The YY1 Transcription Factor is a Component of Ribonucleoprotein Complexes in *Xenopus laevis* Oocytes and Embryos.

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Abstract

Yin Yang 1 (YY1) is a multifunctional transcription factor that is known mainly for its ability to activate or initiate transcription of a wide assortment of genes involved in cellular growth and differentiation. In this study, *Xenopus laevis* oocytes and embryos were used as a model to identify and characterize a potential developmental role for YY1. Northern and Western blots of oocyte and embryonic extracts showed YY1 mRNA and protein is expressed from the earliest stages of oocyte development through to tadpole stages. Examination of the transcriptional activity of YY1 in both oocytes and embryos using reporter gene constructs containing YY1-binding elements demonstrated that YY1 does not act as a repressor or activator of transcription either in oocytes or in embryos. Sub-cellular fractionation of oocytes and Western blot analysis showed YY1 is localized almost exclusively to the cytoplasm of oocytes and in cells of early embryos. Sequence analysis of YY1 revealed that it contains an established RNA binding motif located within the zinc fingers. A series of biochemical assays were performed to address the possibility that YY1 functions as a component of mRNPs in the oocyte cytoplasm. RNA gel mobility shift analyses using *in vitro* synthesized histone H2A transcripts and supershifts using YY1-specific antibodies suggested that YY1 or YY1-containing complexes in cytoplasmic extracts were able to bind RNA. Chromatographic analysis of oocyte lysates showed YY1 was specifically retained on oligo (dT) cellulose columns. Treatment of the same lysates with RNase abolished binding to oligo (dT), indicating that retention is dependent on the presence of intact polyadenylated RNAs. This suggested that YY1 may be a component of messenger ribonucleoprotein particles (mRNP). Separation of oocyte lysates by size exclusion chromatography (SEC) revealed that YY1 was present in large complexes with an approximate molecular mass of 480 kDa. RNase or phosphatase treatment of oocyte extracts released YY1 from high mass complexes. Analysis of phosphatase or RNase-treated extracts for DNA binding activity showed that monomeric YY1 was able to bind DNA with high affinity. Immunoprecipitation of YY1 complexes followed by cDNA synthesis and sequencing revealed that YY1 is associated with both ribosomal and messenger RNAs in the cytoplasm of the oocyte. These results indicate a novel function for YY1 as a component of messenger ribonucleoprotein particles. It is hypothesized that YY1 plays a role in regulating the storage and metabolism of maternal RNAs in oocytes and embryos.

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List of Abbreviations

APS	ammonium persulfate
ADP	adenosine diphosphate
ATCC	American Type culture collection
ATP	adenosine triphosphate
A-V axis	animal-vegetal axis
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumen
C_2H_2	2-cysteine, 2-histidine
c-AMP	cyclic-adenosine monophosphate
CAT	chloramphenicol acetyl transferase
CSD	cold shock domain
cDNA	complimentary deoxyribonucleic acid
CBTF	CCAAT box Transcription Factor
CHO	Chinese hamster ovary
CIAP	calf intestinal alkaline phosphatase
CIRP	cold inducible RNA binding protein
CRAS	coding region activator sequence
CRE	c-AMP response element
CREB	c-AMP responsive element binding protein
CPE	cytoplasmic polyadenylation element
cps	counts per second
CTD	carboxy terminal domain
C-terminal	carboxy terminal
CTP	cytidine triphosphate
˚C	degrees centigrade
DEAE	diethylaminoethyl
dH_2O	distilled (single) water
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
dsRBD	double stranded RNA binding domain
dsRNA	double stranded ribose nucleic acid
DTT	dithiothreitol
E1A	early 1A
EDTA	ethylene diamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
GTP	guanosine triphosphate
H2A	histone 2A
HEPES	hydroxyehtylpiperazine-*N*'-2-ethanesulfonic acid
HAT	histone acetyltransferase
HCG	human Chorionic gonadotropin
HDAC	histone deacetylase

HIV human immunodeficiency virus
HPV human papilloma virus
HRP horseradish peroxidase
hr hour
J/cm^2 joules per square centimeter
kb kilobase
kDa kilodalton
KH K-homology
L liter
LTR long terminal repeat
M molar
mM milimolar
MBT midblastula transition
mg milligram
min minutes
ml milliliter
MOPS 3-(N-Morpholino) propanesulfonic acid
mRNA messenger ribose nucleic acid
mRNP messenger ribonucleoprotein particle
NCBI National Centre for Biotechnology Information
ng nanogram
nr non-redundant
nt nucleotide
NF- B nuclear factor- B
ORF open reading frame
PAGE polyacrylamide gel electrophoresis
PAP poly(A) polymerase
PBI Plant Biotechnology Institute
PBS phosphate buffered saline
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
*p*g picogram
PVDF polyvinylidene difluoride
RAP55 RNA-associated protein of 55 kDa
RGG arginine-glycine-glycine
RPM revolutions per minute
RRM RNA recognition motif
RNA ribose nucleic acid
rRNA ribosomal ribose nucleic acid
RNA Pol II RNA polymerase II
rpm revolutions per minute
RT room temperature
SEC size exclusion chromatography
sec seconds
SDS sodium dodecylsulfate
SRF serum response factor

st	stage
Taq	*Thermophilus aquaticus*
TAE	TRIS-acetic acid-EDTA
TBE	TRIS-borate-EDTA
TBP	tata binding protein
TBST	TRIS buffered saline/Tween-20
TE	Tris-EDTA
TEEMED	N,N,N',N'-tetra methyelenediamine
TFIIIA	transcription factor IIIA
TK	thymidine kinase
TLC	thin layer chromatography
tRNA	transfer RNA
TTP	thymidine triphosphate
U	units
*u*g	microgram
*u*m	micrometer
UTR	untranslated region
UV	ultraviolet
V	volts
VLE	vegetal localization element
(v:v)	volume to volume
(w:v)	weight to volume
YY1	Yin Yang 1
Zn	zinc

1.0 Objectives

Over the last decade, YY1 has been implicated in both activation and repression of a variety of genes and has been shown to play a role in development and differentiation. The vast majority of literature has focused on its function as a transcriptional activator or repressor. Although several groups have identified a role for YY1 in developing tissues, little research has been aimed at defining its role during embryonic development. The goal of this study was to characterize and identify the transcriptional activity and developmental significance of YY1 during oocyte and embryonic development in the *Xenopus laevis* model system. The initial hypothesis was that YY1 controls gene expression differentially during development through its capacity as a transcriptional repressor and/or activator.

The expression pattern of YY1 was analyzed by Northern and Western blot analyses of RNAs and proteins isolated from oocytes and embryos. Gel mobility shift assays were used to examine the ability of YY1 to bind DNA during development and the transcriptional activity of YY1 was assessed by reporter gene expression assays. Subcellular localization of YY1 was determined by manual dissections and biochemical fractionation of oocytes and embryos followed by Western blotting. The original hypothesis was disproven by data obtained from the above described experiments. YY1 was found to be expressed through development in both oocytes and embryos however it remained in the cytoplasm and appeared not to play a role in transcriptional regulation.

Since YY1 contained an RNA binding motif the subsequent hypothesis was that YY1 could function in the regulation of mRNAs in the cytoplasm. To test this, the *in vitro* and *in vivo* RNA binding ability of YY1 was determined by RNA binding assays and Oligo (dT) chromatography. Size exclusion chromatography was used to determine the molecular mass of YY1-containing complexes. The identity of RNAs associated with YY1 complexes was determined by immunoprecipitation of YY1 including associated RNAs. These RNAs provided templates from which cDNAs were synthesized and subsequently sequenced.

2.0 YY1

2.1 YY1 Introduction

Yin Yang 1 (YY1), previously identified as FIII, NF-E1, , F-ACT1, CF1, and UCRBP, is a member of the GLI-Krupple family of transcription factors that is capable of functioning as a repressor, activator or initiator element binding protein depending on its cellular context (reviewed in Shi et al., 1997). YY1 is a ubiquitously expressed protein that has been shown to regulate a variety of genes in a number of cell and tissue types. Structural analyses of mammalian YY1 have revealed a number of prominent features such as four carboxy terminal C_2H_2 zinc fingers and an amino terminal bi-partite transcriptional activation domain (Shi et al., 1997). Also distributed throughout the protein are a number of protein-protein interaction domains which have been shown to modulate transcription (Austen et al., 1997b; Bushmeyer et al., 1995). It is a highly conserved protein, consisting of 414 amino acids in humans and mice and 373 amino acids in *Xenopus* (Bushmeyer et al., 1995). While *Xenopus* YY1 is somewhat smaller, it retains all functional motifs known for the mammalian protein (Eliassen et al., 1998; Flanagan et al., 1992; Pisaneschi et al., 1994; Seto et al., 1991).

A study conducted by Berns and Bohenzky (1987) of the P5 promoter from the adeno-associated virus (AAV), a defective human parvovirus, was among the first to uncover the dual modes of YY1 transcriptional activity. Under normal circumstances, the P5 promoter is inactive, and binding of the E1A

oncoprotein to the promoter is required for transcriptional activation (Chang et al., 1989). Analysis of the AAV P5 promoter indicated the presence of a *cis* element that is responsible for mediating the E1A-induced transcriptional activation. In the absence of E1A, the *cis* element is capable of regulating repression of basal as well as enhancer-mediated transcription. YY1 was isolated on the basis of its ability to bind to the P5 *cis* element, which was used as an affinity reagent to isolate a protein with an approximate molecular mass of 68 kDa from HeLa cells (Seto et al., 1991). Subsequent analysis indicated the protein was capable of repressing transcription in the absence of E1A, yet was able to activate transcription in its presence. The name of the protein was derived from its dual functionality depending on the cellular context. Other early studies identified YY1 as a transcriptional repressor. For example, YY1 was found to bind the upstream conserved region (and thus was referred to as UCRBP) of the Moloney Murine Leukemia Virus (MuLV) and down-regulated promoter activity (Flanagan et al., 1992). YY1 was also found to bind and down-regulate the immunoglobulin 3 enhancer (Park and Atchison, 1991). Also in early studies, YY1 was identified as a transcriptional activator known as , involved with the transcriptional activation of L30 and L32 ribosomal protein genes in mouse (Hariharan et al., 1991).

YY1 has been shown to influence expression of a number of developmentally regulated genes in primary tissue culture model systems. In differentiating cardiac myoblasts, the expression of dystrophin is controlled by YY1-promoter interactions (Galvagni et al., 1998). YY1 was implicated in type switching of globin genes in mouse embryos from (embryonic) to (fetal)

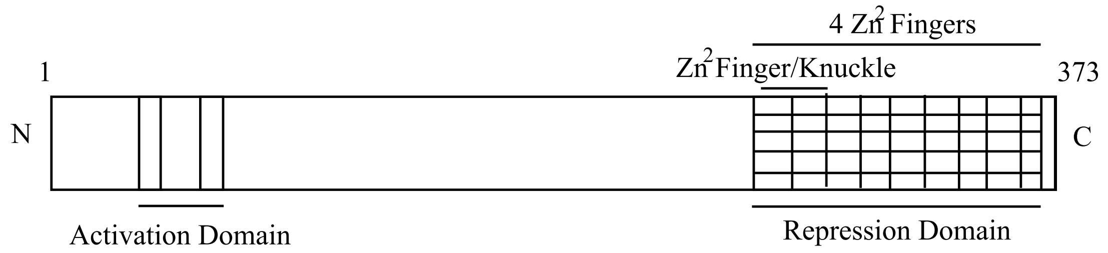
(Gumucio et al., 1993; Peters et al., 1993; Raich et al., 1995; Zhu et al., 1999). In this case different sets of genes are expressed at different times during development. YY1 is a transcriptional repressor shown to bind a *cis* element located in the first intron of the -fetoprotein gene which is expressed in the yolk sac, visceral endoderm and fetal hepatocytes (Scohy et al., 2000). YY1 has been found to influence immune cell differentiation through interactions with the immunoglobulin 3 enhancer (Park and Atchison, 1991) the pre-T-cell receptor (Reizis and Leder, 1999) and the interferon gene (Gilly et al., 1996). Organ specific gene expression has also been attributed to the transcriptional activity of YY1 in tissues such as bone (Guo et al., 1997), testes (Schulten et al., 1999), and liver (Du et al., 1998). In addition to these examples, YY1 has been shown to be responsive to perturbations of the endoplasmic reticulum (Li et al., 1997b; Roy and Lee, 1999), which induced binding of YY1 to promoter elements of the glucose response proteins (GRPs) (Li et al., 1997b; Roy and Lee, 1999). Thus, it has emerged that YY1 is a multifunctional transcription factor with a variety of roles in cell growth and differentiation.

2.2 YY1 structural overview

Mammalian YY1 is a highly conserved 414 amino acid protein with a predicted molecular mass of 44 kDa (Bushmeyer et al., 1995). Analysis of YY1 by Western blots in several laboratories, however, indicates that it migrates at 65-68 kDa, larger than its predicted molecular mass based on the size of the open reading frame (reviewed in (Galvin and Shi, 1997). This discrepancy in size has been attributed to structural anomalies of the protein. In *Xenopus,* the amino acid

Figure 1

Schematic diagram of the *Xenopus* YY1 protein. Amino acid positions and N and C-terminal ends are indicated. The four C^2H^2 type zinc fingers at the C-terminus are indicated. The approximate positions of the activation and repression domains are shown below the figure. The position of the zinc finger/knuckle is shown above the diagram. Adapted from Shi et al., 1997.



sequence of YY1 is very similar to that of the mammalian form, however, it is somewhat smaller at 373 amino acids (Pisaneschi et al., 1994). Both mammalian and *Xenopus* YY1 contain the major structural elements discussed below (shown in Figure 1). The most notable structural elements of YY1 are four C_2H_2-type zinc fingers located in the C-terminus of the protein between amino acids 298-397 of human YY1 (Hariharan et al., 1991). Deletion analysis has indicated that all four zinc fingers are required for DNA-binding activity (Austen et al., 1997b; Bushmeyer et al., 1995). Progressive carboxyl-terminal deletions have shown that the activation domains are located toward the amino-terminus (Austen et al., 1997b; Bushmeyer et al., 1995; Lee et al., 1995b; Lee et al., 1994). This region of the protein contains sequences that are able to form an acidic amphipathic helix (residues 16-29) as well as 11 consecutive acidic residues (43-53), 11 consecutive histidines (70-80) as well as a region that is rich in glutamine and proline (81-100) (Bushmeyer et al., 1995). Austen and co-workers (Austen et al., 1997b) constructed a series of mammalian YY1 deletion mutants and found that two amino terminal acidic regions (residues 2-62 and 92-153) each contribute approximately half of the activating potential of the protein. They also found that the spacer region located between the two acidic regions was essential for full activator function however it was not able to function independently as an activating region (Austen et al., 1997b). They proposed that the spacer region may be required for proper folding of the activation domains (Austen et al., 1997b).

Several groups have found that the repression domain of YY1 is located towards the carboxy terminus of the protein (Lee et al., 1995b; Lee et al., 1994;

Seto et al., 1991). Deletion of residues 397-414 was not sufficient to convert YY1 from a repressor to an activator (Bushmeyer et al., 1995) when the protein was expressed in 3T3 cells. However, deletion of an additional 27 amino acids to residue 370 was found to convert YY1 to a strong activator (Bushmeyer et al., 1995). These deletions demonstrated that residues located between amino acids 370-397 were sufficient to block YY1 activator function. Further analysis of the repressor function of YY1 by fusion of residues 201-414 or 333-414 to the GAL4 DNA binding domain resulted in repression of the GAL 4 reporter by the 333-414 fusion (Bushmeyer et al., 1995). These results indicate that the repression domain is located between residues 333-397 (Bushmeyer et al., 1995). The identification of separate activation and repression domains clearly indicates that the protein is multifunctional (Bushmeyer et al., 1995; Lee et al., 1995b; Lee et al., 1994), however, the actual mechanisms by which its differing transcriptional activities are modulated are not fully understood.

Austen and co-workers (Austen et al., 1997b) demonstrated that the second and third zinc fingers are important for nuclear localization of YY1 in RK13 cells. Analysis of amino acids in the second and third zinc fingers revealed the presence of a number of basic amino acids that may function in nuclear localization (Austen et al., 1997b). While a conventional NLS is not present in YY1, protein-interactions may account for nuclear translocation of YY1. A yeast two-hybrid screen has demonstrated that YY1 is able to bind the nucleolar phosphoprotein B23 which is involved in subcellular trafficking and ribosome assembly (Borer et al., 1989; Busch et al., 1984; Inouye and Seto, 1994; Prestayko et al., 1974; Yung et al., 1985). Since YY1 is able to bind B23

(relieving YY1 mediated repression) (Inouye and Seto, 1994), and since B23 is involved in shuttling between the cytoplasm and the nucleolus, Austen and co-workers (Austen et al., 1997b) hypothesized that in the absence of a consensus NLS, B23 may transport YY1 to the nucleolus. A study conducted in HeLa and Saos cells demonstrated that a subset of YY1 are co-localized in the nucleolus with B23 (McNeil et al., 1998).

Mammalian and frog YY1 proteins are highly similar with only 1 amino acid change found in the last 186 residues (Pisaneschi et al., 1994). More substantial differences have been noted in the N-terminus of frog YY1 in which there is a higher ratio of acidic residues compared to mammalian YY1 (Pisaneschi et al., 1994). The run of glycines that is present in mammalian YY1 (residues 176-194) is not well conserved and the series of histidines (residues 70-80) is completely missing in the frog protein (Pisaneschi et al., 1994; Shi et al., 1997). Yet another prominent tract of amino acids located between residues 156-201 of mammalian YY1 is absent from the frog protein (Pisaneschi et al., 1994).

2.3 Gene structure of YY1

The promoter of YY1 does not contain a recognizable TATA box (reviewed by Shi et al., 1997), but it contains multiple transcriptional start sites as well as G+C rich regions and potential Sp1 binding sites (reviewed by Shi et al., 1997), promoter features that are similar to many housekeeping and growth-regulated genes. The mammalian gene has been found to contain 5 exons that encode the full length 414 amino acid protein (Safrany and Perry, 1993). The majority of the protein is encoded within the first exon including the region

containing the acidic and histidine rich regions. The four zinc fingers are encoded on three different exons (Safrany and Perry, 1993). The intronic separation of the zinc fingers is not unprecendented, and it is unclear whether this is significant or imparts any regulatory advantage.

2.4 Transcriptional regulation by YY1

YY1 consensus sequences were identified using bacterially expressed YY1 in conjunction with the PCR based binding site selection technique (Hyde-DeRuyscher et al., 1995). The currently accepted consensus sequence is 5'-(G/c/a)(G/t)(C/t/a)-CAT(X)(T/a)(T/g/c)-3' (the upper case indicates preferred nucleotides), (Hyde-DeRuyscher et al., 1995).

2.4.1 YY1 mediated repression.

The transcriptional function of YY1 as a activator or repressor is thought to be controlled either by a variety of factors in different cellular contexts including relative YY1 concentration (Bushmeyer et al., 1995), the presence of specific cellular proteins (Bauknecht et al., 1996; Inouye and Seto, 1994; Lee et al., 1995a; Shrivastava et al., 1993; Yang et al., 1995) or the promoter sequences surrounding YY1 binding sites (Shrivastava and Calame, 1994).

Several models have been proposed to explain YY1-mediated repression including displacement, interference and interactions with co-regulators (reviewed by Thomas and Seto 1999; Shi et al., 1997). The displacement model is based on displacement of transcriptional activators by YY1. For example, overlapping binding sites have been observed for YY1 and serum response factor

(SRF, a 67 kDa phosphoprotein) on the c-fos serum response element (SRE) (Gualberto et al., 1992; Natesan and Gilman, 1995), and the skeletal and smooth muscle -actin muscle regulatory elements (MRE) (Bushel et al., 1995; Gualberto et al., 1992; Lee et al., 1992; MacLellan et al., 1994) as well as the muscle creatine kinase CArG motif (Vincent et al., 1993). An example of this is the competition for binding on the -actin promoter MRE which is important for muscle differentiation (Lee et al., 1992). During myoblast differentiation to myotubules in culture, the levels of YY1 decrease while those of SRF are observed to increase (Lee et al., 1992). Myoblasts treated with BrdU (a nucleotide analog that inhibits myoblast differentiation) expressed high levels of YY1 and low levels of SRF suggesting a correlation between relative levels of these factors and muscle differentiation (Lee et al., 1992).

The significance of YY1 binding to the SRE of c-*fos* however is unclear. The c-*fos* SRE shares homology with the MRE of the -actin promoter (Walsh, 1989). While YY1 and SRF have similar affinities for the -actin SRE (Lee et al., 1992), Gualberto and co-workers (Gualberto et al., 1992) have shown *in vitro* that SRF has a much higher affinity for the c-*fos* SRE than does YY1. Furthermore, *in vivo* footprinting analysis has shown that both YY1 and SRF co-occupy the SRE (Natesan and Gilman, 1995). YY1 has been shown to repress the serum amyloid A1 (SAA1) promoter which responds to acute inflammation with a 200 fold increase in expression of SAA1 (Lu et al., 1994). Located within the promoter is a cytokine response unit (CRU) to which NF- B and C/EBP bind (Lu et al., 1994) both having a synergistic effect on SAA1 transcription. Methylation interference studies indicate that YY1 binds within the CRU,

overlapping the binding sites for both NF- B and C/EBP (Lu et al., 1994). Gel mobility shift assays showed that NF- B binding to the CRU was inhibited by YY1 (Lu et al., 1994). The inhibition of NF- B binding to the CRU resulted in lowered SAA1 expression (Lu et al., 1994). When the binding site for YY1 in the CRU was disrupted, basal as well as serum induced expression of the SAA1 promoter was elevated (Lu et al., 1994).

Interference of activators may repress transcription by YY1. The interferon promoter (IFN-) has been shown to be repressed by YY1 (Ye et al., 1994). A search of the IFN- promoter for consensus YY1 binding sites revealed that three YY1 binding sites (Y1, Y2, and Y3) are present in the promoter (Ye et al., 1996; Ye et al., 1994). Gel mobility shift assays demonstrated that YY1 was able to bind two of these sites with high affinity (Ye et al., 1996). One of the YY1 binding sites (Y3) overlaps an AP-1 site. Both YY1 and AP-1 were found to compete for binding to their respective elements (Ye et al., 1996). Repression of the IFN- promoter was observed when YY1 alone was bound (Ye et al., 1996). Binding of YY1 to the Y2 binding element was found to repress expression only when an AP-2-like protein was present adjacent to YY1 (Ye et al., 1996). Both YY1 and the AP-2 like protein are able to bind the IFN- promoter separately, however, repression of the promoter was seen only when both proteins were present (Ye et al., 1996).

The c-*fos* promoter contains two additional YY1 binding sites (in addition to the one discussed above in the SRE) located between the cAMP responsive element (CRE) and the TATA box (Zhou et al., 1995a). E1A activation of c-*fos*

is mediated by a 22bp E1A response element (ERE which is made of a CRE and a neighboring YY1 binding site (Zhou et al., 1995b). Both YY1, which represses transcription, and members of the ATF/CREB family, which activate transcription are capable of binding to the ERE (Berkowitz et al., 1989; Lee and Lee, 1994; Natesan and Gilman, 1993; Zhou et al., 1995a). Zhou and co-workers (Zhou and Engel, 1995) have shown both *in vivo* and *in vitro* that YY1 can physically interact with ATF/CREB members. The interaction between YY1 and the ATF/CREB members has been proposed to be responsible for the ability of YY1 to repress transcription from the c-*fos* promoter (Natesan and Gilman, 1993; Zhou et al., 1995). The importance of the YY1/CREB interaction in c-*fos* repression is further illustrated by the ability of adenovirus E1A to relieve YY1 mediated repression and to disrupt the YY1/CREB interaction *in vitro* (Zhou and Engel, 1995).

The fact that proteins interacting with YY1 bind in one or both of the repression domains suggests quenching as a possible explanation for alleviation of YY1-mediated repression. Galvin and co-workers have investigated this possibility and found that YY1 mediated repression is activator specific (Galvin and Shi, 1997). Data from this study also showed that binding of Sp1 or CREB is not required for repression. They suggested that in this case, YY1 is interacting with the targets of the activators instead of binding CREB or Sp1 (Galvin and Shi, 1997).

2.4.2 Protein-protein interactions

Repression by YY1 may be accomplished through interactions with co-regulators. p300 which has been shown to bind both YY1 and CREB, may be essential for YY1 repressor activity (Lee et al., 1995a; Lundblad et al., 1995). p300, originally described as an adenovirus E1A-associated protein (Harlow et al., 1986; Yee and Branton, 1985) has been identified as a transcriptional co-activator that is related to, and functionally interchangeable with the CREB-binding protein, CBP (Arany et al., 1995; Chrivia et al., 1993; Eckner et al., 1994; Kwok et al., 1994; Lundblad et al., 1995). E1A mediated repression by YY1 has been shown to require p300 which appears to function as an adaptor protein between E1A and YY1.

The activity of the YY1 binding site in the c-*fos* promoter is orientation dependent, and it has been proposed that YY1 mediates c-*fos* transcription by bending DNA and modulation of interactions with other proteins such as CREB and the general transcriptional machinery (Natesan and Gilman, 1993). This model relied on orientation specific binding of YY1 to the promoter the result of which is bending of the DNA in a particular direction. The DNA bending model of YY1-ediated repression is challenged by a study that identified YY1 binding sites by PCR assisted site selection. The YY1 sites were tested for their ability to regulate transcription of a synthetic promoter (Hyde-DeRuyscher et al., 1995). These experiments showed that all of the YY1 binding sequences were capable of repressing the promoter regardless of orientation (Hyde-DeRuyscher et al., 1995). Also arguing against the DNA bending model is a study in which the co-crystal structure of the zinc fingers of YY1 binding to the AAV P5 promoter was

analyzed. The results of these experiments did not indicate that YY1 was capable of inducing DNA bending (Houbaviy et al., 1996). However it is also possible that YY1 is only capable of bending DNA when it is bound to certain recognition sites.

2.4.3 Repression via recruitment of co-repressors

YY1 has been shown to bind p300, (an acetyltransferase) which also binds another acetyltransferase, PCAF(p300 CBP associated factor) (Austen et al., 1997b; Lee et al., 1995a; Yang et al., 1996). Acetylation is known to regulate a number of transcription factors such as p53 (Gu and Roeder, 1997), GATA-1 (Boyes et al., 1998; Hung et al., 1999), E2F (Martinez-Balbas et al., 2000; Marzio et al., 2000), MyoD (Sartorelli et al., 1999), human immunodeficiency virus Tat (Kiernan et al., 1999), E1A (Donohoe et al., 1999), TFIIE, TFIIF (Imhof et al., 1997) and HNF-4 (Soutoglou et al., 2000). A recent analysis of the amino acid sequence of YY1 revealed several lysine residues that may act as potential acetylation substrates (Yao et al., 2001). To test for acetylation of YY1, several deletions were constructed and *in vitro* acetylation reactions were performed on the deletions using GST-tagged bacterially produced p300 and PCAF (Yao et al., 2001). Two regions of YY1 were shown to be acetylated. The first region lies between amino acids 170-200 and was acetylated by p300 as well as PCAF (Yao et al., 2001). The second region, the C-terminal zinc fingers were acetylated only be PCAF (Yao et al., 2001).

Acetylation of these regions produced significantly different functional effects *in vivo*. When residues 170-200 were acetylated, YY1 became a more

effective transcriptional repressor in HeLa cells (Yao et al., 2001). Additionally, acetylation of these residues resulted in YY1 binding to histone deacetylases (HDAC) which had the dual effect of histone H4 deacetylation and subsequent deacetylation of YY1 (Yao et al., 2001).

The link between YY1 and histone acetylation is interesting for its global control of transcription. Histone acetylation is associated with activated genes (Hebbes et al., 1988; Sealy and Chalkley, 1978; Vidali et al., 1978). The addition of acetyl groups to the lysine residues of the histones permits access of transcription factors to the DNA by disrupting the packaging of the chromatin. This outcome is caused by the neutralizing effect of acetyl groups on the positive charge of the histones, which reduces their affinity for DNA (Hong et al., 1993). Conversely, deacetylation increases histone affinity for DNA and reduces access of transcription factors. Thus it is possible that acetylation of YY1 and the resulting association with histone deacetylases confers a histone H4 deacetylase activity to YY1 and promotes repressor activity by YY1 by virtue of packaging of the DNA such that transcription factors cannot access the (Yao et al., 2001).

Adding to the complexity of YY1 regulation, acetylation of the zinc finger domain was demonstrated to decrease DNA binding activity (Yao et al., 2001). In situations were YY1 functions primarily as a repressor, acetylation of residues 170-200 and the zinc finger domain creates a network of negative feedback (Yao et al., 2001). While acetylation of residues 170-200 increases repressor potential through deacetylation of histone H4, it also results in the deacetylation of YY1 itself and thus its histone H4 deacetylation activity. Acetylation of the zinc fingers appears to have the dual effect of stabilizing the

interaction between YY1 and the histone deacetylases (presumably maintaining the packaged chromatin), and decreasing DNA binding activity of YY1. This results in YY1-mediated repression by two modes, YY1 in this state has a lower DNA binding affinity, and stabilization of the HDAC activity ensures tighter association of histones, thus impeding access of transcription factors (Yao et al., 2001).

2.4.4 Transcriptional repression of YY1 via Poly(ADP-Ribosylation)

YY1 has been shown to physically interact with the poly(ADP-ribose) polymerase (PARP-1) (Griesenbeck et al., 1999; Oei et al., 1997; Oei et al., 1998) an enzyme involved in the regulation of several nuclear processes including DNA repair, replication, recombination, transcription and apoptosis (D'Amours et al., 2001; Dantzer et al., 2000; Oei et al., 1997). PARP-1 has been shown to induce poly(ADP) ribosylation of YY1 which interferes with its ability to bind its recognition sequence (Oei et al., 1998). Oei and Shi, (2001) showed that DNA damage induced poly(ADP) ribosylation of YY1 and brought about repression of its DNA binding activity. This provides evidence for a mechanism by which the cell is able to recognize DNA damage and cease transcription until the lesion(s) are repaired.

2.5 YY1-mediated transcriptional activation

YY1 has been shown to activate a wide variety of genes including c-myc (Shrivastava et al., 1996) HMG-CoA reductase (Gauthier et al., 1999), cardiac - actin (Chen and Schwartz, 1997), INF (Gilly et al., 1996) histones (Eliassen et

al., 1998; Wu and Lee, 2001), p53 (Furlong et al., 1996), myelin PLP (Berndt et al., 2001), AAV P5 promoter (Seto et al., 1991), ribosomal proteins rpL30 and rpL32 (Hariharan et al., 1989), rpL14, rpL1 (Pisaneschi et al., 1994), ST5 (Lichy et al., 1996), Surf-1 (Vernon et al., 2000; Cole et al., 1997; Gaston et al., 1995), Surf-2 (Gaston and Fried, 1994; Gaston and Fried, 1995), gp91^{phox} (Jacobsen and Skalnik, 1999), Col1a1 (Riquet et al., 2001), proacrosin (Schulten et al., 1999), Globin (Yant et al., 1995), ribonucleotide reductase R1 (Johansson et al., 1998), DR- (Hehlgans and Strominger, 1995), uterglobin (Klug and Beato, 1996), osteocalcin, (Guo et al., 1997), grp78 (Li et al., 1997b) and -1 acid glycoprotein (Lee and Lee, 1994).

The mechanisms by which YY1 is able to activate transcription are not fully understood. but several models of YY1 mediated activation have been proposed (reviewed by Thomas and Seto, 1999). YY1 may interact directly with general transcription factors in the pre-initiation complex to initiate transcription. YY1 contains two N-terminal acidic activation domains, and has been shown to interact with general transcription factors TBP, TFIIB and TAFII55 (Austen et al., 1997b; Chiang and Roeder, 1995; Usheva and Shenk, 1994). Inhibition of repressor domains and unmasking of the activator domains may be involved. Two groups (Lee et al., 1995b; McNeil et al., 1998) suggested that the N-terminal activation domains of YY1 are masked and may be exposed through interactions with an unknown factor. Structural and functional mapping has suggested that the extreme C-terminal region of YY1 may regulate transcriptional activation through interactions with the N-terminal activator domain (Austen et al., 1997b; Bushmeyer and Atchison, 1998; Lee et al., 1995b).

The only protein thus far demonstrated to interact with an activator domain of YY1 is E1A, however it is also capable of interactions with the C-terminal domain (Lee et al., 1995b; Lewis et al., 1995). To accomplish this it is possible that E1A binds the repression domain to block it while exposing the activation domain (reviewed in Thomas and Seto, 1999).

YY1 may require co-activators to elicit a response on particular promoters (Thomas and Seto, 1999). This model has gained validity through the findings that YY1 is able to interact with p300 and CBP (Austen et al., 1997b; Lee et al., 1995a). Both of these proteins are known to have histone acetyltransferase activity and are known to function as co-activators in other model systems (Boyes et al., 1998; Goldman et al., 1997; Kwok et al., 1994; Lee et al., 1995a; Ogryzko et al., 1996; Yuan et al., 1996; Zhang and Bieker, 1998). p300 and CBP are closely related in sequence and function (Goldman et al., 1997) and have been shown to interact with a wide variety of DNA-binding proteins such as, CREB, E1A, Jun, Fos, and TBP where they function as co-activators (Bannister et al., 1995; Facchinetti et al., 1997; Goldman et al., 1997; Kwok et al., 1994; Lee et al., 1995a; Lundblad et al., 1995; Sartorelli et al., 1999; Swope et al., 1996; Yuan et al., 1996). It is possible that recruitment of p300 by YY1 causes relaxation of chromatin conformation (through histone acetylation) and promotes transcription factor access to the promoter. While it is clear that YY1 is able to activate a number of promoters, the exact role of p300/CBP or how YY1 is able to gain access to DNA in areas where the chromatin is condensed in not understood (Thomas and Seto, 1999).

2.6 YY1 activity as an initiator element binding protein

YY1 has been found to direct and initiate transcription *in vitro* through binding to initiator elements (Inr) (Smale and Baltimore, 1989). The first data indicating that YY1 binds an initiator sequence was gathered from analyses of the AAV P5 promoter (Seto et al., 1991). The initiator function at this site was shown *in vitro,* and further experiments demonstrated that YY1 binding to the AAV P5 promoter was crucial for transcription, since HeLa cells depleted of YY1 no longer were transcriptionally competent (Seto et al., 1991). Mechanisms controlling the Inr binding activity of YY1 have not been fully elucidated. It is possible that YY1 functions similar to TBP with regard to the recruitment of RNA polymerases to the initiation site (reviewed by Shi et al., 1997). Studies of YY1 initiator function in a reconstituted *in vitro* system using purified proteins demonstrated that YY1, TFIIB and RNA polymerase II were sufficient to direct basal transcription from a supercoiled plasmid template (Usheva and Shenk, 1994). YY1 has been shown to physically interact with TFIIB and the large subunit of RNA polymerase II both *in vivo* and *in vitro* (Houbaviy et al., 1996). Further, strand separation of the AAV promoter resulted in transcriptional activation by YY1 from single stranded DNA, indicating that YY1 is able to operate in a manner similar to TBP *in vitro* (Houbaviy et al., 1996).

2.7 Interactions between YY1 and cellular proteins

A large number of proteins have been documented to interact with YY1; the majority of these are co-activators/co-repressors or transcription factors that are essential in regulating a variety of promoters. As well, YY1 has been found

to interact with components of the nuclear matrix which may function to direct YY1 to certain locations within the nucleus (Bushmeyer and Atchison, 1998).

In general there are two general domains at which protein-protein interactions are known to occur: residues 150-200 including the repression domain (Thomas and Seto, 1999), and the C-terminal region and including the four zinc fingers as well as the second repression domain. A number of proteins such as the polycomb group protein EED, TBP, CBP/p300, TFIIB, E1A as well as c-Myc have been found to interact with both domains (Austen et al., 1997a; Galvin and Shi, 1997; Lee et al., 1995a; Lewis et al., 1995; Satijn et al., 2001; Shrivastava et al., 1993). Proteins such as histone deacetylase 2 (HDAC2), Sp1, and ATF/CREB have been shown to bind only the C-terminal domain (Galvin and Shi, 1997; Lee et al., 1995a; Seto et al., 1993; Yang et al., 1996). Recently pRb has been shown to interact with YY1 however the interaction domain(s) have not yet been identified (Petkova et al., 2001). Thus, a wide variety of proteins can interact with YY1, expanding the regulatory potential of this protein.

2.8 Interactions between YY1 and viral promoters

YY1 is a transcriptional regulator of herpesvirus (Chen et al., 1992), papillomaviruses (Bauknecht et al., 1992; Pajunk et al., 1997), polyomaviruses (Martelli et al., 1996), adenoviruses (Zock et al., 1993), parvoviruses (Momoeda et al., 1994; Seto et al., 1991) and retroviruses (Knossl et al., 1999; Mobley and Sealy, 1998). As discussed previously, one of the first promoters in which YY1 was observed to have translational activity was P5 from the adeno-associated

virus (Seto et al., 1991). Under normal circumstances, the promoter is transcriptonally silent, but is activated in the presence of the E1A oncoprotein (Chang et al., 1989). In the absence of E1A, binding of YY1 to its *cis* element was able to repress transcription. YY1 which is a repressor of the P5 promoter, was revealed to bind E1A and in this context it was able to activate transcription (Seto et al., 1991). YY1 has also been found to bind to the upstream conserved region of the Moloney Murine Leukemia Virus (MuLV) which down-regulates the MuLV promoter activity (Flanagan et al., 1992).

The gene regulatory role of YY1 in different viruses has important implications. Park and colleagues (Park et al., 1999) have shown that a point mutation in the YY1 binding site of the p97 gene promoter of HPV-16 virus is present in 78% of the neoplasms they examined. This mutation leads to a loss of E2 gene expression which subsequently up-regulates the E6 and E7 genes (Park et al., 1999). The protein products of E6 and E7 are required for integration of episomal HPV DNA into chromosomal DNA. Thus YY1 plays an important role in viral recombination.

Moriuchi and co-workers (Moriuchi et al., 1999) have shown that YY1 influences the invasiveness of HIV. A number of cellular factors have been demonstrated to directly reduce HIV expression at the promoter level, among them is YY1. For HIV-1 invasiveness, the CD4 molecule is required as well as a fusion/entry co-factor (Margolis et al., 1994; Romerio et al., 1997). Chemokine receptors serve as the fusion/entry co-factors among which CXCR4, a receptor for CXC chemokine stromal-derived factor-1 (SDF-1), is a major co-factor for T-cell tropic HIV-1 (Bleul et al., 1996; Oberlin et al., 1996). The association of

YY1 and c-myc has been shown to mediate activity of the CXCR-4 promoter. Up-regulation of the promoter is influenced by c-myc, while YY1 is able to repress promoter activity. Under normal circumstances, YY1 represses expression of CXCR-4 thereby inhibiting invasiveness. However mutation in the YY1 binding site results in a loss of the repressor activity of YY1 which facilitates HIV-1 invasiveness (Moriuchi et al., 1999).

YY1 is the first known cellular protein found to interact with the vaccinia virus late promoter (Zhu et al., 1998; Zhu et al., 1999). Vaccinia virus is the best studied member of the poxvirus family whose members characteristically have large genomes and replicate in the cytoplasmic compartment of infected cells. Zhu and co-workers (1998) initially identified an unknown cellular protein that was capable of vaccinia virus promoter binding activity. Initial data indicated that the protein was able to bind the promoter at the transcriptional start site and was capable of stimulating transcription *in vitro*. YY1 was identified as the promoter binding protein through promoter binding analysis and SDS-PAGE (Broyles et al., 1999). Mutation of the promoter abolished YY1 binding implicating it as a possible regulator of vaccinia virus late gene transcription (Broyles et al., 1999). An interesting aspect of vaccinia infection that is pertinent to the data presented later in this thesis is that vaccinia replicates in the cytoplasm. Prior to infection, YY1 is located exclusively in the nucleus. Immunofluoresence analysis of vaccinia infected BSC-40 cells indicate that following infection, YY1 is localized to the cytoplasm within the virosomes (Broyles et al., 1999). The mechanism regulating the translocation of YY1 was not identified, and analysis of other transcription factors did not reveal

cytoplasmic translocation suggesting that YY1 was specifically redistributed to the cytoplasm (Broyles et al., 1999).

2.9 Developmental function of YY1

The vast majority of research aimed at deducing the function of YY1 has been carried out in tissue culture model systems. Donohoe and co-workers (Donohoe et al., 1999), were the first to examine the *in vivo* function of YY1 during early development by creating YY1 knockouts in mice and demonstrating an essential role for YY1 in vertebrate development. Embryos (at the blastocyst stage) found to be $YY1^{-/-}$ implanted into uterine tissue but failed to develop to the gastrulation stage thus knockout of YY1 resulted in embryonic death around the time of implantation (Donohoe et al., 1999). Blastocysts that were $YY1^{-/+}$ survived but displayed a variety of defects such as lowered implantation efficiency, neurulation defects, and exencephaly (Donohoe et al., 1999) demonstrating a requirement for both alleles of YY1 for proper development.

Donohoe and co-workers analyzed the nuclear/cytoplasmic distribution of YY1 in the mouse. They showed that YY1 is localized to the cytoplasm of mouse oocytes and remains in this cellular location until the 2-cell stage, at which time zygotic transcription is initiated. At stage E3.5, YY1 is present in the nucleus and cytoplasm of blastocysts as well as the inner cell mass and the trophectoderm of the developing embryos.

Examination of YY1 expression patterns in heterozygous embryos demonstrated that it may be required for later differentiation of mouse tissues (Donohoe et al., 1999). YY1 was found to be highly expressed in somite, tail,

limb bud and ectoplacental cone (Donohoe et al., 1999). While Donohoe and co-workers were able to demonstrate a requirement for YY1 during mouse development, they did not determine its transcriptional activity during development.

3.0 Messenger Ribonucleoprotein Particles

Overview

During oogenesis, which may take up to two years in the frog, a vast supply of $2x10^{11}$ mRNAs and $\sim 10^{12}$ ribosomes are synthesized (Davidson et al., 1982). This pool of mRNAs and ribosomes are necessary to supply the developing embryo with new templates for protein synthesis during early embryonic development until zygotic transcription is initiated at the midblastula transition (MBT), approximately 12 hours post-fertilization (Davidson, 1986). During early oogenesis, less than 2% of poly$(A)^+$ RNA is assembled in polysomes, this amount increases to 20% by stage VI (Smith, 1985). The remaining poly$(A)^+$ RNA is maintained in a stable untranslated state during this period of development by associating with a specific set of masking proteins in early stage oocytes, which form messenger ribonucleoprotein particles (mRNP) (Smith, 1985; Sommerville, 1990; Spirin, 1966). Packaging of the mRNAs into these particles serves an important function, which is to maintain the RNA in a stable and untranslated state until they are required by the developing embryo for new protein synthesis.

3.1 Messenger RNA binding motifs

Thus far a small number of motifs have been described for proteins that bind RNA in the cytoplasm (reviewed by Burd and Dreyfuss, 1994; Cusack, 1999). Although the current knowledge of RNA binding motifs is limited,

structural similarities among different motifs exist. RNA-binding proteins are characteristically modular in structure consisting of defined RNA binding motif(s) as well as auxiliary domains that are able to perform additional functions (Cusack, 1995; Nagai et al., 1996).

3.1.1 RNA Recognition Motif (RRM)

The most common and best characterized RNA binding domain is the RNP domain which is also referred to as the RNA recognition motif (RRM) (Kenan et al., 1991; Query et al., 1989). This motif, first described in the yeast polyadenylate-binding protein, consists of approximately 90-100 amino acids which form the core of the RNA binding domain (Burd and Dreyfuss, 1994a; Mattaj, 1993; Nagai et al., 1995). This motif has been discovered in over 200 proteins such as hnRNP A1 and hnRNP C1/C2 (Dreyfuss et al., 1993), CStF (Takagaki and Manley, 1997), Drb1 (Tamada et al., 2002), U2AF (Kellenberger et al., 2002), TIAR and TIA-1 (Li et al., 2002) that are involved with pre-mRNAs, mRNA, small nuclear RNA (snRNA), pre-ribosomal RNAs ribosomal RNAs (rRNA) and viral RNAs (Bandziulis et al., 1989; Birney et al., 1993; Nagai et al., 1990). This motif is highly conserved amongst animal, plant, fungal, and bacterial proteins suggesting an evolutionary importance in the regulation of RNA (Kenan et al., 1991; Mattaj, 1993).

Characteristic among RRM containing proteins is the RNP consensus sequence (RNP-CS) which is composed of two short regions, RNP1 and RNP2 as well as a number of other predominantly hydrophobic amino acids spread throughout the motif (Bandziulis et al., 1989; Dreyfuss et al., 1988a; Kenan et al.,

1991; Swanson et al., 1987). Some RRM containing RNA-binding proteins lack conserved RNP1 and RNP2 domains, however they contain other well conserved essential residues producing an overall domain framework that is sufficiently similar to the RNP1 and 2 that they retain their function (Birney et al., 1993; Dreyfuss et al., 1988b; Kenan et al., 1991). Structurally the RNP domain consists of a four-stranded antiparallel sheet flanked on one of its sides by two helices (Garrett et al., 1994; Gorlach et al., 1992; Hoffman et al., 1991; Lu and Hall, 1995; MacLellan et al., 1994; Nagai et al., 1990) The RNP1 and RNP2 motifs, located in the two middle sheets are responsible for direct contact with RNA (Burd and Dreyfuss, 1994).

A subset of RRM-containing proteins are able to bind different RNA sequences simultaneously (Dreyfuss et al., 1988a; Swanson et al., 1987). When RNA is bound to the RRP motif, there is little change in the shape of the protein and the RNA is left relatively exposed making it accessible for interaction with other RNAs or RNA binding proteins (Gorlach et al., 1992). This permits the possibility of complimentary nucleic acid annealing, an activity that can change RNA structure in a manner similar to protein folding by chaperones (Krainer et al., 1990; Kumar and Wilson, 1990; Munroe and Dong, 1992; Pontius and Berg, 1990; Portman and Dreyfuss, 1994).

3.1.2 RGG Motif

The RGG RNA binding domain is a 20-25 amino acid long motif that appears to bind RNA non-specifically and is typically found in proteins that contain other RNA binding domains (Kiledjian and Dreyfuss, 1992).

Structurally, the motif contains closely spaced Arg-Gly-Gly (RGG) repeats that are interspersed with other predominantly aromatic amino acids (Burd and Dreyfuss, 1994a). This domain was first identified in hnRNP U which is involved in mRNA biogenesis and is able to bind both RNA and ssDNA (Kiledjian and Dreyfuss, 1992), and has since been identified in Nucleolin (Dreyfuss et al., 1993). The number of RGG repeats required for RNA binding varies, from six repeats in hnRNP A1 to eighteen repeats in the yeast protein GAR1 (Girard et al., 1994).

3.1.3 K-homology (KH) RNA binding domain

The K homology (KH) domain is a motif common among a number of diverse RNA binding proteins (reviewed by Nagai, 1996). First identified in hnRNP K (Siomi et al., 1993), this domain is present in proteins such as ribosomal S3 (Gibson et al., 1993; Siomi et al., 1993), Mer1p, (Engebrecht and Roeder, 1990), FMRP (Ceman et al., 1999; Reyniers et al., 1996), NusA (Gibson et al., 1993), QK1 (Chen and Richard, 1998), Xqua (Zorn et al., 1997) and NOVA (Buckanovich et al., 1993; Ramos et al., 2002) is able to directly bind RNA. Mutation of conserved residues within the domain has been shown to abolish binding to single stranded RNAs (Siomi et al., 1993). Reminiscent of other RNA binding motifs, the KH domain is found alone or in multiple copies or in conjunction with other RNA binding motifs (Ceman et al., 1999 ; Dreyfuss et al., 1993; Gibson et al., 1993; Siomi et al., 1993). The presence of KH domains in a number of diverse organisms suggests that it is an evolutionarily conserved motif with essential cellular functions. For example, a link between human

cognitive function and RNA binding proteins was established by the finding that mutation of the FMR-1 gene whose protein product is FMRP (which contains an RGG domain as well as the KH motif) is responsible for fragile X mental retardation, the most common form of hereditary retardation in males (Eberhart et al., 1996). FMRP has been found to bind its own RNA as well as a subset of brain mRNAs (Ashley et al., 1993; Brown et al., 1988; Siomi et al., 1993; Ceman et al., 1999). As well, FMRP in mRNP complexes has been observed to associate with ribosomes in an RNA-dependent manner (Eberhart et al., 1996, Tamanini et al., 1996, Ceman et al., 1999). The exact mechanism by which FRMP mutation results in mental retardation is not fully understood.

3.1.4 Double stranded RNA binding domain

The double stranded RNA binding domain (dsRBD) is a short sequence motif of approximately 65 amino acid residues (Gibson et al., 1994; St. Johnston et al., 1992) that is found in proteins involved in RNA processing, localization, maturation and most recently post-transcriptional gene silencing (Green and Mathews, 1992; Grishok et al., 2001; Knight and Bass, 2001; Nicholson and Nicholson, 2002; St Johnston et al., 1992). This motif, first discovered in the *Drosophila* protein Staufen is found either in single or multiple copies, and has since been described in a number of eukaryotic and prokaryotic proteins such as; Xlrbpa (Hitti et al., 1998; Eckmann, et al., 1997), hsTRBP (Cosentino et al., 1995), ADAR1 (Brooks et al., 1998), and *Xenopus* RNA binding protein A (Krovat and Jantsch, 1996).

Staufen plays a critical role in the anterior-posterior axis determination in *Drosophila* and was the first protein deemed to be essential for mRNA localization within the developing embryo (St. Johnston, 1995). Staufen associates with two sets of mRNAs that are the axial regulators of the embryo. During oogenesis, Staufen binds *oskar* mRNA and transports it to the posterior pole of the oocyte an event that demarcates the location of the abdomen and future germline (Ephrussi et al., 1991; Kim-Ha et al., 1995; St Johnston et al., 1991). Once the egg has been laid, Staufen accumulates at the anterior pole where it anchors *bicoid* mRNA, which is the anterior determinant of the embryo (Ferrandon et al., 1994; St Johnston et al., 1989). As well, Staufen plays a role in mRNA relocation in somatic cells. During the asymmetric cell divisions of the embryonic neuroblasts, Staufen associates with *prospero* mRNA and mediates its segregation into the apical crescent of neuroblasts (Broadus et al., 1998; Li et al., 1997a; Schuldt et al., 1998).

The mammalian form of Staufen contains 4 dsRBDs, only one of which (dsRBD3) is able to bind RNA with high affininity, although dsRBD4 was shown to have weak RNA binding activity (Wickham et al., 1999). Additionally, Staufen was shown to bind tubulin *in vitro* (Wickham et al., 1999). Examination of Staufen distribution in neurons and fibroblasts showed that it co-localized with markers for the rough endoplasmic reticulum (RER) (Kohrmann et al., 1999; Marion et al., 1999; Wickham et al., 1999). Cell fractionation of Hela cells showed that Staufen was associated with ribosomes and polysomes (Marion et al., 1999). Kohrmann and co-workers (Kohrmann et al., 1999) demonstrated that in humans, Staufen (hStau) is a component of RNA containing granules in

hippocampal neurons. Luo and co-workers (Luo et al., 2002) examining the role of Staufen in COS7 cells have shown that it is predominantly associated with ribosomes. To demonstrate this, point mutations were introduced into either dsRBD3 or both dsRBD3 and dsRBD4. The RNA binding capability of the mutant proteins was analyzed by Northwestern blot analysis which showed that while mutations of both domains were sufficient to eliminate dsRNA binding activity, mutation of the dsRBD3 is essential for RNA binding (Luo et al., 2002). This mutation reduced but did not eliminate the association of Staufen with ribosomes indicating that protein-protein interactions may also play a role as its ability to associate with the ribosomes (Luo et al., 2002). RNase treatment of cell lysates was not sufficient to dissociate Staufen from the ribosomes, suggesting that Staufen-ribosome interactions are not dependent on intact mRNAs (Luo et al., 2002).

The role of Staufen illustrates the essential function that dsRBD proteins play during development. Similar to studies of mRNA localization in other systems, the cis-acting sequences that are necessary for *oskar, bicoid,* and *prospero* reside within the 3'untranslated regions (Kim-Ha et al., 1993; Macdonald and Kerr, 1997). Mutation of several amino acid residues located in the region of protein-RNA interaction result in a blockage of Staufen-dependent localization of RNA within the embryo (Ramos et al., 2000). These results do not indicate whether there is a direct interaction between Staufen and the target RNA, however it does suggest that essential amino acid residues lie within the dsRBD (Schuldt et al., 1998).

Recently a second Staufen homologue (Stau2) has been isolated (Buchner et al., 1999). Stau2 has been found to associate with RNA in cultured neurons suggesting that it may be involved in delivery of RNA to dendrites (Tang et al., 2001). Duchaine and co-workers (Duchaine et al., 2002), showed there are three differentially spliced isoforms ($Stau2^{62}$, $Stau2^{52/59}$). Similar to Stau1, Stau2 was found to be located in the somatodendritic compartment of neurons co-localizing with the microtubules (Duchaine et al., 2002). However double immunofluoresence microscopy showed that Stau1 and Stau2 are not located in the same particles (Duchaine et al., 2002). Stau2 isoforms were shown by cell fractionation to be components of large mass complexes that were not affected by EDTA treatment or high concentrations of KCl. Additionally, RNase treatment was found to release only very small amounts of Stau2 isoforms (Duchaine et al., 2002). The different isoforms of Stau2 were found to be distributed in several complexes. $Stau2^{62}$ was found to fractionate (by sucrose gradient fractionation) to low density fractions which also contain RER and ribosomal protein L7, however these complexes do not contain ribosomes. $Stau2^{52/59}$ were found to fractionate with both the 40S and 60S ribosomal subunits (Duchaine et al., 2002). Ohashi and co-workers (Ohashi et al., 2002) have recently shown that Staufen is released from polyribosomes in conjunction with FMRP, Pur , and Myosin Va. Treatment of lysates with RNase A was sufficient to abolish immunoprecipitation of this complex indicating that it is RNA-depdendent.

3.1.5 Zinc finger knuckle binding domain

The zinc finger knuckle RNA binding domain is composed of critically spaced cysteine and histidine residues which can be generalized as $CX_{2-5}CX_{4-12}C/HX_{2-4}C/H$ (where X can be any amino acid) located within a typical zinc finger DNA binding domain (Burd and Dreyfuss, 1994). Proteins that contain this motif include; TFIIIA (involved in ribosome biosynthesis) (Miller et al., 1985; Searles et al., 2000), NAB2 (involved in mRNA biogenesis) (Anderson et al., 1993), and the RSZ family of splicing factors from Arabidopsis (Lopato et al., 1999). At present, this motif has not been characterized further.

3.1.6 Cold shock domain

The cold shock domain (CSD) family of RNA binding proteins is a diverse group of evolutionarily conserved proteins. They are able to bind single stranded RNA and single stranded DNA and have been found in *Xenopus* (Tafuri and Wolffe, 1990), chicken (Grant and Deeley, 1993), rat (Ozer et al., 1990), mouse, and human (Tafuri et al., 1993). These proteins contain a common structure, the OB fold which consists of five antiparallel -strands that form a barrel structure. Proteins containing this motif have been identified through searches for transcription factors that are able to bind the Y box, a promoter element with the sequence, CTGATTGGCCAA, that is present on the non-coding strand of a variety of genes (Didier et al., 1988; Tafuri and Wolffe, 1993; Wolffe et al., 1992). The CSD itself is one of the most highly conserved motifs having more than 60% sequence similarity between bacteria and vertebrates (Graumann and Marahiel, 1998; Tafuri and Wolffe, 1992; Wolffe, 1994).

Proteins containing CSDs bind to non-translating RNAs during development (Sommerville, 1999). The CSD-containing proteins have been found to associate with DEAD-box RNA helicases and the two protein types appear to coordinately function to maintain the structure of single stranded RNA (ssRNA) (reviewed by Sommerville, 1999).

3.2 Major *Xenopus* mRNPs

The *Xenopus* model is one of the best biological systems in which to study the masking and unmasking of stored mRNPs. In *Xenopus*, the zygotic genome is not activated until the MBT which occurs after the 12th round of cell division approximately 7 hours after fertilization at 23˚C (Gerhart, 1980). Due to this, all mRNAs and proteins that are required for assembly of the first 4096 cells must be present in the unfertilized egg. Measurements of RNA synthesis during oogenesis has revealed that the majority of polymerase II transcription occurs during the first two stages of oogenesis (Bacharova, 1966; Dumont, 1972; Gerhart, 1980). Thus there is a requirement for storage and protection of these mRNAs until they are utilized in later stages of development.

3.2.1 FRGY2

Isolation of Poly(A)$^+$ mRNPs from *Xenopus* oocytes has demonstrated that two major mRNPs are present, p50 and FRGY2 (Minich et al., 1993; Richter and Smith, 1984). p50 originally described as the major core protein of inactive globin mRNPs, posesses strong RNA-binding activity and can be phosphorylated *in vivo* and *in vitro* in somatic cells (Blobel, 1972; Evdokimova et al., 1995;

Minich et al., 1993; van Venrooij et al., 1977). The amount of p50 in non-translating mRNPs is twofold higher than in actively translating mRNPs (Evdokimova and Ovchinnikov, 1999; Minich and Ovchinnikov, 1992). At low concentrations (approximately 10 molecules per globin mRNA), p50 can stimulate translation *in vitro*, conversely at high concentrations (approximately 20 molecules per globin mRNA), p50 fully inhibits translation (Davydova et al., 1997; Evdokimova et al., 1998; Minich et al., 1993). Evdokimova and co-workers cloned rabbit p50 and revealed that it is a member of the Y-box family of transcription factors binds the Y-box in a sequence specific manner but binds binds RNAs without sequence specificity (Evdokimova et al., 1995). The molecular mass of free p50 in solution is 800 kDa (the monomeric form is 35 kDa) however it is not known whether this large complex persists when binding RNA, or if it dissociates into subunits which then bind the RNA. P50 has been shown to promote annealing of complimentary RNA and DNA strands and its DNA binding is mediated throough phosphorylation carried out by casein kinase II (Skabkin et al., 2001).

FRGY2 is the second major component of ribonucleoprotein storage particles in *Xenopus* oocytes. It binds ssRNAs and has been shown to play an essential role in managing masking and transcriptional silencing of maternal mRNAs (Bouvet and Wolffe, 1994; Deschamps et al., 1991; Deschamps et al., 1992; Marello et al., 1992; Murray, 1994; Murray et al., 1992; Ranjan et al., 1993; Tafuri and Wolffe, 1990; Tafuri and Wolffe, 1993; Yurkova and Murray, 1997).

FRGY2 was first identified due to its abundance as indicated by coomassie blue staining of isolated mRNP proteins (Darnbrough and Ford, 1981), and was originally referred to as mRNP3+4 (partnered with several polypeptides). These polypeptides have subsequently been named p54 and p56 (Murray et al., 1991; Richter and Smith, 1983) and pp56 and pp60 (Cummings and Sommerville, 1988), and most recently FRGY2a+b (Tafuri and Wolffe, 1990). mRNP3 and mRNP4 (FRGY2) are closely related polypeptides (85% amino acid homology) and are thought to represent pseudoalleles in *Xenopus* (Murray et al., 1992). Currently mRNP3+4 complexes are known to contain additional polypeptides such as nucleolin, a protein kinase activity and mRNA binding polypeptides of approximately 34 and 36 kDa (Yurkova and Murray, 1997). FRGY2 (and its somatic homolog FRGY1) is particularly interesting as it has dual functions, in RNA binding and transcription through interactions with the Y-box (CTGATTGGCCAA) promoter element in MHC class II genes (Didier et al., 1988; Wolffe et al., 1992) as well as a number of genes selectively active in oocytes (Marello et al., 1992; Tafuri and Wolffe, 1990; Tafuri and Wolffe, 1992; Wolffe et al., 1992).

FRGY2 is highly expressed in the cytoplasm of oocytes, but is absent in somatic cells (Wolffe et al., 1992). Tafuri and Wolffe (1993) demonstrated that FRGY2 binds RNAs non-specifically however, it does not associate with 5 S, 18 S and 28 S rRNAs or tRNAs *in vivo*. FRGY2 accumulates to maximum levels in oocytes by stage II, however storage particles containing FRGY2 assemble later in oogenesis reaching maximum levels by stage IV and persisting until stage VI (Tafuri and Wolffe, 1993). Following fertilization, FRGY2 levels decline

through early development correlating with the utilization of stored maternal mRNAs (Tafuri and Wolffe, 1993).

FRGY2 is phosphorylated early in oogenesis, and its ability to mask mRNAs depends on its phosphorylation state (Dearsly et al., 1985; Kick et al., 1987; Yurkova and Murray, 1997). Several groups have observed casein kinase II-like proteins to be associated with mRNPs (Marello et al., 1992; Murray et al., 1991), which have been proposed to phosphorylate FRGY2. Deschamps and co-workers (Deschamps et al., 1997) indeed showed that FRGY2 is phosphorylated by casein kinase II, however phosphorylation (or dephosphorylation by calf intestinal alkaline phosphatase) did not have any effect on the binding affinity of FRGY2 for RNA (Deschamps et al., 1997).

Reconstitution experiments demonstrated that FRGY2 repressed translation of mRNA templates *in vitro* (Kick et al., 1987; Matsumoto et al., 1996; Ranjan et al., 1993; Richter and Smith, 1984; Yurkova and Murray, 1997). Expression of FRGY2 in somatic cells had two effects, an increase in mRNA accumulation in transcripts expressed from Y-box containing promoters and translational silencing of these mRNAs (Ranjan et al., 1993). Overexpression of FRGY2 in oocytes translationally silenced mRNAs that were synthesized *in vivo* (Bouvet and Wolffe, 1994). Microinjection of antibodies against FRGY2 was sufficient to relieve transcriptional silencing mediated by FRGY2 (Braddock et al., 1994; Gunkel et al., 1995). This data demonstrated that FRGY2 functions in two separate cellular compartments and interacts with both DNA and RNA either as a transcription factor or a translational regulator.

Similar to other RNA binding proteins, FRGY2 is modular in structure. The most prominent nucleic acid binding structure is a cold shock domain near the N-terminus (Bouvet and Wolffe, 1994). Contained within this domain is an RNP-1 RNA binding domain which is able to confer sequence specific RNA binding to FRGY2 (Bouvet et al., 1995; Burd and Dreyfuss, 1994; Schindelin et al., 1993). A second RNA binding domain is near the C-terminal end of FRGY2 (Murray, 1994) which consists of islands of basic/aromatic amino acids that are able to bind RNA non-specifically (Bouvet et al., 1995; Marello et al., 1992; Tafuri and Wolffe, 1990). Matsumoto and co-workers (Matsumoto et al., 1996) showed that both the cold shock domain and the basic/aromatic islands are necessary for stable association of FRGY2 with RNA. FRGY2 has been detected in ribosome containing fractions from sucrose gradients which suggests that its presence did not prevent association of mRNAs with ribosomes (Matsumoto et al., 2000).

There are several aspects of FRGY2 that make it a good model for comparison in this study of YY1. It contains RNA binding domains, and is able to bind both RNA and DNA. FRGY2 protein is present in early oocytes and is assembled into mRNPs during mid to late oogenesis. Following fertilization, FRGY2 levels decline as development proceeds and stored mRNAs are utilized.

3.2.2 CCAAT box transcription factor (CBTF)

CBTF contains two dsRBDs and is a transcriptional activator of the hematopoietic regulatory factor GATA-2. GATA-2 expression has been shown to be necessary for hematopoietic and urogenetical development in mouse (Tsai

et al., 1994; Tsai and Orkin, 1997; Zhou et al., 1998), and has been implicated in the formation of ventral mesoderm in *Xenopus* (Sykes et al., 1998). The binding site for CBTF in the promoter of GATA-2 is evolutionarily conserved, and is required for the proper expression of GATA-2 transcription in amphibian embryos and in avian and murin hematopoietic cells (Brewer et al., 1995; Fleenor et al., 1996; Minegishi et al., 1998; Nony et al., 1998). In *Xenopus*, the 122 kDa subunit of CBTF (CBTF^{122}) changes its subcellular localization through development (Orford et al., 1998). In oocytes, CBTF^{122} is nuclear, relocates to the cytoplasm after germinal vesicle breakdown, and remains in this location until the midblastula transition at which time it relocates to the nucleus (Orford et al., 1998). Orford and co-workers (Orford et al., 1998) have proposed that the subcellular sequestration of CBTF^{122} is essential for proper GATA-2 expression.

Analysis of the amino acid sequence of CBTF^{122} has revealed that it contains two regions that have a high degree of homology to the dsRBD (Brzostowski et al., 2000) and an RGG RNA binding domain in the C-terminal region (Brzostowski et al., 2000). A nuclear localization signal has also been located preceding the first dsRBD (Brzostowski et al., 2000). Northern analysis of RNAs isolated from oocytes and embryos showed that CBTF^{122} mRNAs are present during development, and Western blot analysis demonstrated that CBTF^{122} levels accumulate through oogenesis (Brzostowski et al., 2000). Co-immunporecipitation experiments using CBTF^{122} antibodies followed by Western blotting indicated that CBTF^{122} is part of mRNP3+4 complexes (Brzostowski et al., 2000; Yurkova and Murray, 1997).

Brzostowski et al, examined three potential mechanisms for cytoplasmic retention of CBTF^{122}; phosphorylation state of the protein, binding to the cytoskeleton, and RNA anchoring (Brzostowski et al., 2000). Neither disruption of the cytoskeleton nor phosphorylation were able to bring about translocation of CBTF^{122} to the nucleus of embryos (Brzostowski et al., 2000). However, degradation of endogenous RNAs in pre-MBT embryos was sufficient to cause nuclear accumulation of CBTF^{122} (Brzostowski et al., 2000). Thus it was postulated that degradation of maternal mRNAs at the MBT (Duval et al., 1990) is likely to cause release of CBTF^{122} from the cytoplasm and nuclear relocation (Brzostowski et al., 2000). This mechanism appears to couple activation of the zygotic genome with the depletion of maternal mRNAs.

3.2.3 Cold inducible RNA binding protein

The cold inducible RNA binding protein (CIRP) which contains both RRM and RGG domains has recently been identified as a major cytoplasmic RNA-binding protein in *Xenopus* oocytes (Matsumoto et al., 2000). CIRP2 was first identified in mouse by cDNA cloning of an RRM containing RNA binding protein (Nishiyama et al., 1997). In mouse CIRP is found to be highly expressed in testis where the temperature is maintained below that of the body. Levels of CIRP mRNA were found to increase upon temperature downshift from 37˚C to 32˚C (Nishiyama et al., 1997). Matsumoto and co-workers (Matsumoto et al., 2000) found that the *Xenopus* form of CIRP (xCIRP) is abundantly expressed in testis, ovary, brain, liver, kidney, and heart. The subcellular localization of xCIRP was determined by Western blotting of oocyte cytoplasmic fractions.

These experiments showed that xCIRP is predominantly localized to the cytoplasm (Matsumoto et al., 2000). This is in contrast to mammalian cells where CIRP2 is localized to nuclei (Nishiyama et al., 1997). This discrepancy may be due to cell type; CIRP may be nuclear in somatic cells and cytoplasmic in germ line cells (Matsumoto et al., 2000). Nishiyama and colleagues (Nishiyama et al., 1998) have provided evidence for this hypothesis by showing that in mouse testis, CIRP was cytoplasmically localized, while it was found to be nuclear in round spermatids. In *Xenopus,* it is also possible that xCIRP2 does not possess a nuclear localization signal that is present in the mammalian form of the protein despite the high degree of amino acid conservation (82-83%) between the mammalian and amphibian forms of the protein (Matsumoto et al., 2000).

Gel retardation assays demonstrated that both xCIRP2 and FRGY2 were able to bind the same histone H1 mRNA simultaneously (Matsumoto et al., 2000). Interestingly, density gradient and Nycodenz gradient fractionation of oocyte lysates revealed that xCIRP2 associates with ribosomes in the cytoplasm (Matsumoto et al., 2000). The association between cytoplasmic RNA binding proteins and ribosomes has been observed previously with FRGY2 (Sommerville, 1990; Tafuri and Wolffe, 1993). Separation of oocyte lysates by sucrose gradient centrifugation revealed that xCIRP2 was associated with monosomes (Matsumoto et al., 2000).

The finding that both xCIRP2 and FRGY2 are associated with ribosomes in oocytes is intriguing. FRGY2 displays a 43% amino acid homology with the bacterial cold shock inducible protein CspA, as well as a C-terminal translation repression domain (Graumann and Marahiel, 1998; Matsumoto and Wolffe,

1998; Sommerville and Ladomery, 1996). Matsumoto and co-workers have proposed a function for the association observed between xCIRP2, FRGY2 and ribosomes. They propose that similar to the function of the bacterial cold shock protein CspA which acts as a ribosome bound modulator of translation, xCIRP2 may function in the regulation of translational efficiency of specific mRNAs by associating with ribosomes while FRGY2 acts to repress overall transcription in the oocyte (Matsumoto et al., 2000).

3.2.4 TFIIIA

The best studied protein containing a zinc finger-knuckle motif is TFIIIA which contains 9 zinc fingers, the middle three of which contain the zinc finger RNA binding motif (Miller et al., 1985; Searles et al., 2000). This transcription factor has two essential functions in *Xenopus* which exhibit its versatility to interact with both DNA and RNA. TFIIIA is a transcription factor that is required for the expression of 5S ribosomal RNA (5S rRNA) genes. Binding of TFIIIA to the internal coding region of the 5s rRNA gene is mediated through binding of the first three zinc fingers (Clemens et al., 1993; Hamilton et al., 2001). The second role that TFIIIA plays is as an RNA binding protein in the cytoplasm of developing oocytes. The association of TFIIIA with 5S rRNA (within the central half of the molecule) forms the 42S ribonucleoprotein particle which functions to stabilize the 5S rRNA until it is required for ribosome assembly (Baudin et al., 1989; Baudin et al., 1991; Bogenhagen et al., 1992; McBryant et al., 1995; Rawlings et al., 1996; Setzer et al., 1996; Theunissen et al., 1998; Searles et al., 2000).

3.2.5 RNA-Associated protein 55

RNA-associated protein (with a molecular mass of 55 kDa, RAP55) is a recently discovered protein found to associate with RNAs in *Xenopus* oocytes (Lieb et al., 1998). Sequence analysis of RAP55 has indicated that it contains two RGG domains located at the C-terminus (Lieb et al., 1998). An examination of the subcellular localization of RAP55 has show that it is located in the cytoplasm in oocytes, however following fertilization, the levels of RAP55 decrease until stage 6 after which it is no longer detectable (Lieb et al., 1998). Interestingly, following the loss of detection of RAP55, a 70 kDa protein was detected in its place. Lieb and co-workers consider it possible that the 70 kDa protein represents a post-translationally modified form of RAP55, however they did not fully investigate this possibility (Lieb et al., 1998). Initial characterization of RAP55 interactions with RNA has suggested that it does not directly bind cytoplasmic RNAs (Lieb et al., 1998). The exact function that RAP55 plays in the developing frog is not currently known however Lieb and co-workers (Lieb et al., 1998) postulate that it may function as an inhibitor of cytoplasmic polyadenylation element binding protein (CPEB) thus blocking polyadenylation and the resulting translational activation.

3.2.6 RNA helicases

RNA helicases catalyze the unwinding of RNA duplexes in an ATP-dependent manner and are implicated in many cellular processes that require reorganization of RNA structure, such as transcription, mRNA splicing and

editing, translation initiation, export and degradation (reviewed by Lorsch, 2002). Single stranded RNAs are able to form double helical regions, the minimum requirement being the presence of complimentary sequences within the same molecule which can form double helices (reviewed by Lorsch, 2002). The longer the RNA molecule, the higher the chance that two regions within the same molecule are able to hybridize. Karpel and co-workers (Karpel et al., 1982) were the first to identify a class of RNA binding protein that are able to identify errant double helical RNA regions, and return them to their native single stranded state. These proteins termed nucleic acid destabilizing proteins, function by destabilization of RNA structures, and inducing a single stranded molecule in their place. The DEAD box class of RNA-dependent ATPases contain conserved motifs, including a conserved NTPase 'A' (AXXGXXGKT) and 'B' (DEAD) motifs that are required for RNA binding and hydrolysis. (Schmid et al., 1992; Staley and Guthrie, 1998; Tanner and Linder, 2001).

In *Xenopus* an abundant helicase activity has been observed to associate with the major RNA binding complex, mRNP3+4 (Ladomery et al., 1997). Photocrosslinking of [-^{32}P] ATP to mRNPs in solution showed that only two proteins having molecular masses of 54 and 68 kDa were covalently labeled (Ladomery et al., 1997). Isolation of the 54 kDa ATP-binding protein followed by cyanogen bromide digestion resulted in a series of polypeptides that were partially sequenced. These sequences were aligned with the human p54 helicase, and were found to be a perfect match (Ladomery et al., 1997). The full length *Xenopus* homolog was subsequently cloned by PCR using degenerate primers (Ladomery et al., 1997). Xp54 possess an ATP-dependent RNA helicase activity

and belongs to a family of DEAD box helicases which includes human p54 (Lu et al., 1992), mouse p54 (Akao et al., 1995), *Drosophila* ME31B (de Valoir et al., 1991), *S. pombe* Ste13 (Maekawa et al., 1994), and *S.cerevisae* DHH1 (Strahl-Bolsinger and Tanner, 1993).

Western blotting for protein levels in developing oocytes showed that Xp54 levels are highest in stage I oocytes and remain constant throughout the remainder of oogenesis, fertilization and to blastula stages after which they decline (Ladomery et al., 1997). Immunostaining of oocytes showed that Xp54 is predominantly localized to the cytoplasm. When *Xenopus* tissue culture cells were examined a small portion of Xp54 was found to be localized to the nucleus (Ladomery et al., 1997). In order to determine whether Xp54 was associated with mRNPs in oocytes, lysates were separated through glycerol gradients. Analysis of gradient fractions by Western blotting indicated that Xp54 sediments with FRGY2 suggesting that both proteins are able to bind the same population of mRNAs (Ladomery et al., 1997).

Two possible scenarios may explain the function of Xp54 in the oocyte. With regard to its presence in mRNPs, Xp54 may be required for efficient translational recruitment of stored mRNAs (Ladomery et al., 1997). In this case, Xp54 is thought to facilitate efficient translation by unwinding RNAs mobilized from mRNPs. Akao and co-workers (Akao et al., 1995) have observed mouse p54 to be present on the rough endoplasmic reticulum, consistent with such a role in translation. Alternatively, Xp54 may be involved in the formation of mRNPs which occurs in the nucleus (Sommerville and Ladomery, 1996). In this scenario, Xp54 would participate in RNA unwinding resulting in single stranded

RNAs, to which FRGY2 (which has a binding preference for single stranded RNAs) would then bind (Marello et al., 1992). Immunostaining of *Xenopus* tissue culture cells revealed some reactivity to Xp54 in nuclei (Ladomery et al., 1997). Analysis of Xp54 expression and distribution in oocytes and early embryonic stages showed that Xp54 levels are highest in stage I/II oocytes, and decline through oogenesis such that by stage VI, no nuclear signal is present (Smillie and Sommerville, 2002). Since RNA transcription from lampbrush chromosomes is highest in stage I/II oocytes, and the majority of stored mRNPs are established by the end of stage II, it is possible that Xp54 is incorporated into mRNP particles as they are formed in the nucleus (Smillie and Sommerville, 2002).

4.0 *Xenopus laevis* Development

4.1 Fertilization and developmental outline

Xenopus laevis embryos and oocytes are a widely used and well characterized model system amenable to a variety of experimental studies. From a single female frog, large numbers of oocytes or embryos can be obtained and easily cultured in simple salt solutions. Eggs can be fertilized *in vitro*, and embryos develop outside the mother therefore making then easily accessible for experimentation. The oocytes, eggs and embryos are large (0.5mm-2mm), which provide ample amounts of cellular material for biochemical analyses, and allowing for experimental manipulations such as microinjection and microsurgery. In addition to these morphological aspects, *Xenopus* development has been fully characterized from fertilization to maturity (Nieuwkoop and Faber, 1967).

4.2 Oogenesis

The ovaries of mature *Xenopus* females consist of 24 lobes which vary in size throughout the year (Dumont, 1972). During oogenesis, which may last 6-8 months or longer, a large reservoir of cellular materials essential for early embryonic growth accumulates. The majority of this accumulation occurs during the extended meiotic prophase I. Cellular components synthesized during this time include general and specific transcription factors, RNA and DNA polymerases, tRNA, deoxyribonucleic triphosphates, yolk proteins, and ribosomes, as well as a large and diverse store of mRNAs which are sufficient to

direct protein synthesis to the blastula stage (Davidson and Hough, 1971; Bravo and Knowland, 1979; Woodland, 1980; Laskey 1974). Reorganization of cytoskeletal elements also occur during oogenesis (Gard et al., 1995; Klymkowsky and Karnovsky, 1994). These rearrangements influence the distribution of many organelles and molecules that are transported, anchored, sequestered or otherwise organized along the cytoskeleton.

When the oocytes have matured to stage VI they arrest in metaphase I. Before the oocytes mature further (into a fully developed egg) completion of the cell cycle is required. Progesterone secreted by the ovarian follicle in response to gonaotropic hormones released by the pituitary induce completion of the cell cycle (Dumont, 1972). Responding to the progesterone, the oocytes undergo several biochemical changes, including a decrease in cAMP levels, increases in both protein synthesis, and total protein phosphorylation levels along with activation of maturation promoting factor (MPF) (Maller et al., 1979; Wasserman et al., 1982; Maller et al., 1977; Wu and Gerhart, 1980). Approximately 6 hours following progesterone release, the germinal vesicle begins to degrade, followed by passage of the oocyte through the oviduct (ovulation). At this point, the oocyte has progressed to metaphase II of meiosis and is termed an egg. Fertilization stimulates the egg to complete the second meiotic division at which time embryonic development begins.

4.2.1 Stages of oocyte development

Oocytes have been divided into 6 distinct developmental stages based on both morphological and physiological changes. Stage I oocytes which are

arrested in prophase I, are transparent and are 50-300*u*m in diameter. At this stage both the germinal vesicle (nucleus) and mitochondrial mass are visible and the chromosomes are very active in tRNA transcription (Dumont, 1972). Vitellogenin (the yolk precursor protein which is synthesized in the liver) begins to accumulate and the oocyte is tightly associated with the follicle cells (Dumont, 1972). This is the period during which mRNA transcription and mRNP accumulation is at its highest levels (Wahli et al., 1981). Stage II oocytes assume an opaqueness and increase in size to 300-450*u*m. During this stage the oocyte begins to accumulate large amounts of vitellogenin, and its chromosomes begin to exhibit a lampbrush morphology permitting a high rate of transcription (Dumont, 1972). The follicle cells begin to separate and numerous microvilli form on the surface of the oocyte. Stage III oocytes show extensive lampbrush chromosomes, absorption of vitellogenin continues, and the size of the oocyte increases to 450-600*u*m. Pigment begins to appear and is dispersed uniformly on the entire oocyte surface. By stage IV, oocytes (600-1000*u*m in diameter) begin to show separation of animal and vegetal poles; the darkly pigmented animal pole is separated from the lightly pigmented vegetal hemisphere. At this point, vitellogenin uptake is maximized with the majority deposited in the vegetal hemisphere. Stage V oocytes are 1000-1200*u*m in diameter with clearly separated animal and vegetal hemispheres. The chromosomes are fully condensed, and vitellogenin uptake ceases (Dumont, 1972). Once the oocytes have progressed to stage VI they are 1200-1300um in diameter. The animal and vegetal hemispheres are separated by an unpigmented equatorial band approximately 0.2mm wide. The cytoplasm is highly stratified with

mitochondria and cortical granules (organelles containing proteolytic enzymes) located at the oocyte surface, while the yolk platelets aggregate toward the vegetal pole. Glycogen granules and endoplasmic reticulum amass in the animal hemisphere where the large nucleus (germinal vesicle) is located near the surface. Total protein synthesis during oogenesis increases 127 fold from 0.18ng/hr^{-1} in stage I oocytes to 22.8ng/hr^{-1} in stage VI oocytes (Taylor and Smith, 1985).

4.3 RNA synthesis during oogenesis

Early research focussed on the rate and nature of RNAs synthesized in the oocyte. RNA synthesis in oocytes is initiated during stage I at which time lampbrush chromosomes facilitate high rates of transcription (Pardue and Gall, 1969; Thomas, 1970;Hill and Macgregor, 1980;Ficq, 1970; Gerhart, 1980). It was previously believed that mature lampbrush chromosomes reach a maximum level at stage III oocytes then decline, however Martin and co-workers (Martin et al., 1980) have demonstrated that they persist in fully mature stage VI oocytes. Thus, lampbrush chromosomes are present throughout oogenesis, however they are only readily observed in stages III-IV.

Anderson and Smith (1977; 1978) measured synthesis rates of RNA in both stage III and stage VI oocytes. They found that the transcription rate in oocytes is approximately 10-20 nt sec^{-1} (Anderson and Smith, 1978). Oocytes of both stages are capable of synthesizing unstable high molecular weight (<7kb) RNAs as well as a greater amount of unstable, smaller RNA. The half lives of the RNAs in stage III is shorter than in stage VI, the result of which is an

accumulation of RNAs in the latter stage oocytes (Anderson, 1977). They showed that accumulation of RNA in the cytoplasm proceeds at 1.7-2.2 pg min^{-1} (Anderson, 1982). The total amount of RNA in an oocyte is seen to increase from 0.04*u*g in stage I to at least 4.3*u*g in mature stage VI oocytes (Rosbash et al., 1974). Of the total amount of RNA in a mature oocyte, 0.7-1.0% (~40*n*g) is $poly(A)^+$ RNA representing approximately 20,000 different species of mRNA (Perlman and Rosbash, 1978). Ribosomal RNA comprises the major portion (80-90%) of the remainder of total RNA (Rosbash et al., 1974).

4.3.1 Splicing

The capacity of *Xenopus* oocytes to carry out splicing reactions was discovered by injecting cloned genes that included introns followed by analysis of proper translation of the protein. Wickens and co-workers (Wickens et al., 1980) injected clones that coded for ovalbumin which was correctly expressed. Proper expression of ovalbumin required that seven introns be removed in order to produce the functional protein. Rungger and Turler (1978) were able to inject oocytes with clones that coded for the SV40 T antigen. In this case the mRNA was spliced correctly with the removal of one intron producing the correct protein. Additionally, several groups were able to show accurate splicing of yeast $tRNA^{tyr}$ (Melton et al., 1980; Nishikura and De Robertis, 1981). This splicing reaction involves the removal of a 5' leader sequence as well as nucleotides at the 3' in addition to several base modifications. The enzymes required for these reactions are located in the nucleus (De Robertis et al., 1981).

4.3.2 Ribosomal RNA accumulation

In the developing oocyte, approximately 10^{12} ribosomes are assembled for use during early development of the frog. Synthesis of the ribosomes is initiated in stage I oocytes whose primary transcription products are tRNAs and 5S RNAs (Thomas, 1974). Thomas (Thomas, 1974) conducted experiments which showed that following 24 hours of labeling, 24% of the labeled RNA were tRNAs while 39% is 5S rRNAs. Confirming this data, Rosbash and Ford (Rosbash et al., 1974) showed that 5S RNAs and tRNAs account for approximately 80% of the total RNA present in previtellogenic oocytes. Synthesis of 18 and 28S RNA are initated at stage I, and their levels increase through to stage VI, while the synthesis of 5S RNA and tRNA declines through the remainder of oogenesis (Davidson, 1986). Approximately 50% of the 5S RNA synthesized in previtellogenic oocytes is stored in a 7S particle where it is complexed with TFIIIA (Hamilton et al., 2001). The remaining 50% is present in 42S particles which also contain TFIIIA, and tRNA (Barrett et al., 1983; Denis et al., 1972; Guddat et al., 1990; Hamilton et al., 2001; Picard et al., 1980; Picard and Wegnez, 1979). 42S particles have a half life of 4-36 hours depending on the species of associated tRNA (Denis and le Maire, 1985). Following synthesis of 18/28S rRNAs, they are packaged with a number of ribosomal proteins and then translocated to the cytoplasm as 40S and 60S ribosomal subunits. However storage of ribosomal precursors in the cytoplasm has not been fully characterized.

The requirement of the developing oocyte for 18 and 28S rRNA is the same as that for 5S rRNA however it is accumulated in a different mechanism.

While there are approximately 20,000 5S rRNA genes, there is a significantly smaller number of genes that code for 18 and 28S rRNAs (approximately 600 per haploid genome) (Brown and Weber, 1968; Davidson, 1986). In order to obtain the correct amount of 18 and 28S rRNAs, their genes are amplified during early oogenesis and packaged into extrachromsosmal circular DNA molecules (rDNA) that are then packaged into nucleolar structures (Hourcade et al., 1973; Peacock, 1965). Transcription of the ribosomal DNA during oogenesis thus occurs from the amplified ribosomal DNA. The amplification process results in approximately 2.5 x 10^6 rRNA genes (Thiebaud, 1979), resulting in an approximate 1400-fold increase in gene number.

Table 1. RNA amounts in stage VI oocytes.

Type	Amount	Reference
mRNA	70*n*g	(Dolecki and Smith, 1979)
rRNA	3.8*u*g	(Scheer, 1973)
tRNA	60*n*g	(Gordon and Wickens, 1993)
Ribosomes	10^{12}	(Davidson, 1986)

4.3.3 Polyadenylation

The majority of masked RNAs in the oocyte have short poly(A) tails. As oocyte maturation proceeds, these tails lengthen concomitant with unmasking and translational activation (McGrew et al., 1989; Stebbins-Boaz et al., 1999; Wickens et al., 1997). It is generally believed that polyadenylation of mRNAs confers translational activation while deadenylation results in translational

silencing. A number of laboratories have established that the translation of many *Xenopus* maternal mRNAs such as cyclins A1 and B1 (Sheets et al., 1994; Stebbins-Boaz et al., 1996), B4 (Paris and Richter, 1990; Stebbins-Boaz et al., 1996), c-mos (Sheets et al., 1994; Stebbins-Boaz et al., 1996), and cdk2 (Stebbins-Boaz et al., 1996; Stebbins-Boaz and Richter, 1994) is dependent on polyadenylation. The 3' untranslated regions contain critical information directing adenylation and deadenylation of mRNAs in oocytes (Barkoff et al., 1998; Richter, 1991; Wormington, 1993).

4.3.3.1 *Cis* Elements required for polyadenylation

Two sequences in the 3' UTR are responsible for polyadenylation. The first sequence which is nearly ubiquitous, AAUAAA is also required for nuclear pre-cleavage and polyadenylation, and a U-rich region (cytoplasmic polyadenylation element, CPE) that is usually located approximately 20 nucleotides 5' of the AAUAAA (reviewed in (Richter, 1999). During maturation, the polyadenylation of differing mRNAs may vary. This is controlled by additional regulatory information contained in the CPE, additionally the location of the CPE in relation to the hexanucleotide influences the timing of 3' end modification (Ballantyne et al., 1997; de Moor and Richter, 1997). For example, maturation of the oocyte is dependent on Mos which is an activator of mitogen-activated protein kinase kinase (MAPK) (Posada et al., 1993; Shibuya et al., 1996). The translational control of Mos is controlled by polyadenylation of Mos (Sheets et al., 1994; Sheets et al., 1995). In the immature oocyte Mos mRNAs have short poly-A tails which lengthen as Mos protein is needed by the

oocyte (Sheets et al., 1994; Fox et al., 1989; McGrew et al., 1989; Paris et al., 1991).

4.3.3.2 Cytoplasmic factors required for polyadenylation

Three factors are necessary for polyadenylation to be carried out on transcripts during maturation. The cytoplasmic polyadenylation element (CPE) is bound by cytoplasmic polyadenylation element binding protein (CPEB), which is an RNA recognition mofit (RRM) containing protein (Bally-Cuif et al., 1998; Gebauer and Richter, 1996; Hake and Richter, 1994; Minshall et al., 1999). It is believed that CPEB functions to recruit and/or stabilize the cytoplasmic form of CPSF which binds the hexanucleotide sequence (Dickson et al., 1999). CPSF functions to bind Poly(A) polymerase (PAP) which is necessary to synthesize the addition of the adenine residues to the transcript (reviewed in (Stebbins-Boaz et al., 1999). Dickson and co-workers (Dickson et al., 2001), have recently shown that CPSF and PAP are able to initiate *in vivo* polyadenylation of a subset of mRNAs in the absence of CBEP.

4.3.2.3 Elements required for deadenylation

Cytoplasmic deadenylation is associated with translational silencing of mRNAs in the oocyte. The mRNAs encoding ribosomal proteins act as a good example of this. During oocyte maturation, these transcripts are translationally silenced (Varnum et al., 1992; Varnum and Wormington, 1990). Deadenylation of these mRNAs is not controlled by a specific signal, rather it is controlled by the lack of a CPE therefore the deadenylated state is reached by default (Fox and

Wickens, 1990; Varnum and Wormington, 1990). A contrasting scenario exists in the embryo which requires a specific mRNA deadenylation signal. During oocyte development, cdk2 mRNAs are adenylated (Stebbins-Boaz et al., 1996; Stebbins-Boaz and Richter, 1994) through the standard CPE-mediated pathway, however soon after fertilization these transcripts are deadenylated. The deadenylation step requires two sequences, a 58 nucleotide element immediately upstream of the CPE and a 14 nucleotide element situated downstream (Stebbins-Boaz and Richter, 1994). Each of these is capable of promoting a partial deadenylation of transcripts, but together they facilitate a complete deadenylation of transcripts and the consequent translational inactivation (Stebbins-Boaz and Richter, 1994). While the deadenylation signals in cdk2 have been identified, they are not inherently obvious in other embryonic transcripts.

4.4 Localization of RNAs within *Xenopus* oocytes.

A key feature of *Xenopus* development is that molecular components such as proteins and mRNAs are asymmetrically distributed in oocytes (Carpenter et al., 1982; King, 1985; Moen and Namenwirth, 1977; Phillips, 1982; Sagata et al., 1980). Moen and Namenwirth (1977) were able to show that differences existed in molecular components between animal and vegetal hemispheres by freezing and sectioning the oocytes perpendicular to the A-V axis followed by 2 dimensional gel electrophoresis. King (1985) isolated poly(A)$^+$ RNA from animal and vegetal hemispheres which was used as templates for translation and found that a number of RNAs were enriched in the vegetal hemisphere (King, 1985). Rebagliati and co-workers (Rebagliati et al.,

1985) were the first to identify messages that are preferentially localized to the animal or vegetal poles. This was shown by isolating poly(A)-RNAs from whole oocytes which were used to generate cDNA libraries. Analysis of animal and vegetal cDNA libraries showed that there is a qualitative difference in messages localized within each hemisphere. Four messages, localized to the animal hemisphere were An1, An2, and An3, while Vg1 was sequestered in the vegetal hemisphere (Rebagliati et al., 1985). Northern blot analysis of RNAs isolated from eggs showed that An messages are highly concentrated to the the animal pole of the oocyte while Vg messages are restricted to the vegetal pole and they remain in these locations after fertilization (Rebagliati et al., 1985). Since the discovery of these messages, several others have been identified. The information shown in Table 2 was obtained from Mowry and Cote, 1999 (Mowry and Cote, 1999) and lists RNAs localized to either the animal or vegetal hemispheres.

Table 2. RNAs localized within *Xenopus* oocytes.

RNA	Encoded Protein	Reference:
Localized to the vegetal hemisphere:		
Xcat-2	potential RNA helicase	(Mosquera et al., 1993)
VegT	T-box transcription factor	(Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang et al., 1998)
Xcat-3	*nanos* like zinc finger protein	(Elinson et al., 1993)
Xdazl	related to DAZ	(Houston et al., 1998)
Xlsirts	nontranslated RNA	(Kloc et al., 1993)
Xpat	novel protein	(Hudson and Woodland, 1998)
Xwnt11	wnt protein	(Ku and Melton, 1993)
Localized to the animal hemisphere:		
Oct-60	POU domain transcription factor	(Hinkley et al., 1992)
Xlan4	novel protein	(Reddy et al., 1992)
X121	novel protein	(Kloc et al., 1991)
PABP	Poly(A) binding protein	(Schroeder and Yost, 1996)

Retention of RNAs within the vegetal hemisphere appears to be mediated through binding or attachment of the mRNAs to cortical cytoskeletal elements (Forristall et al., 1995; Kloc and Etkin, 1995; Yisraeli et al., 1990). Localization of mRNAs to the animal hemisphere has not been as well documented however it is believed that mRNAs destined for this region of the oocyte are retained by an entrapment mechanism (reviewed in (Mowry and Cote, 1999). Interestingly, RNAs retained in the vegetal hemisphere appear to be tightly regionalized to the cortex while those located in the animal hemisphere appear to be present in a

graded distribution along the A-V axis (reviewed in (Mowry and Cote, 1999; Perry-O'Keefe, 1990)

Polarity of the developing oocyte and embryo is determined by differential localization of RNAs. Certain subsets of RNAs within somatic cells may encode proteins that play essential roles in the establishment and maintenance of cell motility and may provide the cell with regional functional specification (reviewed by St. Johnston, 1995). For example, Vg1 mRNA is localized to the vegetal pole of the oocyte. Vg1 has been shown to be an inducer of endodermal tissues (Henry et al., 1996). Overexpression of mutant Vg1 results in a disruption of endoderm formation (Joseph and Melton, 1997), suggesting Vg1 acts as a positive regulator of endodermal formation. The specific localization of this message is governed by by a 340 nucleotide sequence (vegetal localization element, VLE) located in the 3' UTR (Mowry and Melton, 1992). This sequence is recognized by Vg1RBP (VERA), which is an RNA binding protein containing two RRMs and four KH domains (Git and Standart, 2002). Retention of Vg1 appears to be governed by Xlsirts which is also localized to the vegetal hemisphere (Reddy et al., 1992). Xlsirts contains a 81 nucleotide sequence that is randomly repeated between 1 and 13 times (Kloc and Etkin, 1994). Destruction of Xlsirts by microinjection of antisense oligonucleotides results in release of Vg1 from the vegetal hemisphere which suggests that Xlsirts is essential for Vg1 localization (Kloc and Etkin, 1994). In the absence of this sequence Vg1 does not localize to the vegetal hemisphere. Conversely if this sequence is placed into other mRNAs, they are localized to the vegetal hemisphere.

Xenopus oocytes are first polarized along the animal-vegetal hemisphere axis (Cheng and Bjerknes, 1989). Axis specification in oocytes begins by mitotic divisions of oogonia which produce 16 cells that are connected by a cytoplasmic bridge (Coggins, 1973). After these cells have been generated the first signs of cellular polarity are present and are dictated by the position of the cytoplasmic bridge, the centriole pair and the chromosomes (al-Mukhtar and Webb, 1971; Kalt, 1973). Gerhart and co-workers (Gerhart, 1986) have proposed that the alignment of these structures within the developing oocyte specifies the animal-vegetal axis that becomes visually evident by mid-oogenesis.

The developmental potential along the A-V axis is coincident with the fate map of the three primary germ layers, ectoderm, endoderm and mesoderm (reviewed by Heasman, 1997). Cells derived from the animal hemisphere give rise to ectodermal structures such as skin and nervous system. Cell arising from the vegetal pole develop into endodermal structures, primarily the gut. Mesodermal structures are derived from cell arising from the marginal zone (Dale and Slack, 1987).

4.5 Embryogenesis

Xenopus development has been very well characterized at the morphological level (Nieuwkoop and Faber, 1967). Newly laid *Xenopus* eggs lie within a clear vitelline membrane encased by a jelly coating. Fertilization occurs when a single sperm enters the animal hemisphere, blocking further fertilization, as well as marking the future ventral side of the embryo. This also releases the embryos from metaphase II thus allowing completion of the second mitotic

division (Gerhart et al., 1980). Following fertilization, the vitelline membrane detaches from the embryonic surface. The A-V axis established during oogenesis is disrupted during the first cell cycle due to cortical rotation of the embryo where the cortex rotates 30° relative to the interior (Vincent and Gerhart, 1987). The first cell division occurs approximately 90 minutes after fertilization followed by 12 subsequent synchronous divisions occurring at 30-35 minute intervals producing an embryo with 4096 cells (Graham and Morgan, 1966). These cell cycles are biphasic, oscillating rapidly between S and M phases (Newport and Kirschner, 1982 a,b; Graham and Morgan, 1966). G1 and G2 phases as well as zygotic transcription are absent at this time (Graham and Morgan, 1966; Newport and Kirschner, 1982 a,b). As the embryo develops, the yolk proteins are degraded and the derived amino acids are used for *de novo* protein synthesis (Tata, 1976; Wahli et al., 1981). The MBT occurs 13 cell divisions after fertilization, at which time the zygotic genome is activated (Newport and Kirschner, 1982 a,b; Brown and Littna, 1964; Bacharova and Davidson, 1966; Gerhart, 1980).

Following fertilization, the first cleavage occurs in the axial plane, separating the embryo into right and left halves. The embryo then divides at right angles to the first, separating future dorsal and ventral aspects of the embryo. The third cleavage is equatorial, separating the animal and vegetal hemispheres. As in other systems, the large cells of the early embryo are referred to as blastomeres. As development proceeds, a cavity is formed within the animal hemisphere called the blastocoel at which point the embryo is referred to as a blastula (Kalt, 1971).

During cleavage stages, the number of cells increases exponentially while the gross size of the zygote remains unchanged (Newport and Kirschner, 1982a,b). Prior to the midblastula transition, there is no measurable zygotic transcription (although there is a low rate of mitochondrial RNA synthesis) (Chase and Dawid, 1972). Therefore the developing zygote must rely on maternally transcribed and stored mRNAs as well as proteins that were translated during oogenesis (Newport and Kirschner, 1982a,b). The midblastula transition marks a dramatic shift in the cell cycle. At this time both G1 and G2 phases are re-acquired, cell divisions become asynchronous, zygotic transcription is initiated, and cells within the embryo become motile (Newport and Kirschner, 1982a,b).

The mechanism(s) controlling the onset of the midblastula transition has been described by several hypothesis. Newport and Kirschner (1982a) demonstrated that the timing of the MBT is independent of time after fertilization, cleavage number, number of nuclear divisions, and RNA synthesis, however it may be dependent on a critical nucleocytoplasmic ratio. They suggested that the nucleocytoplasmic ratio is detected by cytoplasmic factors present in the unfertilized egg which may be titrated by a critical mass of DNA that is reached only at the midblastula transition (Newport and Kirschner, 1982a). Prioleau and colleagues (1994) demonstrated that pre-MBT gene repression was not due to an unavailability of TBP or a transcriptional apparatus. Rather, they showed that the mechanism controlling the onset of the midblastula transition appears to be depletion of the large store of histones accumulated during oogenesis. Previous studies have shown that pre-midblastula transition embryos

contain a very large reservoir of histones (an amount sufficient to package ~ 20, 000 nuclei) (Adamson and Woodland, 1974; Woodland and Adamson, 1977). Prioleau et al (1994) provided evidence that the excess pool of histone could repress gene expression by means of a dynamic competition of between chromatin assembly and the formation of transcription complexes (Prioleau et al., 1994). The depletion of maternally produced histone through packaging of newly synthesized DNA was hypothesized to permit the assembly of transcription complexes at a given point, defining the MBT (Prioleau et al., 1994).

Following the MBT, the process of gastrulation begins. The three germ layers, ectoderm, mesoderm and ectoderm as well as the anterior-posterior axis are formed (reviewed by Slack, 1992). Gastrulation proceed until embryos reach stage 12. The first observable sign of gastrulation is the appearance on the dorsal side of the embryo of an area of condensed pigmentation. The primary motive of gastrulation is the relocation of the mesoderm and endoderm to the inside of the embryo which displaces the blastocoel and forms a new body cavity, the archenteron. At the beginning of gastrulation, boundaries between the germ layers are not visible, however by the time it is complete margins are visible.

During neurulation, (stages 13-20) the central nervous system of the frog is formed. At the end of gastrulation, the neural plate becomes prominent on the dorsal aspect of the embryo. The neural plate is composed of a thickened ectodermal region that forms all of the components of the central nervous system. The edges of the neural plate (the neural folds) give rise to the neural crest cells (Baker and Bronner-Fraser, 1997). The movements of these cells as well as the

underlying mesoderm give rise to the neural tube (reviewed in Smith and Schoenwolf, 1997). The closure of the neural tube marks the commencement of organogenesis, when all of the major body organs are formed.

5.0 Materials and Methods

5.1 Oocyte manipulations

Oocytes were obtained by surgical removal of ovary lobules from mature female *Xenopus laveis*. Frogs were anesthetized in 0.2% 3-aminobenzoic acid ethyl ester methansulfonate salt (Sigma) for 20 minutes at room temperature. Anesthetized frogs were then placed on ice for surgery. An incision was made in the right or left ventral abdomen through the muscle wall and 2-6 lobes were pulled though the incision. Surgical thread (Ethicon silk, 4-0) was then looped around the blood vessels supplying the dissected lobes and tied tightly. The dissected lobes were then cut from the ovary, and the ligated ovary was placed back into the abdomen. The body muscle wall and skin were individually closed with 4-6 sutures each. In order to remove the follicles, dissected ovarian lobes were placed in 0.2% type II collagenase (Sigma), in Ca^{2+} free OR2 (82.5mM NaCl, 2.5mM KCL, 1mM $MgCl_2$, 1mM NaH_2PO_4, 5mM potassium *N*-2-hydroxyehtylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; (pH 7.6)), 10mg/L streptomycin sulfate, 10mg/L benzyl penicillin) and placed on a gyrotory shaker at 100 rpm at 23˚C for 2-3 hours (hr). The oocytes were then washed several times in OR2 (same as above + 1 mM $CaCl_2$; (Wallace et al., 1973)), and stored at 18°C. Oocytes were staged according to Dumont (1972).

5.1.1 Oocyte enucleation

Nuclei of st VI oocytes were obtained by manual dissection. Oocytes were scored at the centre of the animal pole with Dumont No. 5 forceps. Nuclei were discharged by gently applying pressure to oocytes at the equator while simultaneously applying equal pressure on the animal hemisphere next to the perforation with a second pair of Dumont No.5 forceps. Cytoplasmic debris was removed from the nuclei by careful pipetting in and out of a Pasteur pipette 3-4 times. Nuclei and enucleated cells were then transferred to separate 1.5 ml microfuge tubes and residual OR2 was aspirated.

5.2 Embryo manipulation

Eggs for fertilization were collected in petri dishes from mature *Xenopus laevis* (from *Xenopus* I, Ann Arbor, Michigan) by manually squeezing females 12 hours after they had been injected in the dorsal lymph sac with 1000*U* Human Chorionic Gonadotropin (HCG) (Sigma). Testes were obtained by surgical removal from adult males and stored in 200% Steinberg's solution (0.12 M NaCl, 1.3 mM $MgSO_4$, 0.46 mM $Ca(NO_3)_2$, 9 mM Tris (pH7.4)) for up to 1 week at 4°C. Sperm suspensions for fertilization were prepared by crushing 1/2 of one testicle in 80% Steinberg's solution. Eggs were fertilized by pouring an aliquot of the sperm suspension over the eggs. After incubation for 10 minutes (min) at RT, the dishes were flooded with 20% Steinberg's solution. Fertilized eggs (visualized after cortical rotation) were de-jellied by agitation in a solution of 2% (w/v) L-cysteine in 20% Steinberg's for 5 min. Embryos were then washed

extensively in 20% Steinberg's and incubated at 18°C throughout development. Embryo stages were determined according to Nieuwkoop and Faber (1967). Only normally developing embryos, as judged by established morphological criteria (Nieuwkoop and Faber 1967), were used in all experiments.

5.2.1 Whole cell oocyte/embryonic protein extracts

Embryos or oocytes (at the desired developmental stage) were placed in 1.5 ml microfuge tubes to which buffer C (50mM Tris-HCl (pH 7.8), 20% glycerol, 50 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10 ug/ml aprotinin, 10 *ul*/ml leupeptin) was added (10 *u*l per embryo/oocyte) and were homogenized at 4°C by pipetting several times with a Gilson Pipettman (P200) or in some experiments in a Dounce homogenizer with a tight fitting pestle on ice. Homogenates were centrifuged (Eppendorf model 5415C) for 5 min at 14,000 x g at 4°C, supernatants were removed, placed in fresh microfuge tubes and stored at -80°C.

5.2.2 Cytoplasmic/Nuclear embryonic protein extracts

For embryonic cytoplasmic/nuclear extracts, primary crude extracts were prepared using the method of LeMaitre *et al* (1995), and were subsequently used for separation of nuclear and cytoplasmic fractions. Embryos at the desired developmental stage were isolated and homogenized by centrifugal crushing for 10 min at 14,000 x g at 4˚C producing the primary extract. Nuclear extracts were

prepared by a 10 fold dilution of the primary extract with N buffer (20 mM HEPES (pH 7.6), 2% w/v sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 5*u*g/ml leupeptin, 5*u*g/ml pepstatin). Nuclei were collected by centrifugation for 8 min at 5,000 x g at 4˚C, then pelleted by centrifugation through a 0.8 M Sucrose cushion in N buffer. Pelleted nuclei were extracted for 45 min at 4˚C in E buffer (20 mM HEPES (pH 7.6), 2% sucrose, 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, 5*u*g/ml leupeptin, 5*u*g/ml pepstatin). Insoluble material was separated from the nuclei by centrifugation for 5 min at 14,000 x g at 4˚C, and the supernatants were discarded. Nuclear extracts were stored at -80˚C.

For isolation of cytoplasmic fractions, the primary extract was diluted 5 fold in E buffer that did not contain Nonidet P-40, and was centrifuged 10 min at 14,000 x g at 4˚C to pellet the nuclei. The supernatant was transferred to 1.5 ml microfuge tubes and diluted 1:1 in E buffer containing 1% Nonidet P-40, and gently mixed for 45 min at 4˚C. Samples were centrifuged for 10 min at 14,000 x g at 4˚C and supernatants were transferred to 1.5 ml microfuge tubes and stored at -80˚C.

5.2.3 Oocyte nuclear protein extracts

Nuclear extracts were made by homogenizing nuclei in 2 *u*l Buffer C, followed by snap freezing in liquid N_2, and samples were stored at -80°C. Cytoplasmic extracts were made by adding 10 *u*l Buffer C per enucleated cell, followed by homogenization at 4°C. Homogenates were centrifuged (Eppendorf

model 5415C) for 5 min at 15,000 rpm at 4°C, and supernatants were transferred to microfuge tubes and frozen at -80°C.

5.3 ^{32}P-labeling of YY1 and AP-1 binding elements

Oligonucleotides containing the YY1 and AP-1 binding elements were purchased from Life Technologies (Gibco BRL). The positive strand oligonucleotides for YY1, [5'-ggatcCTCGGCCTCATGCGGCTGCAGAGGC-3'] were mixed with the negative strand [3'-GAGCCGGAGTACGCCGACGTCTCCGggatc-5'] (*Bam*HI overhang is represented by the lower case letters) oligonucleotides in a solution containing 100 mM NaCl, and annealed by heating to 100˚C then allowing the solution to cool to 22˚C overnight. Annealing of the AP-1 positive [5'-ggatcCCGGAAAGCATGAGTCAGACAC-3'] and negative [3'-GGCCTTTCGTACTCAGTCTGTG-'5] strands was carried out as described above. The oligonucleotide duplexes containing the YY1 binding element were labeled with [-^{32}P] dCTP (Mandel) for use in gel shift assays. End labeling reactions contained 5 U Reverse Transcriptase (Promega), 100 ng YY1 oligonucleotides, 2 *ul* 5x Reverse Transcriptase reaction buffer (Promega), 1 *ul* of 1 mg/ml bovine serum albumin (BSA), 2 *ul* of a mixture containing 10 mM dATP, dGTP, and dTTP, and 2 *ul* [-^{32}P] dCTP (Mandel). Following incubation for 45 min at 37˚C 90 *ul* of dH_2O was added and labeled probes were extracted with 90 *ul* phenol/chloroform then precipitated at -20°C with 2 ug of

carrier tRNA, 10 *ul* of 5M ammonium acetate, and 300 *ul* ethanol. Precipitated oligonucleotides were collected by centrifugation for 10 min at 14,000 x g at 4˚C, the ethanol was removed and pellets were dried at 23˚C then, re-suspended in 30 *ul* dH_2O and stored at -20°C.

5.4 DNA-Binding reactions and gel mobility shift assays

DNA binding reactions contained 1 oocyte or embryo equivalent (20 ug of protein in 10 *ul*) mixed with a solution containing 0.5 *u*g poly *dI-dC* (Sigma), 100 cps labeled probe (section 5.3) and binding buffer (10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 5% v/v glycerol) then incubated at 4˚C for 20 minutes. Samples were mixed with 5 *ul* non-denaturing loading dye (50% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 0.005% (w/v) xylene cyanol), loaded onto 5% non-denaturing polyacrylamide gels (5% 29:1 acrylamide (v/v), 4 ml 10X TNANA) and electrophoresed in Tnana buffer (6.7 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3.3 mM sodium acetate) for 2 hours at 150 volts (V). Gels were dried at 80°C on a Savant Slab Gel Dryer (model SDG2000) for 70 min and exposed to Kodak X-OMAT AR x-ray film overnight at -80°C with an intensifying screen (Fisher).

Oligonucleotides for competition experiments were as follows; AP-1 [5' ggatcCCGGAAAGCATGAGTCAGACAC-3'] (positive strand). Competitor concentrations were determined spectrophotometrically using a Pharmacia GeneQuant spectrophotometer and confirmed visually after gel electrophoresis on 16% non-denaturing polyacrylamide gels in TBE (890 mM Tris, 890 mM Boric acid, 20 mM EDTA) followed by staining with ethidium bromide (10

*p*g/ml). Competitor oligonucleotides were added to binding reactions immediately prior to the addition of radiolabeled oligonucleotide probes, and binding reactions were carried out as described above.

5.5 Phosphatase treatments

In some experiments, embryonic or oocyte extracts were treated with phosphatase. 1U Calf Intestinal Alkaline Phosphatase (CIAP) (Boehringer Mannheim) was added directly to extracts (1 oocyte or embryo equivalent) with 10x reaction buffer (100 mM TRIS-acetate, 100 mM magnesium Acetate, 500 mM potassium acetate) and the mixtures were incubated either at at 4°C for the specified pre-incubation times. Treated extracts were then used for DNA-binding reactions, immunoblots or fractionated by size exclusion chromatography (SEC).

5.6 *In vitro* transcription

5.6.1 Radiolabeling of histone H2A

Plasmids for the synthesis of histone H2A probes for RNA binding reactions were a gift of Dr. A. Johnson, Florida State University, Tallahassee Florida. Plasmids were prepared by insertion of a 393 nucleotide (nt) histone H2A PCR product into the *Sma*I site of Bluescript KS^+ (Promega). Plasmids were linearized at the 3’ end by digestion with *Bam*HI for 2 hours at 37˚C.

Probes for RNA binding assays were synthesized from the T7 promoter of bluescript KS^+ and *in vitro* transcription reactions were assembled with the following components in 25 *u*l reactions; 100mM DTT, 1mg/ml BSA, 5mM ATP, GTP, UTP, 50 *u*M CTP, 1U RNAGuard (Pharmacia), 50 *n*g H2A plasmid, 5*u*l [-^{32}P]-rCTP (Mandel), 4*u*l T7 reaction buffer (40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) 250 *u*g/ml BSA, and 10U T7 RNA polymerase (Pharmacia). Reactions were incubated for 2 hr at 37˚C, followed by addition of 10U DN*ase* (Pharmacia) and further incubation for 20 min at 37˚C. Labeled probes were extracted once with phenol, twice with phenol/chloroform (1:1), and once with chloroform followed with recovery by precipitation at -20˚C in 1/10 volume 3M sodium acetate, and 2 volumes ethanol. Probes were centrifuged for 30 min at 12,500 x g at 4˚C, resuspended in 50*u*l TE (10 mM Tris-HCl, 1 mM EDTA), 1U RNAGuard was added and samples were stored at -20˚C. Labeling of probes was confirmed by electrophoresis of a 5*u*l aliquot on 5% (w/v) acrylamide/8.3 M urea gels in TBE, and transcript length was measured against co-electrophoresed ^{32}P-labeled *MspI* (New England Biolabs) digested PUC19 marker.

5.6.2 Synthesis of capped YY1 mRNAs

Plasmids used for the synthesis of YY1 mRNAs were a gift of a gift of Dr. Elena Beccari, Universita di Roma La Sapienza, Rome Italy, Accession #X77698. YY1 cDNAs were inserted into the *Eco*RI site of pBluescript KS^+ (Promega). YY1 RNAs were synthesized from the T7 promoter of bluescript

KS^+ and *in vitro* transcription reactions were assembled with the following components in 25 *u*l reactions; 100mM DTT, 1mg/ml BSA, 1U RNAGuard (Pharmacia), 50 *n*g YY1 plasmid, 10 *u*l of 5X NTP capping solution (5 mM each ATP, CTP, UTP, 0.25 mM GTP, 5 mM m^7GpppG) 4*u*l T7 reaction buffer (40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) 250 *u*g/ml BSA, and 10U T7 RNA polymerase (Pharmacia). Reactions were incubated for 2 hr at 37˚C and reactions were stopped by the addition of 10U FLPC-pure DNase I (Pharmacia) and subsequent incubation at 37˚C for 20 min. Capped mRNAs were extracted once with phenol, twice with phenol/chloroform (1:1), and once with chloroform followed with recovery by precipitation at -20˚C in 1/10 volume 3M sodium acetate, and 2 volumes ethanol. Following precipitation, mRNAs were pelleted by centrifugation for 30 min at 12,500 x g at 4˚C, resuspended in 50*u*l TE (10 mM Tris-EDTA) and concentrations were determined by spectrophotometric analysis and diluted to 2 mg/ml.

5.7 RNA binding reactions

RNA binding reactions were performed as previously described by Bouvet *et al* (1995) with minor modifications. Binding reactions were carried out for 20 min at 4˚C in RNA binding buffer (20 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 100 mM KCl, 30% v/v glycerol, 1mM DTT, 20 *u*g/ml BSA) with 0.5 *n*g of labeled RNA probe and 20 *u*g soluble oocyte protein in a total reaction volume of 20 *u*l. In some reactions antibodies against YY1 (Santa Cruz Biotechnology, Santa Cruz California, polyclonal, CAT# sc-281) and Proliferating Cell Nuclear Antigen (PCNA) (Santa Cruz Biotechnology, monoclonal, CAT# sc-56) were

added to cell lysates at 4˚C for 20 minutes prior to the binding reactions in a range of concentrations (25-400 *n*g). Following binding reactions, samples were loaded onto non-denaturing 5% polyacrylamide gels containing 6.7 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3.3 M sodium acetate, 5% v/v Glycerol. Gels were electrophoresed for 2.5 hr at 160 volts in a chromatography refrigerator maintained at 4˚C, and were dried at 80°C on a Savant Slab Gel Dryer (model SDG2000) for 70 min and exposed to x-ray film overnight at -80°C with an intensifying screen.

5.8 RNA isolation

TRIzol (Life Technologies) RNA isolation reagent was used to isolate total RNA from *Xenopus* oocytes and embryos. Batches of 30 oocytes/embryos at the desired developmental stages were transferred to 1.5 ml microfuge tubes and were homogenized on ice in 1 ml TRIzol reagent using an Eppendorf p1000 pipette. Homogenates were incubated for 5 min at 23˚C followed by the addition of 200 *u*l chloroform, vortexed for 15 sec and incubated for 3 min at 23 ˚C. The mixtures were centrifuged for 15 min at 14,000 x g at 4˚C and the upper aqueous phases were transferred to 1.5 ml microfuge tubes, 450 *u*l isopropyl alcohol was added, and samples were incubated for 10 min at 23˚C. Samples were centrifuged for 10 min at 14,000 x g at 4˚C, supernatants were discarded, and pellets were washed with 1 ml 70% ethanol. Residual ethanol was evaporated and RNA pellets were resuspended in 50 *u*l dH_2O, and stored at -80˚C as ethanol

precipitates after the addition of 150 *u*l ethanol (95%), 5 *u*l ammonium acetate, and 1 *u*g tRNA.

5.8.1 PolyA$^+$RNA isolation

PolyA$^+$RNA was isolated from selected total RNA samples by Oligo(*dT*) cellulose chromatography (Aviv and Leder, 1972). For batches of total RNA containing no more than 150 *u*g, 100 *u*g (dry weight) of Oligo$(dT)_{25}$ cellulose (New England Biolabs CAT# 1401) was resuspended in 200 *u*l loading buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA). The bead slurry was equilibrated by centrifugation in a 1.5 ml microfuge tube for 20 sec at 5,000 x g at 23˚C. The loading buffer was aspirated and the beads were resuspended in 500 *u*l loading buffer. Pellets containing 150 *u*g of total oocyte/embryo RNA were dissolved in 200 *u*l loading buffer, heated for 5 min at 65˚C then immediately chilled for 5 min at 4˚C. RNA mixtures were added to equilibriated Oligo$(dT)_{25}$ cellulose beads and slurries were gently agitated for 5 min at 23˚C followed by centrifugation for 20 sec at 5,000 x g at 23˚C. Unbound RNA was decanted, re-heated, for 5 min at 65˚C, then chilled for 5 min at 4˚C and re-applied to the Oligo$(dT)_{25}$ cellulose beads and gently mixed for 5 min at 23˚C. Beads were washed 5 times with 500 *u*l loading buffer followed by a wash in 500 *u*l low salt buffer (0.1 M NaCl, 20 mM TRIS-HCl (pH 7.5), 1 mM EDTA). PolyA$^+$ RNA was eluted from the cellulose beads with 2 x 250 *u*l washes of elution buffer (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA) at 70˚C, and precipitated overnight at -20˚C by the addition of 1/10 volumes of 3 M sodium

acetate and 2.5 volumes ethanol. $PolyA^+$ RNA samples were collected by centrifugation for 30 min at 14,000 x g at 4˚C and the resulting pellets were resuspended in 10 *u*l formamide and stored at -80˚C.

5.9 Random prime labelling

Probes for Northern blots were synthesized from *Xenopus* YY1 cDNAs (a gift of Dr. Elena Beccari, Universita di Roma La Sapienza, Rome Italy, Accession #X77698) or immunoprecipitated cDNAs; 051 (527 bp), 060 (203 bp), 164 (522 bp), 121 (679 bp), 039 (581 bp), 005 (493 bp) and 121 (600 bp). Random primed reactions were assembled in 25 *u*l volumes containing 50 *ng* DNAs (boiled 10 minutes, then immediately chilled on ice), 5 *ul* dH_2O, 3 *ul* of a mixture containing 10mM ATP, GTP, UTP, 2 *u*g random hexamer mix (GibcoBRL), 50 *u*Ci [-^{32}P] dCTP (Mandel), 3 *u*l Klenow reaction buffer (670 mM HEPES, 170 mM TRIS-HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA), 3 U DNA Polymerase I (Klenow) (Pharmacia). Reactions were incubated for 1 hr at 37°C and terminated by the addition of 175 *ul* STE (100 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 8.0)). Reactions were passed through G-50 Sephadex columns (equilibriated with 200 *ul* STE) then boiled 5 minutes, chilled at 4˚C and added to hybridization solutions or stored at -20˚C.

5.10 Northern blot analysis

Northerns were performed as previously described (Sambrook, 1989) with modifications. 10 *u*g of RNA was suspended in 20 *u*l formamide and

diluted with an equal volume of sample diluent (7.35% formaldehyde (v/v), 10 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 0.1 M sodium acetate, 10 mM EDTA) and heated for 15 min at 55˚C then allowed to cool for 5 min at 4˚C prior to gel loading. Samples were mixed with 8 ul loading dye (1x MOPS, 18.5% formaldehyde, 10% formamide, 0.05% bromophenol blue) loaded onto 1% agarose/formaldehyde gels (1% agarose, 1x MOPS, 0.6 M formaldehyde) and electrophoresed for 2h at 100 V in 1x MOPS buffer (1x MOPS, 40 mM sodium acetate, 5 mM EDTA (pH 8.0). Following electrophoresis, the RNA ladder (New England Biolabs, Cat# N0362S, 9000bp, 7000bp, 5000bp, 3000bp, 2000bp, 1000bp, 500bp) was excised with a scalpel blade (Beaver #32), stained with ethidium bromide (10 pg/ml) (Sigma) 1 hour, then destained with dH_2O for 3 hours at 23˚C. The remainder of the gel was soaked for 20 min in 0.5x TBE and RNA molecules were electrophoretically transferred at $3mA/cm^2$ to Hybond N membranes (Amersham) using a Trans-Blot SD (Bio-Rad) semi dry transfer apparatus then UV-crosslinked at $1200J/cm^2$ using a Stratagene Stratalinker 1800. Membranes were prehybridized 4 hr at 42°C in prehybridization solution (500 mM $NaPO_4$, 7% SDS (v/v), 1 mM EDTA) then hybridized overnight at 65˚C in hybridization solution (500 mM $NaPO_4$, 7% SDS (v/v), 1 mM EDTA) containing 1 x 10^6 cpm/ml labeled probes. Membranes were washed 5 times for 5 min at 23˚C in wash solution (40 mM $NaPO_4$, 0.1% SDS (v/v), 1 mM EDTA) then 2 times for 15 min at 55˚C. Membranes were exposed to Kodak X-OMAT film overnight at -80°C.

5.11 Immunoblotting

Protein samples were prepared by mixing equal volumes of protein extract with 2x Laemli buffer (100 mM Tris-HCl (pH 6.1), 200 mM DTT, 4% SDS (v/v), 0.2% bromophenol blue (w/v), 20% glycerol (v/v)) followed by heating for 5 min at 100°C. Samples were separated on 10% SDS-polyacrylamide gels (30% acrylamide (33.5g acrylamide: 0.8g bis-acrylamide), 0.38 M Tris (pH 9.1), 5 ul TEEMED, 0.2% APS (v/v), 0.1% SDS (v/v)) for 45 minutes at 150 V in 1x SDS tank buffer (0.2M Tris, 0.76 M glycine, 14 mM SDS, pH 8.5). Separated proteins were electrophoretically transferred using a semi-dry transfer apparatus (Trans-Blot SD, Bio-Rad) for 20 min at 15 V to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) which were prepared immediately prior to transfers by soaking in methanol for 5 min at 23°C, then in transfer buffer (25 mM Tris, 47 mM glycine, 0.8 mM SDS, 20% methanol (v/v)) 15 min at 23°C. Membranes were blocked 2hr at 23°C in TBST (20mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20 (v/v)) containing 5% milk powder (w/v) (Bio-Rad). Primary antibodies: rabbit anti-human YY1 polyclonal, (Santa Cruz Biotechnology Cat # sc-281), mouse anti-human PCNA monoclonal, (Santa Cruz Biotechnology Cat # sc-56), rabbit anti-*Xenopus* FRGY2 polyclonal (Gift of Dr. K. Matsumoto, RIKEN, Saito, Japan), rabbit anti-*Xenopus* polyclonal nucleolin (Gift of Dr. Patrick DeMario, Louisana State University) were diluted 1:3500 in TBST containing 2.5% (w/v) milk powder and incubated with membranes for 2h at 23°C. Membranes were washed 3 x 5 min at 23°C with TBST followed by incubations with secondary antibodies (HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, Bio-Rad) diluted 1:10,000

in TBST with 2.5% milk (w/v) for 2h at 23°C. Membranes were washed 3 x 5 minutes at 23°C in TBST with 2.5% milk (w/v), followed by 3 x 5 min in TBST. Proteins were visualized by chemiluminescence (Renaissance system; Dupont NEN Life Science Products) and autoradiography using Kodak X-OMAT film.

5.11.1 Coomassie Blue Staining

For detection of total proteins, SDS gels were soaked in 0.005% bromophenol blue (w/v) (Bio-Rad), 10% methanol (v/v), 10% acetic acid (v/v) for 2 hr at 23˚C. Gels were de-stained for 18 hr in a solution containing 10% methanol (v/v), 10% acetic acid (v/v).

5.12 Microinjections

Oocytes and embryos were visualized using a Leica Wild 3X microscope. All microinjections were performed using a Narishge IM 300 microinjector and a Narishge micromanipulator (model MN-151).

5.12.1 Plasmid microinjections

Equal amounts of CAT plasmids (20 pg for oocytes, and 100 pg for embryos in a volume of 20 *n*l) were microinjected into the nuclei of stage VI oocytes or the animal hemisphere of stage 2 embryos at 18˚C. Reporter plasmids (courtesy of Dr. M. Hurt, Florida State University, Tallahassee, Florida)

contained YY1 binding elements as follows; pCAT, no promoter elements ahead of the CAT gene, TKCAT, contains a thymidine kinase promoter ahead of the CAT gene, YY1 TKCAT, contains 6 copies of the YY1 binding element upstream of the TK promoter and CAT gene, YY1R-TKCAT , identical to YY1 TKCAT except that the YY1 consensus element is in the reverse orientation, CRAS TKCAT, contains the coding region activating sequence from histone H2A upstream of the TK promoter and CAT gene, YY1M-TKCAT, contains a 6 copy repeat of a mutated YY1 binding element.

Following injections, oocytes were placed at 18°C for 18 hours in OR2 to allow for CAT expression. Oocytes were then homogenized in 250 mM Tris (10*u*l/oocyte) on ice and centrifuged for 6 minutes 12,000 x g at 4°C. Supernatants were placed in 1.5ml microfuge tubes and stored at -80°C.

Embryos for microinjections were placed in 20% steinberg's solution containing 4% Ficoll (w/v), and injected with test plasmids. Following microinjections embryos were allowed to recover at 18°C in 4% Ficoll for 3 hours, after which the Ficoll concentraion was reduced 1% per hour. Embryos were allowed to develop until stage 10 at which time extracts were prepared as described above.

5.12.2 RNase microinjections

200 *n*g RNase A (Sigma) in a volume of 20 *n*l was injected into the animal hemisphere of stage VI oocytes and incubated at 18°C for 8 h.

Enucleations were performed as described in section 5.1.1. Nuclear and cytoplasmic extracts were prepared as described in section 5.2.1.

5.12.3 YY1 mRNA microinjections

For expression of YY1 in oocytes, 200 *p*g of *in vitro* synthesized mRNA (described in section 5.6.2) was injected into the animal hemishpere of stage VI oocytes which were incubated for 18 hours at 18˚C followed by preparation of protein extracts as described in section 5.2.1.

5.13 CAT assays

CAT assays were performed as described previously by Gorman *et al*. (1982). Reaction mixtures were assembled with 10 *u*l of protein extract, 20 *u*l dH_2O, 1 mM acetyl CoA, 0.5 *u*l ^{14}C-labelled Chloramphenicol (Mandel) (final ^{14}C-Chloramphenicol concentration of 0.2 mCi per reaction). Positive control reactions contained 0.5 *u*l Chloramphenicol Acetyl Transferase in place of sample protein. Reactions were incubated for 60 min at 37˚C then extracted with 250 *u*l cold ethyl acetate by centrifugation at 14,000 X g for 5 min. Supernatants were placed in 1.5 ml microfuge tubes and lyophilized. Dried pellets were resuspended in 10 *u*l ethyl acetate and spotted on silica Thin Layer Chromatography (TLC, Whatman) plates. Plates were placed in a closed TLC chamber containing 95% chloroform/5% methanol (v/v). Separation of reaction products was allowed to continue until the solvent front reached the top of the

plate at which time plates were removed from the chamber and dried at 23°C then exposed to Kodak X-OMAT 5 film overnight at 23 °C.

5.14 Cell culture, stable transfections and CAT assays

Chinese hamster ovary (CHO) cells were grown in McCoy's 5A medium supplemented with 10% calf serum, and stable transfections were performed as described by Bushmeyer and colleagues (Bushmeyer et al., 1995). CAT assays with CHO cells were performed with the CAT enzyme assay system (Promega) using liquid scintillation counting as described in the Promega technical bulletin (part number. TB084).

5.15 Oligo(dT)-cellulose chromatography

5.15.1 Batch oligo(dT)-cellulose chromatography

Lysates were prepared by homogenization of oocytes at 4˚C in 4 volumes of buffer (8% glycerol (v/v), 50 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl (pH 7.5), 100 U/ml RNAGuard (Promega)). Homogenates were centrifuged for 12 min at 14,000 x g at 4˚C supernatants were transferred to fresh tubes and were centrifuged a second time under the same conditions to remove residual cell debris. Yolk proteins were removed by extraction with an equal volume of 1,1,2-trichlorofluororethane (Freon), and supernatants were adjusted to 100 mM NaCl. Lysates (300 *u*l) were applied to 100 *u*l aliquots of Oligo(*dT*)-Cellulose (New England Biolabs) that was pre-equlibriated with 10 volumes of binding buffer

(100 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl (pH 7.5)) and incubated for 2.5 hr at 4˚C with gentle rotation. The Oligo(dT)-Cellulose was washed with 6 volumes binding buffer and bound proteins were eluted with 400 *u*l binding buffer containing 25% formamide (Fluka).

5.15.2 High pressure oligo(dT)-cellulose chromatography

Columns maintained at 4˚C were run at a flow rate of 0.1ml/min using a BioLogic HR (Bio-Rad) high pressure chromatography control unit. Oligo-(dT)-cellulose pre-equlibriated in buffer (100 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl (pH 7.5)) was packed into 5 ml Econo-Column Chromatography columns (Bio-Rad) with a resulting bed volume of 1ml. Lysates (300 *u*l) were applied to columns and $PolyA^+$ bound proteins were eluted in 200 *u*l fractions with binding buffer containing a range of NaCl from 100 mM to 2M. Proteins were precipitated in 5 volumes of acetone at -80˚C, and pelleted by centrifugation for 30 min at 14,000 x g at 4˚C and analyzed by Western blotting as described in section 5.11.

5.16 Size exclusion chromatography

5.16.1 Lysates

Lysates for size exclusion chromatography (SEC) were prepared by homogenization of oocytes at the desired developmental stages in buffer C containing 100 U/ml RNAGuard (Promega). Homogenates were centrifuged 7

min at 14,000 x g at 4˚C and supernatants were filtered through 0.2 *u*m nylon membranes (Gelman Laboratories) and stored at -80˚C. HepG2 cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were washed, and lysates were prepared by three freeze-thaw cycles in 5 volumes of lysis buffer (50 mM TRIS-HCl, 50 mM KCl, 20% glycerol(v/v), 100 *u*m EDTA, 10 *u*g/ml aprotinin, 10 *u*g/ml leupeptin). Homogenates were centrifuged for 5 min at 4000 x g at 4˚C and supernatants were filtered through 0.2 *u*m nylon membranes (Gelman Laboratories) and stored at -80˚C.

For RNase treatments, 300 ug/ml RNase A was added to lysates (that were prepared as above but did not include RNAGuard) and incubated for 30 min at 23˚C. For phosphatase treatments, lysates were incubated with 33U/ml Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim), 10x reaction buffer (100 mM TRIS-acetate, 100 mM Mg Acetate, 500 mM potassium acetate), and were incubated for 30 min at 23˚C.

5.16.2 Column running conditions

Columns maintained at 4˚C and run with column buffer (50mM TRIS-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA) at 0.4ml/min using a BioLogic HR (Bio-Rad) high pressure chromatography control unit. Lysates (300 *u*l) were separated on either Bio-Sil 250-5 (Bio-Rad, separation separation range 10-300 kDa) or Superdex 200 HR 10/30 (Amersham Biosciences, separation range 10-

11,000 kDa). Fractions (200 *u*l) were precipitated in 5 volumes acetone for 2 hr at -80˚C, and proteins were pelleted by centrigfugation for 30 min at 14,000 x g at 4˚C and resuspended with 60 *u*l 2x Laemli buffer. Columns were calibrated using gel filtration standards (Bio-Rad, Cat# 151-1091) containing the following markers: thyroglobulin (670 kDa), globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), vitamin B-12 (1.35 kDa).

5.17 Immunoprecipitation of endogenous YY1 containing mRNP complexes

Overview of the procedure;

RNAs for cDNA synthesis were obtained by immunoprecipitation of YY1-mRNPs using polyclonal YY1 antibodies (a flow chart of this procedure is shown in Appendix 1). Immunoprecipitated RNA was dissociated from YY1-mRNP complexes by Protinase K digestions and precipitated in ethanol and used as templates from which cDNAs were synthesized. The cDNAs were digested with H*ae*II (a four cutter) which produced a varied number of fragments with blunt ends. Adaptors that contained the restriction site for M*lu*I were ligated to the cDNA fragments, digested with M*lu*I, and inserted into the B*ss*HI (an isoschizomer of M*lu*I) site of modified Bluescript plasmids. DH5 cells were transformed with the plasmids, positive colonies were identified then plasmids isolated from the cells. Plasmids containing inserts were verified by X*ho*I digestions and PCR from the M13 foreward and reverse sites diluted to 100*ng*/*u*l and sent for external sequencing at the Plant Biotechnology Centre DNA Sequencing Facility. Returned sequences were converted to Microsoft Word

documents the 5' and 3' plasmid sequences were removed and the sequences were used to search databases at the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

5.17.1 Immunoprecipitations

Immunoprecipitations were performed as described by Tenenbaum et al (2000) with modifications using Protein-A Sepharose beads (Sigma). Stage VI oocytes were homogenized in lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40 (v/v), 1 mM DTT, 100 U/ml RNAguard (Pharmacia), 10 ug/ml Aprotinin, 10 *ul*/ml Leupeptin, 1 mg/ml pepstatin A) and centrifuged for 7 min at 15,000 x g at 4˚C. Protein-A beads were swollen overnight 1:5 (w/v) in NT2 buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40 (v/v)) supplemented with 5% BSA (w/v). 300 *u*l aliquots of the pre-swollen slurry were used for each immunoprecipitation. Antibodies (rabbit anti-human YY1 polyclonal, Santa Cruz Biotechnology Cat # sc-281, anti-human PCNA monoclonal, Santa Cruz Biotechnology Cat # sc-56) were added in excess (2000 *n*g) to the Protein-A Sepharose beads and were incubated overnight at 4˚C with slow rotation. Antibody coated Protein-A beads were washed 5 times with 1 ml of ice-cold NT2 buffer, and centrifuged following each wash for 30s at 500 x g at 23˚C, then resuspended with 850 *u*l NT2. Immunoprecipitation reactions were assembled by adding 20 mM EDTA, 100 U/ml RNAGuard, 1 mM DTT, 200 *u*l cell lysate to the washed Protein-A beads and incubating the mixtures for 3 hr at 23˚C with slow rotation (45 rpm).

Reactions were washed 5 times with 1 ml NT2 buffer and centrifuged 30s at 500 x g at 23˚C discarding the supernatant after each wash. The beads were resuspended with 100 *u*l NT2 buffer supplemented with 0.1% SDS (v/v), 30 *u*g Protinase K (Sigma) and incubated for 30 min at 55˚C. Reactions were centrifuged 1 min at 14,000 x g at 23˚C and supernatants were transferred to 1.5 ml microfuge tubes. The beads were washed 3 times with 200 *ul* NT2 buffer followed by centrifugation for 1 min at 14,000 x g at 23 ˚C to collect residual RNAs. Immunoprecipitated RNAs were extracted once with phenol/chloroform (1:1) and precipitated overnight with 1/10 volume 3M sodium acetate and 2 volumes ethanol. RNAs were recovered by centrifugation for 30 min at 14,000 x g at 4˚C, ethanol was aspirated and pellets were washed with 1 ml of 70% ethanol then centrifuged 2 min at 14,000 x g at 4˚C. RNAs were re-suspended with 5 *ul* DEPC treated dH_2O supplemented with 100 U/ml RNAGuard and used immediately in cDNA synthesis reactions.

5.18 cDNA synthesis.

RNAs were heated for 5 min at 65˚C then immediately placed on ice. First strand reactions were assembled in a total volume of 200 *u*l as follows: 500 *uM* dNTPs (dATP, dCTP, dGTP, dTTP), 40 *u*l RT buffer (250 mM TRIS-HCl (pH 8.2), 250 mM KCl, 30 mM $MgCl_2$), 10 mM DTT, 10*u*g random hexameric primers (GibcoBRL), and 10 U RNAGuard (Pharmacia). Chilled RNAs were added to reaction mixtures on ice and 200 U AMV Reverse Transcriptase (Promega) was added then gently mixed. The reactions were incubated 10 min at

23˚C, then 1.5 hr at 37˚C, and were terminated by the addition of 10 mM EDTA. First strand cDNAs were extracted with 200 *u*l Phenol (1:1) and were centrifuged 5 min at 14,000 x g at 4˚C. The phenol was re-extracted with 100 *u*l TE (pH 7.6) centrifuged 5 min at 14,000 x g at 4˚C and both the phenol and TE extractions were pooled then precipitated 24 hr at -80˚C in 2 M ammonium acetate and 950 *u*l of 95% ethanol. Precipitates were centrifuged 15 min at 14,000 x g at 4˚C, supernatants were discarded and pellets were washed with 1 ml of 70% ethanol and centrifuged for 2 min at 14,000 x g at 4˚C. Pellets were dried 10 min at 37˚C and resuspended with 284*u*l dH_2O.

Second strand reactions (400 *u*l total reaction volume) contained 284 *ul* first strand DNA, 50 uM dNTPs (dATP, dCTP, dGTP, dTTP), 80 *u*l second strand reaction buffer (100 mM Tris-HCl (pH 8.2), 500 mM KCl, 25 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 50 mM DTT, 250 *u*g/ml BSA), 150 *u*m NAD^+, 1 mM ATP, 10 U/ml RNase H, 50 U/ml *E. Coli* DNA ligase, 250 U/ml *Ecoli* DNA polymerase and were incubated for 16 hr at 14˚C. Second strand reactions cDNAs were extracted with 400 *u*l phenol (1:1) and centrifuged 5 min at 14,000 x g at 4˚C. Residual phenol was re-extracted with 200 *u*l TE (pH 7.6) and centrifuged 5 min at 14,000 x g at 4˚C and both phenol and TE extractions were pooled then extracted with 300 *u*l chloroform (1:1) and centrifuged 5 min at 14,000 x g at 4˚C. Extracted cDNAs were equally divided into two 1.5 ml microfuge tubes both of which were precipitated 24 h at -80˚C in 2.5 M ammonium acetate and 950*u*l 95% ethanol. cDNAs were pelleted by centrifugation for 30 min at 14,000 x g at 4˚C, washed with 1 ml of 70% ethanol,

centrifuged 5 min at 14,000 x g at 4˚C, resuspended with 10 *u*l dH_2O and stored at -80˚C.

5.18.1 cDNA enrichment and cloning

5.18.1.1 Adaptor ligation and polymerase chain reaction

To produce blunt ends (necessary for adaptor ligation), cDNAs were digested with *Hae*III (New England Biolabs). Reactions were assembled with the following components, 10 *u*l cDNA, 1.5 *u*l 10x *Hae*III reaction buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl), 1U *Hae*III, and were incubated for 1 hr at 37˚C. Adaptors containing a restriction enzyme recognition site for *Mlu*I (5'-A/CGCGT-3'),

were ligated to the blunt digested cDNAs. Ligation reactions (total volume 20 ul) containing 10 *u*l digested cDNAs, 1 mM ATP, 2000 U/ml T4 DNA ligase (Promega), 1 *u*g MluI adaptors were assembled and incubated 20 hr at 20˚C. The ligation reactions were were transferred to 500 *u*l microfuge tubes and polymerase chain reactions (PCR) were assembled (50 *u*l) with; 10 *u*l adaptor ligated cDNA, 5 *u*l 10x *Taq* reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.0)), 200 *ng* primer (5'CTCTTGCTTACGCGTGGACTA - 3'), 0.2 mM dNTPs (dATP, dCTP, dTTP, dGTP), and 2.5 U *Taq* DNA polymerase (GibcoBRL). Reactions were thermocycled in a MJ Research PTC-150 Minicycler which was programmed as follows; pre-amplification

denaturation, 5 min at 95˚C, (1) 30 sec at 95˚C, (2) 1 min at 60˚C, (3) 3 min at 72˚C, (4) cycle 24 times to step (1), (5) 10 min at 72˚C, (6) hold at 14˚C, (7) end of protocol. After thermocycling the reaction volumes were increased to 200 *u*l with dH_2O. DNAs were recovered by extraction with 200 *u*l phenol (1:1) and centrifugation for 5 min at 14,000 x g at 23˚C. The phenol phase was then re-extracted with 100 *u*l TE (pH 7.5) and centrifuged for 5 min at 14,000 x g at 23˚C. The phenol/TE extracted DNA was next extracted with 300 *u*l chloroform (1:1) followed by re-extraction of the chloroform with 100*u*l TE (pH 7.5) by centrifugation for 5 min at 14,000 x g at 23˚C. Amplified cDNAs were precipitated 20 hr at -80˚C with the addition of 100 mM NaCl and 1 ml 95% ethanol. Precipitates were centrifuged for 30 min at 14,000 x g at 4˚C, ethanol was aspirated and pellets were re-suspended in 20*u*l dH_2O.

5.18.1.2 DEAE electroelution

DEAE electroelution was performed as described by Dretzen (Dretzen et al., 1981) with modifications. Amplified cDNAs were digested with *Mlu*I for 1 hr at 37˚C. Reactions were assembled with; 20*u*l PCR amplified cDNA, 2.4*u*l 10x *Mlu*I buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl), 1 U *Mlu*I, and 0.6*u*l dH_2O. Digested fragments were mixed with non-denaturing loading dye (50% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 0.005% (w/v) xylene cyanol) and separated for 1 hr at 5v/cm on a Mini-Sub electrophoresis system (BioRad) through 0.8% Agarose (BioRad)/ethidium bromide (10 *p*g/ml) (Sigma) gels run in 1x TAE buffer (0.04 M Tris-acetate,

0.001 M EDTA). DEAE membranes were cut into 1 cm^2 squares, and were prepared for electro-elution by soaking in 1x TAE 24 hr at 4˚C. Separation of digested DNAs was monitored using a handheld longwave UV lamp. DEAE membranes were inserted into the gel at the 500 bp level (as judged by comparison to co-electrophoresed *Hind*III digested marker) and the DNAs were electrophoresed onto the membranes for 45 min at 5V/cm. Membranes containing bound DNAs were removed from agarose gels and placed in 15 ml Falcon tubes containing 5ml NET (150 mM NaCl, 0.1 mM EDTA, 20 mM TRIS-HCl (pH 7.5)) and washed overnight at 4˚C by repeated inversion. DEAE membranes were then transferred to 1.5 ml microfuge tubes (the NET was discarded) containing 200 *u*l HSNET (1 M NaCl, 0.1 mM EDTA, 20 mM TRIS-HCl (pH 7.5)) and were washed for 45 min by rotation at 65˚C. The initial 200 *u*l HSNET was removed and saved. Membranes were washed a second time with 100 *u*l HSNET by rotation for 10 min at 65˚C and the HSNET was pooled with that from the first high salt wash 900 *u*l dH_2O-saturated butanol was added and DNAs were extracted by vortexing 30 sec. The residual dH_2O-saturated butanol was extracted by the addition of 100 *u*l dH_2O followed by vortexing for 30 sec, and both extractions were pooled then precipitated overnight at -20˚C by the addition of 1 ml 95% ethanol. DNAs were collected by centrifugation for 30 min at 14,000 x g at 4˚C, ethanol was aspirated and the pellets were washed once with 800 *u*l ice cold 70% ethanol and centrifuged for 5 min at 14,000 x g at 4˚C, and pellets were resuspended with 20 *u*l dH_2O and stored at -80˚C.

5.18.1.3 Ligations

cDNAs containing MluI ends (an isoschizomer of BssHII) were ligated into the BssHII site of modified Bluescript KS^+vectors, multiple cloning site shown below;

5'-GAATTCCTCGAGGCGCGCCTCGAGGGATCCTCTAGAGTCGACCTGCAGGCAAGCTT-3'
EcoRI XhoI BssHII XhoI BamHI XbaI SalI PstI SphI HindIII

Ligation reactions were assembled with 2.5 *u*l DNA, 2 *u*l T4 DNA Ligase buffer (100 mM TRIS-acetate, 100 mM magnesium acetate, 500 mM potassium acetate) 1 *u*l pBluescript KS^+ (25 ng/*u*l), 1 mM ATP, 2.5 U T4 DNA Ligase (GibcoBRL), 5 U *Mlu*I, and were incubated overnight at 20˚C. Circularized plasmids were precipitated for 30 min at -20˚C by the addition of 5 *u*l tRNA (1*u*g/*u*l), 20 *u*l ammonium acetate (5M), 70 *u*l 95% ethanol, then centrifuged 20 min at 14,000 x g at 4˚C. Ethanol was discarded and the pellets were washed with 100 *u*l ice cold 70% ethanol and centrifuged 2 min at 14,000 x g at 4˚C. Pellets were air dried for 30 min then resuspended in 5 *u*l dH_2O.

5.19 Transformations

DH5 cells were transformed with plasmid DNAs by the method described by Sambrook (Sambrook, 1989). Competent cells for transformations were prepared by inoculating 10 ml culture tubes containing 5.0 ml of YT-media (5 g yeast, 10 g Tryptone, 5 g NaCl, 250 ml dH_2O) with DH5 cells and grown

overnight at 37°C with shaking at 200 rpm. 100 *ul* aliquots of this cell suspension were transferred to new culture tubes containing 5 ml of YT-media, and grown for 6 hr at 37°C with shaking at 200 rpm. Cells were pelleted by centrifugation for 10 min at 4000 x g at 23˚C, supernatants were discarded, and 2.5 ml of cold $CaCl_2$ (50 mM) were added. The cells were gently re-suspended, incubated on ice for 1 hr then centrifuged for 10 min at 4000 x g at 23˚C. Supernatants were discarded and cell pellets were re-suspended in 500 *ul* of $CaCl_2$ (50 mM) and put on ice. Transformation mixtures were assembled with 1 *ul* of plasmid DNA, 9 *ul* dH_2O, 20 *ul* TCM buffer (10 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, 10 mM $MgCl_2$) and 100 *ul* aliquots of the competent cell suspension were added to the transformation mixtures and incubated on ice for 1 hr. Transformation mixtures were heat shocked for 2 min at 42°C then allowed to recover for 15 min at 23˚C. 300 *ul* prewarmed (37˚C) YT-media was added, and mixtures were incubated for 30 min at 37°C, then plated on S-Gal/ampicillin plates (Sigma) and grown overnight at 37˚C.

5.20 Screening of immunoprecipitated YY1-associated RNAs

5.20.1 Plasmid preparations

For preparation of plasmid DNAs for sequencing, culture tubes (10 ml, Falcon) containing 5 ml of YT-Broth (5 g yeast, 10 g Tryptone, 5 g NaCl, 250 ml dH_2O) and 375 ug ampicillin were innoculated with positive colonies from S-Gal plates and grown overnight at 37˚C x 200 rpm. Bacterial cells were collected

by transferring media to 1.5 ml microfuge tubes which were centrifuged 20 sec at 14,000 x g at 23˚C and the media was discarded. Plasmids were isolated by alkaline lysis using Promega Wizard Miniprep Kits. Plasmids positive for insert were identified by PCR and restriction enzyme digestion using *Xho*I.

5.20.2 Polymerase chain reaction screening.

Polymerase chain reactions were assembled in 500 *u*l microfuge tubes with the following components in a total reaction volume of 41 *u*l; 4.1 *u*l 10x *Taq* reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.0)), 0.75 *u*l M13 foreward/reverse primers (Invitrogen, Cat#18432-013, 100 *n*g/*u*l), 0.2 mM dNTPs (dATP, dCTP, dTTP, dGTP), 2.5 U *Taq* DNA polymerase (GibcoBRL), 32.5 *u*l dH_2O. Reactions were thermocycled in a MJ Research PTC-150 Minicycler which was programmed as follows; pre-amplification denaturation, 2 min at 94˚C, (1) 1min at 94˚C, (2) 50 sec at 61˚C, (3) 90 sec at 72˚C, (4) cycle 29 times to step (1), (5) 5 min at 72˚C. Products were analyzed on 1% Agarose (BioRad)/ Ethidium bromide (10*p*g/ml) gels run in 1x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), and gel images were captured on ScionImage (V1.1) software.

Plasmids containing inserts were diluted to 100 *n*g/*u*l and sent for sequencing from the M13 reverse primer at the DNA Sequencing Laboratory/National Research Council (Saskatoon, Saskatchewan). Sequence data was converted into Microsoft Word™ documents and the 5' and 3' sequences were removed yielding the sequenced insert. Insert sequences were

copied into the Basic Local Alignment Search Tool (BLAST) (located at http://www.ncbi.nlm.nih.gov/BLAST/) run by the National Centre for Biotechnology Information (NCBI). The BLAST was used to identify the insert sequences by comparison to those existing in the NCBI database. Virtual digestions were carried out using software located at: http://tools.neb.com/NEBcutter/index.php3. Searches for open reading frames (ORF) were conducted using software (open reading frame finder (ORF Finder)) located at the NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) using standard genetic code.

5.21 BLAST searches and parameters

Sequences were compared to both the nucleotide (nr) and expressed sequence tag (EST) databases using the BLAST. This allows comparison of input nucleotide sequences against those in the database. The parameters used were as follows; Low complexity filter, Expect Value – 10, Word Size – 11.

6.0 Results

6.1 DNA gel mobility shift assay shows specific YY1 binding activity

Previously in our laboratory, a DNA binding activity was observed in *Xenopus* embryos that was specific for the internal coding region activator element (CRAS) of replication-dependent histone genes, however the protein(s) in these complexes were not identified (Ficzycz et al., 1997). Subsequently, a yeast one-hybrid screen of a HeLa cell cDNA library performed in the laboratory of Myra Hurt established that the protein interacting with the histone regulatory sequence in mammalian cells was the Yin Yang 1 (YY1) transcription factor (Eliassen et al., 1998).

In our previous study, YY1 was referred to as p85 which was derived from its approximate molecular mass as determined by ultraviolet cross-linking (Ficzycz et al., 1997). In order to confirm that the p85 binding activity was indeed YY1, gel mobility shift assays were performed using the YY1 binding element and specific YY1 polyclonal antibodies (sc-281, Santa Cruz Biotechnology). The YY1 antibodies used in this study are directed against the mammalian form of YY1. In order to ensure that the YY1 antibodies would recognize the frog form of YY1, an amino acid sequence alignment was performed by Santa Cruz Biotechnology (Santa Cruz, California) with the peptides used to make the YY1 antibodies and homologus regions of the frog protein. This showed a 100% alignment between homologus regions of human

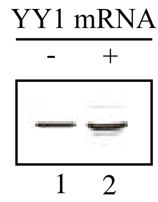
Figure 2

Developmental profile of YY1 DNA binding activity.

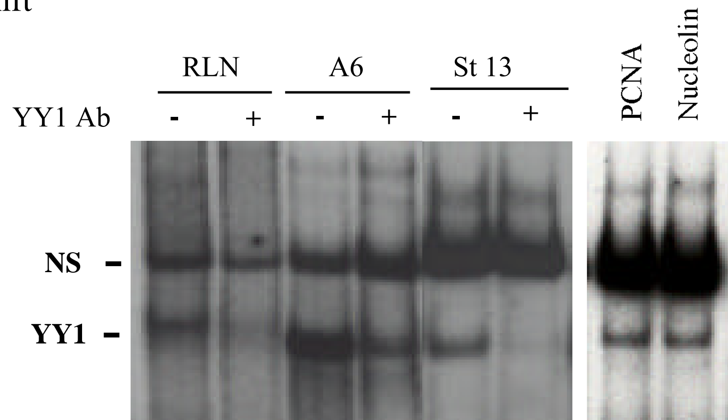
A) Western blot of extracts prepared from stage VI oocytes that were microinjected with mRNAs encoding *Xenopus* YY1. Microinjection of mRNA is indicated at the *top of the panel*. **B**) Gel mobility shift assay of rat liver nuclear (RLN), A6 and neurula stage embryo (St 13) extracts using the YY1-binding element. Antibodies (recognizing YY1, nucleolin or PCNA) were added to binding reactions as indicated *above the panel*. The positions of non-specific (NS) and specific (YY1) bands are indicated at the left. **C**) Gel mobility shift analysis showing relative YY1-DNA binding activity in staged oocyte and embryonic lysates. Developmental stages are indicated *above the panel*. The addition of competitor YY1 antibodies is indicated *above the panel*. **D**) Gel mobility shift analysis of AP-1 DNA binding activity during development. The addition of unlabeled competitor oligonucleotides (50-fold molar excess) is indicated below the panel.

Figure 2

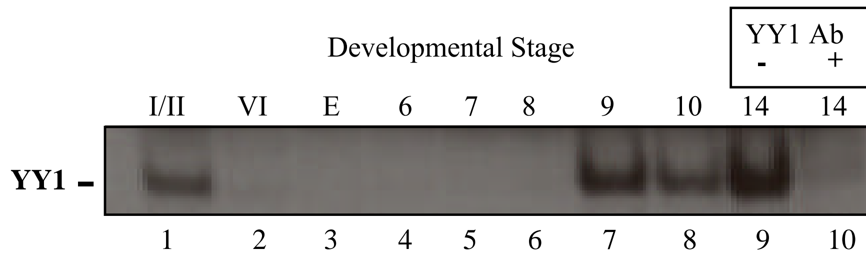
A Western blot



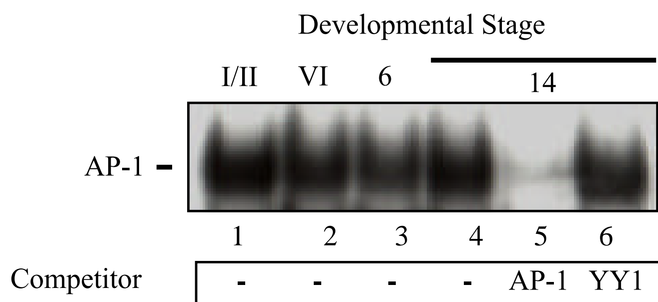
B Gel Shift



C Gel Shift



D Gel Shift



and frog YY1 (the alignment was performed by Santa Cruz Biotechnology; the sequences of epitopes against which the antibody was made is proprietary). Therefore it was expected that these antibodies would recognize the frog protein. To provide further evidence that these antibodies recognize frog proteins, *in vitro* synthesized YY1 mRNA encoding the frog protein were microinjected into stage VI oocytes and incubated for 16 hr at 18˚C to allow for translation. Analysis of protein extracts by Western blotting indicated an increased detection of the YY1 signal (Figure 2A, *lane* 2) versus uninjected controls (Figure 2A, *lane* 1).

To confirm the identity of the *Xenopus* factor, gel mobility shift assays were performed using the CRAS element which contains the known YY1 consensus sequence (5'-CATGGCG-3' (Figure 2B) (Ficzycz et al., 1997). Multiple complexes were detected after incubation of stage 13 embryonic extracts with the YY1 binding element (Fig 2B, *lane* 5). The addition of polyclonal anti-YY1 (*in vitro*) antibodies to binding reactions disrupted the lower complex indicating that it is the YY1-specific binding complex (Figure 2B, *lane* 6). Similar YY1 complexes and specific antibody disruptions were observed with rat liver nuclear extracts (Fig 2B, *lane* 2) and crude whole cell extracts from the A6 *Xenopus* kidney epithelial cell line (Fig 2B, *lane* 4). The prominent slower migrating complexes were not recognized by YY1 antibodies and are thought to represent non-specific interactions that occur between the DNA probe and nuclear factors present in *Xenopus* oocyte and embryo extracts (Ovsenek et al., 1991). The addition of polycloncal antibodies against PCNA or nucleolin did not have any effect on the YY1 complex (Figure 2B, *lanes* 7 and 8).

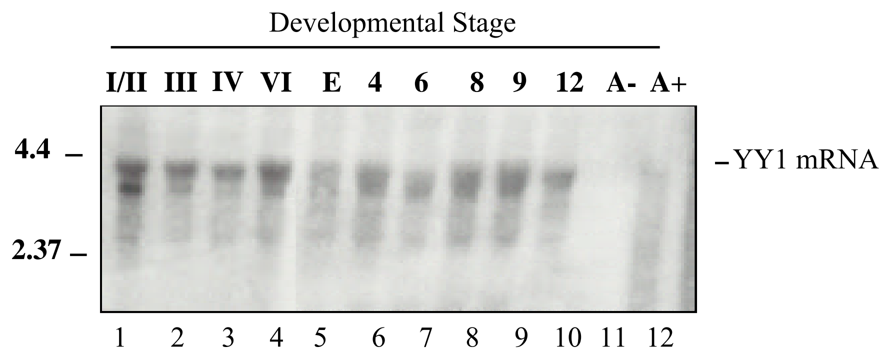
To examine YY1 DNA binding activity during development, crude whole cell extracts were prepared from staged oocytes, eggs and embryos and subjected to gel mobility shift assays using the YY1 binding element probe (Figure 2C). The specific YY1 complex was detected in stage I and II oocytes (*lane 1*) as well in embryos after the MBT (lanes 7-9) however no DNA binding activity was seen in extracts taken between oocyte stage I/II and embryo stage 9 (lanes 2-6). Addition of YY1 antibodies to binding reactions (lane 10) resulted in disruption of the complex confirming the identity of YY1 complexes in these assays.

Controls were performed in order to test the possibility that the fluctuations seen in the DNA binding of YY1 may be due to non-specific biochemical properties of the extracts at different stages of development. The DNA-binding activity of the AP-1 transcription factor has been shown to be stable through this period of development (Gordon et al., 1996). Gel shifts using the same extracts as in Figure 2C and the AP-1 consensus site probe demonstrated that the DNA binding activity of AP-1 was stable throughout the developmental stages analyzed (Figure 2D). The addition of unlabeled AP-1 competitor oligonucleotides resulted in a complete competition of the binding complex (*lane* 5) while the addition of YY1 competitor oligonucleotides (*lane* 6) had no effect on AP-1 binding activity.

6.2 Northern blot analysis of YY1 mRNA expression during *Xenopus* development

Northern blot analysis was performed to examine the pattern of YY1-mRNA expression during early development. Data shown in Figure 2C indicated

Figure 3 Northern Blot



Northern blot analysis of YY1 during *Xenopus* development.

Northern blot analysis of YY1 mRNA expression during oocyte and embryonic development. Each lane contains 10*ug* total RNA isolated from developing oocytes (Roman numerals), unfertilized eggs (E) and embryos (Arabic numerals, *shown at top of panel*). Poly(A)$^{+}$and Poly(A)$^{-}$ RNA is shown at the top of the panel (*lanes* 11 and 12). The positions of the RNA markers (Kb) are shown at the left side of the panel. The position of the YY1 mRNA is indicated on the right side of the panel.

that the DNA binding activity of YY1 was not constant throughout development, thus YY1 mRNA levels were analyzed to determine whether these fluctuations may be attributable to changes in the levels of YY1 mRNA expression during development. Total RNA was isolated from oocytes and embryos at various stages of development, and electrophoresed through agarose/formaldehyde gels, transferred to nylon membranes and probed with ^{32}P-labeled YY1 cDNA probes. A 4-kilobase mRNA transcript was detected (Figure 3). Relatively constant amounts of YY1 mRNA signal were detected in early and late stage oocytes (lanes 1-4), unfertilized eggs (lane 5), in cleavage (lanes 6-8), and early neurula stage embryos (lanes 9-10). Due to the fact that zygotic transcription does not commence until the MBT (Newport and Kirschner, 1982a) it appears that transcription of the maternally encoded YY1 gene occurs very early during oogenesis. These blots also indicate that the maternal transcripts are relatively stable throughout fertilization and early embryonic development. The YY1 mRNA transcripts detected in Northern blots migrated just below the 28S ribosomal RNA bands. To rule out the possibility of cross-hybridization to ribosomal RNA, hybridization of the YY1 probe to Poly-(A)$^{+}$ (*lane 12*) and Poly-(A)$^{-}$ (lane 11) RNA isolated over oligo(dT) columns was analyzed. The 4 Kb YY1 mRNA signal was detected in Poly-(A)$^{+}$ samples containing mRNA, but not in the Poly-(A)$^{-}$ sample containing ribosomal RNA.

6.3 Western blot analysis of YY1 protein levels during development

Protein expression during development was characterized by Western blot analysis. Whole cell lysates were prepared from normally developing oocytes and embryos. Aliquots were separated on 10% polyacrylamide gels, transferred to nylon membranes and probed with anti-YY1 antibodies. A single prominent 59 kDa protein was detected in unfertilized eggs and developing embryos from cleavage through to neurula stages (Figure 4A). The relatively constant levels of YY1 protein are consistent with the constant levels of mRNA detected on Northern blots (Figure 3). The constant levels of YY1 observed during early development by this assay provided evidence that the changes seen in the DNA binding activity of YY1 (Figure 2C) are not due to fluctuations in the absolute levels of maternally expressed YY1.

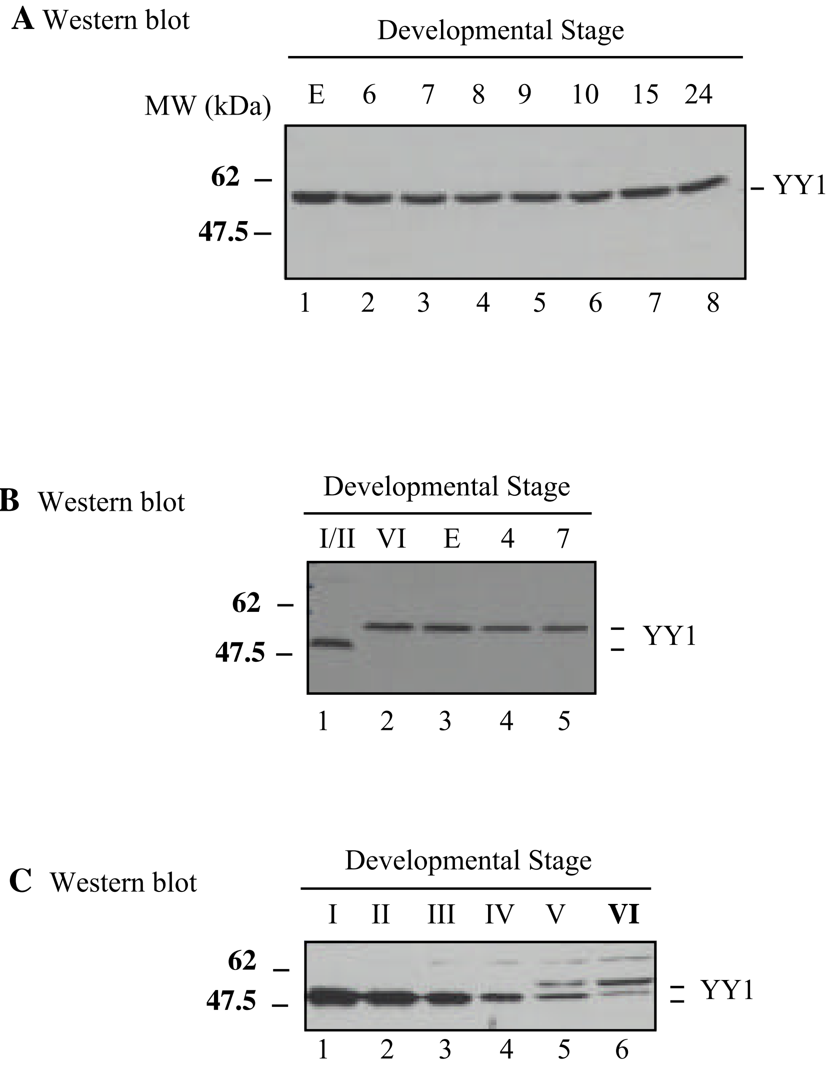
Protein levels of YY1 were also examined during oogenesis and through oocyte maturation, fertilization and early embryogenesis (Figure 4B). Similar amounts of YY1 protein were observed in extracts from oocyte stages and embryonic stages. However, in stage I and II oocytes, the YY1 signal was significantly smaller with an estimated molecular mass of 50 kDa (Figure 4B, *lane* 1). A more detailed analysis of of YY1 expression during oogenesis is shown in Figure 4C. This showed that YY1 is predominantly a 50 kDa protein in stage I oocytes (*lane* 1) but by stage VI (*lane 6)* the majority of YY1 migrates as a 59 kDa species. The molecular mechanism resulting in the shift in mass is not presently known.

Figure 4

Western blot analysis of YY1 protein during development.

Each lane contains 10 *u*g oocyte/embryonic protein isolated from devlopmental stages as indicated at the top of each panel. In each blot, the samples were obtained from the same batch of oocytes/embryos to reduce variability between different females. **A**) Western immunoblot of staged extracts from eggs (E), and embryos at various stages of development. **B**) Western immunoblot showing a comparison of YY1 expression in early and late oocytes and at stages of development around fertilization. **C**) Western immunoblot showing comparison of YY1 expression during each stages of oogenesis. Positions of protein molecular weight markers are indicated at the *left* of the panels.

Figure 4

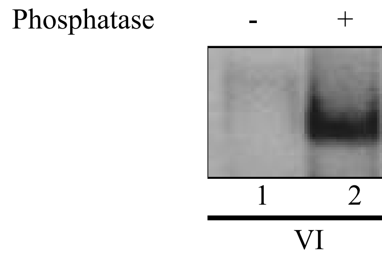


6.4 Phosphorylation state affects DNA binding activity of YY1

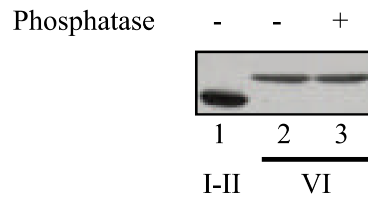
Next, the effects of phosphorylation on the DNA-binding activity and relative molecular mass of YY1 were examined. A correlation was observed between appearance of the 50 kDa isoform of YY1 and a strong DNA binding activity in early stage oocytes (Figure 2B). As the oocyte matures, both of these attributes change, that is the molecular mass increases to 59 kDa, concomitant with the reduction of DNA binding activity. Dephosphorylation by *in vitro* phosphatase treatment of cleavage stage embryonic extracts was known to result in recovery of DNA binding (Ficzycz et al., 1997). In addition, Becker and colleagues (Becker et al., 1994) identified 8 consensus phosphorylation sites within human YY1 and showed that in Jurkat T-cell nuclear extracts, phosphorylation was sufficient to cause an increase in the molecular mass detectable by Western blotting. Thus it was hypothesized that hypophosphorylation of YY1 could contribute to both the high level of DNA binding activity and appearance of the smaller 50 kDa isoform found in early oocytes. As oogenesis proceeds, an unidentified kinase might phosphorylate YY1 resulting in both an increase in the molecular mass of YY1 and a loss of DNA binding activity. This hypothesis was tested by gel mobility shift assays and Western blotting using stage VI oocytes. Phosphatase treatment resulted in a partial recovery of DNA binding activity (Figure 5A, *lane* 2). This suggests that YY1 binds DNA more efficiently in the dephosphorylated state and raised the possibility that DNA binding activity of YY1 might be differentially regulated by phosphorylation during development. Also, it was possible that the increase in the apparent molecular weight of YY1 during oocyte development might be

Figure 5

A Gel Shift



B Western Blot



Effect of phosphatase treatment on YY1 molecular mass and DNA binding affinity.

A) Stage VI oocyte extracts were treated with calf intestinal alkaline phosphatase prior to binding reactions and were analyzed by gel mobility shift assays for DNA binding activity with the YY1 probe. **B**) Western immunoblot of stage VI oocyte extracts (the same extracts that were analyzed in Figure 5 A) treated with CIAP and analyzed for changes in the molecular mass of YY1.

attributable to increased phosphorylation. However, Western blots showed that YY1 was detected at 59 kDa in both phosphatase-treated (Figure 5B, *lane* 2) and untreated extracts (*lane* 3). This indicates that the increase in the apparent molecular weight of YY1 in stage VI oocytes is probably not attributable to phosphorylation *in vivo*.

Additionally, these assays do not necessarily indicate that the dephosphorylation event brought about by the addition of CIAP occurs directly on the YY1 molecule.

6.5 Transcriptional activity of YY1

Given that relatively constant levels of YY1 with fluctuating DNA-binding activity are present in oocytes and embryos, it was of interest to analyze the transcriptional activity of this factor during development. This was examined using a series of reporter constructs containing the thymidine kinase (TK) promoter linked to YY1 binding sites upstream of the Chloramphenicol Acetyl-Transferase (CAT) gene. Six reporter constructs were used in these experiments (Figure 6A). Control constructs contained only the CAT reporter with no promoter elements (pCAT), or the thymidine kinase promoter located immedately upstream of the CAT reporter (TK-CAT). Previous experimental data had shown that YY1 elements placed in front of the TK promoter were able to activate CAT activity in mouse cells (Eliassen et al., 1998; Palla et al., 1989). YY1-TKCAT contains 6 consecutive YY1 binding elements from the histone CRAS (including flanking regions) of the mouse H3.2 gene (Eliasson et al., 1998) in the forward orientation inserted upstream of the TK promoter. YY1R-

TKCAT contains the same YY1 binding elements in the reverse orientation upstream of the TK promoter. YY1M-TKCAT contains 6 repeats of a mutated YY1 binding element (5'-tgagctat***TGGTGCT***ttgcag-3'), and CRAS-TKCAT contains a single YY1 binding site upstream of the TK promoter.

Batches of at least 100 embryos were injected at the 2 cell stage or 100 oocytes at stage VI were injected individually with 200 *p*g of each construct DNA. Embryos were allowed to develop at 18˚C to stage 10 (gastrula) and oocytes were incubated for 18 hours at 18˚C to allow sufficient time for reporter expression. Extracts were prepared from oocytes and embryos and CAT activity was analyzed by thin layer chromatography. No
CAT activity was observed in either oocytes (Figure 6B, *lane* 1) or embryos (Figure 6C, *lane* 1) that had been injected with constructs that did not contain a promoter (pCAT). Oocytes or embryos injected with the TK promoter upstream of the CAT reporter showed a detectable level of reporter activity (Figure 6B,C, *lane* 2). Relative to this level of expression, none of the combinations of YY1 binding elements were able to stimulate or repress activity from the TK promoter in oocytes or post-MBT embryos (Figure 6B,C, *lanes* 3-6). Control experiments using Chinese Hamster Ovary (CHO) cells (Figure 6D) demonstrated that YY1 binding elements situated upstream of the TK promoter are able to influence CAT activity (Ficzycz et al., 2001) resulting in a 3-fold increase over basal promoter activity indicating these constructs are responsive to YY1 binding elements. These data indicate that YY1 is transcriptionally inactive in oocytes and post-MBT embryos.

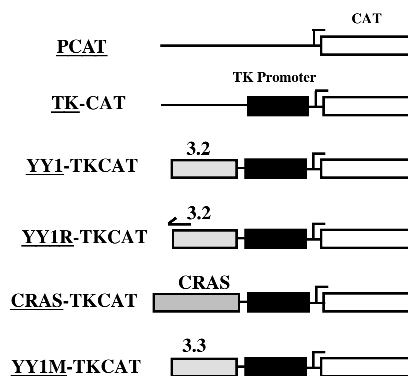
Figure 6

Transcriptional activity of YY1 in oocytes and post-MBT embryos.

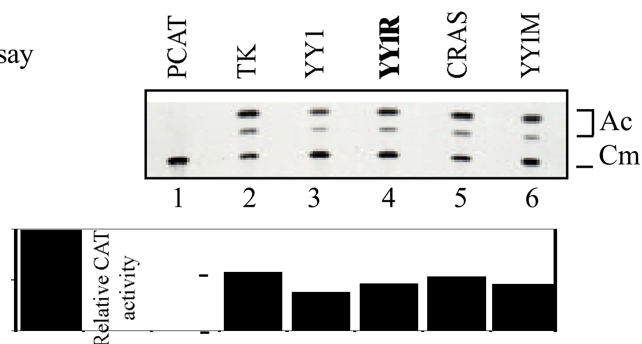
A) Diagram of plasmid constructs containing the CAT gene driven by the TK promoter and YY1 elements. **B**) Stage VI oocytes were microinjected with the indicated plasmids and incubated for 8 hours at which time CAT assays were performed. (*upper panel*). The experiment was repeated at least 5 times with different batches of oocytes and representative data is shown. **C**) Embryos were microinjected after fertilization with the indicated plasmid constructs and then allowed to develop to the gastrula stage at which point CAT assays were performed (*upper panel*). The experiment was repeated at least 5 times with different batches of embryos; representative CAT assays are shown. CAT activity is expressed as a perecentage of the highest relative activity (TK-CAT) which was arbitrarily assigned a value of 100 (*lower panel*). **D**) CAT assays of stable transfectant CHO cells. Constructs are indicated *above the panel*. Activity is presented as percent acetylation. The experiment was performed with triplicate sets of plates.

Figure 6

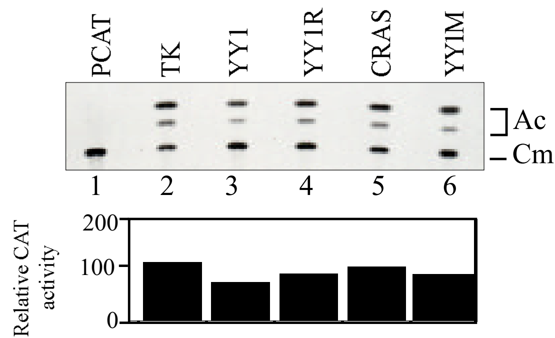
A



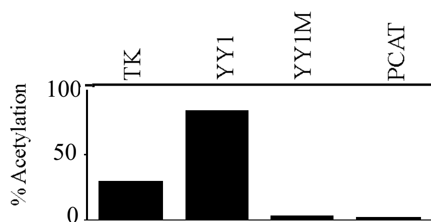
B CAT assay



C CAT assay



D CAT assay



In stage VI oocytes, the finding that YY1 was transcriptionally inactive was not surprising given the absence of DNA binding activity during this period of development (Figure 2C, *lane* 2) (Ficzycz et al., 1997). In addition, these findings are in agreement with the results of others showing that the YY1 binding site from the L1 and L14 ribosomal protein genes did not affect transcription of reporter constructs in oocytes (De Rinaldis, et al., 1998). However it is interesting that YY1 binding elements did not have any effect on transcription in post-MBT embryos given that DNA binding activity at these stages of embryonic development is restored to high levels (Figure 2C, lanes 7-9l) (Ficzycz et al., 1997). The lack of transcriptional activity of YY1-containing constructs seen in this experiment is explained later in this thesis by exclusion of YY1 from the nucleus during development.

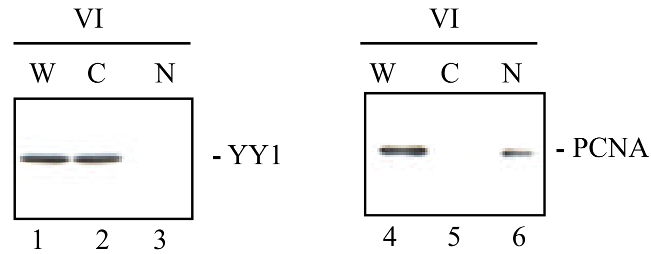
6.6 Subcellular localization of YY1

A recent study of the developmental role of YY1 in mouse embryos has revealed that it is localized to the cytoplasm of unfertilized mouse oocytes and displays a mosaic pattern of sub-cellular localization in E3.5 blastocysts (Donohoe et al., 1999). This was the first study to indicate that YY1 may not be exclusively localized to the nucleus, and the results in mouse suggested that nucleocytoplasmic partitioning could play a role in YY1-regulation in *Xenopus* embryos.

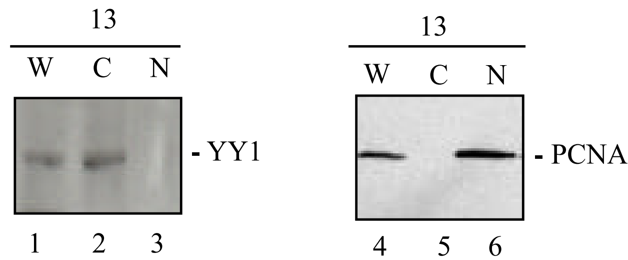
The subcellular distribution of YY1 in the cells of the developing frog was next analyzed. Stage VI oocytes were manually dissected into nuclear and

Figure 7

A Western blot



B Western blot



Detection of YY1 in cytoplasmic fractions from oocytes and post-MBT embryos.

A) Western immunoblot analysis of YY1 in Stage VI oocytes. Oocytes were manually dissected into nuclear (N) and cytoplasmic (C) fractions, (W) indicates whole cell extracts. Blots were probed with anti-YY1 polyclonal (*lanes* 1-3) and Anti-PCNA (*lanes* 4-6) monoclonal antibodies (indicated at the *right of each panel)*. **B**) Western immunoblot analysis of YY1 in stage 13 embryos that were fractionated into nuclear (N) and cytoplasmic (C) fractions, (W) indicates whole embryonic extract. Blots were probed with anti-YY1 polyclonal (*lanes* 1-3) and anti-PCNA (*lanes* 4-6) monoclonal antibodies .

cytoplasmic fractions which were analyzed by Western blotting. YY1 was present in cytoplasmic (Figure 7A, *left panel*, lane 2), but not nuclear fractions (Figure 7A, *left panel*, lane 3) indicating that YY1 is a cytoplasmic protein in later stage oocytes. Control blots were analyzed for the presence of proliferating cell nuclear antigen (PCNA) which is an exclusively nuclear DNA binding protein. As seen in the *right panel* of Figure 7A, PCNA is detected only in the nuclear fractions (*lane* 6) confirming that the enucleation procedure was carried out with no cross contamination between nuclear and cytoplasmic fractions.

Biochemical fractionation into nuclear and cytoplasmic components was employed to determine the subcellular localization of YY1 in embryos. Western blotting showed that YY1 was detected in cytoplasmic fractions (Figure 7B, *left panel*, *lane* 2), but not in nuclear fractions (Figure 7B, *left panel*, *lane* 3) obtained from neurula stage embryos (stage 13). In control blots PCNA was detected only in nuclear fractions (Figure 7B, *left panel*, *lane* 6) again indicating that the fractionation was completed without contamination of nuclear or cytoplasmic fractions.

An immunocytochemical study of the subcellular distrubution of YY1 in *Xenopus* oocytes and embryos carried out by C. Eskiw in our laboratory showed that YY1 is present exclusively in the cytoplasm from stage I oocytes through to neurula stage embryos (Ficzycz et al., 2001). When adult frog liver sections were analyzed for YY1 by immunocytochemistry, it was found to be present in the nuclei, however the precise time and location of YY1 translocation from cytoplasm to nuclei during development has not been determined (Ficzycz et al., 2001). These data suggest that the cytoplasmic localization of YY1 in the

developing frog is likely to account for the complete absence of transcriptional activity seen in Figure 6.

6.7 YY1 Contains a Known RNA-Binding Domain

Structural analysis of mammalian YY1 has shown that it contains four C_2H_2 zinc finger domains that are essential for DNA binding activity (Galvin and Shi, 1997, Thomas and Seto, 1999). The presence of zinc fingers in YY1 provided an initial clue as to a potential function for this protein in the cytoplasm. Certain RNA-binding proteins such as TFIIIA, which is known to bind 5S rRNA (Brown et al., 1988), display some sequence similarity to zinc finger DNA-binding transcription factors, particularly in the spacing of key cysteine and histidine residues (Burd and Dreyfuss, 1994). The amino acid sequence of *Xenopus* YY1 was analyzed for similarity to established RNA binding motifs. Figure 8 shows an amino acid alignment of *Xenopus* YY1 and TFIIIA between residues 255 and 287. Located within the zinc fingers is a known RNA binding motif, the Zinc Finger Knuckle. No significant similarities were found between YY1 and any other RNA binding motifs.

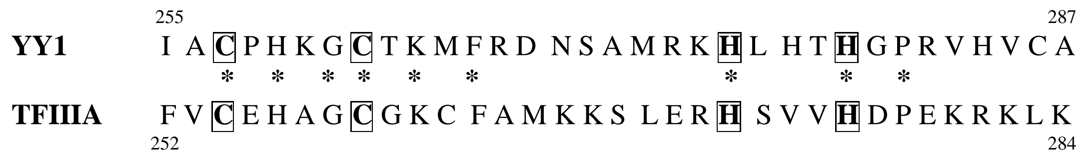
6.8 Endogenous YY1 interacts with maternal mRNAs.

The conserved spacing of key residues within the zinc finger knuckle binding domain of YY1 suggested that it might bind RNA in the oocyte and could therefore be a component of mRNP complexes. To investigate this, *Xenopus* oocyte lysates were analyzed for their capacity to be retained by oligo-

Figure 8

Conservation of a zinc finger/knuckle RNA-binding domain in YY1

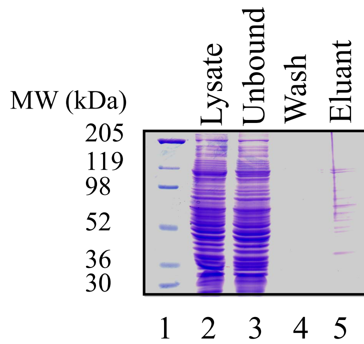
A partial amino acid alignment of *Xenopus* YY1 (PDB accession number JC2426) and TFIIIA (PDB accession number TWXL3) are shown. Key cysteine and histidine residues are *boxed*. Asterisks denote conserved residues.

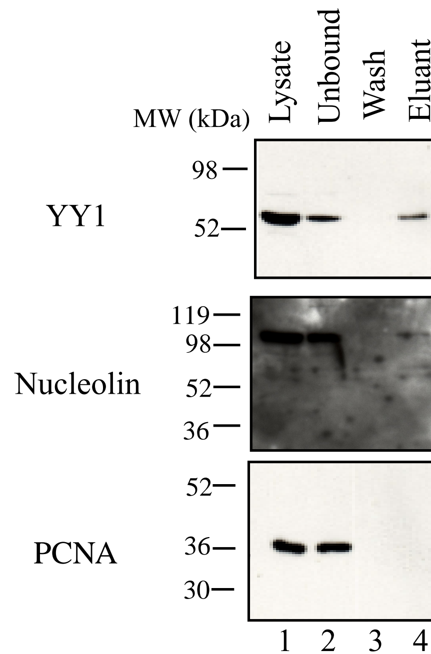


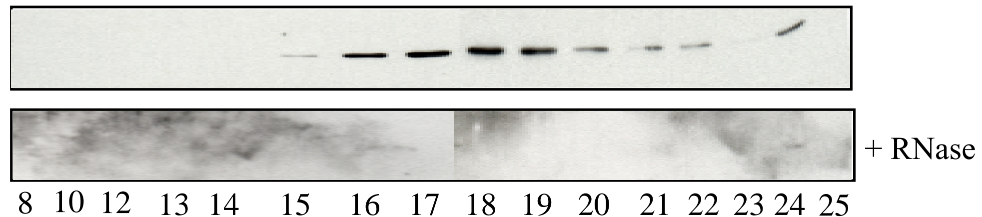
(dT)-cellulose columns. Previous studies have exploited the ability of native mRNP particles containing maternal mRNA to bind selectively to oligo-(dT) through the 3'-poly(A) tail (Marello et al., 1992, Matsumoto et al., 2000, Cummings and Sommerville, 1988). Stage VI oocyte lysates (treated with Rnase inhibitors immediately after they are homogenized to protect endogenous RNAs from degradation) were mixed with oligo-(dT)-cellulose beads, and following extensive washing of the cellulose matrix, bound proteins were eluted in a buffer containing 25% formamide by conventional gravity based chromatography. Analysis of eluates on coomassie blue stained polyacrylamide gels indicated that a small subset of total protein was retained on the oligo-(dT) matrix following extensive washing (Figure 9A, compare lanes 2, 3 and 5). The relative amount of total protein retention is consistent with the results of others attempting to isolate mRNPs from endogenous lysates by this method (Matsumoto et al., 2000). Western blots of the same eluates showed that YY1 was present in the oligo-(dT)-bound fractions (Figure 9B, *top panel, lane* 4). Nucleolin, a known component of mRNPs (Yurkova and Murray, 1997) was detected in bound fractions by Western blotting indicating that mRNPs were successfully isolated in this experiment (Figure 9B, *centre panel lane* 4). When fractions were assayed for PCNA, no protein was detected in the eluates, indicating that there was very little or no non-specific retention of proteins on the oligo-(dT) columns (Figure 9B, *bottom panel*, *lane* 4).

This experiment was also carried out on oligo-(dT) cellulose columns that were run using a high pressure liquid chromatography (HPLC) control unit. This permitted the precise controlling of elution parameters and tracking of protein

Figure 9







Endogenous YY1 interacts with maternal mRNA.

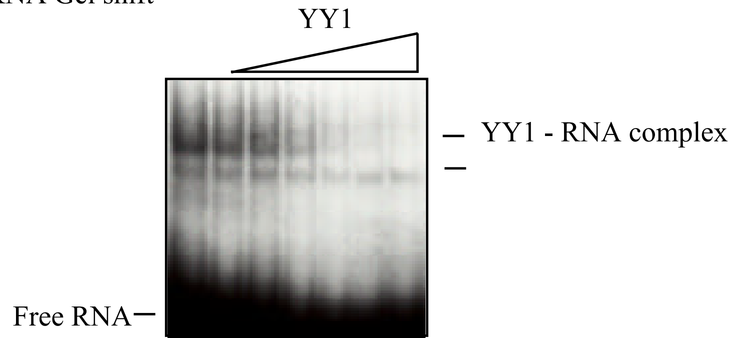
A) Oocyte lysates were incubated with oligo(dT)-cellulose and following extensive washing with binding buffer, bound proteins were eluted with 25% formamide. Aliquots of the original lysate, flow-though, wash and eluant were separated by10% SDS-PAGE, and proteins were detected by coomassie blue staining. **B**) The same fractions were analyzed by Western immunoblotting using antibodies against YY1, Nucleolin, and PCNA as indicated at the *left* of the panel. **C**) Oocyte lysates were applied to to oligo(dT)-cellulose columns and proteins were eluted with a gradient of increasing NaCl concentration. Fractions (indicated at the b*ottom of the panel*) were analyzed by Western blotting for YY1. Samples in the *lower panel* were from lysates that were treated with RN*ase* prior to loading onto the column.

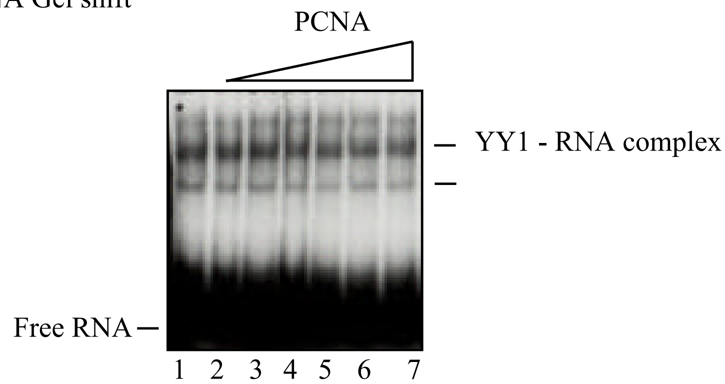
released from oligo-(dT). Stage VI lysates were loaded onto columns at 4˚C and the flow through was re-applied 3 times to ensure maximal RNA/mRNP binding. Bound proteins were eluted from columns using a salt gradient (0.5M-2.0M). YY1 was eluted from the columns at 600-750 mM NaCl (Figure 9C, *top panel*). This reproduces the findings shown in Figure 9B, and supports the conclusion that YY1 is retained on oligo-(dT) by virtue of its interaction with maternal poly(A)$^+$ mRNAs in the cytoplasm. To eliminate the potential for non-specific binding of YY1 to oligo-(dT), lysates were treated with RNase prior to chromatography (these lysates were not supplemented with RNase inhibitors). RNase treatment abolished the retention of YY1 on oligo-(dT) cellulose columns (Figure 9C, *bottom panel*). This demonstrated that the affinity of YY1 for oligo-(dT) cellulose is dependent on intact RNAs in the cytoplasm and provided compelling initial evidence that YY1 associates with maternal mRNA in the cytoplasm and that it may be a component of mRNP complexes.

6.9 *In vitro* YY1 RNA-binding activity

I*n vitro* RNA mobility shift assays were employed to test for the ability of YY1 to bind directly to RNA. A radiolabeled 393 nucleotide histone mRNA was synthesized *in vitro* and used as a probe. The rationale for using the histone mRNA probe in this experiment was that it is a small message amenable to mobility shift assays, and that it is an abundant message in the oocyte (Woodland, 1980) providing a biologically relevant binding target. In addition to this, previous studies have used histone mRNA probes to demonstrate *in vitro* binding activity of mRNPs (Matsumoto and Wolffe, 1998). Stage VI oocyte

Figure 10





RNA gel mobility shift assay of endogenous YY1.

RNA mobility shift assays were performed using oocyte lysates and ^{32}P-labeled H2A probes. Increasing amounts of antibodies against YY1 (**A**) or PCNA (**B**) were added directly to the binding reactions as indicated. The positions of free mRNA, an unidentified complex, and the specific YY1-mRNA complex are indicated at the *right* of the panels.

lysates (supplemented with RNase inhibitors) were combined with radiolabeled histone mRNA probes *in vitro* and the RNA binding reactions were incubated at 4˚C for 20 min then separated on 5% acrylamide/glycerol gels maintained at 4˚C for the duration of electrophoresis. Two distinct protein-mRNA complexes were observed (Figure 10). Antibody competition experiments using antibodies against YY1 and PCNA added individually to RNA binding reactions were performed in order to establish the specificity of these complexes. The addition of anti-YY1 antibodies to binding reactions resulted in a concentration-dependent abolishion of the upper complex, indicating that this band is a specific YY1 containing complex (Figure 10A). No effect was seen on the formation of the upper or lower bands upon addition of anti-PCNA antibodies (Figure 10B). This suggests that abolishment of the upper complex produced by the addition of anti-YY1 antibodies was the result of specific antibody recognition of YY1 in an RNA-protein complex. These data indicate that YY1 is able to associate with RNA, however they do not identify whether YY1 binds directly to RNA or to other proteins that may be present in the shifted complex. As well, these data do not distinguish whether YY1 binds RNAs with any sequence specificity. Previous studies have shown that the major mRNP proteins in *Xenopus* (eg, FRGY2) do not bind RNA with any degree of sequence specificity (Yurkova and Murray, 1997), however this issue still remains to be addressed in the context of YY1.

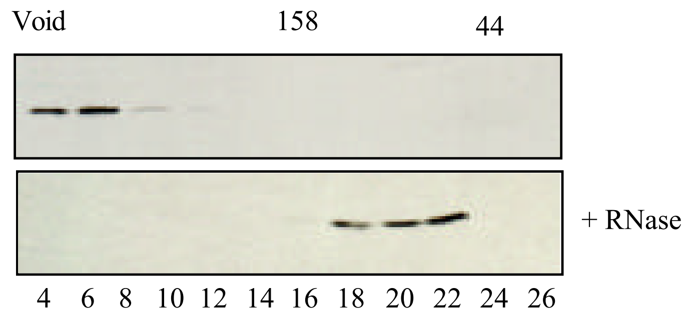
6.10 Size exclusion chromatography of native mRNPs

The results presented in Figures 9 and 10 suggested that YY1 may be a component of cytoplasmic mRNPs. Previous studies have shown the molecular mass of *Xenopus* oocyte mRNP complexes range from 350 kDa to 1000 kDa (Yurkova and Murray, 1997; Matsumoto, 2000). I next examined the molecular mass of YY1-containing complexes. Lysates were prepared from stage VI oocytes, treated with RNase inhibitors to prevent the degradation of endogenous RNAs and fractionated by size exclusion chromatography (SEC). Two columns were used for this analysis, one with a separation range of 10,000-300,000 daltons (Bio-Select 250, Bio-Rad) and one with a separation range of 10,000 – 1,100,000 daltons (Superdex 200 HR 10/30, Pharmacia). Aliquots (200 *u*l) of the oocyte lysates were applied to columns run in column buffer at 0.4ml/min and eluted proteins were collected in 200 *u*l fractions which were precipitated in acetone at -80˚C.

Lysates were first applied to Bio-Select 250 columns (Figure 11), fractions were separated by SDS-gel electrophoresis and the size distribution of eluted proteins was analyzed by Western blotting. All of the YY1 was detected in the column flow-through void volume (greater than 300 kDa) (Figure 11, *upper panel*). The presence of YY1 in the void fractions suggested that in its native state, YY1 exists in complexes that are larger than 300 kDa in the cytoplasm.

To investigate whether the association of YY1 in large molecular mass complexes occurred through interaction with mRNPs, endogenous RNAs were degraded by *in vitro* RNase treatment (lysates used for these experiments were

Figure 11 Western blot/SEC



Presence of YY1 in high molecular mass complexes and the effect of RN*ase*.

Size exclusion chromatography of native (*upper panel*) and RNase treated (*lower panel*) stage VI oocyte lysates was performed. The fractions (indicated *below the panel*) were analyzed for YY1 by Western immunoblotting. The positions of molecular mass standards is indicated *above the panel*.

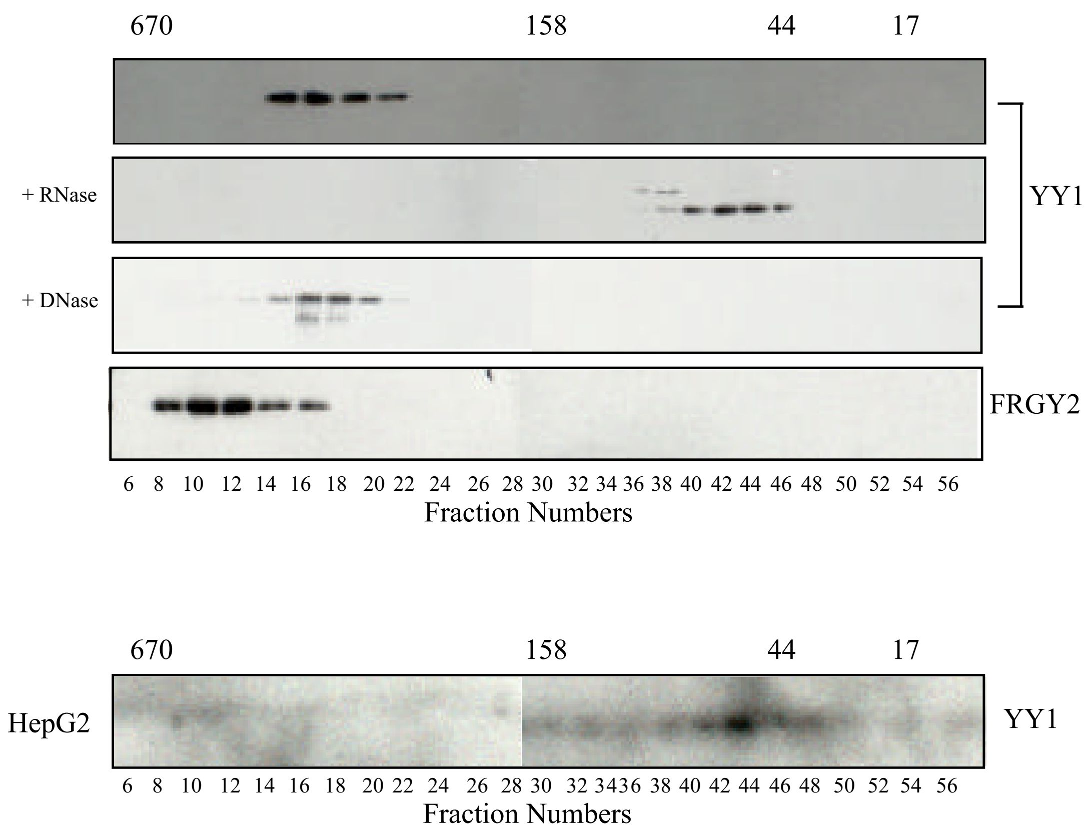
prepared without the addition of RNase inhibitors) prior to chromatography on Bio-Select 250 columns. Western blotting of separated proteins indicated that YY1 was eluted in fractions close to its expected monomeric molecular mass (59 kDa) (Figure 11, *lower panel*). Thus, *in vitro* degradation of RNAs appears to destabilize the large molecular mass complexes permitting YY1 to separate in fractions at or near its expected monomeric mass. The integrity of large YY-containing complexes was dependent on presence of intact maternal RNA suggesting that YY1 associates with or is a component of mRNPs in the oocyte cytoplasm.

6.11 Determination of the Mass of YY1-mRNP complexes

To accurately determine the molecular mass of YY1-associated mRNP complexes, a different column was employed (Pharmacia SuperDex 200 HR10/30) which has a separation range of 10,000 – 1,100,000 daltons. This column was run with the same parameters and calibrated identically to the Bio-Select column resulting in consistent data comparable between the columns (both columns are packed with the same type of cellulose bead, differing only in pore size).

YY1 was detected in fractions with an approximate molecular mass of 480 kDa by Western blotting (Figure 12, *top panel*). Monomers were not detected in untreated native lysates using either column (figures 11 and 12, *top panel*) suggesting that the majority of endogenous YY1 is bound in large molecular mass complexes. FRGY2, the major mRNP component in *Xenopus* oocytes (O’Connor et al., 1996, Tafuri and Wolffe, 1990, Deschamps et al.,

Figure 12 Western blot/SEC



Identification of 480 kDa YY1-mRNP complexes.

Size exclusion chromatography of oocyte lysates (*top four panels*) and liver cell lysates (*bottom panel*) were analyzed for the presence of YY1 or FRGY2 (indicated on the *right of the panel*) by western immunoblotting. The positions of the molecular mass markers is indicated at the top of the panels. Oocyte lysates were treated with RN*ase* or DN*ase* prior to loading as indicated at the *left of the panel*.

1991, Deschamps et al., 1992, Murray et al., 1992) was isolated in a higher mass range (around 500 kDa) in comparison to YY1 (Figure 11, indicated at *right of panel*). The elution profiles of YY1 and FRGY2 were partially overlapping, but not identical.

To confirm that the 480 kDa YY1 containing complex is an mRNP, lysates were treated with RNases prior to separation to degrade endogenous RNAs (Figure 12, indicated on the *left of panel*). SEC separation of these lysates resulted in a redistribution of YY1 to fractions at approximately 59 kDa which is close to its predicted molecular mass as a monomeric protein (Figure 12). In control experiments, aliquots of the same lysates were treated with DNase prior to size exclusion chromatography. No effect on the distribution of YY1 was observed upon DNase treatment (Figure 12, *indicated at left of panel*). Therefore, RNase-mediated destabilization of 480 kDa YY1 complexes was likely a direct result of RNA degradation rather than an artifact of the experimental protocol. The finding that YY1 is isolated in high molecular mass, RNase-sensitive complexes is consistent with the findings that YY1 was isolated from Oligo(dT)-cellulose (Figure 9) and that it has mRNA binding activity *in vitro* (Figure 10). Thus, YY1 behaves biochemically like a *bona-fide* component of mRNP complexes with potential RNA-binding activity.

Comparison of the relative size distribution of cytoplasmic and nuclear YY1 was carried out using size exclusion chromatography. Concurrent studies in our laboratory have established that YY1 is nuclear in adult liver cells (Ficzycz et al., 2001), however the molecular mass of YY1 localized to the nucleus had not been established. Therefore, HepG2 cells (a liver cell line) were used as a source

of nuclear YY1 from which it was possible to determine its molecular mass. HepG2 lysates were passed over the SuperDex 200 HR10/30 (Pharmacia) column which was run using identical parameters as in the experiments described above. YY1 eluted around its predicted molecular mass as a monomer, and no high molecular mass complexes were detected (Figure 12, *bottom panel*). This suggested that when YY1 is localized to the nucleus, it is present as a monomer and when present in the cytoplasm (Figure 12, *top panel*) it associates with or is a component of large molecular mass complexes. Previous data from our laboratory has shown that YY1 is competent to bind DNA in rat liver nuclear extracts (Ficzycz et al., 2001). The findings that YY1 is monomeric and is competent to bind DNA in HepG2 cells but is in large complexes and incompetant for DNA-binding in stage VI oocytes suggests DNA-binding activity is masked by its association with mRNPs in the cytoplasm.

6.12 Microinjected RNase destabilizes YY1-mRNPs

To analyze the association of YY1 with mRNPs *in vivo*, the effects of maternal RNA degradation were tested by microinjection of RNase directly into the cytoplasm of stage VI oocytes. Similar techniques have been used previously to examine the behaviour of the CBTF transcription factor in early *Xenopus* embryos (Brzostowski et al., 2000). Microinjection of RNase resulted in a redistribution of YY1 from high to low molecular mass fractions after SEC on SuperDex 200 HR10/30 columns (Figure 13A, indicated at *right of panel*) confirming that association of YY1 in mRNP complexes is dependent on intact maternal mRNA.

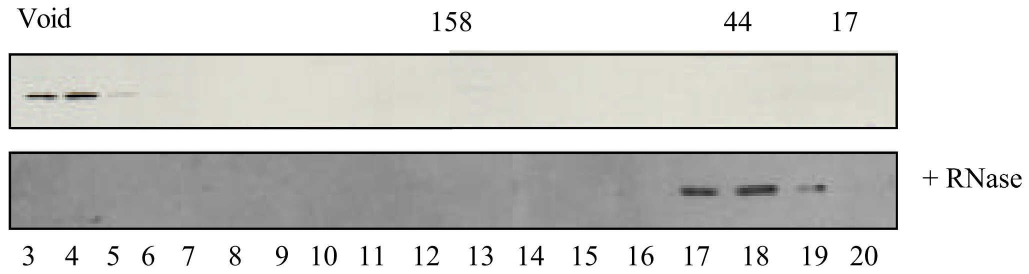
Figure 13

Microinjected RNase destabilizes YY-mRNPs and unmasks DNA binding, but does not promote nuclear translocation.

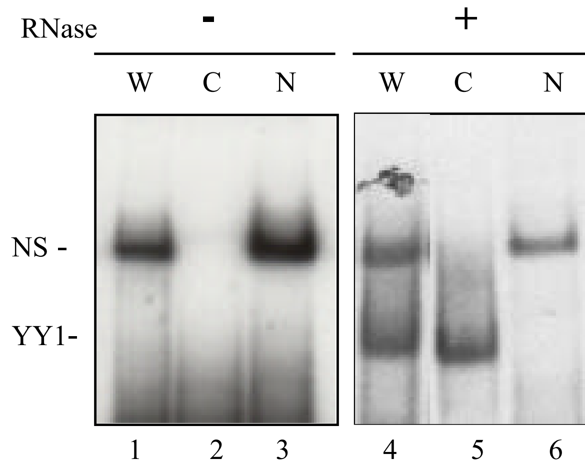
A) Lysates from untreated oocytes (top panel) or oocytes microinjected with RNase and incubated from several hours (bottom panel) were analyzed by SEC and Western immunoblotting for YY1. The positions of size standards are shown above, and fractions are indicated below. **B**) Oocytes were microinjected with RNase (indicated above the panel) prior to the preparation of cell lysates, and gel mobility shift assays were performed on whole cell lysates (W), cytoplasmic fractions (C) and nuclear fractions (N) using ^{32}P-labeled YY1 DNA probes. The postions of nonspecific (NS) and specific YY-DNA (YY1) complexes are indicated at the left of the panel. **C**) The same samples were analyzed by Western immunoblotting using antibodies for YY1 (top panel) or PCNA (bottom panel).

Figure 13

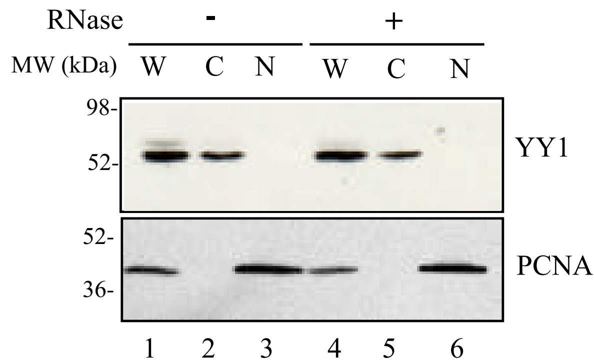
A Western blot/SEC



B Gel shift



C Western blot



YY1 is localized to the cytoplasm through early development (Figure 7A) and displays high levels of DNA-binding activity in early stage oocytes (Figures 2C) (Ficzycz et al., 2001). DNA-binding activity is masked through subsequent oogenesis and reactivated at the midblastula stage after 12 hours of embryonic development (Figure 2C). Therefore it was possible that the DNA-binding activity of YY1 may be blocked by virtue of direct mRNA-binding and/or association with mRNPs. Since RNA degradation releases YY1 monomers from mRNP complexes (Figures 11, 12, 13A) it was possible that RNA degradation may also unmask YY1 DNA-binding activity. To test this, stage VI oocytes were microinjected with RNase, incubated for 8 hours at 18˚C then manually dissected into nuclear and cytoplasmic fractions which were then analyzed by gel mobility shift assays using the ds-YY1 DNA probe. The specific YY1-DNA complex was not detected in untreated lysates (Figure 13B, lanes 1-3) consistent with previous findings that its DNA-binding activity is masked in later oocyte development (Ficzycz et al., 1997). In contrast, high levels of the specific YY1-DNA complex were detected in both whole cell lysates (Figure 13B, lane 4) and cytoplasmic fractions (Figure 13B, lane 5) from RNase-injected oocytes, but not in the nuclear fractions (Figure 13B, lane 6). These data suggest that the DNA-binding activity of cytoplasmic YY1 was unmasked upon release of YY1 monomers from mRNP complexes.

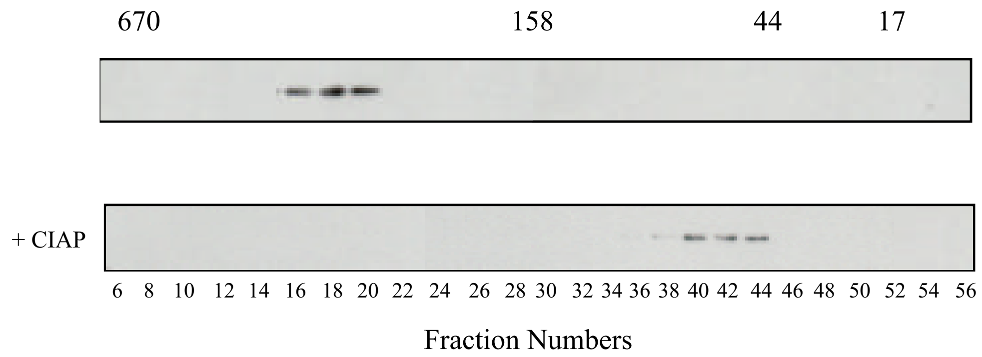
Recently it has been shown that a subunit of the CCAAT box transcription factor, $CBTF^{122}$ is tethered to the cytoplasm through its association with mRNPs, displaying a novel mechanism by which the gene regulatory

activity of a transcription factor may be regulated during development (Brzostowski et al., 2000). Since YY1 is excluded from the nucleus in oocytes and during early embryonic development (Figures 7A,B) it was possible that YY1 might be subject to a similar RNA-dependent cytoplasmic anchoring mechanism. To test this, maternal mRNAs were degraded *in vivo* by RNase microinjection into the cytoplasm of oocytes. It was hypothesized that this would release YY1 monomers from 480 kDa mRNPs, unmask DNA-binding and might also result in nuclear translocation. Aliquots of the same extracts used for SEC and gel mobility shift assays in Figure 13 (A,B) were analyzed by Western blotting for subcellular redistribution of YY1. YY1 was detected in the cytoplasm but not in the nucleus in both uninjected controls (Figure 13C, *left panel,* lanes 1-3) and in RNase-injected oocytes (Figure 13C, *left panel,* lanes 4-6). This suggests that cytoplasmic localization of YY1 is not solely dependent on the stability of maternal RNA. Further, it appears that interaction with mRNPs is not sufficient to prevent nuclear translocation. The control blots showed complete nuclear localization of PCNA (Figure 13C, *right panel*) confirming the viability of nuclear extracts as well as the efficiency of the manual dissection used to obtain the subcellular fractions in this experiment. Overall the results of the RNase microinjection experiments support the conclusion that YY1 associates with mRNP complexes *in vivo*. Destruction of maternal RNAs results in release of YY1 monomers and unmasking of DNA-binding activity, but does not promote nuclear translocation.

6.13. Phosphatase sensitivity of YY1-mRNP complexes

Data presented in Figure 13 provided evidence that YY1 associates with mRNPs which inhibits its ability to bind DNA. It is unlikely that mass RNA degradation is the mechanism which regulates the activity of YY1-mRNPs in the cytoplasm *in vivo*. However, phosphatase treatment of stage VI oocyte lysates is known to upregulate the DNA binding activity of YY1 (Figure 5A) (Ficzycz et al., 1997; Ficzycz, et al., 2001) and mRNPs are known to be regulated by phosphorylation (Deschamps et al., 1997) therefore, it was possible that the upregulation of YY1-DNA binding in response to phosphatase treatment seen in Figure 4 was due to mobilization of YY1 from mRNPs brought about by dephosphorylation. To evaluate this possibility, it was necessary to establish whether YY1-mRNPs are sensitive to phosphatase treatment. Lysates were prepared from stage VI oocytes and treated with RNase inhibitors (identically to those used in experiments described in Figure 12). For this experiment it was essential to have the endogenous RNAs intact as this experiment was designed to test whether YY1 could be liberated from intact endogenous RNAs by dephosphorylation. Lysates were then treated with Calf Intestinal Alkaline Phosphatase (CIAP) and incubated at 4˚C for 45 minutes (using the same conditions as those in Figure 5A to produce the increase in DNA binding activity). Phosphatase-treated lysates (200*u*l) were loaded onto the Superdex 200HR 10/30 column and the proteins were separated at 0.4 ml/minute. Western blots of fractionated untreated lysates indicated that YY1 was maintained in high molecular mass complexes (Figure 14, *top panel*). Analysis of phosphatase-treated lysates indicated that YY1 eluted around its predicted molecular mass as

Figure 14



YY1 containing mRNP complexes are sensitive to phosphatase.

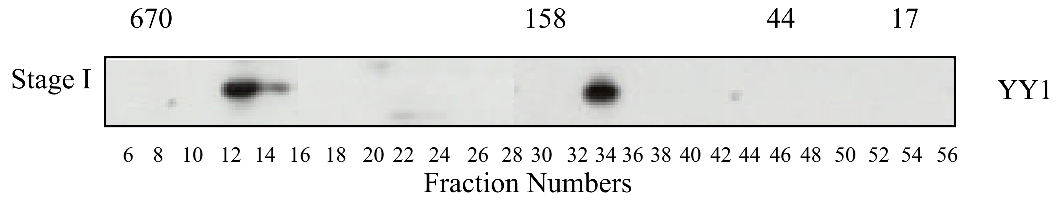
Stage VI oocytes were treated with phosphatase *in vitro* (indicated at the *left* of the panel) then separated by size exclusion chromatography. YY1 was detected by Western immunoblot analysis. Fraction numbers are shown at the *bottom of the panel*, molecular mass markers are shown at the *top of the panel*.

a monomer (Figure 14, *bottom panel*). Thus, dephosphorylation appears to release YY1 from high molecular mass mRNP complexes such that YY1 is redistributed to its monomeric form. It is hypothesized that release or disassembly from mRNP complexes results in unmasking of DNA binding activity. The results of this experiment suggest phosphorylation as the endogenous mechanism for the regulation of YY1 in mRNP complexes in *Xenopus* oocytes.

6.14 YY1 is monomeric in stage I oocytes

Stage I oocytes display high levels of DNA binding activity which decrease by stage III and remain low until after the MBT (Figure 2C) (Ficzycz et al., 1997; Ficzycz, et al., 2001). It is known that mRNPs assemble during early stages of oogenesis (Davidson, 1986). The high level of YY1-DNA binding activity in stage I oocytes could therefore be attributable to the presence of monomeric YY1 prior to association into mRNPs in the cytoplasm. It was therefore of interest to establish the molecular mass and hence, the possible association of YY1 with mRNPs in stage I oocytes. SEC of lysates prepared from stage I oocytes (treated with RNase inhibitors) indicated that YY1 was distributed into both high molecular mass complexes around 600-670 kDa (Figure 15) as well as near its monomeric mass of approximately 50 kDa. It is possible that the two masses observed in this blot indicate the ongoing assembly of YY1 into mRNPs. The presence of monomeric YY1 in stage I oocyte extracts is consistent with the observation of YY1 DNA binding activity in gel mobility shift assays at this stage (Figure 2C).

Figure 15 Western blot/SEC



Size distribution of YY1-complexes in stage I oocytes.

Stage I oocyte lysates were treated with RNase inhibitors and separated by size exclusion chromatography. YY1 was detected by Western immunoblot analysis. Fraction numbers are shown at the *bottom of the panel*, molecular mass markers are shown at the *top of the panel*.

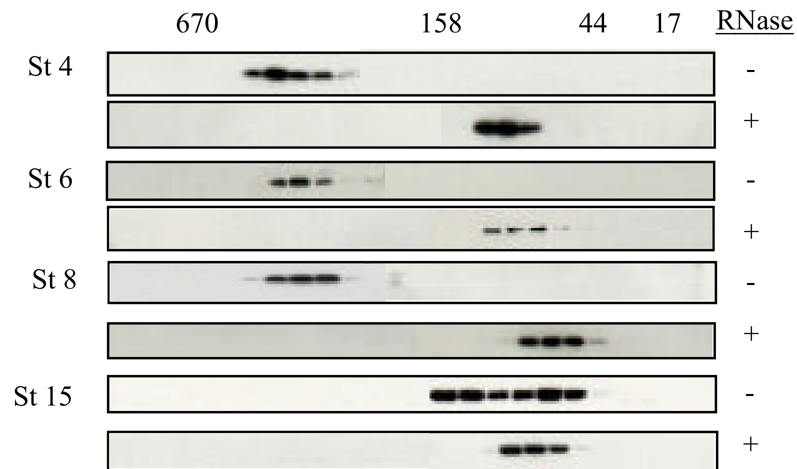
6.15 Dynamic association of YY1 with mRNP complexes through development

An analysis of the molecular mass distribution of YY1 in early embryos was carried out to determine whether it remained associated with mRNP complexes during this period of development. Lysates from embryonic stages 4, 6, 8, and 15 were prepared (supplemented with RNase inhibitors) then loaded onto the Superdex 200HR 10/30 column for SEC chromatography (Figure 16). The mass of YY1-mRNPs in stage 4-8 embryos is approximately 480 kDa which is consistent with the mass of these complexes in late stage oocytes. However by stage 15, the mass of the YY1-mRNP complexes shifted to a lower mass centered around approximately 100 kDa. Extracts were treated with RNase to degrade endogenous RNAs prior to separation by SEC (shown at the bottom of each panel). YY1-mRNPs in pre-MBT stages were RNA dependent and behaved similarly to complexes observed in stage VI oocytes. YY1-mRNPs appear to disassemble after the MBT suggesting that maternal RNAs may be released for translation after the MBT.

6.16 Identification of RNAs associated with YY1-containing mRNPs

The evidence presented in this thesis demonstrates that YY1 is a component of cytoplasmic mRNP complexes, a novel role for YY1. I next set out to determine the functional significance of this by identification of the RNA species associated with YY1-mRNP complexes. It was hypothesized that YY1-mRNPs may associate with a subset of RNA(s) reflecting a specific biologic role (ie, TFIIIA interacting with 5S rRNA) or that they may associate with a variety of RNAs and play a general role in mRNA metabolism and storage (ie, FRGY2).

Figure 16 Western blot/SEC



YY1 appears as a monomer after the MBT.

Analysis of embryonic lysates for the presence of YY1 by Western immunoblotting blotting following size exclusion chromatography. Developmental stages are indicated at the *left of the panel*. RNase treatment of lysates (*in vitro*) is indicated at the *right of the panel*. The positions of the molecular mass markers are indicated at the *top of the panel*.

Previous studies indicated it is possible to immunoprecipitate cytoplasmic mRNPs in order to identify their associated RNAs (Tenenbaum et al., 1999). Aspects of this methodology were used for isolation of YY1-associated RNAs from oocytes (A flow chart of the procedure is shown in appendix 1).

RNA associated with YY1 was immunoprecipitated using YY1 polyclonal antibodies that were bound to protein-A Sepharose beads. Stage VI oocyte lysates were initially analyzed by SEC and gel mobility shift assays to ensure that high molecular mass YY1 containing complexes were not degraded during lysate preparation (data not shown). Lysates prepared from stage VI oocytes supplemented with RNase inhibitors were mixed with YY1 antibody-bound beads. Following immunoprecipitation, proteins were degraded with Proteinase K releasing the captured RNAs, which were then ethanol-precipitated and used as templates for cDNA synthesis.

Precipitated RNAs were used as templates from which random primed cDNAs were synthesized. The result shown in Figure 9 suggests YY1 associates with polyadenylated RNAs, so initial attempts used oligo-d(T) to prime cDNA synthesis however this resulted in undetectable yields (data not shown). The next attempts involved the use of random primers which would also allow for cDNA synthesis from non-polyadenylated messages that would have been missed if only oligo-d(T) primers were used. Control immunoprecipitations using protein-A beads alone as well as PCNA antibodies bound to protein-A were also performed however no detectable levels of cDNAs were synthesized. Following completion of synthesis reactions, cDNAs were digested with H*ae*III, producing a number of fragments with blunt ends. This aspect of the procedure is an

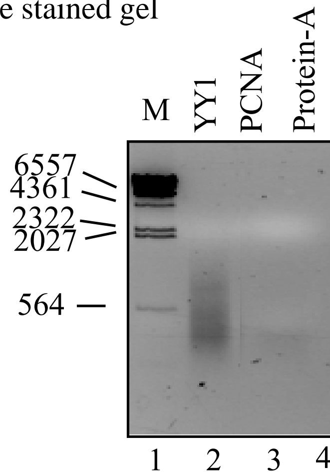
important consideration to the subsequent data because separate cDNA fragments may have arisen from the same RNA template, but may have been generated by digestion of the full length cDNA (see discussion).

Since the immunoprecipitation step isolates very small amounts of RNA, it was necessary to amplify the cDNA population prior to cloning. Following blunt end digestions, adaptors were ligated onto the fragments in order to provide primer annealing sites so that polymerase chain reactions could be employed to amplify the cDNAs. The use of random primers followed by H*ae*III digestion and adaptor ligation resulted in the synthesis of a population of cDNAs with variable sizes that was detectable by ethidium-bromide staining after electrophoresis of aliquots on agarose gels (Figure 17A). The adaptors included a restriction site for the enzyme *Mlu*I, which was essential in the subsequent cloning stages of this procedure. The digested linker-containing cDNAs were PCR amplified then digested with *Mlu*I to produce sticky ends for ligations. The cDNAs were isolated by DEAE electroelution, and ligated into the *B*ssH I (an isoschizomer of *Mlu*I) site of modified Bluescript plasmids. DH5 cells were transformed with the vectors and colonies that contained inserts were identified by colour selection. Colonies that were positive for inserts (white) were picked from plates, grown in liquid media and amplified plasmids were isolated using Promega Wizard miniprep kits.

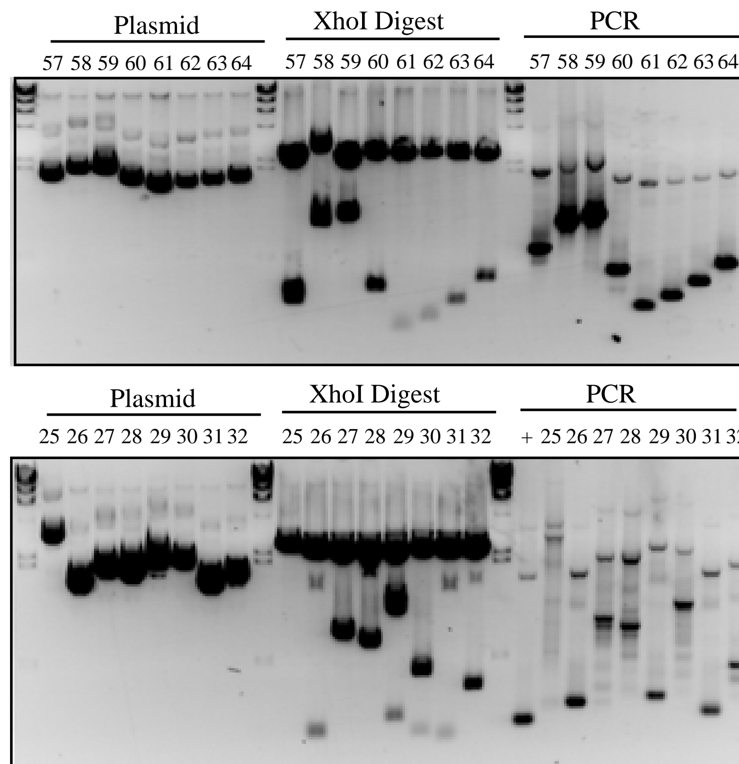
Two methods were used to verify that isolated plasmids contained insert cDNAs. Plasmids were digested with *Xho*I to release inserts, and inserts were amplified by PCR from the M13 forward and reverse primer sites within the vector. The products of both PCRs and restriction digestions were analyzed by

Figure 17

A Agarose/ethidium bromide stained gel



B Agarose/ethidium bromide stained gel



Enrichment PCR and representative screening gels.

A) PCR products using specific foreward and reverse primers recognizing adaptor sequences ligated to random prime synthesized cDNAs. The cDNAs were synthesized from RNAs immunoprecipitated using YY1 (lane 2) PCNA (lane 3) and the protein-A sepharose matrix (lane 4). Size markers (l HindIII) are shown in lane 1 and are indicated at the left of the panel. **B**) Representative screening data. Colonies that were positive by colourimetric screening (white) were processed and plasmids isolated then electrophoresed in 1% agarose/ethidium bromide gels (indicated at the top of each panel). Plasmids were digested with X*ho*I (indicated at the *centre top* of each panel) and inserts were amplified by PCR (indicated at the *top right* of each panel). Sets of 8 plasmid preparations, restriction digests and PCRs were run on each gel. Size markers (l H*ind*III) were loaded at three intervals on the gels and are indicated at the *left of the panel*.

ethidium bromide-stained agarose gels and only plasmids that contained inserts shown by both the PCRs and restriction digests were considered to be viable for sequencing (representative data shown in Figure 17B). Inserts in the PCR amplified panels appear to be larger than in the restriction digested panels. This is because the sites for the M13 foreward and reverse primers flank the X*ho*I digestion sites resulting in the generation of slightly larger fragments. These plasmids were sent for DNA sequencing at the National Research Council-Plant Biotechnology Centre (NRC/PBI), Saskatoon, Saskatchewan. The clone numbering system is based on the total number of colonies that were picked (200).

106 plasmids were evaluated to be suitable for sequencing, 100 of which were returned with high quality sequence data (based on chromatographic data included with the sequence data). All sequences are shown in *Appendix 2*.

Data obtained from the DNA Sequencing Laboratory were converted into Microsoft Word documents and insert sequences were separated from 5' and 3' vector sequences. The insert sequences were then used to search the non-redundant (nr) and expressed sequence tag (EST) databases at the National Center for Biotechnology Information (NCBI) for sequence alignments using the Basic Local Alignment Search Tool (BLAST).

The criterion for considering a valid match was that alignment of the YY1-precipitated cDNA clone with the sequence in the database have an E-value of 1×10^{-5} or less. The E-value is a statistical determinant that evaluates the possibility that a particular aligned sequence could occur randomly within the database. The calculation of the E-value is based upon the length and complexity

of the sequence that is being used for the search. This does not eliminate short sequences provided they are sufficiently complex. A perfect alignment will result in an E-value of 0.0. The E-values are generated by the BLAST and are not produced or altered independently of the database search tool.

The data obtained from this analysis are divided into four tables. Table 3 summarizes the major categories of matches found by alignment using BLAST. Table 4 shows sequences that produced positive matches between YY1-precipitated cDNA clones and ribosomal RNAs while Table 5 contains sequences that produced positive matches to known *Xenopus* protein coding sequences. Listed in Table 6 are the YY1-precipitated cDNA clones that were found to match sequences in the NCBI (EST) database.

The remaining 71 YY1-precipitated cDNA clones that were sequenced did not produce any significant alignments with sequences in either the nr or EST databases. However analysis of these cDNA for open reading frames (ORF) indicated that 70 of 71 did contain ORFs suggesting that they represent uncharacterized mRNAs in the oocyte.

Table 3. Results of Sequence Alignments to the NCBI Database.

Alignment of Clone	Number of Clones in Alignment
Xenopus Ribosomal RNA	18
Xenopus protein coding genes	3
Xenopus EST	8
No Matches	71
Total Sequences Aligned	**100**

Since the cDNAs were developed using Hae*III* to create blunt ends, multiple predictable fragments belonging to the same cDNA are not unexpected. Thus the large number of sequences could have been generated by the Hae*III* blunt-end digestion in the procedure and does not necessarily reflect on the relative affinity of YY1 for rRNA.

Table 4. cDNAs aligned with *Xenopus* Ribosomal RNAs.

Sequence Alignment	E-Value (maximum)	Accession Number	Matching Clones
18s rRNA	$3x10^{-15}$	XLRRN18S	044, 060,119 102, 146, 108
28s rRNA	$3x10^{-10}$	XL28SR	051,063, 114, 153,059, 118, 126, 020, 068 104, 117, 161

Table 5. cDNAs aligned with known *Xenopus* protein-coding genes

Sequence Alignment	E-Value (Maximum)	Accession Number	Matching Clone(s)
epithelial sodium channel, (alpha subunit)	0.0	XLU23535	121
Prickle	$4x10^{-37}$	AF387815	164
Sodium-phosphate co-transporter	$1x10^{-23}$	L78836	039

Table 6. cDNAs aligned with *Xenopus* Expressed Sequence Tags.

Accession Number	E-Value (Maximum)	Matching cDNAs
BG233124	$1x10^{-74}$	004
BJ075077	$2x10^{-12}$	072, 115
BF845685	0.0	086
BJ041818	$2x10^{-8}$	181
BG810899	$5x10^{-46}$	143
BJ038912	$2x10^{-85}$	030
BG234080	$2x10^{-5}$	010

Figure 18 shows Northern blots and schematic representations of YY1-precipitated cDNA clone alignments within selected frog sequences (Figure 18 A-E). These schematics were generated by virtual digestions using H*ae*III of the indicated *Xenopus* sequences using software located at (http://tools.neb.com/NEBcutter/index.php3). Shown in Figures 18A and 18B are the alignments of YY1-precipitated cDNA clones within both 18s and 28s rRNAs. A number of overlapping cDNAs were obtained that produced alignments within 18 S rRNA (102, 146, 108) (Figure 18A) and 28s rRNA ((051, 059)(063, 114, 118)(020, 104)) (Figure 18B). This is due to either cDNA synthesis from multiple rRNA templates or amplification of the templates via PCR. Northern blots of stage VI oocyte total RNA separated on 1% agarose/formaldehyde gels, transferred to nitrocellulose membranes and probed with with cDNA #108 (18S rRNA) and #051 (28S rRNA) are shown above each schematic diagram. These blots indicate hybridization to RNAs of the expected size (1.8 and 4.0 Kb respectively) in total RNA.

Of the 100 YY1-precipitated cDNA clones, 3 matched *Xenopus* protein coding genes. The proteins identified were; the alpha subunit of the epithelial sodium channel, Prickle, and *Xenopus* sodium-phosphate co-transporter.

A single cDNA clone (164) was found to align with Prickle. A schematic alignment of cDNA 164 within *Xenopus* Prickle mRNA is shown in the *lower panel* of Figure 18C. A Northern blot is shown in the *upper panel* of the figure. Total oocyte RNA was separated on 1% agarose/formaldehyde gels, transferred to nitrocellulose membranes and probed with radiolabeled YY1-precipitated cDNA clone 164. A single prominent signal was detected at approximately 4.4 Kb which is consistent with the expected size of Prickle mRNA according to the submission in the NCBI database (shown above each diagram, Accession #AF387815). This result confirms the efficiency of the YY1-IP protocol for identification of YY1-associated mRNAs, since the sequences isolated are able to hybridize with mRNA isolates from a stage VI oocyte.

The upper panel of Figure 18D is a Northern blot of total stage VI RNA probed with radio labeled cDNA clone #121. A prominent signal was detected migrating at approximately 3 Kb, which is consistent with the predicted size of the mRNA according to data submitted to the NCBI database. Shown in the *lower panel* is the schematic alignment between YY1-precipitated cDNA clone #121 and the *Xenopus* Epithelial Sodium Channel.

The *upper panel* of Figure 18E is a Northern blot of total stage VI RNA probed with radio-labeled cDNA clone #039. A strong signal is detected at approximately 4.5 Kb which is consistent with the size of the sodium phosphate co-transporter mRNA according to data submitted to the NCBI database. Shown

in the lower panel of Figure 18E is an alignment of clone 039 within a schematic of the *Xenopus* sodium phosphate co-transporter (Accession #L78836).

Two clones of the 71 which did not align with sequences in the database were chosen for Northern blots analysis to test for possible hybridization to oocyte mRNA.

Shown in Figure 18 (F and G) are Northern blots of total oocyte RNA separated on 1% agarose/formaldehyde gels, transferred to nitrocellulose membranes and probed with radiolabeled YY1-precipitated clone 005 (Figure 18 F) and 060 (Figure 18 G). These cDNAs did not align with sequences in the NCBI database however they contain open reading frames and thus could code for *Xenopus* proteins.

Figure 18

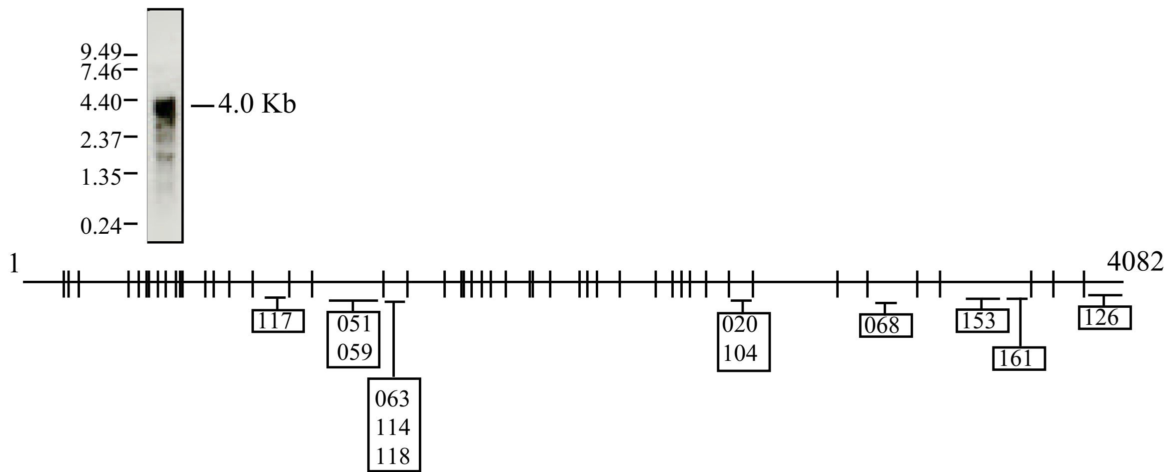
Northern blot analysis of total oocyte lysates probed with select YY1-immunoprecipitated probes.

Northern blot analysis confirming that YY1-precipitated cDNAs hybridize with RNAs in total RNA (10*u*g) isolated from stage VI oocytes. Probes used were (**A**) 060, aligned with 18S rRNA, (**B**) 051, aligns with 28S rRNA, (**C**) 164, aligns with Prickle (XP*k*), (**D**) 121, aligns with sodium epithelial channel (ENaC) and (**E**) 039, aligns with sodium-phosphate co-transporter. Blots shown in **F** and **G** were probed with immunoprecipitated cDNAs that were not aligned with *Xenopus* sequences in the NCBI database. Size markers are shown at the *left of each panel (*in Kb*)*. Shown below each of the panels (A-E) is a schematic diagram of alignments of immunoprecipitated cDNAs with each of their RNAs as determined by the BLAST search. Nucleotide numbers are shown at the top of each schematic. Vertical lines indicate locations of H*ae*III digest sites as found by virtual digestions of each sequence (see Materials and Methods). Boxed numbers indicate identities of cDNAs that were found to align with the sequence.

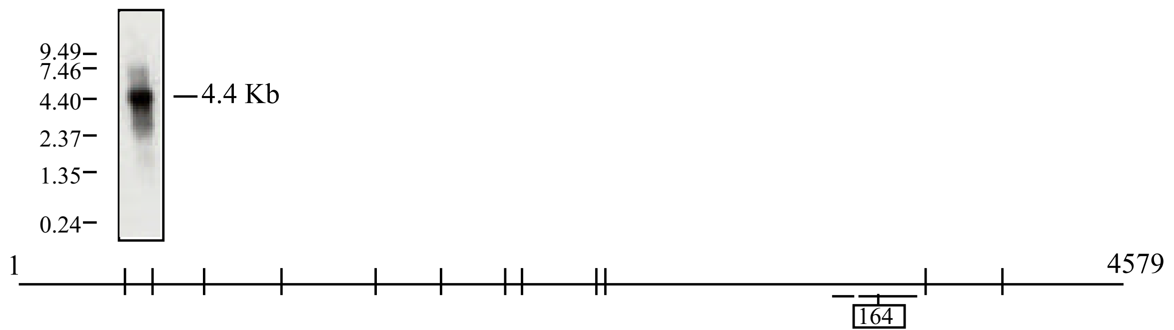
Figure 18

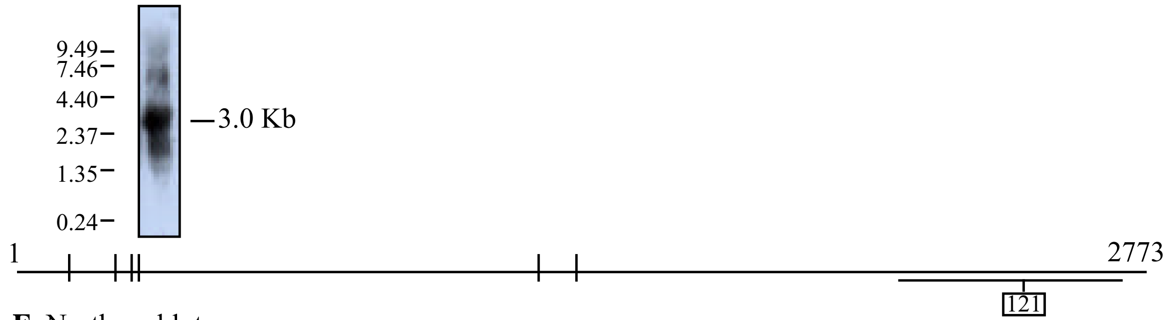
A Northern blot

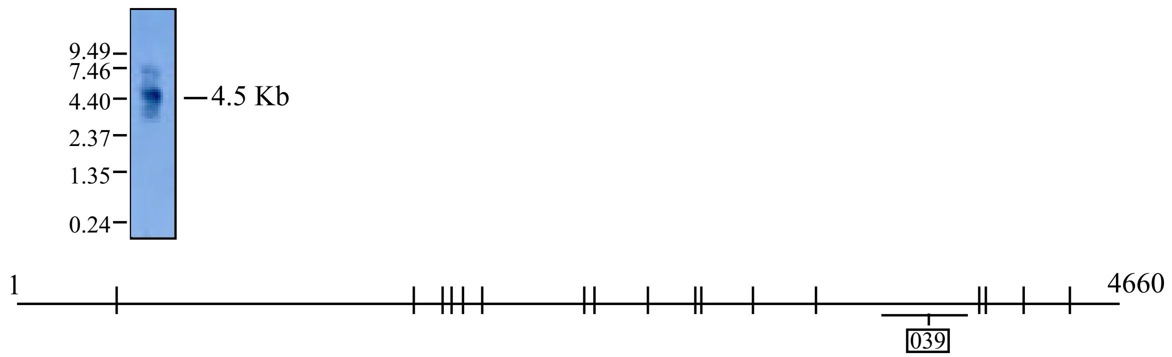
B Northern blot



C Northern blot




D Northern blot
9.49
7.46
4.40
2.37
1.35
0.24
3.0 Kb
1
2773
121


E Northern blot
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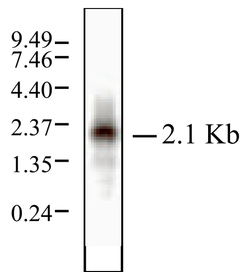
F Northern blot
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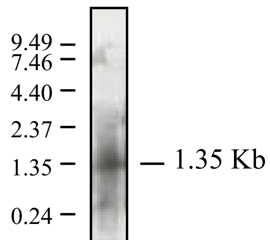
G Northern blot
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1.35 Kb

7.0 Discussion

The initial objective of the research presented in this thesis was to examine the role of the YY1 transcription factor in the *Xenopus* model system. Numerous studies indicated its versatility in transcriptional activation and repression in a variety of model systems and suggested a role in cell growth and differentiation (Shi et al., 1997). While this study began with the intent of examining the transcriptional role of YY1, data obtained from initial experiments resulted in a shift in the focus of the research. The data presented here indicate a novel role for YY1, as a component of mRNPs.

The first major finding is that, even though YY1 is abundantly expressed throughout oogenesis and embryogenesis up to swimming larval stages, it does not play a direct transcriptional role in early *Xenopus* development. This finding was somewhat surprising given the wealth of literature indicating the transcriptional activity of YY1 in a variety of cell growth and differentiation contexts. YY1 mRNA and protein are present at relatively constant levels throughout oocyte and embryo development through to neurula stages (Figures 3 and 4). Analysis of cytoplasmic and nuclear subcellular fractions showed that YY1 was present exclusively in the cytoplasm in both oocytes and embryos (Figure 7). These findings were confirmed in our laboratory by immunocytochemical analyses of the subcellular distribution of YY1 through development (Ficzycz et al., 2001). Consistent with the cytoplasmic localization of YY1, transcription assays showed YY1 has no stimulatory or repressive effect

on basal transcription from the TK promoter in either oocytes or post-MBT embryos (Figure 6).

I conducted several experiments in which YY1 was overexpressed in embryos. No overt morphological deformaties or defects were observed in the developing embryos (data not shown). Exogenous cytoplasmic YY1 may have been incorporated into existing mRNPs or may have remained monomeric. The subcellular localization and incorporation into mRNPs was not monitored.

Remarkably, YY1 was never localized in nuclei throughout early development. However, analysis of adult frog liver sections by immunocytochemistry indicated that YY1 is present in nuclei later in development, but the time at which nuclear translocation occurs is not presently known (Ficzycz et al., 2001). This suggests that maternally expressed YY1 may function in transcriptional regulation later in development, perhaps during organogenesis. It is not known when zygotically expressed YY1 appears in the embryo or whether maternal and zygotic forms play similar or different roles.

The second major finding is that YY1 is a constituent of mRNP complexes in the developing frog. This is a novel and distinct activity that has not been previously described for this protein. A number of observations support this finding. YY1 has a conserved RNA-binding zinc finger/knuckle motif in the DNA-binding domain (Figure 8). YY1 in its native state in stage VI oocyte lysates displays RNA-binding activity in RNA gel mobility shift assays (Figure 10), and specifically binds to oligo(dT)-cellulose (Figure 9) suggesting that it associates with poly(A)$^+$ mRNA *in vivo*. Similar to other mRNP proteins, YY1 in stage VI oocytes is present in high molecular weight complexes of

approximately 480 kDa (Figure 12). The integrity of YY1 complexes is sensitive to RNase treatment both *in vitro* and *in vivo* (Figures 11, 12, 13, 16). Phosphatase-treatment of lysates resulted in release of YY1 monomers from high mass complexes (Figure 14). Thus the cytoplasmic location and its association with mRNPs suggest that similar to FRGY2, YY1 functions in the storage and translational control of maternal mRNA in the oocyte and developing embryo.

Amino acid sequence analysis of YY1 revealed that the spacing of key histidine and cystine residues in the DNA binding domain of YY1 are conserved with the zinc finger/knuckle RNA binding motif (Figure 8), suggesting that YY1 has the potential to bind RNAs in the cytoplasm. RNA mobility shift experiments using stage VI oocyte lysates demonstrated that YY1 binds radiolabeled histone mRNA *in vitro* (Figure 10). YY1 present in native oocyte lysates was shown to be retained on oligo(dT)-cellulose columns suggesting it is associated with poly(A)$^+$ mRNAs *in vivo* (Figure 9). Degradation of endogenous RNA by RNase treatment of extracts *in vitro* prior to chromatography abolished retention of YY1 by the oligo(dT) matrix (Figure 9) providing key evidence in supporting of YY1 as an mRNP component. Thus YY1 may directly contact RNA through the zinc finger/knuckle however, the RNA binding assays and oligo(dT) cellulose chromatography do not indicate whether YY1 present in mRNP complexes makes direct contact with RNA, or associates with RNAs indirectly through interactions with other proteins in mRNP complexes such as FRGY2 and nucleolin. Furthermore, these assays do not indicate whether YY1 or YY1 containing mRNPs recognize and bind select RNA sequences.

The data shown in Figure 10 indicates that YY1 or YY1-mRNP complexes associate with non-adenylated messages (non-adenylated probes were used). Thus the presence of YY1 in the column eluates of oligo(dT) is probably through interaction of YY1 with internal sequences, 5'-cap or other proteins that bind in these regions.

As shown in Figure 9B (*lane* 2), not all of the YY1 bound to the oligo(dT) column. There are two possibilities that may account for this observation. It may be that there are more YY1-mRNP complexes containing poly(A)$^+$ mRNA than the column has capacity to bind. Secondly YY1-mRNP immunoprecipitations indicate that YY1 complexes interact with 18S and 28S rRNAs which would not be retained on the column thus accounting for the lower YY1 signal in the eluant. It is also possible that YY1-mRNPs interact with poly(A)$^-$ RNAs. This suggests that YY1-mRNPs are able to interact with divergent RNAs in the cytoplasm.

The specific retention of YY1 on oligo(dT)-cellulose and its presence in protein-mRNA complexes provided initial evidence that YY1 in its native state in the oocyte cytoplasm associates with or is a component of mRNPs. These methods have been used by several laboratories to identify mRNP proteins such xCIRP2 (Matsumoto et al., 2000), and FRGY2 (Matsumoto et al., 2000; Tafuri and Wolffe, 1993). Futher confirmation of this was obtained through SEC, which showed that YY1 in native lysates is present in large complexes with a molecular mass of approximately 480 kDa (Figure 12). The presence of YY1 in high molecular mass complexes was abolished by degradation of RNAs in stage VI lysates resulting in the release of monomeric YY1 (Figure 11, 12, 13 and 16).

SEC analysis of stage I lysates indicates that YY1 is present at two distinct molecular masses (Figure 15). In stage I oocytes, formation of mRNPs are at their highest levels (Davidson, 1986), thus detection of YY1 both as monomers and in complexes at this stage most likely reflects ongoing incorporation of YY1 into mRNPs. The significance of the monomeric YY1 in these blots will be discussed later.

Several similarities exist between YY1 and FRGY2 suggesting that their biological roles in the developing frog may be similar. Both FRGY2 and YY1 were originally identified as transcription factors recognizing similar consensus ds-DNA binding elements (FRGY2; CTGATTGGCCAA (Wolffe et al., 1992) and YY1; GGCCATTT (Hyde-DeRuyscher et al., 1995) and were later assigned the role of mRNP proteins in the oocyte (Tafuri and Wolffe, 1992; Tafuri and Wolffe, 1990; Richter, 1991; Galvin and Shi, 1997; Thomas and Seto, 1999; Ficzycz and Ovsenek, 2002). Although the amino acid sequences differ, both proteins are highly expressed in the cytoplasm of oocytes. Consensus RNA binding domains (Wolffe et al., 1992, Figure 8 this thesis) have been identified in both proteins; FRGY2 contains the RNP-1 domain, and YY1 contains the zinc finger/knuckle. FRGY2 and YY1 are able to associate with poly-A^+ RNA, and can shift the mobility of RNA *in vitro* (Matsumoto et al., 2000; Tafuri and Wolffe, 1993). Furthermore both of these proteins are assembled into high mass mRNP complexes during early oogenesis in the oocyte (Darnborough, 1981; Yurkova and Murray, 1997, Figure 15 this thesis). Several groups have indicated that phosphorylation increases the RNA binding affinity *in vitro* of FRGY2 (Dearsly et al., 1985; Kick et al., 1987; Braddock et al., 1994).

Dephosphorylation of stage VI oocyte lysates by phosphatase treatment *in vitro* resulted in release of YY1 from mRNP complexes (Figure 14). This does not demonstrate that dephosphorylation of YY1 itself is responsible for the release of monomers, however it does indicate that the affinity of YY1 for RNA or mRNPs is negatively affected by dephosphorylation and thus its RNA affinity may be regulated similarly to FRGY2.

The behaviour of FRGY2 and YY1 are not completely identical and it is likely that FRGY2 and YY1 do not necessarily assemble together in the same mRNPs, but rather that they are exclusive or only partially overlapping. While FRGY2 is observed to dissociate from mRNPs following fertilization (Tafuri and Wolffe, 1992), YY1 is not released from mRNPs until after the MBT (Figure 16). The molecular masses of the respective complexes as determined by SEC differed slightly; YY1 complexes are approximately 480 kDa, while FRGY2 complexes are over 500 kDa. The SEC elution profiles for both of these proteins partially overlap (Figure 12), thus it is possible that certain subsets of mRNP complexes might contain both YY1 and FRGY2. The constituent proteins of YY1-containing mRNPs have yet to be identified, and the possibility that YY1 may functionally associate with FRGY2 awaits further study. Yurkova and Murray (1997) reported that FRGY2 mRNPs had a mass of 320 kDa as determined by SEC, which differs from the mass for FRGY2 (500 kDa) presented in this thesis. The reason for this is most likely due different methodology used in their study. Yurkova and Murray determined the mass of FRGY2-RNP complexes without their associated RNAs. They isolated the FRGY2-mRNP first by sucrose gradients then digested the mRNP with

micrococcal nuclease to degrade the RNAs. They then followed this by ion exchange chromatography and next analyzed mRNPs by SEC. The data shown in this thesis indicating that FRGY2 is present in complexes of approximately 500 kDa is a measurement of the mass of the complex including associated RNAs.

7.1 Cytoplasmic retention of YY1

Given the large amount of literature indicating that the primary function of YY1 is as a transcriptional regulator, the findings here that YY1 is exclusively cytoplasmic in oocytes and through early embryonic development, (Figure 7) and is a component of mRNPs (Figure 12) are somewhat surprising. It is known that the CBTF^{122} transcription factor is tethered to the cytoplasm through its association with RNAs and migrates to the nucleus in response to RNA degradation (Brzostowski et al., 2000). This suggests RNA-association as a novel mechanism to regulate transcriptional activity of CBTF^{122} during development (Brzostowski et al., 2000). Thus it is reasonable to speculate that YY1 may be regulated by a similar mechanism. However, unlike CBTF^{122}, YY1 remains cytoplasmic in response to RNAse microinjection (Figure 13). CBTF^{122} contains a bi-partite nuclear localization signal (NLS) (Robbins et al., 1991) which was hypothesized to be masked through its interaction with RNAs in the cytoplasm. Since the addition of an extra NLS to CBTF^{122} did not promote nuclear localization, retention in the cytoplasm was attributable to RNA anchoring and not NLS masking (Brzostowski et al., 2000). YY1 does not appear to have a consensus NLS, however, portions of the second and third zinc

fingers have been shown to be necessary for nuclear localization in RK13 cells (Austen et al., 1997b). A yeast two-hybrid screen showed that B23, which contains an NLS and is involved in subcellular trafficking and ribosome assembly, was able to bind YY1 the zinc fingers (Inouye and Seto, 1994; Yung et al., 1985). Thus is is possible that interaction between YY1 and B23 (or some similarly functioning protein) is necessary for establishing subcellular localization patterns in the oocyte.

Although there are differences in this aspect of the regulation of CBTF[122] and YY1, the ability of transcription factors to bind RNA and associate with mRNPs may be an important regulatory mechanism during development.

It may be that the primary biological function of cytoplasmic localization of YY1 is to sequester it until its transcriptional activity is required at some point later in development. Immunocytochemical analysis of frog liver sections revealed that YY1 is localized to the nuclei of this tissue (Ficzycz et al., 2001). SEC of HepG2 lysates revealed that YY1 was present in these cells as a monomer (Figure 12). These data indicate that at some point during development, monomeric YY1 relocates to the nucleus. It is assumed that in the nucleus, YY1 would be transcriptionally active (although this has yet to be tested).

A precedent for cytoplasmic localization of YY1 has been established during early mouse development in which YY1 is localized to the cytoplasm in oocytes. YY1 translocates to the nucleus by the two cell stage which is coincident with the onset of zygotic transcription and displays a mosaic pattern of sub-cellular distribution in E3.5 blastocysts (Donohoe et al., 1999). This

suggested that YY1 may function to regulate gene expression early in mammalian embryonic development, however its transcriptional capability has not been analyzed. Donohoe and colleagues suggest it is possible that YY1 is required for the development of more highly specialized cell types and localization to the nucleus merely places YY1 in the correct environment to become active when the appropriate developmental cues are received. The finding that YY1 homozygous knockout results in peri-implantation lethality suggests that the YY1 plays an important function during early development.

It is interesting that the pattern of nuclear localization in *Xenopus* and mouse embryos are somewhat different. While in mouse embryos YY1 relocates to the nucleus with the onset of zygotic transcription at the 2-cell stage, YY1 remains cytoplasmic in the frog well after the MBT (Figure 7). It would appear that YY1 plays a different role in the development of mouse and frog embryos.

7.2 Dynamic changes in the association of YY1 with mRNPs during development

Taken together, the results of DNA-binding assays (Figures 2, 5, and 13) and the profile of YY1 association with large mRNP complexes during development (Figure 12) suggest that YY1 is available to bind to its cognate dsDNA element only when it is released from, or not complexed with mRNPs. DNA-binding activity (Figure 2) appeared only in stage I oocytes when assembly of YY1-containing mRNPs is incomplete, and after the MBT when YY1-mRNPs might disassemble (Figure 16). Consistent with this, both RNase and phosphatase treatment of native oocyte extracts releases YY1 monomers from

complexes and results in the acquisition of DNA-binding (Figures 5 and 13). Therefore, the working hypothesis is that association of YY1 with mRNPs masks DNA-binding. When YY1 is complexed with mRNPs the DNA binding structures in the zinc finger domains are blocked or masked, and release of YY1 monomers from mRNPs exposes the DNA binding portions of the zinc fingers, permitting association with ds-DNA. It is important to note that from a biological perspective, the ability of YY1 to bind DNA detected in oocyte and embryonic extracts in this thesis is biologically irrelevant, since endogenous YY1 remains cytoplasmic (Figures 7 and 13) and has no gene regulatory activity (Figure 6). Interestingly, the RNA-mobility shift experiment (Figure 10), suggests that YY1 in mRNP complexes are able to associate with exogenous RNA, suggesting either that the zinc finger/knuckle domain remains exposed, or other RNA-binding proteins in the YY1-mRNP complexes in the oocyte retain additional RNA-binding capacity.

Dephosphorylation of stage VI oocyte lysates *in vitro* resulted in release of YY1 monomers from high mass complexes as assessed by SEC (Figure 14). This suggests as discussed above that the phosphatase-induced recovery of DNA binding (Figure 5) is the result of liberation of YY1 monomers from YY1-mRNPs. However, phosphorylation sites on *Xenopus* YY1 have yet to be identified, and release of monomeric YY1 from mRNPs following phosphatase-treatment may not be due to dephosphorylation of YY1 itself. It is possible that other proteins in YY1-containing mRNP complexes are dephosphorylated resulting in release of YY1. The effect of changes in phosphorylation state on mRNP-protein affinity for RNA and integrity of mRNP complexes have been

well documented (Kick et al., 1987; Herbert and Hecht, 1999; Murray, 1991). For example, FRGY2-mRNPs have been shown to be phosphorylated by an mRNP associated kinase which increases its RNA binding affinity (Yurkova and Murray, 1997; Kick et al., 1987; Dearsley et al., 1985; Cummings and Sommerville, 1988; Marello et al., 1992; Murray, 1994; Murray, 1991). These findings suggest that phosphorylation plays a crucial role in regulating mRNPs and raises the possibility that YY1 may be regulated in this manner.

7.3 Changes in molecular mass of YY1 during development

In stage I and II oocytes, the molecular mass of YY1 as indicated by SDS-PAGE/Western analysis appeared to be significantly smaller (50 kDa) than observed in later stage oocytes and embryos (59 kDa) (Figure 4). The underlying molecular mechanism or the biological significance of this mass shift is not currently understood. Phosphatase-treatment of stage VI extracts had no effect on the migration of YY1 on Western blots (Figure 5) and so it is unlikely that the change in size during oocyte development is due to phosphorylation. Another post-translational modification that may account for the shift in molecular mass of YY1 in oocytes is poly-ADP-ribosylation. In HeLa cells, YY1 is subject to poly-ADP-ribosylation in response to DNA damage which results in decreased DNA-binding activity (Griesenbeck et al., 1999). The effect of ADP-ribosylation on YY1 in oocytes was not examined in this thesis.

Another possibility is that YY1 may be encoded on two genes expressed differentially during development, one coding for the smaller 50 kDa isoform expressed only in early oogenesis, reflecting a distinct developmental role. By

stage IV when the mass shift is observed to occur, the second gene coding for the larger form of YY1 may be upregulated and then expressed through the remainder of development while the 50 kDa form is degraded. This hypothesis has not been tested and a review of the literature has not indicated the presence of two genes coding for YY1 in other organisms. Additionally, when *Xenopus* YY1 was initially characterized, there was no evidence indicating that there were two genes coding for YY1 (Pisaneschi, 1994). It is possible that the 50 and 59 kDa isoform are due to differential splicing of mRNAs. The Northern blot shown in Figure 3 indicate the presence of a second smaller RNA band at 3.8 Kb. Thus it is possible that the two splice variants exist in the developing however further study will be required to resolve this issue. What might be the functional significance of the appearance of two isoforms at different points in development? It is possible that in stage I oocytes, the smaller form of YY1 is produced very quickly and is immediately incorporated into mRNPs protecting the large quantity of nascently synthesized RNA from degradation.

7.4 Identification of RNAs immunoprecipitated with YY1-mRNPs

A method based on immunoprecipitation was used to isolate and identify the RNA species associated with YY1-mRNP complexes. This is not a widely used method but has proven successful in the isolation of mRNP bound RNAs from mouse P19 embryonal carcinoma cells (Jain et al., 1997; Tenenbaum et al., 2000). Tenenbaum and co-workers (Tenenbaum et al., 2000) isolated mRNAs bound to ELAV/Hu RNA binding proteins from P19 cells. These proteins bind to elements in the 3'-UTRs of proto-oncogene and cytokine mRNAs, increasing

expression of these transcripts. Their study was designed to identify changes in ELAV/Hu protein bound mRNA subsets in response to differentiation; HuB was immunoprecipitated, and the associated mRNAs were used as templates for synthesis of cDNAs used to probe microarrays. They found that HuB, which contains three RNA-recognition motifs (RRM), associated with specific sequences in the 3'UTR of mRNAs that encoded cell-cycle regulators, transcription factors, as well as other early response gene (ERG) products (Tenenbaum et al., 2000). To my knowledge, this method has not been used to identify RNA species from *Xenopus* mRNP proteins.

Sequences of 100 YY1-precipitated cDNAs were used to search the NCBI database for matches to known *Xenopus laevis* sequences. Of these, 3 produced significant alignment with protein coding mRNAs, 18 with 18S, 28S ribosomal RNAs, 8 with *Xenopus* ESTs, and the remaining 71 sequences did not align significantly with *Xenopus* sequences in the database (Table 4). The most obvious conclusion that can be drawn from this data is that YY1-mRNP complexes associate with both messenger and ribosomal RNAs.

Table 5 lists the three YY1-precipitated cDNAs that aligned with *Xenopus* protein mRNAs. These encode the alpha subunit of the sodium epithelial channel (ENaC), prickle (XP*k*) and the sodium-phosphate co-transporter.

The *Xenopus* epithelial sodium channel (accession #XLU23535) is a 632 amino acid protein that was first cloned by Puoti and colleauges (Puoti et al., 1995). These channels mediate sodium re-absorption by a sodium-selective sensitive channel that is located in the apical membrane facing the external

compartment of many tight epithelia (Garty, 1994; Garty and Benos, 1988). These channels are responsible for the rate-limiting step in sodium reabsorption and control of its activity is essential to the maintenance of the global sodium balance and blood pressure. Expression of this channel has been documented late in frog development (stage 25), once the internal organs have formed (Puoti, 1995), however there is no evidence indicating earlier developmental expression. Thus, the appearance of its mRNA in oocytes is a novel finding.

The *Xenopus* sodium phosphate (Na^+/PO_4^{3-}) co-transporter is a 674 amino acid protein that was first cloned by Ishizuya-Oka et al., (1997). It is expressed in the differentiated intestinal epithelial cells at low levels in pre-metamorphic frogs and at high levels in post-metaporphic frogs, and it functions to regulate co-absorption of Na^+ and PO_4^{3-} in intestinal tissues (Ishizuya-Oka et al., 1997). Again, there are no previous reports of this gene being expressed in oocytes.

Xenopus XP*k* is an 835 amino acid protein that was first cloned by Wallingford and colleagues (Wallingford et al., 2002). It is a member of a family of proteins that are responsible for the planar polarity of the organism (Adler et al., 2000; Gubb et al., 1999). In vertebrates, planar polarity is essential for proper development of skin, scales or feathers, and internal organs. In *Drosophila* for example, planar polarity is essential in the development of proximal-distal orientation of wing cells as well as cells in the leg and leg segments themselves (Adler, 1992; Gubb, 1993; Struhl et al., 1997). Wallingford and co-workers (Wallingford et al., 2002) have found XP*k* is expressed maternally in *Xenopus* while zygotic expression was detected at the start of gastrulation in the dorsal marginal zone and steadily increases until tadpole

stages. They also found XP*k* expression later in development in the pronephric duct, otic vesicle and the retina (Wallingford et al., 2002).

These proteins are of divergent function. This indicates that YY1-mRNPs are capable of associating with a variety of RNAs which perform diverse functions within the developing frog at varying times during development. The presence of the sodium channel mRNA in stage VI oocytes (Figure 18 D, 2.7 Kb) suggests that at least a portion is maternally formed and stored until the protein is needed by the developing frog. Data presented in this thesis indicating that YY1 is cytoplasmically localized in neurula stage embryos and complimentary data from our laboratory indicating that it remains in this location into at least larval stages, allow for the possibility that maternal mRNAs could be stored for this length of time (Ficzycz et al., 2001). However, data shown in Figure 16 indicating that YY1-mRNPs disassemble by neurula stage argues that maternal mRNAs are released for potential translation soon after the MBT. It is possible that some YY1-mRNP complexes persist to store mRNAs until they are required later in development. It is also possible that the mRNAs coding for the sodium epithelial channel are released from mRNPs during gastrulation for translation into functional proteins or that YY1 monomers are released from mRNPs alone, but the remainder of the mRNP proteins and possibly the associated mRNAs remain complexed until needed later in development. Still another possibility is that mRNAs may be released concurrently with YY1 but are assembled into new mRNPs that do not contain YY1 and are then stored until needed later in development.

Northern blots indicate the presence of Na^+/PO_4^{3-} co-transporter RNAs in the oocyte at the expected size (4.6 Kb) (Figure 18 E). This indicates that Na^+/PO_4^{3-} co-transporter RNAs are expressed in the oocyte and associate with YY1-mRNPs until they are required later in development. Organogenesis commences at approximately stage 20 once the neural tube closes and it is possible that the Na^+/PO_4^{3-} cotransporter mRNA is translated once the gut begins to form. Given the data suggesting that YY1-mRNPs disassemble after the MBT it is also possible that the cotransporter is released from YY1-mRNPs reflecting a necessity for co-absorption of Na^+ PO_4^{3-} earlier in development.

The finding that *Xenopus* Prickle (XP*k*, 835 amino acids) mRNAs are stored in mRNPs in the cytoplasm of oocytes is interesting. Data shown in Figure 18 C, demonstrate the presence of an RNA species in total *Xenopus* RNA that is of the expected size (4.5 kb) for XP*k*. The presence of Prickle mRNA in YY1-mRNPs could reveal a mRNA-positioning mechanism associated with pattern formation in early development. However, immunocytochemical data indicates that YY1 is uniformly distributed in the cytoplasm of oocytes and all embryonic cells through to organogenesis (Ficzycz et al., 2001). The association with YY1 is probably not a mechanism to localize XP*k* mRNA to certain parts of the developing embryo, as is the function of Staufen with respect to *oskar* and *bicoid* localization. Thus YY1-mRNPs serve to protect XP*k* mRNA from degradation and regulate the timing of its translation during development. It would be interesting to assess the developmental consequences of disruption of YY1-XP*k* interactions. If the biological function of YY1 is similar to that of FRGY2 in translational masking and protection of mRNAs from degradation, it

is possible that premature release of XP*k* may perturb establishment of planar polarity.

Of the 100 cDNAs isolated in the YY1 immunoprecipitations, 18 matched with 18S or 28S rRNAs. What is the significance of YY1 in association with rRNAs? There are two hypothesis that account for this observation. First, it is possible that YY1 associates with rRNA and functions in the storage of ribosomal RNA in the oocyte prior to assembly into ribosomes. Second, it is possible that YY1 associates with intact ribosomes in the oocyte and is able to mediate translation.

It is possible that YY1 interacts with the ribosomal precursors as they enter the cytoplasm for final assembly into functioning ribosomes. *Xenopus* contains approximately 400-600 copies (per haploid genome) of the ribosomal gene that encodes the 40S precursor, which following processing results in the 18S, 5.8S and 28S ribosomal RNAs (Pardue, 1973). Amplification of these genes occurs at the pachytene stage in oocytes (although the process begins in premeiotic oogonia) (Gall, 1968; Pardue and Gall, 1969; Van Gansen and Schram, 1974; Kalt and Gall, 1974) resulting in approximately 2.5×10^6 genes per oocyte. Under optimal conditions (high levels of gonadotropic hormones), the midvitellognic oocyte is able to synthesize 3×10^5 rRNA molecules (18S and 28S) per minute (Davidson, 1986; Keem et al., 1979; Wallace and Misulovin, 1978) which then exit to the cytoplasm for assembly into the 1×10^{12} ribosomes that are necessary for the developing frog (Davidson, 1986). It is possible that YY1-mRNPs associate with rRNA precursors in order to regulate their metabolism. Such interactions may be similar to the regulation of 5S rRNA by

TFIIIA. However despite conservation of key residues in the zinc finger/knuckle between TFIIIA and YY1, no evidence was found that would indicate YY1 associates with 5S rRNA.

Perhaps YY1-mRNPs are a structural component required for regulation of the intact ribosome. In this role, YY1-mRNPs associating with ribosomes may function to unmask incoming mRNAs prior to translation. However the difficulty with this interpretation is that ribosomes have a molecular mass of approximately 4.1 x 10^6 daltons (Spirin, 1969) in which case if YY1-mRNPs were to have eluted with ribosomes, they would have appeared in much higher molecular mass complexes. This was not observed.

It is important to note the absence of 5S rRNA in the sequences isolated from the YY1-mRNP immunoprecipitations. Two possibilities might account for the lack of 5S rRNA cDNAs. The number of these transcripts expressed in oocytes equals that of 18S and 28S rRNAs (Davidson, 1986) and their absence implies that YY1-containing RNPs do not interact with 5S rRNAs, and thus most likely not with ribosomes themselves. Relative to the six 18S and twelve 28S cDNAs, isolation at least some 5S cDNAs would have been expected if YY1-mRNPs were indeed interacting with intact ribosomes. Rather the lack of 5S rRNA isolation supports the notion that YY1-mRNP complexes are involved in storage of maternally derived 18S and 28S rRNA prior to their assembly into functional ribosomes. Second, it is possible that 5S rRNA were singly missed in the screen. 5S rRNAs are small (120 bp) relative to 18S (1826 bp) and 28S (4046 bp) and may not have had been recognized by the random primers used to generate the cDNAs (Miller and Brownlee, 1978). It is possible that clones were

not selected from plates at the initial screening and that 5S rRNAs may be identified if more clones are sequenced. It is also interesting and perhaps significant to note that no tRNAs were not immunoprecipitated.

A number of cDNAs (8) were found to align with *Xenopus* ESTs in the NCBI database (Table 6). These sequences represent *Xenopus* mRNAs that have yet to be identified and may code for novel frog proteins. Anaylsis of ESTs that were found to align with *Xenopus* immunoprecipitated cDNAs for sequence matches amongst themselves using the BLAST did not reveal and sequence homology indicating that they represent distinct RNAs in the oocyte. In one case (Table 5, accession #BG233124), a putative identification of the EST was submitted by the author (Robert Strausberg, *unpublished*). He suggests that a portion of the EST is similar in sequence to that coding for the type B bovine gustatory receptor (accession #P35350) which belongs to a superfamily of G-protein coupled receptors (Matsuoka et al., 1993). These receptors have been implicated in signalling bitter and sweet taste stimuli (Striem et al., 1989; Hwang et al., 1990). It is possible that this EST represents either an amphibian homolog of the gustatory receptor or a yet to be identified G-protein. While the mouth and oral plate of the frog begin to develop at stage 32, the possibility exists that gustatory receptors are expressed earlier. The isolation of clone BG233124 strengthens the conclusion that YY1 interacts with mRNAs encoding functionally divergent proteins, and plays a general role in mRNA storage/metabolism in the oocyte.

Analysis of ESTs that were found to align with *Xenopus* immunoprecipitated cDNAs for sequence matches amongst themselves using the

BLAST did not reveal any sequence homolgy indicating that they represent distinct RNAs in the oocyte.

A total of 71 cDNAs that were synthesized from immunoprecipitated YY1-associated RNAs and did not produce any significant alignments to sequences in the NCBI database. Analysis of the sequences indicated that 70 of 71 contained open reading frames. This indicates that they may represent unknown mRNAs in the oocyte. Of the cDNAs isolated containing ORFs, only 3/74 matched to identified proteins coding sequences. This is perhaps not surprising given the how little of the frog genome that has been sequenced to date. Presently, approximately 254,000 *Xenopus* ESTs have been identified however less than 2000 are full length or have been characterized (Klein et al., 2002). Analysis of these sequences indicated that 70 of 71 contained open reading frames. This indicates that they may represent unknown mRNAs in the oocyte.

Two of the unidentified cDNAs were chosen at random and used as probes for Northern blots to test for potential hybridization to oocyte mRNAs and provide evidence of their validity. The finding that both probes produced discrete bands on the Northern blots (Figure 18F,G) provide compelling evidence that some of the unidentified cDNAs correspond to protein coding messages in the developing oocyte.

In addition to identification of YY1-bound RNAs the immunoprecipitation method provided additional insights into whether YY1-mRNP complexes associate with a single species of RNA or a variety of RNAs. I conclude that YY1 plays a general role in the storage, metabolism or

translational masking of maternal RNAs in the oocyte. The original intent was not to assess RNA binding sites for YY1-mRNPs on associated RNAs, and conclusions in this regard are not made in this thesis.

It is essential to not make any attempt to draw statistical conclusions from the distribution of sequences that were obtained (ie. 18 of 100 clones were rRNAs therfore 18% of rRNAs are assocated with YY1-mRNPs). The clones were picked randomly and do not necessarily represent the actual mRNP distribution within the cell. In order to have ample amounts of cDNA for efficient cloning, PCR was used to amplify the synthesized cDNAs. The PCR step will not representatively amplify all sequences that were immunoprecipitated which ultimately skews the results. Furthermore, the goal of the study was to survey the types of RNAs that are present in YY1-mRNPs and that no attempt was made to design the experiments such that conclusions as to the abundance of a particular RNA sequence in the YY1-mRNPs could be drawn.

7.5 Biological function of YY1-mRNPs

The reults presented in this thesis document the discovery of a novel role for YY1, that of a component of mRNPs. What is the major biological function of YY1-mRNPs in the developing frog?

One hypothesis is that YY1 functions in the cytoplasm as an RNA masking protein similar to FRGY2 (Bouvet and Wolffe, 1994; Deschamps et al., 1991; Deschamps et al., 1992; Marello et al., 1992; Murray, 1994; Murray et al., 1992; Ranjan et al., 1993; Tafuri and Wolffe, 1990; Tafuri and Wolffe, 1993; Yurkova and Murray, 1997). Given the divergent nature of RNAs found in

association with YY1 in the oocyte (Table 3) it is likely that YY1-mRNPs associate non-specifically with RNAs as a global repressor of translation. YY1 may associate with ribosomes themselves as has been demonstrated for a number of other mRNP proteins (eg, FRGY2, xCIRP2, FMRP). In this role, the association between YY1 and the ribosome may function to regulate the timing and rate of translation of YY1-mRNP bound RNAs.

The presence of the zinc finger/knuckle binding domain in YY1 is similar to that found in TFIIIA. As such, it is possible that YY1 functions in the metabolism of 18 and 28S rRNAs in a similar fashion to how TFIIIA regulates 5S rRNA. Both mRNA and rRNAs were isolated in the YY1-mRNP immunoprecipitation, suggesting that YY1 is not an exclusive regulator of rRNA metabolism. It is possible that YY1 may have several distinct biological roles. It may mask and protect maternal RNAs during development as well as regulate their translation at the proper time by associating with ribosomes. As the frog matures, the role of YY1 may change to that of a transcription factor necessary for regulating gene expression. The possibility exists that YY1 may continue to function as a mediator of RNA metabolism in adult tissues, although this remains to be examined.

There are several issues that need to be addressed to unravel the biological function of YY1. Preliminary experiments involving overexpression of YY1 in developing embryos by microinjection of YY1-vector DNA did not result in overt morphological defects. The tentative conclusion consistent with the lack of transcriptional activity of YY1 in oocytes and embryos was that YY1 played no significant role in early development. However given the subsequent

findings that YY1 associates with mRNPs in the cytoplasm, it would be interesting to repeat this experiment ensuring high exogenous expression levels relative to endogenous protein, and monitor subcellular localization of exogenous proteins and morphological effects.

The exact timing of disassembly of YY1-containing mRNP particles and release of associated mRNAs and rRNAs have not been established.

Analysis of SEC fractions from embryos at closer time intervals around the MBT would indicate whether this landmark is significant in the regulation of YY1-mRNPs.

The finding that YY1 associates with rRNAs in the cytoplasm was interesting however the result of the immunoprecipitation-screen did not indicate whether YY1 associates with the rRNAs or ribosomes themselves. Sucrose and nycodenz fractionation could be used to reveal whether YY1 is associated with intact ribosomes in oocytes and embryos. Co-immunoprecipitation of YY1 and analysis of precipitates for ribsomal proteins by Westen blotting would indicate whether YY1 associates with ribosomes.

The immunopreciptiation and identification of YY1-bound RNAs would be better assessed using microarray technology. At the time that YY1-mRNP bound RNAs were identified for this thesis, *Xenopus* gene chips were not fully available. The use of microarrays would be much more efficient in assessing the types of RNAs that associate with YY1-mRNPs potentially leading to a better understanding of the biological role of YY1 during frog development.

The molecular mass of YY1-mRNPs is 480 kDa. To better understand the biological function of these complexes it will be necessary to identify all of

the component proteins. A number of similarities exist between YY1 and FRGY2 however the presence of both of these proteins in the same complex has yet to be demonstrated. Co-immunoprecipitations followed by Western blotting for YY1 and FRGY2 would indicate whether these proteins are part of the same complex, and could be used to identify other proteins in YY1-associated mRNPs.

7.6 Summary

In summary, the work presented in this thesis reveals a novel role for YY1 in the storage and metabolism of maternal RNA. Thus YY1 can be classified along with FRGY2 as a transcription factor with a dual role as an mRNP constituent. Future work should reveal the constituents of YY1-mRNPs and uncover the recise role in RNA metabolism.

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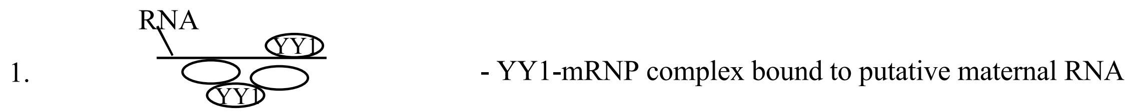
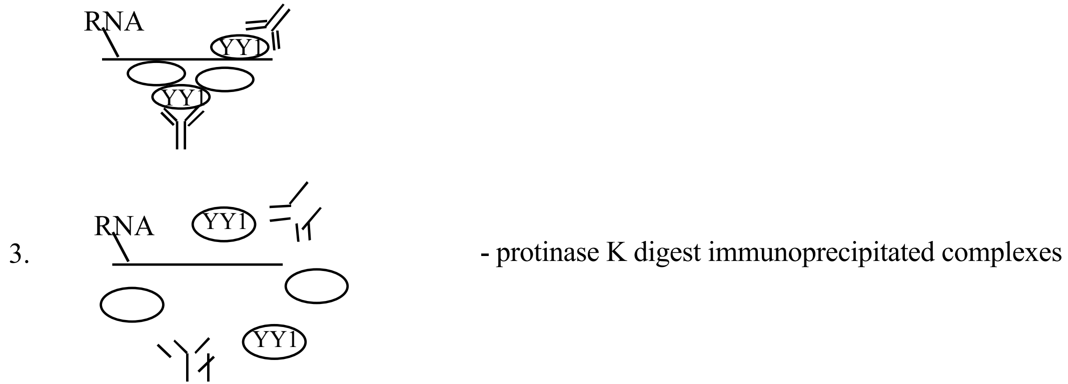
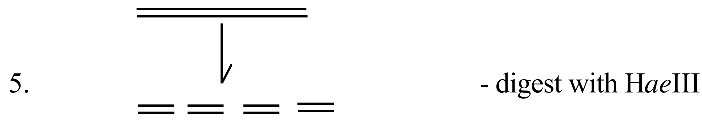
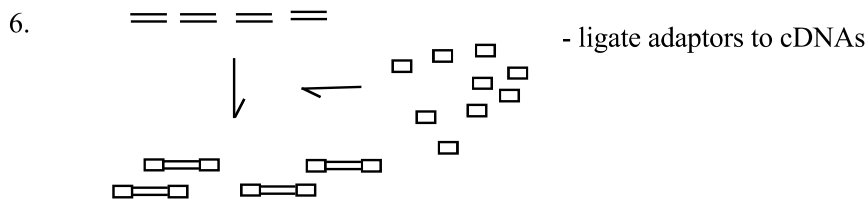
Appendix 1.
Schematic diagram of the immunoprecipitation procedure employed to obtain YY1-mRNP associated RNAs.

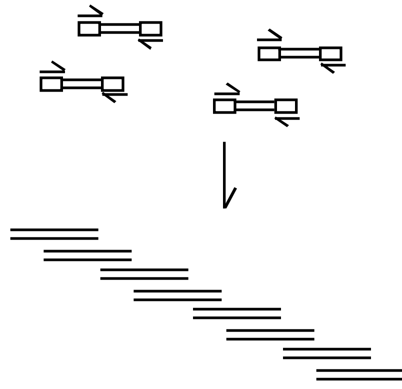




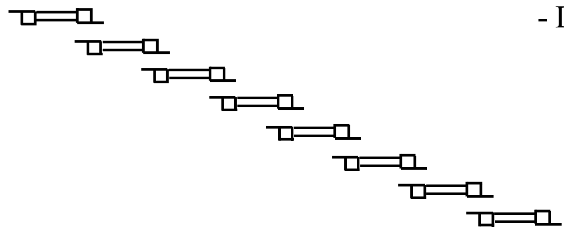




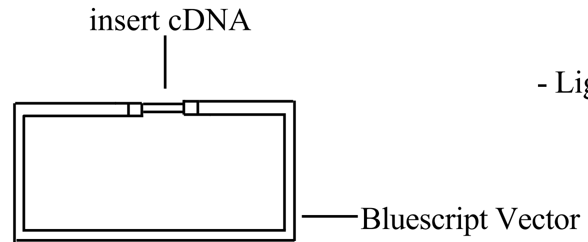
7. - PCR the cDNAs



8. - Digest with M*lu*I



9. - Ligate into pBluescript



Appendix 2. Sequences obtained from the immunoprecipitation of YY1 containing messenger ribonucleoprotein omplexes.

Sequences shown below were obtained from the immunoprecipitation of YY1 containing mRNP complexes. The clone numbers are indicated *above* the sequence. Clone numbers are assigned arbitrarily from the original plates they were picked from. Sequences are shown as single strands only in 5’-3’ as indicated in *bold.*

<u>001:</u>
5’GCAGCAATATCCAGACGAGGGATTAGAAGATCCTTGTGCATCACCGCCC
GAGCGACTTGGTAACGAGACCAGGTATCACGGGTTTCGGCATCATCAAAT
GCATTAAGCACTCGACGAAGTTCCAGTTCATCCGCTTCGTTATCCATCACT
GCGGACAGCGATTCCTGCAGGGCATCACGACTCATGGCGGTTCCTCTCTT
GGCTGTCGCCGCTGTCTCAGTTTTCCTGCAACAACGGCTGCAGGGCTTTAT
CGATGGCTTCCCGAGCCCGGAAGATCCGGGAGCGTACGGTACCCACCGG
ACATTGCATGACGCTCGCAATGTCTTCGTAACTCAGACCATCGAATTCAC
GTAAAGTTAACGCCGTACGCAAATCTTCTGGCAGTTGTTGAATAGTGCGA
TGGACGGTTCCTTCGATCTCGTCGCGCAGCAATGCACGCTCCGGCGACTC
GAGATCCTTGA**3’**

<u>002:</u>
5’CTTGGTGGCGCAATAGGCGATGTGCTGCCGGCTGCCCTTGCGCACCACG
TCATCGCTGATATGCACGATATCGGCAGGTGTCGAGCGTTGCAGCAAAGG
TGAACAATGCAGGTTGATCAGGTACGGCGCAAGCATGTGCACGCTGAACA
TGTCGATAAAGGCACGGCTTTCGTCGCCCGGGGTTTCGGCGACCCAGGCT
GAAGCGTTGTGGATGATTGCGCGCAGGCTTTGGGTATGGGTTTTGACTGC
GCCAATGAAAGCCGGCACGCCGGCTTCGCTGGAGAAGTCGGCAAACACG
CCGATGGCACCCCGTTCACGTAGCGCCTGCACGCCGGGGCGTTCGCTGCG
GTAGCTGAAAATCACGGCT**3’**

<u>003:</u>
5’AGGATCCCTCGAGGCGCGTAAGCAAGCAGCACATATTCAAACGAGAAC
TTTGAAGGTAGTCCACGCGTAAGCAAGAGCACATGCGGATATGTTCCAGC
CGCCGCGTATCCCCCACGTGCCCAAGGGGGATGTCACCCAGCCGCTGATT
GTCGCCGTGTTCGCGCATGGTCAAGGTGTCGGCGATCATCTGGGTCGGGT
GGAATTCAGCGGTGAGG**3’**

<u>004:</u>
5’GCAGGGAGGAAAATCTGTGCAAACTGTCGAACTCCCTTCTTCTCACAGA
GCTCTTCGTAATTACCATAGTCAAAGGTGGTGTCTCCATATTCATCATAGT
TTTCTGTTGTTGCTCTTATTGTGGTACTGATTTTTTCCTCCATGGTGGTTTC
TTTAATAAAGCAAAAATAATGTCATATTACATCATTGTAATTAACCAAGC
CATTAATCTGAAATATATTCATATACGTTTTGGTTAGTGAATTCACCAATT
GTGGGCTGGGTCTATTGATGCCATTGTGTCACTTTTGTTATTAAAACTTGT
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ATCAATATTTAGTTTTGACCACTAGCAAACATCTGGTACATGTAAAGCCA
AGAAAACCAATTTCTACAATAAAATGACTTACTTTACATACATACATACA
TAATGCCAGGGTGGTCTCTAGTTACACCAAAGATTGTTAAATAATGCTAC
AAGCCAACAGTTTATTAGTATTTGCAGGGGTGAT**3'**

005:
5'ATATGGGCACTAAGGGGTATAGGAAACATTTTCATTCTCTTCATAGTTG
GTATCTATACTGTTCGAATTACTATGTGTCTCATGTACAATTTAGAGGCTG
TGTCCATTTCGTGGACTATAACCCAGTGTCGCCCACACTGATTTTACAGTC
ACGATGTTGAGTGAAGGGGCACAGAGAGGCAGCATTGTCAGTCATTCGTG
TCGGTCTGTGGAACAATGACATTGAGCGGGATGGAAAAGGCTAATAGCAT
ATCCCCCTCATCAAGGCTCATGGGGGCACCTGTAACAGAACGAGGGGCTG
ATTCTCGACCCCTTGATTTTGGTTCCCAGATGAAATTTCAACATGGAAGGA
AAGTGCTGCTGTGGACACAGAGGTTGTCATTCATCTGGTTTCGGTTGCCAA
ATAGGGAGTATAAGAATTGGCGTTGATTAGAGACCCCTCCATCGAATATT
3'

007:
5'TCGAGGCGCGTAAGCAAGAGCACATATTCAAACGAGAACTTTGAAGGT
AGTCCACGCGTAAGCAAGAGCACATGCGGATATGTTCCAGCCGCCGCGTA
TCCCCCACGTGCCCAAGGGGGATGTCACCCAGCCGCTGATTGTCGCCGTG
TTCGCGCATGGTCAAGGTGTCGGCGATCATCTGGGTCGGGTGGAATTCAG
CGGTGA**3'**

010:
5'TAACTCGTGTAGAGCAATGCTTCCCCTAATTTTTCTATAAGCTTACACAA
ATATGGATTTTCATGCAGTGACTATACTTCTGCCTATTGTGTCATTCACTTT
GAGTATTTCTGGAAACTTTGAGAAGTAGTCCACACAAAGAAGGTATTCTG
ACCCATTAAAACAAAATGTCACTTCCTACCTTTCCCATGGTCGTCTTGGAA
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AAAGGTTTTAAATATCTGAGCTATCAATCAATTATTACGTGCCCCACCTCT
ATGCTCAAGGCATAGACCTTCAATGACTTTCACTTTCCATTCAGCACTCCC
TAGATGTCACTAGGAATATACTACAGATATTGATGACATTTTTCACACCA
ATATATTATGTTAAGTAGTTATTTTTCATAT**3'**

017:
5'CAAGGTCAGAATTCCCGCCCAGGACTTTGACACGCCGGCGCAGAACCT
GGCGTGCGATAACCAGTCGTTCAACCCCTGGTTCGGTTTGGAGGCGCACC
GCCCCATCGGCGGCATCAATCGGCTGCGCAAAGCGGTGTACGAAGCCGTC

AGCGACTACCGCCACAGCCGCAACGCACCGTAAGGTGCGTTAAATTGCAG
GCGAAAAAAAACCCGCTCAATGAGCGGGTTTCTTGTGGCTACCTGGCGTT
CAGTGTTCCAGGTTACCGAATATGGCGCAGCGGACGGGACTCGAACCCGC
GACCCCCGGCGTGACA**3'**

018:
5'ACTACTATTGTCCTAGGAAGTCTATAGTCTACAGTTTGTATTCTCTATTC
GTTAGCAGCAAAAGCGCAGCAATTAGGTATATGTTAAATAAAAACATACT
GCAAATTAATCTTGCAAAACTGAAAAATGTTTTATTGTTGGGAAATGGA
ATATGTTTGGAACTGATGAAGAGTCATGTCTTCTACAGGGAAGTGGGGCA
TTTAAACGAAAAAAGTCCATTCTTTGAACCACTGCTGCAATACTTTACACT
AATTACATTTAAAATGTTCTGCTGCTGTGCTGACCCTCTTATACTTTAACT
GCTTTGCATCACTCCCTTCTCACTTACTGTGACATCACTTGGGGGTTTGCT
ATCAGGAAAATGGTTTCATGTTGTGGATCTATTTGTGGGCAGGTTGTAAAT
ATCTATTGAGAATGGTGATCTTCTTTTTTTTATGCTTGCTCCTCTTCAGGTG
TGAGGACTGTAAGTTTTATTTTAACCTGACGT**3'**

020:
5'TTTAGCGCCGAGCTGATCGAAGCCTGCCGTTATCGCTTCGAATCTCCTTA
TGTCCATCTGAAGAAAACCTTCGTCGAGTTCTCAGAAAGCTGCTTCCCGC
CCGGCGAGCCTTTACTGCTGGGCACGCAGGCATTGATGGAGAAGATCTTC
AGCGAATTTACCTTCGATGCCGAAGCCACCCAGGTCGCCACACCGCTGGT
GGAAGTGCTGGAGCGCCGTCGCGGCGTGTGCCAGGACTTCGCTCACCTGA
TGCTTGCCTGCCTGCGTTCCCGTGGTAGTCCACGCGTAAGCAAGAGGACA
TTGATTTTCTTAATGCTTATTTTTCGTCACAGACACCAGAAACTGTTCCTCT
GAAGTATTACTAAGAAACATCCAAATTGAAGGTTGAGAGAAAACGAACT
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AGCAAGAGCACACCGCCCCGCCGCCTAGCAGCTGACTTAGAACTGGTGCG
GACCAGGGGAATCCGACTGTTTAATTAAAACAAAGCATCGCGAA**3'**

022:
5'AGGGGGGGACAAATATGGGATACCTNTTCCACAAATGGATCTACGAAG
TTCTGAGNTACANGAAGACCATCNGTCTATACTAGNCCATGAANAATTAC
CTAGGCACATGTTTTTCNAAGGATTNCTTTTTCTCTGCAATANGAAAACNA
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AATTCTGTTGGGTNTATTTAANGTTNAAATGATTTTTAGAANAGATGGGC
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GCGACTATTCTGACGCACAACATTTTTTTTAGTCACGCANGATTTTTTTGC
CAACAAATTTTTGGNGCAGTTTCATGAATTNATTCGCTGGCNG**3'**

023:

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029:
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032:
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037:
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T**3'**

039:
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TGAAATGCCTT**3'**

041:
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042:
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GGGGATATGGTTCTCAAATGGGTGTTCAGCAGTCAACAAGCTGGAAAGCG
TTCAAGATCATCGTCTCAGTCGATGGCTTGGTTCGCAG**3'**

043:
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CCGCGCTCCAGGATTTGCGCCAGTTCAGTCCAGTATTCGAGGGTGTTGTA
GTGGGTGGACGTGTCCCGTGGGTGGGTCCACAAACCGTGGTTGATGTGCC
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GATGGTCCCCCGCAGCGGCGGGTTTTCATCGTTGAGGTAGTAGTTGCCGA
CCGCATGGTATTTCCAGCGGACCGGGTCGTGCAGGGTGTGCACCCGGGCG
TTGCGCCAGTGGCGGTCC**3'**

044:
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TTAGGATTTTTCGGCACGATTAGGTGCTCCTGTATCTAGAAAAAATCGAGT
AGGCAGGAGCAACCCTTGAGCGGAAAGCCGACCTGAATGGTTGACATCC
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CTGACCTATCATCACGCCAGGCGGTGATGGTCGCCTGGCGCCTGTTTCGC
GAAGCAATAGCCCATCTGTGACACCCTTCAGTTTTACATCGGCATGTCCCC
TAACCAGACGCTGCC**3'**

045:
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GGTAGCCATTACCTGTACATAAAAATGCTTGCTACGGTGTGCCAGATTCA
CATATAGGGGCTGATTTATTATGCTGTGTAAAAAAACAGTGACAAAAATT
TGGCATAATTTTATAATGCGTTTTTTATTAGAGCAACTTCCACCAGAACTC
ACTTAAAAATGTCTGAAAATGTGTAAAACAATGGAATAAAATCTGGCATT
TGCACTGTGTAAAATAAAACACACGGTGATAAATTCGCCATTTATTTTAC
ACAGCGGTTTTTTTTTTTTCAGAAAATTTTACACAGCGTAATAAATAGGCG
TATATAGGGGTAATTTACTGAGACCCTGGACACAAGTGCAAGATCAGTTA
AGGACACAAATTTATCCCCTTGGCACTCATTTAATAAGCATATAAATATA
ACATATAAAAATTTACAAGGTTTANAGTGCAGAATCAGCCTCCCACATGG
GCAGAGGCTAAGCCTCCCCAAACCTCTCTGCTACATAAATAAAAATAAGA
AAAGAGGGGGGAAATGTGATGCCTTACTGAGTGGTGTCACTGACAGGTG
GTACAATTCCGTTTTGTAGTCAGCTCTTATTCAAGTGATTGTCTGATGTCT
ACCTGCCTAAGGGTCCTTANGCTTTGGGAGGGAGCCCTATATAGCTCCGG
TTA**3'**

048:
5'TGCGCGGCAAGGCTGCTGGCGGCGAGCGACAAGGCGAGACTCAGGCGG
GCTCCAAACACGGTAAAACCTCAATAAGGGCGGATACGGGTAGACCTTG
CGCCAGCCTTGCGGGTTCAGCATGGAAAAAACCGGAACACTGGAGTGCC
AGCATGCTCTATAACTGCATGACCTTCATCCTGCGCCCTTACGAGGTTTGC
CATGCCTGTTACCGTCAATACGCTCAACGCTGAAAACTTCCGTCACAGCG

TGAATATCAATAATCACGAGCTGTTTACCGACCTGCCCAAGAGCCTGGGC
GGTGACGATTC**3'**

049:
5'AGGCGCGTAAGCAAGAGCACAGCGATAGTCGCTGTGACCGGGCTCGAT
ACCCGCCTCGACACTGCGTCGCCAGGAATCCCAGATGACCTTGCGCACCC
CGTCCGGACGCACGACTTGATAGTCCACGCGTAAGCAAGAGCACATACAC
CAAATGTCTGAACCTGCGGTTCCTGCTCAGTACGAGAGGTAGTCCACGCG
TAAGCAAGGGCACAGGCGGTAAGCGGCATCCACGATCAGTGCCTGGCGC
TCGCGGGGCAGGGCTTTGAATGCAGGCTCCTGCAGTTGCTTTTGGTCATCG
CTGATTTTCAGCACCCACTGTTGCTCATCGCTATCGAGCGGCTTGGCGCGT
TCCAGCAGTTCACGTTCACGGGATGGGCGATAGTCGATCTTCTCCACCAG
CCCAGCGTCTTTTACCGCTTTGACCGTGTCGGTAGGGATCGCGGTGAGCG
GGAACTGTTCGGTCAGGCGCAGGTAGTCCACGCGTAAGCAAGAGCACAT
AGTCCACGCGTAAGCAAGAGCACATATTCAAACGAGAACTTTGAAGGTA
GTCCACGCGTAAG**3'**

051:
5'ACCGTCCTGCTGTCTATATCAACCAACACCTTTTCTGGGGTCTGATGAGC
GTCGGCATCCGCTAAGGAGTGTGCTCTTGCTTACGCGTGGACTAACAGTT
GCCTTGAAGGAAGGAGAAAAATACCGTGACTTTTTGCAAGAAAAAATCAT
TGCCTTGAATTTGCAAAAGCATGTGAAATTCGTAAACAAATATTTGCCTTT
GGACGAACTTTTAGAATACCTGCAAATGACGGATGTTTATTTGTTTACTTC
CAAAGATCCAAATCAAGCGGTTAGCGGAACTTTTGCTTACGCTTTAAGCT
GTGGATGTCCTGTGGTTTCCACCCCAATTCCTCATGCAAAAGAATTTTTGT
CGGGCGATACGGGTTTGCTTTCTGATTTTGGAAATTCGGCAGCATTAGCG
GAATGTGTAAACCGTTTATTATTTGATGTTGAATTGAAACAGAATATCATC
AATAATGGTTTACATAAAATTACCTGTACGGCTTGGGAAAACTCAGCTGT
AGCTCATGCCCAATTTTTACAAAAGGTTTCGGATAATCAAATTGAATTGC
ATTATAGAAATCCAGACATCAACTTAGATCACATCAAAAAAATGACAACT
GATTTTGGAATGTTGCAATTTTCAATTCTAAACCAG**3'**

053:
5'TCTCCAGTCCGATGAGTTGGTCTCCAAGAAGAAGAACAAGAAGCGCAA
GAACCGAAATTTGGGCAATGCTGACAATACAGCTTGTGCTCTTGCTTACG
CGTGGACTATGTGCTCTTGCTTACGCGTGGACTAACTGACAAATCCTGGGT
CTGTATCTCCCAAGCAGCGCTTAGATTAAATGCATCTCCAAAACCAATTTT
AGGGGAAAAATCTCCAGATTCACTAAAATCAGTGAGAAAAGCAAAACCT
GCTAGTCTGGGGGGGCATCACCCAACTTTCCCCTCTTTTTTGCACTAAAA
TAAGGGTTATTTGCCAAAAAATCTCTCAGAGGGAATATACATTTAACACA
GTGCCAACCAACGGACAAAACCAACAGTCTAAACCCATGGGTTACAGTC
ACCTCTCTTACAGTATATGGGATCCTGCTATTTGTCCCCCAATCATTTGCT
ATCACCGCTTAACTGTCCCCATGGCACGTGCCTCTGCTGCCCTCCCCTAGT
TCCACACTTTGATAAAGCGAGCATGTATTTAGCTACAATGTTGGTAGATA
AGAGCAGGGCAGATACCCCAGGGCTGAGGGGCAAAACTCTAAGGAGCCT

GTTAATACTGGGAACAAAAATTAAGCACCTCATGGTGTAGTACAGGGGTC
CCCAAATGCTCCCTACAGTAGAAAATGTGTATATTTGCTGGCAGCCCTGC
ATAACATCCAAATCCTGGGT**3'**

054:
5'TGATTAAGAGGGACGGCCGGGGGCATTCGTATTGTGCTCTTGCTTACGC
GCCTCGAGGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAA
CCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAG
CTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGC
GCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATC
TGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTG
ATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCG
CCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGAC
CGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAAC
GCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTC
ATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGT
GCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCC
GCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGA
AGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCTTTTTTGCGGC
ATTTTGCCTTCCTGGT**3'**

056:
5'AGCCTTGTCGTACCGCCGAGTACAACTGCATCTCGGCGCGCTGTTTAAA
GCCTCGATCAATCAGCGCCTGAGACAACCCGAAGGACGAGCCAGGGCTC
GGATAAAAAGCCCTACGTATATGCTTGCCGTATTCGCCCCCCACATTCAG
GGATGCGATCGTTTTGATCAGGTAGCTCACACTCAAGCGTTCTTTCCAGAC
GGGTTCCAGCCAGCGCGCCCGGTTCAAGCGCCAGCGGGTCCAGGGCGCTT
CAGGGTCGAGGTTGNGCAACGCCAATTGCAGCATGCTGAAGGTGGTACGT
TCCTTACTGACCACCGTTTTAATCTGACGATCACCCACCGCCCCCTGGCTC
GTCCAGCCCTCGTAGNGAGNGCTGACATCATCGGGCATGTCGAACAGGG
GGGTATCGATATCCAATTGCGGGTAGAAACCGTCCTCGGTCAATTTGGCA
ATCAGGGCTTTGCGAGCAAAGGGCTCCAAGTCAGGCAAGTCCTGCCAGA
GTTTGGTTTCCAGTGCAAAAAT**3'**

057:
5'TGGGGCAGCCGAGCCAATCATCGGTGTCCATGTCTACGTACATAACGCG
AACCTCAAATGCTGTATGTGCGTACAGTAATCGAGGTTTGGACGTGACGC
GATTTGAGCCGACGAGCTGTATGGGCTTGCGACTATTCCGACTCTATAAG
CACGGCGAGAGTCATTTTGATGAACTCTTCGTTGTGTCCGATTGTGAAGA
GGGCGCCTCGCACGTTGTCTGCCACCTCGGCGGAGCCACGTTGCTCAACC
CAATTTGACAGCTCCAGGATGGACGCCTCGAGTGCAAGTTGGTTTTCGTA
GAGTTTGAAAAGCAGGGAAGGGATTAGGTCTGAGTTCGGCATCGTTGTTC
CTCCGTGGAGCGAACAGCGTAGCAGGCAAATTATGGAGTTGGGAGATCGT
TCGGCAGGACGCCGGAGGAGGGGTAAATCATTTCCGCAACCCAATGCAC

GCCCCTTGATCCATGCGGGCTGTAGGCTGGCTAAAGCTCTAAGTTGCGGA
AAC**3'**

058:
5'GTATTCGGTGCATGTGGCAAATAAAGTTTGATTTGATTTATGTCTGTCTT
AGGTCAAATCAAATGTCATTTGTCGCATGCGCCGAATACAACAGGTATTA
GACCTTACAGTGGAATGCTTACGTACAAGTCTGTAACCAACAATGCAGTT
TTAAGAAAATACCCCCCAAAAAGTAAGAGACAAGAATAACAAAAAAATT
AAGAGCAACAGTAAAATAACAATAGCGAGACTATATACAGGGGGTACCG
GTACAGAGTCAATGTTCGGGGGCAGAGGTTAGTCGAGGTAATATGTACAT
GTAGGTAGAGGTACAGTAACTATGCATGGCTAATAAACAAGAGTAGCAG
AACCGTAAGGGGAGGTACAAAGTTAAAGGATTCTGACAGGTTAAGAAAC
TCCCCAGCTAGAACATCATGAATGAATGTTCTCCAAGAATAATAAGCCCT
AGTGGTTAGAGTGTAGAGGCGGCAGGGTAGCCTAGTGGTTAGAGCGTAG
AGGCGGCAGGGTAGCCTAGTGGTTAGAGCGTAGAGGAGGCAGGGTAGCC
TAGTGGTTAGAGCGTAGAGGCGG**3'**

059:
5'GTCCTGCTGTCTATATCAACCAACACCTTTTCTGGGGTCTGATGAGCGTC
GGCATCCGCTAAGGAGTGTGCTCTTGCTTACGCGTGGACTAACAGTTGCC
TTGAAGGAAGGAGAAAAATACCGTGACTTTTTGCAAGAAAAAATCATTGC
CTTGAATTTGCAAAAGCATGTGAAATTCGTAAACAAATATTTGCCTTTGG
ACGAACTTTTAGAATACCTGCAAATGACGGATGTTTATTTGTTTACTTCCA
AAGATCCAAATCAAGCGGTTAGCGGAACTTTTGCTTACGCTTTAAGCTGT
GGATGTCCTGTGGTTTCCACCCCAATTCCTCATGCAAAAGAATTTTTGTCG
GGCGATACGGGTTTGCTTTCTGATTTTGGAAATTCGGCAGCATTAGCGGA
ATGTGTAAACCGTTTATTATTTGATGTTGAATTGAAACAGAATATCATCAA
TAATGGTTTACATAAAATTACCTGTACGGCTTGGGAAAACTCAGCTGTAG
CTCATGCCCAATTTTTACAAAAGGTTTCGGATAATCAAATTGAATTGCATT
ATAGAAATCCAGACATCAACTTAGATCACATCAAAAAAATGACAACTGAT
TTTGGAATGTTGCAATTTTCAATTCTAAACCAGCCCGACATCAATTCAGGA
TATACTATTGATGACAATGCCAGAGCGATGATTGCCATGTGTCAG**3'**

060:
5'TAACACCGTCCGCGGGCTGTGCTCTTGCTTACGCGTGGACTACCAGGCG
GACGAATATGTTCGTGATAATCCGTGGACTGGTATCGGTATCGGCGCCGC
TGTCGGTGTGCTCTTGCTTACGCGTGGACTACCGCGTCGCCTGGATACTTC
AGCTANGAATAATGGAATANGACTCCNGNTCTATTTTGTTGGTTTTCGGA
ACT**3'**

061:
5'GCTTCATGCCTGTCGGTCGACTCTAGAGGATCCCTCGAGGCGCGTAAGC
AAGAGCACAAG

AGGTAGTCCACGCGTAAGCAAGAGAACAGACAAGTTCCATAGGTCCACT
ACTCTCCATTTTACCTAATGGGGTTAGTCCACGCGTAAGCAAGAGCACAC**3**
’

062:
5’GGGGGCATTCGTATTGTGCTCTTGCTTACGCGCCTCGAGGAATTCACTG
GCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACT
TAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAG
AGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAA
TGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACAC
CGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAG**3**
’

063:
5’GCTTCATGCCTGTCGGTCGACTCTAGAGGATCCCTCGAGGCGCGTAAGC
AAGAGCACAAGAGGTAGTCCACGCGTAAGCAAGAGAACAGACAAGTTCC
ATAGGTCCACTACTCTCCATTTTACCTAATGGGGTTAGTCCACGCGTAAGC
AAGAGCACACCCGACGCTCCAGCGCCATCCATTTTCAGGGCTAGTTGATT
CGGCAGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGTA
GTCCACGCGCC**3’**

064:
5’GACCATTTCCCGCGAACACCCCACAATACGTCCGATCTCCTGGCGCGTG
ACTTTGATCTGCATGCCGTCGGGATGGGTCATGGCATCGGGTTGCTTGCAT
AGCTCCAGCAGGCAGCGAGCGACGCGACCGGTGACATCGAAGAACGCCA
GGTCGCCGACTTTGCGCGTGGTGTCGCGCAGGCGCTGGGCGATCTGTCCG
CTCAGGGCATACAAGATATCCGGATCATGCT**3’**

065:
5’GATAGCCAGCTTCATGCCTGNAGGTCGACTCTAGAGGATCCCTCGAGGC
GCGTAAGCAAGAGAATCGTTAGCTAAATTAGTTTCGCCAATTTTATAAAG
AATAATTTCAAGTCTTTAGTCCACGCGTAAGCAAGAGCACACCTGGCGCC
CGGTGCCTCCCAAGACCGCCTGCGCTTTGTCACCACCATCGAAGACTGCG
TAAAAGACGCCGACTTCATCCAGGAAAGCGCCCCGGAACGCCTGGAACT
GAAGCTGCAATTGCACAGCCAGATCAGCGCCGC**3’**

067:
5’ACGCGTAAGCAAGAGCACACAGGAACGAGGGCAGGTCGCTGGTGCGTT
CGAAGATCACCAGGGTCGCCATCGGGACCACCATGGCGACCGCAAGGGT
GATCAGGAAGATGCCGATGATGAAACCGATGATACGCAGGGTCGGCAAC
GCCATGAAGTTCGCTCGGGTGATTGGGCTGAGGCGCCATTCTACCTGCGG
TGCAGGTCTTGTAAACCAAGGGCTGCGGTGGCTTGGTGTCTACACAACTT
CGCAGCGACGCTGAAGCTGTGGCGAGGGAGCTTGCTCCCGTGACGACCTA
CCCACTGGCGCCTATCTACCTGACACACCACGATCCAAATGTGGGAGCTG
GCTTGCTCGCG**3’**

<u>068:</u>
5'GCACAGAAGGGCAAAAGCTCGCTTGATCTTGATTTTCANCATGAATACA
GACCGNGAAAGC
GGGGTAGNCCACGCGCCTCGAGGAATTCACTGGNCGNCGTTTTACAACGG
CGNGACTGGGAAAACCCTGNCGTTACCCNACTTAATCGCCTTGCANC**3'**

<u>069:</u>
5'CAAGCGATGGATCAGCCGACCAACAATAGCGCTGCACCGGCAACCGGC
GAAATACCGTCTGGTCATCGCCAAACACCAGCAGATGATTCTCCGCGCTG
TGGCGAAAGTGGTAGTGAATACCTTCCTCTTCACACAGCCGCTGGATGAA
ATGCAGGTCGGACTCCCTGTACTGCACGCAAAATGTGTGCTCTTGCTTACG
C**3'**

<u>071:</u>
5'ATTTCTGACGGNTTTATCCGCGTGGGTGATGCTTGGCGTGCATGTCCTGC
AAACGTGCGCGTGCCACATGGGTGTAGATCTGGGTTGTGGATAAGTCGCT
GTGCCCAAGCAGCATTTGCACCACGCGCAAATCCGCACCGTGGTTGAGCA
AGTGGGTGGCGAACGCATGGCGCAGGGTGTGGGGTGACAGCGCCTTGCC
GATCC**3'**

<u>072:</u>
5'AAGTTCTGATAATACGCAAACTTACTCAACATATTGCAAAGCTGAAATC
TAAAAAGTTAGTTCATGCTTGTATATATCTGATACTAAAGAAAAAAAAGT
ACCAGGAATGCCCTGAATTGATAACATGTATCATGAGACCCAATATTACG
TTACAATTCTTCAAAACCAAAGCGATTGCTATTGACTATTACCCCTTGCAT
TTGTAGATTTCTTATAACATATACATTCCTAAACTGATCCTCTGTTCCTTTC
TCTATTTCTATTTGTTGCAACATATTTCTCATTTTCCTGTTCTGGATTATTC
ACACATAGCCGTATTTACTTTTACAGGTTTTGTTCATAGGACTCAATACAT
ATTATGTCAGTCTAGTATGCGTCATTCTTTCATTTAATTTTATCTGACAGTC
TATATTTAATCTCACTATTTTGACTGGATTCTAAACGTGTTTTATCTTTTTT
TAAGTAACTCTGTAGTCAGTTACTTGAGCTGAAACCGGATTATAAAATTC
ACAATGCAAATTCATGAAGAAGAAAAAAAAAAACACTGTTAGAGTGCTG
CTGAACCTCTGGGCTATAACAGTAAGTTAATTATATTAAAAACTGCATTTC
TAGCTATGTTCTGTTTTGNGTGTTTTAGTTCTCCCTAAACAATTCACAGATT
AAAATACGTAATATTTTTCAGGGTCTACCCCCCCCTTAG**3'**

<u>075:</u>
5'TGTTCAGCGGCTGGCAGGGCAAAAGCGCTGGAAGCCAGGACAGATAAC
AGAACGGTAGCGAGTAATTGGCGTTTCATGATGGTTGCTCCTTGGGAGGG
CGATAAAGTGGGTACAGGGCTAATGTTACCCTTGATAAGTCGATATAAAA
GTTCATAAACACAATGGTAATAATCAACAGAATTGATTGTTCTCGCCGGA
GGCTCTAGACCGAGCCTCTCAAGCCCGCGGCTTTGCACTGATGTGGGTAT
TTTCGACACGAACCTGGGTAACAATGGTGCGTCGTCGATGGGGAAAATGG
TCTGGTTGATCAGGAAAATCGCTTTTTTCGGCTAAATCCGCCGTTGCGTTA

AACCCCCAGGCACTTTGCCAGTCGTAGTCCTATAAGCTGTTTAAAAGGCT
GTTGCCCAGCCAGTCGTATTCAGGAGTCCTGTGCAATGACGCGCACTCGT
AAAATTGTCGCTTGGAGCTGCGCCAGCTTCGTTCTGTTGATTGCCATCGTG
GTACTGGTGTTGGTGTTCTTCGACTGGAACCGCATCAAGCCGCCCCTCAAT
GCCAAGGTCTCCGAAGAACTGCATCGTCCGTTCGCCATTAACGGCAACCT
CGCCGTGGTATGGCAGCGTGAGCCCGATGAAGCGGCTGGCGCGCCTGGGT
GCC**3'**

076:
5'AAGCGCCTGCTGTTGTTTACCGGCAGTTCGGCAAATGAAATCTGCTACC
AACTGGGGTTCAAGGACCCGGCGTATTTCAGCCGGTTTTTCTTGCGGTATG
CGCAGATGACGCCGGGGGAGTATCGGGTACGGCAGGCGGGGATGCGCTG
ATAAGGGATCGGACAAAAAATGCAACGTTGAGGAATTGGCTGATAGTGG
TCCGTTGAACGAGAAGGATGGTGTTACCTGTCGAGCCGGAACTTTTCTGA
CCGTC**3'**

079:
5'TACTGATTGCAGAACGGTGACCGTCCATCCGCAACCTTAATGTCTGGTC
GGTTTTCCCAACATATGAGAGTCCACAGGGGCATGTTATTAGGTATATCA
CATGAGTGGTTGTGCAGGTAATGTGATGTCTAATGGAGAATTTTTACCCG
TTTGGGGATGATGAAATGATGTACCCGGTGTCATATACCTGCAAGATGTA
CAATTTGGACATTTGTGACACCCGGGTCTCCCTCCACTTAGCCATCCTTTC
TGGGTTTTGGAATAGCATTTAATTGGATCGCTTTTGACTAAAACGTCTCTC
AAATTGGTACCTCGTCTGTAGCACATCATAGGAGGTCCTTTCACAACTTTT
CCCAATGCAGTATCTATCTCTACGATCTTCCAAAGATCCCTGACCCCCTTA
TTAATGATCTTAGCACCAGTATTAAAGGTAGTGGTAAAGGTAAGTGCTTG
ATCTTTTTTCACTTGTATAGCCTTCTCACTCACCCTACACCTGGCAATCTCC
AAAGCATTTAATAGCACTGTGTCTGGGTATCCTCGCTTCTTAAAGCGAAA
C**3'**

084:
5'ACGAGTAGGTGTGCTCTTGCTTACGCGTGGACTACCAGTGTTTGACAAC
ACAAAGATGTGCTCTTGCTTACGCGTGGACTAAGGTTGTTTATCTTATGTT
TGCTTTTCAACTGCTAGGGAAGAGAAGACAATGCTTCTACTAATCCGACT
CAATCGCACAAGCACAAACATGACTTAAGTCACACTATCATGGTAACCTC
CTGCCCTTAAAGGAGCAGGTGAAAATGTTTGTCTCATCTGTGATATTTTGA
TAAATGTGATAAATTACTCATAAATTACTCATGTCATAAAAAATACATATT
ATTCAATATACCACATTAGTTTCTTCTTACTAGTAATACATGTATTGTTTTA
GAAACATAACAAAGCATGCTCATCTGTTTTTTTGTGTTTTGTTAGTATAAT
TTTCTCTGCATTTTTTTTTTTATCTACCTGCCAGTGACTGGTTGACCAAAAT
ATTAAATTTATTGCCATGTTCCTCACCTTGAAAGTTGCCTATGGGCAAGGA
GAAACTGAAATTCATGGCTTGATTTTATTTAAACAGCCATTGCAAACG**3'**

086:

5'CCCAGTTCCGAAAACCAACAAAATAGAACCGGAGTCCTATTCCATTATT
CCTAGCTGAAGTATCCAGGCGAAGCGTTTACTTTGAAAAAATTAGAGTGT
TCCAAGCAGGTGTGCTCTTGCTTACGCGTGGACTACCGTACTCCCCAGGC
GGTCAACTTAATGCGTTAGCTGCGCCACTAAAAGCTCAAGGCTTCCAACG
GCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT
GCTCCCCACGCTTTCGCACCTCAGTGTCAGTATTAGTCCAGGTGGTCGCCT
TCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAA
ATTCCACCACCCTCTACCATACTCTAGTCAGTCAGTTTTGAATGCAGTTCC
CAGGTTGAGCCCGGGGATTTCACATCCAACTTAACAAACCACCTACGCGC
GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCGCG
GCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAAT
TGCAGAGTATTAATCTACAACCCTTCCTCCCAACTTAAAGTGCTTTACAAT
CCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCA
TTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCG**3'**

087:
5'GTCTGGAAATATAGGGGCAAATCCAATAAACAATTGGAATTTACTTCCA
TTGATCTGTTTACTCAGTGGTTGTCACTTTTATCGAGAAAGGTTTGCAGAA
CGCGGGTTTTTTTATAAGGTTCCTGATGTACTAAGGGATTATTTATCAGCT
ATACCGCTGGAAATAAATGAAAAGGCACGATACAAACCCGGCATCGCTA
ACTATCACAATATCATCACATGTGGTTTTTCAACGTTGTTACCCTATATAA
GGCAACAACCATTGGCAATGCAGCAACGATTTAATTTATTATTTCCTGATT
TTGTTGACCACATACAATCGCCGTTACCACTTGCATCAACACTTTTAGAGA
GAATTACATTTTATGCGAAAAAAAATAGAGATGAGCTTGATGAAATCTCC
TGCAAATGGTGTTGTGATTAAAAGAAAAATTTCTGATGGATTGAAAGAAA
TAGTTTCTCTTAAAGAAAAAATCCTTCTTGAAACCACCGCAAAAATTCAG
AGTATTGAGGAAAAGAGAGAAGAAAAATTTATTCAAGGTTATTATGATGG
CTATACAAAAGGCATTATTGATGAGATGGATAATTTTATACCCTTGATAA
GCTTGCTATGCTCAGAACTTGAAAAAAAAGAATAAACATGATAAACCGAT
CTGAAGAGTATATTGTTAAAACCTTCAGAAAGAAGTTGATGTTTTTTAGT
CCCCCGCGCCTCGANGGAATTCACCTGGNCGTCGTTTTACAACCGTCCGT
GGA**3'**

088:
5'TCTTTATGTCATAAATTTACTTTAGTTTTCCGGTCCATTCATCGGTCAGG
TTAACAGGTTATTATGTTGAGATACAATTAACATTAACTTAATGAGGAAG
CCTTTACTTTTGTGATAAAGTTATGGGTACTTCAGCGGATCAGTTATTATT
TCATGCCGTACAACAAAATGACCGCAGGGCATTTGACCGTCTCTATCTTAT
GTATTGGGAACAAATGTATATCAGGGCATGGAATCGTACGGGTGATGAGC
AAACGGCTCAGGATATTGTCCAGGAACTTTTCATTAATCTGTGGGAACGA
AGGGGAGAGATTCTGATTCAGACCAGTTTCCTGCAGTACTTATCAGGAGC
CTTAAAGTACAGACTGATCAATCATTTTCAATCCGAAAAAGTTAAGCGAA
GGGTATTAGAAAAAGTATTGCACAGAATGGCTGAACTTTCTGGTGGAATA
GATGATCTTGCAGATTATAAACATCTTGAACAAGCACTGGATGATGAATT
GGAGAAAATGCCGAAAAACATGAAAAACTCTTTGCTTCTTCGGCTTGATA

ATCTGTCTGTTAAAGATATTGCCCTCCGCCTGGACCTGGCAGAACAAACC
GTTTCCAATAACTTAAGTGCTGCCAGTAAACGCTTGCATAAGAATCTGGG
TTCTA**3'**

089:
5'AGTGCGGCAAATTCGAAATCGCTTAGCTTTTTCACAGGAATGACCTCGT
ATCTAGTTGTTTAACAAAGCTTTCGAGAGCGTCNGTCNGTCTTCGTCGATT
GCTTTCGCTATCCCTTCCATGCATCCCACGTACTCCTGCGCTTTTTGCNTGT
GTGGGGATCGCAATCTGGCTTGCGAGCAGATCAATGATCGAGCACACCTC
CCTGAACTGTGCTGCTATTTGTGCGTACCGTCGGGTTTTCAAGCTTCAGGA
CGCTAATAGAATAGTTTTTCGCTTGGTGCAGAACACGGTTTGCAGCATGT
AGCACATTATCGTACAGAATTGCAACCGTTTTTTGCTTCTGATTAAGCTCT
TCCTGAACCATTCCCGACGATCCTGATGTTTATAAAAGGTCGCGGGATTCT
ATAGCTATTGGTGAGACTGTCAACCTTCGCCAAAAGTCACAATCCAAATA
TGGGCTACCGTTAGTGGTCTCGTCAATACGATAAACCTAACAGTTTTATGA
AGCTTTTCTATGAAGAGAAATCGTAGCGAAACACCGATGAATCCTACGTT
ACGGCTGTTTCATTTCCTCTACAGCGTTAGTCAATTTGGGCGT**3'**

091:
5'GATGGTCANGGTGGCATGTGACTAACTGTCAGGATAATAGTGCAGGTGC
TACATGGAGAGTAACACTCAGGAGGGAGCAGTTCAGGGGCTGGATGAGT
CTCAGAAGACTTGGTGTGTGTACTCTTGCTTACGCGTGGACTACCTTGCTG
TCTAGTAGCTTCTTGTAGCTCAACGCACGTTCGAAGTCTCTTGCTTACGCC
GTGGACTACCAGCGTGGTTTGACNGGGGCGCTTAAGATCAAGATCAAAAG
CAGATCAAGAGCGACTCGTTCGATCGTGGTTACCGTCCGCTGCTACAGCC
CACTCAGTTGTGTAGATACCTATGCCGCTATGGGGCAA**3'**

092:
5'GGTCANGGTGGCATGTGACTAACTGTCAGGATAATAGTGCAGGTGCTAC
ATGGAGAGTAACACTCAGGAGGGAGCAGTTCAGGGGCTGGATGAGTCTC
AGAAGACTTGGTGTGTGTACTCTTGCTTACGCGGTGGACTACCTTGCTGTC
TAGTAGCTTCTTGTAGCTCAACGCACGTTCGAAGTCTCTTGCTTACGCCGT
GGACTACCAGCGTGGTTTGACGGGGCGCTTAAGATCAAGATCAAAAGCA
GATCAAGAGCGACTCGCTTCGATCGTGGTTACCGTCCGCTGCTACAGCCC
ACTCAGTTGTGTAGATACCTATNCCGCTATGGGGCAAGTTGTGGATATGC
TGACCGGCTCTCAAACAGTGATTGCCAAGGACGCCGACCATGACTGCCTT
GACCCTTGCTGCGGCNCAAACCGTTTCCATCCCGGGCGATGTCGCTGCCA
ATATCCAGCGGCACCTGGCGTTGATGTGTGCTCTTGCTTACGCGTGGACTA
CCCGCCCCATCGCCGTCCCCATGCCGTGACTGGAAAAT**3'**

093:
5'TGCTCTTGNTTACNCGCCTCGTAGGAATTCACTGGCCGNCGNTTTACAA
CGNCGCGACTGGGAAAANCCTGGCGTTACCCAACTTAATCGCCTTGCAGC
ACATCCCCCTTTCGCCAGCTGGCGTAATANCGAAGAGGCCCGCACCGATC
GCCCTTCCCAACAGNTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGG

NATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACT
CTCAGNACAATCTGCTCTGATGCCGCA**3'**

094:
5'CGCANCCACCTGACAGCATCATCCTGATGGATCGCTGGTACCCTTCTGA
TGCCGCGTTTCGTCGATTGATACCCTTTGCAGAGATTCTTGAGCTGAACAT
AGATCGACAGGTGCAAGTGCCGGATCTTCATGTGGGGGTTGTCACCGCGG
CGCAAATATCCTGGGCGCGGGCGGCAACACGACGGCGCGGGCTGAGCAG
TACGGTGATGTCTAAGCTGGAGGAGCAAGTTGTGTGTACCGAGGCGTTTG
AGCAAGCGGCTGTGGGGCAGGGGTGGGTGATGTGTCGCAATGAAGGGAC
GATTGAAGAGGCGATGAGGCGGGTGGTTTCGCAGATATATAAAGTCCTTT
GATCTAATTTGATCTGGACCAAGTGAGGTTCATCAATTTGAACTAATCACT
AAGCCTGCCCACCTAACGCTTTGCACGTTGCCTCAAGCCCAGGTTTTCCTG
GGCTACCCAATGACACACGAAGGTTTGGTTAATCATGAAGAGCGAAC**3'**

097:
5'TCATAGCTGGGCAACGGCGCATCCATCGGCAATTGCTGAAAAGCCAAC
AGCGCGTCGATGGCATCGGCAAATAATTGGTCGGCGTTTTCACCGTCAAT
CACGTCCAGATAGGTTTTTGTGCCCAGGTCATTGAGCAAAAGAAAGCCTC
GGGGCAAATCTTCTGCATAAATTTTTGGCACATTTATTCCCGATTTCGCGA
GCAAATGCGCGATATCGACGAAAGGTTTGCAGTTTTCCTGGGGGGGAGGC
GCGTCCATGACGACGAACGTATGGGCACCGCCCTCCCATCGGAAGTAACG
CCTGAAACTCGCGTCGCTG**3'**

101:
5'TGGATCGCTCCACCTGTGTAAATATCAAAACTCGCAATGTTGCTGAAAT
CATCGTTACCGGTAAAAAACATCAACCTGACACTTGCACCAACATACACA
TCGTCCATACGTATAACAATTTGATCCTGATCTGAATAAACCAGAAAAGT
TTCTAATTGGTTATTAAATGGCGCGGTGTACACGTTGTTTACGTTTGCACT
TCGGGCGATTCCTGGCGAAGTGATTATGTGTGCATTCTTAGCTAGCGTAA
AGTCATTGGCAGAGCTATAAATCGAGTCCCGGACCATGTGCCCCCATGCA
GCCAGCCCATATGGGTTCTTGTATGCATAGTCGGCGGGGAGCAGTGGCGT
GCTGCTTGGGAACTGAAAGGCGTTGAACGGCAGAGAGTCGCCAGTGATG
ACAACCTTGTAGCCCGAAGTGATCTTCGATGTTAGATCTTCCTTCTTTGTG
GCGCTTGGAACGTATGCCGAGAGGTTTTCACCGGCGCGAATCCAGGTGTT
TGCAGGCTCAGCTTCAAAGCTGCCTGTAATGGATTTCGGAAGAGTTGCAC
CGATGTATTTGTAAAGCGTTCCCCCGCTCAGCACCAGTTGGTCGTAAGTCG
TGATGCTGCCGGTACCCCAGGGCTTTGCAGCAGACGAGTATTCAAACCCA
ACAGGGAATGGGTCTGGATCGCGCTGTCCAGGCTGCCAAATACCGATTCC
ACCANGCTCGCCCCATCTGGCTNCGCAAGANCCGCACGCNAAGAAGCAT
CGCCGACAGCGGNGAGCTTTGGGCATNCGTANCCCATACTTCCGGNTACG
3'

102:

5'CTCGATCTCGTGTGGCTGAACGCCACTTGTCCCTCTAAGAAGTTGGACG
CCGACCGCCGGGGGTCGCGTAACTAGTTAGCATGGAGGAGTCTCGTTCGT
TATCGGAATTAACCAGACAAATCGCTCCACCAACTAAGAAC**3'**

104:
5'TTCGCGATGCTTTGTTTTAATTAAACAGTCGGATTCCCCTGGTCCGCACC
AGTTCTAAGTCAGCTGCTAGGCGCCGGGGCGGTGTGCTCTTGCTTACGCG
TGGACTACCTTTATGACCAACATCACTGTGCTCTTGCTTACGCGCCTCGAG
GAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG
TTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTA
ATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTG
AATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGT
ATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCA
TAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACG
GGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCG
GGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGA
CGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGA**3'**

105:
5'GCTTTGAAAAACTCCTGTCTAGTCTTAAGTGTTCGGAAAGCTTCAGCCA
ATCCAAAGTCTGAACCTATTGCCTCCACCAAATCGTTACAAAGAGGTTCA
TAAGCTTCCGGCTCGGGTAAAGCAAGATTGATAATTTCATCTTTTTTGTTT
TTTAAATTGGTACAAGCTTTGTCGTAAGTTACTTTGCTCTCGTCTTGCATG
AATTGCCAGAAGCGCTCAAGACGATCTTTTGCATGGTCGTCAAGTGGTTG
CATACAAAGGACACACAACGCCTCTTCGCTAGTGTTCGGAAATTCGCTTT
CGGTGTAGGCATGAGAGGTGGAGAAATCTTTGGCTGCGGTGAAAAGAATT
TTCCAAGGGTCCGAGTGAACTCCCTCTAACGGGTCTGCTTTGTCAAAAGTT
GTGAGTGAAAAGTCGTAAGCTTTTTTTGCGGTGACTTCAGTAACCTGTAA
GTTATCAATTTCATCAGGTTTTGTTTCGTTTAATATTTTTGCGGCAGCAGCT
AGTTTTTGGACTGCTCGGGGAAGCCGCTCCGTCGAGATCTTTGTTAATTTC
GCAATGGTTTTTACTGTAGTGCCATTTTCGTAGTCGGCAACGGTGCCTGTT
AAGTTTGTTAGGTCGCTTGAATCTTGCTCACTCCAAGTGAGAGTGAGTTTC
ACATCGGCACTATTGGTTTTGTCCGACATACGATTTAACCAGG**3'**

107:
5'GCCCAATGCCCCAGTAACCGCCGGTGCTGTGCTCTTGCTTACGCGTGGA
CTACCCACGGATACTCTTTCACCATTTCACCCAGTACGCCCTGAATTTCAG
ACATATGCGCACCGAACATGGTTTCTGCCATCCATGCGATACCGTACACC
GCCACGATGGCGATCATACCGGAACGGAAGACTTCGTTTTTTGAGATAGA
CGCGGGATTGGTTTTGGTCAGGATAATAATCAGCGCCCCGGTCAGCAGCA
TAAACATCTGAATAACCAGTACCATCGACAGCGGTTTGCCGCCGAAGGAT
GGACGCAGGTCCGAATCAGCACCAAGAAGGGCGACTACAGCGATTGCCC
CGAGGAAAATCCACATTGCCAGCCAGTTGCTTTTCGGCAGTTTTTTATCCA
GCAGCGTCGCGGTATCACCGTAAACATACTCACGGTTTTCCGGTACGGAG
ATGAATTTCTGGAACTCTTCGTCTTTATCCAGATCTTTACCGCGGAACCAG

CTGAAGATACCGATCGCCAGGATACCGATTAACGTCGATGGAATGGTGAT
TGCCAGCAGATCGAGGAACTCAAGATGGCGACCATCAAAGGTGACATTA
CCCAGCATCGCAACCAGAGACACGACCGCAACCGACACCGGACTGGCGA
TAATCCCCACCTGTGCACCGATAGAACTTGCCGCCATCGGACGTTCCGGA
C**3'**

108:
5'GGGACTTAATCAACGCGAGCTTATGACCCGCACTTACTGGGAATTCCTC
GTTCATGGGAAATAATTGCAATCCCCGATCCCTATCACGAACGGGGTTCA
GCGGGTTACCCGCACCTGTCGGCGCAGGGTAGACACACGCTGATCCGTTC
AGTGTAGCGCGCGTGCAGCCCCGGACATCTAAGGGCATCACAGACCTGTT
ATTGCTCGAT**3'**

110:
5'GGGACTTAATCAACGCGAGCTTATGACCCGCACTTACTGGGAATTCCTC
GTTCATGGGAAATAATTGCAATCCCCGATCCCTATCACGAACGGGGTTCA
GCGGGTTACCCGCACCTGTCGGCGCAGGGTAGACACACGCTGATCCGTTC
AGTGTAGCGCGCGTGCAGCCCCGGACATCTAAGGGCATCACAGACCTGTT
ATTGCTCGAT**3'**

112:
5'AGCTGTACGGTAGTGACAATACCGGCGGCGATCAGCAGTAAATTCAGC
GACATCGGGTTTTGCCCCATATGGCTGGTTGAGCTGTCGGCAATAGCAAA
CAGGTAAATTGCCGCCACGGGCAGCAGCCACATGGTTTCGATTAACATGC
CGGTTTGCGCTTCAACGGCAATCTTCTTGCGTACCAGACCGTAGAAGGCA
AAACTAAATGCCAGTCCCAGCGCGATAATAGGTAGCGAACCAAAAGTCC
ACAGCTGGACTAACACGCCACATATCGCCAGAATCACCGCCAGCCATTGC
ATCCGGCGGAATCGCTCGCCGAGGAAAATCATCCCCAGCACAATGTTCAC
CAGCGGGTTAATAAAGTAACCAAGGCTCGCTTCCAGCATATGGTGATTGT
TCA**3'**

113:
5'TGATCGCCGACGGCACCATCTACGGCGGCATGCTGCCCAAGATCCGCTG
CGCGCTGGAAGCAGTGCAAGGCGGCGTCGGCAGCTCGCTGATCCTGGAC
GGTCGGGTACCGAATGCAATCCTGTTGGAAATCTTCACCGATACCGGTGT
GGGTACGTTGATCAGCAACCGCAAGCGTCCTTAATCGCTGCAAACAAAAA
AGCCCCGCTCAATTCAATTGAGCGGGGCTTTTTTGCATAGAGCGTGAGGA
CAATGGAGATCCCCTGTGGGAGCGGCCTTG**3'**

114:
5'ATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAAT
CAACTAGCCCTGAAAATGGATGGCGCTGGAGCGTCGGGTGTGCTCTTGCT
TACGCGTGGACTAAGTTGCTGCGCCGCTTTGAGAAATCGACGAAGTCTTA
ATCAGTGTAATGTCGGATGCGTTTCGCTTATCTGACCTGGCATCGCGTGTC
CTCTTGCTTACGCGCCTCGAGGAATTCACTGGCCGTCGTTTTACAACGTCG

TGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATC
CCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCT
TCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTT
TCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAG
TACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCC**3’**

115:
5’AGCTCTGATAATACGCAAACTTACTCAACATATTGCAAAGCTGAAATCT
AAAAAGTTAGTTCATGCTTGTATATATCNGATACTAAAGAAAAAAAAGTA
CCAGGAATGCCCTGAATTGATAACATGTATCATGAGACCCAATATTACGT
TACAATTCTTCAAAACCAAAGCGATTGCTATTGACTATTACCCCTTGCATT
TGTAGATTTCTTATAACATATACATTCCTAAACTGATCCTCTGNTCCTTTCT
CTATTTCTATTTGTTGCAACATATTTCTCATTTTCCTGTTCTGGATTATTCA
CACATAGCCGTATTTACTTTTACAGGTTTTGTTCATAGGACTCAATACATA
TTATGTCAGTCTAGTATGCGTCATTCTTTCATTTAATTTTATCTGACAGNCT
ATATTTAATCTCACTATTTTGACTGGATTCTAAACGTGTTTTATCTTTTTTT
AAGTAACTCTGTAGTCAGTTACTTGAGCTGAAACCGGATTATAAAATTCA
CAATGCNAATTCATGAAGAAGAAAAAAAAAAACACTGTTAGAGTGCTGC
TGAACCTCTGGGCTATAACAGTAAGNTAATTATATTAAAAACTGCATTTCT
AGCTATGTTCTGTTTNGGGGGGTTNTAGTTCTCCCCTAAAACAATTCCACA
GGATTAAAAAATTACGTNAATAATTTTTCAGGGGTTCCTA**3’**

117:
5’GGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTGCAAATCGGTCGT
CCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGT
TCCCTCCGAAGTTTCCCTCAGGATAGCTGGCGCTCGTCCGTCGCAGTTTTA
TCCGGTAAAGCGAATGATTAGAGGTCTTG**3’**

118:
5’GACGCTCCGGCGCCATCCATTTTCAGGGCTAGTTGATTCGGCAGGTGAG
TTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGTAGTCCACGCGCCT
CGAGGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCT
GGCGTTACCCAACTTAATCGCCTTGCAGCACAT**3’**

119:
5’GAATTCTGCTTCACAATGATAGGAAGAGCCGACATCGAAGGATCAAAA
AGCGACGTCGCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGA
ATCGAACCCTGATTCCCCGTTACCCGTGGTCACCATGGTAGGCGCAGAAA
GTACCATCGAAAGTTGATAGGGCAGACATCCGAATGTATCGTCGCCGTCA
CGGGGACGTGCGATC**3’**

120:
5’CCAATCCTTCAAGCCTTGTCGTACCGCCGAGTACAACTGCATCTCGGCG
CGCTGTTTAAAGCCTCGATCAATCAGCGCCTGAGACAACCCGAAGGACGA
GCCAGGGCTCGGATAAAAAGCCCTACGTATATGCTTGCCGTATTCGCCCC

CCACATTCAGGGATGCGATCGTTTTGATCAGGTAGCTCACACTCAAGCGT
TCTTTCCAGACGGGTTCCAGCCAGCGCGCCCGGTTCAAGCGCCAGCGGGT
CCAGGGCGCTTCAGGGTCGAGGTTGTGCAACGCCAATTGCAGCATGCTGA
AGGTGGTACGTTCCTTACTGACCACCGTTTTAATCTGACGATCACCCACCG
CCCCCTGGCTCGTCCAGCCCTCGTAGTGAGTGCTGACATCATCGGGCATG
TCGAACAGGGGGGTATCGATATCCAATTGCGGGTAGAAACCGTCCTCGGT
CAATTTGGCAATCAGGGCTTTGCGAGCAAAGGGCTCCAAGTCAGGCAAGT
CCTGCCAGAGTTTGGTTTCCAGTGCAAAAATGCTGACCAGATAGCGGCGT
AACAAACGCTTATAGTGTTTGCGCTCAGCGGGCGATGCGATGCCGAGCCA
AGAAGGGAGTTTTT**3’**

121:
5’TGCTGTTCACATGGAACCCTTCTCCACTTCGGTAGTCCCGCGTAAGCAA
GAGCACAATTCCATATTTACATAATGAATTTATTGCACCAGCCCAAATTT
TCAGCTTGTCAATAACAGCAACGATCCAAACTTGTCACAGGGGGTCACCA
TCTTGGAAAGTGTCTGTGACACTCACATGCTCAGTGGGCTCTGATTGGCTG
TTGAGAAGCTAAGCTTAGGGCTCGTCACTAATTATCCAGCAGAAAATGAG
CTTCCCTGGCTGTAATATAAGCTGATGCTACAGGTTTGCTGATTATTAAAT
TCTGATGCTAATTGCACTGGTTTCTGTGCTGCCATGTAGTAATTATCTGTA
TTAATTACTAATCAGTCTTATATTGTGACATTTCTATTC**3’**

122:
5’GACGAAAGCCTGCTGCTGGACGCCCTGACCCAACTGGTGGTCAAGCAC
GGCATCCTGCGGGTCAAGGGCTTCGCGGCGATCCCGAACAAGCCGATGCG
CCTGTGCTCTTGCTTACGCGCCTCGAGGAATTCACTGGCCGTCGTTTTACA
ACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAG
CACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGAT
CGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCG
GTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCAC
TCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACC
CGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCC
GCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTT
TTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCC
TATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGT**3’**

123:
5’CNCGATNCGNGCCCAANCNCATGCNAAACTCGCTGTTGATCTTCNTCNC
GGNTGAAATGGNAATNNAATAATCTTCCATTACAAGATCNTAAGTCCCGN
CACCCCCTGGGCATTCCCTCTAGCAGGTCTTGCTTCACAACTTCGACAGCA
CCGGACAGTCCGGCTTCCTCAACACNCCTACGTGTGCTCTTGCTTACGCGC
C**3’**

124:
5’TACGTTCGTTAACTTTTCAGACGCGGGTCCAGCCATACCAACACAAGGG
TGGAGAGCGAACACATCAACAAAATGCCGACGAACCAAAGTAAATACCA

CATTGTTTCTGCTCCTTAGTACAACTCGTTTTCGTTACGGCGGAGAGTTTC
TGTTGTCATGCGCCCCCACATTTTGTAGTAGCTCCAGAGAGTGTAGAGCA
ACACAATGGGCAAAAATATCAGCACGATTACCAACATAATGCTCAGCGTC
AGCTGACTGGAAGTACTGTCCCACAACGTCAGGCTGGAGATCGGACTCAC
GCTTGACGGCATGACAAAGGGGAACAGCGTGATGCCTGCCGTGAAAATC
ACGCCAAATTGCACCAATGATGCCATCAAAAATCCCCAACCCGGGCGACC
ACGATAAATCGCCATCACCGTAAGCAATGGGCAGAAGAATCCCAGCAGC
GGGAAGATCCAC**3'**

126:
5'GCACGTTCGTGGGGAACCTGGTGCTAAATCATTCGTAGACGACCTGATT
CTGGGTCAGGGTTTCGTGCGTAGCAGAGCAGCTACCTCGCTGCGATCTAT
TGAAAGTCATTAGTCCACGC**3'**

128:
5'TCGGACCCACGCCTCGCGTTTTGAAAGCAACATGCTTGGTCTATTCTCGC
ATCGCAGATACGGCACCTGTATACGTCTATGTAGCCAATGACGGACCTAA
GCCTCATGTAATGTCACAAATCTCTTGCTCACCACCCAACACAGCCTACA
CCATTACGCCGAATCCAAACGCCCCCAAAAAGGGACAAATCATGGAACA
CAGAAGGAAGGACGTTGAACGCGGGGGTGTGTGTAGGAGCAGAGGTCAG
AAAGCAATGACACTGTGCTAGTGGCTTGTCAGCAGCCCAGGTCGTTTTTT
GGAGCCGATGGAATCCACCAGTCCG**3'**

130:
5'GCCGTGGACCGTGCCGTGCGCCGCAAGGAAGCCGTGGTGTTCTTTGGTT
GGGCGCCGCACCCGATGAACGTCAACGTCGAGATGACTTACCTCACCGGC
AGCGAAGACGCCCTGTTCTCTTGCTTACGCGTGGACTACCGACTGCACGC
GGGTGTTGAAGCTGATGTCACGGCTCAAGTCCAGCTTGCGGTCCACGAAG
TGGAAATATTCGCGCATCTGCCCCCAGTCGGGGAAGAGCTCTTTCCAGTT
GTACTCCTGCCACAGTTCGGGGATCGAGTACTGATAGATTTGGCAGTGCG
TATCCACCCGGGCGCCGGGATAGCAGTTCCAGTGCCAGATTCCGCCAACA
TCGCTACCGGCATCCAACAGATGCACTTTATAGCCGCGTTTGCGGAGGTG
ATAGAGTTGGTACAGACCTGAGAAGCCTGCGCCGATGAGCAGGACGTCG
AGCTTCTCGTTGACCGAGTTGTTCATGCCTTTGCTGTTCATGTCGCGTTTCC
CCTTGAGGGTTATAGTTGGAAGGGTAGCATTGTTTTCCGCCGTCTCGACGA
TGCGGACTCACTGTCGATGATCGCGTCTCGATAAGCCGCGTACGCTTAT**3'**

132:
5'GGGTCTGGGCATGCATTTGCTGCCAGCCACAAAAGCCATTCCTAAAGAG
ATGCTACCGATCGTCGACAAGCCGATGATTCAATACATCGTCGATGAGAT
TGTTGCTGCAGGTATCAAAGAAATCGTCCTGGTTACACATTCTTCCAAGA
ATGCAGTAGAGAACCACTTCGACACCTCTTATGAACTCGAAGCGTTGCTT
GAGCAGCGCGTAAAGCGTCAGCTGCTGGCGGAAGTTCAGTCTATCTGTCC
TCCTGGCGTGACCATCATGAACGTGCGTCAGGCGCAGCCGCTGGGTCTGG

GGCACTCCATTTTGTGCGCTCGCCCGATTGTCGGTGACAATCCGTTTATCG
TGGTACTGCCGGATATCATTATCGATAGTGCTTCTGCAGATCCGCTGCGT**3'**

136:
5'TGGTCTTCGGCGGAAGTGTTTTCGTGTTCGGTAGCCATTACATGTCCTCG
GCGCAGCAAACAATCATGAATTAGGGTTGAATCAGCGGCGGTTGATCGGC
TCAACCACTTGCAGCGCCAGGTTGCCCTTGTGGGAACCGAGCTTGACCTT
GAATGACGGCACGCCATTGGCGCGCATGATCAACTCTTCCGGCAAGTCGA
CGGGGATGATGTCCCCAGGCGCCATGTGCAAAATGTCCCGCAGGCGCAAC
TGGCGGCGGGTAGTCCACGCGTAAGCAAGAGAACATATTCAAACGAGAA
CTTTGAAGGTAGTCCACGCGTAAGCAAGAGCACACGCACCCGACTCTGCA
CGAGTCTGTGGTTAGTCCACGCGTAAGCAAGAGCACAGACAATGAATCAT
TGATATCGCGGTCTGGCGTAGTACCGCGTCAAGCTCGCTGCACAACACGA
CTGCACGTACCTTTGCGCTGTTGCTGGCATGACGCAGGAAAGCCAGCCCA
TCCATTCCTGACATTTTCAGATCGCAAAGAAGAATATCCACATGAGTGCT
GGCAGCCAGGATAGCCACGGCTTCGGCGCCATCCGCCGCTTGAAGAACCG
G**3'**

139:
5'TCATGTTACACAATACATGTATGTTTTGTTTGATAAAGTTCATATTTATC
CAAATATGAGTTTACATTGGCGCGTTACATTCACTAGTTCCAAAAACAAC
CCGTGATTTTGCATCGCCACATCATTCAACAGAAATACTCATCATAATTGT
AGATGATAATACAAGTTATACACATGGAATTATAGATATACCTCTCCTTA
ATGCAACCGCTGNGTCTGATTTCAAAAATACTTTACAGAATAAGCCAGCC
ATGCAATAATCTGAGACGGCGCTCAGAAATATTTGTCACNATAGCCGCCA
TCTTGACGTCAACATAAACAAGAAATGACATGATAAATATTCCCTTACCT
TTGATGATCTATATCAGAAAGCACTCCAGGAATCCCAGGTCCACAATACA
TGTTTGTTTTGTTCGATAATGTCCAAATACCTTATTTTGTTAGCGCGTTTGA
TATACATATCCAAACGCTCATTCTGGTCAGCGTTATATCCGACAAAAACG
TTAAAAAGTTATATTACAAGAAACATTTCNAACTAAGTACAGAATCAATC
ATTAGGATGTTTTTAACATATAGCTTCAATAAAGTTCCAACCCGAGTTCTC
CCTTCTTACCATGAAGAATAAGCATGGATCAAGAAAATGGCTGGACT**3'**

142:
5'GGGTGAAGAAGGGCGACAAGATTTTGGTCAAGTCCACCGGCAAGATCC
ACCTGGTGGACAGCGTCGGTGTATTCAACCCGAAACACACCGCCACCGTT
GACCTGAAAGCCGGTGAAGTAGGCTTCATCATCGCCGGTGTCAAGGACAT
CCATGGTGCGCCGGTCGGTGACACCCTGACCTTGAGCTCCACCCCTGACG
TCGATGTGCTGCCGGGTTTCAAGCGCATCCAGCCTCAGGTCTATGCCGGT
CTGTTCCCGGTCAGCTCCGATGACTTCGAAGATTTCCGCGAAGCCCTGCA
AAAACTGACCCTCAACGACTCGTCGTTGCAGTACACCCCGGAAAGCTCCG
ACGCCCTGGGCTTCGGCTTCCGCTGCGGGTTCCTGGGCATGCTGCACATG
GAAATCATCCAGGAGCGCCTTGAGCGCGAATACGACCTGGACCTGATCAC
CACTGCACCGACCGTTATTTTTGAGCTGGCGCTGAAAACCGGTGAAACGA
TTTACGTCGACAACCCGTCCAAGCTCCCAGACCTGTCTTCCATTGAAGAC

ATGCGCGAGCCCATCGTGCGCGCCAATATCCTTGTGCCGCAGGAACACCT
GG**3'**

143:
5'TATATTTTTAGAGAGACCTACTTGTTTGAGGGGTATAGTTTTCCTTTAAG
TCTACTAGAAAATCATGTAAACATTAAATAAACCCAATAGGCTGGTTTTG
CTTCCAATAAGGGTGAATTATAACTTAGTTTGGATCAAGTACAAGGTATT
GTTTCATTATAACAGAGAGAAAGGGAATTGTTTTAAAAATGAGTCTATG
GGGAATATTTATCAAAGAGTGAAGTTAGAGATCACCACAG**3'**

144:
5'TCGATCAGAAGGACTTGTGCTCTTGCTTACGCGTGGACTAGTACTCAGT
ATTCGTGTCGTGTATAAACTTTAGTCTATGTATATTGCCTCGTAAACCAAG
AACCCAAGCCTACAAAAGTGAGTAGGGGTACTATCGTAAAGGATATATA
ATCCATTTATTTTTTAAAAACAACAGTCATTTTCGAGAAACATAAATGCGC
TTATAAATTGGAAAGACATAAAGAAAATAAAACAAAAATAAACATTTGTT
CTCTTCCACTGTCTTGTCTAATGTCTATAGAGTAAAGAGATAGAGACGAC
GTTGCAGTATCTCTCTCTCTCACACGATCAAGAAAACTCTCACGTTCACTG
GAGAAAACTATTATTCCGGTAAGATCTATTCAAATGGAGGATTCTAGAGC
TTTTCCTTTCGCCGGGAATCTAGACCCTCGAGCTCAAGCGTTCGTACCACT
AAACCCTATGTCTTCACGTTTTTACTTTCCGTACACTTCCCTGCCACCGCC
GCTTCTGCCACCTCCTCCGTCGGATCCAAGGATGTTCACGTTCTTTAATAT
CCCACCACATCCGATGAT**3'**

146:
5'AATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCTA
CACTGAACGGATTTNCGTGTGTCTACCCTGCNCCGACAGGTGCGGGTAAC
CCGCTGAACCCCGTTCGNGATAGGGATCGGGGATTGCAATTATTTCCCAT
GAA**3'**

148:
5'ACCTGCGTTCTCATCAGNACCTGGTGGCTTCGGTGTGGATGGGGCTGGG
TATCGCGTTNATGCACTACGTGGGCATGTCAGCCATGCGTTCCAGGTAGT
CCACGCGCCTCGAGGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTG
GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACAT**3'**

153:
5'GGGGTTTTCTGAATGCTGCTGCGAAAGCCGACCATGCCGAAGCTGACGC
TGTCCAACTCGCCGCGCTCGGCAATGCGGGTTTGCAGCTCATGTGCTCTTG
CTTACGCGTGGACTAACTGAGCAGGATTACTATTGCGACAACACATCATC
AGTAGGGTAAAACTAACCTGTCTCACGACGAGAGGAACCGCAGGTTCAG
ACATTTGGTGTATGTGCTCTTGCTTACGCGCCTCGAGGAATTCACTGGCCG
TCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATC
GCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGT**3'**

161:
5’CGAGGCGCGTAAGCAAGAGCACATACACCAAATGTCTGAACCTGCGTA
GTCCACGCGAAGCAAGAGCACACCGCCACAAGCCAGTTATCCCTGTGGTA
ACTTTTCTGACACCTCCTGCTTAGTCCACGCGTAAGCAAGAGCACAGCAT
GGCGGTGCCGGGGGCGATGCCCACCAGCGCGGCGAACTCGTCCGAGGCA
TTGATCGCCTGGATGTGCTGCAAGGCGCGCACGATGGGCTTGCCGATGCT
TTCCAGGTATTCGTACAACGAATTGCCGATGGTAGTCCACGCGTAAGCAA
GAGCACATACACCAAATGTCTGAACCTGCGGTTCCTCTCGTAG**3’**

164:
5’AAATTTTTCAGACAAGCGGAAAAAAAAAGGATAAATAAATTTAAATCA
GCCCAATAGGAGCAGCGCAGCTCACATTGACTTCTAAAGGACCTCCATAA
CTTTTACTTGATAACATTTTGTATTAGAGGTTTTCATGGTTTTTGCACTTAG
GGGGTTATCTATTAAAGTCAGAATGCCAAAAACGTGAAAAATCTTAATTT
TACTATAAAATCTGAATTTTTAGTGGAAAAAAAAACCCTTGAATTTTTTT
GATGATTTTGATTTATTATAACCTGAATCTGAAAAAAAAATCTCCAAGTG
GTCGGGGTCCTGTAGAAGTCAATGGCAAAGGTCCAATTTCAAATTTGACG
ATATCCTGGTCTGCGCAGAGTTTTGGACAAAAATTTCAAAAAAAAAAAAC
GGGGGTTTCGATCGATATCGCATTTGCAGGCATAAAACTCCAAAATTTGG
AAAAACGCTAATTTTTAGAGTTTTTTTCCAAACCGATTTTATCAAGTTTTTT
CAATCATAAATAAGGTCAA**3’**

168:
5’GCGCCTCGAGGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGA
AAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGC
CAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT
TGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGC
ATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCT
CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGAC
GCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGT
GACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGA
AACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAAT
GTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAA
TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA**3**
’

170:
5’GGAGACTATAACTCAGGATTAATAGGTCATTTTAAACCTTTAAGTCACG
CAATGTTTTCGCAGCATGAGATGGTGGAGGAAATCGGTTCAATGTTCGAG
TTGGACCTGTGGGGGGGCAATTCCCTTGATGCGTCTCAATGGGAAGCCTA
CTGTAGGATTGTGCTGATGACGTACAAGGACAAGGTTAACAGCGACTTCT
GGGGTAGTTCAAGATCTCTTTGGCATGCCTTTGATAATATTGAGAGCATG
GTTAGCGACTTATATAGACTTAACGGAGTTGTCGATTATTCAATAAATGAT
GATTCAATAAAAAAATTGCGCGTTGCAGTGAGTTTTATACAGAGCATTAT
AGCCGCATTAGAAACTCTTGGTGTTCCTAAGCATTTGACACTTAAGAAAG

ACGGCGTAGTTGGAAATCACTACGATCAAATTGCAAATTTAATTTTTGAG
ATTATTCAAAGGGCTGCTGCAATCAAGGAGCCTTGGGCGCTGGCATGGTG
GATACACCACAACACATTATGGGGAGAAATATTCGGCGCATTTTCTTCTA
AAGGACCGGCGGCTAAGATTATTATGTTTAAGTTTAGACGCCTTGTCTATG
AAGAGTTCAAAAGAATGGAAGAATTTCCAAATTTCAAAGGAGCGAAATT
CGCAGGGT**3’**

171:
5’CNCTAGAGGATCCCTCGAGGCGCGTAAGCAAGAGCACATAGTCCACGC
GTAAGCAAGAGCACACTCCTTAGCGGATTCCGACTTCCATGGTAGTCCAC
GCGCCTCGAGGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA
AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCC
AGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTT
GCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGC
ATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCT
CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGAC
3’

172:
5’TGCGTTTGGCAACCACCACTTCACCGTCACCCACTGAAAGCACTGGCGT
ACCCTGCGGCATGGCGAAATCAACACCTCTGTGTGGTGCAACGCGACCGG
TCACCGGATTAGTACGACGCGGGTTAAAATTAGACGAGATACGGAACTGT
TTCGCCGTCGGGAATCGCAAGAATCCTTTCGCCAGACCAGTACCGTTACG
ATCGTAGAATTTGCCATCTTCAGCGCGGATAGCGTAATAATCTTTACCTTC
TGAACGCAAACGTACGCCCAGCAGCTGGCTTTGCTCACGTTTACCATCAA
GCATTTCTCGAGACATTAACACCGCAAATTCATCGCCTTTTTTCAGTTTGC
GGAAATCCATTTGCCACTGCATGGCTTTAATCACTGCGCTCACTTCGGCGC
TGGTTAAACCGGCGTTTCTGGCGCTGGCAACAAAGCTTCCCCCGACGGTA
CCTTTCAGCAGATTGTTAACCCACTCTCCTTGCTGCATTTCGCTGGTCATTT
TAAAACCGTTAGCGGCAGTACGGTCATAGGTTCGGGTTTCACGACGAGAC
ACTTCCCAGGTGAGGCGCTGCAGTTCGCCGTCCGCGGTTAATGTCCAGGA
GAG**3’**

176:
5’TGCAAAGAGTGTACCACTGTAGCTAGATGCAGAAGCTGGAATAATGTTA
ACTGTACTAGCCACTTTACTATGATGTTTATTGGTTTTGTTAGTGCTTGGTT
CAAAGTTCCATAGGCTTTGAGTCATCTGCCCCCAAATCTGTTTTTTCCACA
AGCCCTGATATTTTTAGCATACAATTTTGTAGAACTATGGTTGTAGCTAGA
ACGCCTGTATTTTATTTTCACACAGCAGCCAAAGCACCTGTGTGACCCAG
AGAAGGGTAGTGTCCTGGAGAGGGGTCTTAGAAGGTTGTTAGAAGGAATT
GTAATTGGGTTTGCAGCGGCATCTAGAGGAAATGGCTCCTGATGTGATCT
GTTTCTTGGCATTAAAGGGTCTCTGGCTTGGAGAAAGGCAATGCATCAAG
CTATAGTAATTTAATGGGAAAGGAGAGAAAAAAAACAGTAAAGAATATT
TAGTTCCCTCATTTCTAATTATGTGTTCCTTCTGTAATCTTCTAATCCAGGG
TTGGTATATAGAATTCCTCTCAAATGCCAACTCCAATTGACTGGTGGTGGC

TGCCTGAGGCACGGAAGAGAGAAGGATGCTGAGACCCTGTTGGGCTTCCT
GGAAAGAATGCCACAATTGATTAGTGATGTCTGTCAGGAGTTGCTGCACC
TGTGTCACCCCCATAA**3'**

177:
5'CATGCGCTGAACATGTTTCCTCAAAAAGTGCTGCCGGATCATCTCCAGT
GATGGTGAACGCACTTTCATAAATCAAGATCGGCAGGAGAGGGTTATTCG
GCACCCAGTCATTTGATTTGAGCATCAATGTCTTCAGGTTACTTACCAACT
GGCGAGTATCTTTCATCTAAGCATCTCCCGCATGGGCAATCACATCGCTTG
AATGTAGGACGGATAGCAGGATCCCATATTCCTACATGGGGTTGAGCCAA
GGTGAACATTCATTCCCCACTCTAGAGCGGGTTAGGGTCGCCCCAGATAA
TTTAAACCTTCTGCATGAGCGCAGTGTCGATTGCTGAAGGCTCTGTGCCAG
GCTGAGGGAATGTCAATGATAGGGATCGTGATACCTGCCCATAACGAAGA
AAGGCTTATAGGCGATTGCCTGAACGCCGTGGTGATTGCCGCTGAGCATC
CTTCTCTTCAAGGGCAACCGGTCGAGATACTCGTCGTATTGGATCATTGCT
CCGACAACACGGGAGCAGTGGTAACAGCGAAGGGGATAACGTCGGTAGA
TGTCTGCTTTCGAAACGTGGGTAAGGCAAGANCCGTTGGGGCTGAGCAGT
TACTTAAAGCAGGTGCTCGGTGGCTCGCATTCACGGA**3'**

178:
5'GGCTTGCCCGCCGACCAGCGTGACGAGGTCATGAAACGCGGATCGCGG
CTGGATGAAAGCGCGCCGGGTTCGGGGCTGTTCTCTTGCTTACGGCGTGG
ACTATGTGCTCTTGCTTACGCGTGGACTACCGGATTCCACCAGGGGCGGG
CTGTCGAGGTCGACGCTCAAGCGCTCGGTGTGCTCTTGCTTACGCGCCTCG
A**3'**

181:
5'TTACACCAATAAAGATCTGGTTAAATACGTTGATATTGGTGCGACCTAC
TACTTCAACAAAAACATGTCCACCTATGTTGATTACAAAATCAACCTGCT
GGACAACGACGATGACTTCTACGAAGCAAACGGCATCGCTACCGATGAT
ATCGTTGCTGTGGGCTTAGTCTACCAGTTCTAATCAGTCAACTCGCTGCGG
CGTGTTACTGTAAAACTGAACACTAGCCTGAGTTCGTCCCTCATCGAACTC
AGGCTTTTTTATTGGCATCTCATCTCCATCTGCGGCACACGCAATTGCCGA
AGCATATCGATACAGCACCTTACCCTGCCCCTCCTGGCACAATAACATCA
ACCAGCTACTCTTCAAGCTAACACCCAAATGCATTTCTTTATGAATTAATC
CGTAACAAACACGCTTCCCATATAGAAATATTTGCAAAAATAGTGCATGA
AATAAGCTTTTTCTCTACCAATCCGGGATATAAATATTCATCAGCACC**3'**

183:
5'AGTTTGCCCATCGAATTTGCGCGAGTCGGTGATGGTGGAGAGCTCGCAA
CGGCAATCTGCGCGAATGCGCATCCATGCGCTGTTCTATGGGCAGTGTTG
AATGTAAAAAACCTGGAAGAGGCATGCGAGATAGTCCACGCGTAAGCAA
GAGCACAGTTTTGTCCGTCACGCCCCAGAACATCCGTGCAACGCTGCTGC
TGCGCCAGGAGATCGCGCTGCTCGACGTCAGGCACGAGGCGGAATTCGCC
ACC3'

184:
5’TCCCGAGCCATTGGCTGCCAGTCCAATACTGTTTAGAGGTATAAAGGTA
TTTTCTGTCAGGGCTGAAGTTTCCAGCGGAGTTACTGGTGCTGGTATGGGG
GACTGCAATGCTAGCAATGACTTCATCGCTATTTGTGTTAACCACCAAAA
GGCTGTCCTGCCCCGTTACGCATATTCTCTGTCCGTCTGGTGAGCACGTAA
TAGCGGTCTGTGCAACGCCAATGATTATTTCTTTCATGGCACCAGTGGTTA
AGTCAACTTTTACCAGTCGATTGATATTTGTCACGCAAACGTAGGCATAC
AATCCGTTCGGCGTGATAGCAATGCTGCCCGGACTCCCTCTGAAATTAAA
CACCGACCCCGCTGGTAACAACGACTTGACATTAACTTCGCTCCTGGCGG
AATAACCACCACTGTTTGCCCTCAACTCATAAGCCCCCACTTCCCCGGCGC
TGCGAATGCCCTCAACCGTCAACGCTCCATGCTCATCCGAAAGAAACGCC
TTGGTCCCGGTCGTCCCATCGTCAAAGGTAAAACC**3’**

185:
5’AGTCAGGAAGTGCGTGGGCGTCGCCAGAGCAGCAGAGAGCAGAAGGA
AGACGGAGGAGCTGGTAAGTTGCTTCTTTTTGCGATTTTAGGTCGATCCTG
CAACAATCCTGTGCTCTTGCTTACGCGCCT**3’**

186:
5’ACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGC
GAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTC
ACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTT
AAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTT
GTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGC
TGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAA
GGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGT
TTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTA
TTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
AACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATT
CAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTT**3’**