Translational Regulation of the C-Jun Proto-Oncogene

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TRANSLATIONAL REGULATION OF THE
C-JUN PROTO-ONCOGENE

by

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The \textit{v-jun} oncogene was originally isolated from the ASV17 virus in 1987. Ever since its isolation, extensive work has been done to understand the role of the \textit{v-jun} oncogene in cell transformation. The c-Jun protein is a transcription factor which binds to the DNA target TGACTCA. The c-Jun protein binds to DNA in the form of dimers. It can form homodimers with itself and heterodimers with Jun family (JunB and JunD), Fos family (FosB, Fra1 and Fra2), or with CREB family members through the leucine zipper motif. Because the \textit{c-jun} proto-oncogene plays an important role in cell transformation, extensive work has been done to understand how it is regulated. Previously, it has been shown that \textit{c-jun} transcription can be activated by growth factors, tumor promoters and other oncogenes such as \textit{ras} and \textit{src}. Regulation of \textit{c-jun} activity has been studied at the
level of transcription, dimerization, DNA binding and post-translational modification. I report here that the c-jun proto-oncogene can also be regulated at the translational level.

A closer look at the c-jun transcript revealed the presence of a 313 bases long 5' untranslated region (5'UTR) that is 81% GC rich. Long 5'UTRs are also present in a number of proto-oncogenes including int-2, myb, ets-2, erbB, junB and junD. The c-jun 5'UTR has potential to form a hairpin loop structure. This hairpin loop like secondary structure may prevent the translational machinery from reaching the translational initiation site. I have examined the role that 5'UTR can play in translational regulation of c-jun by creating deletion mutations. Complete removal of the 5'UTR results in a 25 fold increase in protein synthesis. A number of other mutations were constructed within this region to determine the minimum structure required for translational repression. A number of deletion mutations were then assayed for translational activity in vitro. In vitro analysis of these mutations has demonstrated that as the complexity of secondary structure increases translational efficiency decreases. I have also demonstrated that translational suppression caused by the c-jun 5'UTR is not due to an increase in the distance between the 5'CAP and AUG. Using a set of deletion mutations in the 5' and 3'UTRs of c-jun, I have demonstrated that when both the 5' and 3'UTR were present in the c-jun mRNA more translation suppression was observed. The c-jun 5'UTR, when cloned upstream of a heterologous CAT gene, it causes a 30 fold decrease in protein level. Secondary structure analysis of other jun and fos family members 5'UTRs indicate that they may also be regulated translationally. The role of the c-jun 5'UTR in translational regulation in vivo is under investigation.
DEDICATION

This work is dedicated to my loving parents, brother, sister and my beautiful wife Kanchan for their love, support and appreciation during this study.
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I. INTRODUCTION

1. Oncogenes: General Information

Oncogenes are the genes that can cause cancer. Many oncogenes were discovered in acutely transforming retroviruses. These viruses carry altered versions (v-onc) of the cellular genes which are called proto-oncogenes (c-onc). These proto-oncogenes are highly conserved and can be detected in species as divergent as humans, Drosophila and yeast. Proto-oncogenes play an important role in normal cell growth and differentiation. Proto-oncogenes by themselves are not oncogenic until they are activated by genetic alterations that can alter their function. It has been demonstrated that a number of specific tumors have activated proto-oncogenes. More than 50 oncogenes have been discovered to date. Depending upon their specific function, oncogene encoded proteins are distributed in different regions of a cell. These include the plasma membrane, cytoplasm and nucleus. Nuclear oncogenes have been studied extensively in the past few decades to understand their role in cell transformation. These oncogenes encode for transcription factors which may regulate the gene expression of a number of target genes involved in normal cell growth and differentiation. Some examples of transcription factor encoding oncogenes are fos, myc, myb, ski, maf, ets and jun (1). The c-jun is among the most extensively studied proto-oncogene to date. The protein product of c-jun plays an important role in normal cell growth and development. The viral Jun protein (v-Jun) on the other hand causes sarcomas in chickens and transforms cells in vitro.
2. Discovery of the \textit{jun} Oncogene

In 1983, a new virus was isolated from a spontaneous sarcoma of an adult chicken (2). This isolate was termed Avian Sarcoma Virus 17 (ASV17). ASV17 is a retrovirus capable of causing fibrosarcomas in chickens (3,4). This virus can also induce oncogenic transformation of chicken embryo fibroblasts (CEFs) in cell culture. The range of cell types transformed by ASV17 in cell culture are limited. Other than CEFs, ASV17 can transform chicken embryo neuroretina cells and myoblasts.

Sequence analysis of the ASV17 genome has revealed the presence of a cellular sequence. This cellular sequence has replaced part of \textit{gag}, \textit{env} and complete \textit{pol} sequences of ASV17. This presumptive cellular sequence of ASV17 was given a new name, \textit{v-jun}. This name was derived from "Ju-nana" which is Japanese for 17, alluding to ASV17 and to the ground breaking work of a research associate from Japan (2). The ASV17 version of \textit{jun} is referred to as \textit{v-jun} and its counterpart in the vertebrate cellular genome as \textit{c-jun}. The \textit{c-jun} proto-oncogene encodes a 39kd protein which is a transcription factor. The c-Jun protein has several domains which carry out different functions.

3. Characteristics of the Jun protein

The protein encoded by the \textit{c-jun} gene is a transcription factor of the basic leucine zipper family (5,6). It is concentrated in the nucleus in different phases of the cell cycle (7). The c-Jun protein contains two major functional domains. The amino terminus is highly acidic and it plays an important role in transcriptional activation. The carboxy terminus, on the other hand, has DNA binding properties. The DNA binding domain contains three subdomains. The proline rich domain provides a hinge in the protein. This
hinge gives c-Jun protein an ideal three dimensional structure to carry out its DNA binding function. The carboxy terminus of c-Jun protein has a major DNA binding domain. Within this DNA binding domain there are two subdomains, the basic domain and the leucine zipper domain. The basic domain comes in direct contact with DNA and the leucine zipper domain is involved in protein dimerization. Recently, it has been shown that the basic domain of c-Jun includes a nuclear localization signal. This signal sequence is required for entry of c-Jun into the nucleus (8).

The leucine zipper domain is one of the major functional domains of Jun and is necessary for dimerization. The amino acid sequence in the leucine zipper domain forms an α helical structure in which every seventh amino acid is a leucine. These leucines stick out of the major backbone of c-Jun and can hydrophobically interact with other proteins that have a leucine zipper motif. Other than the Jun family members, the leucine zipper motif is also present in a number of other proteins of the Fos and CREB families (9-17). Unlike v-Jun which was isolated from ASV17 transformed cells, c-Jun was first identified as a major component of the AP-1 complex. The AP-1 complex is a mixture of Jun, Fos and CREB family proteins.

4. AP-1 Complex

AP-1 is a mixture of polypeptides originally isolated from HeLa Cells (18,19). Using a series of immunoprecipitation reactions and DNA binding assays, it was demonstrated that Jun and AP-1 are closely related antigenically. The AP-1 complex binds to a DNA consensus sequence called the AP-1 site (TGACTCA). In addition to the Jun family members (JunB and JunD), AP-1 preparations also contain products of the fos related genes such as c-fos, fra-1, fra-2 and fosB as well as several other still unidentified components (20-23). The AP-1 site is present in a number of cellular and viral genes (24-27). Binding of the AP-1 complex to the AP-1 site can cause transcriptional activation of a gene. Some examples of genes activated by AP-1 include metallothionein IIA,
collagenase and stromelysin (24-27). AP-1 activity of certain cell types can be increased by tumor promoters such as TPA (12-0-tetra decanoyl-phorbol-13-acetate), serum, growth factors and calcium ionophores (20,24,26,28,29).

Like c-Jun, v-Jun can also bind to the AP-1 site after dimerization with other proteins. The v-jun and c-jun have several structural differences. Some of these structural differences may be responsible for the oncogenic potential of v-Jun.

5. Structural differences between v-jun and c-jun

The c-jun proto-oncogene encodes a 39kd protein which is a part of the AP-1 complex previously isolated from the human system. This protein complex contains products from the c-Jun and c-fos family members. The v-Jun protein on the other hand is a gag-Jun fusion protein of 65kd isolated in ASV17 transformed cells. The protein encoded by v-jun differs from c-jun in several aspects.

The amino terminus of v-Jun includes a 220 amino acid gag encoded leader sequence that is fused in frame with cell derived c-Jun sequences. The v-Jun also has a 27 amino acid deletions at the amino terminus (amino acids 32-58) and three point mutations in the carboxy terminus. These three point mutations result in non-conservative amino acid substitutions (at c-Jun position 185, Glycine to Arginine position 226 Serine to Phenylalanine and position 252, Cysteine to Serine). Except for these differences the rest of the v-Jun and c-Jun proteins are identical (7).

The mRNA encoded by v-jun and c-jun differ in two major aspects. Firstly, v-jun mRNA lacks most of the 3'UTR that is about 667 nucleotides long. The 3'UTR contains two poly(A) addition signals and two AUUUUA like motifs that can cause destabilization of the RNA (31,33). Secondly, c-jun mRNA has a 313 nucleotide long, 81% GC rich 5'UTR. This GC rich 5'UTR is absent in the v-jun mRNA. GC rich 5'UTRs are thought
to be involved in the translational regulation of several other genes such as \textit{c-myc} and \textit{bcr-abl} (30,31).

What structural differences between v-Jun and c-Jun are responsible for transforming activity? It has been shown previously that the gag sequences of v-Jun are neither necessary nor essential for cellular transformation (7). Conversely, deletion of the transcriptional activation domain of v-Jun or c-Jun completely abolish their transforming activity. This suggests that the transactivation domain of v-Jun and c-Jun is essential for cell transformation. Recently, it has been shown that deletion of 27 amino acids in the transactivation domain of v-Jun and 3'UTR of \textit{c-jun} are responsible for full oncogenic activation (7).

The \textit{c-jun} plays an important role in normal cell growth and development. Its altered function can lead to cell transformation. Thus, it is important to understand how \textit{c-jun} is regulated. Several levels of regulation have been studied to date. These include transcriptional regulation, protein dimerization and post-translational modification (phosphorylation).

\textit{a. Transcriptional Regulation of c-jun}

Like many other nuclear oncogenes, \textit{c-jun} is tightly regulated at transcriptional and post-translational levels. Transcriptional regulation in response to PDGF, TGFβ, EGF, NGF, TPA, retinoic acid, UV radiation, TNFα, IL-1, cAMP and mitomycin C has been observed in a number of different cell types (26,32-34). The majority of these agents can cause a transient increase in \textit{c-jun} mRNA level. This induction occurs rapidly and does not require \textit{de novo} protein synthesis (25,35,36). The mRNA induction of \textit{c-jun} lasts considerably longer than \textit{c-fos} mRNA induction (25,37,38). This is probably due to the increased stability of \textit{c-jun} mRNA. Recently, it has been shown that \textit{c-jun} can autoregulate its own synthesis at the transcriptional level.
A close examination of the human \textit{c-jun} gene has revealed the presence of two TATA sequences located about 24-30 bases upstream of the transcription initiation site. These elements are also present in the chicken \textit{c-jun} gene (39). Upstream of these TATA elements, an AP-1-like sequence (5'GTGACATCAT-3') is also present. Except for an additional adenine, this sequence is identical to the consensus AP-1 site (5'TGACTA3'). Because this AP-1 like site is different from the classical AP-1 site it is thought that only a subset of c-Jun homodimers or heterodimers will bind to this sequence. Upstream of this AP-1 site, a CAAT box and a GC box is also present. These sequences are shown to be recognized by the transcription factors CTF and SP-1 respectively. The AP-1 site of the \textit{c-jun} promoter appears to be the key element responsible for induction of \textit{c-jun} in response to TPA. The presence of the AP-1 site in the \textit{c-jun} promoter suggests that transcription of \textit{c-jun} could be the subject of a positive autoregulatory loop (25). This positive autoregulatory loop of \textit{c-jun} alone can cause constitutive expression of c-Jun which can lead to cell transformation. In order to autoregulate its own synthesis, interaction with the AP-1 site requires c-Jun protein dimerization. Thus, it is important to understand how c-Jun protein is regulated at the dimerization level.

\textit{b. Dimerization of Jun}

The protein encoded by the \textit{c-jun} oncogene can form dimers with other transcription factors which have a leucine zipper. It can form homodimers with itself and heterodimers with Jun family (JunB and JunD), Fos family (FosB, Fra1, Fra2) and CREB family members (CREBP-1 and CREBP-2), through the leucine zipper motif (9-17).

Recently, it has been shown that c-Jun not only interacts with proteins which have leucine zippers, but can also interact with non leucine zipper proteins. These proteins include myoD (40), NF-kB (41), TATA box binding protein (42) and the Epstein Barr
Virus gene product, BZLF-1 (43). Interaction of c-Jun with these proteins indicates that it can also modulate their activity through protein-protein interactions.

The c-Jun heterodimers are much more stable than c-Jun homodimers. Jun homodimers and heterodimers bind to DNA at a consensus AP-1 site (TGACTCA). AP-1 and AP-1-like sites have been shown to be present in the promoters of a number of other genes such as collagenase and transin/stromelysin. Dimer formation between Jun, Fos and CREB family proteins is driven by the relative abundance of each member at a specific time point in the cell (44). Stimulation of the cell with growth factors or other physical agents may result in different proportions of Jun-Jun, Jun-Fos or Jun-CREB dimers. These c-Jun dimers are likely to regulate a number of common genes and some unique ones. Thus, changes in the relative abundance of these protein dimers can induce subtle changes in gene expression. Recently our lab has shown that c-Jun does not require heterodimerization with other Fos or CREB family members to cause cell transformation in vitro (45). This implies that homodimerization of the c-Jun is sufficient for cell transformation.

Like many other oncoproteins, c-Jun can be regulated at the level of phosphorylation. The relative abundance of c-Jun in its phosphorylated or dephosphorylated form in a given cell type can modulate its function. Thus, it is important to understand that how the c-Jun function is regulated at the level of phosphorylation.

c. Post-translational regulation of Jun

The c-fos and c-jun are immediate early genes that were stimulated by growth factors. Under normal circumstances, c-jun is expressed at relatively low levels in cells, although it can be induced rapidly and transiently by extracellular stimuli. Once synthesized, DNA
binding activity and transcriptional activation of c-Jun protein can be modulated by phosphorylation.

In non-stimulated fibroblasts and epithelial cells c-Jun is phosphorylated on two or three sites adjacent to its DNA binding domain (46,47). Serine 246 of c-Jun is among the most extensively studied phosphorylation sites. This site is substituted by phenylalanine in v-Jun. Substitution of Serine 246 by phenylalanine causes at least a 10 fold increase in c-jun activity, in vivo. Thus, Casein Kinase II which causes phosphorylation of Ser 246 is a negative regulator of the c-Jun DNA binding activity (48).

The amino terminus of c-Jun can also be post-translationally modified by phosphorylation. Serine 63 and 73, which are present in the amino terminus of Jun are the major sites of phosphorylation. PKC, p34cdc2 and mitogen activated protein kinases (pp54 and pp42/44), specifically phosphorylate these sites (46,49,50). The v-Jun however is not phosphorylated on these residues. Recently, it has been demonstrated that lack of phosphorylation of v-Jun at serine 63 and 73 is due to the absence of binding by a kinase which binds to the c-Jun specific δ domain (49). This domain is deleted in v-Jun. Phosphorylation of c-Jun causes an increase in its transactivating property. Serine 63 and 73 of c-Jun are also shown to be phosphorylated in cells transfected with activated Ha-Ras. Mutation of either serine 63 or 73 of c-Jun inhibits its transcriptional activation property by Ha-Ras and PMA respectively (47). These findings suggest that phosphorylation at serine 63 and 73 plays an important role in the post-translational regulation of c-Jun.

So far, I have discussed three different levels at which c-jun can be regulated. These three levels of regulation are key elements which may help us understand how c-jun plays an important role in normal cell growth. Two major levels of c-Jun regulation, dimerization and post-translational modification described above, requires c-Jun protein synthesis. I have discovered that c-jun can be regulated at a new level, the translational
level. Translation of RNA into protein is a complex process and it requires several factors that can be regulated in several different ways.

6. Mechanism of translation: General information

Translation of mRNA into protein is a multistep process. It involves a number of specific factors which play important roles at specific steps of the translation process. The mechanism of translation initiation in eukaryotes has been studied extensively. It involves a series of steps leading to protein synthesis. At present some of the specific steps involved in translation are still not clear.

Briefly, 40S and 60S ribosome subunits exist either in complex or as dissociated subunits. The 40S subunit can bind to eIF3 and start the process of assembly of subunits and factors. The eIF-4C and eIF-GTP-met-tRNA complex then binds to the 40S subunit of the ribosome. At this stage it is ready for binding to the 5'CAP of mRNA. This is mediated by binding of eukaryotic factors eIF-4A, eIF-4B and eIF-4F binding to the 40S subunit. This step is immediately followed by the scanning of the 40S subunit along the mRNA. Once the 40S ribosomal subunit reaches a Kozak consensus AUG, the 60S subunit can bind and protein synthesis can begin (51).

Regulation of translation is a complex event which may involve phosphorylation of translation initiation factors. Most research in understanding translational regulation has been focused at the level of translation initiation. There are several features within the mRNA that can control the process of translation initiation. These include the 5'CAP, (m7G(5')PPP(5')N; where N is any nucleotide) and the primary or secondary structure of untranslated regions (UTRs) (52,53).

At the translation initiation level, eIF-4E is among the most extensively studied initiation factors (54,55). With the help of three initiation factors eIF-4A, eIF-4B and eIF-4F, the 5'CAP of the mRNA binds to the 40S ribosome subunit. The eIF-4F initiation
factor is composed of three subunits eIF-4E, p44 and p220. The eIF-4E subunit is a 25kd protein that recognizes the CAP when it is already associated with two other subunits, eIF-4A (p44) and a 220kd protein (p220). There are two major features of eIF-4E that are significant for activity of the eIF-4F complex. The active eIF-4E concentration in the cell is limiting as compared to the other initiation factors as well as the sum of total mRNAs present in a cell. Thus, the mRNAs present in a cell compete for binding to active eIF-4E. The eIF-4E can be activated by phosphorylation, in cells that are treated with growth factors and mitogens. It has been shown specifically that eIF-4E can be activated by phosphorylation in cells stimulated with TPA (56), insulin (57), Tumor Necrosis Factor (TNF) (58), serum (59), PDGF (60) and v-Src (61). Conversely during mitosis and in cells responding to heat shock, eIF-4E is present in a dephosphorylated form (62,63,64).

Overexpression of eIF-4E causes transformation of NIH 3T3 and RAT-1 cells. The eIF-4E can collaborate with EIA or c-myc to cause transformation of rat embryo fibroblasts (65).

One possible way by which eIF-4E causes cell transformation is by enhancing translation of mRNAs coding for those proteins that are involved in the normal control of cell growth. Recently, it has been shown that overexpression of eIF-4E can facilitate the translation of mRNAs with extensive secondary structure within their 5'UTR (66).

Thus, it is important to know that how much and how active eIF-4E is present in the cells. In addition to the translation initiation step where eIF-4E is a crucial factor, structural features within the mRNA can also regulate the process of translation initiation. These include the AU rich 3'UTRs and the GC rich 5'UTRs.
a. **AU rich 3' Untranslated region of c-jun mRNA**

Interestingly *c-jun* mRNA also has a very long 3'UTR that is AU rich. Several other genes that code for cytokines, oncoproteins and growth factors also contain long 3'UTRs (67). These 3'UTRs have AU rich sequences that have been shown to be the major determinants of mRNA stability. In addition to AAUAAA (polyadenylation signal), multiple copies of the AU rich octanucleotide, UUAUUUAU are also present. These sequences have been shown to be present in *c-myc* and *c-fos* transcripts (68), and are highly conserved in transcripts coding for proteins related to the inflammatory response (69). Removal of these octanucleotides confer a greater stability to the mRNA. What is the role of the AU rich sequences in translation? The polyA tail has been postulated to be involved in determining the stability of RNA and in the translation initiation process. This may occur by long range interaction between the 3' and 5' ends of the mRNA. The UUAUUUAU motif is shown to have inhibitory effects on the translation of *c-fos* (68). The actual mechanism of inhibition caused by these AU rich sequences is still not known. There is some evidence that instability of mRNA containing AUUUA motifs is mediated through translation dependent assembly of a >20S degradation complex (70).

Previous experiments have shown that stimulation of cells with the tumor promoter TPA, has resulted in a rapid and transient increase in *c-jun* message. However the increase in *c-jun* transcripts is not followed by a similar increase in c-Jun protein synthesis suggesting that there might be a block in *c-jun* mRNA translation (33). The structural elements involved in this translational block are the subject of this thesis.
b. **GC rich 5' Untranslated region of c-jun mRNA**

A close look at the *c-jun* proto-oncogene transcript revealed the presence of a long 5'UTR that is 81% GC rich (Figure 1). Long 5'UTRs such as this one, are present in a number of other transcripts coding for oncoproteins, growth factor receptors, G proteins and growth factors. Some specific examples include *fes*, *int-1*, *int-2*, *c-jun*, *junD*, *junB* adenergic receptor, estrogen receptor, NGFR, SRE-BF, TFIIS, NF-kB, GAP, PKCs, cAMP, thymidine synthetase and dihydrofolate reductase (67). The 5'UTR of the *c-jun* proto-oncogene is 313 nucleotides long and is predicted to form a long hairpin loop structure with a free energy value of -252.7Kcal/Mole. This free energy is very low indicating high structural stability (Figure 1). I have speculated that this hairpin loop secondary structure may prevent the translational machinery from reaching the translation initiation site thereby suppressing translation.

The suppressive role of the 5'UTR on translation has also been studied in genes such as *c-sis/PDGF-2*, thymidine kinase of the herpes simplex virus, human ferritin H chain gene and the *bcr-abl* oncogene (31,71-73). Recently, translational inhibition by the 5'UTR secondary structure in alpha globin was shown to be determined by its proximity to the translation initiation site, AUG. If the secondary structure is very close to the translation initiation site, translation repression is much more effective, than if it is located further downstream (74). Interestingly, it has been shown that the thymidylate synthetase gene product can regulate its own translation initiation by binding to its own mRNAs 5'UTR secondary structure (95). Thus, GC rich 5'UTRs can play an important role in translation regulation. The presence of a long loop secondary structure in the 5'UTR of the *c-jun* proto-oncogene and its proximity to the AUG makes it a good candidate for translation suppression.
Figure 1  Schematic of the c-jun 5'UTR secondary structure. Panel A shows a stick diagram of various regions of the c-jun message. Sequences are numbered from +1 (CAP) through +313 (AUG). Shaded box is the c-jun coding region. The translational start site is indicated by ATG and an arrow. The sequence of the 5'UTR is shown directly below it. Guanines and cytosines are shown as capital letters. Panel B shows the secondary structure of the c-jun 5'UTR which was predicted using Zuker's RNA fold method with the help of Mac-DNAsis program obtained from National Bioscience Incorporation.
A

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\[
\begin{array}{cccccc}
+1 & +140 & +193 & +259 & +313 & \\
\text{BgII} & \text{SacI} & \text{BstHII} & & & \\
\hline
\text{5'UTR} & \text{CODING REGION} & & \text{WILD TYPE} & & \\
\text{3'UTR} & & & & & \\
\end{array}
\]

\[
\begin{align*}
+1 & \quad \text{GC GCC CG aG} \\
+140 & \quad \text{GC GCC CG aG} \\
+193 & \quad \text{GC GCC CG aG} \\
+259 & \quad \text{GC GCC CG aG} \\
+313 & \quad \text{GC GCC CG aG}
\end{align*}
\]

\[ \Delta G = -252.70 \text{ Kcal/Mole} \]

B

\[ 313 \text{ AUG} \]

\[ \begin{array}{c}
100 \\
150 \\
50 \\
200 \\
250 \\
300 \\
\end{array} \]

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In this study, I have attempted to address the role of the GC rich 5'UTR on translation regulation of the c-jun message. Using a series of in vitro experiments, I have demonstrated for the first time, that c-jun can be regulated at the translational level. Serum stimulation of Rat-1 cells and CEFs has resulted in a several fold increase in the RNA level but no significant change in protein synthesis (25). This indicates that there is a translational block in the c-jun message. This translational block could be due to the presence of the GC rich 5'UTR. When I have removed the 5'UTR from the c-jun message it resulted in a several fold increase in the level of c-Jun synthesis in vitro. Several deletion mutations in the c-jun 5'UTR were constructed and tested in vitro for their translation efficiency. Our results show that there is a good correlation between complexity of the 5'UTR secondary structure and translation efficiency. As the complexity of c-Jun 5'UTR secondary structure increases translation efficiency decreases. Interestingly, the 5'UTR of c-jun not only suppresses its own translation but also that of a heterologous gene. I have demonstrated that sequences within the 5'UTR are sufficient for translational suppression. Thus, the overall conclusion which can be drawn from this study is that c-jun can be regulated at the translational level and that the GC rich 5'UTR is a major player in translational regulation.
II. MATERIALS AND METHODS

1. Construction of c-jun plasmid with (pG5'CJ3) and without (pGCJ1) 5'UTR

The chicken genomic c-jun gene is contained within a 3.2kb XbaI fragment in pUCGCJ1. The 5'UTR of c-jun was cloned into the in vitro expression plasmid pGCJ3 by a multistep process. The pUCGCJ1 plasmid was cut with HincII restriction enzyme. Two bands of 3.2 and 3.0kb were obtained. The 3.2kb band was further digested with HindIII. The resulting 1.5kb fragment was digested with HinfI. Three bands of 1.0, 0.2 and 0.013kb were obtained. The 1.0kb band was treated with Klenow and then digested with NcoI. The resulting 0.7kb was ligated with the EcoRI, NcoI fragment of pGCJ3 to get pG5'CJ3. To construct a c-jun plasmid without the 5'UTR, the BamHI and EcoRI fragment of A2 (7) was ligated with the BamHI, EcoRI fragment of pGEM3. The pGCJ3 retains 3'UTR whereas pG5'CJ3 lacks the 3'UTR of c-jun.

2. Construction of c-jun 5'UTR mutations

The pG5'(A1-193)CJ3 plasmid was constructed by ligating a 1.2kb SacI, HindIII fragment of pG5'CJ3 with the SacI and HindIII fragment of pGEM4. This plasmid lacks 193 bases from the 5'end of the c-jun 5'UTR. For the pG5'(A1-259)CJ3 construction, a 1.2 kb (BssHII Pol HindIII) fragment of pG5'CJ3 was cloned into the Smal and HindIII sites of pGEM4. This plasmid lacks 259 bases from the 5'end of the c-jun 5'UTR. For construction of pG5'(A143-193)CJ3, the pG5'CJ3 plasmid was digested with SacI and BglII. The resulting plasmid fragment was ligated in the presence of an adapter, ad-1. The ad-1 adapter was prepared by annealing two synthetic sequences (5'TAGCAGAGCT3' and 5'CTGCTAGCGG3'). This adapter has compatible cohesive ends for SacI and BglII.
restriction enzymes. This plasmid lacks 50 bases between +143 and +193 of the c-jun 5'UTR. In a separate set of reactions, pG5'CJ3 plasmid was cut with BgII and BssHII. The resulting large fragment was ligated in the presence of an adapter ad-2, to construct pG5'(Δ143-259)CJ3. The ad-2 adapter was constructed by annealing two synthetic sequences (5'TGAGACTA3' and 5'CGCGTAGTCTAGCGG3'). This adapter has compatible cohesive ends for BgII and BssHII restriction enzymes. This plasmid lacks 116 bases between +143 and +259 of the c-jun 5'UTR.

3. Construction of pG5'(Δ1-259)CJ3+S

The pG5'(Δ1-258)CJ3 plasmid was cut with SacI and BssHII. The resulting plasmid fragment was ligated in the presence of an adapter, ad-3 to form pG5'(Δ1-259)CJ3+S. The ad-3 adapter was constructed by annealing two synthetic sequences (5'CAAAAAA(GGGGGGAAAAAA)4GGGGGAAAAG3' and 5'CGCGCTTTTCCCCC(TTTTTTCCCCC)4TTTTTTGAGCT3'). This adapter has compatible cohesive ends for SacI and BssHII restriction sites. This plasmid lacks 193 bases from the 5'end of the c-jun 5'UTR and is predicted to form a secondary structure with a free energy of -34.5Kcal/Mole.

4. Construction of pGCAT and pG5'J CAT

The pGEM4 plasmid was cut with HindIII and BamHI. The resulting linear fragment was ligated with a 1.6kb fragment containing the CAT coding region. This 1.6kb fragment was obtained by cutting pSV2CAT with HindIII and BamHI. The resulting plasmid was named pGCAT. This plasmid has the CAT gene cloned into the HindIII and BamHI sites of pGEM4. pG5'JCAT was constructed by restriction digestion of pGCAT with HindIII and ligating the c-jun 5'UTR PCR product (see below) previously cut with HindIII. The orientation of the 5'UTR was confirmed by Southern blot analysis.
This plasmid has the *c-jun* 5'UTR cloned upstream of the CAT gene in a pGEM4 backbone vector.

5. Construction of pSV2JCAT

The pSV2CAT plasmid was obtained from Dr. R. Stenberg. To construct pSV2JCAT, pSV2CAT was cut with HindIII, phosphorylated and then ligated with the *c-jun* 5'UTR PCR product previously cut with HindIII. Orientation of the *c-jun* 5'UTR was confirmed by Southern blot analysis. This plasmid has the *c-jun* 5'UTR cloned upstream of the CAT gene in a pSV2CAT backbone.

6. Construction of pG5'3'CJ3

The pGCJ3 plasmid which has the complete 3'UTR, was cut with NcoI and PvuI. The resulting 2.5kb fragment was ligated with the 2.30kb NcoI and PvuI cut fragment of pG5'CJ3.

7. PCR amplification of the *c-jun* 5'UTR

The 5'UTR of *c-jun* was PCR amplified as follows. Fifty nanograms of pG5'CJ3 digested with NcoI was mixed with 200uM dNTPs, 2mM MgCl$_2$, 8% DMSO, 5ul of 10X Vent buffer, five microliters BSA (1mg/ml) 0.5uM Bos-as-4 primer (5'GCTCAAGCTTATAGAATACACGGAATTACT3') and 0.45uM Bos-as-5 primer (5'ATGGAAGCTTAGAAGACAGGCCCCGGA3') in a 50ul reaction volume. The HindIII site in the primers is indicated by an underline. After boiling for two minutes, two units of vent polymerase was added and PCR was carried out as follows: two minutes at 94°C, one minute at 43°C and two minutes at 74°C for eight cycles. Next 30 cycles were carried out the same way except annealing was done at 65°C for one minute instead of 43°. This method was based on a protocol described previously for PCR amplification of very high GC rich sequences (74).
8. Quantitation of protein using *in vitro* translation

Plasmids pG5'CJ3, pG5'Δ1-193)CJ3, pG5'Δ1-259)CJ3, pG5'Δ143-193)CJ3, pG5'Δ143-259)CJ3 and pG5'3'CJ3 were digested with HindIII. The pGCJ1 plasmid on the other hand was digested with MluI or EcoRI. The pGJCAT plasmid was digested with BamHI. One microliter of the cut plasmid (1μg/μl) was mixed with 6μl DEPC (diethylpyrocarbonate) water, 5X transcription buffer, two microliters of 100mM DTT, 20 units RNasin, NTP mix1 (10mM ATP, CTP, UTP 10μl each, 1μl of 10mM GTP and nine microliters DEPC water), two microliters of five millimolars 7mGpppG cap analog and 20 units of SP6 RNA polymerase. Incubation was done at 37°C for 30 minutes. Four microliter of NTP mix two (10μl each of 10mM ATP, CTP, UTP and GTP) was added and incubated again at 37°C for 30 minutes. For translation, one microliter of freshly transcribed RNA product from the above reaction was mixed with 17.5μl of nuclease treated rabbit reticulocyte lysate, 0.5μl of one millimolar amino acid mix minus methionine and 14.5μl of 35S methionine (1175 Ci/mmole). Incubation was done at 30°C for 60 minutes. Five microliters of the translated product mixed with one microliter of 10mg/ml RNase was incubated at 30°C for 15 minutes. Five microliters of 2X sample buffer (125mM Tris pH 6.8, 4% SDS and 5.76M 2-mercaptoethanol) was added to the translated product and incubated at 90°C for 5 minutes. After two minutes of centrifugation at room temperature, six microliters of protein dye (1mg/ml bromophenol blue, 62.5mM Tris pH 6.8 and 50% glycerol) was added to the translation product and loaded on to a 10% SDS-PAGE gel. The gel was run overnight at 60 volts. It was then dried and exposed to a Phosphor-Image screen.
9. Quantitation of RNA using *in vitro* transcription

In order to study RNA levels from the above mentioned templates, transcription was done in the presence of \(^{32}\text{P}\) (UTP or CTP) (94). Prior to *in vitro* transcription each of the templates were linearized with restriction enzymes as mentioned in the above section. Each of the linearized plasmids had a concentration of 1ug/ul. One microliter of the linearized plasmid was used for transcription. The transcription reaction was done as mentioned in the above section, except that the 2.5ul of \(^{32}\text{P}\) UTP (10mCi/mole) was used. After 60 minutes of transcription an equal volume of RNA loading buffer was added and boiled for five minutes. Three to five microliter of this reaction mix was loaded on to an 8% polyacrylamide gel and run for several hours. The gel was then dried and exposed to a Phosphor-Image screen.

10. Preparation of RNA and Northern blot analysis

Total cellular RNA was isolated from 3X10^6 cells by Guanidium Thiocyanante Phenol Chloroform extraction (75). In brief, cells were lysed in solutionD (4M guanidinium thiocyanate 25mM sodium citrate, pH 7.0, 0.5% Sarcosyl, 0.1M 2-mercaptoethanol). Lysis product was then extracted with phenol-chloroform and isoamyl alcohol. The aqueous phase was then removed and precipitation of RNA was carried out using an equal volume of isopropanol at -20°C for one hour. After doing a couple of rounds of extraction, the RNA pellet in the final step was washed in 75% ethanol and then dried. RNA was then resuspended in an appropriate volume of DEPC water. After quantitating the RNA, an equal amount from different samples was run on a 1.2% agarose gel containing 2.2M formaldehyde and transferred onto a Hybond-N membrane (Amersham). Before blotting, the gel was soaked for 15 minutes in depc treated water.
followed by two washes in 10XSSC. After transfer, membranes were baked for two hours at 80°C and prehybridization was done at 60°C for 24 hours in prehybridization solution (25mM K₂HPO₄ pH 7.4, 5XSSC, 5xDenhardt's solution, 50ug/ml salmon sperm DNA, 50% formamide and 10% dextran sulfate). Hybridization was done using ³²P UTP labelled antisense chicken c-jun probe at 60°C for 24 hours. After hybridization, blots were washed at room temperature, 42°C or 60°C in 0.1XSSC+0.1%SDS solution. Relative amounts of RNA were quantitated on a Molecular Dynamics Phosphor-Imager.

11. Cell labelling and immunoprecipitation of Jun Protein

Chicken embryo fibroblasts or Rat-1 cells were grown in 1XDMEM media containing 10% NUV or 10% FBS serum. These cells were then stimulated after starvation for 48 hours. Cells were then labelled with 133uCi/ml (1174Ci/m mole) of ³⁵S methionine for the last 15 minutes of serum stimulation. After labelling, radioactive solution was removed and cells were washed two times in 1XTBS buffer. Cells were scraped and centrifuged at 14,000 rpm at 4°C for two minutes. The pellet was resuspended in lysis buffer (25mM Tris pH 8.0, 150mM NaCl, 1% NP40, 0.2% SDS, 1.05mM leupeptin, 1mM PMSF, 1.457mM peptatin and 0.153mM aprotinin) and passed through a 25 gauge needle five or six times until the solution is no longer viscous. Cell extracts were set on ice for 20 minutes and then centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatants were then removed and placed in a new tube. Five microliters of labeled protein extract was then counted in a Beckman scintillation counter. Approximately 2X10⁶ counts from each sample was suspended in a final volume of 500ul diluted with RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1% SDS and 1.2 mM sodium deoxycholate). Thirty microliters of previously washed Immobilized protein A™ (from Repligen) was added and incubated on ice for one hour. Protein extracts were then centrifuged at 4°C for 5 minutes at 14,000 rpm. Supernatants were removed into a new tube. One microliter of PEP-1 (Goat anti rabbit IgG 0.1ug/ul),
Jun specific antibody was added and incubated on ice for three hours. After three hours, 60ul of protein A was added and mixed every five minutes for an hour. Protein extracts were then centrifuged at 14,000 rpm for five minutes at 4°C. Supernatants were discarded and pellets were washed three times with ice cold RIPA buffer and with 100mM Tris pH 7.4. Protein extract was then resuspended in 70ul 1XSample buffer (62.5mM Tris pH 6.8, 2% SDS and 2.88M 2-mercaptoethanol) and boiled for five minutes. The supernatant from the protein extract was transferred to a new tube after centrifugation for 5 minutes. Ten microliters of protein dye (1mg/ml bromophenol blue, 62.5mM Tris pH 6.8 and 50% glycerol) was added, loaded onto a 10% SDS-PAGE gel and run for 12 hours at 50 volts. The protein gel was then dried for 2 hours at 80°C in a Bio-Rad gel drier. Quantitation of protein was done on a Molecular Dynamics Phosphor-Imager.

12. Quantitation of RNA using RT-PCR

In order to quantitate the CAT RNA, quantitative RT-PCR was done exactly as described previously (65).

13. Quantitation of protein using CAT assay

To study the effect of the c-jun 5'UTR on the CAT gene, quantitation of CAT protein was done using a standard CAT assay procedure (76).

14. Cell culture and transfection

Rat-1 cells were grown to confluence in 100mm petri dishes in IXDMEM media. Cells were split 1/6 and transfected with three micrograms of pSV2CAT, pSV2JCAT-R or pSV2JCAT-W plasmids along with one microgram of CMV-ßgal to check for transfection efficiency. Transfection of these plasmids was carried out using the DMSO shock method described previously (77). Twenty two hours after the transfection, cells were harvested to quantitate RNA and protein levels.
15. Computer Analysis

The secondary structure of the 5'UTR of the \textit{c-jun} gene and \textit{c-jun} 5'UTR mutations, as well as \textit{jun} family and \textit{fos} family members, was predicted using the MacDNAsis program. This program determines the secondary structure of RNA by free energy minimization, using the Zuker's RNA fold program (78). This program was obtained from the National Bioscience Corporation.
III. RESULTS

1. Characterization of the translational response of the $c$-$jun$ proto-oncogene in vivo

It has been demonstrated previously that when HeLa cells are stimulated with the tumor promoter TPA, there is a transient increase in $c$-$jun$ mRNA. However, c-Jun protein synthesis does not follow a similar increase. This may indicate that there may be some kind of translational block in the $c$-$jun$ message (25).

To explore this possibility, I have studied the effect of 20% fetal bovine serum (FBS) stimulation on endogenous rat $c$-$jun$ RNA and protein synthesis. In a similar set of experiments, chicken embryo fibroblasts were stimulated with 20% NUV serum for different time lengths. In detail, confluent CEFs and Rat-1 cells were serum starved for 48 hours in a serum free 1XDMEM media, followed by stimulation with 20% fetal bovine serum (FBS) or 20% NUV serum. Total RNA was harvested at 0, 30, 60, 180, 240, 360 and 480 minutes post-stimulation. Quantitation of RNA was done by Northern blot analysis using antisense chicken $c$-$jun$ as a probe. The blot was stripped and then hybridized with human $G3PDH$ to normalize the RNA level in each lane (Rat-1). For CEFs RNA loading in each lane was confirmed by ethidium bromide staining (data not shown).

In identically treated sets of cells, C-Jun protein levels were assayed by pulse labeling of cells for the last 15 minutes of stimulation with $^{35}$S methionine (133uCi/ml). Cells were then serum starved in methionine free media for 30 minutes before labeling. Cells were then lysed in a lysis buffer containing protease inhibitors and counted on a
scintillation counter (for details see Materials and Methods). Equal counts of radioactivity were used for immunoprecipitation using PEP-1, a c-Jun specific antibody. Experiments were done in duplicate and average values from CEFs and Rat-1 cells serum stimulation are shown in Figure 2.

When Rat-1 cells were stimulated with 20% FBS, within 30 minutes a 16.2 fold increase in the RNA level was observed. This RNA level dropped quickly to three fold by one hour and then did not change significantly, even after eight hours of serum stimulation. Conversely, protein synthesis increased only 1.9 fold after 30 minutes or one hour after serum treatment. After two, four and eight hours of serum stimulation no significant change in c-Jun protein synthesis was observed. Thus after 30 minutes of serum stimulation a large amount of RNA was present but only a very small percentage of that may have been translated. This result indicates that there is some kind of translational block in the c-jun message (Figure 2A). Similar results were observed in an independent cell system.

CEF's when stimulated with 20% NUV serum resulted in a different kinetics of RNA but similar kinetics of protein synthesis. After 30 minutes of serum stimulation, the RNA level increased six fold. An increase in RNA level was observed up to 8.5 fold after one hour of stimulation and then it drops. The c-jun RNA level dropped almost to basal level after six hours of serum stimulation. The c-Jun protein synthesis on the other hand, does not change significantly after 6 hours of serum treatment. Thus, the main conclusion that can be drawn from this experiment, is that both rat and chicken c-jun message appears to have some kind of translational block in vitro (Figure 2B).

The kinetics of chicken c-jun RNA is different from that of the rat c-jun RNA in response to serum stimulation. There can be several reasons for this difference. Firstly, the serum used for these two experiments were different. Epidermal growth factor (EGF)
Figure 2  Effect of Serum stimulation on c-jun RNA and protein synthesis.  90-100% confluent Rat-1 cells and chicken embryo fibroblasts (CEFs) were serum starved for 48 hours. These cells were then stimulated with 20% (Fetal Bovine Serum) (Rat-1) and 20% NUV serum (CEFs) for 0.5, 1, 2, 4, 6, 8 hours. One set of plates was used for RNA extraction. The second set of plates were pulse labeled with 200uci of S35 for the last 15 minutes of stimulation. Both set of plates were treated identically. Protein synthesis was measured by immunoprecipitation from equal counts of lysates of each time point with PEP-1 antibody. Immunoprecipitated product was run on a 10% SDS-PAGE gel. The gel was then dried and Jun protein was quantitated by phosphorimage analysis. For studying the accumulation of RNA, RNA was extracted from each time point. Equal quantities of RNA were run on a 1.2% agarose gel and transferred onto nitrocellulose membrane. Prehybridization was done for 24 hours. Hybridization was done using antisense c-jun as probe. Blots were washed and quantitated by phosphor Imager analysis or by scanning on a densitometer after autoradiography.

Panel A shows the effect of 20% FBS stimulation on c-jun RNA and protein synthesis in Rat-1 cells. Empty circles show RNA levels and filled triangles show protein synthesis.

Panel B shows the effect of 20% NUV serum stimulation on c-jun RNA and protein synthesis in chicken embryo fibroblasts. Empty circles shows RNA levels and filled triangles show protein synthesis.

Panel C shows the quantitation of data from Panel A and Panel B.
A

Fold Increase

Time (Hours)

B

Fold Increase

Time (Hours)

C

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is enriched in NUV serum and not in FBS. This can lead to stimulation of different signal transduction pathways that may have contributed to differences in kinetics. Secondly, Rat-1 is an immortalized cell line whereas CEFs are normal diploid fibroblasts. It is possible that there are certain cell specific factors in the CEFs that are responsible for stabilizing c-jun RNA under serum stimulation conditions. Lastly, the rat and chicken c-jun 5'UTRs differ considerably in their primary and secondary structure. The 5'UTR of rat c-jun is 917 bases long and it can form a very complex secondary structure with a standard free energy of -517.6Kcal/Mole. The chicken c-jun 5'UTR on the other hand is only 313 bases long and forms a less complex secondary structure of -252.7Kcal/Mole free energy (Table 3). Because of the differences in secondary structure of the 5'UTRs, these RNAs may differ in their stabilities. This difference in stability of these RNAs from these two species may have lead to differences in kinetics of mRNA induction. The actual reason for this difference is not known at present.

The overall conclusion that can be drawn from this set of experiments is that there is a translational block in both rat and chicken c-jun messages. Both rat and chicken c-jun have a very long and highly structured GC rich 5'UTR. A number of other cellular proto-oncogenes that are involved in normal cell growth and development have highly structured 5'UTRs (37). In some of these genes it has been shown that the 5'UTR plays an important role in translational suppression (66). Thus, it is possible that other growth related genes can also be regulated in a similar manner. With this in mind, I was interested in analyzing the role of the 5'UTR secondary structure in translational regulation of chicken c-jun.
2. Secondary structure analysis of the chicken c-jun 5'UTR

The basic structure of the chicken c-jun message is shown in Figure 1A. The 5' UTR is 313 nucleotides long and 81% of the nucleotides are guanines and cytosines. Computer analysis of the 5'UTR was done using Zuker's RNA fold method in the MacDNAsis program (78). Results from this analysis are shown in Figure 1B. The 5'UTR of c-jun has the potential to form a complex secondary structure. This secondary structure is extremely stable with a theoretical standard free energy value of -252.7Kcal/Mole. The 5'CAP and translation initiation AUG are very close to the hairpin structure. In other systems, the relative position of the secondary structure as well as the hairpin stability have been shown to influence translational efficiency (73). From this structural analysis, I predicted that the wild type chicken c-jun message was translated inefficiently. The next step would be to study the effect of the chicken c-jun 5'UTR on its own translation in vitro.

3. Effect of GC rich 5'UTR on the translation of c-jun mRNA in vitro

In order to study the effect of the 5'UTR on the translation of c-jun mRNA, I have utilized an in vitro transcription and translation assay. The c-jun cDNA with and without the 5'UTR was cloned into pGEM4 such that it was under the control of the SP-6 promoter. The 3'UTR of c-jun has been implicated in mRNA instability. Thus, I have prepared these templates for transcription such that transcripts made would run off shortly after the stop codon (Figure 3). These mRNAs were then translated in rabbit reticulocyte lysates in the presence of 35S methionine. Equal volumes of protein were run on 10% SDS-PAGE gels. These gels were dried and c-Jun protein levels were quantitated using a Molecular Dynamics Phosphor-Imager. Results from this experiment are shown in Figure
Figure 3  Schematic of the c-jun 5'UTR mutations, tested in vitro. The +1 site indicates the 5'CAP. The 5'UTR of c-jun spans from +1 through +313. The shaded box represents the c-jun coding region.
4A. The c-Jun protein appears as a set of two tight doublets. It has been shown previously by other investigators that c-Jun protein translated \textit{in vitro}, appear as multiple bands. These multiple bands may have resulted from differential phosphorylation. Identity of the c-Jun protein was confirmed from translated products by immunoprecipitation (data not shown). RNA levels, on the other hand, were assayed by carrying out \textit{in vitro} transcription in the presence of $^{32}$P UTP followed by separation on an 8% acrylamide gel (Figure 4B). Protein values were normalized to the amount of starting RNA, which was quantitated by phosphor-Image analysis. The fold increase in relative protein level is shown in the form of a histogram in Figure 3C. It appears that complete removal of the 5'UTR from \textit{c-jun} mRNA results in a 25 fold increase in relative c-Jun protein level (Figure 4, Lanes 1 and 6). This result indicates that the 5'UTR of \textit{c-jun} has translational suppression property \textit{in vitro}.

In an effort to map the sequences within the 5'UTR that are responsible for this suppressive effect, I constructed four deletion mutations. In these mutations different regions within the 5'UTR were removed (Figure 3). Secondary structure analysis of the 5'UTR of these deletion mutations were done and are presented in Figure 4. Each of these mutants retains the ability to form secondary structures with different complexity and stability (Figure 3). The stability of each of these structure is however, dramatically less than wild type. The pG5'(Δ1-193)CJ3 mutant can form a structure with a theoretical standard free energy of -87.10Kcal/Mole. The pG5'(Δ1-259)CJ3 mutant on the other hand can form a very weak secondary structure with a standard free energy of -21.70Kcal/Mole. This structure can be easily melted by helicase activity of translation initiation machinery. Two internal deletions on the other hand, pG5'(Δ140-259)CJ3 and pG5'(Δ140-193)CJ3, form much more complex secondary structures with free energy values of -153.0 and -215.10 Kcal/Mole respectively.
Figure 4  Diagrammatic representation of secondary structure of the c-jun 5'UTR mutations. Panel A, B, C and D shows the two dimensional secondary structures of the c-jun 5'UTR mutations tested in vitro. The '+1' site in each structure represents the transcription start site and the arrow represents the translational start site. The ΔG values of each secondary structure are shown below each secondary structure. Secondary structure analysis was done as described in materials and methods.

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Figure 5 Translational efficiency of the c-jun 5'UTR mutations in vitro. Mutants indicated in panel A were cut with HindIII or MluI. In vitro transcription and translation was done as described in materials and methods.

Panel A shows the results from an in vitro translation assay. Jun protein is indicated by two arrows.

Panel B shows the results from an in vitro transcription assay. Jun RNA is shown by an arrow.

Panel C shows quantitation of results obtained form panel A and B in the form of a histogram. Protein and RNA levels in panel A and B are quantitated using phosphorimage analysis. Protein synthesis was normalized to RNA levels (panel C). Numbers above each bar of the histogram represent fold increase over wild type.
Table 1  Quantitation of the relative protein levels from various deletion mutations of the 
c-jun tested *in vitro*.
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</tr>
</tbody>
</table>
Except for pG5'(Δ1-259)CJ3, all of these mutants are predicted to form a complex secondary structure. Once constructed, these mutants were then cloned into the pGEM4 vector. Transcriptional and translational efficiency was measured in vitro. Results from this experiment are shown in Figure 5 and Table 1. As we can see, mutant pG5'(Δ1-259)CJ3, which has a deletion of 259 nucleotides, translates 25.0 fold more than the c-jun with complete 5'UTR. On the other hand pG5'(Δ1-193)CJ3 which has a much more complex secondary structure than pG5'(Δ1-259)CJ3 translates only 3.8 fold more than pG5'CJ3. This result indicates that as the complexity of secondary structure within the 5'UTR increases, translation efficiency decreases. Mutants, pG5'(Δ140-259)CJ3, pG5'(Δ140-193)CJ3 and pG5'CJ3 can form very complex secondary structures. Their secondary structures differ within a range of three fold of their ΔG values but they do not differ much in their relative protein level. These 5'UTR mutations have retained sequences that can form the base of hairpin loop like secondary structure (Figure 4). This may imply that the two sequences which make up ends of the 5'UTR may play a major role in translation suppression caused by the c-jun 5'UTR. This was further supported by the fact that when 140 nucleotides were cloned at the 5'end of pG5'(Δ1-259)CJ3 mutant, to make pG5'(Δ140-259)CJ3, a sudden drop in the relative protein level was observed (Figure 5).

These results indicate that there is a good correlation between the linear length of the 5'UTR and the relative protein level observed (Table 1). As the linear length between the 5'CAP and AUG in these mutations increases, translation efficiency decreases. This could be either due to structural complexity of 5'UTR or due to increase in the distance between the 5'CAP and AUG. This may imply that translational suppression caused by the c-jun 5'UTR can be due to the increase in the distance between the 5'CAP site and the AUG. This possibility is addressed below.
4. Translational suppression caused by 5'UTR is not due to an increase in the distance between the 5'CAP and AUG

In the previous set of experiments, I have demonstrated that the GC rich 5'UTR of c-jun can suppress its own translation in vitro. Using 5'UTR mutations, our in vitro results have also demonstrated that as the length between the 5'CAP and AUG increases, translational efficiency decreases. One possible mechanism by which the 5'UTR may have caused translational suppression is due to an increase in the linear distance between the 5'CAP and AUG.

In order to address this question, I made two mutations of c-jun such that they had equal lengths of 5'UTR, but differ considerably in their secondary structure complexity. To accomplish this, I have designed the mutant, pG5'(Δ1-259)CJ3+S (Figure 6A). This mutant has a 70 base pair synthetic sequence that was prepared by annealing two synthetic oligonucleotides. The sense strand which would transcribe RNA, is a stretch of adenines and guanines. I have selected a stretch of adenines and guanines in the sense strand so that the resulting RNA will not fold to form a secondary structure. The nonsense strand is a stretch of thymines and cytosines. Once constructed, this synthetic sequence was then cloned between the SacI and BssHII site of pG5'(Δ1-193)CJ3. The pG5'(1-259)CJ3+S mutant now has the same 5'UTR length as pG5'(Δ1-193)CJ3. The secondary structure analysis of (5'UTR only) mutants pG5'(Δ1-259)CJ3+S and pG5'(Δ1-193)CJ3 was done and is shown in Figure 6B and 6C. pG5'(Δ1-193)CJ3 is predicted to form a secondary structure that is much more complex than the pG5'(Δ1-259)CJ3+S mutant. If the translation suppression caused by the 5'UTR is due to the increase in distance between the 5'CAP and AUG, then I expected to see similar levels of relative protein levels from pG5'(Δ1-193)CJ3 and pG5'(1-259)CJ3+S. These mutants were cloned into pGEM-4.
Figure 6  Schematic of the c-jun 5'UTR mutations tested in vitro to demonstrate the effect of increase in the length between 5'CAP and AUG on the translation efficiency of c-jun mRNA in vitro.

Panel A shows schematic of mutants pG5'(Δ1-193)CJ3 and pG5'(Δ1-259)CJ3+S. The dark box in pG5'(Δ1-259)CJ3+S represents the synthetic sequence which was used for construction of this mutation. The synthetic sequence in detail is also shown.

Panel B and C show secondary structures of the 5'UTR of the above mentioned mutants. The ΔG values of each secondary structure is shown below each structure.
A

Untranslated Region

Coding Region

BssIII

SacI

PG5'(Δ1-193)CJ3

PG5'(Δ1-289)CJ3+S

5'CAAAAAA(GGGGGGAAAAAA)^GGGGGAAAAG 3'

3'TCGAGTTTTTT(CCCCCCTTTTTT)^4CCCCCCTTTTCGCCG 5'

B

PG5'(Δ1-193)CJ3
ΔG -87.10 Kcal/Mole

C

PG5'(Δ1-289)CJ3+S
ΔG -34.50 Kcal/Mole

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Figure 7  Effect of increase in the distance between 5'CAP and AUG on the translation of c-jun mRNA in vitro. Mutants pG5'(Δ1-193)CJ3, pG5'(Δ1-259)CJ3 and pG5'(Δ1-259)CJ3+S were linearized with HindIII. Transcription and translation was done as described in materials and methods.

Panel A shows the result obtained from in vitro translation. The c-Jun protein is indicated by arrows. RNA levels from each of these mutants was quantitated using an in vitro transcription assay (data not shown).

Panel B shows quantitation of the results obtained from in vitro transcription and translation assays. Protein levels were normalized to RNA levels. Fold increases were calculated and are shown in Panel B in the form of a histogram. Numbers above each bar of the histogram represents the fold increase.
Table 2  Quantitation of the results obtained from the *c-jun* 5'UTR mutations as shown in Figure 8.
<table>
<thead>
<tr>
<th>c-jun 5'UTR Mutants</th>
<th>ΔG Kcal/Mole</th>
<th>Fold Increase</th>
<th>Length of 5'UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG5'(Δ1-193)CJ3</td>
<td>-87.5</td>
<td>1.0</td>
<td>120</td>
</tr>
<tr>
<td>PG5'(Δ1-259)CJ3</td>
<td>-21.7</td>
<td>2.8</td>
<td>54</td>
</tr>
<tr>
<td>PG5'(Δ1-259)CJ3+S</td>
<td>-34.5</td>
<td>4.0</td>
<td>120</td>
</tr>
</tbody>
</table>

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RNA and protein levels were measured using in vitro transcription and translation assay as described in Materials and Methods. Results from this experiment are shown in Figure 7A and 7B.

As shown in Figure 7A and 7B, pG5'(Δ1-259)CJ3+S whose 5'UTR can form a less complex secondary structure than pG5'(Δ1-193)CJ3 translates much more efficiently. I have also compared the translational efficiency between pG5'(Δ1-259)CJ3 and pG5'(Δ1-259)CJ3+S. When the synthetic sequence was absent, pG5'(Δ1-259)CJ3 translated 2.8 fold better than the pG5'(Δ1-193)CJ3 mutant. Data from this experiment is summarized in Table 2. On the basis of secondary structure prediction, I had expected that pG5'(Δ1-259)CJ3 would translate more efficiently than pG5'(Δ1-259)CJ3+S. Instead, it translated 1.2 fold less efficiently. This 1.2 fold difference in translation may not be significant. The overall conclusion which can be drawn from this experiment is that it is the secondary structure complexity and not the distance between 5'CAP and AUG, that is responsible for translation suppression. Similar results have been reported by other investigators (78).

5. Effect of c-jun 5' or 3'UTRs on the translation of c-jun mRNA in vitro

In the serum stimulation experiment mentioned in section 1, I have demonstrated that there is a translational block in c-jun mRNA translation. The c-jun mRNA synthesized in vivo contains both 5' and 3'UTRs. So far, I have demonstrated that the 5'UTR of c-jun can suppress its own translation in vitro. Does the 3'UTR of c-jun have any effect on in vitro translation when present with or without the 5'UTR?

Genes encoding for cytokines, oncoproteins and growth factors also contain long 3'UTRs. There are specific sequences within the 3'UTR that have been shown to be the major determinant of mRNA stability. In addition to the AAUAAA polyadenylation signal, multiple copies of the AU rich octanucleotides, UUAUUUAU are also present.
These sequences are also referred to as the translation inhibitory elements (TIE) in c-fos mRNA (70). These sequences have been shown to be present in c-myc and c-fos transcripts and are highly conserved in transcripts coding for proteins related to the inflammatory response (71). These sequences (from c-fos and c-myc) can have an inhibitory effect on translation but the actual mechanism is not known at present (70).

In order to address the role of the 3'UTR in translation regulation, I have constructed several mutations (Figure 8). These mutants were cloned into pGEM-4. *In vitro* transcription and translation was done as described in Materials and Methods. Results from this experiment are shown in Figure 9. As expected, the presence of the 5'UTR on c-jun translation (pG5'CJ3) has resulted in a 12.3 fold decrease in relative protein levels. Cloning of the 3'UTR in addition to the 5'UTR (pG5'3'CJ3) has caused a further 0.5 fold decrease in the protein level. This 0.5 fold decrease may not be statistically significant. Removal of the 5'UTR from pG5'3'CJ3 has resulted in a 11.2 fold increase in the relative protein level as compare to pG5'CJ3. These results indicate that 3'UTR of c-jun may not co-operate with the 5'UTR to enhance translation suppression. Results from this experiment strongly suggested that it is the 5'UTR of c-jun that is sufficient for its own translational suppression *in vitro*.

6. Effect of GC rich 5'UTR of c-jun on the translation of a heterologous CAT gene *in vitro*

Using a series of experiments, I have demonstrated that the 5'UTR of c-jun can suppress its own translation *in vitro*. In order to demonstrate that the 5'UTR of c-jun does carry translation suppressive sequences, it was cloned upstream of a heterologous CAT gene (Figure 10). The CAT gene was subcloned from the pSV2CAT plasmid. The CAT gene was selected because it can be used conveniently for *in vitro* and *in vivo* assays and it has been used previously by other investigators (30,79).
Figure 8  Schematic of the c-Jun 5' and 3' mutants tested to demonstrate the effect of untranslated regions on the translation of c-jun mRNA in vitro. Numbering was done as described in figure 3.
Figure 9  Effect of 5' and/or 3' UTR on translation of c-jun in vitro. Mutants pG5'3'CJ3 and pG5'CJ3 were linearized with HindIII. pGCJ1 was digested with MluI or EcoRI. In vitro transcription and translation was done as described in materials and methods. Results from in vitro translation is shown in panel A. The c-Jun protein is indicated by arrows. RNA levels from these mutants were analyzed using in vitro transcription (data not shown). Protein levels were normalized to the RNA level. Fold increase was then calculated and is shown in panel B in the form of a histogram. Numbers above each bar of this histogram represent the fold increase.

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The 5'UTR of c-jun was PCR amplified (see Materials and Methods) and cloned upstream of the CAT gene in a pGEM4 backbone to form pGJCAT (Figure 10). As shown in Figure 10, there were 31 nucleotides present before the first AUG of CAT in the pSV2CAT plasmid. The role of these sequences in translational regulation is not known at present. The SV40 polyadenylation sequence, that is present at the 3'end of the CAT gene, may help in the stabilization of mRNA.

In vitro transcription and translation of these plasmids containing the CAT gene with and without the c-jun 5'UTR was done using T7 polymerase. Results from this experiment are shown in Figure 11. As expected, the 5'UTR of c-jun present upstream of the CAT gene caused a 30.7 fold decrease in the relative protein level. This result clearly indicates that the 5'UTR of c-jun has translational suppressive information that can not only suppress its own translation but also of a heterologous gene in vitro. Similar results have been reported previously when the 5'UTR of c-myc was cloned upstream of CAT (30).

Furthermore, the translational suppression caused by the c-jun 5'UTR on CAT is stronger than on its own translation. The reason for this difference is not known at present but it may be due to the difference in complexities in secondary structure of the CAT and c-jun coding regions in relation to the 5'UTR.

With these results in mind, the next obvious step was to study the effect of the c-jun 5'UTR on translation in vivo. However, I wanted to analyze the secondary structures of other c-jun and c-fos family members 5'UTRs.
Figure 10  Schematic of the mutations used to demonstrate the effect of c-jun 5'UTR on the translation of CAT gene. Mutants, pGCAT and pGJCAT are shown in the form of a box diagram. The 5'UTR of c-jun is shown in the form a hatched box. The black box immediately next to the HindIII site represents 31 nucleotides which were present in the original pSV2CAT plasmid. The light box next to the 31 nucleotides indicate the CAT coding region. The SV40 small T intron and polyadenylation sites are indicated by dark and light hatched boxes next to the CAT coding region. The bold arrow represents the T7 promoter used for in vitro transcription reactions.
Figure 11  Effect of the c-jun 5'UTR on the translation of the CAT gene *in vitro*. Plasmids pG5'JCAT and pGCAT were cut with BamHI. Resulting linear plasmids were used for *in vitro* transcription and translation reactions. Panel A shows the result of an *in vitro* translation assay. CAT protein is indicated by the arrow. Panel B shows the result of an *in vitro* transcription assay. CAT RNA is indicated by an arrow. Protein level was normalized to RNA level and fold increase was then calculated and is shown in Panel C in the form of a histogram. Numbers above each bar of this histogram represent the fold increase.
7. Secondary structure analysis of the 5'UTR of other Jun and Fos family members

Previously, it has been shown that genes which play important roles in normal cell growth have highly structured 5'UTR. Translation of these genes may be modulated in a similar manner. Since other Jun and Fos family proteins can modulate c-jun activity and are themselves involved in normal cell growth, I decided to analyze the structure of their 5'UTRs.

In order to do so, the primary sequence of the 5'UTR from each of the c-jun and c-fos family members was obtained from Genebank using GeneWorks program. Secondary structure analysis of the c-jun and c-fos 5'UTRs of different species was done using the Mac-DNAsis program. This program predicts the secondary structure of RNA on the basis of Zuker's RNA fold method. Results are shown in Table 3. As expected most of the c-jun and c-fos family members from a variety of species have long 5'UTRs which range from 121 nucleotides in mouse junD to 1201 nucleotides in mouse fosB. In addition, most species have very high GC contents in their 5'UTR indicating their potential to form stable secondary structures. As expected, all of the jun family members from different species are predicted to form secondary structures with very low theoretical standard free energy. Some of the representative examples are shown in Figure 12 and 13. The c-fos family members are also predicted to form complex secondary structures (Table 3). On the basis of this analysis, I speculate that each of these c-jun and c-fos family members may be regulated at the translational level.
Figure 12  Secondary structure analysis of mouse \textit{junB} and human \textit{junD} 5'UTR.
Mouse JunB
$\Delta G = -214.1 \text{ Kcal/Mol}$

Human junD
$\Delta G = -151.8 \text{ Kcal/Mol}$
Figure 13  Secondary structure analysis of mouse \textit{c-fox} and mouse \textit{FosB} 5'UTR.
Mouse c-fos
$\Delta G = -66.0$ Kcal/Mole

Mouse fosB
$\Delta G = -503.3$ Kcal/Mole
Table 3  Comparison of the 5'UTR of *jun* and *fos* family members.
<table>
<thead>
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<th></th>
<th>Length</th>
<th>% GC</th>
<th>Delta G Kcal/Mole</th>
</tr>
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<td></td>
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<td>81</td>
<td>-252.7</td>
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<td>72</td>
<td>-399.6</td>
</tr>
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<td>rat</td>
<td>917</td>
<td>63</td>
<td>-517.6</td>
</tr>
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<td>mouse</td>
<td>614</td>
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<td>-316</td>
</tr>
<tr>
<td>human</td>
<td>974</td>
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<td>drosophila</td>
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<td>-138</td>
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<tr>
<td><strong>Jun D</strong></td>
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<td><strong>Fra-2</strong></td>
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<tr>
<td>chicken</td>
<td>240</td>
<td>63</td>
<td>-142.5</td>
</tr>
</tbody>
</table>
8. Effect of the c-jun 5'UTR upon translation of CAT gene in vivo

Using a series of in vitro experiments, I have demonstrated that 5'UTR of the c-jun can not only suppresses its own translation but also that of a heterologous CAT gene in vitro. Thus, the next step would be to study the role of c-jun 5'UTR in translational regulation in vivo.

To do so, the c-jun 5'UTR was PCR amplified and cloned upstream of CAT in pSV2CAT in both orientations (Figure 14). This plasmid not only has a constitutive SV40 early promoter but also a long SV40 3'UTR and polyadenylation sequence. These sequences are required for RNA stability. In order to quantitate protein and RNA levels, I have used a standard CAT assay and RT-PCR method respectively. RT-PCR is a very sensitive method to quantitate very small amount of RNA. In order to demonstrate that RT-PCR method is quantitative, I have PCR amplified the CAT gene from increasing quantities of the pSV2CAT template. There appears to be a direct relationship between the quantity of template and PCR product observed (data not shown). Once constructed, these plasmids (pSV2CAT, pSV2JCAT-R and pSV2JCAT-W) were then transfected into Rat-1 cells using the polybrene DMSO shock method (80). Transfection efficiency of each of these plasmids was measured by co-transfection of a CMV β-gal plasmid along with the other plasmids. Cell extracts were assayed for CAT activity and transfection efficiency was normalized by β-gal assay. CAT RNA levels were measured using quantitative RT-PCR described earlier (68). Protein levels were measured using a standard CAT assay (79). Experiments were done in duplicate and the results are shown in Figure 16.
Figure 14  Schematic of the mutations used to demonstrate the effect of the *c-jun* 5'UTR on translation of CAT *in vivo*. Mutants, pSV2JCAT and pSV2CAT are shown in the form of a box diagram. The 5'UTR of *c-jun* is shown in the form of a hatched box. Arrows below it represent orientation of its cloning. The black box immediately next to HindIII site on *c-jun* 5'UTR represents 31 nucleotides which are present in the backbone of the pSV2CAT plasmid. Bold arrows before the beginning of each plasmid represents SV40 early promoter.
GGCGAGATTTCCAGGAGCTAGGAAGCTAAAATG

SV 40 small T intron SV 40 polyA

PSV2J CAT

PSV2CAT

Hind III
Figure 15  Effect of the *c-jun* 5'UTR on translation of the CAT gene *in vivo*. Plasmids pSV2CAT, pSV2JCAT-R and pSV2JCAT-W were transfected into Rat-1 cells. Forty eight hours after transfection protein was extracted and a standard CAT assay was done as shown in panel A.

Panel B shows the quantitation of RNA using RT-PCR. Protein level was normalized to the RNA level and fold increase was calculated.

Panel C shows fold increase in the form of a histogram. Numbers above each bar of this histogram represent the fold increase.
When the *c-jun* 5'UTR was present upstream of CAT in the right orientation it caused a 77.68 fold increase in relative protein level. Likewise when the *c-jun* 5'UTR was present in the wrong orientation it caused a 93.21 fold increase in the relative protein level. This result clearly indicates that the *c-jun* 5'UTR has caused translational enhancement of the CAT gene in Rat-1 cells. This was an unexpected result on the basis of our previous *in vitro* observations. One possible explanation for the translational enhancement by the *c-jun* 5' UTR can be due to the differences between the *in vitro* and *in vivo* systems.

Results like these are not unusual and have been observed in other genes such as *c-myc*. When 240 bases of the *c-myc* 5'UTR were present upstream of the *c-myc* coding region it caused almost complete inhibition of its translation *in vitro*. When the same 240 bases were cloned upstream of CAT, translational repression was observed in a rabbit reticulocyte lysate and Xenopus oocyte system. Conversely, translational enhancement was observed in a HeLa cell extracts. The *c-myc* 5'UTR can also cause translational enhancement of the 70kd heat shock protein of *Drosophila* in HeLa cell extract (30). In another situation, a palindromic sequence that is predicted to form a complex secondary structure (ΔG=-70Kcal/Mole) was cloned upstream of the CAT gene. RNA from these plasmids was transcribed *in vitro* and was injected into the animal pole of Xenopus oocytes, eggs and embryos. Results from these experiments have clearly shown that there was more than a 50 fold increase in CAT activity in fertilized eggs than in the late embryonic stages. No changes in CAT activity was observed from the control plasmids. This experiment has clearly demonstrated that translational enhancement can be caused by the highly structured 5'UTR (83).
Results from these two studies clearly demonstrate that translational regulation can be quite different in different cell types and in different cell lysate systems. At present there is no explicit explanation available to explain the translational enhancement by the \textit{c-jun 5'UTR} on the CAT gene \textit{in vivo}. There can be several possibilities. First, there can be internal attachment and assembly of the 80S ribosomal complex downstream of the secondary structure. Mechanisms like these have been observed in uncapped RNAs (84,85). Another possibility is that the 80S ribosomal complex can jump across the secondary structure. These two mechanisms have been mainly observed in viral genes but no evidence of such occurrence have been reported in cellular genes. The most likely candidate that may explain the translational enhancement, is a family of RNA helicases (eIFs) that are reported in mammalian cells. These translation initiation factors can unwind RNA in either directions. It has been reported that overexpression of one of these factors, eIF-4E can cause transformation of NIH3T3 cells possibly by causing increased translation of essential gene that control normal cell growth and development (67). Thus it would be important to see if eIF-4E is overexpressed in Rat-1 cells as compared to the reticulocyte lysate which we have used in our assays. Another possible explanation for translational enhancement can be that there are cell specific factors in the Rat-1 cells that may have prevented the secondary structure formation of the 5'UTR. This may allow more 40S ribosomal subunits to bind and scan along the 5'UTR. This can cause a burst of translational enhancement. Several possible mechanisms like these may explain how translational enhancement of CAT with the \textit{c-jun 5'UTR} occur \textit{in vivo}. Understanding these mechanisms is one of the major future directions of this project.

Even though I have observed translational enhancement in one situation, it does not contradict all the results that I have obtained \textit{in vitro}. It is possible that the \textit{c-jun}
5'UTR may have translational suppression properties in vivo only under specific and unique circumstances. Experiments are underway to study the effect of the c-jun 5'UTR on CAT and on its own translation in vivo under different growth conditions. Regardless, in vitro experiments strongly suggest that the GC rich 5'UTR of c-jun has translational suppression properties that can not only suppress its own translation but also that of a heterologous CAT gene in vitro.
IV. DISCUSSION

Chicken *c-jun* plays an important role in normal cell growth and development. Deregulated expression of *c-jun* can lead to cell transformation *in vitro* and *in vivo*. As such, *c-jun* is under tight regulatory controls. This regulation is not only exerted at the level of transcription but also at the post-translational level. Transcriptional regulation of *c-jun* is quite complex and it has been studied extensively in response to growth factors and physical agents in the past few years. At the post-transcriptional level, c-Jun is regulated by phosphorylation as well as through protein-protein interaction. The c-Jun protein can not only form dimers with its own family members but also with Fos and CREB family members. The c-Jun protein together with Fos and CREB family proteins forms the AP-1 complex. This complex can then bind to the AP-1 site in promoters of a number of cellular genes. Many of these genes are thought to play a critical role in normal cell growth and differentiation.

Recently, it has been shown that c-Jun not only interacts with leucine zipper containing proteins but also with other proteins without leucine zipper. These include NF-kB, myoD and the TATA box binding protein (40-43). Thus, these proteins which interact with c-Jun not only modulate its function, but their function in turn can be regulated. The main function of c-Jun that can be modulated by dimerization is its DNA binding activity. It has also been shown that DNA binding of c-Jun is necessary to perform its transactivation and transcriptional regulation function. Dimer formation between c-Jun and other proteins depends upon their concentration at a given time point in the cell. Thus, dimerization is a crucial regulation step for *c-jun* function. Furthermore c-Jun activity can also be regulated at the level of post-translational modification.
(phosphorylation). Previously, it has been demonstrated that phosphorylation of c-Jun is important for carrying out its transactivation function. Phosphorylation of c-Jun can be regulated by various kinases or phosphatases that are stimulated in response to events at the cell surface. Thus, regulation of c-Jun is quiet complex and can be modulated at several different levels.

Multiple levels of regulatory controls are critical for normal functioning of c-jun. This is illustrated by the fact that deregulated expression of c-jun can induce oncogenic transformation in avian and rodent cells. This indicates that the proper regulation of c-jun is critical for maintaining normal cellular homeostasis.

Regulation of c-jun either at the transcriptional level, post-translational (phosphorylation) or dimerization level has been studied extensively. I show here for the first time that c-jun can be regulated at the translational level. The results presented here demonstrate an additional layer of complexity to the regulation of c-Jun.

Our first clue that there is translational regulation in c-jun RNA, came from a series of serum stimulation experiments of Rat-1 cells and CEFs. Both rat and chicken c-jun RNA was stimulated several fold in response to serum but a similar increase in protein synthesis was not observed. This indicates that there is a translational block in the c-jun message. The kinetics that was followed by rat and chicken c-jun transcripts in response to serum was different. Rat c-jun RNA increases and then decreases very rapidly in response to serum. Chicken c-jun RNA, on the other hand, increases and then drops slowly to basal level in six hours. Differences in these kinetics can be due to the use of different serums, different cell types or due to different secondary structures formed by the chicken and Rat c-jun message. Overall, this experiment has demonstrated that there is a translational block in c-jun messages in response to serum. What sequences within the c-jun message are responsible for this translational block? A closer look at the c-jun message has revealed the presence of a long GC rich 5'UTR that may be involved in the translational block.
Translation is a process by which a mRNA is translated into a protein. It is a very complicated process involving a series of steps and participation of a number of factors. Translational efficiency of a specific mRNA into protein can be influenced by a number of different factors. Thus, proper regulation of these factors is necessary for normal protein synthesis. Translational efficiency of a specific mRNA into protein is not only modulated by factors which participate in different enzymatic function but also by several structural features within the mRNA. These include the 5'7mGpppN CAP, the nucleotide context around the translation start site, the length of the 5'UTR, the number of AUG start codons in the 5'UTR and the secondary structure surrounding the translation start site. This structure can have a stimulatory or inhibitory effect depending on its location (55).

A close look at the c-jun message revealed that it contains a long 5'UTR which ranges in length from 313 bases in chicken to 974 bases in human. This leader sequence is extremely GC rich (Table 3). This high GC content in the 5'UTR is conducive to creation of a complex and stable secondary structure. Computer analysis has revealed that the 5'UTR of chicken c-jun has the potential to form an extremely stable secondary structure with a standard free energy of -252.7Kcal/Mole. This hairpin loop secondary structure is present very close to the 5'CAP site and AUG. It has been shown previously that if the secondary structure is present very close to the AUG it is very effective in causing translational suppression (76). This extremely stable potential structure in the 5'UTR makes the c-jun message an ideal candidate for translational repression. By creating several deletion mutations within the c-jun 5'UTR, I was able to address two major questions. Firstly, what sequences within the c-jun 5'UTR are responsible for translational suppression and secondly, is the distance between the 5'CAP and AUG is important for translation suppression.
Using a series of mutations, I have demonstrated that it is the complexity of *c-jun* 5'UTR secondary structure that may play an important role in translation suppression. Mutants pG5'(Δ1-193)CJ3 and pG5'(Δ1-259)CJ3, in which 193 or 259 nucleotides from the 5'UTR were deleted, resulted in relief of translation suppression. Two internal deletion mutations in the *c-jun* 5'UTR, pG5'(Δ193-259)CJ3 and pG5'(Δ143-259)CJ3 were also constructed. These internal deletion mutations have retained sequences in their 5'UTR that can form a loop-like secondary structure. When tested *in vitro*, these mutations does not cause a relief in the translational suppression. This implies that the sequences which make up the base of the secondary structure may play an important role in translational suppression caused by the 5'UTR. I have observed a good correlation between the complexity of the *c-jun* 5'UTR secondary structure and translation efficiency (Table 1). Thus, as the complexity of the secondary structure in the 5'UTR increases, translational efficiency decreases. Another critical observation was that as the linear length of the 5'UTR increases, translation efficiency decreases (Table 1). Thus, translation suppression caused by the *c-jun* 5'UTR could be due to an increase in the linear distance between the 5'CAP and AUG. In order to address this question, I have constructed a mutant called pG5'(Δ1-259)CJ3+S. This mutant has a 5'UTR of the same length as that of pG5'(Δ1-193)CJ3 but is predicted to form a secondary structure of less complexity. When compared for relative protein level *in vitro*, pG5'(Δ1-259)CJ3+S translates four times more efficiently than the pG5'(Δ1-193)CJ3 mutant. This implies that it is the complexity of the 5'UTR secondary structure and not the increase in distance between the 5'CAP and AUG that is responsible for translational suppression. Similar results have been reported by other investigators (82). In addition, I have also demonstrated that it is the 5'UTR and not the 3'UTR of *c-jun* that is responsible for translational suppression. Furthermore when the GC rich *c-jun* 5'UTR was cloned upstream of a heterologous CAT gene it caused a 30 fold decrease in CAT translation. These experiments demonstrate that the *c-
jun 5'UTR has the ability not only to suppress its own translation but also of heterologous gene in vitro.

It is important to point out that the translation suppressive property of the c-jun 5'UTR was demonstrated in vitro. In one experiment, I have observed that the 5'UTR of the c-jun gene causes translational enhancement in vivo. The exact reason for this translational enhancement is not known at present. Translational enhancement caused by complex secondary structures is not unusual and has been observed under different in vitro and in vivo situations. For example a GC rich synthetic 5'UTR can cause translation enhancement of the CAT gene in Xenopus oocytes after fertilization (83). In another situation where the c-myc 5'UTR was shown to inhibit its own translation in vitro, translation enhancement was observed in differentiating murine erythroleukemia cells (86). Thus, it is important to note that translational regulation (enhancement or suppression) can be quite different under different growth conditions and in different cell types. The translational enhancement caused by the c-jun 5'UTR on the CAT gene in Rat-1 cells does not necessarily contradict the results that I have obtained in vitro. In fact, the effect of the c-jun 5'UTR on c-Jun translation may not be the same as on CAT. This can be due to different secondary structures formed by the complete c-jun and CAT mRNAs. Alternatively, there may be cell specific factors that can modulate translation of different genes by different mechanisms. Our in vitro experiments strongly indicate that the GC rich 5'UTR of c-jun has the capability to suppress translation.

The translation regulation of c-jun that I have observed in vitro may or may not be the same in vivo. This may have to do with the complexity of a living cell. In vivo, it is likely that there are cellular factors that interact with the c-jun 5'UTR to modulate its translation. In vitro I have demonstrated that it is the secondary structure of the c-jun 5'UTR that plays an important role in translation suppression. Do the same secondary structures are formed in vivo? During mRNA synthesis in vivo, it is possible that cellular factors may interact with the 5'UTR and inhibit or enhance secondary structure formation.
Another level of complexity in understanding the translational regulation of \textit{c-jun} gene is what kind of cell type is used for analysis. Different cell types may have cell specific factors under different growth conditions that may modulate translation differently. Translational regulation may or may not be observed under normal growth conditions. Previously, in a number of other genes, it has been demonstrated that the condition in which cells are grown is crucial for demonstrating translational regulation.

In the past few years PDGF (Platelet Derived Growth Factor) has been studied for its translational regulation. In undifferentiated F9 teratocarcinoma cells, mRNA of PDGF is expressed and is translated to form biologically active PDGF AA homodimers. When differentiation is induced by treatment with retinoic acid and cAMP, the production of PDGF AA protein is terminated. Inhibition of PDGF synthesis is exerted at the post transcriptional level. Both undifferentiated and differentiated F9 cell cultures contain comparable amounts of PDGF AA mRNA. However this mRNA becomes dissociated from polysomes during F9 differentiation. One possible reason for this translation suppression is the presence of a 5'UTR (104). PDGF has a long 5'UTR that may have been modified in some manner during differentiation to bring about translational repression. Thus, it would not only be important to study \textit{c-jun} translation in different cell types but also under different growth conditions \textit{in vivo}. This may help us find a situation where translation regulation of \textit{c-jun} exists.

Under normal circumstances, most of the cellular mRNAs are efficiently translated. They contain 5'UTRs shorter than 100 nucleotides with little secondary structure (55). Interestingly, almost all of the genes shown to be translationally repressed code for critical regulatory proteins and proto-oncogenes such as thymidylate synthetase, PDGF, \textit{c-sis} and \textit{c-myc}. Computer searches have revealed that a large number of other growth factors, growth factor receptors and proto-oncogenes have relatively long 5'UTRs suggesting that
translational control may be an important component to the regulation of their activity (69).

As described earlier, some of these translationally repressed genes can be efficiently translated or repressed under specific growth conditions. The mechanisms by which this occurs is not well understood. There can be several possible mechanisms. Regulated splicing or regulated use of alternate promoters, may result in a message with reduced secondary structure in the 5'UTR. Transcription from the major promoter may create an inefficiently translated mRNA, whereas a regulated switch to a minor promoter may generate an mRNA with reduced secondary structure that is efficiently translated. Thus, a minor mRNA species with little secondary structure in the 5'UTR may become the major mRNA that is translated. A mechanism such as this occurs in the c-sis gene (73).

Translation can also occur through a 5'CAP independent internal initiation mechanism as observed in a number of viral genes (81,82,85). Translation through internal initiation involves much of the same translational machinery but may also require RNA binding proteins that can direct the binding of various helicases and the 40S preinitiation complex. Thus, it is possible that the translational enhancement of CAT mRNA in Rat-1 cells that I have observed could have been due to the presence of specific factors that helps in the internal initiation of CAT message.

Overexpression of the CAP binding translation initiation factor eIF-4E has been linked to increase translation of mRNAs with extensive 5'UTR secondary structure. The eIF-4E is one of the major limiting components of the eIF-4F complex. The mechanisms by which this occurs is not known but may involve increased recruitment of the eIF-4A and 4B helicases to the mRNA. Interestingly, eIF-4E is activated by phosphorylation, that is induced by stimulation of a number of different signaling pathways. Overexpression of eIF-4E has been shown to oncogenically transform rodent fibroblasts. It has been proposed that eIF-4E does this by increasing the translation of those genes that are
expressed at low levels in normal cells and are involved in normal cell growth and development (80-87). It is possible that eIF-4E is over expressed in Rat-1 cells. This may explain the translational enhancement of CAT with the c-jun 5'UTR.

Translational control may be a general property of all of the jun and fos family proteins. As show in Table 3 each of the jun and fos family mRNAs contain long 5'UTR that are GC rich ranging between 63% and 86%. Both jun family members and fos family members are predicted to form stable secondary structures. All of these structures however, have the potential to impede scanning by the 40S ribosome subunit, resulting in translational repression. Thus both jun and fos family members may be regulated at the translational level.

It appears that regulation of translation is an important element in controlling the activity of critical regulatory genes. Using a series of mutations I have demonstrated that there is a translational block in the c-jun message in response to serum stimulation. I have also confirmed that the sequence that is responsible for the translational block in vitro is the 5'UTR. In addition, I have also demonstrated that the translational suppression caused by the 5'UTR is not due to an increase in the distance between the 5'CAP and AUG but is probably due to the complexity of secondary structure. The GC rich 5'UTR of c-jun has translational suppression property that can not only suppresses its own translation but also of a heterologous CAT gene in vitro. In an unexpected result I have shown that the GC rich 5'UTR of c-jun causes translational enhancement of the CAT gene in Rat-1 cells. Translational enhancement by GC rich 5'UTR, is not unusual and has been reported in other cases under specific conditions. In addition, I have shown that other members of the Jun and Fos families have the potential to form complex secondary structure making them ideal candidates for translational repression.
Thus, on the basis of results discussed above, it would be crucial to study the effect of the c-jun 5'UTR on its own translation in vivo in different cell types and under different growth conditions. This would help us understand how c-jun is translationally regulated under these situations and may help us explain some of the mechanisms responsible for translational regulation. The mechanism by which c-Jun protein synthesis is regulated in vivo is not known but is the subject of our current investigation.
V. FUTURE DIRECTIONS

The overall objective of this project was to demonstrate that the GC rich 5'UTR of c-jun can play an important role in its regulation. Once this is demonstrated, it will lay the groundwork for future studies to understand its mechanism of action.

1. Demonstration of translational regulation of c-jun in vivo

The experiments that I have described until now, dealt with studying the effect of the 5'UTR on c-jun translation in vitro. I have clearly shown that the GC rich 5'UTR can not only inhibit its own translation but also that of a heterologous gene in vitro.

The next step in this study will be to study the effect of these translational control elements in vivo. I have generated some preliminary results from which future studies will be pursued.

In order to study the role of the c-jun proto-oncogene UTRs on its own translation in vivo, the following approach was undertaken. The c-jun 5'UTR, coding region and 3'UTR were PCR amplified and cloned into the pSVSPORT-1 shuttle vector (Figure 16). The main advantage of using this approach is that the coding and non-coding regions of c-jun can be precisely PCR amplified and cloned into convenient restriction sites. Some unique features of the pSVSPORT-1 vector are that it has an SV40 early promoter followed by a polylinker site with convenient restriction sites for cloning. Another advantage of using this vector is that different regions of c-jun (coding or non-coding) can be replaced or removed conveniently. It also has an SP6 promoter next to the SV40 promoter which can be used for in vitro transcription and translation. Since the c-jun mRNA has its own polyadenylation sites in the 3'UTR, the SV40 splice site and...
Figure 16 Construction of c-jun mutations used for in vivo studies.

Panel A shows a schematic for PCR amplification of the different regions of c-jun cDNA. Primers used for PCR amplification are indicated by arrows below the stick diagram of the wild type c-jun message.

Panel B shows schematic of pSVS 5'Jun3' and pSVS Jun3' constructs used in vivo.
polyadenylation site were replaced with c-jun 3'UTR. I made two sets of plasmids. One of them had the complete c-jun message (pSVS5'Jun3'), whereas the other one lacked the 5'UTR (pSVSJ3'). Once these plasmids were constructed, two major experiments were done. Firstly, these plasmids were linearized with BamHI and in vitro transcription, and translation was done using SP6 polymerase. Results from this experiment indicate that the c-jun 5'UTR causes about an 8.4 fold decrease in Jun protein synthesis (data not shown). This suppression is much more modest than observed previously in section 3 of Results. Secondly, these plasmids were transfected into Rat-1 cells using the DMSO shock method. The main advantage of using Rat-1 cells is that the chicken c-Jun product can be distinguished from endogenous rat c-Jun protein on the basis of size. Transfection of these plasmids was carried out along with pSV2NEO plasmid in a ratio of 30:1. Cells were then selected in G418 media to yield neomycin resistant colonies. Several colonies were picked and analyzed for the presence of chicken c-jun using southern blot analysis (Figure 17). Clones 106-4, 106-7, 108-7 and 108-8 show the presence of chicken c-jun (data not shown). These colonies were then picked and grown in 1XDMEM + 10% NUV serum media for several days. RNA and protein were isolated from these clones. To analyze the protein levels, western blot analysis was done using PEP-1, Jun specific antibody. Chicken c-Jun was used as a size marker to distinguish between endogenous Rat c-Jun and chicken c-Jun. As shown in Figure 18, clones 106-2 and 106-7 which do not have the c-jun 5'UTR express more c-Jun protein than clone 108-8 (clones without c-jun 5'UTR). Clone 108-7 on the other hand does not express any chicken c-Jun. The actual reason for this is not known at present.

In order to analyze RNA levels from these clones, Northern blot analysis was done using chicken antisense c-jun as probe. As shown in Figure 19, c-jun mRNA was detected only in clones 106-2 and 106-7. In clones 108-7 and 108-8 which do not have c-jun 5'UTR, no chicken c-jun mRNA was detected. From our in vitro experiments we know that c-jun message is synthesized and protein is being made. This indicates that no
Figure 17  Southern blot analysis of G418 resistant clones. Genomic DNA from clones 106-2, 106-4, 106-7 and 108-7, neo (pSV2NEO transfected) was isolated. One hundred nano grams of DNA template was used to PCR amplify c-jun, using its coding region specific primers. PCR amplified product was run on 1% agarose gel and transferred onto nitrocellulose membrane. After prehybridization for 24 hours, hybridization was done using c-jun multiprime labeled probe for 24 hours. After washing in 0.1XSSC+0.1%SDS, the membrane was exposed to X-ray film. Clones 106-2, 106-4 and 106-7 are c-jun clones without the 5'UTR where as clone 108-7 contains c-jun 5'UTR. One set of clones was serum starved (indicated by the - sign) for 48 hours where as the other set was grown normally (indicated by the + sign) in 1X DMEM+10%NUV Serum.
Chicken c-jun DNA
Figure 18 Western blot analysis of G418 resistant clones. One hundred micro gram protein was isolated from these clones with and without 5'UTR. Protein from each clone was run on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After blocking, c-Jun protein was detected by treating the blot with PEP-1 Jun specific antibody. Chicken Jun protein is indicated by an arrow.
Chicken JUN Protein
Figure 19  Northern blot analysis of G418 resistant clones. RNA from clones 106-2, 106-7, 108-7 and 108-8 was isolated and Northern blot analysis was done as described in materials and methods. Antisense chicken c-jun was used as probe. Chicken c-jun RNA is indicated by an arrow.
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mutations were created during PCR amplification of the different regions of c-jun for construction of the plasmids used here. Northern blot analysis of clone 108-7 and 108-8 indicate no c-jun mRNA accumulation. There can be several reasons for that. Firstly, the Northern blot analysis technique which we have used here may not be sensitive enough to detect the chicken c-jun RNA from clones 108-7 and 108-8. Another more likely possibility could be that the RNA with c-jun 5'UTR in clones 108-7 and 108-8 is not very stable and is rapidly degraded. At present it is not known that if the presence of the GC rich 5'UTR can cause destabilization of RNA in other genes. This may indicate that synthesis of c-jun RNA with the 5'UTR may be regulated at the transcriptional level.

Thus, at present I am unable to demonstrate that the GC rich 5'UTR of c-jun mRNA has a translational suppression effect in vivo. But our in vitro experiments strongly suggest that the GC rich 5'UTR has translational suppression properties in vitro. Now the next step would be to study if the 5'UTR of c-jun causes destabilization of its own RNA. Now, because the translational suppression of c-jun under conditions of serum stimulation was observed, I will start investigation of translation regulation of c-Jun under these conditions in near future.

2. Conditions under which translational regulation exists

The c-jun proto-oncogene plays an important role in normal cell growth development and progression through the cell cycle. Thus, it will be important to search for conditions under which translation regulation occurs. Changes in c-Jun level in cells can be very important from the point of view of growth, cell cycle or differentiation. Overproduction of c-Jun by relief of translation suppression can lead to cell transformation.

Different cell types and cell lines can be tested for the presence of c-Jun translation repression or relief of repression in the presence of growth factors, tumor promoters or physical agents such as UV radiation. If one can find conditions under which translation
regulation exists, the next step would be to search for specific mechanisms that are responsible for translation regulation:

a. Characterization of proteins binding to untranslated regions, conditions under which their activity is regulated by phosphorylation or interaction with other proteins.

b. To study if translation of c-jun mRNA is modulated by internal ribosomal entry under specific cell growth conditions.

3. **Demonstrate existence of secondary structure in vitro and in vivo**

I have demonstrated that GC rich 5'UTR can suppress translation under *in vitro* conditions. The main mechanism by which this can occur is due to the formation of a complex secondary structure in the c-jun 5'UTR. If that is the case, then it would be important to demonstrate that this secondary structure does exist *in vivo*. Once we can find a situation where secondary structure does exist *in vivo*, then it would be interesting to see if this secondary structure is melted under specific growth conditions or in certain cell lines. With this information one can search for and characterize factors that are responsible for this mechanism. If factors like these can be characterized, their normal levels or activated levels (such as phosphorylated forms) can be studied in different tumors. If over expression of these factors lead to cell transformation then they can be potential targets for therapy.

4. **Demonstrate similar mechanism of translation regulation in other Jun and fos family members**

Like chicken c-jun mRNA, quail, rat, mouse human and *Drosophila* c-jun mRNA have long GC rich 5'UTR. Their 5'UTRs are predicted to form complex secondary structures. I have done a survey of 5'UTRs of other c-jun and c-fos family members (Table 3). Most of them have long 5'UTRs which are GC rich. Thus it would be
interesting to demonstrate that they can also be regulated at the translational level. This is important because these transcription factors interact at the level of protein dimerization. If translation of one $c\text{-}jun$ and $c\text{-}fos$ species is repressed than target recognition can be altered. This is important because stimulation of different targets by different $c\text{-}jun$ and $c\text{-}fos$ species can lead to changes in cell physiology, growth, cycle regulation or transformation.
VI. REFERENCES


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AUTOBIOGRAPHICAL STATEMENT

I was born in a small town of Baroda (Gujrat) in INDIA on the 17th of January 1966. I did my schooling in Government Schools in the city of New Delhi, the capital of INDIA. After completing high school, I pursued Bachelors of Sciences Degree in Zoology in the Hindu College, a prestigious college of University of Delhi and completed the same in the year 1986. I completed my first Masters of Sciences Degree in Zoology in 1988 from University of Delhi. During my Masters program, I was accepted in MS Biology (Biotechnology Emphasis) program in Old Dominion University. During this study, I gained extensive experience as Teaching Assistant for a period of three semesters teaching 'Introductory Biology for non Biology Majors'. I successfully got my second Masters Degree in 1990. During this program I published one Research Article in the journal of 'Environmental and Molecular Mutagenesis'. The title of my article was 'Aneuploidy in Drosophila. III: Aneuploidogens Inhibit In Vitro Assembly of Taxol-Purified Drosophila Microtubules'. I also won Graduate Student Research Award in 1990.

In 1990, I was accepted into the Doctor of Philosophy program in Biomedical Sciences, joint program between Old Dominion University and Eastern Virginia Medical School. During my Ph. D. program I worked on several projects to understand the role of c-jun in normal cell growth and development. I presented my work in several Regional, National and International meetings. I also won Best Poster Award for two consecutive years in 1992 and 1993 for my work presented on the Research Day of Eastern Virginia Medical School.
During this program, I again gained Teaching Experience for the first year in 1990-1991 and was awarded with Graduate Student Fellowship for the next three consecutive years. I published one Research Article in the journal of 'Cell Growth and Differentiation' with the title as 'Heterodimerization of c-fos is not required for cell transformation by jun'. I submitted another Research Article to same journal with a title 'Translational Regulation of c-jun proto-oncogene: Suppression by untranslated sequences'. Recently, I have joined a position of Post Doctoral Fellow in the Institute for Human Gene Therapy, University of Pennsylvania.