THE UNIVERSITY OF CALGARY

Post-Transcriptional Regulation by Non-Coding Sequences of the c-myc Transcript in *Xenopus laevis*

by

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A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JANUARY, 1997

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ABSTRACT

Expression of messenger RNA (mRNA) may be profoundly influenced by the untranslated sequences at the 5' and 3' ends. For example, mammalian oncogene mRNAs may contain long G/C-rich 5' UTRs that fold into secondary structures with the potential to impede translation. In addition, elements in the 3' UTR can control translation, RNA stability and cytoplasmic polyadenylation. Previous evidence shows that both the 5' and 3' UTRs of the c-myc messengers may be important to the regulation of expression. I have examined the effects of different first exons of c-myc and the 3' UTR of Xenopus c-mycI on the expression of a chloramphenicol acetyltransferase (CAT) reporter.

In vitro-transcribed mRNA was microinjected into Xenopus oocytes or early embryos. RNA stability, cytoplasmic polyadenylation and translation were monitored by measuring mRNA recovery and CAT activity. Similar to the murine c-myc 5' first exon, the human c-myc 5' first exon inhibited reporter translation in both Xenopus oocytes and early embryos. However, unlike their mammalian counterparts, the Xenopus c-mycI 5' non-coding elements that were initiated at either of the two promoters (P1 or P2) did not impede translation. I conclude that the previously-reported inhibition of translation by the Xenopus c-mycI 5' UTR was due to the inclusion of non-transcribed and intronic sequences. This also implies that the 5' UTRs of c-myc are not conserved between Xenopus and mammals and that secondary structure may have recently evolved as a mechanism for translational regulation, possibly linked to the advent of homeothermy.

In contrast, the Xenopus c-mycI 3' UTR enhanced CAT activity in Xenopus embryos, independent of the 5' UTR. This enhanced expression was not due to increased stability over the control mRNA. However, RNase H-mediated degradation indicates that the Xenopus laevis c-mycI 3' UTR can induce the cytoplasmic polyadenylation of CAT mRNA. This result suggests that the enhancement of translation caused by the c-mycI 3' UTR may be a consequence of post-fertilization cytoplasmic polyadenylation.
ACKNOWLEDGEMENTS

I would like to thank Dr. L. Browder for his guidance, advice and understanding throughout the course of my degree and in the editing of this dissertation. I believe that my experiences under the supervision of Dr. Browder have prepared me for a future career in research and teaching. I could not have learned from a better teacher.

I would also like to thank the members of my committee, Dr. R. Johnston, Dr. S. Lees-Miller and Dr. G. Schultz for their advice and assistance through the last five years. Many of their suggestions and constructive criticisms were very valuable to the progress of my research.

Dr. L. Gedamu and Dr. M. Lohka were also remarkably helpful to my research and to my education thanks to their willingness to allow me access to the equipment in their labs and to their ideas and advice.

I would also like to thank Dr. C. Spencer and Dr. P. Mains for agreeing to serve as my external and additional examiners on my examination committee.

Finally, I would like to thank all of the others in the Departments of Biological Sciences and Medical Biochemistry for their advice and support, especially Ms. Jillian Wilkes-Johnston, Mr. Tod Strugnell, Ms. Lauri Lintott, Ms. Lauryl Nutter, Ms. DeeAnn Warren and Dr. Cheryl Wellington. I would also like to thank Dr. Michael Carpenter for his excellent technical advice.

I would also like to express my gratitude to the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research for financial support during the course of my degree. In addition, I would like to thank the Faculty of Graduate Studies, the Department of Biological Sciences, the Graduate Students Society and the International Society for Developmental Biology for additional financial support in terms of other scholarships and awards to assist in my research and attendance at conferences during my degree.
DEDICATION

To Geoff, for everything.
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<tbody>
<tr>
<td>15-LOX</td>
<td>15-lipoxygenase</td>
</tr>
<tr>
<td>4EBP</td>
<td>eIF4E binding protein, also known as PHAS-1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AURE</td>
<td>AU-rich element</td>
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<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CF</td>
<td>cleavage factor</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CLP</td>
<td>Clipper</td>
</tr>
<tr>
<td>CPE</td>
<td>cytoplasmic polyadenylation element</td>
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<tr>
<td>CPSF</td>
<td>cleavage-polyadenylation specificity factor</td>
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<td>CTP</td>
<td>cytosine triphosphate</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dsRAD</td>
<td>dsRNA adenosine deaminase</td>
</tr>
<tr>
<td>dsRBP</td>
<td>dsRNA binding protein</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diaminetetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>eEF</td>
<td>eukaryotic elongation factor</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>ERCC</td>
<td>excision repair cross-complement rodent repair deficiency</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GVBD</td>
<td>germinal vesicle breakdown</td>
</tr>
<tr>
<td>hGH</td>
<td>human growth hormone</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>hp</td>
<td>hairpin</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IP</td>
<td>imaging plate</td>
</tr>
<tr>
<td>IRE</td>
<td>iron-responsive element</td>
</tr>
<tr>
<td>IRE-BP</td>
<td>IRE binding protein</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosomal entry site</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>m7G(5')ppp(5')N</td>
<td>5' cap structure. 7-methylguanosine joined to RNA by a 5'-5' linkage bridged by three phosphate groups.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
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<tr>
<td>m\textsuperscript{7}GTP</td>
<td>cap analog</td>
</tr>
<tr>
<td>MBT</td>
<td>mid-blastula transition</td>
</tr>
<tr>
<td>MMR</td>
<td>modified Marc’s Ringer’s</td>
</tr>
<tr>
<td>MPF</td>
<td>maturation promoting factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NPE</td>
<td>nuclear polyadenylation element</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P1</td>
<td>promoter 1</td>
</tr>
<tr>
<td>P2</td>
<td>promoter 2</td>
</tr>
<tr>
<td>PABP</td>
<td>poly(A) binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAN</td>
<td>poly(A) nuclease</td>
</tr>
<tr>
<td>PAP</td>
<td>poly(A) polymerase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PF</td>
<td>polyadenylation factor</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-piperazine-diethanesulfonic acid</td>
</tr>
<tr>
<td>PTB</td>
<td>pyrimidine tract binding protein</td>
</tr>
<tr>
<td>REF</td>
<td>rat embryo fibroblast</td>
</tr>
<tr>
<td>RLP</td>
<td>ribosomal landing pad</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSL</td>
<td>suppresser of stem loop</td>
</tr>
<tr>
<td>S\textsubscript{SOD-1}</td>
<td>somatic cell SOD</td>
</tr>
<tr>
<td>TAR</td>
<td>trans-acting responsive</td>
</tr>
<tr>
<td>TAT</td>
<td>trans-activator</td>
</tr>
<tr>
<td>tCK</td>
<td>trout creatine kinase</td>
</tr>
<tr>
<td>TFIID</td>
<td>transcription factor IID; also known as TATA-binding protein</td>
</tr>
<tr>
<td>Tfr</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>TIE</td>
<td>translational inhibitory element</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>tRNA\textsubscript{Met}</td>
<td>initiator methionyl tRNA</td>
</tr>
<tr>
<td>T\textsubscript{SOD-1}</td>
<td>testis-specific SOD</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
</tbody>
</table>
UTR  untranslated region
UV   ultraviolet
xbra  Xenopus brachyury
XDD  Xenopus c-myc1 Dpn I restriction fragment
XFGFR  Xenopus FGF receptor
xg   number of times gravity
INTRODUCTION

The primary control over gene expression occurs at the level of transcription. However, many post-transcriptional mechanisms also regulate the expression of mRNA. Model systems in which transcription does not occur are often used to gain an understanding of the processes involved in post-transcriptional regulation. Examples of such systems are reticulocytes, which have no nucleus but continue to synthesize protein actively, or early embryos, which divide rapidly and in which transcription is excluded. The use of these systems has fostered an understanding of the regulation of translation and mRNA stability.

In 1936, E.B. Harvey discovered that an enucleated, parthenogenetically-activated, sea urchin egg could initiate cell division before becoming developmentally arrested (Harvey, 1936). Later studies confirmed this observation by showing that *Rana* eggs that are enucleated and fertilized with ultraviolet (UV)-irradiated sperm could also divide several times before stalling (Briggs *et al*., 1951). Later it was found that even by preventing transcription with Actinomycin D, early development could still proceed, demonstrating that early development does not require transcription (Gross *et al*., 1964). However, inhibition of protein synthesis is lethal, causing inhibition of cleavage before the first cell division (Hultin, 1961; Ecker and Smith, 1971). These experiments indicated that there must be a store of mRNA in the egg that can be used by the early embryo during the initial stages of development. This store of mRNA has come to be known as maternal mRNA (Richter, 1993). The concept of maternal mRNA was a key discovery in the modern study of translational regulation. It led to the idea that some system of regulation must exist to ensure correct expression of maternal mRNA during development.

In *Xenopus laevis*, during oocyte growth, the cell rapidly accumulates mRNA for use after fertilization. When fully grown, the *Xenopus* oocyte is competent to undergo a process called maturation. During maturation, the oocyte completes meiosis I and arrests
at metaphase II as a mature egg that is ready to be fertilized. Transcription appears to stop upon germinal vesicle breakdown during maturation (Davidson et al., 1964; reviewed in Browder et al., 1991).

Upon fertilization, there is a rapid cascade of physical changes in the egg and molecular changes in the cytoplasm that allow the zygote to proceed through early cleavage. During early cleavage, the cell must function solely on proteins made either during oocyte growth or from the stockpile of maternal mRNA. In Xenopus, embryonic transcription does not resume until several hours post-fertilization, at the mid-blastula transition (MBT; Newport and Kirschner, 1982a)

During development, despite the presence of excess polysomal components, the Xenopus oocyte has a translational capacity of about 2 ng of RNA. However, the oocyte is estimated to contain at least 50 ng of poly(A)+-containing mRNA (Rosbash and Ford, 1974; reviewed in Richter, 1993). This indicates that maternal mRNA is stored for use during early embryogenesis.

Studies have been conducted into how specific mRNAs are selected for translation at specific times (Richter and Smith, 1984; Richter et al., 1984; Smith et al., 1984; reviewed in Curtis et al., 1995). One such mechanism utilizes proteins that mask the maternal mRNA. Masking was discovered by comparing the proteins made in an oocyte to those translated in a reticulocyte lysate from oocyte RNA. No differences were noted, except when the mRNA was deproteinized before being added to the reticulocyte lysate. Upon deproteinization, additional proteins were made that are not seen in oocytes or in a lysate using native oocyte mRNA. Readdition of these proteins to the pure, deproteinized RNA also restored the translational inhibition (Richter and Smith, 1984; Richter et al., 1984; Smith et al., 1984). Additional mechanisms for RNA selection include the regulation of mRNA cytoplasmic adenylation and deadenylation during different stages of development, the specific and non-specific degradation of mRNA at
different points in development and the localization of mRNA in the cell. All of these mechanisms can dramatically influence the ability of a messenger to be expressed during development. The majority of these mechanisms affect the translation of the mRNA. By studying mechanisms regulating the developmental expression of mRNA we can gain insight into both general translational regulation and the regulation of aberrant mRNA expression.

1. Translation

There are three steps in the translation of mRNA: initiation, elongation and termination. Although regulation can occur at all of these steps, initiation has been the main focus of study because it is the rate-controlling step (De Benedetti et al., 1991; reviewed in Merrick and Hershey, 1996). Translation initiation can occur by two mechanisms: the "Scanning Model of Initiation" (reviewed in Kozak 1989a) and "Internal Initiation" (Pelletier and Sonenberg, 1988). Although the scanning model seems to be accurate for the majority of cellular messengers, there are several viral messengers and some eukaryotic messengers that do not behave by the rules of the scanning model (Pelletier and Sonenberg, 1988; Timmer et al., 1993). It was these exceptions that led to the proposal of the internal initiation model. In this section, I shall discuss the current models of translation initiation, elongation and termination.

1.1. Scanning model of translation

The basis of the scanning model is that the 40S ribosomal subunit and the eukaryotic initiation factors (eIFs) bind at the extreme 5' end of the molecule at a structure called the cap (see Section 2.2 for a detailed description). The eIFs and the 40S subunit then proceed along the mRNA in the 5' to 3' direction in an effort to "scan" for the initiation site, or AUG codon. At the AUG codon, the process of elongation of the protein begins. Termination is the final stage in translation; termination occurs when the correct termination codon is found.
1.1.1. Initiation

I shall review all of the steps in the sequence of initiation as they are currently thought to occur (Figures 1A and 1B). However, most of the studies that have elucidated the steps of initiation have been biochemical studies with little in vivo confirmation (reviewed by Merrick and Hershey, 1996). Initiation by the scanning model is thought to proceed by the formation of the ternary complex and recycling of the eIF2 subunit, binding of the mRNA by the 43S pre-initiation complex, scanning to locate the AUG and finally recruitment of the 60S subunit.

The 80S ribosomal subunits are in excess in the cell and must be dissociated into their constituent 40S and 60S subunits for initiation to proceed. There are at least three proteins or complexes that are required to dissociate and prevent the reassociation of the 80S complex. The 40S subunit is bound by eIF1A and eIF3. eIF1A not only binds the 40S subunit and prevents it from re-associating with the 60S subunit, but it may also stabilize the binding of the 40S subunit to methionyl-transfer RNA (tRNAiMet). eIF3 is a complex of at least eight polypeptides. It functions to dissociate the 40S subunit from the 80S complex, to stabilize the binding of the 40S subunit to tRNAiMet, to stabilize the binding of eIF2 in the ternary complex and to bind GTP. eIF3 may also recognize the AUG codon. Like eIF3 and eIF1A, eIF6 prevents the re-association of the 60S subunit with the 40S subunit, but by binding to the 60S subunit (Merrick and Hershey, 1996). After dissociation of the 80S subunit, the 40S subunit is free to bind the “ternary complex”, forming the 43S pre-initiation complex. The initiator ternary complex is made up of the initiator tRNAiMet and eIF2 bound to GTP.

The formation of the ternary complex, which can bind the 40S subunit and begin translation initiation, is a highly-regulated step during translation. Initiation cannot begin without eIF2, the factor that binds and hydrolyzes GTP. eIF2 is made up of three subunits: eIF2γ may bind the GTP, eIF2β contains a zinc finger that may affect AUG
43s pre-initiation complex

SCANNING (see Fig. 1B)

mRNA+eIFs (see Fig. 1B)

m^7GTP

AUG

(A)_n

eIF1A + eIF3
+ eIF6
+ eIF4A
+ eIF4E
+ eIF4G
+ eIF4B

Binary Complex

GDP

GTP

40S

60S

80S

eIF6

eIF1A + eIF3

Ternary Complex

Met

GTP

eIF2C

Met

m^7GTP

AUG

(A)_n

eIF2B

GTP

GDP

Figure 1: Scanning model for translation initiation. (A) Ternary complex formation and recycling, 43S pre-initiation complex formation and 60S junction. (B) Cap-binding, secondary structure unwinding and initiation of scanning for the first AUG. (Based on information in the review by Merrick and Hershey, 1996).
recognition, and phosphorylation of the eIF2α subunit (at serine 51) regulates the recycling of the eIF2 complex. When eIF2 is released from an initiation complex, it is tightly bound to GDP. This prevents its re-use in a new initiation step until the GDP is exchanged with GTP. Recycling is facilitated by the eIF2B complex, a guanine-nucleotide exchange factor. Although it is unknown why, this complex can bind GTP, ATP and NADPH. eIF2B may be regulated by phosphorylation on its ε subunit. Once eIF2B has exchanged GDP with GTP on eIF2, eIF2 is then capable of binding tRNA Met to form the ternary complex. The ternary complex can then associate with the 40S subunit to form the 43S preinitiation complex.

The next step in initiation is the binding of the mRNA by initiation factors and the 40S ribosomal subunit and the subsequent scanning to the initiation codon (see Fig. 1B). The binding of eIF4E to the mRNA is the rate-determining step in initiation (De Benedetti et al., 1991). Initially, the mRNA is bound by the cap-binding complex, eIF4F, which is comprised of three subunits, eIF4A, 4E and 4G. eIF4A is the prototype of the DEAD-box helicases and is involved in the unwinding of secondary structure present in the 5′ untranslated region (UTR) of the mRNA using an RNA-dependent ATPase (Blum et al., 1992). The second subunit is eIF4G (previously p220). eIF4G is digested in poliovirus-infected cells allowing translation by only a cap-independent mechanism. eIF4G appears to act as a bridge protein between eIF4A or eIF4E and eIF3 (reviewed by Merrick and Hershey, 1996).

eIF4E is the cap-binding factor. Phosphorylation of eIF4E causes an increase in in vivo translation rates, whereas dephosphorylation causes a reduction in translation. Changes in the phosphorylation state of eIF4E correlate well with changes in cellular states: phosphorylation increases upon induction by growth factors, heat shock and during mitosis and phosphorylation decreases during the host-shutoff of protein synthesis by several viruses (reviewed in Sonenberg, 1996). eIF4E is phosphorylated in a
Ras-dependent manner by protein kinase C (and probably other kinases), resulting in an increase in the cap-binding efficiency of eIF4E (Tuazon et al., 1990; Frederickson et al., 1991; 1992; reviewed by Sonenberg, 1996). A protein, called PHAS-1 or 4E-BPI, can bind to eIF4E and prevent its association with eIF4F, thereby preventing the highest efficiency cap-binding (Haystead et al., 1994; Lin et al., 1994). eIF4E is also considered to be an oncogene, because when overexpressed in tissue culture cells, it can cause malignant transformation mediated by Ras (Lazaris-Karatzas et al., 1990; 1992; Lazaris-Karatzas and Sonenberg, 1992; De Benedetti and Rhoads, 1990). This overexpression also facilitates the increased translation of messengers that contain extensive secondary structure in their 5’ UTRs (Koromilas et al., 1992), which implies that it may be oncogenic in nature when overexpressed by virtue of the fact that it allows translation of growth-related mRNAs that are normally inhibited (Fagan et al., 1991; Koromilas et al., 1992). Injection of eIF4E into early Xenopus embryos can also cause the induction of mesoderm without the generalized stimulation of protein synthesis (Klein and Melton, 1994). These results taken together point to a role for eIF4E in the specific expression of certain mRNAs that have important oncogenic and developmental consequences.

Association of the 43S preinitiation complex with the mRNA is facilitated by the cap-binding complex and possibly another factor called eIF4B. eIF4B associates with eIF4A (either alone or as part of eIF4F) and stimulates the helicase activity of eIF4A. It is hypothesized that eIF4B may recognize the junction between single-stranded and double-stranded RNA (Méthot et al., 1994). At some point either during or after the eIF4A helicase has unwound the secondary structure, the 43S preinitiation complex associates with the mRNA, apparently via eIF4F; this entire complex is called the 48S initiation complex.

After landing on the messenger, the 43S preinitiation complex then proceeds in a 5’ to 3’ direction on the mRNA, “scanning” for the first initiation codon (AUG) that is in
the correct sequence context. Recognition of the first AUG in the correct sequence context is facilitated by the tRNA\textsubscript{i}\textsuperscript{Met} (Cigan et al., 1988). The association of the 60S subunit appears to be facilitated by the factor eIF5, which induces the GTPase of eIF2 and decreases the affinity of binding of the factors already associated with the 40S ribosomal subunit. When eIF1A and eIF3 have been released from the 40S subunit, the 40S subunit is free to bind the 60S subunit, completing initiation (Merrick and Hershey, 1996).

Most of the progress on the timing of initiation events is based on in vitro studies using sucrose gradients to isolate translation intermediates. This methodology has left some unanswered questions, including if the tRNA\textsubscript{i}\textsuperscript{Met} binds to the subunit before or after the mRNA binds the 40S subunit; if eIF2 recycles free or bound to the 40S subunit; if the eIFs function separately or in a complex; what steps, other than eIF4E binding, are rate-limiting; and the order of binding and the movement of the initiation factors near the cap and the 43S pre-initiation complex. Work is continuing on these problems, and in vivo studies are underway to confirm this model (reviewed in Merrick and Hershey, 1996).

1.1.2. Elongation and Termination

Although translation initiation is a highly-regulated stage of translation, elongation and termination are the steps that actually produce the protein (Merrick and Hershey, 1996). Elongation is the step in which amino acid residues are added to the carboxy-terminal end of the growing peptide chain. Studies in prokaryotes have led to the characterization of four steps during elongation: (1) the binding of the amino-acyl tRNA to the ribosome; (2) the hydrolysis of GTP and the action of the guanine-nucleotide exchange function of eEF1A (eukaryotic elongation factor 1A); (3) the formation of the peptide-bond between the amino acids; and (4) the translocation of the mRNA and peptidyl-tRNA on the ribosomal surface. Translational inhibition can occur by phosphorylation of eEF2, which inhibits this translocation step (Hershey, 1993).
Termination occurs when the first of three tRNA-binding sites on the ribosome is exposed, because no tRNA anti-codon can recognize the termination codon. A release factor can then bind and promote hydrolysis of the peptidyl-tRNA and release the new protein (Merrick and Hershey, 1996).

1.2. Internal initiation model of translation

A second model exists for translational initiation of certain viral and eukaryotic messengers. This mechanism has been termed "internal initiation" of translation. Pelletier and Sonenberg (1988) first reported the use of a mechanism by poliovirus that allowed cap-independent translation to occur. The mechanism of internal initiation was first demonstrated using the 5' UTR of the poliovirus messenger as an intercistronic element between two different coding sequences in a bicistronic messenger. Upon transfection into COS cells, both cistrons were translatable in bicistronic transcripts, regardless of the presence of the poliovirus 5' UTR in the intercistronic region. However, in poliovirus-infected cells that were transfected with these same constructs, translation was only observed from the second cistron of a messenger that had the poliovirus 5' UTR in its intercistronic region. Deletion constructs identified a sequence that was necessary and sufficient to confer internal initiation to a messenger; this sequence was initially coined the "Ribosomal Landing-Pad" (RLP; Pelletier and Sonenberg, 1988), but has since become known as the "Internal Ribosomal Entry Site" (IRES; see Jackson et al., 1990). Since then, several IRESs have been identified from other viral RNAs, such as infectious bronchitis virus and encephalomyocarditis virus (EMCV; Liu and Inglis, 1992; Borovjagin et al., 1994).

The highly-conserved secondary structure of the 5' UTR of picornaviruses is involved in the recognition of the IRES by the ribosome (Andino et al., 1990; reviewed in Meerovitch and Sonenberg, 1993). It is predicted that accessory factors bind to the conserved secondary structure, while the 40S ribosomal subunit binds 3' to this at a
polypyrimidine tract, upstream of an AUG codon (Meerovitch and Sonenberg, 1993).
Interestingly, it appears that picornavirus internal initiation requires factors that are
already present inside the eukaryotic cell. It is this observation that led to the discovery
of the first eukaryotic messenger that contains an IRES; this mRNA is from promoter 2
of the Drosophila Antennapedia gene (Oh et al., 1992). Other cellular mRNAs may also
be translated in a cap-independent manner; for example, many heat shock mRNAs are
translated preferentially in an extract that is deficient in cap-binding factors eIF4E and
eIF4G (Joshi-Barve et al., 1992). Other examples of eukaryotic mRNAs with IRES
sequences include those encoding TFIID (TATA-binding protein), fibroblast growth
factor 2 (FGF2), Ultrabithorax, eIF4G and immunoglobulin heavy-chain binding protein
(Macejak and Sarnow, 1991; Oh et al., 1992; Iizuka et al., 1994; Teerink et al., 1995;
Vagner et al., 1995; Gan and Rhoads, 1996).

As mentioned above, internal initiation does not seem to require any factors that
the normal cell does not have. Paradoxically, internal initiation appears to require the cap-
binding complex eIF4F (Tahara et al., 1991; Scheper et al., 1992; Pause et al., 1994). A
factor called the pyrimidine tract binding protein (PTB) has also been identified that can
specifically bind to EMCV IRES and can enhance the internal initiation of RNAs
containing this binding site (Borovjagin et al., 1994). Recently, in vitro internal initiation
has been accomplished using purified factors. Using only purified tRNA\textsubscript{\text{Met}}, EMCV
RNA, 40S subunits and the initiation factors eIF2, eIF3 and eIF4F, IRES-mediated 48S
initiation complexes were formed. Stimulation of these complexes occurred when purified
eIF4B and—to a lesser extent—PTB were added to the in vitro system (Pestova et al.,
1996a). In this same in vitro reconstitution assay, 48S initiation complexes could also be
formed using only eIF4A and the middle of the three domains of eIF4G instead of eIF4F
(Pestova et al., 1996b). eIF4G acts as a bridge between eIF3 and both eIF4E and eIF4A.
This bridge activity is disrupted during poliovirus infection by the cleavage of eIF4G
between the eIF4E and eIF3 binding sites. The observation that a portion of eIF4G alone could enhance cap-independent translation explains previous observations that eIF4E was not necessary for cap-independent initiation (Altmann et al., 1989) and why the eIF4G subunit could be digested by poliovirus infection even though eIF4F was necessary for cap-independent translation (Pause et al., 1994; Pestova et al., 1996a; 1996b; reviewed in Merrick and Hershey, 1996).

M. Kozak (1992) raised some serious concerns regarding the validity of the experiments done to demonstrate internal initiation. Her biggest concern was the use of the dicistronic messengers, mainly the lack of good evidence that there were no monocistronic messengers produced and that the dicistronic mRNAs were the messengers that were being translated on the polysomes. These concerns have not been addressed directly, but acceptance of the concepts of an IRES and of internal initiation and their use in research, have become so widespread that the subjects warrant their own symposia at scientific meetings (e.g., “Translational Control”, Cold Spring Harbor Laboratory, N.Y., Aug. 24-28, 1994).

2. Elements and factors influencing translation

The basic elements of the mRNA molecule (from 5' to 3') are the 5' cap structure, the 5' untranslated region (UTR), the initiation codon (AUG) and the context it is in, the coding sequence, the 3' UTR and the poly(A) tail. As more research is done on the mechanisms of RNA expression, it is becoming evident that every one of these elements is involved in the regulation of RNA expression. In this section I shall discuss some of the best-understood regulatory mechanisms of mRNA expression.

2.1. The initiation codon (AUG) and the 5' untranslated region (UTR)

The scanning model of translation initiation asserts that the 40S ribosomal subunit and the associated initiation factors will scan the 5' UTR of the messenger in order to find the first AUG and initiate translation at that site (Kozak, 1989a). One of the predictions
of this model is that the first AUG encountered must be the site of initiation; however, this is not always the case (reviewed in Geballe, 1996; Hinnebusch, 1996; Jackson, 1996). A literature search has demonstrated that proto-oncogene encoding messengers contain multiple AUG codons present in their 5' UTRs that are not the authentic site of translation initiation. However, only 9% of other cellular mRNAs contain upstream AUG codons (Kozak, 1987). Often, these same proto-oncogene encoding mRNAs contain long 5' UTRs with high G+C contents (Kozak, 1987). These observations taken together with the fact that many oncogenes are activated by loss of the 5' end of their genes may indicate that proto-oncogene messengers are normally repressed due to the presence of these sequences at their 5' end, quite possibly at the level of translation initiation.

Adherence to the first AUG rule of the scanning model is an important defense of the model. However, there are at least two situations (other than internal initiation) in which initiation can occur at an AUG other than the first one. These situations are when the first AUG is followed by an in-frame termination codon and when there is an unfavourable context surrounding the first AUG (Kozak, 1987; Kozak, 1995).

When an upstream AUG codon is utilized, translation is initiated and then terminated at an in-frame termination codon before the authentic initiation codon. By terminating translation before reaching the correct initiation codon, the 40S subunit is released from the ribosome and—as long as the next initiation codon is close enough—reinitiation can occur at the correct AUG. This results in the production of a short peptide from the upstream open-reading-frame (ORF) and a full-length protein from the second ORF (Kozak, 1987).

Initiation-codon context was proposed as a regulatory mechanism when it was observed that many mRNAs contain not only the conserved AUG of the initiation codon but also a conserved sequence that surrounds that codon (Kozak, 1987). Analysis of
hundreds of vertebrate mRNAs resulted in the elucidation of a consensus sequence of \( \text{ccc}^A/G\text{cccAUGG} \), where upper case letters are the consensus sequence and lower case letters are the preferred sequence. A purine at position -3 (when the A of AUG is +1) and a G at +4 are very important in the recognition of the initiation codon (Kozak, 1987). If the consensus of the first AUG is poor, "leaky scanning" can occur. Leaky scanning assumes that if the first AUG is not in a favourable context, especially with a purine at -3 and a G at +4, then the 40S subunit may skip that AUG and continue scanning for one in a more favourable context (Kozak, 1987). An exception to this context is in the yeast *Saccharomyces cerevisiae*, in which the context consensus is quite different; unlike the highly C-rich sequence near the vertebrate consensus AUG, the yeast sequence is A-rich: aaaaaaaAaaAUGuc (Oliveira et al., 1993).

In addition to the context of the AUG in the 5’ UTR, the composition of the entire 5’ UTR can have a dramatic impact on the expression of the mRNA. Sequence elements and secondary structures are involved in the regulation of translation by the 5’ UTR. When a "synthetic" hairpin element (made up of inverted repeats of a sequence, such as a restriction enzyme sequence) is placed in the 5’ UTR of an mRNA, this hairpin can inhibit translation of a reporter gene that has been placed downstream of it in *in vitro* lysates or upon injection into *Xenopus* oocytes (Pelletier and Sonenberg, 1988; Kozak, 1989b; Fu et al., 1991). Further characterization has shown that a "moderately stable" hairpin with a thermal stability of -30 kcal/mol can inhibit translation only when it is near the cap structure. A "very stable" hairpin with a thermal stability of -61 kcal/mol can inhibit translation no matter where it is in the 5’ UTR (Kozak, 1989b). Secondary structure in an mRNA can also inhibit translation when it is formed between the 5’ and 3’ UTRs or between the 3’ UTR and the coding sequence, but only when the secondary structure is close to the AUG or if it overlaps with the 5’ UTR (Kozak, 1989b; Liebhaber et al., 1992).
Several molecules have been identified that can unwind secondary structures. The ones most likely involved in translation are members of the DEAD-box family of helicases; eIF4A is the prototype member of this family of helicases, and it is required as a member of eIF4F for initiation of translation (Pause and Sonenberg, 1993; Fuller-Pace, 1994; Pause et al., 1994). Other helicase or unwindase functions have been identified in cells that could be involved in translation and perhaps mediate translation of specific messengers. Examples include the DEAH-family member, SSL2 (suppressor of stem loop), which is the yeast homologue of the human ERCC-3 protein (excision repair cross-complement rodent repair deficiency; reviewed in Pause and Sonenberg, 1993), or the developmentally-regulated unwinding/modifying activity, called the double-stranded RNA adenosine deaminase (dsRAD), from *Xenopus* that can unwind secondary structures and then permanently modify the RNA to prevent the reformation of secondary structures (Bass and Weintraub, 1987; 1988). Further work with dsRAD has identified a dsRNA-binding protein (dsRBP) that binds to dsRNA in *Xenopus* oocytes and protects it from modification (Saccomanno and Bass, 1994). This may be a potential masking protein like those discussed earlier, which functions to prevent the translation of mRNA in the oocyte.

There are several examples of natural mRNAs with secondary structures that are involved in the in vivo regulation of the expression of these messengers. Some of the best-studied examples include ornithine decarboxylase mRNA, which is regulated by polyamines, ferritin mRNA, which is regulated by iron conditions, the *trans*-acting responsive (TAR) region of HIV mRNA, which is regulated by the *trans*-activator (TAT) protein and a superoxide dismutase (SOD-1) mRNA, which is subject to testis-specific expression.

Ornithine decarboxylase (ODC) is an enzyme in the polyamine biosynthetic pathway. Polyamines can regulate their own synthesis by influencing the expression of
ornithine decarboxylase. By adding polyamines (such as spermidine) to cells expressing ODC, that expression was decreased (Kahana and Nathans, 1985; Fonzi, 1989). Early experiments also indicated that this regulation may be at the level of translation (except in yeast), possibly due to the 5' UTR (Kameji and Pegg, 1987; Fonzi, 1989; Autelli et al., 1990; Bassez et al., 1990; Kashiwagi et al., 1991). Deletion of the 5' UTR of rat ODC mRNA demonstrated that removal of the 5' UTR allowed the translation of the ODC mRNA in rabbit reticulocyte lysates, which contain high levels of polyamines (Manzella and Blackshear, 1990; Ito et al., 1990). When the ODC 5' UTR was subcloned upstream of the human growth hormone (hGH) coding sequence, it conferred repression onto hGH upon transfection into fibroblasts (Manzella and Blackshear, 1990). Although unconfirmed, there was a prediction that the inhibition by polyamines was due to the presence of stable secondary structure and that release of the inhibition in the absence of polyamines was because of a reduction in the secondary structure. The only evidence for this regulation comes from the observations that the regulatory sequence in the ODC 5' UTR is a G+C-rich sequence (Manzella and Blackshear, 1990; Kashiwagi et al., 1991).

The ferritin transcript is a better example of how secondary structure can be used to regulate the expression of an mRNA. Ferritin protein is responsible for detoxifying sequestered iron. It may also be involved in the regulation of iron availability and uptake. Two observations indicated that ferritin mRNA is subject to a translational regulatory mechanism: Actinomycin D (a transcriptional inhibitor) did not inhibit the stimulatory effect that iron has on ferritin expression, and ferritin mRNA shifts to polysomes in the presence of iron (Hentze et al., 1987). Experiments that deleted a portion of the 5' UTR demonstrated that iron regulation was eliminated upon transfection of the truncated 5' UTR into fibroblasts (Hentze et al., 1987). Chimaeric constructs were made that placed the ferritin 5' UTR (or portions of it) upstream of a chloramphenicol acetyltransferase (CAT) reporter. When these mRNAs were expressed in tissue culture cells, they were
translationally regulated by iron (Aziz and Munro, 1987; Hentze et al., 1987). Using deletions, the translational regulatory sequence was mapped to a portion of the 5' UTR and was termed the iron-responsive element (IRE; Aziz and Munro, 1987). Further work has identified a protein termed P-90 or IRE-BP (IRE-binding protein) that binds to the IRE in the absence of iron and inhibits translation of ferritin mRNA. It was also found that there was secondary structure present in the 5' UTR. Mutations that disrupted the secondary structure also eliminated the translational control, demonstrating the importance of the secondary structure. Disruption of translation could be rescued by complementary mutations that allowed the secondary structure to reform, but with the opposite sequence (Bettany et al., 1992). It is likely that IRE-BP works by increasing the stability of the 5' UTR secondary structure (Harrell et al., 1991). This was confirmed by using gel shift assays that demonstrated the importance of the stem-loop structure to the binding of IRE-BP (Jaffrey et al., 1993).

All HIV mRNAs contain a region called the trans-acting responsive (TAR) region. The sequence in this region is poorly conserved, although its predicted secondary structure is highly conserved. The TAR region and its 3'-flanking sequence contain inverted repeats that generate a stable secondary structure (Parkin et al., 1988a; Baudin et al., 1993). When the TAR region of HIV mRNA was placed upstream of a CAT reporter, it inhibited translation in cis in rabbit reticulocyte lysates or Xenopus oocytes. Mutations that were predicted to destroy the secondary structure also eliminated the inhibition, and complementary mutations that replaced the secondary structure (but not the sequence) also replaced the inhibition of translation (Parkin et al., 1988a). The trans-activator (TAT) protein could relieve this inhibition, but only when it was coinjected with mRNA containing the TAR region into the nucleus of the cell (Braddock et al., 1993). The current model for TAT function is that TAT binds TAR via its secondary structure, but nuclear factors are also required to facilitate this binding (Baudin et al.,
1993). The role of cellular factors in TAT-activation was confirmed using the anti-viral drug Ro74-7429, which blocked TAT-specific activation by interacting with TAR loop-binding cellular factors (Braddock et al., 1994).

Recently, a sequence element has been characterized that can inhibit translation in testis-specific superoxide dismutase (SOD-1) mRNA. Two SOD-1 mRNAs (S_{SOD-1} and T_{SOD-1}) exist in testes but T_{SOD-1} is specifically expressed in male postmeiotic germ cells. T_{SOD-1} contains 114 additional nucleotides in its 5' end by virtue of transcription from a different promoter than S_{SOD-1}. Unlike S_{SOD-1}, T_{SOD-1} is translationally regulated during spermiogenesis. In vitro there is no difference in the translation between the mRNAs, but a protein has been purified from testes that specifically binds to the longer 5' UTR of T_{SOD-1} and that inhibits translation of T_{SOD-1} mRNA when added to the in vitro lysate (Gu and Hecht, 1996).

Finally, a very exciting report described the translational regulation of insulin-like growth factor II (IGF-II) mRNA in a cell-type specific fashion (Nielsen et al., 1995). The G+C-rich leader of the IGF-II mRNA inhibited translation in the stationary phase of cell culture. However, during cell growth, the level of inhibition was lower (Nielsen et al., 1995). This indicates that with this mRNA, the translational inhibition could be relieved upon induction of growth.

By understanding how translation is affected by elements in the 5' UTR, we can hopefully gain insight into the general mechanism of translational regulation by this region. This is especially relevant for the 5' UTRs of oncogenes, growth factors and growth factor receptors, which often contain a number of upstream AUGs and/or are highly G+C-rich, with the potential to form extensive secondary structure (Kozek, 1987).

2.2. The 5' cap structure

The cap is a 7-methylguanosine residue joined to the 5' end of eukaryotic mRNAs by a 5'–5' linkage bridged by three phosphates. This is abbreviated as
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m7GG(5')ppp(5')N, where “N” is the first nucleotide of the mRNA and can be any nucleotide. The first known function of the cap was to provide the mRNA with increased longevity. Synthetic mRNA was injected into _Xenopus_ oocytes and then recovered and analyzed by gel electrophoresis. mRNA that had either a “monomethylated” cap [m7GG(5')ppp(5')G] or a “dimethylated” cap [m7G(5')ppp(5')Gm] was stable after six hours post-injection and reduced by only about 50% after 24 hours post-injection compared to uncapped mRNA that was reduced by 40% at six hours and 90% by 24 hours (Drummond et al., 1985). This six hour experiment was repeated to analyze the translation of mRNAs with different cap structures. Even taking the differences in longevity into account, the capped mRNAs were translated much more efficiently than were the uncapped mRNAs. Monomethylated-capped RNA was translated 25-fold more efficiently than uncapped mRNA and 5-fold more efficiently than dimethylated-capped RNA (Drummond et al., 1985). Similar results were obtained using the yeast _S. cerevisiae_ (Gerstel et al., 1992).

Once it was established that the cap not only affects the stability of mRNA, but also impacts its translation, research was undertaken to determine how the cap functions. Most of this work was done by isolating factors that bind to the cap. Two mechanisms have been used to crosslink proteins to a cap, cap analog or mRNA. Chemical crosslinking allows crosslinking of proteins to the cap that are near the cap but not necessarily bound to it. It was with chemical crosslinking that an association between the cap and eIF4E, eIF4B and eIF4A was first noted (Pelletier and Sonenberg, 1985; van Heugten et al., 1992; Berben-Bloemheuvel et al., 1992). The other technique, UV crosslinking or photochemical crosslinking, has the advantage of using labeled cap structures only, so that only factors that bind directly to the cap are identified. UV crosslinking has shown that only eIF4E or eIF4B associate directly with the cap. Thus, the previously observed crosslinking of eIF4A to the cap was because it was bound very close to—but not at—
the cap (Pelletier and Sonenberg, 1985). eIF4B can only crosslink to the cap in the presence of ATP and not at all if there is secondary structure near the cap, as is also the case for eIF4F (Pelletier and Sonenberg, 1985; Berben-Bloemheuvel et al., 1992). When different 5' UTRs are used, the binding of eIF4E can be affected, but it appears as though secondary structure itself may have no impact on its cap-binding ability (Pelletier and Sonenberg, 1985; Berben-Bloemheuvel et al., 1992).

More recent work has analyzed how eIF4A is involved in the association of eIF4E or eIF4B with the cap. eIF4A and eIF4B can interact with the cap in an ATP-dependent manner, but when eIF4A is part of eIF4F, eIF4B can still interact with the cap, but not as efficiently as it does with free eIF4A. In fact, eIF4B functions with eIF4A, which is an ATP-dependent RNA helicase (Jaramillo et al., 1991; van Heugten et al., 1992). In addition, eIF4A can enhance the binding of eIF4E to the cap in an ATP-independent manner, implying that this assistance is distinct from its role as a helicase (van Heugten et al., 1992). Therefore, secondary structure can impact the functioning of the cap-binding complex (eIF4F), but it does not impact the binding of eIF4E alone (Carberry et al., 1992). However, when eIF4E is overexpressed in cells, it can lead to malignant transformation (Lazaris-Karatzas et al., 1990; De Benedetti and Rhoads, 1990). It can also increase the translation of messengers that are otherwise poorly translated, such as mRNAs containing extensive secondary structure (Fagan et al., 1991; Koromilas et al., 1992).

The extent of methylation of the cap can also influence mRNA translation. There are three forms of cap methylation found in eukaryotic messengers: (1) cap0 is the previously mentioned m7GpppN, which prevails in lower eukaryotes; (2) capI has a methyl group on the 2' oxygen of the penultimate ribose (giving m7GpppN1m); and (3) capII has a methyl group on both the penultimate and subpenultimate riboses (giving m7GpppN1mN2m). CapI and CapII are the predominant forms in higher eukaryotes
(Sonenberg, 1988; Kuge and Richter, 1995). Recent work in *Xenopus* indicates that methylation of endogenous RNA does occur in response to progesterone-induced oocyte maturation. Synthetic RNA that is injected into *Xenopus* oocytes becomes cap-ribose methylated in a polyadenylation-dependent manner during maturation. If this cap-ribose methylation is prevented, translational recruitment of the injected mRNA is decreased (Kuge and Richter, 1995). Either the extent or action of methylation at the 5' cap can influence the expression of the mRNA.

2.3. The 3' untranslated region (UTR)

The 3' UTR of the mRNA has recently been receiving considerable attention for its ability to regulate mRNA expression. Elements in the 3' UTR could regulate polyadenylation, translation and/or mRNA stability. As with the 5' UTR, much of what is known about the 3' UTR is due to the examination of specific mRNAs.

The β-interferon (β-IFN or IFN) mRNA contains a negative regulatory element in its 3' UTR called the AU-rich element (AURE). The AURE is the same element that is known to regulate messenger stability of some highly unstable messages in tissue culture cells, such as the *c-fos* and *c-myc* mRNA (see Section 4; reviewed in Jackson and Standart, 1990). However, when these elements are downstream of a coding sequence of mRNA that is injected into *Xenopus* oocytes, they do not change the stability of the mRNA but instead cause a dramatic decrease in mRNA translatability compared with mRNAs that lack these elements. These elements also inhibit translation in rabbit reticulocyte lysates, but not in wheat germ extracts (Jackson and Standart, 1990). More recent work with the IFN 3' UTR has demonstrated that the AUREs can inhibit translation of a reporter in *Xenopus* eggs after fertilization or in oocytes before maturation. Three contiguous elements consisting of UUAUUUAU are required anywhere in the 3' UTR for AURE function (Marinx *et al*, 1994). These elements affect poly(A)+ or poly(A)− mRNA to the same extent (Marinx *et al.*, 1994).
Another mRNA that has 3' UTR elements that inhibit translation is the 15-lipooxygenase (15-LOX) mRNA. 15-LOX is required for lipid degradation; the expression of this mRNA is limited to the mature reticulocyte when it reaches the peripheral blood even though the mRNA is transcribed in the bone marrow. The 15-LOX 3' UTR contains an unusual element consisting of ten tandem copies of a 19-nucleotide pyrimidine-rich motif. Further characterization has identified a protein factor that binds to the 3' UTR at the pyrimidine tract and inhibits translation of mRNA until expression is appropriate (Ostareck-Lederer et al., 1994).

Similar to the 15-LOX mRNA, the Xenopus fibroblast growth factor receptor-1 (XFGFR-1) mRNA is translationally inhibited, apparently due to the presence of a cis-acting translational inhibitory element (TIE) in the 3' UTR. The XFGFR-1 mRNA is a maternal mRNA that is not expressed in the developing oocyte but is released from this inhibition at maturation. This release is not polyadenylation-dependent, but it may be dependent on a cytoplasmic protein that has yet to be well characterized (Robbie et al., 1995).

Several other mRNAs are known to be translationally repressed during development via elements in their 3' UTR. An example is the glp-1 gene in C. elegans. The correct spatial and temporal pattern of expression of glp-1 is important for orderly development of the C. elegans embryo. The GLP-1 protein is expressed only in the anterior blastomere of the two-cell embryo, even though the RNA is present in both cells. Two elements have been identified in the 3' UTR of the glp-1 mRNA that inhibit translation in a temporal- and localization-dependent manner; one inhibits translation until the 2 to 4 cell stage and the other represses translation in the posterior cells (Kimble, 1994).

The transferrin receptor (TfR) mRNA is another example of a messenger with elements in the 3' UTR that regulate expression, but in this case, they regulate messenger
stability. The TfR mediates uptake of iron into the cell. Coincidentally, the elements that regulate mRNA stability in this messenger are the same elements that are present in the 5' UTR of the ferritin mRNA and regulate translation of that messenger: the IRE. The TfR mRNA contains five IREs in its 3' UTR. It is thought that the IRE-BP binds to these elements and prevents the degradation of the TfR mRNA when there is insufficient iron in the cell (Horowitz and Harford, 1992). The regulation of the stability of this mRNA is also responsive to the growth factor interferon γ (IFNγ; Bourgeade et al., 1992).

Another example of mRNAs that contain 3' secondary structures that are important to their regulation are certain plant viral mRNAs. These mRNAs contain a sequence at the 3' terminus that mimics the tRNA structure, complete with pseudoknots, even though the sequence is not conserved. Depending on the mRNA, there are other elements further upstream that are thought to regulate translation and that also appear to fold into unusual secondary structures. These elements are still poorly understood, but they may function to stabilize the RNA in lieu of a poly(A) tail, which these viral mRNAs do not have (Gallie and Kobayashi, 1994; reviewed by Gallie, 1993). Secondary structure instead of a poly(A) tail may also function in some mammalian mRNAs. This was first proposed because it was noticed that certain messengers that had lost their poly(A) tails were not degraded, unlike most other mRNAs (Bandyopadhyay and Brawerman, 1992).

Recently, it has been demonstrated that the length of the 3' UTR may be responsible for some translational regulation. The firefly luciferase (Luc) mRNA contains a very long 3' UTR. Tanguay and Gallie (1996) used deletions of this 3' UTR to analyze what effect they would have on the expression of the mRNA. When the 3' UTR was reduced to four nucleotides, there was very little translation from the Luc mRNA. When this was increased to 24 nucleotides, there was still very little translation, but as the length was increased beyond this, there was a concomitant increase in the translatability
of the mRNA. When a poly(A) tail was added to an mRNA with a seven nucleotide 3’ UTR, there was very little increase in expression, but when a poly(A) tail was added to an mRNA with a 27 nucleotide 3’ UTR, there was a 50-fold increase in translation as compared to a similar mRNA with no poly(A) tail. Interestingly, the effect of poly(A) was differential, with the optimal effect observed on intermediate length 3’ UTRs (Tanguay and Gallie, 1996). This effect of the 3’ UTR may not be due to specific sequences in the 3’ UTR. When a small, independently ineffective portion of the 3’ UTR was repeated a different number of times as the 3’ UTR, translational stimulation was noted at similar levels as with a 3’ UTR sequence of approximately the same length. The conclusion was that the length of the 3’ UTR alone was responsible for the increased translation of the luciferase reporter (Tanguay and Gallie, 1996).

2.4. The poly(A) tail

Early work on the role of the poly(A) tail in eukaryotic mRNA expression was very controversial. Many researchers believed that it had no effect, except perhaps to slightly increase the stability of the mRNA, but that the 5’ cap structure was much more important in the regulation of translation. It seemed strange that so much cellular effort would go into the production of an (A) tail on nearly every eukaryotic mRNA and that each mRNA could have a different length of (A) tail, with some mRNAs lacking an (A) tail altogether. It was this observation that led to the work on the effect of the poly(A) tail on stability and translation. In agreement with previous observations, a poly(A) tail had very little effect on mRNA stability in the short term (several hours), but it could impact stability over a very long term (several days; Drummond et al., 1985). The big breakthrough in poly(A) studies came when it was found that poly(A) could promote a 10- to 20-fold stimulation of the translation of an mRNA that was injected into *Xenopus* oocytes (Drummond et al., 1985).
Xenopus oocytes are commonly used in poly(A) studies; they have given repeatable results, the system is well understood and it is easy to use. A reporter that had a poly(C) tail, in addition to a 3' UTR, showed no significant increase in translation over one with nothing in the 3' end in Xenopus oocytes. However, the same reporter with an (A) tail with or without a 3' UTR showed a large increase in the translation of the reporter, regardless of the status of the 3' UTR (Galili et al., 1988). Furthermore, there was a greater stimulation of translation if the mRNA was allowed to reside in the oocytes longer before assaying for protein synthesis. This appeared to be because the polyadenylated mRNA had a competitive advantage over mRNA without an (A) tail; poly(A)+ mRNA became associated with larger polysomes as the incubation time increased (Galili et al., 1988). The observation that poly(A)+ mRNA is associated with larger polysomes has since been confirmed using reticulocyte lysates (Munroe and Jacobson, 1990). Munroe and Jacobson also examined the specific role of the poly(A) tail in translation. When they used anisomycin and cycloheximide, which both allow the accumulation of 80S initiation complexes, they found that there was a 2- to 2.5-fold increase in the efficiency of poly(A)+ mRNA in accumulating 80S complexes as compared to poly(A)-. This indicated that the poly(A) tail may influence the association of the 60S subunit with the 40S subunit during the initiation stage of translation. Similar results have been obtained in the yeast S. cerevisiae (Sachs and Davis, 1989; Gerstel et al., 1992).

Protein factors have been identified that are involved in the recognition and function of the poly(A) tail in the regulation of mRNA expression. The factor that appears to mediate a large number of the effects of the poly(A) tail is the poly(A)-binding protein (PABP). When excess, free poly(A) was added in vitro, it was capable of inhibiting translation of poly(A)+ mRNA, but not poly(A)- mRNA (reviewed in Munroe and Jacobson, 1990). A limiting factor was probably being depleted when excess external poly(A) was added. When purified PABP was added back into the in vitro translation
mix, it stimulated translation (Munroe and Jacobson, 1990). Depletion of PABP can also cause a general inhibition of translation; it appears to affect mRNA at the level of recruitment of the 60S subunit (Sachs and Davis, 1989). Finally, the PABP/poly(A)$^+$ mRNA complex is resistant to degradation by partially purified Xenopus deadenylase. Hence, PABP may protect RNA from degradation, or it may induce ribosomal recruitment to enhance mRNA translation.

2.5. Combinatorial effects of multiple elements

There is evidence that many of the elements of the mRNA and the factors that interact with them may work together to regulate translation. The first evidence for this effect came from work using the 5' cap and the 3' poly(A) tail to study the roles of these distant elements on translation (Gallie, 1991). A poly(A) tail could enhance the translation of uncapped mRNA by about 1.5-fold \textit{in vivo}. The cap alone enhanced translation by about 21-fold, but by putting both a cap and a poly(A) tail on the mRNA, the translation was stimulated by 297-fold. If the effects were only additive, the stimulation was expected to be much lower than the observed 297-fold. Thus, the stimulation by the cap and the poly(A) tail are synergistic, meaning that they specifically enhance the translation of mRNA more when they are together than alone (Gallie, 1991). Further work on this subject has indicated that this effect may be at the level of cap-binding. The addition of exogenous poly(A) to an \textit{in vitro} lysate inhibited translation of uncapped mRNA regardless of its (A)-tail status. This effect could be reversed by adding back a combination of eIF4F, eIF4A and eIF4B. In addition, gel shift assays demonstrated that eIF4B and eIF4F could bind directly to poly(A). The conclusion was that poly(A) in excess could sequester factors required for general translation. It is likely that these factors recognize the poly(A) tail, implying that the poly(A) tail may be directly involved in translation initiation (Gallie and Tanguay, 1994).
3. Polyadenylation and deadenylation

Proudfoot (1994) suggested that the (A) tail on the mRNA may be a signal to ribosomes that the RNA is full-length and acceptable for translation. This system would allow only full-length mRNAs to be translated, possibly signaling the cell to remove mRNAs that are not full length and therefore lacking potential regulatory sequences. If this theory is true, then the regulation of polyadenylation could be vital to the healthy function of a cell.

3.1. Nuclear polyadenylation

Post-transcriptional processing of primary transcripts is necessary to produce a “mature” mRNA. This processing includes the capping, splicing, base methylation, 3’ terminal cleavage, polyadenylation and transport of the mRNA to the cytoplasm. Cleavage and polyadenylation of mRNA are closely-linked processes, and both appear to require an element consisting of AAUAAA (the nuclear polyadenylation element; reviewed by Wickens and Stephenson, 1984).

Cleavage occurs on RNA that is still being transcribed in the nucleus. The results of this cleavage leave a 3’ hydroxyl and a 5’ phosphate group by cleavage of a phosphodiester bond downstream of the AAUAAA. RNA that is not properly cleaved is not polyadenylated (Wickens and Stephenson, 1984). Point mutation studies have demonstrated that the AAUAAA must be conserved to allow cleavage. A small number of the RNAs with point mutations in this AAUAAA sequence were cleaved, and of these, over 90% were polyadenylated. This implies that the AAUAAA is important for correct 3’ end formation, but it is most important for the correct cleavage of the message to occur, with its requirement for polyadenylation being less stringent (Wickens and Stephenson, 1984).

Studies of polyadenylation have led to the identification of elements involved in 3’ processing. Some of these elements have been characterized by studying the activities
that function through them. These activities have been characterized as containing either polyadenylation factors or cleavage factors. Many of these factors either have not been purified to homogeneity, or they have been purified, but not characterized.

Purified nuclear fractions have been used to test for the ability to cleave and adenylate mRNA \textit{in vitro} (Gilmartin and Nevins, 1989). Two fractions each for the cleavage and polyadenylation of pre-mRNA have been identified; they were termed cleavage factors I and II (CFI and CFII) and polyadenylation factors I and II (PFI and PFII). CFI is responsible for the specific recognition of an element that is G/U-rich downstream of AAUAAA. This G/U-rich element may regulate the “strength” of the cleavage site and may explain why AAUAAA elements that have been found in introns do not permit polyadenylation. The different CF and PF activities are also closely linked. For example, PFII crosslinks to mRNA in an AAUAAA-dependent manner and is required for both cleavage and polyadenylation (Gilmartin and Nevins, 1989). This crosslinking may be due to a small nuclear ribonucleoprotein (snRNP) activity. SnRNPs have been well characterized due to their role in splicing of pre-mRNA (Gilmartin and Nevins, 1989; Wickens, 1990). When the CFII fraction is added to PFII, the result is the endonucleolytic cleavage of pre-mRNA. This result indicates that CFII may be the nuclease itself (Gilmartin and Nevins, 1989).

Upon successful cleavage, the messenger is competent to be polyadenylated. Polyadenylation occurs in the presence of PFI [the poly(A)-polymerase activity], magnesium ions and ATP. Polyadenylation requires the AAUAAA element, which may also be sufficient for polyadenylation. Short substrates (as short as 11 nucleotides) support adenylation if this hexanucleotide (AAUAAA) is present in them (Wickens, 1990). The first step in polyadenylation is the addition of a short oligo(A) tail to the 3’ terminus of the cleaved RNA (Wickens, 1990). The oligo(A) is then recognized by an oligo(A)-binding protein that encourages the conversion of the oligo(A) into a full length
(A) tail in an AAUAAA-independent manner (Proudfoot, 1994). Once the (A) tail reaches a determined length, it can apparently grow no longer. The regulation of the termination of polyadenylation is unclear, but it is possibly due to the presence of PABP or another, unidentified termination factor (Wickens, 1990).

The choice of polyadenylation site can also be important to the regulation of gene expression and the disease process. Amyloid protein precursor (APP) is the protein that builds up in lesions in the Alzheimer brain. Two different mRNAs encoding this protein have been identified. Each of them utilizes a different polyadenylation site. Translation from these two mRNAs is different, with the longer mRNA producing more protein than the shorter one. It is possible that the different polyadenylation sites produce different 3' UTRs that regulate protein synthesis differentially, because of different elements in the 3' UTR (see Section 2.3 for examples). Investigation is underway to determine if the longer mRNA for APP is more commonly used in Alzheimer's brains (de Sauvage et al., 1992).

3.2. Cytoplasmic polyadenylation

Until recently, it was supposed that the mature mRNA was polyadenylated in the nucleus and that this was the only time an mRNA could receive a poly(A) tail. However, it was noticed that mRNAs that are adenylated in the nucleus all receive approximately 250 adenylate residues as an (A) tail, but in the cytoplasm, poly(A)⁺ mRNAs can have (A) tails ranging from 15 to 400 adenylate residues (Fox and Wickens, 1990). It has since been shown that during certain stages in development, adenylation and deadenylation of mRNA can also occur in the cytoplasm. Cytoplasmic polyadenylation is dependent upon the presence of a cytoplasmic polyadenylation element (CPE) in addition to the nearly ubiquitous nuclear polyadenylation element (NPE). mRNA deadenylation has also been shown to be an important and highly-regulated event, especially during development.
Work done predominantly in *Xenopus laevis* has led to the discovery that the state of the poly(A) tail may change during development, but not until after oogenesis (Golden *et al.*, 1980; Paris and Phillippe, 1990). *Xenopus* oocytes arrested at prophase I are stimulated to complete meiosis I and arrest again at meiotic metaphase II as an egg. This process can also occur in enucleated eggs. Hence, although translation is necessary for maturation, transcription is not. Maturation of *Xenopus* oocytes can be stimulated by either progesterone treatment of oocytes or injection of maturation promoting factor (MPF). Cytoplasmic polyadenylation is one of the processes that is stimulated at maturation. Maturation-induced polyadenylation differs from nuclear polyadenylation in several respects. Most notably, it occurs in the cytoplasm, it is not coupled to 3' end cleavage, it requires sequences in addition to the NPE and it is highly regulated, whereas nuclear adenylation appears to be constitutive (Fox *et al.*, 1992). Two elements are required for cytoplasmic polyadenylation: the nuclear polyadenylation element (NPE) and another element that was first identified as a U-rich element upstream of the NPE, termed the cytoplasmic polyadenylation element (CPE).

Two activities have been identified that are necessary for cytoplasmic polyadenylation: a poly(A) polymerase (PAP) and an RNA-binding activity, which recognizes the NPE and the upstream CPE (Fox *et al.*, 1992). Recently, it has been shown that the RNA-binding activity may not be novel with respect to cytoplasmic versus nuclear polyadenylation. The nuclear cleavage-polyadenylation specificity factor (CPSF), which recognizes the NPE, and a poly(A) polymerase can cause the activation of maturation-dependent cytoplasmic polyadenylation *in vitro* (Bilger *et al.*, 1994). Bilger *et al.* (1994) hypothesized that the CPE affords increased specificity for the binding of CPSF in the cytoplasm. Without this increase in specificity, cytoplasmic polyadenylation would not occur on any mRNA because of the large number of mRNAs in the cytoplasm. If CPSF is present in limiting amounts, only mRNAs with an increased
specificity for it would be polyadenylated. This theory is supported by the observation that the addition of CPSF to oocyte cytoplasmic extracts stimulates polyadenylation (Bilger et al., 1994).

Although much of the work on cytoplasmic polyadenylation has been done using maturing oocytes, cytoplasmic polyadenylation also occurs post-fertilization on mRNAs such as *Xenopus* C11 and C12 mRNAs (Simon et al., 1992; Simon and Richter, 1994). Generally, mRNAs that are polyadenylated and translationally activated at maturation are not polyadenylated and activated at fertilization and *vice versa*. This temporal regulation of cytoplasmic polyadenylation appears to be dependent on the type of CPE present and the distance between the NPE and the CPE. Maturation-specific polyadenylation tends to require the consensus sequence of the CPE, UUUUUUAU (Richter, 1996). However, cytoplasmic polyadenylation of the C12 mRNA after fertilization is regulated by dodecauridine [(U)_{12}; Simon et al., 1992] The distance between the CPE and NPE also tends to be greater in mRNAs that are polyadenylated during embryogenesis than those that are adenylated during oocyte maturation (Simon et al., 1992).

Much of the work that has been done to study cytoplasmic polyadenylation has also demonstrated that the changes in adenylation status of the mRNA may regulate coincident changes in polysomal recruitment and thus translation. The discovery of cytoplasmic polyadenylation of the mouse tissue plasminogen activator (tPA) mRNA during oocyte maturation established a correlation between the poly(A) tail and translation (Vassalli et al., 1989). mRNA that contained either a poly(A) tail or a CPE injected into mouse oocytes enhanced tPA translation. Thus, the presence of the poly(A) tail itself controls the recruitment of tPA mRNA onto polysomes. The presence of a poly(A) tail is also known to enhance translational regulation of the *Xenopus* B4 mRNA in oocyte maturation (Paris and Richter, 1990). In contrast, the maturation-dependent cytoplasmic polyadenylation and translational recruitment of the *Xenopus* G10 mRNA
appears to be via the process of adenylation itself and not simply the presence of the poly(A) tail, based on the observation that mRNA injected with a pre-existing (A) tail does not enhance translation (McGrew et al., 1989; reviewed in McGrew and Richter, 1990). The process of polyadenylation also stimulates translation of *Xenopus* CI1 and CI2 mRNAs, but this stimulation occurs during early embryogenesis, rather than at oocyte maturation (Simon et al., 1992; Simon and Richter, 1994). Thus, the translational regulation by polyadenylation is dependent upon either the process of polyadenylation or the presence of an elongated poly(A) tail and is stage-dependent.

Recently, a new technique has been employed to demonstrate that translation is affected by cytoplasmic polyadenylation. The 3’ UTR of the *Xenopus c-mos* mRNA contains a CPE that, when linked to a reporter, can stimulate its translation (Sheets et al., 1994). By injection of antisense DNA to the 3’ UTR of endogenous *Xenopus c-mos* mRNA, the endogenous RNase H could then digest the RNA portion of the RNA/DNA hybrid. This “amputation” resulted in the inability of the cells to undergo maturation, for which *c-mos* mRNA polyadenylation is required. After amputation, a subsequent injection of “prosthetic RNA” rescued maturation; prosthetic RNA contains polyadenylation signals and a sequence that can hybridize to the remaining 3’ UTR of the natural mRNA. By using the prosthetic RNA to replace the amputated adenylation signals, it was shown that the cytoplasmic polyadenylation of the *in vivo c-mos* mRNA was necessary and sufficient to induce maturation (Sheets et al., 1995).

Translation is required for maturation to proceed, specifically translation of the *c-mos* mRNA (Sagata et al., 1988). It has also been shown that *c-mos* mRNA must be cytoplasmically polyadenylated in order for it to be translated (Sheets et al., 1994; 1995). The stimulation of cytoplasmic polyadenylation during maturation by progesterone treatment of the oocytes also requires protein synthesis. So, Mos protein must be synthesized for maturation to proceed, but the *c-mos* mRNA must be cytoplasmically
polyadenylated for translation to occur, and the stimulation of cytoplasmic polyadenylation requires translation. Thus, another protein must be synthesized before c-Mos. It is possible that this other protein is directly involved in the induction of cytoplasmic polyadenylation during maturation. (McGrew and Richter, 1990; Kuge and Inoue, 1992; Paris et al., 1991; Vassalli and Stutz, 1995; Sheets et al., 1994; 1995).

Alterations in translation due to changes in cytoplasmic polyadenylation can also impact differentiation and pattern formation (Simon et al., 1996). Recently, it has been shown that the embryonic cytoplasmic polyadenylation of the *Xenopus* activin receptor mRNA was necessary for correct expression of that transcript. Overexpression of this mRNA produced morphological defects because of competition for a CPE-binding protein. When the CPE was removed from the activin receptor mRNA, no defects were seen when this mRNA was overexpressed. The increased competition for the CPE-binding protein disrupted mesoderm formation by inhibiting muscle actin and *xbra* mRNA synthesis (Simon et al., 1996). In conclusion, translational regulation by the CPE can have significant developmental consequences.

### 3.3 Deadenylation

The processes that regulate the deadenylation of an mRNA are as important as those that regulate the adenylation of the mRNA in the first place. Regulation of deadenylation is important because mRNAs that become deadenylated are removed from active translation and shortening of the poly(A) tail is often the first step in the degradation of the mRNA.

Deadenylation can either be specific to certain mRNAs, or it can occur by default during certain stages of development (Fox and Wickens, 1990; Sagliocco et al., 1994). Default deadenylation has been suggested to explain what appears to be a non-specific, large scale deadenylation of mRNAs at oocyte maturation. This default deadenylation is prevented on mRNAs that contain a maturation-specific CPE (Fox and Wickens, 1990;
reviewed in Richter et al., 1990). In fact, the presence of a maturation-specific CPE may also be a signal for specific deadenylation and subsequent translational quiescence after fertilization (Wormington et al., 1996). Deadenylation can be as potent a translational regulator as polyadenylation, because the deadenylation of an mRNA would result in the loss of a poly(A) tail and therefore its removal from the pool of translatable mRNA (Paris and Richter, 1990).

The mechanics of deadenylation and the role of various factors in the regulation of deadenylation and translation are poorly understood. In yeast, the poly(A) tail that is added post-transcriptionally is recognized by a cytoplasmic poly(A) nuclease (PAN) that slowly shortens it in a PABP-dependent manner (Sachs and Deardorff, 1992). PABP-deficient yeast strains do not translate mRNA, nor do they shorten poly(A) tails. To demonstrate that PAN is also required for translational stimulation, temperature-sensitive PAN strains were made. At the restrictive temperature, these strains were blocked for both translation and poly(A)-shortening. The implication of this work is that in yeast the shortening of the poly(A) tail is required for the translation of the mRNAs (Sachs and Deardorff, 1992). This is not the case in metazoans, where PABP does what it is predicted to do: protect mRNAs from deadenylation. *Xenopus* oocytes have a large pool of mRNAs and a low concentration of PABP. During *Xenopus* oocyte maturation, the overexpression of PABP did not interfere with the polyadenylation of CPE-containing mRNAs, but it did reduce their recruitment onto polysomes in mature oocytes. In addition, a PABP-poly(A) tail complex was resistant to partially-purified oocyte deadenylase. The overexpression of PABP allowed mRNAs that would normally become deadenylated after maturation to escape deadenylation. The translational recruitment of CPE-containing mRNAs was reduced due to increased competition from other mRNAs that were not properly deadenylated (Wormington et al., 1996).
Deadenylation, unlike cytoplasmic polyadenylation, does not require de novo protein synthesis, but deadenylation does not occur in oocytes that have been enucleated before maturation. In addition, the deadenylation step occurs late in maturation, after germinal-vesicle breakdown (GVBD). Taken together, this may mean that the deadenylase is present and sequestered in the oocyte nucleus before maturation to be released only upon GVBD (Wormington et al., 1996).

In summary, there appears to be a complex series of mechanisms that regulate the polyadenylation and deadenylation of mRNAs in a developmentally-dependent manner. It may be this regulation, at least in part, that is vital in the regulation of translation of mRNAs at the appropriate times during oocyte maturation and early embryogenesis.

4. Messenger RNA stability

The mechanisms regulating mRNA instability and degradation are not very well understood. Investigators are still trying to understand what elements in the mRNA affect stability and by what mechanisms degradation proceeds. Much of the progress in this field has been made in yeast. However, work done in vertebrates tends to agree with that done in yeast.

There are at least three mechanisms by which mRNA degradation is initiated. In the first, deadenylation occurs followed by decapping and then 5' to 3' exonucleolytic cleavage. In the second, deadenylation is followed by 3' to 5' exonucleolytic cleavage. The third known mechanism is by direct endonucleolytic cleavage, usually at a cleavage site in the 3' UTR of the mRNA (reviewed in Decker and Parker, 1994; Beelman and Parker, 1995; Ross, 1995). Although the mechanism by which a pathway is selected is unclear, elements in the mRNA have been identified that can “predispose” the mRNA to one or the other forms of degradation, but individual mRNAs can be degraded by more than one mechanism.
Decapping and deadenylation may be involved in regulation of mRNA stability, rather than simply being removed as a consequence of degradation. In fact, the cap and poly(A) tail were initially thought to be exclusively involved in mRNA degradation. It has been known for some time that synthetic mRNAs lacking a 5' cap are unstable when injected into cells or used in in vitro extracts. There has been very little additional research into this subject, and the conclusion so far is that the cap acts as a physical impediment to the 5' to 3' exonucleases (Decker and Parker, 1994; Ross, 1995). The poly(A) tail is presumed to be involved in mRNA stability because many mRNAs become deadenylated before degradation and because poly(A) bound by PABP is known to protect mRNAs (Ross, 1995).

One possible role for deadenylation in mRNA instability may be due to the ability of the poly(A) tail to bind to the cytoskeleton (Taneja, 1992). By loss of the poly(A) tail, the mRNA moves to a different sub-cellular compartment, which may contain decapping enzymes. After decapping, degradation proceeds from the 5' to the 3' direction. Another explanation is that poly(A) could inhibit decapping by virtue of the binding of PABP by poly(A) and the resultant recruitment of eIFs to the cap. The presence of the eIFs on the mRNA is sufficient to protect the mRNA from being decapped and thus degraded. Finally, it is possible that PABP is important in stabilizing the mRNA. When the poly(A) tail is reduced to a critical size, PABP no longer protects the mRNA. The effective length of the poly(A) tail is the same length that allows PABP to bind in sufficient quantity to protect the mRNA (Sachs and Davis, 1989; Decker and Parker, 1994; Beelman and Parker, 1995). The concept of PABP as a protective protein against degradation is supported by the observation that it is synthesized in HeLa cells when they have been heat shocked, implying that it is a necessary factor to prevent cellular damage during heat shock (Schönsfelder et al., 1985).
Other portions of the mRNA molecule can also impact its stability. AU-rich elements (AUREs) were first identified in the tumour necrosis factor (TNF) mRNA 3' UTR and were found to regulate the stability of this mRNA (Caput et al., 1986). Known AUREs can be either the consensus AUUUA or they can be longer, containing predominantly (U) residues (Caput et al., 1986; Ross, 1995). Whenever these elements are found in the 3' UTR, the mRNA in which they were found is highly unstable. In addition, when an AURE is placed downstream of the coding sequence of a stable mRNA, the previously stable mRNA becomes unstable. Unfortunately, different AUREs function differently, and it is still unclear exactly how they function or what kind of degradation they induce. Degradation of both c-myc and c-fos is preceded by a deadenylation event, so it seems likely that the AURE could be functioning to induce one of the two deadenylation-dependent degradation pathways (Laird-Offringa et al., 1989; Brewer and Ross, 1988; Shyu et al., 1991; Ross, 1995). Recently, several protein complexes have been identified that can bind to the AURE of tumour necrosis factor α (TNFα), although the factors have yet to be characterized (Hel et al., 1996).

Other 3' UTR elements have been identified that cause an mRNA to become unstable. Two of these elements, the IRE in the transferrin receptor mRNA and a 3' terminal stem-loop in histone mRNAs, also form secondary structures (Ross, 1995). The function of these specific elements is to induce the cleavage of the mRNA at that site by an endonuclease. This cleavage then facilitates the further degradation of the mRNA. It has been proposed that although these elements seem to induce degradation by a pathway other than the deadenylation-dependent pathway, they still produce an mRNA that is functionally equivalent to a deadenylated mRNA due to the cleavage site in the 3' UTR (Ross, 1995).

Stability or instability elements can also reside in the coding sequence or in the 5' UTR. Examples include c-fos and c-myc mRNAs. Sometimes, mutations in the coding
sequence of these messengers result in different half-lives than their normal counterparts (Shyu et al., 1991). In addition, the half-lives of c-fos and c-myc mRNAs that lack their 3’ UTRs or the AUREs are still short compared to other mRNAs, and the mRNAs produced from translocated myc genes that lack the 5’ UTR are stabilized (Jones and Cole, 1987; Ross, 1995). At first, it was thought that the c-myc 5’ UTR contained destabilizing elements, but no evidence of this has been found (Jones and Cole, 1987). However, this translocation usually results in the c-myc coding sequence and 3’ UTR being brought under the control of an immunoglobulin gene. The half-lives of immunoglobulin mRNAs are different depending on the stage of cell differentiation (Jäck and Wabl, 1988; Ross, 1995). This could mean that the combination of the c-myc 3’ UTR with the immunoglobulin 5’ end may stabilize the mRNA.

In addition to specific elements that may function through the binding of specific factors, it has been noticed that most mRNAs are stabilized when exposed to translational inhibitors and that some mRNA half-lives are altered by changing mRNA secondary structures such that they affect translation (reviewed in Brown, 1993; Ross, 1995). Thus, by preventing translation, mRNAs can be stabilized. This mechanism could function through ribosome-associated mRNases that become activated by specific stimuli. One of these stimuli could be the presence of premature STOP codons in the mRNA. In yeast, it has been found that premature STOP codons in the 5’ end of the coding sequence are more destabilizing than those at the 3’ end. However, this mechanism could be regulated differently than the previously mentioned degradative mechanisms, because a mutation in the UPF gene in yeast stabilized mRNAs with premature STOP codons but had no effect on most wild-type mRNAs (Brown, 1993).

Germ cells and embryonic cells may also utilize different mechanisms of degradation than somatic cells. mRNAs are very stable in Xenopus oocytes, but after fertilization, mRNAs become less stable, and most are degraded by the time zygotic
transcription begins at the mid-blastula transition (MBT). However, the mechanism governing degradation still appears distinct during early development. Specific mRNAs that are deadenylated during maturation or after fertilization are not immediately degraded (Richter et al., 1990; Bouvet, et al., 1991). This implies—at least in eggs and early embryos in *Xenopus*—that the degradative pathway and the deadenylation pathway are uncoupled (Richter et al., 1990). In early development, components of the degradative machinery may be absent and must be synthesized or activated when transcription starts at the MBT in order to allow degradation of deadenylated mRNAs (Richter et al., 1990). This supports the idea that the mRNAs containing AUREs or U-rich elements are stable during early development but by the MBT stage these messengers become susceptible to deadenylation and then degradation.

Recently, a genetic screen in *Drosophila* has identified a new gene from ovarian cDNA that encodes the Clipper (CLP) protein (Bai and Tolias, 1996). CLP is a developmentally-regulated RNase that specifically cleaves RNA hairpins. The region that cleaves the RNA uses five copies of a CCCH zinc finger motif. CCCH zinc fingers are very rare, and until their characterization in the CLP protein, have never been found to mediate any kind of RNase activity (Bai and Tolias, 1996). There are indications that the developmentally-specific expression of this gene is regulated both at the transcriptional and at the post-transcriptional levels, possibly by phosphorylation at eleven putative phosphorylation sites (Bai and Tolias, 1996). More proteins like CLP must be identified in order to understand the mechanisms of RNA instability.

5. The c-myc messenger RNA

The *myc* gene is considered to be one of the “immortalization” class of oncogenes. In tissue culture cells, when an oncogene from the immortalization class such as *myc* is activated, it cooperates with activated *ras* to transform the cells in the culture. *myc* can rescue primary cultured cells from senescence without inducing tumourigenesis; the cells
become “immortal” (Piechaczyk et al., 1987). The function of the Myc protein has been difficult to characterize. Myc is thought to be a transcription factor because it binds DNA in a sequence-specific manner. Myc can dimerize with itself and with other factors such as Max; this dimerization is also known to be important to the function of Myc in transcriptional activation, induction of cell cycle progression and apoptosis (Amati et al., 1992; 1993). Myc may also be involved in DNA synthesis and in the synthesis and accumulation of rRNA (Piechaczyk et al., 1987; Marcu et al., 1992; Gibson et al., 1992; 1993).

The c-myc gene was first discovered due to its homology to v-myc in avian retroviruses of the MC29 family (Bernard et al., 1983). Activation of the c-myc gene can result in aberrant cell growth. Integration of the avian leukemia virus near the c-myc locus can cause chicken B lymphomas by stimulating c-myc expression. Similarly, stimulation of c-myc expression by chromosomal translocations in mammals can result in B lymphoid neoplasms, such as murine plasmacytomas or human Burkitt lymphomas. These translocations often occur between c-myc and the immunoglobulin heavy chain or light chain loci (Bernard et al., 1983). The breakpoint in myc is often near the 5’ portion of the transcriptional unit with some translocations cleaving the myc transcriptional unit (Bernard et al., 1983).

The c-myc gene contains three exons, two of which contain the coding and 3’ UTR sequences, whereas the upstream exon (exon 1) contains the majority of the 5’ UTR (Saito et al., 1983). The first exon of the mRNA can be initiated from either of two promoters, generating two different 5’ UTRs (Saito et al., 1983). Two observations indicate that loss of the 5’ UTR deregulates c-myc: (1) many chromosomal translocations in tumors result in the cleavage of the myc gene within the first intron; and (2) the first exon encodes only the 5’ UTR. The mammalian c-myc mRNA has the potential to form a large stem loop encompassing the first and second exons, with the initiation codon being
in the loop of the structure (Saito et al., 1983). It was proposed that this stem loop is responsible for regulating the translation of the c-myc mRNA, and by the removal of the first exon in many translocations, the translational repression offered by this structure would be eliminated, causing the dramatic stimulations of myc expression often observed in tumours (Saito et al., 1983). Other possible explanations are that the loss of the 5’ portion of the gene simply removes transcriptional inhibitory elements, the elements responsible for transcriptional attenuation, the loss of sequence element inhibitors of translation or the loss of elements responsible for the instability of the c-myc messenger.

5.1. Translation control of mammalian c-myc mRNA and its alternatives

Various authors have discussed the possibility that the mammalian c-myc 5’ UTR can inhibit translation. Both in vitro and in vivo approaches have been used with different results. The systems have included rabbit reticulocyte lysates (RRL), Xenopus oocytes, HeLa cell extracts and various tissue culture and tumour cell lines. The RNAs used often contained partial 5’ UTRs, varying lengths of the 3’ UTR, first introns instead of first exons, no second exon or 5’ ends from myc coupled with a CAT coding sequence. Despite all of the variation, there are some generalizations that can be made. Whenever a significant portion of the mammalian myc 5’ UTR is present upstream of any coding sequence (CAT or myc), there is always translational inhibition in RRL (Darveau et al., 1985; Parkin et al., 1988b; Lazarus et al., 1988). Additional experiments have shown that this is also true for certain myc constructs when translated in Xenopus oocytes or wheat germ lysates (Parkin et al., 1988b; Lazarus et al., 1988). However, when mammalian myc mRNA translation has been examined by transfection into mammalian tissue culture cells, the 5’ UTR does not inhibit translation (Persson et al., 1984; Butnick et al., 1985; Parkin et al., 1988b). Similarly, myc mRNA that lacks its 5’ UTR is not differentially recruited onto polysomes, compared to cells with a normal myc mRNA (e.g., Burkitt lymphoma; Nilsen and Maroney, 1984).
From these observations it would appear that mammalian myc mRNA is not translationally regulated \textit{in vivo} in mammalian cells. However, two recent papers describe an increase in the translation of c-myc in Bloom's syndrome and multiple myeloma cells (West \textit{et al.}, 1995; Paulin \textit{et al.}, 1996). In both cell types, there was an increase in Myc protein levels compared to mRNA levels. The authors demonstrate this increase by showing an increase in the recruitment of myc mRNA onto polysomes. However, in both cases, no translocations were found. In Bloom's syndrome, it appears as though a defect in DNA ligase I is responsible, but it is still unknown what cellular pathway could be responsible for this regulation (West \textit{et al.}, 1995). However, in multiple myeloma cells, a single base mutation in the 5' UTR is associated with a large number of cell lines that overexpress myc. It is still unknown if this mutation is responsible for the increased expression (Paulin \textit{et al.}, 1996). At this point, it is unclear if the mammalian c-myc 5' UTR can regulate the translation of the mRNA \textit{in vivo} or only \textit{in vitro}.

Other research has been conducted to determine if stimulation of expression by loss of c-myc transcript 5' ends could be the result of the loss of another regulatory mechanism. A block to transcriptional elongation, called transcriptional attenuation, has been previously identified for the mammalian c-myc gene. Transcriptional attenuation describes a mechanism in which transcription is initiated and then pauses and is prematurely terminated because some signal is encountered by the RNA polymerase (reviewed in Marcu \textit{et al.}, 1992). Further work has characterized a potential difference between the products of the two main promoters of c-myc in transcriptional attenuation when studied using a \textit{Xenopus} oocyte transcription assay (Meulia \textit{et al.}, 1992). However, upon further study, the increased attenuation seen with transcripts from P2 appeared to be because of a limit in the transcriptional machinery in \textit{Xenopus} oocytes; when injected RNA levels were decreased, the differential attenuation was lost. These results demonstrated that it is possible to change expression from different promoters by
changing availability of transcription factors (Spencer and Kilvert, 1993). Therefore, transcriptional attenuation can affect c-myc P1 and P2 differently in different cell types, providing an explanation for some observed differences in myc expression.

Knowing that translocations can shorten or eliminate the 5' UTR, it is possible that these truncations could impact mRNA expression. Xu et al., (1993) noted that when myc was co-transfected with ras, it could transform a Rat Embryo Fibroblast (REF) cell line. Transformation capabilities of a full-length myc or a myc gene with a 5' truncation were tested in this system. In the cells transfected with the 5' truncated myc, more foci formed sooner and were more readily established as a permanent cell line than in cells transfected with the full-length myc (Xu et al., 1993). This is in agreement with the observation that a truncated myc gene is formed by chromosomal translocations in some tumours. Further analysis demonstrated that even though the RNA turnover was the same for cell lines expressing either full-length or truncated myc mRNA, the truncated myc was expressed at higher steady-state levels. The lines expressing full-length myc displayed a block to transcriptional elongation (Xu et al., 1993). The conclusion from these results was that the tumourigenicity caused by removal of the 5' end of the myc gene could be due to an increased number of full-length transcripts being made from these genes because of the loss of the block to transcriptional elongation, not because of the loss of a translational inhibitory element. However, the two experiments mentioned earlier that described a translational stimulation of c-myc did not find any evidence for a loss of transcriptional attenuation in these cells (West et al., 1995; Paulin et al., 1996).

A protein has been identified in HeLa, multiple myeloma, Chinese hamster ovary (CHO) and mouse embryonal carcinoma cells that can bind to exon 1 of human c-myc RNA (Parkin and Sonenberg, 1989). However, the authors concluded that this protein was not involved in translational regulation because mRNAs lacking the binding sequence do not change their translational behaviour (Parkin and Sonenberg, 1989). These
experiments show that an increase in the level of Myc protein can result from several
different mechanisms.

5.2. Translation control of Xenopus c-myc1 mRNA

Due to the advantages of using the Xenopus system to study the regulation of
translation, Xenopus c-myc RNA was identified and characterized and cDNA and genomic
clones for two Xenopus c-myc genes were isolated (Taylor, et al., 1986; Godeau et al.,
1986; King et al., 1986; Vriz and Méchali, 1989; Vriz et al., 1989; Principaud and Spohr,
1991; King, 1991). During development, the majority of transcripts are from the c-myc1
gene. Similar to the mammalian c-myc gene structure, the Xenopus c-myc1 gene is
composed of three exons and two introns, with exon 1 encoding the majority of the 5’
UTR (King et al., 1986; Vriz et al., 1989; Principaud and Spohr, 1991). However, there
are significant differences between the two Xenopus c-myc genes and their mammalian
counterparts, especially in the untranslated regions. The 5’ UTR of Xenopus c-myc1
mRNA is much shorter than that of the mammalian c-myc mRNAs and shares no
sequence similarity with them. The 3’ UTR of Xenopus c-myc1 mRNA is much longer
than that of the mammalian c-myc mRNAs, and the only 3’ UTR homology seen among
seven different c-myc mRNAs from six species (Xenopus, chicken, mouse, rat, cat and
human) was in three identical blocks of sequence (King et al., 1986; Vriz and Méchali,
1989). One of these three blocks contains an AURE. Other than the coding sequence, the
basic structure of the gene and limited 3’ UTR homology, there is very little similarity
between the c-myc genes from Xenopus and mammals.

Even if sequence similarity is not conserved, other conserved mechanisms could
regulate the expression of c-myc; recall that HIV TAR is conserved primarily in its
secondary structure, not its sequence (Parkin et al., 1988a). This indicates a precedent for
the involvement of conserved secondary structures in the regulation of c-myc gene
expression.
Some analyses have been conducted into the levels of c-myc protein and mRNA expression during Xenopus development. Taylor et al. (1986) noted that Xenopus c-myc mRNA is abundant during oogenesis, with a peak in mid-oogenesis, but after fertilization there is a degradation of 90% of the mRNA until the gastrula stage when new synthesis appears to occur and the mRNA level stabilizes until at least the feeding tadpole stage. However, two other papers report quite different observations and interpretations. Godeau et al. (1986) report that the level of Xenopus c-myc mRNA per oocyte or embryo does not change dramatically during development. They demonstrate that the mRNA content in the embryo stays the same during early development, but that there would be a decrease in the amount of c-myc mRNA per cell due to cell division. King et al. (1986) claim that the level of c-myc mRNA in the oocyte and embryo remains the same through oogenesis and the rapid cleavage stages of development to the MBT. At MBT there is a rapid decline in the level of mRNA over several stages, then the mRNA level begins to gradually increase again, presumably due to the onset of zygotic transcription (King et al., 1986; Schreiber-Agus et al., 1993).

Myc protein is found in the oocyte and early embryo (Taylor et al., 1986; Godeau et al., 1986; King et al., 1986). Gusse et al. (1989) also show that the level of Myc protein is high in oocytes and embryos until at least MBT. However, it is unknown why the level stays high. There are two alternative explanations: (1) the Myc protein that was made during oogenesis could be very stable, or (2) the protein could become unstable after fertilization and Myc protein levels stay high because of increased translation. This, and the lack of consistent RNA data makes predictions about translational regulation difficult.

Given the evidence that the mammalian c-myc 5' first exons could inhibit translation in Xenopus oocytes and the possibility that there may be an increase in translation of myc post-fertilization, experiments were done to determine if the Xenopus c-myc 5' UTR could regulate translation. Lazarus (1992) subcloned portions of a
Xenopus c-myc genomic clone (King et al., 1986) upstream of a CAT reporter in order to determine if any portions of the 5' UTRs of the different mRNAs could affect translation differentially during development. Several of the sequences used were found to inhibit translation while two others did not. It was concluded that the mRNAs containing sequences transcribed from P1 or P0 (a putative upstream promoter) inhibited translation during oogenesis and after fertilization. This claim was based on the observation that one of the mRNAs that inhibited translation had a 5' UTR containing P1 and P0 transcribed sequences in addition to non-transcribed sequences. When the sequences that corresponded to the mRNA-encoding portion were removed, the inhibition by the remaining non-transcribed sequence was lower than with the entire sequence (Lazarus, 1992). However, the apparent inhibitory sequence that was removed was never tested to determine if it could inhibit translation by itself, without the non-inhibitory, non-transcribed sequence. Other than this experiment, there has been no work on the regulation of Xenopus c-mycI translation during Xenopus development.

5.3. c-myc messenger stability

c-myc mRNA has long been known to be unstable in somatic cells (Dani et al., 1984). Pulse-chase experiments in HeLa cells resulted in the assignment of a 15 to 20 minute half-life for the c-myc mRNA. It is still unclear how c-myc mRNA stability is controlled. It is possible that different cell types regulate c-myc mRNA stability differently. In HeLa or HL60 cells, addition of cycloheximide or emetine (protein synthesis inhibitors) prevented the degradation of c-myc mRNA and resulted in the apparent stimulation of the expression of myc. However, in MRC5 or Daudi cells the addition of protein synthesis inhibitors did not stabilize the c-myc mRNA (Dani et al., 1984).

Much work has gone into the characterization of the elements in the c-myc mRNA that may regulate its stability. All portions of the c-myc mRNA may be involved. The 5'
UTR may regulate the stability of c-myc mRNA, because the mRNA was stabilized in some neoplasms in which the 5' end of the c-myc gene has been removed by translocation (Jones and Cole, 1987). However, when the 5' UTR was placed upstream of a stable mRNA, no destabilization was noted (Jones and Cole, 1987). The apparent instability conferred by the 5' UTR could require an interaction with another portion of the mRNA. It is also possible that the truncated c-myc mRNA in tumour cells is stabilized by another change in the cells that could result in the loss of the degradative mechanisms that normally function with the c-myc mRNA.

Other elements that could regulate instability of the c-myc mRNA are the AUREs in the 3’ UTR, a U-rich element in the 3’ UTR or a sequence element near the 3’ end of the coding sequence (Jones and Cole, 1987; Brewer and Ross, 1988; Wisdom and Lee, 1991; Laird-Offringa et al., 1991; Bernstein et al., 1992; Morello et al., 1993). Some experiments indicate that the 3’ UTR is dispensable, whereas others show that the 3’ UTR alone can account for the destabilization. It is possible that the coding sequence and 3’ UTR elements may be redundant. This could explain why either can enhance degradation of the c-myc mRNA. Another explanation is that the regulatory mechanisms may be cell- or tissue-specific.

Degradation of the c-myc mRNA via the 3’ UTR seems to occur through a deadenylation-dependent mechanism (Brewer and Ross, 1988). The regions of the 3’ UTR apparently responsible for this degradation correspond to the area around the previously mentioned AURE; an additional U-rich element in this region may also be involved in the stability of the c-myc mRNA (Jones and Cole, 1987). An RNA-binding activity has been identified that can bind to elements in the 3’ UTR at the areas characterized as putative degradation elements (Brewer, 1991; Alberta et al., 1994).

c-myc mRNAs that lack all UTR sequences remain unstable (Wisdom and Lee, 1991; Laird-Offringa et al., 1991; Morello et al., 1993). The regulation of stability by a
putative coding sequence element is translation-dependent, even though translation through the putative element does not appear necessary (Laird-Offringa et al., 1991; Morello et al., 1993). Finally, an RNA-binding activity has been identified in the 3' region of the c-myc mRNA coding sequence. When this activity is competed out using this region of the c-myc mRNA, an even greater destabilization of the mRNA is observed. This activity may protect c-myc mRNA from degradation by blocking an instability element (Bernstein et al., 1992).

6. Objectives

Many cancers display regulatory mechanisms that are characteristic of regulation in a developing organism. By studying how normal gene expression is regulated during early development, one may gain insight into the mechanisms allowing aberrant gene expression in cancers. In my Ph.D. research, I studied the role of the Xenopus c-myc 5' and 3' UTRs in post-transcriptional gene expression during the early development of Xenopus laevis.

6.1. The role of the Xenopus c-myc 5' UTR in regulating gene expression

Many oncogenes have highly G+C-rich 5' UTRs with the potential to form stable secondary structures (Kozak, 1987). This indicates that oncogene 5' UTRs could be involved in the regulation of translation (Pelletier and Sonenberg, 1988; Kozak, 1989b; Fu et al., 1991). In addition, the mammalian and Xenopus c-myc 5' UTRs have been implicated in being involved in translational regulation. By understanding how the expression of this oncogene is regulated in development, the mechanisms behind its regulation in both normal and malignant cells can hopefully be elucidated. It was for this reason that an analysis of the role of the Xenopus c-myc 5' UTR in gene expression during early development was undertaken.

PCR was used to amplify the 5' first exons from both promoters of the c-myc1 gene. These PCR products were placed upstream of the CAT reporter in plasmids for
use in *in vitro* transcription. The effects on translation and stability by the 5' first exons of *c-myc* were tested using RNAs that contained no 3' sequences as well as mRNAs that contained a plasmid-encoded (A) tail—the (A)$_{73}$ element. No stability or translational differences were noted when either 5' UTR was placed upstream of CAT. I also investigated whether a mammalian *myc* 5' UTR and *Xenopus c-myc* genomic sequences could inhibit translation in *Xenopus*. These experiments were done as controls to confirm previous observations that the mammalian *c-myc* 5' first exons and portions of the *Xenopus c-myc* genomic clone can inhibit translation in *Xenopus* oocytes (Parkin *et al.*, 1988b; Lazarus *et al.*, 1988; Lazarus, 1992). In both cases, these sequences inhibited translation as was expected. This indicates that my results with the authentic *Xenopus c-myc* 5' first exons are more biologically relevant than those previously reported.

6.2. *The role of the Xenopus c-myc 3’ UTR in regulating gene expression*

Messenger RNA expression is highly regulated. Often different parts of the molecule can be involved in this regulation, and the 3' UTR is frequently involved in the regulation of gene expression during development. Because of this, it was decided to investigate the effect of the *Xenopus c-myc* 3' UTR on the expression of a CAT reporter, both alone and with the 5' first exons. Plasmids were engineered so that RNA could be synthesized *in vitro* that contained the CAT reporter with either the 5' UTRs of the *Xenopus c-myc* gene upstream and/or the 3' UTR of the same gene downstream.

Translation, stability and cytoplasmic polyadenylation were monitored to determine if the 3' UTR could impact any of these processes. It has been noted previously that the *myc* 3' UTR can stimulate rapid degradation. However, it has also been seen that the *Xenopus c-myc* mRNA changes its adenylation status during development, shifting from nonadenylated to polyadenylated after fertilization, but before the MBT (Tchang *et al.*, 1991). These observations made it difficult to predict whether there would be a decrease in translation due to the decreased stability or an increase in translation due to
cytoplasmic polyadenylation. My results indicate that the *Xenopus c-myc* 3' UTR stimulates an increase in translation. I also confirmed the observation of Tchang *et al.*, (1991) that the *Xenopus c-myc* 3' UTR can induce cytoplasmic polyadenylation. However, it is premature to conclude that the translational stimulation is due solely to the cytoplasmic polyadenylation until more experiments are conducted.

6.3. *Translational regulation by a synthetic hairpin element*

As a prelude to these investigations of the roles of the *c-myc* 5' and 3' UTRs, I studied the effects of synthetic 5' and 3' elements on translation. I first undertook to determine if a synthetic hairpin (hp) element placed upstream of a reporter coding sequence was capable of regulating translation in a developmentally specific manner. Previous observations were made that indicated that the hp elements could repress the translation of a reporter in *Xenopus* oocytes, but that this repression was relieved post-fertilization (Fu *et al.*, 1991). I was unable to confirm that observation.

The presence of an (A) tail can influence gene expression of the mRNA at both the level of stability and translation. I also studied how a plasmid-encoded (A) tail could impact hp-containing mRNA. Normally poly(A)-polymerase would be used to enzymatically add an (A) tail onto the end of the *in vitro*-transcribed mRNA. However, this procedure suffers from some serious drawbacks. The enzyme is very expensive, it is difficult to generate a homogenous population of adenylated RNAs, all with the same length of (A) tail and the enzyme is unreliable. To avoid problems associated with the use of this enzyme, other researchers have synthesized mRNA *in vitro* from plasmids that contain a plasmid-encoded (A) tail (Munroe and Jacobson, 1990). I inserted a poly(A) sequence from the plasmid pBS-poly(A) (a gift from Dr. A. Garber) downstream of hp-CAT in our plasmids. Studies were carried out to determine if the (A) tail could change the translational capabilities of the mRNA containing the hp. While examining the effect of the sequences from pBS-poly(A) on the translation of hpCAT mRNA, it was
discovered that what was thought to be only an (A) tail, actually included a portion of the trout creatine kinase (tCK) gene. In addition, the use of the wrong bacterial strain to maintain the plasmid culture resulted in the attenuation of the (A) tail from what was originally 68 (A)s to 17 (A)s. It was decided that any observations that were made using this (A) tail would be suspect for two reasons: (1) the length of the (A) tail was very short and (2) the 300 bases of the tCK mRNA could be responsible for any altered translational activity of the mRNA. However, a very significant stimulation of translation of hp-containing mRNAs was noted by this tCK(A)17 sequence (see Appendix 3 for the results).
METHODS AND MATERIALS

1. Competent bacteria and bacterial transformation

Competent cells were made by the calcium chloride (CaCl₂) method (modified from Sambrook et al., 1989). A starter culture of the desired strain was allowed to grow in LB broth overnight at 37°C. 1 mL of the starter was used to inoculate 50 mL of LB broth the next morning. This culture was allowed to grow at 37°C until it reached an OD₆₀₀ of about 0.6-1.0 by that afternoon. The flask was cooled for 10 minutes on ice, and the cells were centrifuged for 5 minutes at ~1000-1500xg. Cells were resuspended in 20 mL of 50 mM CaCl₂ and placed on ice for 50 minutes. The cells were pelleted for 5 minutes and then resuspended in 2 mL of 50 mM CaCl₂. These cells were then placed at 4°C overnight (12-24 hours). The next morning, cells were aliquoted for use or for long-term storage at -80°C in 15% glycerol.

Transformation of bacteria was done several ways depending on the efficiency needed. The lowest efficiency method was to add DNA in 10 μL to 80-100 μL of thawed competent cells. This mixture was incubated on ice for 30 minutes, then subjected to a 45 second heat shock at 42°C and returned to ice. All of the cells were then plated onto LB agar plates containing ampicillin (50 μg/mL; LB-Amp agar) and allowed to grow overnight.

Transformation efficiency was increased in a variety of ways. After the 30 minute incubation on ice, 100 μL LB broth was added to the cells, and they were allowed to recover at 37°C for 45-60 minutes. 50 to 200 μL of this cell suspension was plated on LB-Amp agar plates and allowed to grow overnight. If even greater efficiency were needed, three things could be done. First, the time on ice could be increased from 30 minutes to 45 minutes. Second, a 45-75 second heat shock at 42°C could be done before addition of the LB broth and recovery. Third, adding 800 μL of LB broth (instead of 100
μL) for recovery also increased efficiency. If this last step was done, the cells were
concentrated by a low speed centrifugation before plating.

2. DNA manipulations

All DNA manipulations were modified from Sambrook et al. (1989) unless
otherwise referenced.

2.1. Agarose gels and gel purification

DNA was resolved and visualized by electrophoresis on a 1.2% TAE-agarose gel
containing approximately 0.3 μg/mL ethidium bromide. DNA was gel purified as per
instructions in the Gene Clean kit (Bio101). If small-size DNA fragments were to be
isolated, two other techniques were used. For DNA between 150 and 500 base pairs
(bp), Gene Clean (Bio101) was used, but 5 μL of 10% acetic acid per 1 mL of sodium
iodide used was added before binding of the Glass milk, as per manufacturer’s
instructions. This allows more efficient binding and release of smaller DNA fragments.
For even smaller fragments, the MerMaid kit (Bio101) was used as per manufacturer’s
instructions.

2.2. Plasmid purification

Plasmids were purified for mid-scale preps (50 mL cultures) in two ways; the first
was by alkaline lysis. 50 mL of culture were pelleted by centrifugation at about 1500xg
for 10-15 minutes. The pellet was resuspended in 1 mL of Solution 1 (50 mM glucose,
10 mM EDTA, 25 mM Tris; pH 8.0). To this suspension, 2 mL of Solution 2 (0.2 N
NaOH, 1% SDS; made fresh) were added. This was mixed well, but not vortexed, and
incubated on ice for 5 minutes. 1.5 mL of Solution 3 were added (60 mL 5M KAc, 11.5
mL glacial acetic acid, 28.5 mL H2O; for 60 mL 5M KAc, 29.5 g KAc was dissolved in
about 54 mL H2O, pH to 7.5 with 2 M acetic acid and final volume was adjusted to
60 mL). The suspension was mixed well and incubated on ice for another 5 minutes.
This mixture was centrifuged for 15 minutes at 12,000xg and the supernatant transferred to a clean tube. If the supernatant was not clear, this spin could be repeated. The clear supernatant was then phenol/chloroform-treated twice and precipitated with 0.1 volumes 3 M sodium acetate (pH 8.0) and 2.5-3 volumes ice cold 95% ethanol for 15-30 minutes on dry ice or at -80°C. The DNA was pelleted with one centrifugation of 30 minutes at 12,000xg. The pellet was washed twice with 70% ethanol, dried and resuspended in approximately 250 µL of H₂O. If desired, RNase A could be added to Solution 1 just before resuspension in order to degrade any RNA in the sample.

The Wizard (Promega) midi-prep was also used for cultures of approximately 50 mL in size to purify plasmid DNA. The Wizard prep is faster, but tends to be less pure than by alkaline lysis purification. If cleaner DNA was needed from this prep, a phenol/chloroform step and ethanol precipitation were done to give similar quality as for alkaline lysis.

Wizard mini-preps were also used for small plasmid purifications (3 mL cultures). This procedure was done to make small amounts of DNA for use in the sequencing of new clones before doing a larger prep.

Rapid screening of a large number of clones utilized the “rapid mini-prep” procedure. 1.5-3 mL of culture were pelleted in a 1.5 mL Eppendorf tube at maximum speed in a microcentrifuge for 5 minutes. The pellet was resuspended in 20 µL STE + lysozyme (STE: 8% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA; a few crystals of lysozyme were added to 250 µL of STE just before use). To this, 100 µL of STET (STE plus 0.5% Triton X-100) and a few microliters of RNase A were added. The tubes were boiled for one minute either while keeping the caps loose or after punching a hole in the lid to allow the tubes to vent. Immediately after boiling, 200 µL of 0.3 M sodium acetate (pH 8.0) were added. Cell debris was pelleted by a centrifugation at 16,000xg. The supernatant was transferred to a clean tube and precipitated with an equal volume of cold
isopropanol for 10 minutes at room temperature. (For slightly better recovery, precipitation could be done at -20°C for 30 minutes.) The pellet was washed with 70% ethanol, dried and resuspended in about 50 µL of H₂O. This prep could not be used for HB101 strains of *E. coli* or others with the *endA* gene unless a phenol/chloroform step was added before precipitation. The *endA* gene produces an endonuclease that is activated in the presence of magnesium, which is common in restriction enzyme buffers.

2.3. PCR

Polymerase chain reaction (PCR) from plasmids was performed using the following formula: 10 µL of commercial *Taq* polymerase buffer (plus magnesium if necessary), 10 µL 10x dNTPs (2.5 mM each of the four dNTPs), 5 µL 20 µM primer #1, 5 µL 20 µM primer #2, 5 µL template DNA (1 ng or less) and H₂O to 99 µL. Five units of *Taq* DNA polymerase (BRL or Pharmacia) were added to the mix that was then overlaid with mineral oil. Generally, the PCR protocol was for one cycle at 95°C for 5 minutes followed by 25-30 cycles of 1 minute at 95°C to denature the DNA, 1 minute at 50°C to anneal primer to the DNA and 1 minute at 72°C for extension. The cycling was followed by a 10 minute “polish” at 72°C. The product was checked on a gel and gel purified if correct. PCR controls included (1) no template, (2) no polymerase and (3) no primers (Eeles and Stamps, 1993).

2.4. DNA ligations and digestions

Ligation of cohesive termini was carried out as described in Sambrook *et al.* (1989). The only exception to this protocol was the use of commercial ligation buffers containing their own ATP (BRL or Promega). Blunt-end ligations used the same protocol except when problems arose. If the usual ligation procedure did not work, the condensing agent PEG8000 was added to the ligation to a final concentration of 50 µg/mL (Sambrook *et al.*, 1989).
Standard DNA digestions were carried out as described by Sambrook et al., (1989). Mung Bean Nuclease (Pharmacia) was used to create blunt-ends from DNA digests as described by Sambrook et al. (1989).

2.5. DNA sequencing

DNA sequencing was carried out using standard protocols (Sambrook et al., 1989; Slatko and Albright, 1991). Sequencing reactions were done with the T7 Sequencing Kit (Pharmacia). The only exception was when the hp element was sequenced. To prevent compressions during electrophoresis, a formamide sequencing gel was used (Slatko and Albright, 1991).

3. Plasmid construction

All of the relevant constructs are diagrammed in Appendix 1 in alphabetical order. Unless otherwise noted, plasmids were transformed into either the HB101A (Munroe and Jacobson, 1990) or the XL1-Blue strain (Stratagene) of E. coli.

To make pSP6-CAT(A)\textsubscript{73}, the (A)\textsubscript{73} sequence from pBSK-As(-Hind III) was placed downstream of the chloramphenicol acetyltransferase (CAT) gene using the enzymes BamH I and Kpn I (pBSK-As was a gift from Dr. J. Richter, Worcester Foundation for Biomedical Research, Shrewsbury, Mass.). In order to examine the effects of c-myc first exons on translation, various c-myc sequences were inserted at the Hind III site that was downstream of the SP6 promoter and upstream of the CAT reporter. PCR primers were designed that would amplify the first exon of Xenopus c-myc I from both promoters 1 and 2. The PCR primers also contained a Hind III cloning site that would be used to insert the amplified sequence into pSP6-CAT(A)\textsubscript{73}. PCR was performed on a Xenopus genomic clone (King et al., 1986). The purified (GeneClean; Bio101) and Hind III-digested PCR fragments were then cloned into pSP6-CAT(A)\textsubscript{73} that had been cut with Hind III. The resultant plasmids were called pSP6-Xc-mycIP1-CAT(A)\textsubscript{73} and pSP6-Xc-mycIP2-CAT(A)\textsubscript{73}. The insert orientation was confirmed by DNA restriction
enzyme mapping, and the PCR fidelity was confirmed by sequencing (\textsuperscript{T7}Sequencing Kit, Pharmacia P-L Biochemicals).

pSP6-Hc-mycP1-CAT(A)\textsubscript{73} was made by subcloning into pSP6-CAT(A)\textsubscript{73} a PCR-amplified human c-myc first exon from promoter 1 obtained from myc1-pIRV (Xu, 1994). Orientation was confirmed by sequencing and DNA restriction mapping.

pSP6-XDD-CAT(A)\textsubscript{73} was made as described by Lazarus (1992) by isolating the 638 bp \textit{Dpn I} fragment from the \textit{Xenopus} genomic clone provided by Dr. M. King (King \textit{et al.}, 1986). This was then inserted into the blunt-ended \textit{Hind III} site of pSP6-CAT(A)\textsubscript{73}. Orientation was confirmed by sequencing and DNA restriction mapping.

In order to generate constructs containing the 3' UTR of the \textit{Xenopus} c-mycI gene downstream of the CAT coding sequence, PCR was used. Primers to the 3' UTR of the \textit{Xenopus} c-mycI genomic clone (King \textit{et al.}, 1986) were designed to amplify the entire 3' untranslated region. These PCR primers were engineered with restriction enzyme sites that could allow the directed insertion of the 3' UTR into pSP6-CAT(A)\textsubscript{73} at its \textit{BamH I} and \textit{Xba I} sites to generate pSP6-CAT-Xc-mycI(A)\textsubscript{73}. Sequencing and restriction mapping were used to ensure PCR fidelity and correct insert orientation. This 3' UTR element was also subcloned into pSP6-Xc-mycIP1-CAT(A)\textsubscript{73} and pSP6-Xc-mycIP2-CAT(A)\textsubscript{73} to generate pSP6-Xc-mycIP1-CAT-Xc-mycI(A)\textsubscript{73} and pSP6-Xc-mycIP2-CAT-Xc-mycI(A)\textsubscript{73}.

In order to make constructs that contained truncations of the \textit{Xenopus} c-mycI 3' UTR, a \textit{Sca I} site in the 3' UTR was utilized (see Figure 2E). To make pSP6-CAT-prox(A)\textsubscript{73}, the plasmid pSP6-CAT-Xc-mycI(A)\textsubscript{73} was digested with \textit{Sca I}. The fragment containing the 3' end of the CAT coding sequence and the 5' end (or proximal sequence; 641 bases) of the \textit{Xenopus} c-mycI 3' UTR was isolated. Linkers for the restriction enzyme \textit{Pst I} were then added onto the \textit{Sca I} blunt-ends. Finally, this
AAGCTTatttt gcgggaggac cgccgctata taaggctcag tcggagcttt 50
ttgtgtgcca gctctggtgc gggaggcag cagggaatgt gggtgtgctag 100
cctgctgccgc agggaggagc agtagtactg gggaaacgaa cgcctattcg 150
acaagggaga gcacacccgttg gatättataaa acgcagcaccag aggaatatgt 200
gactcgtatt atccggggtg tagacggAAG CTT 233

A. Xenopus c-mycI promoter 1 to intron 1

AAGCTTagtc tgtgtgctcgg gacgcgcagac gaagtgtgtg ggtgcagctg 50
cctgctgccgc agggaggagc agtagtactg gggaaacgaa cgcctattcg 100
acaagggaga gcacacccgttg gatättataaa acgcagcaccag aggaatatgt 200
tgtttattttt ctcggaggtg aaggtggagc cgcctattcg 170

B. Xenopus c-mycI promoter 2 to intron 1

C. XDD sequence

D. Human c-myc sequence from promoter 1
E. *Xenopus* c-mycI 3' UTR

**Figure 2:** Sequences of the 5' and 3' inserts. Methods for inserting these sequences are described in the text, and diagrams of the completed plasmids are shown in Appendix 1. (A), (B) and (D) are the PCR-amplified 5' first exons from c-myc genes with the *Hind* III sites shown in capital letters that were used to insert them into the plasmid. (C) The XDD sequence, from two blunt *Dpn* I sites in capital letters, which was inserted at a blunt *Hind* III site in the plasmid. The bases indicated in boldface are the P1 and P2 start sites. The underlined sequence denotes a region that lies either upstream of the mRNA coding sequence or in intron 1. (E) The *Xenopus* c-mycI 3' UTR sequence that was amplified for insertion into the plasmid. The bases in capital letters are the *BamH* I and *Kpn* I sites that were used for insertion. The underlined sequence is the *Sca* I site that was used to generate the prox and dist sequences.
portion of the 3' UTR was cut with *BamH* I to remove the CAT coding sequence portion so it could be cloned back into pSP6-CAT(A)\textsubscript{73} that had been cut at *BamH* I and a new *Pst* I site that replaced *Xba* I. To make pSP6-CAT-dist(A)\textsubscript{73}, pSP6-CAT-Xc-mycI(A)\textsubscript{73} was cut with *Sca* I and *Xba* I to isolate the 3' portion (the distal portion; 314 bases) of the *Xenopus c-mycI* 3' UTR. This was then subcloned into pSP6-CAT(A)\textsubscript{73} that had been cut first with *BamH* I and blunt-ended and then with *Xba* I.

Plasmids pCAT and phpCAT are described in Fu *et al.* (1991), and pLuc is described in Fu (1992). The plasmid phpCAT has an inverted repeat derived from the multiple cloning site (MCS) of pUC-12 inserted at the *Hind* III site of the pCAT plasmid. This element has the potential to form a stable hairpin near the cap site of the 5' UTR of the transcribed RNA.

To generate pSP6-CAT-tCK(A)\textsubscript{17}, the first step was to isolate the CAT coding sequence from CAT-73(*BamH* I) by digesting with *Xho* I and *EcoR* V. This was placed into pSP73 (Promega) cut with *Sal* I and *Pvu* II to make a plasmid called pCAT-Cassette. pCAT-Cassette was designed as a flexible donor for the CAT coding sequence. A CAT fragment was isolated from pCAT-Cassette with Sau3A I and placed into the *BamH* I site of pBS-poly(A)(-*Hind* III). A CAT-tCK(A)\textsubscript{17} fragment was isolated by digestion with *Hind* III and *Kpn* I and placed into pGEM3 cut with the same enzymes. pSP6-hpCAT-tCK(A)\textsubscript{17} was made by insertion of the hairpin sequence into the *Hind* III site of pSP6-CAT-tCK(A)\textsubscript{17}. This was confirmed by sequencing and DNA restriction mapping. All of these plasmids were transformed into DH5\textalpha{} (BRL) *E. coli*.

4. *in vitro* transcription

4.1. Cold capped mRNA

Plasmids were linearized with *BamH* I and used as templates for synthesis of RNA according to standard transcription protocols (Melton *et al.*, 1984; Melton, 1987; Yisraeli and Melton, 1989) using 0.5 mM each of ATP, CTP and UTP, 0.1 mM GTP and
0.5 mM m⁷GTP. Transcription buffer, RNA polymerase and 0.1 M DTT were purchased from BRL or Promega; RNase inhibitor was purchased from Pharmacia or Promega; nucleotides and DNase I were obtained from Pharmacia; and m⁷GTP cap analog was purchased from Ambion or Pharmacia.

The mRNA was cleaned with the RNAid kit (Bio101). A 100 μL transcription reaction was typically adsorbed to 20 μL RNAMATRIX and eluted in 30–50 μL sterile water. The amount of RNA was determined spectrophotometrically, and the quality was determined by running an aliquot through an ethidium bromide-containing 1.1% agarose- TAE gel.

Schematic diagrams of all of the capped mRNAs used are presented in Figure 23 in Appendix 1.

4.2. Radiolabeled capped mRNA

Virtually the same protocol was used as above with a few exceptions. Nucleotide concentrations were the same except that 560 kBq of ³²P-UTP (IsoBlue; ICN) were added to the mix. The total volume of the reaction was only 20 μL, and it was cleaned with 10 μL of RNAMATRIX from the RNAid kit and eluted into 15 μL H₂O. Quantification was also done by spectrophotometer, but quality of the mRNA was checked by resolving 1 μL by 4% Urea-polyacrylamide gel electrophoresis (4% Urea-PAGE). In addition, cpm/μL were measured to ensure sufficient label incorporation.

The plasmids used for RNA synthesis are summarized in Table 1.

4.3. Probes for RNase protection

Probes were synthesized in a 20 μL uncapped in vitro transcription reaction. This reaction is as described for capped in vitro transcription except that no m⁷GTP was used, and the GTP concentration was increased to 0.5 mM. The CAT probe was produced by cutting the pCAT plasmid with Sca I and transcribing using the SP6
Table 1: Plasmids used for synthetic capped mRNAs

<table>
<thead>
<tr>
<th>RNA Synthesized</th>
<th>Plasmid Used</th>
<th>Map Location</th>
<th>Restriction Site Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT(A)(^*)</td>
<td>pSP6-CAT(A)$_{73}$</td>
<td>Fig. 22 E</td>
<td></td>
</tr>
<tr>
<td>CAT(A)$_{73}$</td>
<td>pSP6-CAT(A)$_{73}$</td>
<td>Fig. 22 E</td>
<td></td>
</tr>
<tr>
<td>CAT-dist(A)(^*)</td>
<td>pSP6-CAT-dist(A)$_{73}$</td>
<td>Fig. 22 F</td>
<td></td>
</tr>
<tr>
<td>CAT-myc(A)(^*)</td>
<td>pSP6-CAT-Xc-mycI(A)$_{73}$</td>
<td>Fig. 22 I</td>
<td></td>
</tr>
<tr>
<td>CAT-myc(A)$_{73}$</td>
<td>pSP6-CAT-Xc-mycI(A)$_{73}$</td>
<td>Fig. 22 I</td>
<td></td>
</tr>
<tr>
<td>CAT-prox(A)(^*)</td>
<td>pSP6-CAT-prox(A)$_{73}$</td>
<td>Fig. 22 G</td>
<td></td>
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<tr>
<td>CAT-tCK(A)$_{17}$</td>
<td>pSP6-CAT-tCK(A)$_{17}$</td>
<td>Fig. 22 H</td>
<td></td>
</tr>
<tr>
<td>hpCAT(A)(^*)</td>
<td>pSP6-hpCAT-tCK(A)$_{17}$</td>
<td>Fig. 22 K</td>
<td></td>
</tr>
<tr>
<td>Hc-mycCAT(A)(^*)</td>
<td>pSP6-Hc-mycP1-CAT(A)$_{73}$</td>
<td>Fig. 22 J</td>
<td></td>
</tr>
<tr>
<td>Hc-mycCAT(A)$_{73}$</td>
<td>pSP6-Hc-mycP1-CAT(A)$_{73}$</td>
<td>Fig. 22 J</td>
<td></td>
</tr>
<tr>
<td>Luc(A)(^*)</td>
<td>pLuc</td>
<td>Fu, 1992</td>
<td></td>
</tr>
<tr>
<td>P1-CAT(A)(^*)</td>
<td>pSP6-Xc-mycIP1-CAT(A)$_{73}$</td>
<td>Fig. 22 L</td>
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<td>P1-CAT(A)$_{73}$</td>
<td>pSP6-Xc-mycIP1-CAT(A)$_{73}$</td>
<td>Fig. 22 L</td>
<td></td>
</tr>
<tr>
<td>P1-CAT-myc(A)(^*)</td>
<td>pSP6-Xc-mycIP1-CAT-Xc-mycI(A)$_{73}$</td>
<td>Fig. 22 N</td>
<td></td>
</tr>
<tr>
<td>P2-CAT(A)(^*)</td>
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<td>Fig. 22 M</td>
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<td>P2-CAT(A)$_{73}$</td>
<td>pSP6-Xc-mycIP2-CAT(A)$_{73}$</td>
<td>Fig. 22 M</td>
<td></td>
</tr>
<tr>
<td>P2-CAT-myc(A)(^*)</td>
<td>pSP6-Xc-mycIP2-CAT-Xc-mycI(A)$_{73}$</td>
<td>Fig. 22 O</td>
<td></td>
</tr>
<tr>
<td>XDD-CAT(A)(^*)</td>
<td>pSP6-XDD-CAT(A)$_{73}$</td>
<td>Fig. 22 P</td>
<td></td>
</tr>
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<td>XDD-CAT(A)$_{73}$</td>
<td>pSP6-XDD-CAT(A)$_{73}$</td>
<td>Fig. 22 P</td>
<td></td>
</tr>
</tbody>
</table>
promoter to generate an anti-sense probe to the 3’ end of CAT transcripts (approximately 124 bases were protected). Luc probe was generated by cutting a truncated Luc coding sequence with Xba I and transcribing with SP6 RNA polymerase to generate a probe that protected approximately 540 bases. However, this probe was AU-rich, and a portion of the sequence that was especially AU-rich generated a hypersensitive site for RNase protections because of the localized decrease in melting temperature. Thus, this probe gave two specific protection products.

5. Collection of oocytes

A fully mature female was anesthetized in 0.25% Tricaine (Sigma A5040), and ovarian fragments were removed from the body cavity and placed in Modified Barth’s-HEPES Solution (MBS-H; this and other solutions used for oocytes, eggs, and embryos are described in Kay and Peng, 1991). The body wall was sutured using Ethicon 4-0 plain gut (S101H), and the skin was sutured with Ethicon 3-0 braided silk (A-54). The frog recovered in shallow tap water until she was able to swim. For storage of oocytes for up to 3 days at 18-19°C, the MBS-H was supplemented with 10 $\mu g/mL$ each penicillin-G (Sigma PEN-NA) and streptomycin sulfate (Sigma S6501). Stage VI (Dumont, 1972) oocytes were removed from the ovarian fragments using watchmaker forceps (Dumont No.5) and transferred to a fresh dish of MBS-H until used.

6. Collection of embryos

Females that had not ovulated for at least two months were injected subcutaneously into the dorsal lymph sac with 800 IU human chorionic gonadotropin (Sigma CG 10) approximately 12 hours before the eggs were needed.

Testes were removed from a Tricaine-anesthetized male. They were placed in 4.5 mL 110% Marc’s Modified Ringer’s (MMR) supplemented with 0.5 mL Fetal Calf
Serum and could be stored for several days at 4°C. A testis was macerated in a drop of MBS-H, and the resulting sperm suspension was used to fertilize eggs.

Eggs to be fertilized were squeezed into 34% MBS-H, and sperm suspension was added. The dish was shaken gently for 5 minutes and then kept still for 15 minutes. If the majority of eggs showed evidence of fertilization, the batch was de-jellied with 2% cysteine-HCl in Steinberg’s solution neutralized to pH 7.8 with 10 M KOH. The embryos were washed well with Steinberg’s solution and used as needed (Heikkila et al., 1985).

7. Coenocytic embryos

Fertilized eggs that were dejellied were washed in 110% MMR made with 5% Ficoll. The embryos in 5% Ficoll/MMR were layered over a 50% Ficoll solution in small centrifuge tubes. These were then centrifuged at 500xg for 10 minutes. The packed coenocytic embryos were gently removed from the centrifuge tube, washed in 5% Ficoll/MMR and placed in 5% Ficoll/MMR to allow development. Coenocytes were staged relative to untreated siblings. Only coenocytes that showed evidence of unsuccessful cleavages were injected. This was evidenced by the development of dark lines that indicate failed cleavage furrows upon the initially white surface of the embryo (Newport and Kirschner, 1982b).

8. Translation lysate

An in vitro translation lysate was made from Xenopus fertilized eggs and embryos following a modified version of the protocol of Matthews and Colman (1991). The only changes were: (1) the eggs were squeezed into MBS-H and fertilized as described in Section 6; (2) embryos were incubated in Steinberg’s solution containing 10 μg/mL each penicillin-G and streptomycin sulfate to reduce bacterial growth while embryos were allowed to develop; and (3) embryos were placed into Steinberg’s 5% Ficoll for a few
minutes before beginning the procedure to reduce the volume of the perivitelline space in the hope of reducing the contamination with RNases. 100 μL of translation mix was supplemented with 2.5 μL 350 mM creatine phosphate and 1 μL 120 mM spermidine before RNA was added for translation. The reaction was stopped by adding 0.25 M Tris (pH 7.8) to a volume of 100 μL.

9. Microinjection, in vivo translation and sample preparation

Needles used for microinjections were made with glass capillary tubes (1.5-1.8 x 100 mm, Kimax-51, CanLab). The tubes were siliconized with SigmaCote (Sigma SL2), dried either overnight at room temperature or for 1 hr at 65°C, rinsed with chloroform, dried and autoclaved. They were then pulled to form needles using a Narashige needle-puller (magnet setting 6; 17 amps).

A MicroLab P (Hamilton Instruments) pipettor was adapted for use in microinjection according to Hitchcock and Friedman (1980) and adjusted to deliver 50 nL per cell. In all experiments, molar equivalent amounts of RNA were injected to insure that the amount of CAT coding capacity was equal when injecting RNA with different-sized untranslated elements. After injection, the cells were transferred to medium (MBS-H for oocytes, Steinberg’s solution for embryos and MMR for coenocytes) containing 4–5% Ficoll Type 400 (Sigma F4375) to incubate at room temperature for the desired length of time.

10. CAT assays, thin layer chromatography and quantification

The samples were incubated at room temperature for 2 hours and then washed with 0.25 M Tris-HCl pH 7.8 (CAT assay buffer) in 1.5 mL microtubes. The buffer was removed, and each sample was homogenized in 50 μL CAT assay buffer. The samples were then centrifuged at 14,000 rpm in an Eppendorf microfuge for 5 minutes. The supernatant was transferred to a new tube.
An appropriate amount of each sample was diluted to 120 μL with CAT assay buffer. Care was taken to ensure that all CAT assays were within the linear range of the reaction. To each sample, 40 μL of reaction mix were added [0.67 μL
\[14^C\text{-chloramphenicol (NEN NEC-408), 70 μg acetyl co-enzymeA (Sigma A2181) in CAT assay buffer},\]
] and the reaction was incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 mL of ethyl acetate (BDH B10108). After vigorous mixing, the samples were microfuged at 14,000 rpm for 5 minutes, and 900 μL of the ethyl acetate were transferred to a new tube. The solvent was dried in a Savant SpeedVac attached to a water aspirator.

Each dried sample was dissolved in 10 μL ethyl acetate and spotted onto TLC plates (Whatman PE-SIL-G 4410-221). The reactants were resolved in about 30-50 minutes by ascending chromatography in 95% chloroform, 5% methanol. The plates were dried and exposed to Kodak XARS film or placed on a Fuji BAS-IIIS imaging plate (IP) for the Fujix BAS 1000 phosphorimager.

To determine the amount of chloramphenicol that was acetylated (and hence the amount of enzyme present in the sample), two different methods were used to quantify the percent conversion of \[14^C\text{-chloramphenicol. For scintillation counting, TLCs were quantified by cutting out each lane on the TLC plate. The lower major spot down to the origin (which represents unconverted }14^C\text{-chloramphenicol) was counted separately from the upper two spots up to the solvent front (which represents acetylated forms of }14^C\text{-chloramphenicol). The image from the phosphorimager could be quantified on-screen in the same way; i.e., by quantifying the upper and lower portion of each lane separately. These values were then used to calculate the percentage of total counts present as acetylated forms of }14^C\text{-chloramphenicol. Comparable results were obtained with either method.}

}
11. RNase protection assays

Oocytes or embryos were injected as described above. After a 2 hour incubation they were rinsed in sterile distilled H₂O in sterile 1.5 mL microfuge tubes. The samples were stored at -80°C until needed. Total RNA was isolated by proteinase K and phenol/chloroform, followed by precipitation with ethanol and then with 8 M lithium chloride (Evans and Kay, 1991). The RNA was dried and resuspended in 19 μL hybridization buffer (8.3 mL formamide, 0.8 mL 5 M NaCl, 0.8 mL 0.5 M PIPES pH 6.4, 0.1 mL 0.1M EDTA). 1 μL each of 35S-labeled CAT probe and Luc probe (10⁵ cpm) was added to the RNA, and hybridization was permitted overnight at 47°C.

0.35 mL digestion solution (10 mM Tris-HCl pH 7.6, 5 mM EDTA, 40 μg/mL RNase A) was added to each sample and incubated at 28°C for 30 minutes. 20 μL 10% SDS and 5 μL 10 mg/mL proteinase K were then added and incubated for 10 minutes at 28°C.

One phenol/chloroform extraction was done, and 4 μg yeast tRNA were added to each sample followed by 1 mL 95% ethanol. The samples were chilled at -70°C for 15–20 minutes and microfuged for 15 minutes. The pellet was washed once with 70% ethanol and air-dried. The pellet was resuspended in 2 μL diethyl pyrocarbonate (DEPC)-treated water followed by addition of 2 μL gel dye solution (0.3% each bromophenol blue and xylene cyanol FF; 10mM EDTA (pH 7.5); 97.5% deionized formamide), loaded onto a 4% urea-PAGE and run at 42–45 W until the bromophenol blue had just reached the bottom of the gel. The gel was then dried and exposed to Kodak XAR5 film.

12. Labeled RNA recovery and visualization

Oocytes or embryos injected with ³²P-RNA were collected after a 2 hour incubation and frozen immediately upon dry ice. The samples were stored at -80°C until
needed. Total RNA was isolated using TRIzol®Reagent (GibcoBRL) followed by an additional precipitation using 8 M LiCl, if necessary.

Radiolabeled RNA that was recovered from oocytes or embryos at either 0 hours or 2 hours post-injection was resolved on 4% urea-PAGE to determine the extent of degradation of the mRNA. Dried gels were exposed to a Fuji BAS IIIS imaging plate that was read by the Fujix BAS 1000 phosphorimager. The program MacBAS (version 2.2 by Fuji film) was used to visualize and quantify the gels. This same recovery technique was used to purify RNA that was to be used to monitor for cytoplasmic polyadenylation (see below).

13. Monitoring poly(A) tail length by RNase H analysis

Analysis of the extent of polyadenylation with RNase H was modified from Mercer and Wake (1985). A schematic of the procedure can be found in Figure 16 (page 91 in the Results section. Purified RNA was resuspended in 20 μL of 1 mM EDTA. This was then heated to 80°C for 10 minutes to denature the RNA. Hybridization of 0.3 μg of oligo, and 0.5-2.0 μg poly d(T)15 if necessary, was done by adding the DNA to the RNA and incubating first for 10 minutes at room temperature, then adding 1 μL of 4 M KCl and incubating 10 more minutes at room temperature. After hybridization, 20 μL of TM Buffer (56 mM MgCl2; 40 mM Tris; pH 8.0) and 0.5-1.0 units of RNase H (Pharmacia) were added to the DNA/RNA hybrid mixture. RNase H specifically digests the RNA portion of an DNA/RNA hybrid. Digestion was allowed to proceed for 30 minutes at 37°C. After digestion, the samples were treated with phenol/chloroform once and ethanol precipitated as usual. Cleaned samples were then resuspended in 2 μL of DEPC-treated water and 2 μL loading dye (see above) and loaded onto a 4% Urea-PAGE. Dried gels were then exposed to the imaging plate for one to two days.
Figure 3 outlines the quantification procedure used for RNase H gels. The band and the smear above the band were quantified separately using boxes A and B. Backgrounds for boxes of the same size were subtracted from the values obtained (boxes 4A and 4B). The two areas from one lane (minus background) were then added together to get a total number of units for the lane. The number in box A was then used to generate a number indicating the percent of units in the area above the band; called percent adenylated.

14. Statistical Analysis

The method chosen for statistical analysis was non-parametric statistics because of the large variability in values and the necessity to adjust all activity relative to CAT(A). It is this adjustment and the variability that makes the data unsuitable for standard statistical analysis. The method used was the Mann-Whitney U-test for non-parametric statistics (a two sample version of the Kruskal-Wallis test). This test assigns a rank to all values included and then calculates the probability that the ranked values of the two variables [e.g., CAT(A) and CAT- myc(A)] could occur by chance. It indicates whether there is an overall similarity or difference between the values given for the variables. A computer program called Data Desk® 5.0 Test Flight (©1995) was used to calculate the Mann-Whitney U-tests for the samples. Results are designated as being either statistically significant (p<0.05 that the values ranked could occur by chance), highly significant (p<0.01) or not significant. The results of all statistical analyses are presented in Appendix 2.
Figure 3: Diagram to explain quantification of the RNase H gels. See text for details.

\[ \text{Total units for lane 1} = [\text{Box 1A-(Box 4A)}] + [\text{Box 1B-(Box 4B)}] \]

\[ \% \text{ adenylated} = \frac{[\text{Box 1A-(Box 4A)}]}{\text{Total units for lane 1}} \times 100 \]
RESULTS

1. Effects on gene expression of c-myc 5' elements

Many oncogene, growth factor and growth factor receptor mRNAs are predicted to contain extensive secondary structures in their 5' UTRs (Kozak, 1987). One of the few complete cDNAs that has been cloned for a Xenopus gene is the Xenopus c-mycI gene (King et al., 1986; King, 1991; Principaud and Spohr, 1991). It was for this reason that I chose the 5' UTR of the Xenopus c-mycI mRNA to determine if a naturally-occurring 5' UTR from a growth-related transcript could impact the translation of a reporter. Previous attempts to examine the effects of the myc 5' first exons on translation had produced variable and inconclusive results, likely due to the use of non-homologous systems.

1.1. The authentic Xenopus 5' first exons

When I initiated my studies, Lazarus (1992) had reported translational inhibition by Xenopus c-mycI 5' UTRs. However, none of the constructs that were used by Lazarus (1992) actually tested the authentic 5' first exons without including non-transcribed or intron sequences. I chose to test the effect of the authentic 5' first exons from both promoter 1 (P1) and promoter 2 (P2) of the Xenopus c-mycI gene on the translation of a CAT reporter. I designed PCR primers that specifically amplified the desired sequences and that contained terminal Hind III restriction sites, which were used to place the 5' first exons upstream of the CAT coding sequence. Transcripts from these constructs were microinjected into Xenopus oocytes and early embryos to monitor for their effect on translation or stability.

1.1.1. Effects on CAT activity

To examine the effects of the 5' first exons on translation, mRNA either with or without the Xenopus c-mycI 5' first exons was injected into either stage VI oocytes or
early embryos (Figure 4). No significant effect of the 5' first exons on CAT activity was observed. See Appendix 2 for statistical analysis of these data.

1.1.2. The effect of (A)$_{73}$

The presence of a poly(A) tail is a hallmark of most messenger RNA molecules. Poly(A) is known to enhance both the translation and stability of mRNA. To determine if an (A) tail could potentiate the effects of c-myc 5' first exons, I engineered plasmids containing an (A) tail of approximately 73 (A) residues downstream of CAT, as described in Methods and Materials. To determine if the (A)$_{73}$ element could enhance translation, mRNA was transcribed in vitro either with or without the (A)$_{73}$ element and with the P1 or P2 first exons or with no *Xenopus* 5' sequences. This RNA was injected into *Xenopus* stage VI oocytes and early embryos. The presence of (A)$_{73}$ enhanced CAT activity from every transcript when compared to their (A)$^-$ counterparts. There was a significant difference between CAT(A)$^-$ and CAT(A)$_{73}$ and between both P1-CAT(A)$_{73}$ and P2-CAT(A)$_{73}$ and their non-adenylated counterparts (Appendix 2; Figure 5). However, statistical analysis showed no significant difference between either P1-CAT(A)$_{73}$ or P2-CAT(A)$_{73}$ and CAT(A)$_{73}$. Hence, the (A)$_{73}$ element enhanced CAT activity regardless of the developmental stage or the presence of c-myc 5' first exons.

1.2. The human c-myc first exon

Upon discovery that the *Xenopus* 5' first exons did not inhibit CAT activity, I examined whether a mammalian c-myc first exon was inhibitory upon injection into *Xenopus* oocytes as previously reported (Parkin et al., 1988b). The human c-myc 5' first exon from promoter 1 (P1), which had been amplified by PCR (Xu, 1994), was subcloned from *myc1-pIRV* into a position upstream of the CAT coding sequence in pSP6-CAT(A)$_{73}$ (see Methods and Materials). RNA transcribed from these constructs was then injected into stage VI oocytes or early embryos of *Xenopus*. The human c-myc P1 5' first exon sequence markedly decreased CAT activity in both stages of development.
Figure 4: The effects of the authentic *Xenopus* c-mycI 5' first exons on CAT activity. The experiments were performed as described in Methods and Materials. Diagrams of the RNAs used are in Figure 23 of Appendix 1. The equivalent of 0.5 ng of CAT(A)− RNA was injected into each of five (A) stage VI oocytes or (B) early embryos. A total of $\frac{1}{10}$ of a cell was assayed for CAT activity. Shown here is a representative experiment of the entire data set. No statistical significance was attributed to any differences observed (see Appendix 2).
Figure 5: The effect of the (A)$_{73}$ element on CAT activity. Experiments were done as described in Methods and Materials and in the legend to Figure 4. The (A)$_{73}$ stimulated CAT activity from all transcripts to the same extent (Appendix 2).
(Figure 6). This is in agreement with previous reports that mammalian myc 5' UTRs are inhibitory in Xenopus oocytes (Parkin et al., 1988b). As shown in Figure 6, CAT activity for Hc-mycCAT(A)$_{73}$ was 25.9 fold greater than Hc-mycCAT(A) for stage VI oocytes and 792.0 fold greater in early embryos, whereas CAT(A)$_{73}$ was only 1.7 fold greater than CAT(A) for stage VI oocytes and 3.1 fold greater for early embryos. Thus, Hc-myc had a much smaller inhibitory effect when (A)$_{73}$ was present. This difference was determined to be highly significant (Appendix 2).

The contradiction between the effects of Xenopus and human 5’ first exons suggests that there are substantial functional differences between these 5’ UTRs. An alignment of the 5’ first exons between the two species reveals that there are also significant differences in the composition of these 5’ UTRs (Figure 7). First of all, there is a large difference in the length of the two first exons and, with the exception of the 12-base sequence underlined, there is no sequence homology between them. This indicates that if there are any regulatory sequence or structural elements present in these sequences, they were not conserved during evolution.

1.3. The XDD element

When I discovered that my results with the Xenopus c-mycI first exon sequences conflicted with those previously reported by Lazarus (1992), I repeated a portion of Lazarus’ experiment by isolating a 638 bp Dpn I restriction fragment from the c-mycI genomic clone, which contains sequences not found the native mRNA (see Figure 2C) and placing it 5’ to the CAT coding sequence (pSP6-XDD-CAT). This Dpn I fragment spans a 638 base sequence from 387 nucleotides upstream of the P1 transcription start site to 26 nucleotides into intron 1 (Principau and Spohr, 1991). Figure 8 shows inhibition of CAT activity compared to the CAT control for all stages injected with in vitro-transcribed RNA. The absence of any significant effect by the more biologically relevant Xc-mycI first exon sequences suggests that the inhibition caused by XDD is due to
Figure 6: The effect of the human c-myc first exon on CAT activity. Experiment performed as described in Methods and Materials and the legend to Figure 4. The human c-myc first exon was highly inhibitory at both stages of development. The effect was slightly attenuated when the (A)$_{73}$ element was present.
Figure 7: Alignment of 5' first exons of c-myc. The human c-myc P1 first exon is in normal text on top numbered from 1-578. The *Xenopus* c-myc P1 first exon is in italics on the bottom numbered from 1-221. Where there is no sequence for *Xenopus* there is a (•). A match between *Xenopus* and human sequences is indicated by both sequences being underlined.
Figure 8: The effect of the XDD element on CAT activity. Experiments were done as described in Methods and Materials and the legend to Figure 4. The XDD element is inhibitory, as was previously reported (Lazarus, 1992). In addition, the (A)$_{73}$ element enhanced CAT activity in the presence of XDD to a greater extent than in its absence.
inclusion of upstream genomic elements, rather than the 5' UTR of Xenopus c-myc1 mRNA. Similar to the results for the human c-myc first exon, CAT activity for XDD-CAT(A)\textsubscript{73} was 6.1 fold greater than for XDD-CAT(A)' for stage VI oocytes and 13.4 fold greater for early embryos, whereas CAT(A)' was only 1.7 fold greater than CAT(A)' for stage VI oocytes and 3.1 fold greater for early embryos. These differences were also determined to be highly significant (Appendix 2).

1.4. The stability of the mRNAs with different 5' UTRs

The apparent lack of translational effect by the Xenopus c-myc 5' first exons could be artifactual if these sequence elements affect mRNA stability. Likewise, the apparent translational inhibition of the human c-myc first exon and the XDD element could have been due to effects on stability, rather than translation. Hence, RNase protection assays were carried out to monitor for any differences in stability (Figure 9). As a control, Luc(A)' RNA was coinjected and subjected to an RNase protection assay. There were no differences in stability for P1-CAT(A)', P2-CAT(A)' or CAT(A)', nor were there any differences in the stability of Hc-mycCAT(A)', XDD-CAT(A)' or CAT(A)' RNA, despite the large inhibition of CAT activity noted (Figures 6 and 8). Thus, the differences in the CAT activity with Hc-mycCAT(A)' or XDD-CAT(A)' and CAT(A)' were likely due to translation—not stability—differences. In addition, the lack of stability differences between P1-CAT(A)' or P2-CAT(A)' and CAT(A)' supports the evidence (Figure 4) that the Xenopus c-myc 5' first exons have no impact on gene expression.

1.5. Stimulation of translation by an (A)\textsubscript{73} element

To determine whether the effect of the (A)\textsubscript{73} element on CAT activity was due to an effect on stability or translation, radiolabeled CAT(A)' or CAT(A)\textsubscript{73} was injected into stage VI oocytes or early embryos and recovered either at 0 hours or 2 hours post-injection. The percent of RNA remaining after two hours was used to adjust the CAT
Figure 9: The effects of 5' UTRs on stability. RNase protection assays were carried out as described in the Methods and Materials using Luc and CAT probes. This experiment used stage VI oocytes. There are two specific Luc(A)* protections from the Luc probe. This is because the Luc probe contains a long AU-rich sequence near the middle of the probe; it is likely that this sequence, which by itself has a very low melting temperature, breathes during hybridization and digestion resulting in a hypersensitive site that generates two bands.
activity (Figure 10). The occasional decreased stability of CAT(A) \textsuperscript{+} RNA relative to CAT(A) \textsubscript{73} was not sufficient to account for the differences in translation. Thus, the stimulation of CAT activity by the (A) \textsubscript{73} element was due to an increase in translation.

2. The effects of the Xenopus c-mycI 3' UTR on gene expression

As previously discussed (see Section 3.5 in the Introduction), the 5' and 3' ends of an RNA molecule can cooperate to regulate gene expression. In particular, the poly(A) tail and elements in the 3' UTR that can regulate mRNA stability or polyadenylation can impact translation, probably at the level of initiation (Drummond \textit{et al.}, 1985; McGrew \textit{et al.}, 1989; Paris and Richter, 1990; Marinx \textit{et al.}, 1994). To determine if the Xenopus c-mycI 3' UTR could impact gene expression, it was placed downstream of a CAT coding sequence either with or without the Xenopus c-mycI 5' first exons upstream. This was accomplished by PCR (see Methods and Materials). \textit{In vitro}-transcribed mRNA was injected into cells, and gene expression was monitored by measuring CAT activity and RNA stability.

2.1. Translation and RNA stability

2.1.1. The 3' UTR alone

Stage VI oocytes were injected with RNA and monitored for their ability to translate mRNA either with or without the 3' UTR. Although the c-mycI 3' UTR occasionally stimulated CAT activity slightly, the effect in oocytes was variable (data not shown) and was judged to be statistically insignificant (Appendix 2).

For early embryos (between one and four cells) there was a highly significant stimulation of CAT activity due to the presence of the c-myc 3' UTR (Figure 11A). Late embryos (stage 8; MBT) also showed a striking stimulation due to the presence of the \textit{myc} 3' UTR (Figure 11B). These results were very consistent and were considered highly significant (Appendix 2).
A. Stage VI Oocytes

Figure 10: The effects of an \((A)_73\) element on translation. Experiments were performed as described in Methods and Materials and in the legend to Figure 4. In all stages, there was a stimulation by the \((A)_73\) element on translation, even after accounting for stability differences.
Figure 11: The effects of the *Xenopus* c-myc 3' UTR on CAT activity. Experiments were performed as described in Methods and Materials and the legend to Figure 4. The c-myc 3' UTR significantly stimulated CAT activity in embryos.
2.1.2. The 3' UTR and the 5' first exons

To determine if the *Xenopus c-mycI* 3' UTR could cooperate with the 5' first exons, constructs were made that contained the 5' first exons of *Xenopus c-mycI* upstream and the 3' UTR downstream of CAT. RNA was *in vitro* transcribed that contained CAT alone with or without the 5' first exons or the 3' UTR. Injections into early and late embryos were monitored by CAT activity. Figure 12 shows that the presence of the 3' UTR stimulated CAT activity regardless of which 5' UTR was used. As before, the 5' first exons on their own had no impact on CAT activity.

2.1.3. Deletions of the 3' UTR

The experiments described above confirm that the *Xenopus c-mycI* 5' first exons have no impact on reporter expression either alone or with the 3' UTR. However, they also show that the *Xenopus c-mycI* 3' UTR is a potent stimulator of reporter activity. In an attempt to determine where in the *Xenopus c-mycI* 3' UTR the elements responsible for this effect reside, simple deletions were done. A *Sca I* site was utilized to cut the 3' UTR into two portions (see Methods and Materials) termed the proximal and the distal elements. The proximal element consists of the 641 bases of the *Xenopus c-mycI* 3' UTR closest to the coding sequence. The distal element consists of the 314 bases of the *Xenopus c-mycI* 3' UTR farthest from the coding sequence. *In vitro*-transcribed RNA was injected into *Xenopus* embryos, and translation was monitored (Figure 13). Neither element alone was sufficient to stimulate CAT activity. Thus, either both elements must be present to obtain the effect, or there is a stimulatory element present exactly at the *Sca I* site.

2.1.4. Effects on stability

In order to determine whether the effects seen by the presence of the *Xenopus c-myc 3' UTR* are due to differential stability of the mRNA compared to CAT(A)\(^{-}\), the stability of the mRNA was measured after injection. As with the CAT(A)\(^{-}\) and
Figure 12: The effects of *Xenopus* 5' first exons and the 3' UTR on CAT activity. The experiment was performed as described in Methods and Materials and in the legend to Figure 4. The stimulation by the *Xenopus* c-myc 3' UTR occurred regardless of the presence of the 5' UTR. The variations visible in the expression of the mRNAs are all within the normal variability seen with the 3' UTR alone or the 5' first exons alone.
Figure 13: The effect of truncated *Xenopus* 3' UTRs on CAT activity. Experiments were carried out as described in Methods and Materials and in the legend to Figure 4. The stimulation observed by the *Xenopus c-mycI* 3' UTR was not matched by either of the deletions.
CAT(A)$_{73}$ results in Section 1.5. (Figure 9), radiolabeled RNA was injected and recovered either immediately (0 hours) or 2 hours after injection. The results (Figure 14) show the CAT activity before RNA degradation was taken into account, the RNA-corrected CAT activity and the gel that was quantified. For both stages, any effects seen by the proximal and distal sequences before correction are not considered significant and these differences disappear after RNA correction is applied. The results shown here confirm that the effects seen by the proximal or distal sequences are not sufficient to account for the differences seen by the Xenopus c-myc 3' UTR in its entirety, and the stimulation by the 3' UTR is not due to an increased stability of the myc 3' UTR-containing mRNA.

In summary, the 3' UTR of Xenopus c-myc can stimulate translation. In addition, neither the proximal nor distal portion of the Xenopus c-myc 3' UTR can account for the translational stimulation seen using the entire structure.

2.2. Cytoplasmic polyadenylation

2.2.1. The effects of (A)$_{73}$ on translation of CAT-myc RNA

All mRNAs discussed so far have been translationally stimulated by the (A)$_{73}$ element. Therefore, the effects of the (A)$_{73}$ element on CAT-myc were also tested to determine if the (A)$_{73}$ element could stimulate translation to an even greater extent than the myc 3' UTR alone. As seen in Figure 15, (A)$_{73}$ had no additive effect on CAT activity. In fact, when a statistical analysis was done (Appendix 2), no differences were seen when comparing CAT-myc(A)° to CAT(A)$_{73}$, CAT-myc(A)° to CAT-myc(A)$_{73}$ or CAT-myc(A)$_{73}$ to CAT(A)$_{73}$. Thus, the 3' UTR alone was as effective as the (A)$_{73}$ element alone.

2.2.2. Cytoplasmic polyadenylation by the 3' UTR

The observation that the myc 3' UTR enhances translation to approximately the same extent as the (A)$_{73}$ element suggests that they may stimulate translation by a similar mechanism. Furthermore, the addition of an (A)$_{73}$ downstream of the myc 3' UTR does
Figure 14: The effects of the Xenopus c-myc 3' UTR on translation. The effects on CAT activity are not due to an increased stability of the 3' UTR-containing mRNA. The proximal and distal sequences cannot stimulate translation to the same extent as the entire Xenopus c-myc 3' UTR.
Figure 15: The effect of the \((A)_{73}\) element on the CAT activity of CAT-\textit{myc} mRNA. Experiments were performed as described in Methods and Materials and in the legend to Figure 4. The stimulation seen post-fertilization by the \((A)_{73}\) element was highly significant comparing CAT\((A)^{-}\) to CAT\((A)_{73}\), but it was not significant comparing CAT-\textit{myc}(A)^{-} to CAT-\textit{myc}(A)_{73}.
not significantly enhance translation of CAT-myc(A), unlike all other mRNAs mentioned so far. Thus, cytoplasmic polyadenylation may be the mechanism that is causing the observed translational stimulation by the Xenopus c-mycI 3’ UTR.

The possibility of cytoplasmic polyadenylation was investigated by an RNase H-mediated degradation technique. RNase H analysis utilizes the digestion properties of the RNase H enzyme to reduce the length of the injected RNA so that the addition of an (A) tail is apparent on an acrylamide gel. Figure 16 shows a diagram of the protocol. This approach utilizes a DNA oligonucleotide with a sequence specific to a site in the 3’ UTR of the target RNA. After hybridization, the RNA portion of the DNA/RNA hybrid is digested with RNase H. The resulting products, when resolved on an acrylamide gel, are a long 5’ end of the original RNA and a much shorter 3’ end. If there was any cytoplasmic adenylation, the 3’ end piece would appear smeared on an acrylamide gel. As a control, poly d(T) is added during the hybridization step in addition to the DNA oligo. Poly d(T) can hybridize to a poly(A) tail so that the (A) tail is removed when RNase H is added; this should reduce the 3’ end to the same size it would have been without the (A) tail (at 0 hours).

The results of three RNase H experiments are presented in Figures 17, 18 and 19. Figure 17 shows that the inclusion of the Xenopus c-mycI 3’ UTR is sufficient to allow the addition of a poly(A) tail to the mRNA. The smear due to polyadenylation is reduced upon addition of the poly d(T) (Figure 17, lanes 3-5). A positive control was included in these experiments that contains the cyclin A2 3’ UTR downstream of the CAT sequence, CAT-cyclinA2(A) (Figure 17, lanes 6-8). Cytoplasmic polyadenylation by the cyclin A2 3’ UTR has been observed previously (T. Strugnell, personal communication). Other researchers testing for cytoplasmic polyadenylation often allow longer periods of time for growth of the (A) tail (e.g., Simon et al., 1996). Therefore, a second experiment (Figure 18) was conducted to increase both the period during which
Figure 16: Diagram of an RNase H analysis experiment. See Methods and Materials, and the text for details.
Figure 17: RNase H analysis to test for cytoplasmic polyadenylation of injected mRNAs. Experiments were performed as described in Methods and Materials. Approximately 5-10 ng of RNA were injected into each embryo. The RNA was recovered and purified. RNA recovered immediately (0 hr) from 10 cells was hybridized only to a specific oligo before treatment with RNase H (lane marked “0”). RNA from 20 cells recovered after 2 hr post-injection was split into two samples. One sample was hybridized only to the specific oligo (lanes marked “2”) whereas the other was also hybridized to poly d(T) before RNase H addition (lanes marked “2T”). Lanes 1 and 2 show that CAT(A) does not become adenylated after 2 hours post-injection. Lanes 3-8 show that CAT-myc(A) and CAT-cyclinA2(A) increase in size after 2 hours and that this increase can be reduced by addition of polyd(T).
Figure 18: RNase H analysis to test for cytoplasmic polyadenylation after 3 hours post-injection. Experiments performed as described in Methods and Materials and in the legend to Figure 17, except injections were left for 3 hours post-injection before RNA recovery, and four times as much poly d(T) was added.
Figure 19: RNase H analysis to test for cytoplasmic polyadenylation in stage VI oocytes. This experiment was similar to those in Figures 17 and 18, except that one-tenth the amount of RNA was injected into stage VI oocytes. There is no adenylation of CAT-\textit{myc(A)} in stage VI oocytes.
adenylation could occur and the amount of poly d(T). In both Figures 17 and 18, an approximate length of an 85 residue (A) tail for CAT-myc(A) was estimated by graphing the distance of the bands of known size from the origin and using this graph to determine where RNA of an approximate size would be seen.

As previously shown, stage VI oocytes are incapable of cytoplasmic polyadenylation of mRNA (Fox et al., 1992). This property was used as a control to demonstrate that CAT-myc(A) RNA is not adenylated upon injection into stage VI oocytes (Figure 19).

These experiments demonstrate that the *Xenopus* c-myc I 3' UTR is capable of stimulating cytoplasmic polyadenylation in embryos. Thus, there must be a CPE in the 3' UTR.

2.2.3. Quantification of polyadenylation

To confirm the visual impression of the RNase H experiments for cytoplasmic polyadenylation, quantification was done using the program MacBAS (v. 2.2; see Methods and Materials for procedure). Figure 20 summarizes the results seen in Figures 17 and 18. After 2 or 3 hours, there is a 20-40 percent increase in the amount of RNA above the 0 hour band when RNA containing the myc(A) or cyclinA2(A) 3' UTRs was injected. This percent is reduced upon addition of the poly d(T). The control CAT(A) shows essentially no increase at 2 hours in the counts above the 0 hour band. These results confirm that the presence of the *Xenopus* c-myc 3' UTR downstream of CAT can confer cytoplasmic polyadenylation on the RNA.
Figure 20: Quantification of RNase H gels. Quantification of the gels shown in Figures 17 and 18 was carried out as described in Methods and Materials. The graph shows the percent of total counts that were above the 0 hour non-adenylated band for CAT(A)', CAT-myc(A)' and CAT-cyclinA2(A)'. Each of the sets of bars shows the 0 hour level of "adenylated", the level after a 2 or 3 hour post-injection incubation and the level after the post-injection incubation and hybridization with poly d(T).
DISCUSSION

The importance of the c-myc gene in the regulation of carcinogenesis is apparent from how frequently it is expressed aberrantly in cancers such as Burkitt lymphoma, Bloom’s syndrome and multiple myeloma (Bernard et al., 1983; West et al., 1995; Paulin et al., 1996). Many cancers found in species such as chickens and humans lack the 5’ first exon of the c-myc gene. It was this observation that led to attempts to characterize the role of the c-myc 5’ first exon in the regulation of c-myc gene expression. Although many experiments have attempted to do this, no definitive conclusions can be drawn, because the results are so varied (Nielsen and Maroney, 1984; Persson et al., 1984; Butnick et al., 1985; Darveau et al., 1985; Parkin et al., 1988b; Lazarus et al., 1988).

Because the mammalian c-myc first exons cause a translational inhibition when used in Xenopus, there is the potential for the Xenopus c-myc first exons to function in a similar fashion, provided there is a conservation of regulation in species (Parkin et al., 1988b; Lazarus et al., 1988). However, the Xenopus and human c-myc 5’ first exons are very different in size and share virtually no sequence homology (Figure 7). The reported inhibition (Lazarus, 1992) by the Xenopus c-myCl 5’ sequences was used to confirm the role of the c-myc 5’ sequence in inhibiting translation in Xenopus oocytes and indicated a conservation of regulation throughout species. Unfortunately, the elements used by Lazarus (1992) to demonstrate inhibition by the c-myCl 5’ UTR contained non-transcribed and intron sequences. Thus, the authentic Xenopus 5’ first exons have never been tested for this function.

Transcripts from the Xenopus c-myCl gene—like the mammalian gene—are initiated from two different promoters during development, generating mRNAs with two different 5’ UTRs. It is possible that these differences could be important to the regulation of gene expression of c-myc during development at the level of translation. Thus, I tested for translational regulation by the authentic Xenopus c-myc 5’ first exons
from the two promoters. Two controls were also used: the human c-myc first exon and one of the constructs used by Lazarus (1992). Both of these controls were expected to be inhibitory when used in Xenopus oocytes and early embryos (Parkin et al., 1988b; Lazarus et al., 1988; Lazarus, 1992).

In addition to translational regulation, mRNA expression can be regulated by all parts of the transcript by altering its stability or adenylation status. The 3’ UTR is involved in both of these mechanisms of regulation. The Xenopus and human c-myc 3’ UTRs have both been shown to contain consensus AUREs—known instability elements (Jones and Cole, 1987; Vriz and Méchali, 1989). However, the c-myc mRNA is remarkably stable in some cancers (Dani et al., 1984). A previous report has also shown that the adenylation status of Xenopus c-myc mRNA changes during development (Tchang et al., 1991), possibly indicating that it becomes cytoplasmically polyadenylated post-fertilization. Hence, the roles of the Xenopus c-mycI 3’ UTR were also studied in regulating translation, cytoplasmic polyadenylation and stability.

Finally, the role of poly(A) in the regulation of gene expression is very well characterized, especially in the developing Xenopus embryo. For this reason and because the (A) tail is known to interact with the 5’ end of the mRNA to regulate gene expression, the role of a plasmid-encoded (A) tail—(A)_{73}—was also tested to determine if an (A) tail could interact with c-myc 5’ UTRs.

These studies were designed to gain insight into the regulation of c-myc mRNA expression during Xenopus development. To test the role of the Xenopus c-mycI UTRs in the regulation of gene expression, CAT reporter transcripts were injected into Xenopus oocytes and embryos. This system is useful, because (1) it examines in vivo expression while using exogenous mRNAs; (2) female Xenopus produce large numbers of oocytes and eggs, making harvesting simple; (3) the cells are large and easy to inject; and (4) the RNA and protein recovery techniques are also simple. The CAT reporter is also valuable for
several reasons: (1) it is bacterial in origin, thus unlikely to contain elements that could complicate the study, unlike luciferase (Luc), which is eukaryotic and whose 3' UTR has been shown to impact its expression (Tanguay and Gallie, 1996); (2) there is a sensitive assay for CAT activity with low background, allowing small amounts of RNA to be used, thus preventing RNA toxicity to the cell upon injection; (3) the CAT mRNA is short (approximately 790 bases) compared to either Luc mRNA (approximately 1800 bases) or β-gal mRNA (approximately 3050 bases), making it much easier to synthesize, with less chance of premature transcription termination products. This shorter length also allows smaller amounts of RNA to be injected for the same molar equivalents of reporter, allowing more efficient use of a single batch of in vitro transcribed RNA.

Specific PCR primers were used to amplify the entire 5' first exon or 3' UTR from a genomic clone. The PCR products contained not only the sequences of interest, but also restriction enzyme sites that allowed the products to be inserted into plasmids. By contrast, Lazarus (1992) used restriction enzyme sites in the genomic clone to isolate large sequences, which included regions not present in the native mRNA. I repeated the construction of one of Lazarus' (1992) plasmids as a control (see XDD construction, Methods and Materials). A PCR-amplified human c-myc 5' first exon was used as another control. Both of these controls were used to ensure that previous observations of translational inhibition could be repeated (Parkin et al., 1988b; Lazarus et al., 1988; Lazarus, 1992).

Exhaustive analysis of the role in translation and stability of the two 5' first exons from Xenopus c-myc in reporter constructs demonstrated that these elements had no effects on either translation or stability of mRNA (Figures 4 and 9), nor was there any evidence for interactions between the 5' first exons and the c-myc 3' UTR (Figure 12). The lack of demonstrable effect of the 5' first exons on translation was in direct contradiction with the conclusions made by Lazarus (1992). Thus, I repeated his
experiment in which the sequence between two *Dpn I* restriction enzyme sites of the *Xenopus c-myc* genomic clone was used to inhibit CAT translation in *Xenopus*. This element contains the P1 first exon plus upstream non-transcribed sequences and is predicted to contain extensive secondary structure ($\Delta G = -192 \text{ kcal/mol}$; Zuker and Stiegler, 1981). As with Lazarus’ (1992) results, I have shown that this XDD sequence is inhibitory to translation of a CAT reporter in both *Xenopus* oocytes and early embryos (Figure 8).

The other control used was the human *c-myc* 5' first exon. It was tested to see if it could inhibit translation in *Xenopus* in the same way as that reported for the murine *c-myc* first exon (Parkin *et al.*, 1988b; Lazarus *et al.*, 1988). As shown in Figure 6, the human *c-myc* first exon was a potent inhibitor of translation in stage VI oocytes and early embryos. As with XDD, the human *c-myc* 5' first exon is predicted to form very stable secondary structures ($\Delta G = -231.4 \text{ kcal/mol}$; Zuker and Stiegler, 1981). The extensive secondary structures that are predicted to form in the XDD and human *myc* sequences have much higher thermal stabilities than the predicted secondary structures of the authentic 5' first exons ($\Delta G = -48.8 \text{ kcal/mol}$ for P1 and -31 $\text{ kcal/mol}$ for P2; Zuker and Stiegler, 1981). Thus, they are more likely to cause translational inhibition.

It is also possible that the initiation of translation at upstream initiation codons in XDD and the human *c-myc* exon 1 could be inhibiting the synthesis of the CAT protein. Although the XDD sequence contains two AUGs and the Hc-*myc* sequence contains one AUG, they are all in very poor context making it unlikely that they could efficiently initiate translation. However, there is a CUG in the Hc-*myc* sequence that is in an excellent context and this CUG is known to initiate endogenous *myc* translation under certain conditions (Marcu *et al.*, 1992). Hence, it is possible that translation is initiated at this site, interfering with the correct initiation of translation of the CAT protein. This CUG may explain why Hc-*myc* is more inhibitory than XDD.
Although the c-myc genes of amphibians and mammals share the structural similarities of a non-coding first exon and two coding exons, the *Xenopus* c-mycI first exon does not share any sequence similarity with its mammalian counterparts, nor does it form significant secondary structure. The high sequence conservation of the first exon between mice and humans (70%; Bernard *et al.* 1983) suggests that the first exon sequence plays an essential role in mammals, which may be related to the comparable abilities of the mouse and human c-myc first exons to repress translation in *Xenopus*. Extensive secondary structure in the first exons of c-myc with the potential to inhibit translation may have been newly acquired during evolution, possibly connected with the advent of homeothermy, which may aid in the unwinding of secondary structure. It is possible that a warmer body temperature would have allowed an increased translation of mRNAs such as those encoding oncogenes, growth factors and growth factor receptors. This would have made it necessary for organisms to develop new strategies to prevent the overexpression of the potentially harmful genes. More extensive secondary structure may have been a consequence of the need to have increased control over the expression of certain messengers. It will be interesting to learn whether genes encoding other growth-related proteins show similar differences in length and stability of their 5' UTR between amphibians and mammals.

My results leave unanswered the question of the functions of the two distinct 5' termini of *Xenopus* c-mycI transcripts. From my data, I must conclude that the different 5' UTRs are simply a by-product of the two different promoters and have no differential effects on *Xenopus* c-mycI mRNA translation. One explanation for the conservation of different promoters that produce different transcripts could be that there is a transcriptional mechanism regulating mRNA production. It is possible that the cellular state can regulate the transcriptional attenuation of the two different promoters of c-myc by altering availability of transcription factors, based on studies of the human c-myc gene.
in *Xenopus* oocytes (Spencer and Kilvert, 1993). Because the two c-mycI mRNAs appear to behave identically during development, the regulation of transcription would simply allow either more or less mRNA to be synthesized. Transcriptional attenuation may be the mechanism used to down-regulate production of excess c-myc mRNA when it is not required. This may be supported by the observation that the *Xenopus* c-mycI mRNA from promoter 1 is only present during oogenesis (Principaud and Spohr, 1991). It is during this stage of development that maternal production of RNA and protein is high. It is possible that the extra c-myc transcripts from P1 are there to boost production of c-myc protein. c-myc mRNA from P1 is not present in embryos and may be unnecessary because other mechanisms are active to stimulate production of c-myc protein, such as cytoplasmic polyadenylation.

Many experiments show that poly(A) is capable of enhancing translation (McGrew *et al.*, 1989; Paris and Richter, 1990; Simon *et al.*, 1992; Simon and Richter, 1994; Simon *et al.*, 1996). To analyze how a plasmid-encoded (A) tail could impact expression, the (A)$_{73}$ element from pBSK-As (Simon *et al.*, 1992) was placed downstream of the CAT reporter and/or the 3' UTR. Although the (A)$_{73}$ element behaved like a natural (A) tail by enhancing translation of mRNAs (Figures 5 and 10), the extent of translational stimulation of XDD-CAT or Hc-myc-CAT by (A)$_{73}$ was significantly greater than the effect on CAT mRNA alone (Figures 6 and 8). This suggested that the (A) tail had differential effects depending upon the sequences at the 5' end. Because both XDD and the human c-myc first exon sequences have the potential to form extensive secondary structures, it is possible that an (A) tail preferentially potentiates translation of transcripts with extensive secondary structure.

Four observations have been documented that explain why the (A)$_{73}$ element could preferentially enhance translation of Hc-mycCAT or XDD-CAT mRNAs: (1) the cap and poly(A) tail cooperate to enhance translation (Gallie, 1991); (2) excess eIF4E (the
cap-binding protein) can specifically enhance translation of mRNAs containing secondary structure (Fagan et al., 1991; Koromilas et al., 1992); (3) excess PABP inhibits translation of all mRNAs, presumably by sequestering initiation factors (Gallie and Tanguay, 1994); and (4) poly(A) has been shown to bind directly to eIF4B and eIF4F (Gallie and Tanguay, 1994). If poly(A) and/or PABP can specifically bind cap-binding complexes (eIF4F with eIF4B), it is possible that a poly(A) tail could play a role in enhancing translation of mRNAs with secondary structures by allowing recruitment of the limiting factor eIF4E to the cap, which is known to specifically enhance translation of such mRNAs. This could explain why an (A) tail can stimulate translation of both XDD-CAT and Hc-myc-CAT to a greater extent than CAT alone; i.e., by binding eIFs, specifically eIF4E, which stimulates translation of these mRNAs more extensively than those without secondary structure.

Unfortunately, the (A)\textsubscript{73} element is not exactly like an (A) tail, because it contains vector-derived sequences downstream of it. These sequences may be responsible for effects on translation. (A)\textsubscript{73}-containing RNA transcribed \textit{in vitro} could be hybridized to an oligo specific to the sequence at the 3' of the 73 (A)s, and RNase H could be used to digest the downstream nucleotides. It would be necessary to use radiolabeled RNA to confirm that the digestion by RNase H was complete and produced a homogeneous population of RNA. This RNA ending in (A)\textsubscript{73} could then be used to test for the ability of an (A) tail alone to enhance specifically translation of XDD-CAT or Hc-myc-CAT mRNA.

The \textit{Xenopus} c-mycI mRNA has been shown to exhibit changes in both its stability and adenylation status during development (Tchang et al., 1991; Dani et al., 1984; King et al., 1986; Schreiber-Agus et al., 1993; Godeau et al., 1986; Taylor et al., 1986). Hence, the impact of the \textit{Xenopus} c-mycI 3' UTR on translation, stability and polyadenylation of a CAT reporter mRNA was tested.
The *Xenopus* c-*mycI* 3′ UTR was expected to inhibit translation and promote transcript instability because of the presence of a consensus AURE in its 3′ UTR (Vriz and Méchali, 1989). This is because the AURE of the IFN 3′ UTR has been shown to inhibit translation in *Xenopus* embryos (Marinx et al., 1994) and the level of *Xenopus* c-*mycI* mRNA may decrease after fertilization (Godeau et al., 1986; King et al., 1986; Taylor et al., 1986; Schreiber-Agus et al., 1993). Thus, it was predicted that the *Xenopus* c-*mycI* 3′ UTR should cause the reporter mRNA to become unstable and lower its activity. The translation of mRNAs with or without the c-*mycI* 3′ UTR was monitored by CAT assays in both oocytes and post-fertilization embryos. The results from stage VI oocytes were inconclusive, and statistical analysis inferred that the *Xenopus* c-*mycI* 3′ UTR had no effect on translation during that stage (Appendix 2). However, in post-fertilization embryos, a highly significant *stimulation* of translation by the 3′ UTR was seen (Figure 11), and stability studies showed that the *Xenopus* c-*mycI* 3′ UTR-containing mRNA was very stable (Figure 14), but not more stable than CAT(A)' RNA. This indicated that RNA containing the *Xenopus* c-*mycI* 3′ UTR is not unstable and has enhanced translation.

The *Xenopus* c-*mycI* 3′ UTR stimulation of translation is consistent with an ability to cytoplasmically polyadenylate RNA. Previous evidence exists that adenylation status of the *Xenopus* c-*mycI* mRNA changes during development (Tchang et al., 1991). Thus, RNase H analysis was used to determine if CAT-*myc(A)' was cytoplasmically polyadenylated. This mRNA behaved in a similar fashion as a CAT-cyclinA3(A)' positive control that is cytoplasmically adenylated (Figures 17, 18 and 20; Tod Strugnell, personal communication). On the other hand, a CAT(A)' control did not undergo cytoplasmic polyadenylation. When RNA that had become adenylated by 2 or 3 hours post-injection in early embryos was hybridized with poly d(T) in addition to the oligonucleotide, before RNase H digestion, the length of the (A) tail was reduced
confirming that the increased length was indeed due to poly(A). As expected, CAT-
myc(A)′ mRNA injected into stage VI oocytes did not become adenylated (Figure 19). These experiments show that CAT-myc(A)′ RNA is cytoplasmically polyadenylated in
Xenopus embryos. Furthermore, the (A)73 element did not significantly enhance
translation of CAT-myc (Figure 15). This indicates that this effect may have already been
accomplished by the Xenopus c-myc 3′ UTR. I interpret these observations to mean
that, in spite of the vector-derived sequences at the 3′ end, the (A)73 element mimics the
effects of cytoplasmic polyadenylation of CAT-myc(A)′ mRNA.

Neither of the 3′ UTR truncations was capable of enhancing translation to the
same extent as the full-length 3′ UTR (Figures 13 and 14). This observation implies that
either at least two elements are necessary for this stimulation and that they reside in
different parts of the mRNA or that there is a single element right at the Sca I site used to
make the two truncations. However, this cut site does not resemble any previously
documented CPE. An example of a situation where two elements are found to stimulate
translation is during cytoplasmic polyadenylation, which is regulated by the CPE and the
NPE. Although in the case of the Xenopus c-myc I 3′ UTR it is difficult to link
conclusively the translational stimulation to cytoplasmic polyadenylation, it is possible
that this is the situation and the truncations each contain one of the two elements.
Although these data are preliminary, they suggest that the first place to look for a CPE is
in the 641 bases of the proximal sequence. This means that the Xenopus c-myc I CPE is in
an unusual location compared to those previously identified: over 300 bases away from
the known NPE (Richter, 1996). To isolate the CPE, a series of constructs that contain
nested deletions of the 3′ UTR could be used to narrow down the location of the CPE.
Nested deletions should be done from only the 5′ end in order to remove the CPE without
disturbing the NPE. After a small region of the mRNA that contains the putative CPE is
identified, point mutations done by site-directed mutagenesis should be used to identify
the specific sequence of the CPE. Mutation of the NPE can also be used as a negative control.

Several reports indicate that cytoplasmic polyadenylation can enhance translation (McGrew et al., 1989; Vassalli et al., 1989; Paris and Richter, 1990; Simon et al., 1992; Simon and Richter, 1994; Sheets et al., 1994; 1995). To demonstrate that the observed translational stimulation by the *Xenopus* c-myc 3' UTR was due to cytoplasmic polyadenylation, a block to adenylation should also block the translational stimulation. One way to do this would be to use cordycepin (an analogue of ATP). When cordycepin is incorporated into RNA, it terminates polyadenylation. By using cordycepin in the RNAs mentioned, it may be possible to prevent (A) tail addition and hopefully translational stimulation at the same time. Cordycepin has been used successfully to study maturation-specific polyadenylation (McGrew et al., 1989). Stage VI oocytes that were incubated in cordycepin-containing buffer for several hours before maturation and injection to allow uptake of cordycepin did not facilitate cytoplasmic polyadenylation. However, this same technique cannot be used for injections into embryos, because embryos must be injected shortly after fertilization and cannot be incubated in cordycepin for long enough to allow uptake. Preliminary attempts at coinjection of cordycepin with the mRNA to prevent cytoplasmic polyadenylation in early embryos have been unsuccessful. Poly(A) polymerase (PAP) was also used in attempts to add cordycepin to mRNA before injection. However, because PAP is unreliable, these experiments were also unsuccessful (T. Strugnell, personal communication). We concluded that insufficient cordycepin was incorporated into RNA to be effective. However, further attempts should be made to use cordycepin as an inhibitor of polyadenylation.

One potential approach would be to use an *in vitro* lysate system. Cordycepin could be added to the lysate to block both polyadenylation and translation. Preliminary observations with a translation lysate (Appendix 4) show that the translation of
CAT-myc(A) mRNAs is enhanced compared to CAT(A). If a lysate system could be developed that would reliably facilitate both translation and polyadenylation, the addition of cordycepin to this lysate could be used to test for the role of polyadenylation in translational stimulation. However (as mentioned in Appendix 4), the unreliability of lysate preparations must first be addressed.

Stimulation of translation by polyadenylation can be due either to the process of adenylation or simply the presence of an (A) tail (Richter, 1996). If it is assumed that the translational stimulation is due to cytoplasmic polyadenylation, it seems likely that the stimulation of CAT translation is by the presence of the (A) tail. This is because the (A)73 element alone can stimulate translation to a similar level as that seen by the myc 3' UTR. In addition, stimulation by the myc 3' UTR is not significantly enhanced by the addition of the (A)73 element, despite the fact that further polyadenylation of CAT-myc(A)73 is likely (data not shown). Thus, a minimal poly(A) tail is sufficient to promote translation.

The conclusion that the presence of an (A) tail is sufficient to stimulate myc translation is difficult to make because CAT is the sequence being affected, not the myc coding sequence. Thus, it is necessary to analyze how the endogenous myc mRNA behaves. Northern blots using Xenopus oocyte, egg and embryo mRNA in conjunction with an RNase H analysis (Mercer and Wake, 1985) could be conducted to determine if the endogenous Xenopus c-mycI mRNA is deadenylated in eggs and readenylated after fertilization, as was previously reported (Tchang et al., 1991). Second, in vitro-transcribed mRNA containing the full-length Xenopus c-mycI sequence, Xenopus c-mycI without the 3' UTR or with a mutated CPE or Xenopus c-mycI sequences with an (A)73 could be used in lysates from different developmental stages supplemented with 35S-methionine to monitor levels of myc translation. The levels of translation and
polyadenylation should indicate if the process of polyadenylation or the presence of an (A) tail is sufficient to enhance translation of myc mRNA.

An additional experiment might be to place either the Xenopus c-mycI 3’ UTR or the human c-myc 3’ UTR downstream of either Hc-myc-CAT or XDD-CAT to see if: (1) the human c-myc 3’ UTR can stimulate cytoplasmic polyadenylation in Xenopus; or (2) cytoplasmic polyadenylation by the Xenopus c-mycI 3’ UTR can enhance translation of the Hc-myc- or XDD-containing mRNAs, as the (A)₇₃ does.

The type of CPE and the distance between the CPE and the NPE are known to regulate the timing of adenylation during development (Richter, 1996). The putative location of the Xenopus c-mycI CPE is far from the NPE, making this a potentially novel location for a CPE. The CPE sequence is still unknown, but an analysis of the Xenopus c-mycI 3’ UTR indicates that it is probably oligo(U) and therefore fertilization-specific.

Given the theoretical type and location of the CPE, it is unlikely that mRNA containing the Xenopus c-mycI 3’ UTR could regulate adenylation during maturation. Furthermore, a previous report examined the poly(A) status of the c-myc mRNA in Xenopus, noting that there was no myc in the poly(A)⁺ fraction of the RNA by maturation and this did not change until after fertilization (Tchang et al., 1991). However, this CPE is potentially quite different from those previously characterized and it is necessary to confirm that the Xenopus c-mycI 3’ UTR cannot regulate maturation-specific cytoplasmic polyadenylation.

With the possibility of a CPE in the 3’ UTR of Xenopus c-mycI, it is also necessary to analyze the 3’ UTRs of mammalian myc genes to determine if they contain a CPE. Figure 21 shows the alignment of the human c-myc and Xenopus c-mycI 3’ UTRs. Unlike the 5’ UTRs there are a number of areas of sequence homology (Wilbur and Lipman, 1983). The myc sequences are very (U)-rich, and much of the alignment is in (U)-rich sequences. However, the 3’ UTR of the human sequence is much shorter than
the Xenopus 3' UTR, and it contains a putative (U)-rich CPE within the normal distance from the NPE that is not conserved in Xenopus (bases 376-387 of the human sequence, Figure 21). There is also a stretch of high homology between the two species that is very (U)-rich (see bases 218-231 of the Xenopus c-myc 3' UTR; Figure 21). Thus, there are two consensus CPEs in the human sequence and one in the Xenopus sequence. The latter is one of the most likely sequences that could function as a CPE in Xenopus despite its distance from the Xenopus NPE. Its homolog could also function as a CPE in the human sequence.

Because of the (U)-rich homology and the conservation of a putative CPE between in the Xenopus and human c-myc 3' UTRs, it seems probable that the human c-myc 3' UTR could also stimulate cytoplasmic polyadenylation. If this is confirmed, it could define a new regulatory mechanism for myc mRNA. It would be especially interesting if a correlation existed between (A)-tail status and level of translation of messengers containing high degrees of secondary structure in their 5' UTR.

It is ironic that an oligo(U) sequence can regulate instability in somatic cells (Ross, 1995) and be a CPE that can stimulate translation of certain mRNAs during development by polyadenylation. Thus, the same sequence might be acting to destabilize mRNAs in somatic cells and stimulate their polyadenylation during development. Destabilization of growth-related transcripts such as c-myc in somatic cells prevents their overexpression, which would be oncogenic.

Cytoplasmic polyadenylation may be responsible for the dichotomy, because mRNAs that are cytoplasmically polyadenylated are protected from degradation, probably by their removal from the deadenylation-dependent degradation pathway. In addition, it has been observed that the degradation pathway is uncoupled from deadenylation in Xenopus development (Fox and Wickens, 1990). Elements in c-fos that
Figure 21: Alignment of 3' UTRs of c-myc. The Xenopus c-mycI 3' UTR is in normal font on top numbered from 1 to 951. The human c-myc 3' UTR is in italics on the bottom numbered 1-444. Where there is no sequence for human or when a gap was added by the Bestfit alignment (Wilbur and Lipman, 1983), there is a (*). A match between species is indicated by underlining. Shaded text indicates a putative CPE.
regulate deadenylation have been identified as those that also regulate instability (Shyu et al., 1991).

Because the CPE prevents deadenylation and it is also thought that a fertilization-specific CPE can stimulate deadenylation during maturation (Fox and Wickens, 1990; Wormington et al., 1996), it is possible that the CPE and the AURE are the same or at least are related. The *Xenopus* c-myc1 mRNA is also known to be unstable in later stages of development (King et al., 1986; Schreiber-Agus et al., 1993; Godeau et al., 1986; Taylor et al., 1986), but the 3' UTR does not destabilize it during early development. Therefore, instability elements are differentially regulated during development, and they could also stimulate cytoplasmic polyadenylation in early development. Unfortunately, this observation is not universal, because some mRNAs that are unstable in somatic cells are translationally inhibited in early development of *Xenopus* (Marinx et al., 1994). It is possible that there are different mechanisms or other elements involved in this system, because it is known that not all AUREs function the same (Ross, 1995).

To determine if the CPE and the AURE are related, it is necessary to demonstrate that many of the AURE-containing mRNAs that are unstable in somatic cells are polyadenylated via a functional CPE during development. It is also necessary to determine whether mutations that eliminate instability also eliminate cytoplasmic polyadenylation. This experiment would require that many different RNAs be characterized in order to ensure that differences in regulation are found.

Finally, if some oncogenes contain CPEs, it is possible that one of the mechanisms that cancer cells use to upregulate gene expression is to induce cytoplasmic polyadenylation. To test if cancer cells stimulate cytoplasmic polyadenylation, a detailed study of the adenylation status of certain mRNAs in different cellular states is required. Initially, it may be worthwhile to investigate the adenylation status of mRNAs in cell lines that are known to contain stabilized oncogene mRNAs.
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APPENDIX 1: PLASMIDS AND mRNA DIAGRAMS

This appendix contains diagrams of all original plasmids (Figure 22) that were used in this dissertation and schematic diagrams of the capped mRNAs (Figure 23) that were microinjected. For details on construction and mRNA synthesis, see Methods and Materials.

The legend for Figure 22 is on page 148. The legend for Figure 23 is on page 153.
A. pBS-poly(A)(-HindIII).
B. pBSK-As(-HindIII).
D. pCAT73(BamHI).
E. pSP6-CAT(A)$_{73}$.
F. pSP6-CAT-dist(A)\textsubscript{73}. 
G. pSP6-CAT-prox(A)$_3$. 
H. pSP6-CAT-tCK(A)_{17}.
I. pSP6-CAT-Xc-myc1(A)$_{73}$.
J. pSP6-He-mycP1-CAT(A)$_{73}$.
K. pSP6-hpCAT-tCK(A)₁₇
L. pSP6-Xc-myelIP1-CAT(A)_{73}. 
M. pSP6-Xc-mycIP2-CAT(A)$_3$. 
N. pSP6-Xc-mycP1-CAT-Xc-myc(A)\textsubscript{73}.
O. pSP6-Xc-mycIP2-CAT-Xc-myc1(A)$_{73}$.
Figure 22: Plasmid maps. Restriction enzyme and RNA polymerase start sites are indicated, multiple cloning sites are boxed and important elements are shown. The location of a site is given in small numbers beside the site, when appropriate. AmpR indicates the location of the ampicillin resistance gene, β-lactamase.
A. CAT(A)'

B. CAT(A)_{73}

C. CAT-dist(A)'

D. CAT-myc(A)'
E. \text{CAT-\textit{myc}(A)}_{73}

F. \text{CAT-\textit{prox}(A)}^+

G. \text{CAT-tCK(A)}_{17}

H. \text{bpCAT(A)}^+
I. hpCAT-tCK(A)_{17}

J. Hc-myc-CAT(A)^-

K. Hc-myc-CAT(A)_{73}

L. P1-CAT(A)^+
M. P1-CAT(A)73

N. P1-CAT-myc(A)73

O. P2-CAT(A)'

P. P2-CAT(A)73
Figure 23: Schematics of the mRNAs used. The cap (m^7GTP) in all mRNAs is base #1. Indicated above the mRNAs are significant sites, including important restriction enzyme sites from the DNA sequence, the AUG of the CAT sequence (●), the stop codon of the CAT sequence (■) and the locations of the plasmid-encoded (A) tails. Any 5' UTR sequence is in gray, the CAT sequence is white, any 3' UTR sequence is hatched and the (A) tails are in black. The myc sequences that were amplified by PCR can be found in Figure 2 in the Methods and Materials section.
APPENDIX 2: STATISTICAL DATA

This appendix summarizes the results of the Mann-Whitney U-tests of the data utilizing Data Desk® 5.0 Test Flight (©1995; Table 2). The use of averages and standard deviations is not particularly valuable when considering that the variation is high and the sample size is small; a single value that is aberrant could alter the standard deviation significantly. However these values have been included in Table 3.

I have chosen to apply the Mann-Whitney U-test to my data. This test compares all of the data from one variable to all of the data from another variable. It ranks the data and assigns each data point a value, thus eliminating the effect of the large variations of the sample on the statistical result; it does not look at the actual values, only how they compare to the values from the other variable. The average of the ranked data is then tested to determine if the data in the two variables is significantly different or not. The advantage of this system is that even with the large variation, the values are ranked, so even very large or small values are simply ranked the highest or lowest of the set. This means that the difference is small compared to the effect it would have on the real average.
Table 2: Statistical values for the Mann-Whitney U-test.

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<td>VI oocyte</td>
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<td>Stim by (A)\textsubscript{73} over (A)': CAT</td>
<td>VI oocyte</td>
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<td>Stim by (A)\textsubscript{73} over (A)': XDD-CAT</td>
<td>Stim by (A)\textsubscript{73} over (A)': CAT</td>
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<td>0.7963</td>
<td>9</td>
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</tbody>
</table>

**Figure indicates the figure in the dissertation to which the data are relevant; RNA 1 and RNA 2 are the two RNA species that were compared; Stage is the developmental stage from which the data were taken; p value is the probability that the data could occur by**
chance ($p<0.05$, the value is statistically significant and is indicated by bold face print; $p<0.01$, the value is highly significant and is indicated by bold face and underlined print); $n$ is the number of experiments used to calculate the value.
Table 3: Means and standard deviations for CAT activities relative to CAT(A).*

<table>
<thead>
<tr>
<th>Figure</th>
<th>RNA</th>
<th>Stage</th>
<th>Mean (%)</th>
<th>SD</th>
<th>n</th>
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<tr>
<td>4, 5</td>
<td>P1-CAT(A)</td>
<td>VI oocyte</td>
<td>141.45</td>
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<tr>
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<td>P2-CAT(A)</td>
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<td>124.24</td>
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<td>E. Emb.</td>
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</tr>
<tr>
<td>5</td>
<td>P1-CAT(A)_{73}</td>
<td>VI oocyte</td>
<td>507.61</td>
<td>471.66</td>
<td>14</td>
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<tr>
<td>5, 6, 8, 10</td>
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<td>408.86</td>
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<td>5, 6, 8, 10, 15</td>
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<td>22.61</td>
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<td>11, 12, 13, 14, 15</td>
<td>CAT-myc(A)</td>
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<td>225.53</td>
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<td>428.57</td>
<td>248.1</td>
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</table>

**Figure indicates the figure in the dissertation to which the data are relevant; RNA is the RNA species used to generate the data; Stage is the developmental stage from which the data were taken; Mean is the mean values of the data where CAT(A)\(^-\)= 100%; SD is the standard deviation of the data; n is the number of experiments used to calculate the values.
APPENDIX 3: SYNTHETIC HAIRPIN-CONTAINING mRNA

1. The effect of a synthetic hairpin on translation

Synthetic hairpin elements are known to inhibit the translation of reporters (Pelletier and Sonenberg, 1988; Kozak, 1989b; Fu et al., 1991). A hairpin (hp) was previously reported to inhibit translation in *Xenopus* oocytes when placed upstream of the reporter chloramphenicol acetyltransferase (CAT) (Fu et al., 1991). Interestingly, this same synthetic mRNA was observed to be more translatable upon microinjection into *Xenopus* early embryos. This apparent release from translational inhibition was termed the “potentiation of translation” of secondary structure-containing mRNAs after fertilization (Fu et al., 1991). I endeavoured to repeat the work of Fu et al. (1991) and to extend those results by examining the effects of poly (A) on translation of hp-containing transcripts. As shown below, I was unable to reproduce the potentiation effect on translation of hpCAT mRNA after fertilization. In addition, I constructed a plasmid for *in vitro* transcription that would generate transcripts containing either CAT or hpCAT followed by the tCK(A)17 element. This element slightly enhanced translation of a CAT reporter, but it significantly enhanced translation of hpCAT.

1.1. Translational inhibition by a hairpin

I injected synthetic hpCAT or CAT mRNA into *Xenopus* oocytes or early embryos (one to two cell stage). As can be seen in Figure 24, the translation of hp-containing mRNA is substantially repressed as compared to translation of RNA lacking the hp element. This is true both for stage VI oocytes and for early embryos. This is in contrast to what has been previously reported by Fu et al. (1991). I and others in Dr. Browder’s laboratory have repeated these experiments numerous times and have never confirmed “potentiation”.
Figure 24: The effects of a synthetic hairpin (hp) element on CAT activity. 5 ng of in vitro transcribed mRNA was microinjected into five Xenopus (A) stage VI oocytes or (B) early embryos. Soluble protein was extracted and a CAT assay was performed. All 5 cells were assayed for hpCAT samples, but only 1/50 of a cell was assayed for CAT samples. The CAT activity was calculated and corrected to account for different amounts assayed.
Considering the enormous differences in CAT activity obtained between the hpCAT and CAT transcripts, it was possible that the hp affected the stability of RNA. To examine this possibility, RNase protection assays were conducted (Figure 25). From these results, I concluded that the transcripts are equally stable.

1.2. The effects of 3’ elements on hp

I next examined whether a plasmid-encoded poly(A) tail could affect translation of hpCAT or CAT mRNA. Initially, this was to be done by placing a poly(A) sequence downstream of the CAT coding sequence in the mRNA. The plasmid utilized was pBS-poly(A) obtained from Dr. A. Garber. It was thought that pBS-poly(A) contained only an (A) tail of 68 (A) residues surrounded by a multiple cloning site. Upon sequencing of the constructs containing this sequence, two things were discovered. First, it was found that the (A) tail was truncated from 68 to only 17 (A) residues. This apparently happened because certain bacterial strains are unable to replicate a long stretch of any single nucleotide and after a short period of time, they attenuate homopolymers (Munroe and Jacobson, 1990). Subsequently, plasmids containing long homopolymers were placed into either the HB101A (Munroe and Jacobson, 1991) or the XL-1 Blue bacterial strains (J. Richter, personal communication), which are competent to replicate accurately homopolymers in DNA. The second problem discovered by sequencing was that the poly(A) tail from pBS-poly(A) was preceded by 300 bases of the 3’ end of the trout creatine kinase (tCK) cDNA, which might affect translation or stability.

When the tCK(A)₁₇ sequence was placed downstream of the CAT coding sequence in mRNA, there was very small stimulation of translation compared to CAT(A)ₙ mRNA. However, when the same tCK(A)₁₇ sequence was placed downstream of hpCAT, there was a significant increase in the translation of this mRNA. Figure 26 shows a representative experiment. Although the hp was still inhibitory, CAT activity for hpCAT-tCK(A)₁₇ was 46.2 fold greater than for hpCAT(A)ₙ whereas CAT-tCK(A)₁₇
Figure 25: RNase protection of hpCAT and CAT RNA in stage VI oocytes. Experiment performed as described in Methods and Materials with the exception that no Luc(A)* RNA was used. Despite an enormous difference in the translation due to the presence of the hp element, there was no difference in the stabilities of the mRNA.
Figure 26: The effect of the tCK(A)$_{17}$ sequence on the translation of hpCAT and CAT mRNA. The experiment was carried out as described in Methods and Materials and in the legend to Figure 21 using stage VI oocytes.
was only 1.4 fold greater than CAT(A)⁻.

The unexpected observation that hp-containing mRNA can be partially released from translational inhibition in the presence of the 3' end of a trout creatine kinase (tCK) cDNA and a sequence of 17 (A)s has prompted additional experimentation in Dr. Browder's laboratory.
APPENDIX 4: TRANSLATION LYSATES

There are both advantages and disadvantages to using microinjections to test for RNA translation, stability and polyadenylation. Two things limit the system biologically. One is that embryos develop. Because the embryos are constantly developing, this limits the number of injections that can be done on a single batch of embryos, making it difficult to compare samples. The second problem is the observation that cytoplasmic polyadenylation does not begin in embryos immediately upon fertilization, but can take up to 1.5 hours to begin (McGrew et al., 1992). In fact, the Cl2 RNA in *Xenopus* is not normally maximally adenylated until the 4000-cell fine-blastula stage (McGrew et al., 1992). This means that microinjections must either be done early and left longer, thereby decreasing the percent recovery of RNA due to degradation, or the embryos must be injected later, which is impossible in very late embryos because the cells are too small. One way to get around this problem would be to use coenocytes. However, it is unknown how “normal” coenocytes are. There are other problems with microinjections: (1) Radiolabeled RNA is not as safe as non-radiolabeled RNA; (2) injections are time consuming, thereby limiting the amount that can be done at any one time; and (3) because they require some skill and the embryos are damaged by the injection, there is a chance for errors to be introduced by the procedure.

The use of an *in vitro* lysate system would avoid the problems with development and handling large numbers of radiolabeled samples. However, there are also disadvantages to using a lysate system. It is an *in vitro* system, hence it lacks components that may be important to regulation, such as the cytoskeleton, which is known to be involved in binding of poly(A) and ribosomes (Taneja et al., 1992). This problem can be avoided if appropriate positive and negative controls are used in addition to demonstrating that the system behaves similarly to the endogenous situation. Dr. Browder’s laboratory has previously used a translation lysate made from unfertilized
Xenopus eggs (Matthews and Colman, 1991). I modified the protocol slightly to allow lysates to be made from embryos (see Methods and Materials). Unfortunately, the amount of RNA degradation that was seen was highly variable from batch to batch, making it difficult to get repeatable results. This is less of a problem when using unfertilized eggs. Figure 27 shows an example of an experiment that used lysate made from MBT stage embryos and in which the RNA was stable. The CAT activity in the CAT-\textit{myc}(A)^{-} sample was enhanced over the CAT(A)^{-} sample. This indicates that the MBT lysate, when the RNA is stable, behaves very much like the coenocytic late embryos described in the results Section 2.
B. RNase Protection

Figure 27: Use of an MBT lysate to demonstrate that its use is possible. The experiment was performed as described in Methods and Materials. (A) CAT activity was monitored to demonstrate that the CAT-myc(A)^− RNA could stimulate translation in the lysate as in injections. (B) RNase protection assay to demonstrate that in this particular lysate, the RNA was stable.