

REGULATION OF *junB* GENE EXPRESSION IN  
*v-fos* TRANSFORMED  
RAT-1 FIBROBLASTS AND REVERTANTS

by

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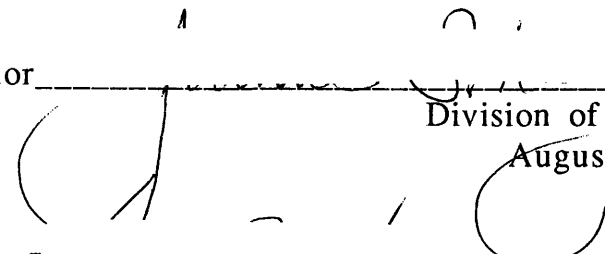
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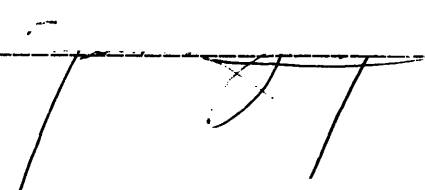
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Submitted to the Division of Toxicology  
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## Abstract

Differential regulation of *jun B* occurred in an FBJ *v-fos*-transformed Rat-1-6 (1302-4-1) cell line. It was hypothesized that *v-fos* mediated transformation of Rat-1 fibroblast cells occurred via TATA-binding protein complexes of the *junB* oncogene (Zarbl, unpublished results). To test this, two promoter fragments of *junB*, Kpn I/Xba I Large and Kpn I/Xba I Small, were combined with the chloramphenicol acetyl transferase (CAT) reporter gene to test for differential regulation of gene expression of *junB* in normal, transformed, and revertant cell lines of rat fibroblasts, isolated by Zarbl *et al* (1987). Specifically, these cell lines are Rat-1-6, 1302-4-1, and EMS 1-19, respectively. CAT activity was normalized with gene copy number using polymerase chain reaction (PCR). It was shown that, contrary to expected results, for the plasmid, pC6-K/X Large, 1302-4-1 gene expression of *jun B* was lower than in normal Rat-1-6 and EMS-1-19 revertants. pC6-K/X Small showed Rat-1-6 having the lowest CAT activity levels, but 1302-4-1 and EMS-1-19 levels were comparable, being only 60% higher than 1302-4-1. For both constructed plasmids, CAT activity levels in 1302-4-1 were lower than expected. In addition, rudimentary electrophoretic mobility shift assays (EMSA), using a 28 base pair fragment of the TATA region of *junB* showed that there is indeed a specific protein-DNA complex being formed.

Thesis Supervisor  
Dr. Helmut Zarbl  
Associate Professor of Toxicology

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## Introduction

Oncogenes are activated homologs of cellular genes that normally function in the regulation of cell proliferation via four possible mechanisms. They can modify the tyrosine or serine/threonine residues of the protein product by phosphorylation. They can control the regulation GTP. They can act in regulating transcription, levels of mRNA present, or DNA replication, copy number of the gene (Bishop, 1987).

Since several oncogenes have been shown to function as transcription factors, it is reasonable to conjecture that transformation by these oncogenes results from aberrant regulation of gene expression. In order to study the mechanism of transformation, revertant (EMS-1-19) cells were generated from FBJ *v-fos*-transformed Rat-1 cells (1302-4-1). Revertants are thought to be a valuable tool in analyzing the biochemical pathway. It is thought that to become revertants of transformed cells, the transforming oncogenes were inactivated, a dominant suppressor gene was activated, or an effector gene of the transforming oncogene was inactivated (Zarbl, 1994). It was hypothesized that the identification of genes that are differentially regulated and the study of the mechanisms underlying this regulation would allow insight into the mechanism of *v-fos* induced cell transformation.

Previous studies in our laboratory were based on the hypothesis that Jun B was an effector of *v-fos*. This hypothesis was based on the fact that *c-fos* encodes a transcription factor which forms a heterodimer with a member of the Jun family of oncoproteins and binds to the AP-1 promoter binding site (TGACTCA). Disabling Fos or Jun mutation that cause a loss of heterodimerization potential, the DNA binding or transcription activation properties also abolished *v-fos* transformation. These findings indicated that members of the Jun family of transcription factors could be *v-fos* effectors.

In revertant cell line EMS-1-19, functional *v-fos* mRNA and protein were present, and the levels were similar to those of transformed cell line, 1302-4-1. Thus either *c-Jun*

and Jun B could be effector proteins of *v-fos* transformation. Loss of the transformed phenotype could therefore result from mutations that decrease the level of Jun function or from decreased Jun expression. Ectopic expression of *c-jun* failed to retransform revertants, making it unlikely that *c-jun* was inactivated in the revertants. Northern blot analysis showed that c-Jun levels were similar in normal Rat-1, transformed 1302-4-1, and revertant EMS-1-19 cell lines. There was no evidence to indicate alterations in *c-jun* and thus we investigated the role of Jun B in transformation.

Northern analyses that *jun B* mRNA levels were 2-4.5 fold higher in 1302-4-1 cell lines than in Rat-1 (Zarbl, 1994). Further investigation showed that levels of Jun B protein corresponded to the levels of mRNA present. Specifically, in EMS-1-19 cells, there was 2.5-fold less Jun B than in rat or 1302 cells. Furthermore, ectopic Jun B via transfection caused retransformation. These results indicated that levels of Jun B protein above a threshold could be required for transformation by *v-fos*. Furthermore, when an antisense Jun B expression vector was also introduced into *v-fos* transformants, revertants could be isolated at a significant frequency (~10%).

In order to study the mechanism underlying the differential regulation of *jun B*, transient transfection studies using construct in which the murine *jun B* promoter was fused to the reporter chloramphenicol acetyl transferase (CAT) were done (van Amsterdam, 1993). Identification of the regulatory elements involved and the transcription factors that bind to these elements should provide insight into the biochemical pathways through which the *v-fos: jun B* heterodimer effects cell transformation. A series of *jun B* promoter deletion constructs were thus generated and their activity assayed in Rat-1 cells, *v-fos* transformants and revertants. Deletions were generated from a 640 base pair promoter region by exonuclease digestion to yield fragments of decreasing length. The smallest promoter construct retained only 71 base pairs in length (pC6-*PvuII/EcoRV*) and still showed gene expression. More importantly, this construct was still differentially regulated



between *v-fos* transformants and revertants. This region included only the TATA box, a sequence of TATAA found just upstream of the start of transcription initiation. The focus of this thesis was to determine if the TATAA box or the region within the 71 base pairs was responsible for the differential regulation of the *jun B* TATAA box. In addition, electrophoretic mobility shift assays (EMSA) were done to detect any differences in DNA/protein interactions of *jun B* among the cell types. Any differences would further implicate the TATA region as the region needed for differential regulation of *jun B* transcription.

## 1. Literature Review

### 1.1. Fos

The *fos* gene was discovered as the transforming genes present in two different osteosarcoma viruses, Finkel-Biskis-Jenkins (FBJ) and Finkel-Biskis-Reilly (FBR) (Woodgett, 1990) and coded for transforming proteins of 55kDa, and 75 kDa (1981, 1984; Curran, *et al*), respectively. *v-fos* could induce chondrosarcomas and was also found in chicken sarcoma virus. FBJ murine osteosarcoma virus was found to contain 1.7kb of sequence unrelated to FBJ-MLV (murine leukemia virus) which contained *v-fos*, the gene responsible for the transformation potency of FBJ-MSV (Curran, *et al.*, 1983).

It was shown that both *c-Fos* and *v-Fos* are posttranslationally modified in the cell, most likely due to phosphorylation. Using alkaline phosphatase, it was shown that most of the modifications seemed to occur because of phosphoesterification on Serine residues. *c-fos* was four to five-fold more phosphorylated than *v-fos*. Phosphorylation was induced by serum or TPA stimulation and was independent of protein kinase C (phosphorylation was  $Ca^{2+}$  and diacylglycerol independent) (Barber *et al.*, 1987).

Fos activates transcription from promoters containing regulatory elements that have the binding site for AP-1, the sequence TGACTCA, which was originally discovered in the simian virus 40 enhancer (Lucibello, 1988; Lewin, 1991). Introduction of five extra copies of the AP-1 binding site of thymidine kinase gave increased activation of thymidine kinase by Fos while addition of anti-sense *fos* mRNA gave reduction of gene expression with a chloramphenicol acetyltransferase (CAT) reporter system. These results suggested that Fos might induce tumorigenesis by binding to the AP-1 site.

Fos protein was shown to repress both the *c-fos* promoter and serum response elements (SRE) and activate sequences, namely TGACTCA, recognized by transcription factor AP-1. Fos proteins with mutations in the leucine zipper region could not induce or inhibit target genes well, indicating that protein interactions were based on these regions.

Surprisingly, Fos proteins that did not have DNA binding ability could *trans*-repress target genes but could not induce gene expression. In contrast, *v-fos* proteins could induce transcription but could not repress it, suggesting that there were different mechanisms for repression and induction. Thus, it was proven that different mechanisms exist for *trans*-activation and *trans*-repression (Lucibello, *et al.*, 1989).

In another study, transcription of *c-fos* was shown to be mediated by the presence of a serum response element (SRE) that was bound *in vivo* to p67<sup>SRF</sup> and p62<sup>TCF</sup> as part of a ternary complex. Mitogen-activated protein (MAP) kinase phosphorylated serine and threonine residues of p62<sup>TCF</sup> 1-5 minutes after induction with mitogens and growth factors, inducing re-entry into the cell cycle. MAP kinase was found to phosphorylate p62<sup>TCF</sup> *in vitro*, which in turn helped ternary complex formation. Swiss 3T3 cells supplemented with epidermal growth factor, showed induction of MAP kinase, expression of *c-fos*, and presence of active p62<sup>TCF</sup> to form ternary complexes. Swiss 3T3 cells supplemented with insulin did not induce MAP kinase, and therefore did not induce *c-fos* expression or ternary complex formation (Gille *et al.*, 1992).

Franza *et al.*, (1988) determined that many proteins, in addition to Fos, interacted with the AP-1 site. Fos and related proteins were found to bind to the HeLa cell activator protein 1 (AP-1) site, which was found in an adipocyte gene (aP2) negative regulatory sequence, a Gibbon ape leukemia virus transcriptional enhancer, and in another negative regulatory sequence -- the long terminal repeat (LTR) of the human immunodeficiency virus (HIV).

In addition to playing a role in transcription regulation, Fos also has a distinct role in cell differentiation. High levels of Fos were found bound to the regulatory site FSE2 of the adipocyte P2 (aP2) gene which was important in adipose cell differentiation. In addition, the complex of FSE2 and Fos correlated well with the amount of Fos being expressed in the cell (Rauscher III *et al.*, 1988).

Metal inducible chimeras of *c-fos* (MT-*fos*) were used to study Fos. In 3T3MT*fos* cells, it was shown that Fos could *trans*-activate an AP-1 site, whereas in F9MT*fos* cells, Fos could not. However, when a c-Jun/AP-1 expression vector was transfected into these cells, detectable levels of AP-1 were measured. By using antibodies to Fos, a protein complex was precipitated. This conglomerate included Fos associated proteins (FAP), one of which was identical to c-Jun/AP-1 (FAP p39). These results indicated that Fos acts by indirect interactions with DNA through transcription factor AP-1 (Chiu *et al.*, 1988).

Fos is found to interact with other oncogenes. *c-fos* and TRE regions are activated by *v-src*, *c-Ha-ras*, *v-mos* and phorbol esters. TRE is activated by high levels of *fos* expression, but *c-fos* is repressed by these levels. Antisense *fos* proteins did not induce TRE regions. In addition, *c-fos* was not repressed. In summary, *fos* acts in *trans* to repress and induce TRE regions (Schonthal *et al.*, 1988). Transcription of *fos* can be mediated by other oncogenes. The *src* protein was seen to activate the *c-fos* promoter by way of tyrosine kinase phosphorylation (Fujii *et al.*, 1989). Activation of protein kinase C was found to increase AP-1 binding to DNA, as did the presence of Fos and Jun. Specifically, presence of *Ha-ras* increased phosphorylation of Serines 63 and 73 of *c-fos* which in turn stimulated the expression of *c-fos* (Smeal, *et al.*, 1991). Furthermore, *c-fos* was implicated as a downstream regulator of *c-H-ras* in a signal transduction pathway. Ledwith, *et al.* (1990), showed that antisense *fos* RNA inhibits *c-fos* gene expression. In NIH 3T3 cells that have the EJ *c-H-ras* oncogene and antisense *c-fos* RNA, the *ras* protein product was overexpressed yet a partial reversion was seen in the phenotype of previously transformed EJ cells.

When the LexA repressor of bacteria was fused to *v-fos* or *c-fos*, transcription was activated in yeast, showing that Fos proteins could activate eukaryotic promoters (Lech *et al.*, 1988). Fos protein was seen to have two domains important for DNA binding and transcriptional activating functions when dimerized with Jun (Neuberg *et al.*, 1989). A alternatively spliced form of the Fos B protein which was missing the C-terminal 101

residues, was not able to activate a promoter with an AP-1 site or inhibit the *c-fos* promoter. By interacting with Jun or Jun and Fos, Fos B protein inhibited transcription and, in addition, by interacting with normal Fos proteins, the truncated Fos B prevented Fos from dimerizing with Jun to activate transcription (Nakabeppu *et al.*, 1991). The *fos* proto-oncogene is negatively autoregulated by its protein product. The same region needed for repression is also responsible for induction (Sassone-Corsi *et al.*, 1988). The protein product of *jun* has been identified as AP-1. Co-immunoprecipitation of Jun and Fos with Fos antibody has shown that Fos modulates Jun function by way of a heterodimer via a leucine zipper (Sassone-Corsi *et al.*, 1988).

## 1.2. AP-1

AP-1 is a transcription factor that was discovered because it stimulated SV40 early promoter activity and recognizes the sequences TGACTCA. It is also known as the phorbol ester element of the collagenase gene. In addition, the presence or absence of this sequence determines a target gene's TPA inducibility. Activation of genes needed for cell transformation could be induced by AP-1 transcription factor, inducible by phorbol ester, TPA (12-O-tetradecanoylphorbol-13-acetate).

AP-1 was also characterized as a 47 kD protein which activates transcription of the wild-type human metallothionein II<sub>A</sub> (hMT II<sub>A</sub>) gene (Lee *et al.*, 1987). AP-1 transcription factor has two subunits, each with domains for DNA binding, dimerization, and transactivation. Mouse epidermal JB6 cells which are P+ (transformation promotion via susceptibility to TPA enhancement) or P- (resistant) were transfected with 3XTRE-CAT which encodes AP-1 *cis*-acting enhancer elements and the chloramphenicol acetyl transferase gene (CAT). Only JB6 P+ cells showed induction of CAT activity. It was concluded that: 1) AP-1 was responsible for the specificity for TPA induction and, 2) AP-1 induction was needed, but in itself, was not sufficient for tumorigenesis (Bernstein, *et al.*, 1989).

Jonat, *et al* (1990), and Radler-Pohl *et al.*, (1992) found that glucocorticoid hormones downregulate inflammation, phorbol ester promoter transcription, and extracellular proteases such as collagenase I. These hormones had a role in inhibiting expression of AP-1, which was found to enhance transcription of the collagenase gene. The hormones do so by interacting with unbound and DNA-bound AP-1 by a direct interaction of the hormone receptor and AP-1. In addition, Jun or Fos overexpression inhibited transcription from hormone-dependent genes.

A 9 base pair sequence that interacts with the AP-1 protein is conserved in the promoter region of TPA-inducible genes (SV40, collagenase, stromelysin) . This provided an indication that AP-1 could be responsible for regulating phorbol ester promoters involved in cell proliferation (Angel *et al.*, 1987). In addition, AP-1, could be the last target of the protein kinase C signal transduction pathway.

Inhibitory protein (IP-1), found in the nucleus and cytoplasm, had an affect on AP-1, since it was found to inhibit DNA binding by AP-1 in nuclear extracts. Protein kinase A (PKA)-mediated phosphorylation blocked the actions of IP-1. From this information, it is possible to hypothesize that IP-1 might function as an anti-oncogene (Auwerx *et al.*, 1991).

An alternatively spliced form of the Fos B protein which is missing the C-terminal 101 residues, is not able to activate a promoter with an AP-1 site or inhibit the *c-fos* promoter. By interacting with Jun or the Jun : Fos heterodimer, Fos B inhibits transcription and, in addition, by interacting with Fos proteins, the truncated FosB prevents Fos from dimerizing with Jun to activate transcription (Nakabeppu *et al.*, 1991).

### **1.3. The Leucine Zipper**

Leucine residues were found as an  $\alpha$ -helix in Jun, Fos, and GCN4 (yeast regulatory protein) via x-ray crystallography. The leucines of the  $\alpha$ -helix occur every seven amino acids. They "line up" and two of these  $\alpha$ -helices form a "zipper" because the leucine residues interdigitate. This zipper is thought to be the domain that interacts with

other DNA binding proteins (Landschulz *et al.*, 1988). Using site-directed mutagenesis in the basic region directly adjacent to the leucine zipper which binds TRE, it was found that this region was needed for the Fos and Jun heterodimer to bind to the DNA (Neuberg *et al.*, 1989) The degree of interdigitating and forming the leucine zipper correlated with ability to *trans*-activate transcription and induce transformation (Schuermann *et al.*, 1989).

The leucine zipper regions of Fos, Jun and GCN4 (yeast) were found to be responsible for differences in dimerization potential through "domain swapping" experiments. The yeast transcription factor GCN 4 DNA binding domain can homodimerize and bind to TRE with high affinity. Unlike GCN 4, Fos could not homodimerize and therefore could not bind DNA well. A 68 residue peptide of containing the basic region motif of Fos and the GCN4 leucine zipper region, FBGZ, was synthesized. Another chimera, GBFZ, of 107 residues was also made. Studies with these peptides revealed that GBFZ could not bind TRE because of the fact that the Fos leucine residue region could not form homodimers. Considering that the basic regions were comparable to one another in DNA binding, the dimerization potential of the proteins was determined by TRE binding affinity to regulate transcription (Kouzarides *et al.*, 1989). Domain swap analysis was done on the leucine zipper domain, thought to be involved in protein-protein interactions, and the basic region just N-terminal to this region, thought to be responsible for the DNA binding to AP-1 sites. It was found that the Jun-Fos leucine zipper interaction was more stable than that of the Jun-Jun (Schonthal *et al.*, 1988).

The leucine zipper region was found to be important in protein binding in Fos, Jun, GCN4 and C/EBP (enhancer binding protein). When the leucine residues were substituted with non-leucine residues, the dimer could not form. When Fos protein leucine region was replaced with that of GCN4, this chimeric protein of Fos was able to form a homodimer. Usually, it was not able to. The chimera could bind with GCN4, not Jun, indicating that the leucine zipper region was important and specific for protein interactions. The Fos protein chimera did not need Jun to bind to DNA. It was therefore deduced that the Fos

chimera protein was in direct contact with DNA when heterodimerized with Jun (Sellers *et al.*, 1989) However, it was shown that the heterodimer formed by Fos and Jun is due to an altered protein conformation, most likely due to an increased  $\alpha$ -helical content (Patel *et al.*, 1990).

#### **1.4. Jun and Fos**

The c-Jun protein homodimer binds to the AP-1 site. c-Fos cannot homodimerize and therefore has no affinity for the AP-1 site. The c-Jun/c-Fos heterodimer shows a twenty-five fold increase in binding affinity relative to that of the c-Jun homodimer (Halazonetis *et al.*, 1988) and its affinity is sensitive to oxidation-reduction of a cysteine residue in the DNA binding regions of the proteins (Abate *et al.*, 1990). In addition, although dimers of Fos-Jun and Jun-Jun were both found to activate transcription, the conformational change of the protein induced in the basic amino acid region or at the AP-1 site of the DNA differed. It was found that the Fos-Jun heterodimer causes a bend in the DNA toward the major groove while the Jun-Jun homodimer causes one toward the minor groove. Although both dimers activated genes, the topological changes that occurred at the site of the protein-DNA interaction was different and may have been the cause of a difference in DNA binding affinity (Kerppola *et al.*, 1991).

mXBP/CRE-BP2 (murine protein mXBP, cyclic AMP response element, human CRE-BP2) forms a leucine zipper complex with c-Jun and can coexist with complexes of c-Jun-c-Fos. mXBP/CRE-BP2 can bind to CRE but cannot interact with TPA (12-O-Tetradecanoylphorbol-13-acetate) response elements (TREs). Fluctuations in relative amounts of mXBP/CRE-BP2, c-Fos, and c-Jun and the complexes made by these components would have an impact on the expression levels of CRE and TPA-responsive genes and thus offer flexibility in gene regulation (Ivashkiv *et al.*, 1990).

Cotransfection of Fos and Jun vectors was seen to inhibit transcription from the osteocalcin gene and stop induction of this gene by both retinoic acid and vitamin D3.



Retinoic acid receptor (RAR) was shown to contain an AP-1 sequence, which was seen to be bound by the Jun-Fos protein complex. Two classes of transcription factors could recognize the same sequence; this was called cross-coupling (Schule *et al.*, 1990). Fos and Jun were also seen to bind to the regulatory region of proenkephalin, produced in the hippocampus. Elevated levels of Fos and Jun were followed by elevated proenkephalin levels and seizure (Sonnenberg *et al.*, 1989).

### 1.5. Jun

The *v-jun* oncogene was discovered in avian sarcoma virus (ASV), a retrovirus which was found to cause sarcomas in adult chickens and transform chicken embryo fibroblasts. It was thought that *v-jun* was the oncogenic component of this virus (Vogt *et al.*, 1987; Maki *et al.*, 1987) because of the inability of the defective virus to replicate and ability to transform cells in culture. It was shown that *c-jun* also has an activator domain, A1, which was necessary for interaction with its inhibitor. While other avian sarcoma viruses resemble protein kinases that recognize tyrosine, Jun is peculiar in that it does not. Jun was found to be involved in transcription induction in the nucleus and also to have considerable homology to yeast transcriptional activator, GCN4 (Angel *et al.*, 1988; Vogt *et al.*, 1987; Struhl, *et al.*, 1987, 1988). Both Jun and GCN 4 were shown to bind to the same DNA sequence, ATGA(C/G)TCAT, although the DNA binding regions are only 45% homologous in amino acid sequence. Specifically, 66 C-terminal amino acids involved with DNA binding function are homologous.

Jun protein of avian sarcoma virus 17 (ASV17) is 65 kDa (p65<sup>*gag-jun*</sup>) made of partial *gag* sequences fused to *jun*. Through immunofluorescence studies, it was shown that v-Jun was localized to the nucleus of chicken embryo fibroblasts (CEF) that were transfected with ASV17 DNA. Footprinting experiments with DNAase I showed p65<sup>*gag-jun*</sup> was bound to sequences of Simian Virus 40 (SV40) which the AP-1 transcription factor recognized; point mutations showed similarities between binding properties of v-Jun, AP-

1, and GCN4. It seemed likely that activation of the *jun* oncogene could be due to other factors besides alterations in the DNA binding properties of the normal cellular protein (Bos *et al.*, 1988).

*v-jun* recognized DNA sequences similar to those recognized by AP-1, a phorbol ester-inducible enhancer binding protein. Fos protein antibodies were seen to immunoprecipitate both Fos and Jun from nuclear extracts. The cellular form of *jun*, *c-jun*, has been found to have approximately 80% homology to *v-jun*.; *v-Jun* is similar to *c-Jun* except that *v-Jun* has a deletion of the  $\delta$  region referring to 27 amino acids (amino acids 40-67 of *c-Jun*). It was shown that *c-jun* also has an activator domain, A1, which was necessary for interaction with its inhibitor. The  $\delta$  region is responsible for stabilizing A1 interaction with the inhibitor of *c-jun*. In addition, there are several point mutations within the *v-Jun* sequence and a lack of the 3' nontranslated region (in *v-Jun*). *v-jun* does not have the  $\delta$  domain. This makes *v-jun* a stronger activator because inhibitor is not stabilized as well. It was also shown that transcription was induced in *c-jun* by preventing the interaction between *c-jun* and its inhibitor (Baichwal, *et al.*, 1990; 1991). Because of the similarities between the two, it can be inferred that *v-Jun* is derived from *c-Jun* (Lewin, 1991).

Because antibodies to protein segments in *v-Jun* reacted with AP-1, purified AP-1 sequences showed similarity to *c-jun*, and *c-jun* seemed to produce a protein identical to AP-1, it was even hypothesized that AP-1 was encoded by *c-jun*. Furthermore, it was thought that other genes were involved in transcriptional regulation by *c-jun* since *c-jun* interacted directly with DNA regulatory sequences (Bohmann *et al.*, 1987). *in vitro* transcription assays showed that *v-Jun* had greater transcriptional activity than *c-Jun*, specifically because of a regulatory sequence in the N-terminal region of *c-Jun* and possibly because of acidic and proline residues in the C-terminal domain, near the DNA binding domain. Because of this, it was thought that a repressor domain, found in *c-Jun* and not in *v-Jun*, was responsible for the constitutive action of *v-Jun* (Bohmann *et al.*, 1989).

Jun/AP-1 was seen to bind to DNA by way of an 8 base pair sequence called the TPA responsive element (TRE). Induction of Jun/AP-1 activity occurred via posttranslational modification or gene activation of *jun*. Site directed mutagenesis within this 8 base pair site prevented transcriptional activation, demonstrating that *jun* was positively autoregulated and possibly explained the prolonged labile messages made by protein kinase C activation (Angel *et al.*, 1988).

Phosphorylation of *c-jun* was found to be mediated by mitogen-activated protein-serine (MAP) kinases (pp54 and pp42/44), which are in turn positively regulated by tyrosine and serine/threonine phosphorylation. When activated, as by phorbol esters, *c-jun* proteins are dephosphorylated (negative regulation in response to phorbol ester presence). (Pulverer *et al.*, 1991). In addition, Lin *et al.* (1992), showed that in normal, nontransformed cells, c-Jun is phosphorylated by casein kinase II (CKII) at two of three sites (Thr-231, Ser-249), inhibiting DNA binding and AP-1 stimulation. Here also, less phosphorylation increased AP-1 activity.

Activation of protein kinase C (PKC) was shown to decrease phosphorylation of c-Jun (Boyle *et al.*, 1991). The cellular Jun protein was found to be phosphorylated on five serine and threonine sites in human epithelial and fibroblast cells; three sites, found in the basic DNA-binding domain (residues 227-252), were phosphorylated by glycogen synthase kinase 3 (GSK-3) *in vitro*. Protein kinase C activation decreased phosphorylated c-Jun at one or more sites and correlated with increased binding to AP-1 sites. When serine 243 was changed to phenylalanine, phosphorylation of all three sites was blocked *in vivo* in resting human epithelial and fibroblastic cells. The mutant c-Jun (Phe 243) was able to activate transcription with a 10-fold increase.

Transcription of *jun* can be mediated by other oncogenes as well. The presence of the Ha-Ras protein caused increased transcription by c-Jun most likely by increased phosphorylation of specific sites of c-Jun protein. The increase in phosphorylation caused by a protein kinase cascade could explain cooperativity between oncogene products in

causing cell proliferation in rat embryo fibroblasts (Binetruy *et al.*, 1991) *src* and *ras* cotransfection increased the transcription level of a GAL4:*c-jun* hybrid which only had the  $\delta$ -A1 domain of *c-jun* (no DNA binding region). When angiotensin (Ang) II causes overstimulation (hypertrophy) of aortic smooth muscle cells, *c-fos* and *c-jun* were seen to be overexpressed and to interact with AP-1 binding site. Although Jun and Fos are implicated in tumorigenesis and cancer, these proto-oncoproteins apparently have a normal cellular function as well (Naftilan *et al.*, 1990).

Threshold levels of Jun protein are necessary for the induction of the transformed phenotype. A mutant Jun protein, lacking an activation domain, was found to act as an anti-oncogene in *ras*-transformed cells, producing revertants of the previously transformed cells. Even in the presence of other oncogene products such as Ras, v-Src, polyoma middle T, c-Jun, c-Fos, and TPA, a revertant phenotype was seen (Lloyd *et al.*, 1991).

In another situation, transgenic mice carrying the *v-jun* oncogene under the control of the MHC class I antigen gene promoter show signs of *v-jun* fibroblast proliferation during wound repair. After 2-5 months, some lesions develop into dermal sarcomas, which is typical of *v-jun* expression. It was hypothesized that there might be a threshold level of *v-jun* expression needed for cell proliferation and that *v-jun* expressing cells must undergo secondary events (mutations) for tumors to progress (Schuh *et al.*, 1990).

## 1.6. The Jun family

*jun-B*, *c-jun*, and *jun-D* are members of a gene family. All were found to bind to AP-1 sequences (TGACTCA) as homo- or heterodimers. The three protein products have differential binding affinity to consensus sequences of. c-Jun homodimers bound ten times stronger to the AP-1 site than either Jun D or Jun B, with Jun B binding the weakest. In addition, sequences flanking the AP-1 sites also influenced how tightly bound the Jun proteins were. However, with CRE (cAMP response element) sites, the Jun proteins bound more uniformly and without influence of adjacent sequences. When

heterodimerized with Fos B or c-Fos, binding affinity to TRE's was even higher and could be attributed to the longer half-lives of the heterodimers as opposed to the homodimers. This, in turn, could account for the weaker transcriptional activation of Jun B as compared to other members of its family (Nakabeppu *et al.*, 1988; Ryseck *et al.*, 1991).

c-Jun and Jun B are highly homologous in the C-terminal region. Both have low basal levels of gene expression and are transcriptionally activated by serum induction. However, Jun D differs in that its endogenous levels are higher and that it is not as well-induced as its counterparts. In addition, Jun D levels have different tissue distribution. The highest levels of Jun D are found in the intestine, thymus, spleen and lung, (Hirai *et al.*, 1989) and could have a possible role in immune reaction. In contrast, c-Jun and Jun B are expressed mostly in the lung and heart. Thus, a possible for the binding affinity and expression of the three oncoproteins could be the distribution of function.

### 1.7. *jun B*

The murine *junB* gene was cloned from a genomic testis library and characterized (de Groot *et al.*, 1991; Bornstein thesis; 1992). It was homologous to *c-jun* and its properties were found to be very similar to those of *c-jun*: activation by serum, TPA, and activated protein kinase A. Interestingly, Jun B was found to be a negative regulator of *c-Jun*.

*Jun B* was found to have no introns; *c-jun* also was discovered to be uninterrupted. de Groot *et al.* (1991) determined that there were several *cis* elements needed for *jun B* to be able to activate transcription. Sequences -91 to -44 were involved in gene regulation. Specifically, a G-C rich region (-100 to -77), an inverted repeat (IR) (-57 to -50), and a CAAT box. It was determined that a protein of 110 kD bound to the IR sequence and was involved in initiation of transcription via TPA and cAMP.

It was found that *junB* was not as able to transform primary rat embryo cells (with *ras*) as *c-jun*, and could not transform Rat-1A cells alone like *c-jun*. *junB* was found to

repress *c-jun*, since the presence of both decreased transformation, whereas *c-jun* alone did not. Although *junB* or *c-jun* alone activated transcription, the presence of both resulted in negative regulation of *c-jun* by *jun B* (Schutte *et al.*, 1989).

It was discovered that Jun B alone could not activate genes with TRE elements. The *jun* family of genes encode proteins which bound to TPA response elements (TRE) as homo- or heterodimers with Fos and homo-or heterodimers with each other or other members of the Jun family. It was shown that the c-Jun protein activated the *c-jun* gene, but not Jun B, gene (positive regulation) and collagenase genes (one TRE). Jun B was able to inhibit formation of c-Jun. In addition, Jun-B was found to activate only promoters with multiple TRE elements. By using protein chimeras, it was shown that differences in these proteins were due to their respective activation domains; Jun-B relied on cooperative interactions (between DNA binding domains or activation domains) whereas c-Jun did not (Chiu *et al.*, 1989).

Four different *v-src*-transformed cell lines from three different species were shown to have higher levels of *junB* protein. Elevated levels of *junB* were specific for *v-src* only. Deletions of the *junB* promoter showed a 121 nucleotide sequence containing both CCAAT and TATAA that were responsive to *v-src*. It was also shown that tyrosine kinase activity of *src* proteins was needed for elevated *junB* levels. Point mutations within the TATAA box deleted *src* responsiveness (Apel *et al.*, 1992). Hence, Jun B was thought to be similar to c-Jun structurally, but functionally, it was found to be very different. Jun B was found to negatively regulate *c-jun*.

## **1.8. TATA and Transcription**

All transcription factors were found to have three domains: the DNA binding domain, the multimeric domain, and the transcription activation domain where the multimeric domain is used to form complexes of transcription factors, such as homo- or heterodimers. Usually, transcription factors aggregate in promoter regions at specific *cis*

elements called enhancers are seen to have transcription factor binding sites (Tjian and Maniatis, 1994).

Binding of TATA binding protein (TBP or TFIID) is thought to be the first step in forming the transcription initiation complex. Other factors of basal transcription found in the initiation complex are TFIIA, TFIIB, TFIIIE, TFIIF, and TFIIH. TFIID was actually TBP and eight TBP associated factors (TAFs): TAFs 250, 150, 110, 80, 60, 40, 30a, 30b. Different classes of TAFs interact with different classes of transcription activators. For example, the glutamine-rich activation domain of Sp1 was found to bind well to the glutamine-rich domain of TAFII 110. The C-terminal activation domain of VP16 was found with TAFII 40. In addition, some TAFs act as coactivators. In some cases, TAFs are needed for basic functions or "housekeeping" and not just transcription activation (Tjian and Maniatis, 1994).

TATA Binding Protein (TBP) subunit, TFIID, was determined to have a saddle-like shape, by x-ray crystallography. The DNA binding surfaces of this protein have an antiparallel  $\beta$  sheet structure (Nikolov *et al.*, 1992). TFIID was seen to activate transcription from even TATA-less promoters whereas TBP was not (Tjian and Maniatis, 1994).

Taggart *et al.*(1992) discovered that there were many parallels in transcription activation needed by the three RNA polymerases, I, II, and III. Polymerases I-III share five identical subunits. They all needed promoter specific TBP for transcription initiation. It was thought that these complexes were functionally similar. Specifically, SLI bound to UBF for polymerase I transcription, TFIID bound to Sp1 for polymerase II transcription, and TFIIB bound to TFIIC for polymerase III transcription. TBP addition to polymerase II could restore transcription activity, whereas, for polymerase III, TBP and TBP associated factors were needed.

It was discovered that RNA polymerase II needed more than twenty proteins assembled at the promoter. The initial committed step of transcription occurred when TFIID

formed at the TATA box of the promoter. Subsequently, TFIIB bound to this complex and recruited polymerase II and TFIIF. TFIIE and TFIIH were then seen to bind to the complex. After assembly of the transcription initiation proteins, ATP-dependent activation occurred (Buratowski, 1994).

TBP was needed for RNA polymerase III function, although some templates did not have a TATA box (adenovirus VA1). Interacting with TBP were other transcription factors, TFIIB and TFIIC, where TFIIB was complexed with TBP in solution and may have been the reason why TATA-less promoters could attract TBP (White *et al.*, 1992). TATA binding protein alone was found to be responsible for basal transcription levels in a U6 promoter containing the TATA sequence. In contrast, sequences that did not have TATA required more than just TBP (Lobo *et al.*, 1992). In addition, SL1, TFIID, and TFIIB are similar to TBP (TATA binding protein)-TAF (TBP-associated factors) complexes that are needed for polymerase I, II, and III transcription, respectively (Taggart *et al.*, 1992).

## 1.9. Revertants

In 1987, Zarbl *et al* isolated two classes of FBJ MSV (*v-fos*) revertants that were isolated using rhodamine 123 retention in mitochondria. Class I was shown to have mutations in cellular genes, and on the most part, could not be retransformed (except with *trk*, polyoma middle T antigen). The revertant phenotype was recessive to the transformed. Class II had a defective *v-fos* sequence.

Revertants of *v-fos* transformed fibroblasts (Finkel-Biskis-Reilley (FBR)-murine sarcoma virus-transformed) were found to have sustained levels of *c-jun*, *junB*, and *junD*. Because of the previous information and because transformation could not be induced by an additional *c-jun* vector or *c-Ha-ras* that was transfected into the cells, the reversion was therefore attributed to a nonviral mutation capable of suppressing transformation. Others have isolated revertants of *v-fos*-transformed cells that harbor dominant mutations capable



of suppressing transformation even in the presence of elevated *jun* levels (Wisdom *et al.*, 1990).

## Materials and Methods

### Construction and Cloning of Plasmids

The *jun B* promoter region from the pC6-Bam/Bam plasmid (obtained from R. Bravo at Squibb) was used to make pC6-K/X Large. The *jun B* promoter region of this plasmid was ligated to the CAT gene also derived from pC6-B/B. pC6-B/B (Figure 2) is a plasmid consisting of two parts: 1) a 1.9 kb segment of the *junB* promoter isolated by restriction digest with *Bam*HI, a polylinker region for restriction engineering, and 2) pCAT (Promega), a vector containing the Chloramphenicol Acetyl Transferase gene.

In making the smaller of the two plasmids, pC6-Kpn/Xba Small (Figure 8), two oligonucleotides, 19 mer and 28 mer (the small insert), were ordered from Integrated DNA Technologies and annealed to form a double stranded piece of DNA with *Kpn*I and *Xba*I compatible overhangs at the ends. pC6-B/B was cut with *Kpn*I and *Xba*I (New England Biolabs) to obtain the CAT gene. The 4.2 kb segment of the CAT gene (the vector) was isolated using a 1% agarose/1X TAE gel (molecular size markers included were  $\lambda$  phage DNA digested with *Hind*III (New England Biolabs) and  $\phi\chi$ 174 digested with *Hae*III (Gibco BRL)). The DNA was then purified from the gel slice by spinning in a microcentrifuge tube having a hole at the bottom and plastic wool to separate the agarose from the DNA, which was collected in another, larger tube. Centrifugation was at 20,000g for 30 seconds or as needed. The two pieces of DNA, the vector and the small insert, were then ligated for 1 hour at room temperature using T4 DNA ligase (New England Biolabs), and digested with *Kpn*I to prevent formation of concatamers (Figure 7). The linear fragment containing the CAT gene and the *junB* promoter region were gel purified as described before and ligated again to form the plasmid subsequently used to transform a competent cell line, DH5 $\alpha$  (Promega).

DNA for both the larger promoter region and the CAT gene of the larger plasmid were derived from pC6-B/B. The 1.9 kb Bam/Bam *junB* promoter insert was cloned into a

*Bgl* II site of the pCAT/polylinker vector. pC6-B/B was again restriction digested with *Kpn*I and *Hind*III to isolate the promoter sequence. Since the *Hind*III site did not complement with the vector with *Kpn*I and *Xba*I sites, an adaptor was made for the *Hind*III and *Xba*I sites. Hence, a three-piece ligation was done in a stepwise fashion where: 1) adaptor (12 mer) and promoter sequence (0.5kb) were ligated, 2) the ligation product was digested with *Xba*I and *Kpn*I to get rid of any concatamers formed, and 3) the product was further ligated to the vector containing the CAT gene (4.2kb) in a one-to-one molar ratio at low dilution. The plasmid was then electroporated into competent cells.

### **Transfection of Bacteria**

1  $\mu$ l of each plasmid (0.1mg/ml) was electroporated (resistance=200 W; voltage=2.5 V; capacitance=25mF) into *E. coli* DH5a and grown for one hour in 1 ml SOC. 200 ml was then plated onto Luria-Bertani (LB) plates containing LB medium, ampicillin (Sigma; 100 $\mu$ g/ml), and top agar. Plates were incubated overnight or 24 hours at 37<sup>o</sup>C to obtain single colonies. Colonies were picked and grown overnight in 5 ml LB and ampicillin (100mg/ml) in a 37<sup>o</sup>C shaker. Small scale plasmid preparations were then made, followed by restriction analysis of the larger construct with *Bcl* I, *Kpn*I, *Xba*I, and both *Kpn*I and *Xba*I. The smaller construct was analyzed with *Xba*I and *Kpn*I. These two clones were grown overnight, and plasmid preparations were done.

### **Plasmid Preparations**

Small scale bacterial cultures were used to inoculate 1 liter cultures of LB/ampicillin (100ug/ml). The bacteria were grown overnight in a 37<sup>o</sup>C shaker and cells were spun in a Sorvall centrifuge for 15 minutes at 4<sup>o</sup>C. No chloramphenicol was added while cultures were growing. The supernatant was drained and the cells were resuspended in GTE (50 mM glucose, 25 mM TrisCl pH 8.0, 10 mM EDTA pH 8.0). The mixture was kept at room temperature for 10 minutes. 120 ml of 0.2 M NaOH/1% SDS was added, the

suspension was mixed gently, and it was iced for 10 minutes. 40 ml of 3.0 M potassium 5.0 M acetate (60 ml of 5.0 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml water) was added. The liquid was mixed well and iced for another 20 minutes. The cellular debris was pelleted at 4000 rpm for 15 minutes at 4°C and the supernatant was filtered through cheesecloth and into Sepcor tubes. 0.6 volumes of isopropanol was added, and the DNA was kept at room temperature for 45-60 minutes after mixing well. The DNA was then pelleted for 15 minutes at 4°C. The pellet was resuspended in 7.5 ml of TE and transferred to a 30 ml Corex tube. 2.5 ml of 10.0 M ammonium acetate was added, mixed in, and iced for 20 minutes. The suspension was again pelleted. This time, however, the supernatant was kept and transferred to another tube, after which 2.0 volumes of ethanol and 0.1 volume of 3.0 M sodium acetate were added. This was kept on ice for 20 minutes. The DNA was spun down for 15 minutes at 4°C, and the pellet was resuspended in 6.93 ml TE and 70 µl of RNase stock (20 mg/ml). The solution was kept at 37°C for 15 minutes for the RNase to take effect. Three phenol/chloroform extractions were done and the DNA was ethanol precipitated with salt. The pellet was washed two times with 70% ethanol and dried. Finally, the pellet was resuspended in 0.5 ml and DNA was quantitated, either by running on an agarose gel against standards or by OD<sub>260</sub> on a spectrophotometer.

### **Cell Lines**

All cell lines were obtained at confluence from Robert Sullivan and Yan Wang (laboratory of Helmut Zarbl). Cells were cultured using 9 ml of Minimal Eagle's medium (MEM; Gibco BRL) supplemented with calf serum (ICN Flow) to 10% and penicillin-streptomycin (10,000 U/ml in 85% saline; Gibco BRL) to 1% on 10 cm plates. Cells were split or harvested after washing 2 times with 9 ml of phosphate buffered saline (PBS), adding 1-2 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Gibco BRL) and finally, complete medium to stop trypsinization.

## **Transfection of Rat Cells by Electroporation**

Cells were prepared as described by Maniatis (1989). Cells were trypsinized and pelleted by centrifugation. Cells diluted in 19.5 ml of 0.9% NaCl were counted using a Coulter Counter and resuspended to a concentration of  $10^7$  cells per transfection. This corresponded to approximately 18 plates of Rat-1-6 cells, 13 plates of 1302-4-1, and 30 plates of EMS-1-19. All plates were assumed to be at about 90% confluence. They were then electroporated with 10  $\mu\text{g}$  of construct of interest at  $C=960\mu\text{F}$ ,  $V=340\text{V}$  in 0.4cm cuvettes (BioRad and BTX). The DNAs used were: 1. 1  $\mu\text{l}$  of pC6-P/E [10.4 $\mu\text{g/ml}$ ] (J. van Amsterdam); 2. 7  $\mu\text{l}$  of pC6-B/B [1.58  $\mu\text{g/ml}$ ] (J. van Amsterdam); 3. 8  $\mu\text{l}$  of pC6-K/X Small [1.33  $\mu\text{g/ml}$ ]; 4. 8  $\mu\text{l}$  of pC6-K/X Large [1.35  $\mu\text{g/ml}$ ]; 5. 1  $\mu\text{l}$  of pBAG [11.0  $\mu\text{g/ml}$ ];(the negative control containing no CAT gene); and 6. 2.8  $\mu\text{l}$  of pCAT [3.8  $\mu\text{g/ml}$ ]. The positive and negative controls were pCAT (constitutive regulation of the CAT gene) and pBAG ( $\beta$ -galactosidase), respectively.

Electroporated cells were allowed to recover for 1-2 minutes in the chamber after which 200  $\mu\text{l}$  was plated onto each of 2 plates with 9 ml of complete media. Cellular debris was cleaned and the cells were refed after 8-12 hours post-transfection. They were then allowed to grow for a total of 48 hours at 37°C.

## **Harvesting of Cells**

Cells were harvested with trypsin and spun into pellets; two plates of each transfection were combined so cell number did not differ because of cell growth on an individual plate. They were then resuspended in 3 ml PBS and divided into two parts. One half (1.5 ml) was transferred to a microfuge tube and these cells were pelleted, drained, and stored at -20°C for CAT assay use as needed. The other half was kept in a 15 ml polypropylene tube and pelleted and drained. These cells were resuspended in the lysis buffer (described in Materials and Methods), kept on ice, spun and drained. These

steps were repeated as needed or until the solution turned from an off-white to a whiter color, indicating that cells had lysed and only nuclei were present. The nuclei were then kept at -20°C until needed for PCR to normalize the CAT activity.

### **Chloramphenicol Acetyl Transferase Assays**

The cells were resuspended in 100µl of 0.25M TrisCl (pH 7.8) and were subjected to 3 cycles of freezing in ethanol/dry ice and thawing in 37°C, respectively. 30µl of the supernatant was used for each CAT assay and the remainder was stored at -20°C for later use.

30µl of cell extract, diluted with 20µl of 0.1M TrisCl (pH7.8), was incubated in a 1.5ml Eppendorf tube for 15 minutes at 65°C to inactivate deacetylases. Afterwards, 200µl of the CAT reaction mixture (25µl 1.0M TrisCl (pH 7.8), 50µl 5mM chloramphenicol, 0.5µl <sup>3</sup>H acetyl coenzyme A (1.4Ci/mmol, 50µCi/ml), 124.5 µl water) was added (Maniatis). The mixture was transferred to a scintillation vial and carefully overlaid with 5 ml of organic (water-immiscible) scintillation fluid (Betafluor; National Diagnostics). This mixture was incubated at 37°C until each reading was taken. By scintillation counting, radioactivity of just the organic phase (CAT induced acetylated chloramphenicol) was measured. Each vial was measured for 1.0 minute every 20 minutes. CAT activity was measured by scintillation counter (Beckman) and then normalized by polymerase chain reaction (PCR) DNA copy number to give the final CAT copy number.

The acetylated chloramphenicol was covered with scintillation fluid that was not miscible with the aqueous phase which contains labeled acetyl Coenzyme A. The chloramphenicol becomes acetylated because of the CAT gene product by the acetyl CoA. Because the acetylated chloramphenicol is more organic, it diffuses into the organic scintillation fluid where the radioactivity is measured by scintillation counter. This reaction

was then measured at timepoints of 0, 20, 40, and every 20 minutes thereof until the measurements failed to increase significantly or reached a linear rate.

### **Nuclei Extraction**

After harvesting, half of the cells were used for DNA purification. These were washed in 2 ml of cold lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40) left at 4° for 15 minutes, pelleted at 1100g for 5 minutes, and drained. Nuclei were then stored at -20°C until needed for PCR.

### **DNA Purification for PCR**

Cells were resuspended in 50µl of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and transferred to a microfuge tube. 0.5 ml of extraction buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, 0.5% SDS) was added along with 2 µl of a 10 mg/ml solution of pancreatic RNase. The mixture was incubated for 1 hour at 37°C. 2.25µl of Proteinase K was added to a final concentration of 100µg/ml and incubated at 50°C for 3 hours. An 18 gauge hypodermic needle was used to shear the genomic DNA after cooling the DNA to room temperature. Twelve times through the syringe was enough to shear the DNA about 80% (B. Bahramian, unpublished). The first precipitation required 32 µl of NaCl, and 720 µl of isopropanol. The tubes were mixed well and left at room temperature for 15 minutes. The DNA was spun for 10 minutes at 4°C at 12,000g, and the supernatant drained. The pellet was then resuspended in 360 µl of TE, after which 40 µl of 3.0 M sodium acetate (pH 5.2), and 400 µl isopropanol were added. The DNA was left at room temperature for 15 minutes and again spun in 4°C for 10 minutes at 12,000g. The pellet was subsequently redissolved in 192 µl TE. 8 µl of 5.0 M NaCl, and 2 volumes of ethanol were added. The tubes were transferred to ice for 15 minutes and then pelleted at 4°C for 10 minutes. The pellet was washed with 0.5 ml of 75% ice cold ethanol and spun at 4°C for 2 minutes. The DNA was drained and allowed to dry before adding 100 µl TE. The DNA was left at 65°C

for 10 minutes to allow the DNA to resuspend properly, and it was kept at 4°C until further use.

### **PCR Preparation**

10 µl (10%) of the prepared DNA was used. Added to this was a PCR mixture containing 10 mM Tris-Cl, pH 8.4, 50 mM KCl, 2.25 mM MgCl<sub>2</sub>, 200mM of each dNTP (Pharmacia), 10µCi of [ $\alpha$  <sup>32</sup>P] dCTP (Dupont/New England Nuclear), 1µM (100 pmol) of each primer (Integrated DNA Technologies), 0.1% gelatin (w/v), and 2 U of Taq DNA polymerase (New England Biolabs, Pharmacia, Stratagene); the total reaction volume was 100 µl. Reactions were carried out in the Perkin Elmer Cetus thermal cycler (TC-1) at the following conditions for 10-20 cycles: 1 minute denaturation at 94°C, 2 minutes annealing at 53°C, and 2 minutes polymerization at 70°C. The primers were extended at 70°C for 3 minutes after the last cycle. 10 µl of each reaction (10%) was loaded with 3.5ml 5X loading buffer (0.1% bromophenol blue, 100 mM EDTA, pH 8.0, 0.5% SDS) and analyzed by gel electrophoresis.

A 7.5% polyacrylamide gel was used to analyze the PCR products. Ethidium bromide was used to visualize the DNA bands, and a 10% acetic acid, 10% methanol solution was used to soak the gel for 15 minute before drying and autoradiographing. To quantitate reaction products, a PhosphorImager (Molecular Dynamics) and software was used.

### **Preparation of Nuclear Extracts**

3 X 10<sup>7</sup> cells were harvested for each cell line using trypsinization. After pelleting for 2 minutes at 20,000g in a microfuge, the pellet was washed with 30 volumes of PBS and the packed cell volume was determined by pelleting at 1200 rpm for 5 minutes. The packed cell pellet was resuspended in 2 volumes of Buffer A ( 2 sources) and allowed to swell on ice for 15 minutes. Cells were lysed rapidly by a narrow gauge (23-26)



hypodermic needle by slowly drawing the suspension into the syringe and rapidly ejected out in one stroke; five strokes are usually enough for about 80% cell lysis. This occurs in a 1.5 ml microfuge tube. This was pelleted in a microfuge at room temperature for 20 seconds (12,000 g). The pellet is resuspended in two-thirds packed cell volume of Buffer C (2 sources) This was kept on ice which stirring for 30 minutes. The nuclear debris was pelleted by spinning for 5 minutes in a microfuge. The supernatant was dialyzed against Buffer D (2 sources) for 2 hours. The dialyzed solution was quick frozen in aliquots and kept at -20°C.

### **Gel Mobility Shift Assay**

The protein-DNA binding reactions were carried out in microfuge tubes and included the following: 10,000 cpm DNA probe, 2µg double stranded poly dIdC-polydIdC (Pharmacia), 300 µg/ml BSA, approximately 15 µg protein from crude cell extract. The binding reaction mix was incubated in a 30°C water bath for 15 minutes. Half of the samples were run on a 7.5% acrylamide gel without any loading buffer, and the other half was kept at 4°C overnight until another gel was run the following morning.

## Solutions

### Buffer A

10 mM HEPES (hydroxyethylpiperazineethane sulfonic acid) pH 7.9 at 4°C  
1.5 mM MgCl<sub>2</sub>  
10 mM KCl  
0.5 mM DTT (dithiothreitol -- a reducing agent)

### Buffer C

20 mM HEPES pH 7.9  
25% glycerol (v/v)  
0.42 M NaCl  
1.5 mM MgCl<sub>2</sub>  
0.2 mM EDTA (ethylenediamine tetraacetate)  
0.5 mM PMSF (phenylmethylsulfonyl fluorid -- a proteinase inhibitor)

### Buffer D

20 mM HEPES pH 7.9  
20% glycerol (v/v)  
0.1 M KCl  
0.2 mM EDTA  
0.5 mM PMSF  
0.5 mM DTT

### LB Broth (1 liter)

10 g Bacto-tryptone  
5 g Bacto-yeast  
10 g NaCl  
NaOH to pH 7.5

### LB/amp plates (1 liter)

15 g Agar  
50 mg ampicillin after autoclaving

### PBS (1 liter) pH 7.4

8.0 g NaCl  
0.2 g KCl  
1.44 g Na<sub>2</sub>HPO<sub>4</sub>  
0.24 g KH<sub>2</sub>PO<sub>4</sub>

### SOC (1 liter)

20 g bacto-tryptone  
5 g bacto-yeast  
0.5 g NaCl

### 50X TAE

242 g Tris base  
57.1 ml glacial acetic acid  
100 ml 0.5 M EDTA pH 8.0

**5X TBE**

54 g Tris base

27.5 g boric acid

20 ml 0.5 M EDTA

## Results

### Construction of Plasmids

In order to investigate the mechanism of differential *jun B* promoter regulation, two plasmids were generated for promoter/reporter gene transfection experiments. In each of the constructs made previously by van Amsterdam (1993), decreasing segment lengths of the promoter region were fused to the CAT reporter gene. In doing so, he determined the contribution of various regulatory elements to *jun B* expression in all the cell lines tested. Each of these previously tested plasmids included the start site of transcription (+1) and approximately 30 base pairs of 5' non-coding sequence. We were thus interested to see the effect that removing the 5' non-coding region would have on *jun B* promoter activity in the normal and transformed cell lines. In addition, we wanted to see if the TATA box region was responsible for the differential regulation of *jun B* expression in these cells.

The larger plasmid of the two new plasmids was generated by ligating the CAT gene of pC6-Bam/Bam and a 500 base pair fragment of the pC6-B/B *jun B* promoter region. Because the restriction enzymes dividing the promoter (insert) and CAT (vector) regions were derived by digestion with *Kpn I* and *Xba I*, this plasmid was named pC6-*Kpn/Xba* Large (Figure 6). Unfortunately, the desired segment of the promoter region was not delineated by an *Xba I* site. Hence, an adaptor fragment (*Hin* dIII and *Xba I*) was synthesized and used in plasmid construction.

*Kpn I* and *Xba I* were used to restriction digest and isolate a 4.3 kb fragment of DNA containing the CAT gene which is referred to as the "vector". In another reaction tube, *Hind III* and *Kpn I* were used to isolate a 500 base pair band coding for the *jun B* promoter and was referred to as the "large insert". This piece of DNA, when ligated to the vector and electroporated into competent cells, did not produce any ampicillin resistant colonies. Aliquots of DNA were thus taken after each step in the ligation

process and examined by agarose gel electrophoresis. These results suggested that the failure to detect viable plasmid was due to the formation of concatamers of the adaptor. To alleviate this problem, the ligation product of the large insert and adaptor were digested with *Kpn* I and *Xba* I and further purified. When this segment was subsequently ligated to the vector in equimolar concentrations, the desired plasmid was formed and was isolated for the transfection studies.

pC6-K/X Small was made in a similar fashion using the same vector (see Figures 7, 8). Because this insert was very small and difficult to isolate, a synthetic double stranded oligonucleotide was used in the construction. Again, it was necessary to solve the problem of concatamer formation before generating a circular plasmid.

To verify that the plasmids generated were the correct plasmids, restriction analysis with *Kpn* I, *Xba* I, and *Bcl* I was done until bands of the appropriate size were visualized.

### **Transfection of plasmids into Mammalian Cells**

Using the Gene-Pulser, plasmids with previously determined promoter activity levels (pBAG and pCAT) in the recipient cells were transfected concurrently with the two new plasmids generated and were used as the negative and positive controls, respectively. In addition, I included the previously studied pC6-B/B and pC6-P/E (Figure 4) as controls. In some cases, cells were transfected, but no plasmid DNA was found within the cells, upon subsequent examination. Data from these transfections was not included in the analyses.

### **CAT Activity**

pC6-B/B gave, by far, the highest levels of CAT activity, even surpassing the positive control (pCAT). pC6-P/E did not show very high activity levels. This low level of activity was due to low gene copy number introduced into the cells, as seen

subsequently by polymerase chain reaction. Even after normalization with gene copy number, pC6-B/B gave much higher activity than pC6-P/E, which was the expected result.

For the newly made plasmids, the results were surprising. After normalization with gene copy number, the larger of the two plasmids gave lower CAT activity in two separate transfection experiments and in of each in each of the three cell lines. The slopes of the CAT reaction curves of these samples were on average, five fold less for pC6-K/X Small after normalization for plasmid copy number (Figure 9).

For the pC6-K/X Large plasmid, there was a 2.2-fold higher CAT activity level for Rat-1-6 cells than for 1302-4-1. In EMS-1-19 revertant cells, the activity of the promoter in plasmid pC6-K/X Large was 2.3-fold higher. All three cell lines showed comparable CAT activities for pC6-K/X Small, with cell lines EMS-1-19 and Rat-1-6 showing only a 1.6-fold higher CAT activity levels than 1302-4-1. These results are in contrast to the previous results where the transformed, 1302, cell line was shown to have the highest activity. These results suggest that sequences in the 5' non-coding region might contribute to differential regulation of *jun B* in revertants and *v-fos* transformants.

### **Optimizing the Polymerase Chain Reaction for Plasmid Quantitation**

The conditions for plasmid quantitation by PCR were defined by B. Bahramian (paper submitted 1994). Reactions were carried out in the Perkin Elmer Cetus thermal cycler. The optimum number of cycles in these experiments was found to be closer to 15 than to 10, since some of the samples could not be visualized with just 10 cycles. The PCR mixture used contained 50% of the previously recommended amount of radioactivity used (B. Bahramian) and still gave very good autoradiograph and PhosphorImager signals. For each cell line, it seemed that the positive control (pCAT) always had the largest number of CAT gene copies per cell, while the negative control (pBAG) never gave a signal. The conditions again were: 1 minute denaturation at 94°C,

2 minutes annealing at 53°C, and 2 minutes polymerization at 70°C. The primers were extended for 3 minutes after the last cycle. Ten cycles were carried out on a preliminary basis. In using these conditions, several of the samples could not be visualized. To visualize, five and ten more cycles were added making the total number of cycles fifteen and twenty, respectively. It seemed that twenty cycles gave too strong of a signal for the bands to be discrete enough to be analyzed by the PhosphorImager. Fifteen cycles was thus determined to be optimal. The gels were vacuum dried for 30 minutes at 75°C and subsequently exposed onto film for about 6 hours with an intensifying screen at room temperature.

### **Nuclear Extracts for Electrophoretic Mobility Shift Assays (EMSA)**

Several attempts at storing nuclear extracts indicated that best results were obtained with freshly prepared lysates. Lysates prepared from  $3 \times 10^7$  cells were sufficient for two sets of assays. There was some problem with the stability of the extracts, however. Usually, it seemed optimal to make extracts and use them for assays on the same day. Extracts (or pelleted cells, for that matter) did not seem to give good results if kept at 4°C or even -20°C. In theory, all of the assays should have worked well, but there were some technical problems were encountered in most experiments.

### **Electrophoresis Mobility Shift Assays**

Nuclear extracts were prepared and combined with *jun* B promoter (28/28) probe coding for the TATA box region. Results were as expected: when the labeled TATA consensus sequence was added to the extracts, a novel band containing the labeled probe appeared in the gel (Figure 10, dark arrow). The novel band showed a slower mobility pattern than probe alone suggesting that it was due to DNA-protein complex formation. In addition, when excess cold probe was added in a 100 and 1000-fold molar excess, the signal of the shifted band decreased as the cold excess amount increased (Figure 10,

lanes 2-4, 5-7, 9-11). These results indicated that the excess unlabeled probe competed away proteins that bound specifically to the labeled probe, suggesting that the binding of the proteins to the probe is specific. The observation that an excess of a mutant TATAA probe (Figure 11A, lane 8) could not compete the labeled probe away from the complex provided further support for specificity. When TFIID consensus and mutant sequences were added in excess, only the consensus sequence was seen to compete with the *jun* B promoter region (Figure 11A, Lanes 8, 9). In order to determine if extracts from the various cell lines were equally active, the experiments were also carried out using Sp1 (Figure 11A, Lanes 10-15) and *oct 1* (Figure 11B, Lanes 1-8) consensus sequences as probes, in experiments that again included the *jun* B TATA binding region (Figure 11A, Lanes 4-9). Binding assays performed with labeled Sp1 probe suggested the possibility that proteins had degraded in extracts from EMS-1-19 revertant cells (Figure 11A, Lanes 10-15), since little binding to Sp1 was seen in the latter extracts. However, analysis with labeled *oct 1* indicated that the nuclear lysate from EMS-1-19 was active (Figure 11B, Lanes 8), because *oct 1* binding was highest in this lysate.

There were quantitative differences in specific protein binding to the *jun* B TATAA box sequence seen with extracts from the three different cells. The amount of protein bound to the TATAA probe in experiments using 1302-4-1 extracts was higher than the amount of complex formed with RAT-1 or 1-19 extracts. This was indicated by the fact that the shifted portion of the labeled *jun* B TATA box region (28/28) yielded a darker band on the autoradiograph (Figure 11A, Lanes 4-6).



**Table 1: *cis* element that react to certain factors (Latchman, 1990; Mitchell and Maniatis, 1989)**

<b>Sequence</b>	<b>Response to</b>	<b>Protein Factor</b>	<b>Genes with Sequence</b>
CTNGAATN TTCTAGA	Heat	Heat shock Trx factor	hsp70, hsp83, hsp27
T/G T/A CGTCA	cAMP	CREB/ATF	Somatostatin, fibronectin, $\alpha$ - gonadotropin, <i>c-fos</i> , hsp70
TGAGTCAG Metallothionein IIA,	phorbol esters	AP1	$\alpha$ 1-antitrypsin, collagenase
GATGTCCATA TTAGGACATC	growth factors TPA insulin	serum response factor (SRE)	<i>c-fos</i> , Xenopus $\gamma$ actin
GGTACANNNT GTTCT	glucocorticoid, progesterone	GR and PR receptors	Metallothionein IIA, Trp oxygenase, uteroglobin, lysozyme
AGGTCAMMM TGACCT	estrogen	estrogen receptor	ovalbumin, conal- bumin, vitellogenin
TCAGGTCATGA CCTGA	thyroid hormone, retinoic acid	TH and RA receptors	growth hormone, myosin heavy chain
TGCGCCCGCC	heavy metals		metallothionein genes
AAGTGA tumor	viral infection		IFN- $\alpha$ and $\beta$ , necrosis factor
GGGCGG		Sp1	multiple sites with strongest to increased glutamine
GCCAAT		CTF/NF-1	CCAAT box
ATTGTCAT		oct-1	ubiquitous in mammals, histone H2B and IgK gene promoters

**Table 2: Jun B promoter activity in Cell Lines for  
pC6-Kpn/Xba Small**

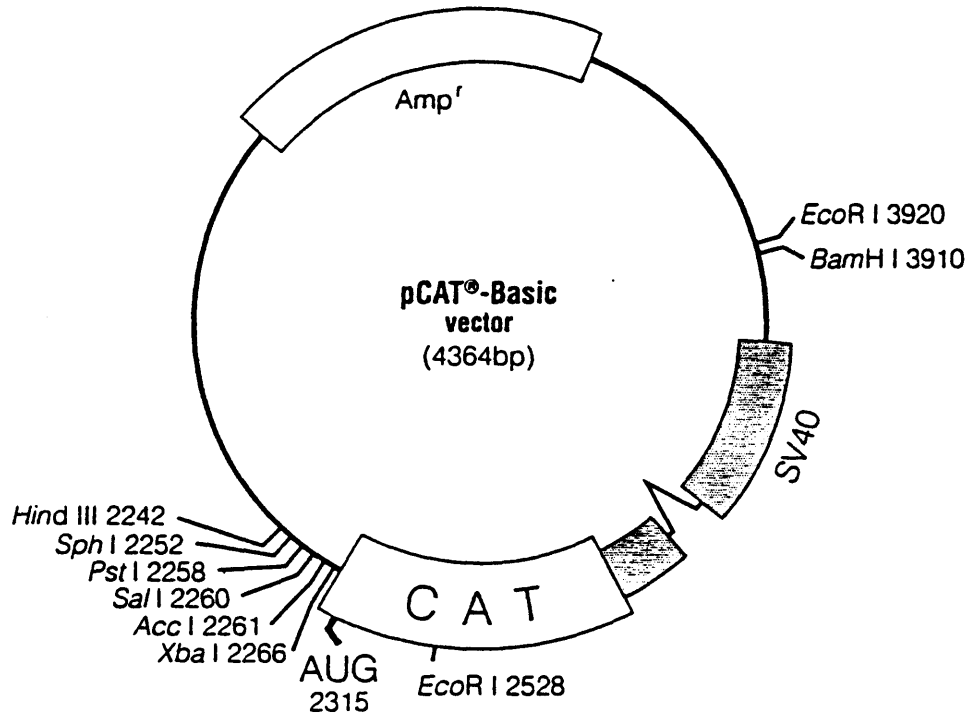
	<b>CAT activity</b>	<b>Fold difference</b>
Rat-1-6	76.9	1.0
1302-4-1	125.5	1.6
EMS-1-19	120.3	1.6

**Table 3: Jun B promoter activity in Cell Lines for  
pC6-Kpn/Xba Large**

	<b>CAT activity</b>	<b>Fold difference</b>
Rat-1-6	24.6	2.2
1302-4-1	11.2	1.0
EMS-1-19	25.9	2.3

**Figure 1. pCAT Basic vector**

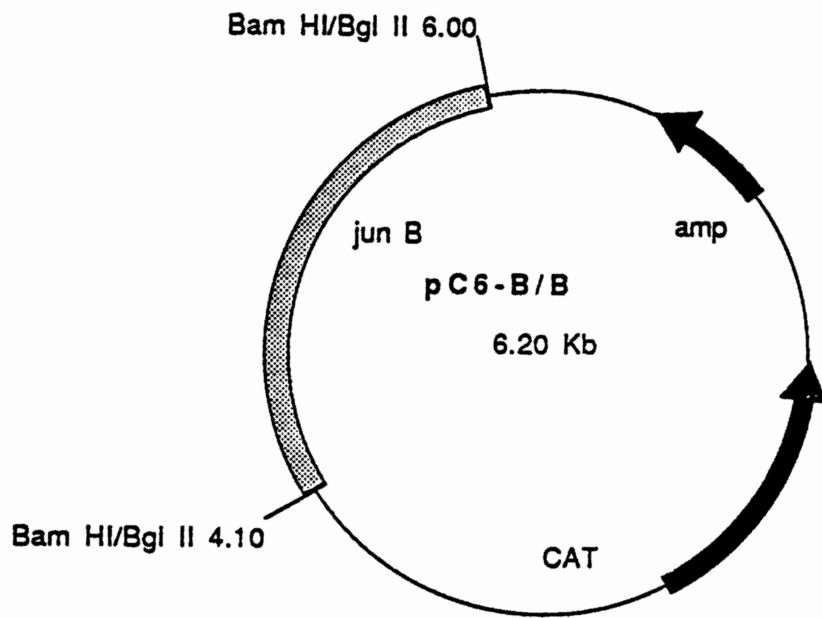
pCAT Basic vector (Promega) was used for the original *jun* B/CAT fusion plasmid made by R. Bravo. It encodes a  $\beta$ -lactamase region from site 209 to site 1069 (ampicillin resistance), and also many cloning sites.



**Figure 2. pC6-*Bam*HI/*Bam*HI**

R. Bravo used *Xba* I at site 2266 and the *Hind*III site at 2242 to introduce a polylinker.

Within the *Bgl* II site of the polylinker, the *jun* B promoter region was fused. This 1.9 kb fragment encoded *Bam* HI at both ends.



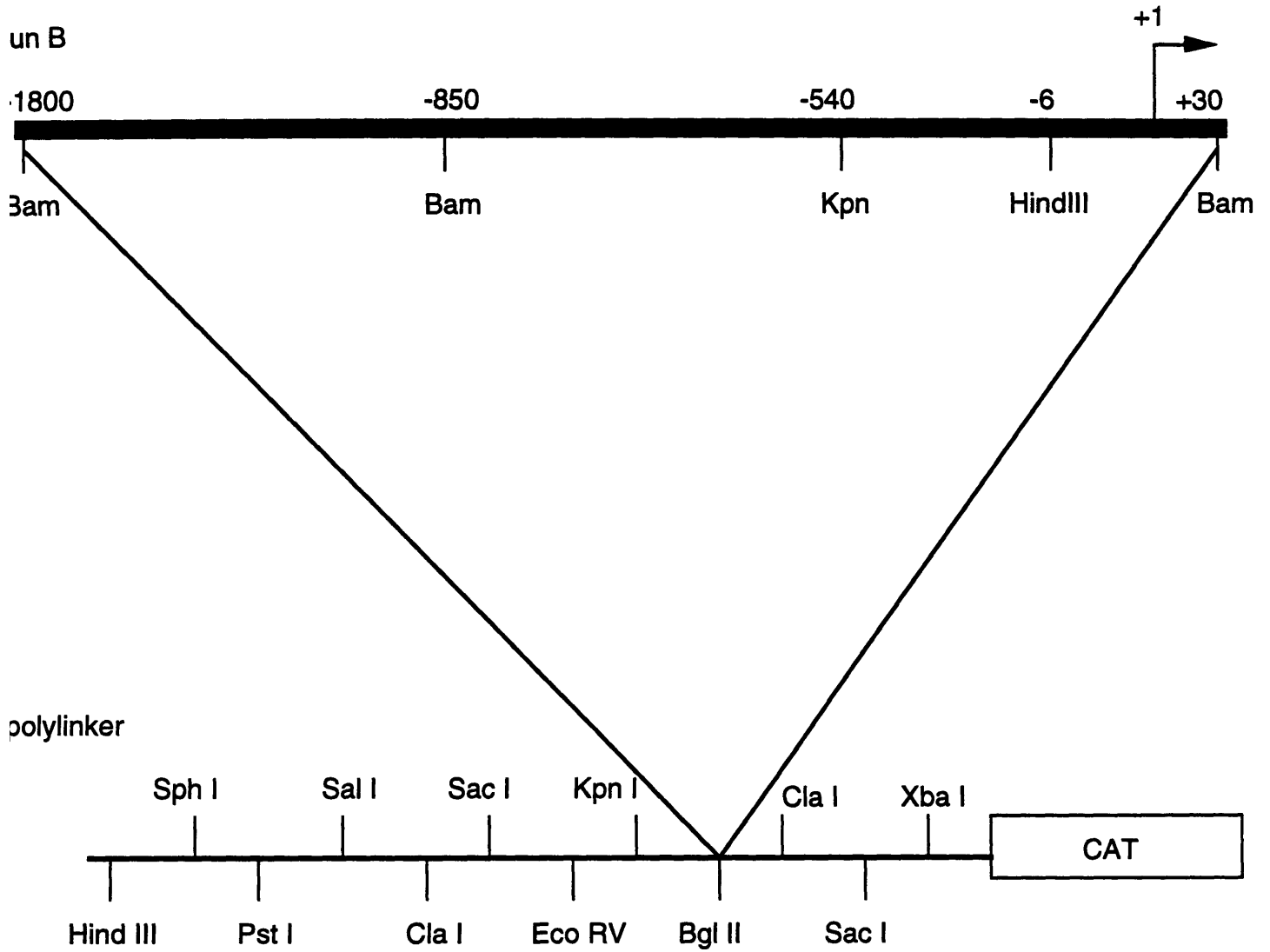
**Figure 3. Schematic *jun B Bam/Bam* promoter and polylinker sequence**

The *Bam*HI/*Bam*HI fragment of the *jun B* promoter contains many important sequences for transcription regulation and initiation. The fragment starts at site -1900 upstream from transcription initiation, and ends at +30, just downstream of it. The CAAT box, which (significance), is located at position -65. The TATAA box is at position -30 and is the region of interest in making the two plasmids.

The polylinker region found in pC6-B/B has twelve different cloning sites available. R. Bravo used the *Bgl* II site to ligate with the *Bam* HI overhanging ends of the promoter fragment.



**Figure 3: Bam/Bam jun B promoter fragment and polylinker**



**Figure 4. The different promoter fragments of *jun B*.**

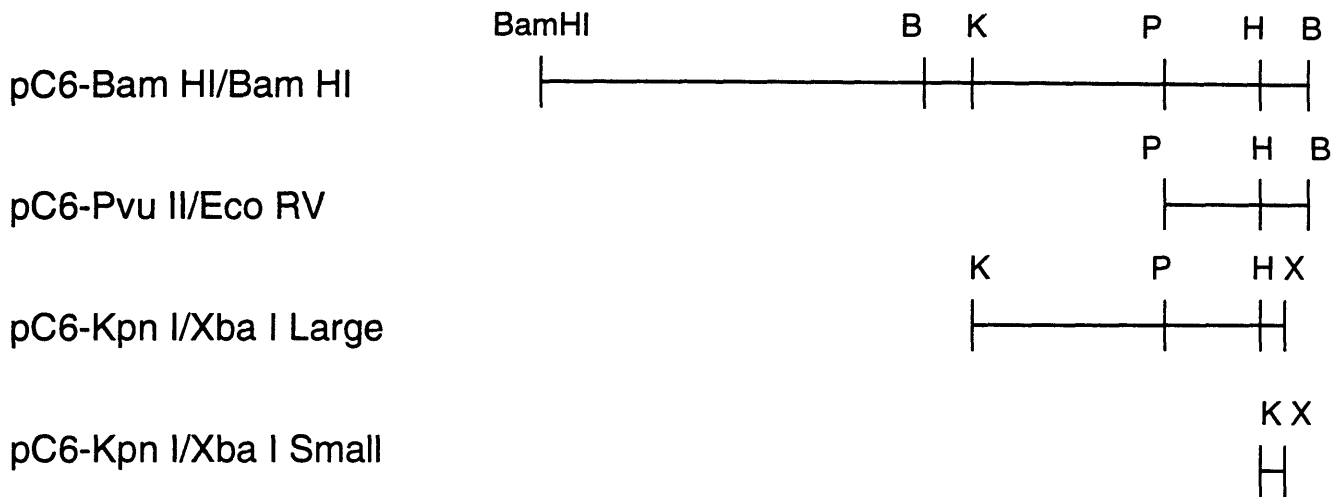
The *Bam* HI/*Bam* HI fragment found in pC6-*B/B* contained more than 1900 base pairs of *jun B* promoter region and was the largest of the four promoter fragments shown and tested.

The next fragment shown, *Pvu* II/*Eco* RV, was the region found in plasmid pC6-*P/E* (J. van Amsterdam), made by deleting out the *Eco* RV to *Pvu* II piece in pC6-*Bam* HI/*Kpn* I (R. Bravo). Since both sites were blunt ended, the plasmid was subsequently circularized by blunt end ligation.

The *Kpn* I/*Xba* I Large fragment was acquired by isolating the *Kpn* I/*Hin* dIII fragment from pC6-*B/B*. This was then ligated to an adaptor DNA with ends of *Hin* dIII and *Xba* I. The significance of this promoter fragment was that it did not code for sequence of the *jun B* gene after the TATAA box, including the +1 or site for initiation of transcription.

Likewise, the *Kpn* I/*Xba* I Small fragment did not code any sequence after the TATAA box. In this case, two oligonucleotides, one of 19 bases and the other of 28 bases, were ordered and annealed. This double stranded DNA with ends *Kpn* I and *Xba* I was ligated to the vector carrying the CAT gene.

**Figure 4: The promoter fragments of jun B**



B = Bam HI

H = Hin dIII

K = Kpn I

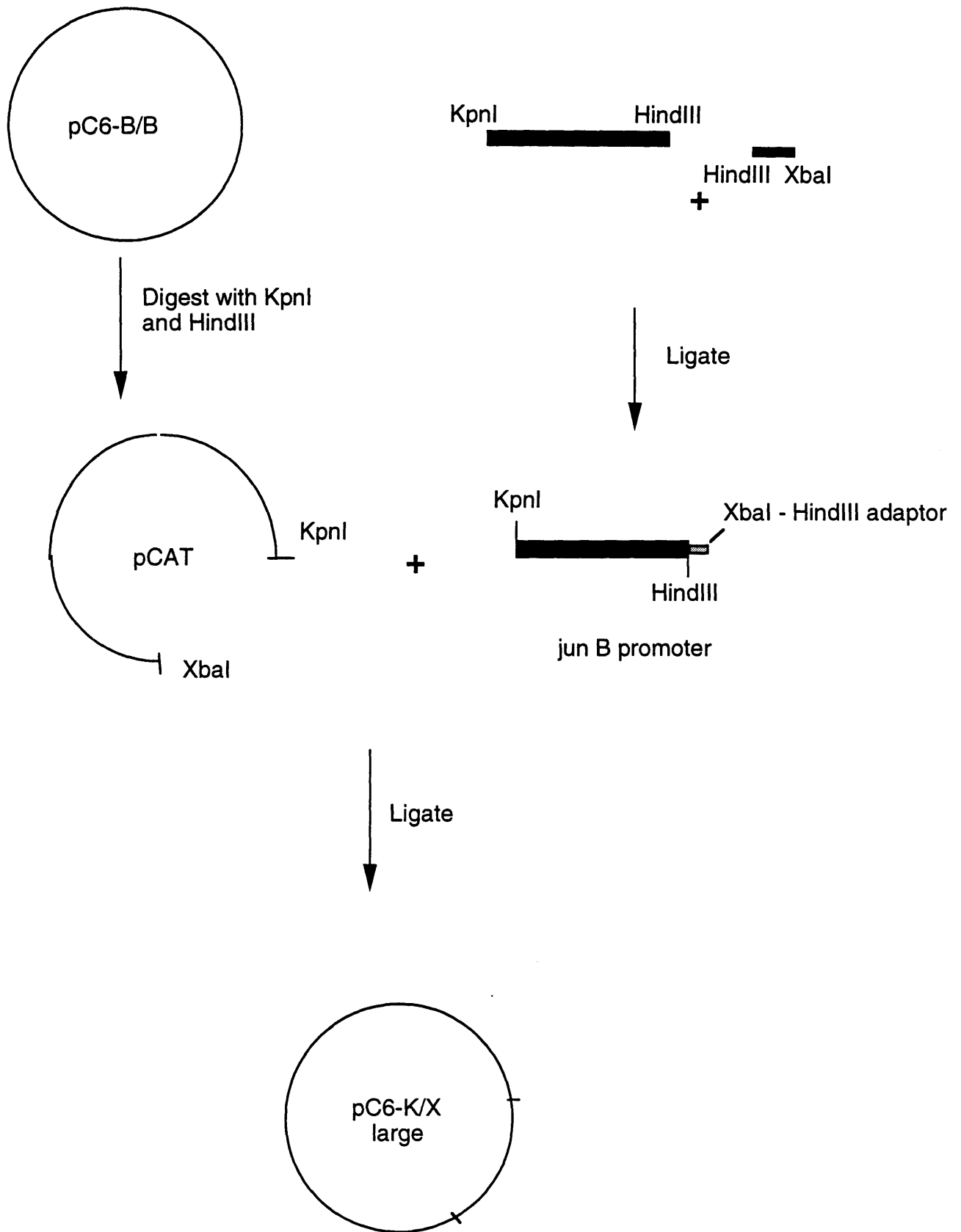
P = Pvu II

X = Xba I

**Figure 5. pC6-Kpn I/Xba I Large flow chart**

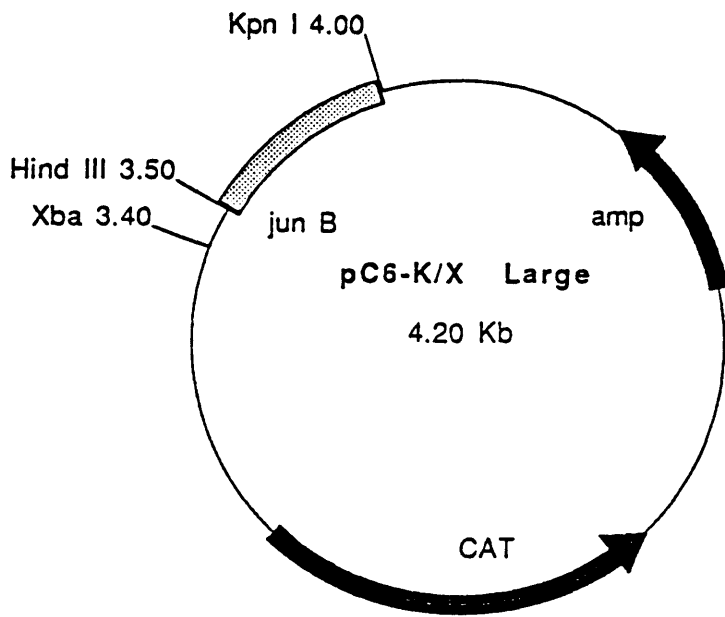
pC6-*B/B* was digested with *Kpn* I and *Xba* I to form the vector containing the CAT gene. In another tube, pC6-*B/B* was combined with *Kpn* I and *Hin* dIII to cut out the insert, which was about 500 base pairs. This insert was then ligated to an adaptor with ends of *Hin* dIII and *Xba* I. The ligation was cut with *Kpn* I and *Hin* dII to get rid of concatamers, and purified using agarose gel electrophoresis. This product was then ligated to the previously isolated vector at room temperature in an equimolar solution.

**Figure 5: Large plasmid flow chart**



**Figure 6. pC6-*Kpn* I/*Xba* I Large plasmid**

The plasmid contains the CAT gene,  $\beta$ -lactamase gene, and a 0.5 kb fragment of the *jun* b promoter.

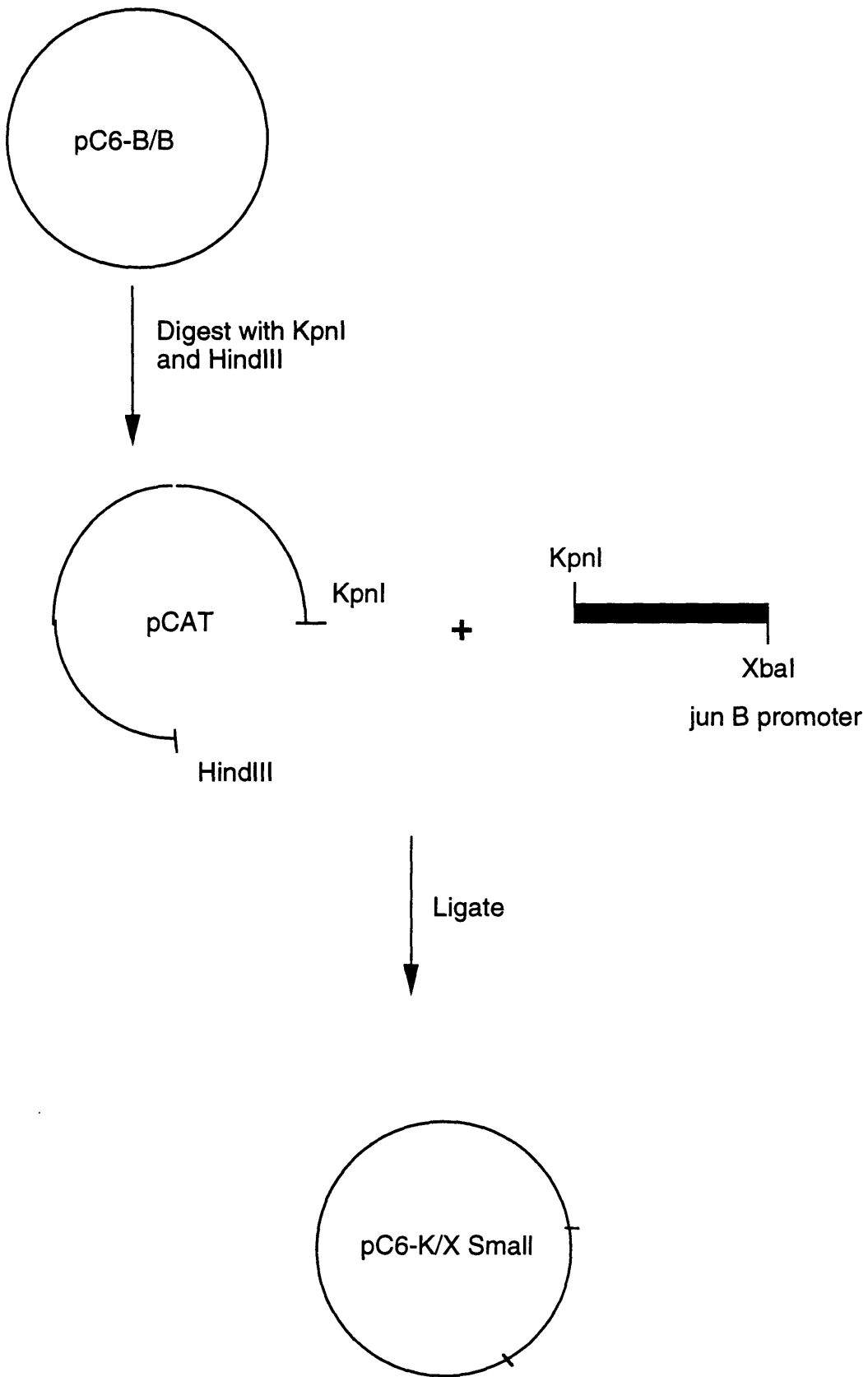


**Figure 7. pC6-Kpn I/Xba I Small flow chart**

The vector was isolated as described for pC6-*K/X* Large. Two oligonucleotides of 19 and 28 mer were annealed and ligated to the vector. This could be done because both the vector and insert had *Kpn* I and *Xba* I sites. The product of this ligation was digested with *Kpn* I to get rid of concatamers, and the DNA was gel purified. Another ligation was done to circularize the DNA.

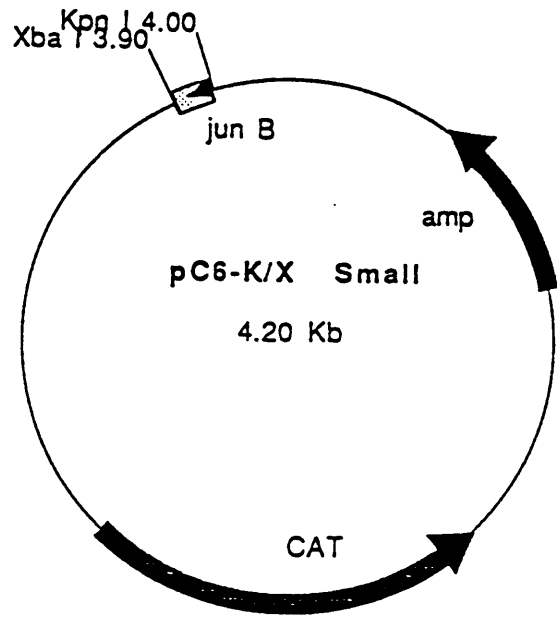


**Figure 7: Small plasmid flow chart**



**Figure 8.** pC6-*Kpn* I/*Xba* I Small plasmid

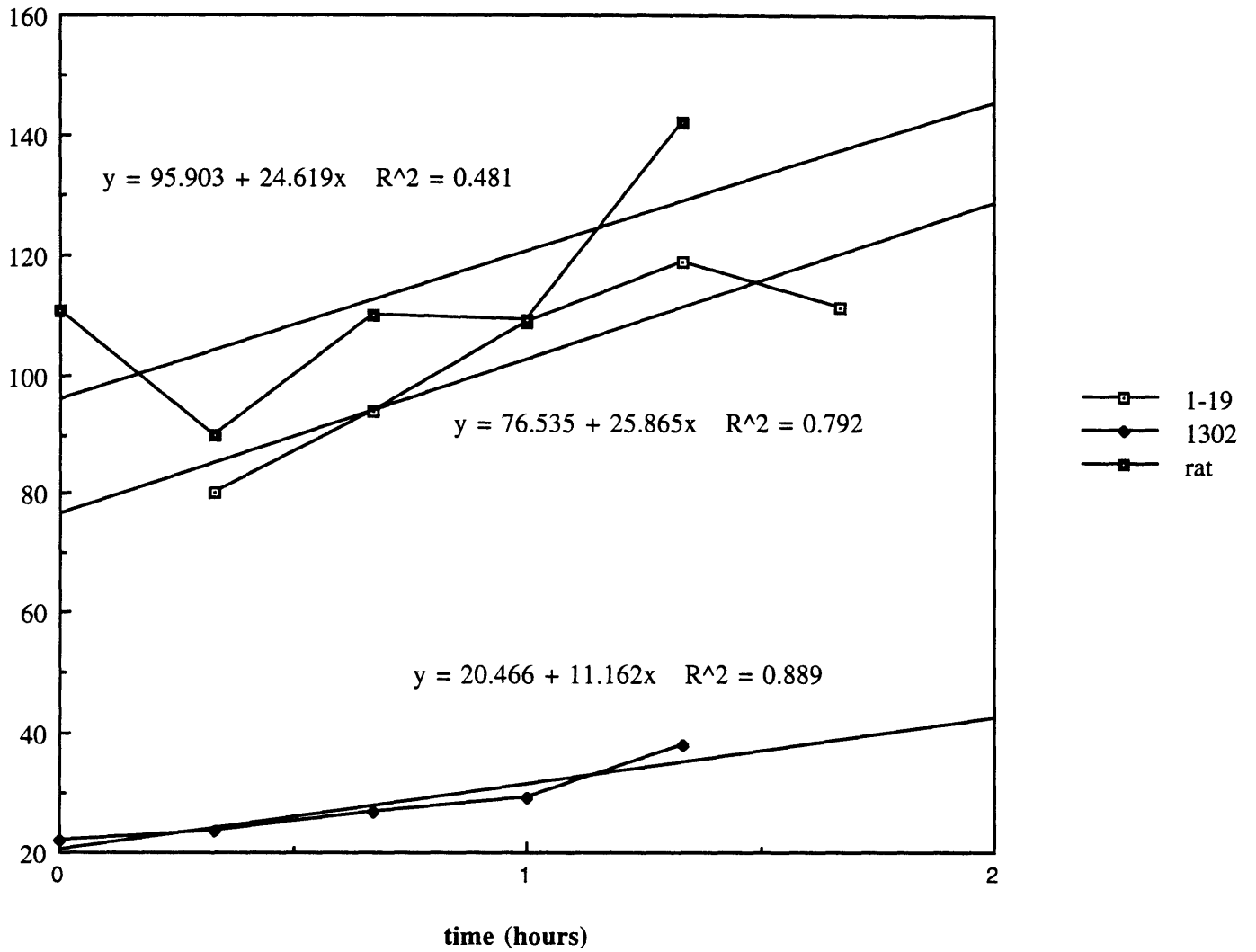
This plasmid contains the CAT gene, the  $\beta$ -lactamase gene, and a 19 base pair fragment of the *jun* B gene which codes for the TATAA box.



**Figure 9a. Normalized Chloramphenicol Acetyl Transferase Activity**

Normalized CAT assay graphs for pC6-Kpn I/Xba I Large

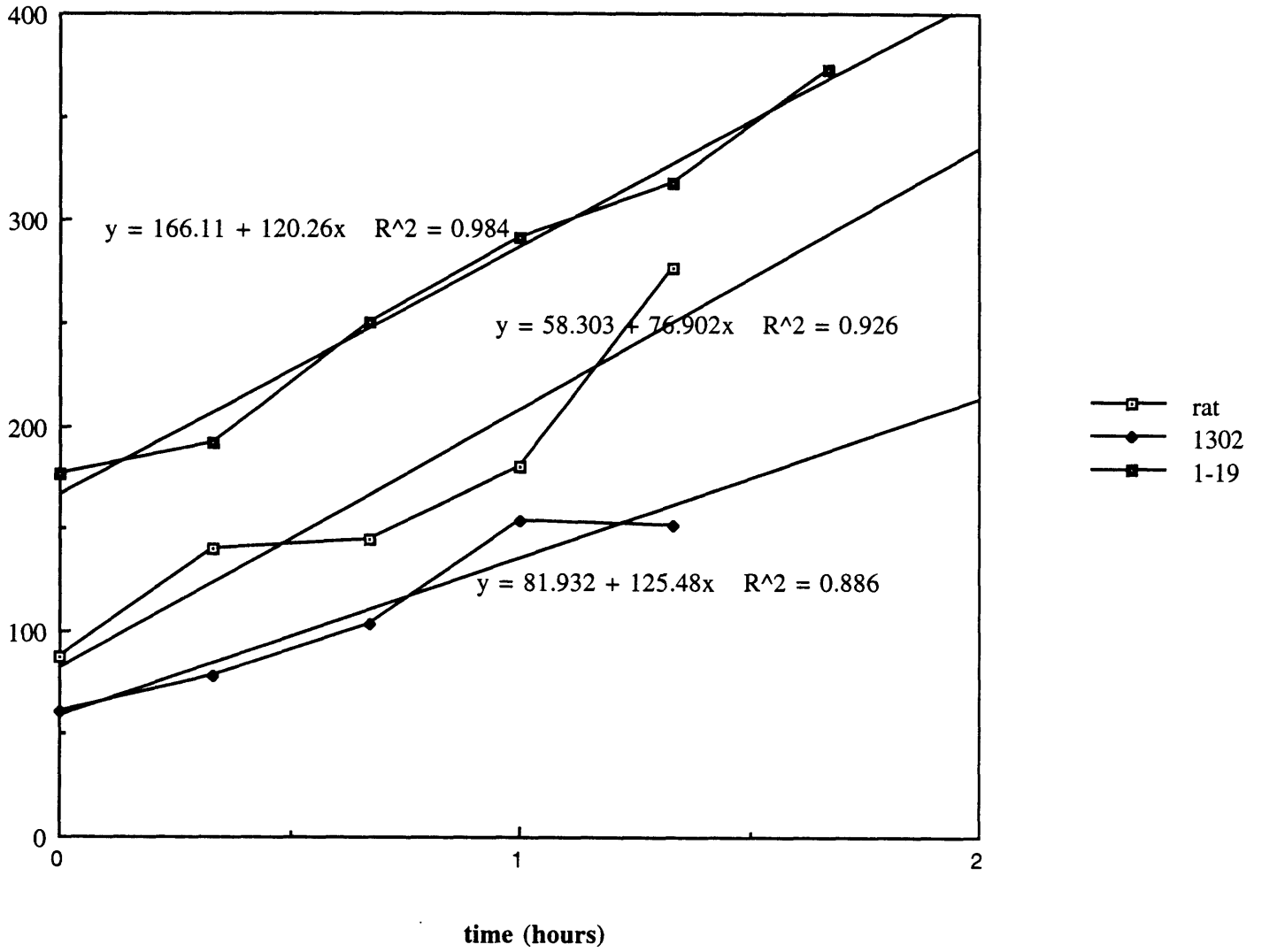
### Data from "large"



**Figure 9b. Normalized Chloramphenicol Acetyl Transferase Activity**

Normalized CAT assay graphs for pC6-Kpn I/Xba I Small

### Data from "small"



**Figure 10. Electrophoresis Mobility Shift Assay (EMSA) of *jun B***

**Lanes** 1 *jun B* 19/28 probe with extracts from Rat-1-6

2 *jun B* 19/28 probe with extracts from Rat-1-6 and 100-fold excess cold probe

3 *jun B* 19/28 probe with extracts from Rat-1-6 and 1000-fold excess cold probe

4 *jun B* 19/28 probe with extracts from 1302-4-1

5 *jun B* 19/28 probe with extracts from 1302-4-1 and 100-fold excess cold probe

6 *jun B* 19/28 probe with extracts from 1302-4-1 and 1000-fold excess cold probe

7 blank

8 *jun B* 19/28 probe with extracts from EMS-1-19

9 *jun B* 19/28 probe with extracts from EMS-1-19 and 100-fold excess cold probe

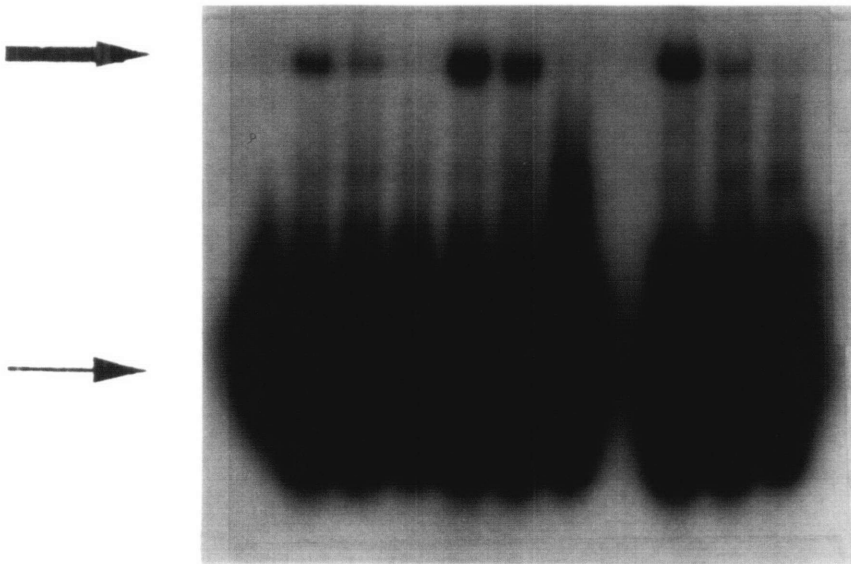
10 *jun B* 19/28 probe with extracts from EMS-1-19 and 1000-fold excess cold probe

The 100 ng of probe was labelled with 50 $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dCTP by random primer reaction and incubated for 1 hour at 37°C. Unincorporated nucleotides were removed by spun column (Sephadex G50).

The binding reactions were incubated at 30°C for 1 hour.



1 2 3 4 5 6 7 8 9 10 11



**Figure 11. Electrophoretic Mobility Shift Assays with *jun B* (28/28), Sp1 and *oct 1***

**A. Binding reaction time was 15 minutes**

Lanes	1	5X loading buffer
	2	<i>jun B</i> probe
	3	Sp1 probe
	4	<i>jun B</i> probe with Rat-1-6
	5	<i>jun B</i> probe with 1302-4-1
	6	<i>jun B</i> probe with EMS-1-19
	7	<i>jun B</i> probe with 1302-4-1 and excess cold <i>jun B</i>
	8	<i>jun B</i> probe with 1302-4-1 and excess cold mutant TFIID
	9	<i>jun B</i> probe with 1302-4-1 and excess cold wild type TFIID
	10	Sp1 probe with Rat-1-6
	11	Sp1 probe with 1302-4-1
	12	Sp1 probe with EMS-1-19
	13	Sp1 probe with 1302-4-1 and excess cold <i>jun B</i>
	14	Sp1 probe with 1302-4-1 and excess cold mutant TFIID
	15	Sp1 probe with 1302 and excess cold wild type TFIID



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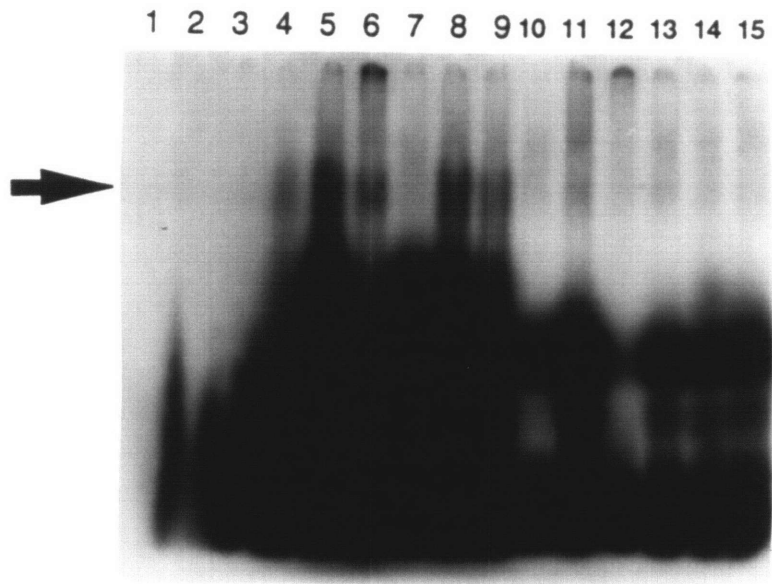
p. 67

**Figure 11. Electrophoresis Mobility Shift Assays (EMSA) with *jun B*, Sp1 and *oct 1***

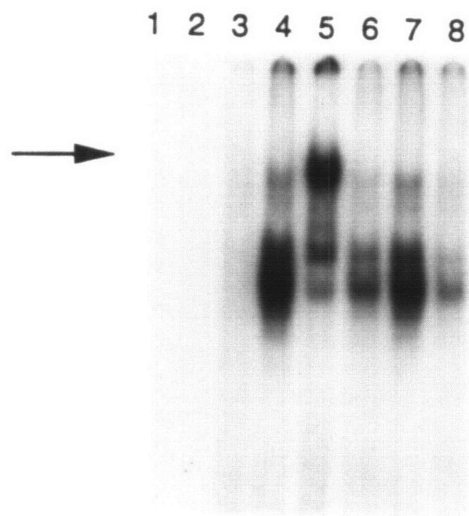
**B. Binding reaction time was 15 minutes.**

<b>Lane</b>	<b>1</b>	<b>5X loading buffer</b>
	<b>2</b>	<b><i>oct 1</i> probe</b>
	<b>3</b>	<b><i>oct 1</i> probe with Rat-1-6</b>
	<b>4</b>	<b><i>oct 1</i> probe with 1302-4-1</b>
	<b>5</b>	<b><i>oct 1</i> probe with EMS-1-19</b>
	<b>6</b>	<b><i>oct 1</i> probe with excess <i>jun B</i></b>
	<b>7</b>	<b><i>oct 1</i> probe with excess cold mutant TFIID</b>
	<b>8</b>	<b><i>oct 1</i> probe with excess cold wild type TFIID</b>

**A**



**B**

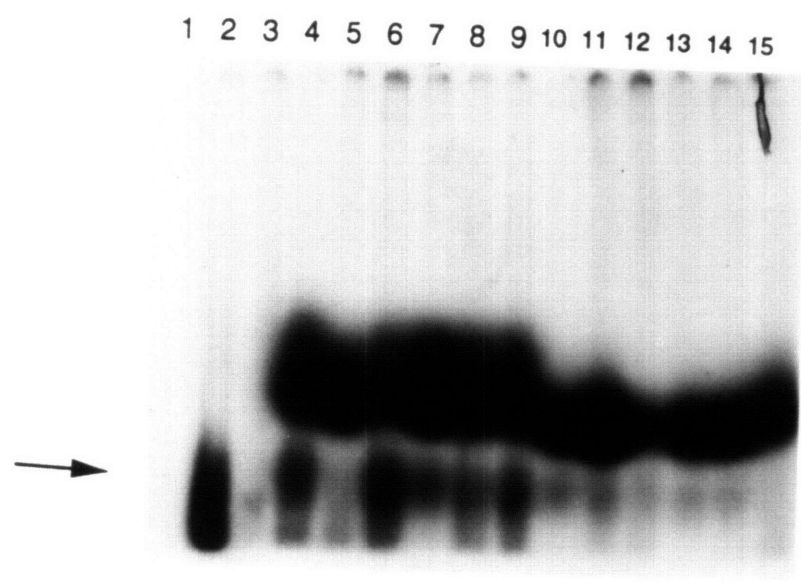


**Figure 12. Electrophoresis Mobility Shift Assays (EMSA) with *jun B*, Sp1 and *oct 1***

**A. Binding reaction time was 24 hours**

Lanes	1	5X loading buffer
	2	<i>jun B</i> probe
	3	Sp1 probe
	4	<i>jun B</i> probe with Rat-1-6
	5	<i>jun B</i> probe with 1302-4-1
	6	<i>jun B</i> probe with EMS-1-19
	7	<i>jun B</i> probe with 1302-4-1 and excess cold <i>jun B</i>
	8	<i>jun B</i> probe with 1302-4-1 and excess cold mutant TFIID
	9	<i>jun B</i> probe with 1302-4-1 and excess cold wild type TFIID
	10	Sp1 probe with Rat-1-6
	11	Sp1 probe with 1302-4-1
	12	Sp1 probe with EMS-1-19
	13	Sp1 probe with 1302-4-1 and excess cold <i>jun B</i>
	14	Sp1 probe with 1302-4-1 and excess cold mutant TFIID
	15	Sp1 probe with 1302-4-1 and excess cold wild type TFIID

**A**



**B**

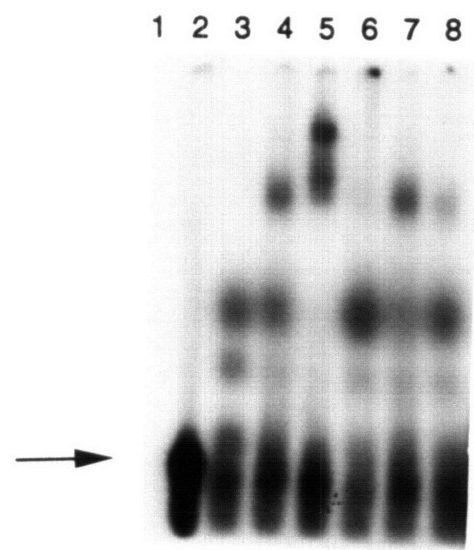


Figure 12. Electrophoresis Mobility Shift Assays (EMSA) with *jun B*, Sp1 and *oct 1*

**B. Binding reaction time was 24 hours.**

Lane	1	5X loading buffer
	2	<i>oct 1</i> probe
	3	<i>oct 1</i> probe with Rat-1-6
	4	<i>oct 1</i> probe with 1302-4-1
	5	<i>oct 1</i> probe with EMS-1-19
	6	<i>oct 1</i> probe with excess <i>jun B</i>
	7	<i>oct 1</i> probe with excess cold mutant TFIIID
	8	<i>oct 1</i> probe with excess cold wild type TFIIID



## Discussion

Previous results demonstrated that regulation of *jun B* gene transcription in FBJ *v-fos* transformed cells (1302-4-1) was different than in revertant (EMS-1-19) and normal Rat-1 cells. Northern analysis mRNA levels of Jun B in transformed cells were 2 to 4.5-fold higher. Jun B protein levels in EMS-1-19 cells returned to levels seen in Rat-1 cells and ectopic expression of *jun B* was able to retransform the revertant cell (van Amsterdam thesis, 1993). These results indicated that Jun B was an effector of *v-fos* transformation and that the mutation that led to the revertant phenotype affected the expression of *jun B*. Preliminary studies (van Amsterdam, 1993) indicated that the mutation blocked the Protein Kinase A induced increase in *jun B* expression seen in normal cell lines. In order to gain further insight into the mechanism responsible for reduced *jun B* expression in revertants, we decided to identify the cis acting regulatory elements in the *jun B* promoter than are responsible for this differential regulation. Previous studies had narrowed the region of the promoter involved to 70 base pairs, including 40 base pairs of the 5' promoter and 30 base pairs of 5' non-coding sequence. To determine if the TATAA box was the element involved, additional *jun B* promoter/reporter gene constructs were made. Fragments of the *jun B* promoter region were fused to the reporter gene for chloramphenicol acetyl transferase (CAT). One of the constructs made coded for about 30 base pairs of the promoter region of *jun B* (pC6-Kpn/Xba Small) and the other one coded for approximately 500 base pairs (pC6-Kpn/Xba).

In hopes of determining differential transcription regulation levels, each *jun B* promoter plasmid was transfected into the three cells lines. Since the regulation of the CAT gene was under *jun B* control, it was hoped that expression levels of the plasmid would correspond to transformation status of the recipient cell (transformed versus untransformed phenotype). The expression levels of the promoters in each plasmid were

determined by CAT assays. There was a difference observed in the CAT activity of transformed 1302-4-1 cells versus normal Rat-1-6 or revertant EMS-1-19 cells. However, 1302-4-1 cells did not give higher activity levels than the normal and revertant cell lines, as expected. The latter hypothesis provided that levels of *jun B* promoter activity and thus CAT activity would be higher in transformed 1302 cells, since *jun B* was implicated as a *v-fos* effector gene involved in tumorigenesis. In actuality, for plasmid pC6-K/X Large, Rat-1-6 and EMS-1-19 cell lines showed a 2.2 fold and 2.3 fold higher activity than 1302-4-1 cells. In addition, for pC6-K/X Small, the highest levels of CAT were seen in the revertant EMS-1-19 and 1302-4-1. The CAT activities of both were 1.6 fold higher than the activity of EMS-1-19.

For each plasmid, the results differed from the expected result. For the larger plasmid, pC6-K/X Large, activity levels were expected to be similar to pC6-B/K which it most strongly resembles. pC6-B/K contains a 600 base pair fragment of the *jun B* promoter region which includes the start site of transcription (van Amsterdam thesis, 1993). The relative activity levels of pC6-B/K showed a 2.5 fold higher activity level for 1302-4-1 and a 1.5 fold activity level for Rat-1-6 as compared to EMS-1-19. In the case of pC6-K/X Large, Rat-1 and 1-19 are comparable with 1302 cells showing the lowest activity. This could be an indication that the transformed cell line responded to the new start site of transcription differently than the nontransformed cell lines. Another interesting observation was that the promoter showed even higher activity in the revertant cell line than the normal Rat-1, possibly because EMS-1-19 have overcome a deficient mechanism (because they are revertants of transformed cells) and do not need the transcription start site for one of many unknown reasons. There are three possible explanation for this difference between expected versus actual results. This includes experimental error, the possibility that the transcriptional start site in the constructs made is somehow different, and finally that the 30 base pairs of 5' non-coding sequence is actually responsible for lower *jun B* expression in revertants.

In experiments using the pC6-K/X Small plasmid, CAT assay results with the revertant and transformed cell lines had similar relative activity which was 1.6 fold above that of the normal Rat-1 cell line. However, since these results were not very different, and with the numerical values of the results being very small, a relative difference of 0.6 could be due to experimental error in CAT activity values or in the normalization process.

In the present experiments, polymerase chain reaction was used instead of  $\beta$ -galactosidase assays to normalize CAT activity, because of the high levels of endogenous  $\beta$ -galactosidase in rat fibroblast cells. The PCR technique requires standardization of the samples, which involves dilutions of a known amount of CAT DNA. It is very possible that in some cases, there was pipetting error in the standards or a faulty technique used in PhosphorImager quantitation, thus accounting for an overall error in normalized CAT activity. Regardless of where the uncertainty lays, the CAT activity levels did not lead to a conclusive result about *jun B* regulation. We therefore decided to test TATAA box activity and participation in differential *jun B* expression by using another approach, electrophoretic mobility shift assays (EMSA).

By using EMSA which take advantage of nondenaturing conditions to test for the presence of protein-DNA complexes, one can determine both qualitative and quantitative differences in complexes formed with the TATA box sequence of *jun B*. The results of EMSA showed that there was a protein or protein complex that bound to the promoter region of *jun B*, specifically, the TATA box region. This labeled TATA box region could be competed away with a cold excess of the same sequence in 100 and 1000-fold. However, by visualization in preliminary experiments, no differences in binding (intensity of autoradiograph band) could be seen among the three cell lines. In these experiments, there was no control for differences in activity (protein degradation) of lysates prepared from the different cell types.

When the same binding assays were repeated in another experiment, the results indicated that there was a quantitative difference in *jun B* probe binding among the three cell lines. The 1302-4-1 cell extract showed the most protein binding, followed by lower levels of Rat-1 and 1-19, which were similar to each other. TFIID consensus sequence in excess was seen to compete away labeled probe (Figure 11A, Lane 9), while the TFIID mutant sequence did not, showing a specificity for the *jun B* (or any) TATA box region (Figure 11A, Lane 8). In addition, protein binding assays were done with Sp1 and *oct 1* consensus sequences. While very little protein bound to Sp1 (Figure 11A, Lanes 10-15), hinting at the possibility of protein degradation in lysates from the revertant, bands of protein-DNA complex were seen with *oct 1* (Figure 11B, Lanes 1-8). EMS-1-19 extracts were seen to form a distinct band when *oct 1* was used as the probe, showing that revertant cell line extract retained binding capacity for *oct 1* (Figure 11B, Lane 5), thereby ruling out inactivation of the lysates as the cause for reduced TATAA binding activity. These results suggested the possibility that differential regulation of *jun B*, specifically, an increased level in transformed cells could in fact be mediated by a decrease in TFIID levels or binding activity.

It was interesting to observe that different incubation times yield differences in the EMSA. After 15 minutes of incubation, some protein complexes were formed. After 24 hours of binding and analysis on a different gel, these complexes disappear, showing that the complexes formed are very labile (Figure 12). Loss of the shifted band after 24 hours of binding could be due to either protein instability or complex instability.

In conclusion, it can be inferred from the CAT assay of transient transfection experiments that the *jun B* promoter region and hence the *jun B* gene, is differentially regulated in normal, transformed and revertant cell lines. However, the levels of *jun B* promoter activity with the two new plasmids yielded results that were different from what was expected, with the revertant cell line having the highest levels of expression and the transformed, the lowest for the pC6-K/X Large plasmid. The results of the

Small plasmid suggested the possibility that the regulatory region involved in differential regulation of *jun B* expression was within the 5' non-coding region. However, since the transcriptional start site was also changed in these constructs, alternative explanations cannot be excluded at this time.

The EMSA showed differential binding of proteins to the *jun B* TATA box region occurred in the three different cell lines, suggesting that promoter binding proteins are present in reduced quantities or have reduced activity in the revertants. The possibility that different proteins are involved in the different cell lines can also not be excluded by these experiments. The results of the EMSA experiments are nonetheless consistent with the hypothesis that the TATAA box in the *jun B* promoter is responsible for the differential expression of *jun B*. The possibility that the block in the protein kinase A signal transduction observed in the revertants is responsible for decreased TATAA binding activity remains to be determined.

### **Future Experiments**

The results presented here help to conclude that one of the mechanisms for transcriptional regulation is within the TATAA box sequence. Not very much sequence remains to be studied by deletion analysis (18 base pairs), but much more can be done to identify the protein(s) involved with the protein-DNA complexes that are being formed in the TATAA box region. In addition, antibodies to TFIID should also be able to bind to the DNA-protein complex and thus "supershift" the complex on a gel.

DNase footprinting experiments could be done on the promoter region to verify that indeed the complexes are being formed in the TATA box region. In addition, site-directed mutagenesis directed at the TATA box could be used to make a mutation in the TATA box sequence to see if any differences in CAT activity or EMSA binding occur.

Much more work could be done with the electrophoretic mobility shift assays. Assay binding time (i.e. stability of the DNA-protein complex) could be tested for, to

see how long the complex is present. Other parts of the *jun B* promoter region could be used to test for protein binding, specifically, to identify any other sequences putatively involved in gene regulation.

## Vendors

### **Boehringer-Mannheim**

1-800-262-1640  
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Indianapolis, IN 46250-0414

### **BTX Inc.**

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11199 Sorrento Valley Rd.  
San Diego, CA 92121-1334

### **Dupont/New England Nuclear (NEN)**

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Boston, MA 02118

### **Gibco BRL**

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Grand Island, NY 14072

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11011 North Torrey Pines Rd.

La Jolla, CA 92037



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