoln *et al.*, 1994), (2) C/EBPβ [Cao *et al.*, 1991; also referred as LAP (Descombes *et al.*, 1990), NF-IL6 (Akira *et al.*, 1990), IL-6DBP (Poli *et al.*, 1990), AGP/EBP (Chang *et al.*, 1990), and CRP2 (Williams, Cantwell and Johnson, 1991)], (3) C/EBPγ [Cao *et al.*, 1991; also referred as Ig/EBP-1 (Roman *et al.*, 1990)], (4) C/EBPδ [Cao *et al.*, 1991; also referred CELF (Kageyama *et al.*, 1991), CRP3 (Williams, Cantwell and Johnson, 1991), NF-IL6β (Kinoshita, *et al.*, 1992)], and (5) C/EBPε [Cao *et al.*, 1991; also referred CRP1 (Williams, Cantwell and Johnson, 1991)].

The original C/EBP protein (now called C/EBPα) was given this name because it can bind to virus enhancer sequences and was originally thought to be a CCAAT-binding protein (Graves *et al.*, 1986; Landschulz *et al.*, 1988b). Although the latter is not the case, the C/EBP nomenclature is still in use. The four kinds of DNA sequence motifs shown to be C/EBP binding sites include (1) a 5'-GCAAT-3' motif (Graves *et al.*, 1986; Vinson *et al.*, 1989; Cao *et al.*, 1991), (2) a 5'-T(T/G)NNG(C/T)AA(T/G)-3' motif (Ryden and Beemon, 1989), (3) a 5'-GTTGCAAC-3' motif (Howell *et al.*, 1989; Lagace *et al.*, 1992; Goping *et al.*, 1992; Goping and Shore, 1994), and (4) a 5'-MTTRCNNMA-3' motif (M is C or A, N is A, C, G or T, and R is G or A; Xu and Tata, 1992). Results, from studies with C/EBPα protein, have shown that these C/EBP binding-sites are capable of driving reporter genes to be expressed in hepatoma cells (Vinson *et al.*, 1989; Ryden and Beemon, 1989; Goping *et al.*, 1992). These observation, coupled with the limited cellular distribution of C/EBPα (liver and fat cells; Landschulz

et al., 1988a) have implicated a role for the mammalian C/EBPα in the transcription of liver and/or fat cell-specific genes (Costa *et al.*, 1988; Cao *et al.*, 1991; Maire *et al.*, 1989; for reviews see Sladek and Darnell, 1992; Noda and Ichihara, 1993).

The fact that the urea cycle enzymes are specific to the liver tissue of most organisms and generally exhibit coordinate expression has led to investigation focused on characterizing the promoter regions of the urea cycle enzyme genes (for a review see Morris, 1992). In mammals, at least, three of these genes, CPS-1, OTC and arginase, have DNA elements that are recognized by C/EBP or C/EBP-related factors. Lagace *et al.* (1987) found that the rat CPS-1 promoter activity in liver nuclear extracts was dependent on a C/EBP element at position -109. Mutagenesis at this site was sufficient to abolish transactivation of the CPS-1 promoter by C/EBPα in co-transfected HepG2 cells (Lagace *et al.*, 1992). Liver-specific expression of the rat OTC gene, as judged by cell transfection assays, is conferred by 222 bp of the 5'-flanking region which contains two C/EBP-binding sites (Murakami *et al.*, 1990; Nishiyori, *et al.*, 1994). Footprinting with a C/EBP-related factor protected a region from -95 to-82 in the rat arginase promoter, thus suggesting a possible functional role for this element (Takiguchi *et al.*, 1988; Takiguchi and Mori, 1991). All of these reports implicate a role for C/EBPα in regulating the expression of the urea cycle enzyme genes in the mammalian liver.

Recently, Xu and Tata (1992) described a gradual increase of a C/EBP-like mRNA in the liver of metamorphosing *Xenopus laevis* tadpoles and suggested that the C/EBPs may be involved in liver-

specific gene expression and maintenance of the adult liver phenotype. With this thought in mind, I isolated and characterized cDNAs (see Chapter 4) encoding *Rana catesbeiana* homologues of the mammalian C/EBPα (Landschulz *et al.*, 1988a; Lincoln *et al.*, 1994) and C/EBPδ (Cao *et al.*, 1991; Williams, Cantwell and Johnson, 1991). Transcription and translation of these cDNAs in cell-free systems resulted in the production of proteins which, when used in gel-mobility shift assays, appeared to recognize and bind specifically to DNA elements with consensus sequences specific for mammalian C/EBPs. Northern- and dot-blot hybridization analyses revealed that mRNA transcripts encoding one of these transcription factors, the *Rana* homologue of C/EBPα (RcC/EBP-1), accumulate in the liver of spontaneously and thyroid hormone-induced metamorphosing tadpoles. In fact, the thyroid hormone-induced upregulation of RcC/EBP-1 mRNA is concurrent with the upregulation of TRβ, a gene presumed to be a direct-responding gene (Tata, 1993; Tata; 1994), and is apparent well before the upregulation of the mRNAs encoding the ornithine-urea cycle enzymes CPS-1 and OTC.

This latter observation reinforced my contention that a C/EBP-like transcription factor, such as RcC/EBP-1, might be involved in the upregulation of the CPS-1 and OTC genes. For this reason, I inserted the open reading frame of RcC/EBP-1 into a pTrcHis vector, transfected it into *E. coli* cells, produced recombinant RcC/EBP-1 protein, and used this protein to assess some of the putative C/EBP binding sites in the promoter regions of the *Rana* CPS-1 and OTC genes (Chapter 5). Gel mobility shift assays, using the recombinant protein and sequences matching the putative C/EBP-binding

elements in the *Rana* CPS-1 and OTC genes, confirmed that the protein product of this gene could bind specifically to the *Rana* sequences in the promoter regions of both the CPS-1 and OTC genes. The fact the C/EBP-1 binding elements in the *Rana* CPS-1 gene promoter exactly match the C/EBPα binding sequence reported in the mammalian CPS-1 promoter (Lagace *et al.*, 1992) adds further support to my hypothesis.

Thus, the observations (1) that the product of the RcC/EBP-1 gene can recognize and bind specifically to DNA elements in the *Rana* CPS-1 and OTC gene promoters, and (2) that RcC/EBP-1 mRNA accumulation in the liver of tadpoles is coincidental with TRβ mRNA accumulation and occurs well before any accumulation of CPS-1 and OTC mRNAs, appeared to support the contention that thyroid hormone may be directly effecting the expression of the gene(s) encoding RcC/EBP-1. In order to appraise the possibility that TH is directly affecting an upregulation of RcC/EBP-1 mRNAs, I isolated and characterized the promoter region of the gene encoding the RcC/EBP-1 cDNA which I had previously isolated. To my amazement, inspection of the promoter region of this gene did not reveal any consensus sequences corresponding to known TREs. However, Southern blot hybridization analyses, using 5' upstream exon regions of RcC/EBP-1 sequences with *Rana* genomic DNA, indicate that this gene is not a single-copy gene. This latter demonstration raised the possibility that another member of this RcC/EBP-1 family may contain TREs and, in fact, be responsible for the TH-induced upregulation in RcC/EBP-1 mRNAs observed in the liver of these tadpoles. Whatever the case, the results presented in this thesis

implicate the product of an RcC/EBP-1 gene in the hormone-induced transcription of the CPS-1 and OTC genes in the liver of this amphibian, Indeed, if RcC/EBP-1 has both the antimitotic and transcription regulatory properties of the mammalian C/EBPα transcription factor (Vasseur-Cognet and Lane, 1993; Umek *et al.*, 1991), then it also could be envisioned to play an early role, perhaps in concert with a TR or other transcription factors, in orchestrating both the terminal differentiation and the reprogramming of gene expression in this tadpole's liver during both spontaneous and thyroid hormone-induced metamorphosis.

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