INITIAL CHARACTERIZATION OF THE Rho B PROMOTER

by

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Date Aug 6 1996
ABSTRACT

Quiescent cells can be stimulated to proliferate or differentiate by extracellular signals which initiate cascades of tyrosine and serine/threonine kinases. Signaling pathways terminate in the nucleus where they can cause activation or suppression of gene expression.

Immediate early genes are the initial genes induced at the level of transcription by mitogenic signals. The transcriptional induction of these genes occurs very rapidly after mitogenic stimulation and does not depend on de novo protein synthesis. This enhancement of transcription is transient producing mRNA species which are labile. In order to understand the signaling processes which lead to cellular proliferation, it is crucial to understand the mechanisms which control transcriptional induction of immediate early genes. Pathways which lead to the final event of transcription can be delineated by identifying and characterizing the factors which directly associate with the promoters of these genes and result in transcriptional induction.

*Rho B* (ras homologous B) is the first and only member of the ras family of GTPases which has been classified as an immediate early gene. Evidence suggests that transcriptional regulation of *Rho B* is unique in comparison with other immediate early genes; therefore, studying its transcriptional induction should lead to a better understanding of signaling pathways which trigger the proliferative cycle of the cell.

Here I show *Rho B* is an intronless gene with a TATA-less promoter containing a consensus initiator core element, -3' CGCAGTCC+5'. *Rho B* transcription initiates at multiple closely spaced nucleotides, CGCA, with the A nucleotide representing the major start site. I describe possible functions for putative protein binding sites in the 1 kb 5'-flanking region and propose that the cis-elements responsible for mitogenic stimulation of *Rho B* transcription are not located within the 3.2 kb 5'-flanking region relative to the start site.
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LIST OF ABBREVIATIONS

μCi  micro curie
μg  micro gram
μl  micro liter
ng  nano gram
A  adenine
AP-2  activator protein 2
bHLH  basic helix-loop-helix
bp  basepair(s)
C  cytosine
cAMP  cyclic AMP (adenosine mono phosphate)
CaPO₄  calcium phosphate
CAT  chloramphenicol acetyltransferase
cDNA  complementary DNA
CF1  common factor 1
cpm  counts per minute
CS  calf serum
CSF-1  colony stimulating factor 1
CTD  C-terminal domain
DEAE  Diethyl amino ethyl
DEPC  diethylpyrocarbonate
DMEM  Dulbecco’s modified Eagle’s medium
DTT  Dithiothreitol
E4F  Adenovirus E4 factor
EDTA  ethylenediaminetetraacetate
EGF  epidermal growth factor
EtBr  ethidium bromide
FCS  fetal calf serum
FSE2  fat specific element
g  gram
G  guanine
GAPDH  glyceraldehyde phosphodihydrogenase
GDP  guanosine 5'-diphosphate
GIF  general initiation factor
GTP  guanosine 5'-triphosphate
Inr  initiator element
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
</tr>
<tr>
<td>LBP-1</td>
<td>leader binding protein 1</td>
</tr>
<tr>
<td>LMP</td>
<td>low melting point</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
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<td>minutes</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>mt</td>
<td>mitochondria</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PEA-1</td>
<td>polyoma enhancer A 1</td>
</tr>
<tr>
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<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
</tr>
<tr>
<td>R</td>
<td>pyrimidine</td>
</tr>
<tr>
<td>Rho</td>
<td>ras homologous</td>
</tr>
<tr>
<td>SIE</td>
<td>sis inducible element</td>
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</table>
SRE  serum response element
SRF  serum response factor
T    thymine
TAF  TBP-associated factor
TBE  Tris Borate EDTA
TBP  TATA binding protein
TBS  Tris buffered saline
TE   Tris-EDTA
TLC  thin layer chromatography
TPA  12-O-tetradecanoylphorbol-13-acetate
ts   temperature sensitive
UV light  ultra violet light
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INTRODUCTION

1- TRANSCRIPTIONAL REGULATION IN EUKARYOTES.

In eukaryotic cells, an enormous number of protein-coding genes must be precisely and differentially regulated to allow for expression in specific cell types, during specific stages of development, or in response to specific extracellular signals. Expression of many genes has been shown to be regulated at the level of transcription. This is in part controlled by DNA binding proteins which are able to bind upstream or downstream of the transcription start site and control the rate of transcription initiation. RNA polymerase II, in conjunction with several initiation factors, is responsible for transcriptional initiation of protein coding genes in eukaryotes.

Initiation is preceded by assembly of RNA polymerase II and its general initiation factors at the core promoter sequences to form a preinitiation complex which is composed of at least 30 different polypeptides; the molecular mass of this complex has been estimated in excess of 2 million Daltons(22). Assembly of this preinitiation complex is triggered by the sequence specific binding of a multisubunit factor called TFIID. This complex factor has a strong DNA binding affinity for a consensus sequence, which in most class II genes is located 30 nucleotides upstream of the transcription start site. In some instances this sequence is TATAAA known as the TATA box. Some promoters do not have a typical TATA box, but rather, contain an element called the initiator element (Inr element) which spans the transcription initiation site. The Inr element has a core sequence of (R)\(_2\)\(\text{C}_{\text{A}}\text{T}_{\text{A}}\)\(\text{A}\)\(\text{R}\) with the A nucleotide corresponding to the transcription start site(56). In higher eukaryotes, native TFIID is a large multisubunit complex composed of a small TBP (TATA binding protein) and several tightly associated proteins commonly referred to as TBP-associated factors (TAFs)(17). In purified reconstituted systems, TBP can function in the absence of the TAFs to nucleate assembly of a complete functional preinitiation complex and promote specific basal transcription from TATA-containing core promoters (50). It has been shown that TAFs are the factors responsible for the effect of signal-regulated enhancer or repressor proteins which bind to sequences upstream or downstream of the core promoter.
sequence (17). However, in some cases it has been shown that activation domains of transcription factors can directly interact with TBP (109).

1.1- Formation of the preinitiation complex on TATA containing promoters.

The first step in formation of the preinitiation complex on TATA box-containing promoters is association of the TFIID complex at the TATA box sequence. This binding is enhanced by the association of TFIID with another general initiation factor (GIF) called TFIIA (11). TFIID's binding to the TATA-box facilitates binding of another GIF named TFIIB (11). Binding of TFIIB is one of the most important steps in the formation of the initiation complex since this factor also associates with the RNA polymerase II. RNA polymerase II is associated with TFIIF (69). Other GIFs recognize this multiprotein complex and associate with it, including TFIIE, TFIIH and TFIIJ. Association of these three GIFs results in formation of a functional preinitiation complex. TFIIH is believed to have kinase activity and is responsible for phosphorylating the C-terminal domain (CTD) of RNA polymerase II (see Figure 1) (4, 23).

Although it is clear that RNA polymerase II and initiation factors TFIIB, TFIIF and TFIIH can enter the preinitiation complex in a stepwise manner, there has been some recent evidence which indicates that most of the GIFs (TFIIB, TFIIF, TFIIH and RNA polymerase II) can assemble in the absence of DNA to reconstitute a RNA polymerase holoenzyme. This complex can bind TFIID at the core promoter sequences directly (62).

1.2- Formation of the preinitiation complex on TATA-less promoters.

Some genes transcribed by RNA polymerase II lack TATA-boxes yet retain the ability to initiate transcription from a single start site or from a few tightly clustered start sites. TFIID is implicated as having a central function in assembly of preinitiation complexes for this class of promoters as well (91). As mentioned before TFIID is comprised of TBP and several associated factors, the TAFs. Although the mechanism of TBP recruitment to TATA-less mRNA coding gene promoters is still unclear, several models have been proposed.
Figure 1. Formation of preinitiation complex on eukaryotic protein coding genes.

The figure depicts RNA polymerase II and the general initiation factors forming a functional preinitiation complex on promoters containing a TATA box along with initiation element, Inr. A, B, F, E, H and J correspond to TFIIA, TFIIB, TFIIF, TFIIE, TFIIH AND TFIIJ respectively.
It has been observed that TBP can bind to the -30 region of several promoters which lack a consensus TATA box. Therefore it has been proposed that although no consensus site for binding of this protein exists at -30 in these promoters, TBP can still bind with a low affinity to this region and hence trigger the assembly of polymerase II and other initiation factors (120). This model suggests that TBP can bind to the -30 region no matter what the sequence in that region might be. Others have suggested that binding of TFIID to the -30 region is influenced by interaction of upstream binding proteins such as Sp1 with TBP itself and/or with TAFs. This affinity of the upstream binding proteins with TFIID would cause the placement of TFIID at -30 region (91). It has also been reported that polymerase II itself has a low affinity binding activity for the initiator sites at the start site; therefore, polymerase II may bind to the start site first and then direct cooperative recruitment of TFIID and other basal initiation factors in the absence of a consensus TATA element (13). In line with the last model, some have suggested that certain proteins called initiators which have specific binding activity for initiator sequences of different promoters can bind to initiator elements at the start site and due to their interactions with TBP protein they can trigger the binding of TFIID to the -30 region (31, 74, 98, 104). It should be noted that all these studies were done using promoters which did not contain a TATA consensus sequence but did contain sequences with low affinity TBP binding activity and hence they can not be considered true TATA-less promoters.

All the above models have one common theme in that they all require TBP’s DNA binding activity for the process of preinitiation complex formation. Recent experiments with true TATA-less promoters convey a completely different role for TBP DNA binding activity and its role in the preinitiation complex formation. These studies suggest that TBP’s DNA binding activity has no role in the formation of complexes and that the ability of specific TAFs to bind initiator sequences either autonomously or in association with initiator binding proteins, nucleates the formation of preinitiation complex at TATA-less promoters (73, 123). TBP by itself can not promote specific initiator-dependent basal transcription from TATA-less promoters unless TFIID-specific TAFs are present. This direct or indirect binding of TAFs to the initiator sequence is in no way influenced by upstream
promoter binding proteins or the sequences at -30 of the promoter (73). In most TATA-less promoters the binding of TAFs to the initiator sequence, directly or indirectly, is only mediated by the initiator sequence. TAFII150 has been implicated in Inr binding, as this protein produces footprints downstream of the promoter start site, indicating that it does bind DNA (113, 114).

In some TATA-less promoters it has been shown that consensus sequences other than the initiator element are involved in correct positioning of the preinitiation complex. For instance, in Drosophila a number of TATA-less promoters have a conserved sequence 30 to 40 bp downstream of the start site which is involved in correct transcription start site selection. This sequence is called the downstream promoter element (DPE) and has the consensus sequence $A/GA/T$CGTG. It has been suggested that DPE acts in conjunction with the initiation site sequence to provide a binding site for TFIID in the absence of a TATA box to mediate initiation at TATA-less promoters (12).

**2- TRANSCRIPTIONAL ACTIVATION AND CELLULAR PROLIFERATION.**

Regulation of cellular growth and proliferation is a complex process involving intricate cellular signal transduction pathways which can be triggered as the result of extra-cellular mitogenic factors such as polypeptide growth factors, stress related signals, or deregulated expression of number of oncogenes. Extracellular growth factor ligands trigger the proliferative signals at the membrane of the cell and initiate a cascade of reactions which terminate in the nucleus of the cell, resulting in transcriptional activation of a subset of genes termed immediate early (48). Proliferative signals can also cause transcriptional repression of another subset of genes called growth arrest specific genes (gas genes). The gas genes are activated in response to growth-arrest, and repressed upon mitogenic stimulation and they are not expressed in continuously growing cells (19).
2.1- Immediate early genes.

For the past decade there has been a lot of interest in identifying the genes which are induced as the result of growth factors and oncogenes. The main approach has been differential or subtractive cDNA cloning employing cDNA libraries from quiescent and mitogen stimulated cells. The first such genes cloned were those induced by the platelet derived growth factor (PDGF) in NIH-3T3 fibroblasts. The genes cloned in that study displayed regulatory properties which are characteristic of immediate early genes described to date.

Transcription of immediate early genes is induced rapidly within one hour of growth factor treatment. This induction is always transient in nature but they do display a variability in the kinetics of their mRNA decay. For instance, \textit{c-fos} (encoding a DNA binding protein) transcription can be detected within 5 minutes post stimulation, but expression is typically shut off at the transcriptional level by 1 hour (55). The messenger RNA for \textit{c-fos} is unstable, and the persistence of the message is less than 2 hours. Contrast this with the JE gene (encoding an excreted cytokine) in fibroblasts, which is transcriptionally induced by 30 minutes and continues to be transcribed up to 6 hours after the induction (20). Absolute levels of induction can vary greatly; for example, the \textit{c-myc} gene (encoding a DNA binding protein) is induced only to a level of 30 to 50 copies per cell, whereas the \textit{c-fos} gene is transcribed transiently at 20 times that level (20, 60). For individuals of the same gene family, such as the \textit{c-fos}-related genes, the time courses can vary also. The time course of the \textit{fra} gene transcription appears to be slower and longer than those of \textit{fos}; this may have a physiological significance for the type of AP-1 complexes present in the cell (63).

All immediate early genes are induced by mitogenic signals in the absence of new protein synthesis. Therefore, the induction of these genes is a primary response to events at the cell surface and not secondary to other waves of gene expression or changes in growth state of the cell. The ability of early-response genes to be induced in the absence of new protein synthesis distinguishes this set of genes from other genes, which are called late-response genes. Expression of late-response genes is dependent on \textit{de novo} protein synthesis. Late or intermediate-response genes are probably regulated
<table>
<thead>
<tr>
<th>GENE</th>
<th>POSSIBLE FUNCTION KNOWN MOTIFS</th>
<th>SUB-CELLULAR LOCALIZATION</th>
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<tr>
<td>C-myc</td>
<td>Transcription factor Helix loop helix</td>
<td>nuclear</td>
</tr>
<tr>
<td>C-fos</td>
<td>Transcription factor Leucine zipper</td>
<td>nuclear</td>
</tr>
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</tr>
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<td>Steroid receptor superfamily</td>
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<td>β-actin</td>
<td>Cytoskeletal component</td>
<td>cytoplasmic</td>
</tr>
<tr>
<td>Rho-B</td>
<td>unknown Ras-like G protein</td>
<td>cytoplasmic</td>
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</table>

Table 1. Immediate early genes and their functions.

The table is a compilation of a few immediate early genes identified to date. Their functions, motifs associated with function (if known), and sub-cellular localization is indicated.
by transcription factors which are encoded by immediate-early genes. A
number of the immediate early genes are cellular homologs of various
retroviral oncogenes such as c-fos, c-jun and c-myc (48). Studies with anti-
sense RNA probes or antibodies to c-fos have shown that expression of this
gene is necessary in order for the cell to leave the resting state and enter the
cell cycle (51). Also, microinjection or overexpression of c-myc by itself can
potentiate or mimic the response of cells to growth factors (57). These results
show that expression of at least some immediate-early genes is necessary for
progression of the cell cycle.

A wide variety of immediate early genes has been identified from
studies of serum growth factor-stimulated fibroblasts. The total number of
such genes is close to 100 and is still growing. Proteins encoded by these genes
have a wide variety of functions and can be divided into 3 groups;
transcription factors or nuclear proteins, cytokines, and structural proteins
(Table 1).

2.1.1. Examples of immediate early genes.

The subset that has attracted most attention is that encoding
transcription factors, which can be divided into distinct categories. The best
studied are c-fos and c-jun which encode DNA-binding proteins. These two
proteins can form heterodimers via their leucine zipper domains. There are
several homologues of each of these genes identified so far; their encoded
proteins all can form heterodimers and in addition c-jun can form
homodimers with members of its own family. Therefore, the number of
possible dimeric complexes formed by this family of proteins is significantly
larger than the number of genes which actually code for them. All of these
heterodimers or homodimers can bind to the AP-1 consensus sequence
(TGACTCA) with different affinities characteristic of each complex.
Moreover, each complex of heterodimer or homodimer can have different
effects on transcription; in some cases the target gene can be negatively
regulated by the dimer. It has been shown also that secondary
posttranslational modification of these complexes, such as phosphorylation,
can influence their activity.
2.1.2. Regulation of c-fos transcription.

As mentioned before, c-fos is the best studied member of the immediate early genes. Its promoter responds to a wide variety of mitogenic signals, and most of the cis-elements responding to these signals have been mapped. Figure 2 depicts the promoter of this gene up to 400 nucleotides from the transcription start site and shows the regulatory cis-elements in this region. Upstream of the transcriptional start site is a TATA-box which is characteristic of most RNA polymerase II promoters.

**CRE site at nt -50 and YY1 element.**

At nt -50 with respect to the transcription start site there is a CRE (cAMP Responsive Element) consensus site, which has several functions. It has been shown that this element is involved in basal level expression of the gene and also is responsive to levels of cAMP in the cell, which indicate that the factors binding to this region can be under the regulation of protein kinase A (PKA) (36). This sequence can also mediate responses to intracellular calcium levels in PC12 cells. It has been shown that the DNA binding protein CREB, which is a cAMP responsive factor, can bind to this site as a dimer. A DNA binding protein named YY1 can bind to the region between the cyclic AMP responsive element (CRE) and the TATA box and repress promoter activity by introducing bends in the DNA that interfere with the productive interaction of proteins bound to the two flanking elements, CRE and TATA-box (81).

**Direct repeats at nt -100 (Rb-responsive element).**

Further upstream at nt -100 there is a direct repeat element which has also been shown to have different functions in the context of the c-fos promoter. There is evidence that this element may be involved in regulation of basal level transcription, and in addition, this sequence may act as a repressor element in cotransfections with retinoblastoma (Rb) expression plasmids (36, 96). It has also been suggested that the transcription factor E2F can bind to the retinoblastoma responsive element (16). E2F was originally characterized as a protein which bound upstream of adenoviruses early genes
**Figure 2.** Signaling pathways leading to stimulation of c-fos transcription.

The c-fos promoter from nt -400 to the transcription start site. The cis-elements and the factors associating with them are indicated. Extracellular signals which can stimulate transcription of c-fos are shown in boxes. The kinase pathway(s) responsive to each extracellular signal is also shown.
which are transactivated by the adenovirus transforming gene, E1A. This is interesting since it has been shown that E2F can form a complex with the retinoblastoma gene product and also that E2F has serum-stimulatable DNA binding activity and is involved in regulation of other immediate early genes such as \( c-myc \) (77). However, the responsive activity of E2F and retinoblastoma gene product have not been shown in the context of the \( c-fos \) promoter. It has also been shown that elements near this region are required for signaling events initiated by PDGF in combination with the SIE sequences at nt -340; this region will be discussed in more detail below.

**AP-1 site at nt -295.**

At nt -295 there is an AP-1 like sequence; it has been shown that this sequence can bind AP-1 and also other jun:fos dimers such as ATF-1, which is a heterodimer of c-jun and CREB (35). It has been shown that due to steric hindrance, factors can only bind to this site if the serum responsive element (SRE) site at -310 is empty. It was suspected that this site could be involved in autorepression of the transcription. However since it has been shown that Fos containing mutations in its DNA binding domain can still cause repression of \( c-fos \) transcription, repression must involve mechanisms other than autorepression (83).

**Dyad symmetry region at nt -310.**

This region centered around nt -310 contains dyad symmetry which has been termed the serum response element, (SRE) (110). It has been shown that this region by itself can confer serum responsiveness to heterologous promoters. The serum response factor (SRF) is a protein of 67 kd that binds to the core of the dyad symmetry element, called the CArG box, which is also found upstream of several other immediate early genes (110). SRF is constitutively phosphorylated and also has constitutive DNA binding activity (47). Phosphorylation of this factor does not affect its DNA binding activity. SRF has been shown to be an anchoring member of a ternary complex that binds to the SRE. This ternary complex includes p62\(^{TCF}\) (Ternary Complex Factor), which makes contacts with the 5' side of the SRE and has been shown to contact the SRF protein. These protein-protein contacts are essential to the
function of serum response element. Mutation of either protein in the regions involved in the interaction results in non-responsiveness of the SRE. p62\textsuperscript{TCF} is a member of the ets family of DNA binding proteins and binds to a region adjacent the CArG box at nt -320. In the absence of the p62\textsuperscript{TCF} binding site, SRE can only respond to stimulation by serum, lysophosphatidic acid (LPA) and Aluminum Fluoride ions (ALF\textsuperscript{-}) (49). LPA is found at high concentrations in serum and both LPA and ALF\textsuperscript{-} act through heterotrimeric G protein-coupled serpentine receptors (49). p62\textsuperscript{TCF} confers responsiveness to other stimuli such as CSF-1 (colony stimulating factor 1), PDGF (platelet derived growth factor) and EGF (epidermal growth factor). In order for SRF to respond to tyrosine kinases, p62\textsuperscript{TCF} has to be phosphorylated, bind DNA, and associate with SRF (39, 40, 72, 124). Therefore, it can be said that p62\textsuperscript{TCF} is the modulator of SRF activity in that p62\textsuperscript{TCF} is the part of the complex which is affected by the majority of cellular signaling pathways and confers those signals to SRF. SRE is also responsible for activating \textit{c-fos} transcription as the result of stress signals such, as UV (Ultra Violet) light, and interleukins.

\textbf{SIE sequence at nt -340.}

Upstream of the SRE at nt -340 is a sequence called the sis/PDGF-inducible element (SIE) (116). A transcription factor with inducible DNA binding activity binds to this element after treatment of fibroblast cells with EGF, PDGF, CSF-1 and interferons IFN-α and IFN-γ (24, 107). It has been shown that proteins which binds to the SIE are Stat proteins (signal transducers and activators of transcription) (106). These factors have been shown to be phosphorylated by Jak family tyrosine kinases in the cytoplasm of the cell and then translocate to the nucleus and directly activate target genes (105). The Jak proteins are activated as the result of tyrosine phosphorylation (106). Activation by the SIE is context dependent in that mitogens can activate STAT DNA binding activity and transcription of SIE-containing reporter genes, but not the \textit{c-fos} promoter. Activation via the SIE in the context of the \textit{c-fos} promoter requires additional signals and the complexes (SRF and p62\textsuperscript{TCF}) at the SRE; the spacing between the two sites is crucial for maximal promoter activation (116). This implies that Stat proteins bound at nt -340 and the SRF-p62\textsuperscript{TCF} complex at nt -310 to nt -320 somehow interact.
2.1.3- Regulation of *Egr-1* transcription.

*Egr-1* is another immediate early gene which encodes a DNA binding protein. Consensus binding sites for this protein exist in several immediate early genes, and in the *egr-1* promoter itself (103). *Egr-1* displays *c-fos*-like induction kinetics in several tissues such as epithelial, fibroblasts and lymphocytes (108). The *egr-1* promoter has 4 SRE/ets binding motifs, 2 CRE/ATF core motifs, an incomplete SRE sequence containing only the core CArG motif, and 2 AP-1 sites (6, 103). It has been shown that oncogenic tyrosine kinases induce *egr-1* transcription through the cluster of SRE/ets sequences (6). These 4 binding sites have been shown to have a synergistic effect in response to *v-fps* and *v-src* tyrosine kinases. Serum and TPA (12-0-tetradecanoylphorbol-13-acetate) also exert their effect through the 4 SRE/ets sites. The CArG box is not involved in binding of SRF or any other known DNA binding proteins (18). The CRE/ATF binding sites are involved in induction of transcription in response to CSF-1 and interleukins. Binding of CREB to the CRE/ATF sites is stimulated by both growth factors; however, these sites require the near by SRE/ets binding sites for growth factor responsiveness (103). The *egr-1* promoter also has a an egr-1 binding site (EBS). Binding of egr-1 to its own promoter has been detected and the protein's DNA binding activity is stimulated by CSF-1, although the importance of this binding activity has not been analyzed yet (103).

2.1.4- Regulation of *c-jun* transcription.

*C-jun* is also encoded by an immediate early gene. Kinetics of *c-jun* induction is different from that of *c-fos* and *egr-1*: relative to *c-fos*, *c-jun* mRNA is maintained at a high level during the G₀ state of the cell, and although the expression of mRNA reaches its maximum levels after 30 minutes of induction, the high level is maintained for 4 hours, indicating a longer half life for the mRNA of *c-jun* in contrast to that of *c-fos* (102). As stated previously, *c-jun* can function as a homodimer or a heterodimer with *c-fos* or, in some cases CREB. Analysis of the *c-jun* promoter by *in vivo* footprinting indicates that there are seven upstream protein binding sites. These include AP-1-like sequences, an NF-jun sequence, SP-1, MEF2 site and a site for an unknown protein (45, 100). The major difference between the
promoter of c-jun and c-fos and egr-1 is the fact that, although this promoter responds to serum and other peptide growth factors, it does not contain an SRE/ets or a CArG box. The MEF2 site is an important element for growth factors and serum induction. MEF-2 sites are bound by a family of transcription factors related to SRF in their DNA binding domain (46). This site is also referred to as the RSRE (related to serum response element). The AP-1 sequences mediate autoregulation of c-jun expression and are responsive to UV light. The Sp-1 site represses transcription; if this site is deleted, c-jun transcription is increased four fold. NF-jun is similar to NF-κB in that it can be translocated from the cytosol to the nucleus upon induction by an external stimulus. This factor is responsive to TPA and tumor necrosis factor alpha (TFNα) (100). All of these binding sites were shown to be occupied in serum-starved cells as well as stimulated cells (99). Therefore, expression of the c-jun gene is likely mediated by secondary modification events of the transactivation domains of promoter-bound transcriptional activators.

I have described in detail the cis-responsive elements in three well studied immediate early gene promoters, which encode nuclear transcription factors. As mentioned previously, the number of immediate early genes identified so far is enormous. For some a function has been determined, but for most immediate early genes, although the gene has been isolated and the promoter sequences are known, the level at which the gene product exerts its function is not known.

3- Rho B AS AN IMMEDIATE EARLY GENE.

The Rat Rho B cDNA clone was initially isolated by screening a plasmid cDNA library enriched for sequences that are more abundant in stimulated than in resting ts v-src-expressing LA23 NRK cells. The screening was done by differential hybridization using a 32P-labelled cDNA probe from resting cells that had been shifted to 34°C to activate the ts v-src protein tyrosine kinase (55). Rho B was one of 9 cDNA clones which were isolated in this study (54). The original Rho B cDNA clone is 2180 bp with one open reading frame of 580 bp coding for a protein of 196 amino acids. The 5' region contains 85% G and C nucleotides. The cDNA has two mRNA instability
sequences positioned 300 nucleotides apart within the long 3' untranslated region. These AU-rich sequences have been found in a number of immediate early genes, and have been shown to be required for mRNA degradation; in the case of Rho B mRNA these are so far only identified as putative instability sequences. This cDNA was used as a probe for further analysis of mitogenic induction of the Rho B transcription.

Rho B shows all of the characteristics of an immediate early gene. Transcription is induced as the result of mitogenic stimulation of resting cells. It reaches maximum levels after 30 minutes with a half life of 20 minutes and returns to basal expression after 1 to 2 hours. In Rat-2 fibroblasts the expression of Rho B is induced in response to growth factors: EGF, PDGF, and the oncogenic tyrosine kinases, v-fps and v-src. FCS-stimulation of quiescent cells also causes induction in Rho B transcription depending on how the cells were rendered quiescent (54). Cells rendered quiescent by serum starvation show a very low level of induction in Rho B transcription; however, in density arrested cells Rho B transcription is not observed. Stimulation of cells with either EGF or PDGF results in a higher level of Rho B transcripts compared to that observed with v-fps and v-src kinases (55). Initial observations indicate that Rho B transcription is regulated differently than other immediate early genes. For instance the level of c-fos RNA in resting cells is undetectable, and transcription of this gene is induced after 30 minutes with very low TPA concentrations; whereas expression of Rho B is easily detectable in resting cells and is more abundant in serum deprived than in density arrested cells (55). Furthermore, no induction of Rho B RNA occurs after 30 minute with very high concentrations of TPA. The only other immediate early gene in fibroblasts that is non-responsive to PKC activity is the KC gene (encoding a cytokine), although KC transcription is non-responsive to EGF stimulation.

Transcriptional induction of Rho B has also been studied in other cell lines. Mitogenic stimulation of Hela cells with EGF or PDGF causes a 3 to 4 fold increase in Rho B transcription; this agrees with the observations in fibroblast cells (122). However, one major difference exists between how Rho B RNA production is regulated in Hela cells relative to fibroblast cells. Treatment of Hela cells with very low levels of TPA caused an increase in
transcription of Rho B. The kinetics of this induction are slightly slower than those determined with growth factors. Rho B message reaches its highest levels after 60 minutes of treatment with TPA, whereas it can reach its highest levels after 30 minutes of treatment with EGF and PDGF (122). It should be noted that the half life of the mRNA is still less than 30 minutes.

Rho B gene expression has also been studied in PC12 cells, a neural crest-derived adrenal chromaffin cell line. In PC12 cells Rho B expression is induced as the result of both proliferating and differentiating signals. In addition to induction by EGF and PDGF PC12 cells, Rho B RNA production is also induced as the result of cells being treated with NGF (nerve growth factor); this factor is a differentiating factor which causes neurite extension from the cells. The kinetics of induction are the same with both differentiating and proliferating stimulatory factors(122). In addition, Rho B RNA expression is also induced by treatment of PC12 cells with insulin. It should be noted that treatment of fibroblast cells with insulin causes no changes in the level of Rho B. (55). In all three cell lines, Hela, PC12 and Rat-2, Rho B expression was unchanged upon activation of PKA (protein kinase A).

Induction of Rho B RNA in response to stress signals such as UV light and other DNA-damaging agents has also been studied. This study was conducted in the mouse fibroblast line NIH-3T3. Rho B gene transcription is induced 3 to 4 fold as the result of UV light treatment of cells and it reaches its maximum levels after 30 minutes, with a half life of 20 minutes (38). This induction is transient in nature since 2 hours after exposure to UV, Rho B mRNA returns to its basal level. This UV response is not dependent on the proliferative state of the cells since the level of Rho B mRNA increased after UV treatment of serum-starved or confluent cells. Kinase activities of both PKC and PKA are required for induction, and tyrosine kinase activity appears not to be involved in the UV-induction of transcription (38). This is in contrast to what has been observed with other immediate early genes induced by UV light. For instance, it has been shown that tyrosine kinase activities, mainly encoded mainly by v-srC, are involved in transcriptional regulation of c-jun in response to UV light (28). Therefore, it can be concluded that stress signaling pathways and the mitogenic signaling pathways which lead to
regulation of *Rho B* gene maybe different than that for other immediate early genes.

Regulation of Rho B protein expression has also been investigated. In both PC12 and Hela cells, the amount of Rho B protein increases as a result of EGF treatment (122). The protein expression reaches its maximum level after one hour. This is also observed in NGF-treated PC12 cells. The increase of the protein abundance is also transient, since it reaches basal levels after 4 hours of treatment, indicating that Rho B protein is unstable. Analysis of the Rho B protein sequence gives no indication as to what mechanism causes this instability. Moreover, Rho B protein accumulation is periodic in nature as the cell progresses through its proliferative cycle. Rho B protein is first detected at the G1/S phase transition; the level of Rho B is maximal during S phase and declines at the S/G2 transition (122). This is not specific to the first cell cycle after the cells are released from a G1/S block, since the levels of Rho B expression are the same in the subsequent cycles. This is an indication that whatever the specific function of this GTPase might be, this function may be important during transition of the cells from the G1 to S and/or during the S phase of the cell cycle. Cellular protein fractionation shows that Rho B, unlike Rho A, is associated with the membrane (122). The different C-terminal modifications on these two proteins is probably responsible for their different cellular localization (see below).

4- CHARACTERISTICS OF THE Rho B PROTEIN.

The Rho B protein is a member of the Ras superfamily of low molecular mass GTP-binding proteins. The common feature of all members of this family is their transition between a functionally active and inactive state depending on their association with GTP or GDP, respectively. These proteins have intrinsic GTPase activity which is under the influence of an associating factor GAP (GTPase activating protein) (64). The activity of GAP, in turn, is under the influence of intracellular and receptor tyrosine kinase pathways. The mammalian Ras superfamily is comprised of an estimated 50 proteins which share a number of structural and sequence homologies. Based on sequence comparison, this superfamily can be subdivided into three major subgroups, the Ras, Rab and Rho subfamilies, which appear to be specifically
involved in different areas of cell physiology (112). The Ras proteins control check points in signal transduction and cell proliferation, whereas the Rab proteins are involved in control of intracellular membrane traffic. The Rho subfamily contains rac1 and rac2, CDC42Hs, TC10s and the Rho1 subgroup, which has 4 members: RhoA, RhoB, RhoC and RhoG (44). All members of this latter family are expressed ubiquitously in cells except rac2 which is specific to cells of hemopoietic lineage. The members of the Rho subfamily share over 85% sequence identity at the level of amino acids. There is close to 30% identity with the products of the \textit{ras} proto oncogenes within their 4 "Ras boxes" which mediate binding and hydrolysis of the guanine nucleotide (112).

The domain which is thought to interact with effector molecules is fairly divergent from the rest of the Ras proteins but highly conserved within the Rho subfamily. The 3 Rho proteins (A, B and C) are unique among the members of the Rho subfamily in that they are substrates for C3 toxin. C3 toxin causes ADP ribosylation of asparagine 41 of all three rho proteins (A, B and C) which inhibits their activity (15, 101). This ribosylation occurs in the effector region of these GTPases; therefore, it is assumed that the ADP ribosyl group might interfere with their ability to interact with down-stream effectors. This specific ribosylation of 3 Rho proteins has been used effectively as an assay for the presence of Rho A, B and C.

Rho proteins share with the ras proteins a C-terminal sequence that is subject to several posttranslational modifications. These modifications affect their intracellular localization (112). The sequences of this region of Rho B differ from that of Rho A and C; this has been shown to influence the secondary modifications and hence its localization within the cell (1). It has been shown that Rho A and Rho C are cytosolic, whereas Rho B protein is associated with membranes, specifically with early endosomes and pre-lysosomal compartments in the cell (122). This points to the fact that the function of Rho B might differ considerably with Rho A and Rho C. In fact Rho A is known to have a central function in the formation of actin stress fibers and assembly of focal adhesion points (93). Rho B is thought to be involved in events during progression of the cell through its proliferative cycle; mainly during the G1 to S transition and during the S phase (122). No function for Rho C and Rho G has been determined.
Rho A and Rho C are expressed constitutively during the progression of the cells throughout the cell cycle, and mitogenic stimulation has no effect on their expression (54, 122). The Rho G gene has been classified as a late early gene since its transcription is induced as the result of several growth factors and mitogenic stimulation of resting cells; this transcriptional induction requires previous protein synthesis and occurs at late G1 of the cell cycle (115).

5- AIM OF THIS RESEARCH.

The aim of my research was to isolate the promoter sequences of the Rho B gene, characterize the core sequences, and study the transcriptional activity of the promoter in response to mitogenic stimulation. Furthermore, attempts were made to study the basal transcription of Rho B and the DNA binding factors involved.
MATERIALS AND METHODS

1- Cell lines and cell culture.

Cell lines and growth conditions:

Rat-2 fibroblasts and the Rat-2 clone CNA7, expressing a ts mutation of Fujinami sarcoma virus (v-fps) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO₂-95% air atmosphere at 37°C. NIH-3T3 cells were provided by Robert Kay (Terry Fox labs) and were grown under the same conditions as above except that 10% calf serum (CS) was used in place of FCS. To render the cells quiescent by serum starvation, cells were plated at 2 x 10⁴ cells per cm² in 0.2% FCS or CS for 48 hours; CNA7 cells were grown at 39°C, Rat-2 fibroblasts and NIH-3T3 cells were grown at 37°C.

Mitogenic stimulation:

EGF from Calbiochem was dissolved in non-supplemented DMEM at a concentration of 100μg/ml and was stored at -20°C in aliquots. For treatment of quiescent cells, EGF was added directly to the medium of cultured cells at the final concentration of 20ng/ml. Temperature shifts of CNA7 cells were performed by taking the cultured dishes from a 39°C incubator and moving them to 34°C for indicated times. Stimulation with CS was achieved by adding additional pre-warmed CS to the resting cells to bring the final concentration to 20% of the media volume.

2- Transfections and Chloramphenicol Tranferase Assays (CAT).

Transient transfections:

Cells were either transfected using the DEAE Dextran or calcium phosphate. Confluent dishes of cells were split 1 to 4 in the case of Rat-2 cells, and 1 to 5 for NIH-3T3 cells at least 16 hours before transfection. Calcium phosphate transfections were done as follows: DNA was added to 450 μl of autoclaved deionized water and 50 μl of 2.5 M CaCl₂, the tube was mixed by
inverting a few times. This solution was added in a dropwise manner to 500 μl of 2x HeBs (2x HEPES-buffered saline pH 7.4); this was done while mixing the tube containing the 2x HeBs very gently by using a pasteur pipette and making bubbles at the bottom of the tube. The mixture was vortexed for 5 seconds, set aside for 20 minutes and the 1 ml resulting DNA CaPO₄ precipitate was added to the plates. The plates were rocked gently and transferred to the incubator for 16 hours. Plates were washed twice with warm TBS (8g NaCl, 0.38g KCl, 1g glucose, 3g Trizma Base in 1 liter, pH 7.0) and full media was added to the cells. Transfected cells were rendered quiescent by removing the transfection media, washing the cells twice with TBS and maintaining them in DMEM containing 0.2% FCS or CS for 48 hours. For DEAE Dextran transfections, growth media was removed, cells were washed with TBS twice and plasmid DNA was introduced to the cells in 5 ml of transfection medium which contained 0.25 mg/ml of DEAE Dextran polymer in DMEM media. Cells were left in the transfection media for 8 hours then the media was removed, cells were washed and complete DMEM media (containing 10% FCS or CS) was added to the plates.

Stable transfection:

To produce stably transfected lines, confluent NIH-3T3 dishes were split 1 to 6 16 hours before the transfection. The dishes were cotransfected with 10 μg of the test plasmid and 1 μg of pSV-2neo, using calcium phosphate precipitation. 20 hours after addition of DNA/calcium phosphate, the cells were washed with warm TBS, and DMEM containing 10% CS was added. Cells were grown for 24 hours, after which they were trypsinized, split 1 to 3 and placed under selection with 400 μg/ml of G-418 (Geneticin from Gibco Laboratories). The selective media was changed every 4 days until resistant clones were observed. The dishes were trypsinized and the resistant cells were pooled; the number of colonies in each plate ranged from 5 to 20. The cells were grown in the selective media containing 200 μg/ml of G-418 for period of 2 weeks and then the amount of drug was decreased to 100 μg/ml. G-418 was made up in DMEM with no CS added.
Chloramphenicol Acetyl Transferase (CAT) assay:

After transfection, cells were washed with ice cold TBS once and scraped off the plates with a rubber policeman into 1 ml of ice cold suspension buffer containing 40 mM Tris-HCl pH 7.5, 1 mM EDTA and 150 mM NaCl. The cells were pelleted and taken up in 100 μl of 0.25 M Tris-HCl pH 8.0. The suspended cells were lysed by three cycles of freezing and thawing. Five μl of the extract was used to determine the protein concentration by a standard Bradford assay. Four μl of 0.25 M Tris-HCl pH 8.0 was added to 50 μl of the extract from cells, and the CAT assays were started by addition of 92 μl of a cocktail containing 70 μl of 1 M Tris-HCl pH 8.0, 2 μl of [14C]chloramphenicol (0.05 μCi/μl) and 20 μl of 4 mM Acetyl Co-A (Pharmacia). Reactions were incubated at 37°C. For extracts from transient transfections the duration of the assay was 90 mins and for extracts from stable transfections the duration was 30 mins. Reactions were stopped by addition of 1 ml of ethyl acetate and vortexing for 3 20 second periods. The tubes were spun for 5 minutes in a microfuge at top speed and 950 μl of the top layer was removed to a fresh tube. The samples were dried in a speed vac, redissolved in 15 μl of ethyl acetate and spotted onto Silica Gel TLC plates. The TLC plates were chromatographed in chloroform: ethyl acetate (75 ml: 25 ml). The plates were air dried in the fume hood and either exposed to Kodak XAR film or phosphor-imager screens. In the case of phospho imager screens the intensity of the acetylated and the non-acetylated spots were measured and the CAT activity was expressed as pmoles of acetylated [14C]chloramphenicol /min/mg protein. In the case of the TLC plates exposed to the Kodak XAR film the amount of protein extract used in the assay was normalized prior to start of the assay.

3- Southern blot analysis.

Phage DNA carrying Rho B genomic sequences was provided by D. Niedbala. The DNA was digested with restriction enzymes overnight in a 37°C incubator and electrophoresed on an 0.7% agarose gel for 2 hours at 80 Volts. The gel was stained with EtBr for 30 minutes and photographed. Gel transferred to nylon membrane using the TE 80 TRANSVAC vacuum blotting unit. After the transfer the nylon membrane was rinsed in 2 x SSC
(17.4g NaCl and 8.82g sodium citrate per liter, pH was adjusted to 7.0) and air dried. The membrane was pre-hybridized for 1 hour at 45°C in 18 mls of hybridization buffer (0.5% SDS, 5x Denhardt's Reagent, 6x SSC, 10% Dextran Sulphate and 250 µl of 1 mg/ml sheared denatured salmon sperm DNA. The 277 and 760 bp SacI fragments from the Rho B cDNA were gel purified on LMP agarose followed by chromatography on NACS columns. DNA (50 ng) was labelled using a random primer labelling kit from Pharmacia. Labelled DNA was dissolved up in 100 µl of deionized water and 25 µl was used for hybridizations, (minimum of 10⁶ cpm/ml). The membrane was hybridized over night, washed with 1x SSC for 30 mins at 25°C then with 0.1x SSC at 65°C for 1 hour and exposed at 25°C for an hour.

4- RNA isolation and analysis.

Total cellular RNA isolation:

Media was completely removed from a plate of adherent cells and 1 ml of Ultraspec RNA (BIOTECX laboratories) was added. Cells were scraped to one corner with a rubber policeman and were pipetted up and down with a P1000 tip to create a homogenate. The sample was removed to an ice cold eppendorf tube and incubated at 4°C for 5 mins. 200 µl of chloroform was added to the cell homogenate and the tube was shaken for 15 seconds, incubated in an ice bucket at 4°C for 5 mins and then spun at 4°C for 15 mins in a microfuge. The aqueous layer was removed and the RNA precipitated by addition of an equal volume of isopropanol and further incubation at 4°C. The precipitate was spun down at 4°C; after washing the RNA pellet with 70% ethanol it was air dried for 30 mins. The pellet was dissolved in DEPC-treated deionized water, the A₂₆₀ of a 500x dilution was measured and the RNA samples were stored at -70°C.

Northern blot analysis:

15 µg of total RNA was aliquoted into tubes and 3 volumes of denaturation buffer (500 µl formamide, 60 µl formaldehyde, 340 µl deionized water and 100 µl of 10x northern buffer ([200 mM Mops, 50 mM sodium acetate, 10 mM EDTA] in total volume of 1 ml) was added. The sample was
heated to 60°C for 5 mins and placed on ice. 6x concentrated northern loading buffer (500 µl glycerol, 100 µl of 10x northern buffer, 40 µl of saturated bromophenol and 360 µl deionized water) was added to the samples and they were loaded on a 1.0 % agarose/16% formaldehyde gel in TBE. The gel was run at 100 Volts for 3 hours. The nucleic acids were transferred for 36 hours by capillary action to nylon membrane in 20x SSC. RNA was cross-linked to the membrane by UV irradiation (120 mJ for 20 seconds). The membrane was pre-hybridized over night at 42°C; the hybridization solution contained 50% formamide, 5x SSC, 0.05 M sodium phosphate pH 6.4, 1x Denhardt's reagent, 50 µg/ml of sheared denatured salmon sperm DNA. The 760 bp SacI fragment was labeled using a random primer labeling kit from Pharmacia. The membrane was hybridized for 36 hours at 42°C, washed at 65°C twice with 2x SSC/0.1% SDS for 15 minutes, and twice with 0.5x SSC/0.1% SDS for 15 mins. The membrane was exposed to Kodak XAR film over night at -70°C.

Ribonuclease protection assay:

Total RNA was prepared as described above. Plasmid pGrb440 and pGrb213 (for description of plasmids refer to section 6) were used to make the antisense RNA probes. Both plasmids are pGEM-Zf7(+) based; the SacI fragment from -90 to +349 of Rho B gene was cloned in the Sac I site of pGEM-Zf7(+) plasmid, pGrb440 and pGrb213 was made by purifying the 213 bp HindIII/BamHI fragment from plasmid pOCrb8 and cloning it directionally into HindIII/BamHI sites of pGEM-Zf7(+). The antisense probe from pGrb440 was made by linearizing the plasmid with Hind III and the antisense RNA from pGrb213 was made by linearizing with EcoR I site; in both cases the SP6 RNA polymerase (GIBCO BRL) was utilized. The reaction for making the probes contained, 1 µg of linearized DNA (digested overnight), 2 µl of 10 mM DTT, 1 µl RNasin (Promega), 4 µl of nucleotide mix (2.5 mM ATP, TTP and GTP, excluding CTP), 4 µl of 5x SP6 buffer [0.2 M Tris-HCl(pH 7.9), 30 mM MgCl2, 10 mM spermidine-(HCl)3], and 2 µl [α32P]CTP (3000 Ci/mmol). The reaction was started by addition of 30 units of SP6 RNA polymerase and incubated at 37°C for one hour. The reaction was stopped by addition of 1 µl RNase free DNase I (Boehringer Mannheim) and incubated at 37°C for an additional 15 mins at which time an equal volume of loading buffer ( RPAII kit from Ambion) was added. The incubation mixture was heated to 90°C for
3 minutes and loaded on an 8M urea 6% acrylamide gel and electrophoresed at 300 volts for 1 hour. The probe was purified from the gel by excising the band using a razor blade, eluted over night by submerging the gel piece in 300 μl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS) and incubating at 37°C. 10 μg of RNA was hybridized to 2 x 10^5 cpm of probe (total volume of 20 μl) overnight in 43°C incubator. 200 μl of a RNase mixture containing 2.5 units/ml of RNase A and 100 units/ml of RNase T1 was added to the hybridized probe/RNA samples and incubated at 37°C for 30 minutes. 300 μl of RNase inactivation/precipitation mixture (solution Dx from the Ambion RPAII kit, patented) was added to the tubes and the RNA was precipitated by placing the samples at -20°C for 15 minutes. The RNA pellet was dissolved in 8 μl of gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA).

RNA was purified from plasmid DNA transfected cells was treated with DNase I as follows: RNA was dissolved in 300 μl of TE pH 7.4 and 0.2 μl of RNasin (Promega), 3 μl of 1 M MgCl2, and 1 μl DNase I (Boehringer Mannheim) was added to the sample. The sample was incubated at 37°C for 20 mins. SDS (6μl of 10% solution) and 6μl of 0.5 M EDTA were added and the sample was extracted with phenol/chloroform twice.

RNA standards were made by digesting pGEM-Zf5(+) with Pvu II, or with Apa I and pGEM-Zf7(+) with EcoRI or with Hind III and NaeI. Equal amounts of these digested plasmids were used to make RNA standards using SP6 RNA polymerase.

5- Dideoxy sequencing.

Dideoxy sequencing was performed on double stranded templates. The plasmid DNA was denatured as follows: 2 μl of 2M sodium hydroxide was added to 1 μg of plasmid DNA in total volume of 10 μl, the tubes were heated to 100°C for 2 mins and transferred to 4°C; 3 μl of 3 M sodium acetate (pH 4.6) was added and the volume was adjusted to 20 μl with water. The DNA was precipitated, washed with 70% ethanol/water, dried and dissolved in 7 μl of water. The annealing reaction was as follows: 0.5 pmole primer, 2 μl of 5x Sequenase reaction buffer [200 mM Tris-HCl (pH 7.5), 100 mM MgCl2, 250 mM
NaCl], was added to the denatured template and the volume was adjusted to 10 μl by deionized water. The solution was heated to 65°C for 2 mins, allowed to cool slowly to room temperature over a period of 30 minutes and transferred to ice. The labeling reaction was as follows: 1 μl DTT (0.1 M), 2 μl labeling nucleotide mix (1 : 5 dilution of 1.5 μM dGTP, dCTP and dTTP), 5 μCi of [a-32P]dATP and 3.25 units of Sequenase Version 2.0 enzyme was added to the annealed template/primer. The reaction was incubated at room temperature for 5 minutes. Termination reaction was as follows: 3.5 μl of the labeling reaction was transferred to 4 prewarmed (37°C) tubes each containing 2.5 μl of termination nucleotide mixtures (80 μM dGTP, 80 μM dATP, 80 μM dGTP, 80 μM dTTP, 80 μM dCTP and 50 mM NaCl; in addition, the "G" mixture contained 8 μM dideoxy-dGTP; the "A" mix, 8 μM ddATP; the "T" mix, 8 μM ddTTP and the "C" mix, 8 μM ddCTP). After 5 minutes of incubation at 42°C, 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) was added to each tube.

The pUC/M13 forward primer (5' GTTTTCCAGTGTCAG-3') was used to sequence the 5' region of all the Bal 31 deletion mutants. The deletion mutants were also sequenced in the reverse orientation by using 4 oligonucleotides located 200 base pairs apart. The position of these oligonucleotides in relation to the promoter region are as follows: seq1, (5'-CTGAGCTCATCGCTGCGGT-3') position -93 to -104 on the promoter; seq2, (5'-CCTTCGTAGCAGCCTCT-3') position -254 to -270 on the promoter; seq3, (5'-AGATCATGTTTTCACTTAAAC-3') position -440 to -460 on the promoter; seq4, (5'-TGAGTCCTCTCTTAGGGCTTTT-3') position -640 to -659 on the promoter.

Each of the sequencing gel was read at least 3 times in both orientations. Sequence data were compiled and analyzed by the Macintosh program DNA Strider.
6- Plasmid construction and Bal 31 deletions:

pGrb11.5 contains all of the *Rho* B genomic sequences and was made by purifying the 11.5 kb Sal I fragment isolated from the Dash vector and cloning it into the Sal I site of pGEM-Zf3(+).

pGrb5 contains the 5 kb EcoR I *Rho* B genomic fragment cloned in the EcoR I site of pGEM-Zf7(+), the 5 kb fragment has 1.8 kb of the cDNA sequence plus 3.2 kb 5' of the cDNA. The orientation of the subcloned fragment was such that the 3' cDNA sequences were close to the Sac I site of the vector. pGrb440 contains the Sac I fragment from -90 to +349 subcloned into pGEM-Zf7(+) vector. The orientation of the subcloned fragment is such that the non-coding DNA sequences are near the BstX I site in the vector.

pGrb3.2 contains the 3.2 kb fragment from -3.2 kb to +121. This fragment was the product of a PCR reaction using IS80 oligo and M13 forward primer with pGrb5 as the template. The purified PCR fragment was blunt-ended and cloned into the Sma I site of pGEM-Zf7(+).

pOCRb2+ was made by purifying the 3.2 kb EcoR I fragment from plasmid pGrb3.2, blunt-ending with the large fragment of DNA polymerase I (Klenow fragment) and subcloning it in the blunt-ended Xba I site of pOCn (constructed by I. Sadowski). pOCRb2+ has the largest promoter fragment cloned in front of the CAT coding sequence in the positive orientation. pOCRb2- is the same as pOCRb2+ but the subcloned promoter region is in the opposite orientation in relation to the CAT coding sequence.

For pOCRb5+; the 440 bp Sac I fragment from plasmid pGrb440 was made blunt with T4 DNA polymerase and cloned in the blunt-ended Xba I site of pOCn. pOCRb5- is the same as pOCRb5+, except that it has the 440 fragment in the opposite orientation in relation to the CAT coding sequence.

pOCRb19 was made by purifying the 3.0 kb EcoR I/Sac I fragment from pGrb3.2. The EcoR I end was blunt-ended with Klenow and the Sac I end was blunt-ended with T4 DNA polymerase. This blunt end fragment was cloned into the blunt-ended Xba I site of pOCn.
pOCrb7 was made as follows: first the 3.0 kb EcoR I/Sac I fragment from pGrb3.2 was subcloned into the EcoR I/Sac I site of pGEM-Zf3(+). The resulting plasmid was cut at the Sac I site and the 440 bp Sac I fragment purified from pGrb440 was inserted giving rise to the plasmid pGrb3.4. After the orientation of the subcloned 440 bp Sac I fragment was verified, pGrb3.4 was cut with EcoRI and BamH I. The 3.4 EcoR I/BamH I fragment was purified, made blunt with Klenow and subcloned into the blunt-ended Xba I site of pOCn.

pOCrb8 was made by cutting pGrb3.2 with Sac I and Kpn I. The resulting 213 bp fragment from -90 to +121 was made blunt with T4 DNA polymerase and cloned in the blunt-ended Xba I site of pOCn.

pGrb213 contains sequences from -90 to +121 of Rho B cloned into the EcoR I/BamH I site of pGEM-Zf7(+). The fragment was obtained by digesting pOCrb8 with Hind III and BamH I. The orientation was such that the -90 position of the Rho B gene was close to the Hind III site of the vector.

pOCrbS resulted from digesting pOCrb2+ with Sph I and then religating.

The PCR reaction to isolate the 3.2 kb genomic fragment containing the Rho B promoter was as follows: each PCR reaction contained 1 μg of BamH I cut pGrb5 (template), 10 μl of 10x NEB Vent DNA polymerase buffer, 12 μl of 4 mM dNTPs, 2 μl of 1mg/ml non-acetylated BSA, 2 μl of 2 mM magnesium sulfate and 4 μl of primers (IS80 and pUC/M13 forward primer both at 100 pmoles/μl). The volume was adjusted to 100 μl with deionized water and 2 μl of Vent(exo+) DNA polymerase (New England Biolabs) was added. PCR conditions were as follows: 96°C for 2 minutes, 15 cycles of 96°C for 2 minutes, 55°C for 1 minute, 72°C for 3.5 mins, and a final extension of 10 minutes at 72°C. The PCR product was purified by phenol/chloroform extraction and precipitation.

The sequence of the IS80 is 5'-GCGCTGCTTTGCGAGTC-3'.
Bal 31 Deletions:

Plasmids, pOCrb833-, pOCrb622-, pOCrb433-, pOCrb337-, pOCrb305-, pOCrb241-, pOCrb156- were made by performing Bal 31 deletions on the linearized pOCrbS plasmid. In order to make pOCrb833-, pOCrb622-, pOCrb433- and pOCrb337-, pOCrbS was linearized at the Sph I site and a time course digestion with Bal 31 was performed. Sph I digested DNA (46 µg), 14 µl of Bal 31 5x buffer (Promega), 9 µl of water and 1 µl of 3 fold diluted enzyme (Promega) were incubated at 30°C. Ten microliter aliquots were taken out at different time points (from 5 minutes to 40 minutes at 10 minute intervals) and added to a tube containing 1 µl of 0.5 M EGTA. The deleted fragments were gel purified and made blunt with Klenow. Hind III linker (New England Biolabs) was ligated to each blunt ended fragment. The products were extracted with phenol/chloroform, precipitated, and the dissolved pellets were digest with Hind III/Nco I. Nco I has a single cut site 400 bp inside the CAT coding sequence. pOCn plasmid was also digested with Hind III/Nco I, and the larger 4.2 kb fragment was gel purified. Deletions products were ligated to the 4.2 kb fragment from plasmid pOCn. The resulting plasmids were all sequenced with PUC/M13 forward primer and the deletion borders were determined. For the second set of deletions In vitro insertional mutagenesis with a selectable DNA fragment was used (90). The Spectinomycin resistant coding sequences were isolated from plasmid pH45Ω by digestion with Hind III. This Hind III fragment was cloned in the Hind III site 5' of the promoter region in plasmid pOCrb337-. The inserted fragment had a Sph I site near its 3' end, 100 bp 5' of the promoter sequences. Therefore, the resulting plasmid was linearized at Sph I and Bal 31 deletions were performed as described above with the exception that after the Hind III linker was ligated to the fragments, they were digested with Hind III and religated; this digestion removed the un-deleted portions of the Spectinomycin resistant sequences.
RESULTS

1- *Rho B* IS AN INTRONLESS GENE.

An 11.5 kb fragment containing the *Rho B* genomic sequence was cloned by D. Niedbala (this laboratory) from a rat liver genomic library. The library was constructed in the λ Dash vector such that the genomic DNA fragments were cloned in the BamHI site, flanked by EcoRI and SalI sites. To define the borders of the cDNA in relation to the isolated genomic fragment, Southern blot analysis of the cloned genomic fragment was performed.

Phage DNA containing the *Rho B* genomic fragment was digested with several restriction enzymes. The two Sac I fragments from the 5' end of the cloned cDNA were used as the probe; these two fragments were the two smallest fragments (277 bp and 760 bp) at the most 5' end of the cDNA (54). When the phage containing the *Rho B* DNA was digested with Sal I, it produced three fragments, (Figure 3 B lane 4). The 20 kb and the 9.1 kb fragments correspond to the phage arms. The 11.5 kb fragment was found to contain *Rho B* genomic sequences since it hybridized to the cDNA probes, (Figure 3 A lane 4). Lane 5 contains EcoR I digested phage DNA. Of the 7 fragments produced, the two largest fragments correspond to the phage vector. The other five are products of the cloned genomic sequences.

The 5 Kb EcoRI fragment is the only EcoRI fragment that hybridized to the cDNA probes in the blot (Figure 3 A lane 5). This 5 kb fragment was subcloned into the pGEM-7Zf(+) plasmid, (Figure 3 C), and the ends of this insert sequenced with both pUC/M13 forward and pUC/M13 reverse primers. The results showed that sequences previously determined for this cDNA are located proximal to the M13 reverse sequencing primer in plasmid pGrb5 (Figure 3, C). Comparison of the restriction fragments of the cDNA and the plasmid pGrb5, indicated that there are no introns in the 1.8 kb region of the cDNA contained within pGrb5, (data is not shown).

In order to manipulate the *Rho B* genomic sequences more easily, the 11.5 kb fragment containing the *Rho B* genomic sequences was sub-cloned into the Sal I site of the vector pGEM-3Zf(+). Restriction site analysis of this
Figure 3. Southern blot of the restriction enzyme digestion of the *RhoB* genomic fragment cloned into bacteriophage λ.

Phage DNA containing the *Rho B* genomic fragment was digested with Sal I and Sac I (lane 2), Sac I (lane 3), Sal I (lane 4) and EcoR I (lane 5); lane 1 is the cloned 5kb EcoR I fragment in pGEM-7Zf(+) plasmid (pGrb5). The samples were electrophoresed on a 0.7% agarose gel. Panel B is the EtBr stain of this gel; marker was λ phage DNA digested with HindIII. A is the Southern blot hybridized with the 761 bp and 277 bp Sac I fragments from the 5' end of the cDNA clone as shown in Panel C. Arrows in panel B indicate fragments which hybridize with the probes.
Figure 4. Restriction enzyme analysis of the 11.5 kb genomic fragment containing Rho B sequences.

The 11.5 kb Rho B genomic fragment cloned in pGEM-3Zf(+) and digested with several restriction enzymes. A, Restriction sites for enzymes: HincII, Smal, EcoRI, Ndel, HindIII, PstI, Sphi, SacI and BamHI (drawn to scale). The cDNA portion of the genomic fragment is shaded and expanded in B.
fragment is shown in Figure 4 A. Comparison of sites in this fragment with the cDNA again suggested that there are no introns in the coding sequence of this gene. Therefore, Rho B can be classified as an intronless gene.

The Rho B genomic sequence isolated here is not a pseudogene since the southern blot of EcoRI digested rat genomic DNA resulted in only one hybridized band; (Figure 5), indicating that there is only one copy of the Rho B gene present in the genome. Since the size of this fragment is the same as the one which was isolated earlier by screening the λ Dash vector, it can be concluded that they represent the same genomic sequences.

2- Rho B EXPRESSION IS INDUCED AS THE RESULT OF MITOGENIC STIMULATION.

The first step in further analysis of Rho B transcriptional induction was to make certain that expression of the gene was mitogen inducible in the cell lines which were being employed in the laboratory. Plates of Rat-2 fibroblasts were rendered quiescent by starvation (Figure 6, lane 3) and stimulated by EGF peptide treatment (Figure 6 lanes 4 to 6).

CNA7 cells were also utilized here. This modified cell line expresses a temperature sensitive mutation of the v-fps tyrosine kinase. This mutated oncogenic tyrosine kinase is enzymatically inactive at 39°C and can regain its activity by shifting the temperature to 34°C. Therefore, to render the cells quiescent plates of CNA7 cells were grown at 39°C in 0.2% FCS (lane 1) and then shifted to the permissive temperature for 60 minutes (lane 2). The blot was hybridized with the 760 bp SacI fragment from the cDNA. The blot was also hybridized with a GAPDH probe as an internal control in order to control for inconsistencies in loading of the RNA samples. Figure 6 shows that Rho B expression was induced in a rapid and transient manner as the result of EGF stimulation. Rho B RNA reached its highest level of expression after 30 minutes and declined to a basal level after 90 minutes, consistent with the data reported by D. Jahner (54).

The induction by v-fps tyrosine kinase is not as marked; nevertheless a higher level of expression is observed in the cells which were shifted to 34°C
Figure 5. Southern blot of rat genomic DNA.

Rat genomic DNA was digested with EcoRI or with HindIII and electrophoresed on a 0.7% agarose gel. The Southern blot was hybridized with the labeled 761 bp and 277 bp SacI fragments isolated from the 5' end of the cDNA clone as shown in figure 1 C. Marker was λ phage DNA digested with HindIII. This experiment was done by D. Niedbala.
**Figure 6.** Induction of *Rho B* expression by EGF and the \( \nu\text{-}fps \) oncoprotein.

Northern blot of total RNA from CNA7 cells, a Rat-2 clone carrying a \( ts \) mutant of the \( \nu\text{-}fps \) oncoprotein (lanes 1 and 2) and from Rat-2 cells (lanes 3 to 6). RNA was prepared from CNA7 cells which had been rendered quiescent by serum starvation at 39°C in 0.2% FCS for 48 hours (lane 1) and from quiescent cells which had undergone a temperature shift for 30' (lane 2). Total RNA was also prepared from Rat-2 cells which had been starved in 0.2% FCS for 48 hours (lane 3) and then stimulated with 20ng/ml of EGF for 30' (lane 4), 60' (lane 5) and 90' (lane 6). The blot was probed with both the 760 bp Sac I fragment from the 5' end of the *Rho B* cDNA and a GAPDH probe in order to control for inconsistencies in loading of the RNA.
as compared to the cells grown at the non-permissive temperature of 39°C. GAPDH mRNA is 2.2 kb and Rho B mRNA migrates at a size of 2.4 kb, consistent with previous reports (54). The initial Rho B cDNA clone had a size of 2.2 kb but it was suspected to be incomplete since the apparent size of the mRNA by northern blots was 2.4 kb (54). Moreover, the precise 5' and 3' end of the gene as well as the position of the poly(A) addition signal were not reported. Although I have been able to map the precise 5' end of the cDNA (Figure 7), the 3' end remains undefined.

3- DETERMINING THE TRANSCRIPTION START SITE.

As mentioned, the start site for transcription of RhoB mRNA has not previously been defined. Primer extension analysis of the mRNA was not a useful approach in this case since the 5' sequences of the cDNA are very G/C rich. The 200 bps at the 5' end of the cDNA have an 80 percent G/C content. Primer extension with two different oligonucleotides made to different regions of the cDNA was attempted but no extension product was observed. As a result, ribonuclease protection was performed to resolve this problem.

Two riboprobes were used for these experiments; one spanned the 440 bp Sac I fragment and the second spanned the 213 bp fragment as shown in Figure 7 B. Total cellular RNA was isolated from resting and EGF stimulated Rat-2 fibroblasts. RNA molecular markers were also made and electrophoresed along the protected RNA samples. Figure 7 lanes 1 to 4 show the 440 bp antisense probe is protected by the RNA from resting, 30', 60' and 90' EGF stimulated cells. It was difficult to measure the size of this protected fragment by comparison to the standards since the size of the fragment was large and the RNA standards were compressed together at higher molecular weights.

Lanes 5 to 8 show the same samples of RNA protecting the smaller 213 nt probe. In this case, RNA protected 121 nt of the 213 nucleotide antisense probe, Figure 7 lanes 5 to 8. There were also minor protected fragments of 122, 123 and 124 nucleotides. Examination of the sequence of the 213 nucleotide probe (Figure 7 C) indicates the 4 nucleotides CGCA correspond
Figure 7. Mapping of the \textit{Rho B} transcription start site.

Ribonuclease protection assay using total RNA prepared from either quiescent Rat-2 cells (lanes 1 and 5) or cells stimulated with EGF for 30' (lanes 2 and 6), 60' (lanes 3 and 7) and 90' (lanes 4 and 8) are shown. The fragments used to make the antisense probe are illustrated in B. These were obtained from plasmids pGrb440 and pGrb213 digested with Hind III and EcoR I, respectively. Sp6 RNA polymerase was used to make the antisense RNA probes. The RNA was either hybridized with the 440 nucleotide Sac I antisense riboprobe (lanes 1 to 4) or with the 213 nucleotide riboprobe (lanes 5 to 8). RNA molecular weight markers were electrophoresed on the gel (not shown). The size of the major protected fragment of the 213 antisense probe was determined to be 121 nt. Panel C shows the sequence of the 213 nt probe and the major start site is in bold. The major and 3 other adjacent minor start sites are underlined.
B

SacI
cDNA

213 bp
440 bp

C

GAGCTCAGCCCTGTGGCTTCCCATTTGGGT
GGCTATATTAAGAAAGTGGCCGGACTCTT
TAAATAGCGGCCGCTAGGGCCGCAGCCGC
TGCGCCCCTGTCACTAGGCTGGAGAAGAA
CCCGCCCCGGAAAGAAGCIAGGCCGGGTC
GCAAAATCAGCCATCGACTCGCACAAAGC
AGCGCACTCCGGGACAG

1
121
100
90
80
60

213
92
with the start site of transcription. Nucleotide A at position 92 of this fragment is the major start site.

4- 5' REGION OF THE CLONED RhoB GENE HAS PROMOTER ACTIVITY.

I set out to clone the promoter of Rho B gene from the isolated genomic fragment. This was done using PCR on the plasmid pGrb5. This plasmid carries the 5 kb genomic EcoRI fragment which contains 1.8 kb of the Rho B coding sequence plus 3.2 kb of 5' end noncoding sequence, (Figure 8 A). The PCR was done using the pUC/M13 forward primer and the IS80 oligonucleotide. IS80 oligonucleotide is a 20 mer which hybridizes in the antisense orientation to the most 5' region of the cDNA sequence (Figure 8A). The resulting PCR product, rb2, was cloned into the vector pGEM-7Zf(+) and the 3' end was sequenced using IS80 to check for fidelity of the fragment. Restriction sites were also compared between this clone and the pGrb5.

Subsequently this fragment was subcloned in both orientations into the plasmid pOCn. Plasmid pOCn is pUC118-based and had the CAT coding sequence plus the SV40 poly(A) signals inserted into its polycloning site. pOCRb2+ and pOCRb2- were the resulting plasmids; pOCRb2+ has the genomic fragment in the positive orientation and pOCRb2- in the negative orientation in relation to the CAT coding sequences (Figure 8 B). The 440 bp SacI fragment (Figure 8 A) was also subcloned upstream of the CAT gene in both orientations, generating pOCRb5+ and pOCRb5-.

These constructs (pOCRb2+, pOCRb2-, pOCRb5+ and pOCRb5-) were transfected into CNA7 cells grown at the non-permissive temperature of 39°C and CAT assays carried out on cellular extracts (Figure 9). The 3.2 kb fragment has promoter activity as shown by high level of acetylated chloramphenicol observed in pOCRb2+ extracts. Since this CAT activity is only seen in the pOCRb2+ transfected cells and not the pOCRb2-, the possibility that the signal is due to cryptic promoter activity in the vector is excluded. Therefore the 3.2 kb fragment in the plasmid pOCRb2+ must contain promoter sequences of the Rho B gene.
Figure 8. Cloning of the Rho B promoter from the plasmid pGrb5.

A shows the 5 kb EcoRI genomic fragment containing 1.8 kb of the cDNA sequence subcloned in pGEM-7Zf(+) vector. PCR was used to isolate 5' flanking DNA from this clone. The position of the oligos used in the PCR reaction, IS80 and M13 forward primer, are indicated. The 3.2 kb PCR fragment was purified, blunt-ended by treatment of DNA polymerase (Klenow fragment) and cloned in both orientations into the blunt ended XbaI site of the vector pOCn. B is the diagram of the resulting plasmid, pOCrb2+. The 440 bp SacI fragment was also blunt-ended and cloned in both orientations in the XbaI site of the plasmid pOCn.
Figure 9. The 5' region of the Rho B genomic clone has promoter activity.

Chromatogram of the CAT assays performed on extracts of CNA7 cells which were transfected with the promoter/CAT fusion constructs, pOCrb2+ (lanes 3 and 4), pOCrb2- (lanes 5 and 6), pOCrb5+ (lanes 9 and 10), pOCrb5- (lanes 7 and 8). CNA7 cells were grown and transfected at the non-permissive temperature of 39°C. Cells in even numbered lanes were shifted to the permissive temperature of 34°C for 4 hours before they were lysed while odd numbered lanes were maintained at 39°C. Lanes 1 and 2 show CAT assays from CNA7 cells which were transfected with RSV2CAT plasmid as a positive control for the transfection procedure.
Figure 10. Plasmids constructed to map promoter activity for the *Rho B* gene using the CAT reporter gene.

pOCrb2+ contains a 3.2 kb of the *Rho B* promoter fragment including a large region upstream of the *Rho B* transcription start site and some sequences which are transcribed and not translated. pOCrb5+ contains the 440 bp SacI fragment from -90 to +349, pOCrb8 contains the 213 bp PCR isolated fragment from -90 to +123, pOCrb19 has 3.0 kb of the promoter from -3.0 kb to -90 and pOCrb7 contains 3.4 kb of the *Rho B* gene from -3.4 kb to +349. All the fusions were made by cloning the promoter fragments in vector pOCn. cDNA portions of the fragments are shown by the shaded area.
Transcription start site
+1
Translation start

EcoRI
IS80
M13 reverse primer
M13 forward primer

3.2 Kb
-90 SacI
349 SacI
EcoRI

3.2 kb
SacI

CAT
pOCrb2+

440 bp
SacI
SacI

CAT
pOCrb5+

213 bp
SacI

CAT
pOCrb8

3.0 kb
SacI

CAT
pOCrb19

3.4 kb
SacI
SacI

CAT
pOCrb7
The same phenomenon is also observed for pOCrb5+ and pOCrb5-.
Furthermore, cells grown at the permissive temperature showed no increase in the level of Rho B expression as measured by CAT activity, a result which is inconsistent with the northern blot data of endogenous Rho B mRNA (Figure 6).

Plasmid pOCrb5+ which contains only 90 bp upstream of the transcription start site and 349 bp downstream of this site showed less promoter activity than the pOCrb2+ plasmid. To assess the importance of the 90 bp region, three additional plasmids were constructed. The resulting plasmids were pOCrb8, pOCrb19 and pOCrb7, respectively (Figure 10). pOCrb8 contains 90 bp upstream of the transcription start site and 123 bp downstream of this site. pOCrb19 contains 3.0 kb of the promoter sequence up to 90 bp upstream of the start site. pOCrb7 contains 3.4 kb of the promoter fragment up to 349 bp downstream of the transcription start site. Rat-2 fibroblasts were transiently transfected with the 5 plasmids, cell extracts prepared and CAT assays carried out (Figure 11). Plasmid pOCrb19 exhibits no detectable promoter activity (lanes 11 to 14), demonstrating the importance of the 213 bp region (nt -90 to 123) which was deleted in this plasmid. Although this was expected since the transcription start site is positioned within this fragment. Plasmid pOCrb8 (lanes 9 and 10) has the same promoter activity as pOCrb5+ (lanes 4 to 6). Also pOCrb7 (lanes 7 and 8) has the same activity as pOCrb2+ (lanes 1 to 3). These results indicate that the 227 bp cDNA region which is present in plasmids pOCrb5+ and pOCrb7 but absent in pOCrb8 and pOCrb2+ does not influence the transcriptional activity. Therefore, the 227 bp 5'-flanking sequences can be considered as the minimal promoter of Rho B. I also stimulated the transfected cells with EGF for 3 hours and again, as was observed with the temperature shift data in transfected CNA7 cells (Figure 6), stimulation of the transcriptional activity was not observed (Figure 11, EGF stimulated lanes).

5- TIME COURSE STUDY OF THE MITOGENIC STIMULATION.

Figures 9 and 11 show that the genomic fragment which was isolated by PCR contains promoter sequences for the Rho B gene. The CAT assays also
**Figure 11.** Presence of the 90 bp genomic region immediately 5' of the cDNA is essential for Rho B transcription.

This shows a chromatogram of CAT assays performed on transiently transfected Rat-2 fibroblasts. The cells were transfected with plasmids pOCrb2+ (lanes 1 to 3), pOCrb5+ (lanes 4 to 6), pOCrb7 (lanes 7 and 8), pOCrb8 (lanes 9 and 10) and pOCrb19 (lanes 11 to 14). Diagram of these constructs are shown in Figure 10. Samples from cells which were stimulated with EGF prior to harvesting are indicated on the figure.
suggest that transcription of the transfected clones is not induced as the result of EGF administration or \( v-fps \) tyrosine kinase activity (Figure 6). These data are inconsistent with previous results (54) and also with the data reported above including the northern blot (Figure 6) and ribonuclease protection assays both show induction of Rho B expression as the result of mitogenic stimulation.

Quiescent cells, cells at the G\(_0\) stage of the cell cycle, exhibit the lowest expression of Rho B mRNA and this expression is stimulated as the result of cells entering the cell cycle. The transition of the cells from the resting state to the growing state is stimulated by administering mitogenic factors such as the growth factor EGF to the cells. In a non-synchronously growing population of cells, only a small percentage of cells are at the Go stage. As a result, it would not be possible to observe induction of the Rho B gene. Therefore, I tried starving the cells after the transfection in order to synchronize the cells at the Go stage. This was not feasible since the transfection method which I was using killed the majority of the cells leaving few viable cells. Therefore I tried transfecting the cells using calcium phosphate precipitates as the carrier for plasmid DNA instead of DEAE dextran. Calcium phosphate is much gentler on the cells, hence I was able to grow the cells in 0.2% FCS for 48 hours after the transfection and still have viable cells arrested at Go for the experiments. Figure 12 illustrates CAT assays for Rat-2 cells which were transfected by calcium phosphate precipitates. Using this procedure cells transfected with pOCrb2+ plasmid (Figure 12A) do not show induction of the Rho B expression when treated with EGF. Similar results were obtained when cells were transfected with pOCrb5+ (Figure 12 B).

The same experiment was also performed using CNA7 cells (Figure 13 A and B). In the case of CNA7 cells, the cells were starved in 0.2% FCS at 39°C after calcium phosphate mediated transfection and then stimulated either by shifting them to the permissive temperature (Figure 13, lanes 2 to 4) or by addition of EGF to the media for 2 hours (lane 6). Again no stimulation of Rho B expression was observed by either the temperature shift or EGF stimulation.
Figure 12. Transient calcium phosphate mediated transfection of *Rho B* promoter plasmids show lack of stimulation by EGF.

Chromatogram of Rat-2 cells transfected with pOCrb2+ (A) and pOCrb5+ (B) plasmids using calcium phosphate as the DNA carrier. Cells transfected with both constructs were rendered quiescent by starvation for 48 hours in 0.2% FCS (A, lane 1 and B lane 1), stimulated with 20 ng/ml of EGF for 30' (lane 2 in A and B), 60' (lane 3 in A and B), 2 hours (lane 5 in A and B) and 3 hours (lane 4 in A and B).
Figure 13. Transiently transfected Rho B promoter constructs are not induced as the result of v-fps activity.

CAT assay of CNA7 cells transfected with pOCrb2+ (A) and pOCrb5+ (B) plasmids using calcium phosphate as the DNA carrier. Transfected cells were arrested at G_0 by growing them at 39°C and 0.2% FCS for 48 hours after transfection (lane 1 in A and B). The cells were shifted to 34°C for periods of 30' (lane 2 in A and B), 60' (lane 3 in A and B), 2 hours (lane 4 in A and B), 3 hours (lane 5 in A and B) or 20 ng/ml EGF was added to the media at 39°C for 2 hours (lane 6 in A and B).
6- TRANSFECTING THE CELLS DOES NOT AFFECT THE TRANSCRIPTIONAL ACTIVATION OF THE ENDOGENOUS Rho B GENE.

The possibility exists that transfection of the cells alone could have caused an increase in the basal level of Rho B transcription hence masking induction by temperature shift or EGF addition. This was a strong possibility since the basal level of Rho B expression as measured by CAT activity in resting transfected cells is higher than that of endogenous gene transcription seen in the Northern blot and ribonuclease protection assays. As can be seen in Figure 6 and 7, transcription of the endogenous Rho B in resting cells is very low whereas the level of expression of the CAT gene as driven by Rho B promoter sequences in the starved/transfected cells is high. Therefore to rule out the possibility that the transfection procedure itself could somehow activate the factors responsible for the transcriptional induction of Rho B, I performed ribonuclease protection assays on total RNA isolated from Rat-2 cells which were transfected with plasmids pGEM-Zf7(+) (Figure 14, lanes 1 and 2) or a plasmid which carries 340 nucleotides of the c-fos promoter fused to the CAT gene (lanes 3 and 4). The Rat-2 cells were transfected using calcium phosphate precipitates, starved for 48 hours (lanes 1 and 3) and then stimulated with EGF for 30 mins (lanes 2 and 4). The level endogenous Rho B message is low (Figure 14 lanes 1 and 3) and comparable to the level from the non-transfected cells (Figure 7, lanes 1 and 5). Also the endogenous gene is clearly inducible by EGF after transfection and starvation (Figure 14 lanes 2 and 4). This experiment establishes that the procedure itself does not influence the expression of endogenous Rho B gene.

7- CHROMOSOMAL INTEGRATION AND TRANSCRIPTION.

Because I observed differences between the regulation of the endogenous Rho B promoter and the transfected Rho B promoter constructs, I examined the possibility that in the endogenous promoter the chromatin structure plays a repressive role which is alleviated as the result of mitogenic stimulation. To address this question I made stably transfected cells. NIH-3T3 cells were used for this purpose and three stably transfected cell lines were isolated. The plasmids used were as follows: pOCrb2+, which carries the largest promoter fragment fused to the CAT gene; pOCrb8, which carries the smallest promoter fragment fused to
Figure 14. Influence of the transfection procedure on inducibility of the endogenous Rho B gene.

Total cellular RNA was isolated from transfected Rat-2 cells and used to protect the 213 nucleotide antisense RNA probe (refer to Figure 4, B). Cells transfected with plasmid pGEM-Zf7(+) were rendered quiescent by starvation in 0.2% FCS for 48 hours after transfection and the stimulated with EGF for 30'. Total cellular RNA was isolated and used to protect the 213 antisense RNA probe (lanes 1 and 2 respectively). Cells transfected with the plasmid containing the c-fos promoter region were rendered quiescent the same way and also stimulated with EGF for 30', total cellular RNA was purified and protected with the 213 antisense RNA probe, (lanes 3 and 4 respectively). Lanes 5 to 8 are the same RNA samples protected with mouse actin antisense probe in order to check for consistencies during loading.
Figure 15. A 1.0 kb genomic region shows full promoter activity.

A) Chromatogram of the CAT assay performed on Rat-2 cell extracts which were transfected with pOCrb2+ (lanes 1 and 2) and pOCrbS (lanes 3 and 4). B) Promoter region in plasmid pOCrbS in relation to the plasmid containing the largest promoter region, pOCrb2+, and the smallest promoter region, pOCrb8.
A

B

3.2 kb

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1.0 kb

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213 bp

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Figure 16. Effect of chromosomal integration of Rho B promoter/CAT chimeras on promoter activity.

Stable transfectant cells using plasmids pOCRb2+, pOCRb5+ and pOCRbS were made. The experiment shown was done using pools of G418 resistant colonies. The graph depicts the level of CAT expression in the quiescent stable transfected cells, starved cells stimulated with EGF for 30', 60' and 90' or starved cells stimulated with with calf serum (CS) for 60'. The results were quantitated using a phosphorimager.
to the CAT gene; pOCrb8, which carries the smallest promoter fragment fused to the CAT gene; and pOCrbS, which contains 1 kb of the promoter fused to the CAT gene (Figure 15 B).

pOCrbS exhibits the same level of CAT activity as pOCrb2+ when transiently transfected into fibroblast cells, (Figure 15A, lanes 3 and 4 versus 1 and 2).

The level of CAT activity in the stable transfectants is shown in Figure 16. In this experiment cells were starved in 0.2% CS for 48 hours and stimulated with EGF for 30', 60' or 90' or with Calf Serum for 60'. It is evident that stimulation of \textit{Rho B} transcription as measured by the level of CAT activity was not observed in these stable transformants. The level of basal transcription of the gene is also similar to what was observed with transiently transfected cells. In both transiently and stably transfected cells the level of CAT activity in pOCrb2+ transfected cells is the same as pOCrbS transfected cells while the CAT level in pOCrb8+ transfected cells is considerably less than both other cell lines.

8- ANALYSIS OF THE CONSTITUTIVE PROMOTER ACTIVITY IN TRANSIENT TRANSFECTIONS.

Plasmid pOCrbS contains 932 bps 5' of the transcriptional start site and exhibits full promoter activity in that the level of CAT expression of this plasmid is comparable to that of the plasmid containing the largest promoter sequence, pOCrb2+, in both transient and stable transfectants (Figures 15 and 16). This 932 bp region can be defined as a region that appears to be constitutive in function in that its activity was not altered during mitogenic stimulation of the cells. Therefore, to elucidate the functional elements responsible for the constitutive expression of \textit{Rho B} promoter, nested 5' deletions of the pOCrbS plasmid were made. The deletions were made using Bal 31 and were constructed such that they all had a common 3' end and variable 5' ends (Figure 17). All fragments were cloned in front of the CAT coding sequence in the pOCn plasmid, hence it was possible to sequence all the 5' ends with the same pUC/M13 forward primer. The 1kb region was also sequenced in the opposite orientation using 4 oligonucleotides corresponding to sequences approximately 200 bps apart. The deleted clones were transiently transfected into Rat-2 fibroblasts and NIH-3T3 cells and the
Figure 17. Deleted promoter/CAT fusion constructs formed by Bal 31 deletions.

The drawings depict the nested deletions made from plasmid pOCrbS. All deletion mutants were cloned in the pOCn vector. The 5' border of each deletion is indicated. See Materials and Methods for explanation on how these mutants were constructed.
promoter activities were measured by performing CAT assays (Figure 18 A and B). It should be noted that as expected EGF treatment of the transiently transfected cells did not result in stimulation of the CAT expression for any of the constructs. Data for these induced samples is not shown.

Figure 18 A shows the CAT activity of NIH-3T3 cells transfected with the deletion mutants. Significant fluctuations in the level of promoter activity were observed as the first 600 bp of the promoter was deleted. The data for pOCrb 833- suggest that the region -932 to -855 contains a suppressive element(s) as the promoter activity is increased by at least 2 fold when this 77 bp is deleted. The next mutant clone, pOCrb622-, has another 233 bp deleted from the 5' end; it exhibits less activity than pOCrb855- but the same amount as the original plasmid, pOCrbS. It is difficult to postulate a mechanism for the increase in promoter activity and then its decrease, but it can be assumed that the first 77 bp of the promoter sequence was masking an enhancer sequence(s) which was present in the 233 bp deleted region. Interestingly, the same phenomenon is also observed for the next 3 deleted clones; an increase in the transcription of the CAT gene is observed as the sequences between -622 to -433 are deleted and then a decrease from -433 to -337. The same explanation as above could prevail in this case as well. There is also a substantial increase in the activity as another further 32 nucleotides are deleted, (region -337 to -305). Further deletions of the pOCrb305- to pOCrb241- shows a decrease in the activity. At this point a steady pattern of decrease in the promoter activity is observed as more sequences are deleted, indicating the presence of positive cis-elements in the region between -305 to -92. Plasmid pOCrb92- contains the minimal promoter elements in that the transcription start site is in this region. Moreover, deletion of this region abolishes promoter activity (Figure 18).

Figure 18, B shows the CAT activity from the Rat-2 fibroblasts transfected with the deleted mutants. The pattern of increase and decrease of promoter activity is very similar to that observed with NIH-3T3 cells (Figure 18A); with one exception. As was the case with the NIH-3T3 cells, deletion of the first 77 bps caused an increase in the CAT expression, but the decrease which was observed as the region from -855 to -662 was removed is not observed in Rat-2 cells. It is possible that the enhancer element(s) present in
Figure 18. Constitutive activity of the Rho B promoter.

A. The CAT activity in extracts of NIH-3T3 cells which were transiently transfected with deletion mutants of the Rho B promoter. Calcium phosphate was used as a DNA carrier in these transfections. Cells were transfected with the plasmid DNA and were rendered quiescent by starvation for 48 hours in 0.2% calf serum (CS). B. The CAT activity in Rat-2 cells extracts which were transiently transfected with the deletion mutants. CAT activity is measured as pmoles of non-acetylated $^{14}$C chloramphenicol converted to an acetylated form per minute per mg of extract protein used. Data represented in the bars are the mean of three independent samples.
the 233 bp region are cell line specific as deletion of this region in NIH-3T3 cells caused a decrease in the promoter activity whereas this was not observed for Rat-2 cells. Hence, control of the constitutive expression in not conserved. Deletion of the next 189 bp resulted in an increase in the CAT activity, this was also observed in the case of NIH3T3 cells but not to the same degree. Removing of the next 96 bp from -433 to -337 caused a large decrease in promoter activity, and a further 32 nucleotide deletion resulted in the further decrease in the activity, pOCrb337- compared to pOCrb305-. The pattern of change in the CAT activity undergoes a steady decrease as more nucleotides are removed, reaching the basal level with pOCrb92-. A summary of the relative promoter activity in NIH-3T3 and Rat-2 cells is shown in Figure 19. The activity of the minimal promoter region is normalized to 1.0.

It should be noted that the differences in the level of expression of CAT protein is not due to the variability in the transfection efficiencies since co-transfections were done with the deletion mutants using pGK-βgal. β-galactosidase assays were performed on the extracts and there was no substantial variability observed in the transfection efficiencies (data not shown).

Figure 20 shows the sequence of the 1kb promoter region in pOCrbS. Putative protein binding sites identified using the Dnasis program are boxed. Table 2 summarizes the location of these putative sites and the effect of their deletion on the constitutive promoter activity in both Rat-2 fibroblasts and NIH-3T3 cells.
Figure 19. Comparison of the Rho B promoter activity in NIH-3T3 and Rat-2 cells.

The 9 deletion mutant plasmids shown in Figure 17 were transfected into RAT-2 cells (black bars) and NIH-3T3 cells (grey bars) and CAT assays were performed after rendering the cells quiescent by starvation in 0.2% FCS and 0.2% CS respectively. Promoter activity is expressed relative to the smallest fusion clone, pOGrb92-.
Figure 20. Rat Rho B promoter sequences and putative protein binding sites.

A total of 932 base pairs 5' of the start site was sequenced in both orientations. The putative protein binding sites are encased in boxes and the start site is underlined with the major start site in bold (+1). The shaded region indicates the TATA-like sequence and Fp stands for footprint; this site was found by footprint analysis. Arrows indicate where the deletion mutants from Figure 17 would be positioned and the name of the plasmids are to the right of the Figure. Putative binding sites are the result of analyzing the sequence with Mac Dnasis program. Indicates the TATA like sequence. The dotted box shows the protected region found by footprinting, data not shown.
<table>
<thead>
<tr>
<th>DELETED REGION</th>
<th>PUTATIVE BINDING SITE</th>
<th>EFFECT OF DELETION IN NIH-3T3 CELLS</th>
<th>EFFECT OF DELETION IN RAT-2 CELLS</th>
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<tr>
<td>-932 to -833</td>
<td>1- bHLH</td>
<td>increase</td>
<td>increase</td>
</tr>
<tr>
<td>-833 to -622</td>
<td>1- CArG Box</td>
<td>decrease</td>
<td>no effect</td>
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<td></td>
<td>2- LBP-1</td>
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<td></td>
<td>3- CF1 (YY1)</td>
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<td></td>
<td>4- E2A</td>
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<td></td>
<td>5- FSE2</td>
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<td></td>
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<tr>
<td>-622 to -433</td>
<td>1- PEA-1</td>
<td>increase</td>
<td>increase</td>
</tr>
<tr>
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Table 2. Putative protein binding sites in Rho B promoter and the effect of their deletion on the promoter activity.

Putative protein binding sites in each deleted region are summarized. The effect that the deletion of each region has on the total constitutive promoter activity in both RAT-2 and NIH-3T3 cells is also summarized. Conclusions as to the summarized effects were made based on the CAT activity results from Figure 18 A and B. Refer to list of Abbreviations for the full name of binding sites.
DISCUSSION

1- THE Rho B IS AN INTRONLESS GENE AS DETECTED BY RESTRICTION ENZYME DIGESTIONS.

An 11.5 kb genomic fragment was identified in a rat genomic library based on its hybridization with the 5' sequences of the Rho B cDNA. There appears to be only one copy in the genome since Southern blot analysis of the EcoR I and Hind III digested genomic DNA shows a single band which hybridizes to the cDNA sequences. Moreover; both the Southern blot of the EcoR I digested genome and the Southern blot of the EcoR I digested cloned genomic fragment produce a 5 kb hybridized band. This indicates that the correct genomic sequences were isolated.

Restriction enzyme digest analysis of the cloned genomic DNA and its comparison with that of the cDNA sequence indicates that the Rho B gene is an intronless gene. Rho B is a small GTPase member of the ras family of GTPases (112). The ras family of GTPases has been subdivided into three subfamilies; Ras, Rho and Rab. Gene structure of a number of Ras subfamilies of GTPases have been determined and all of these genes contain introns (14, 32, 66, 68). Furthermore, gene structure of a few members of the Rab sub-family have been determined and again these all contain conserved intron and exon positions (8, 29). To date, none of the genes of the Rho sub-family has been isolated, hence this is the first report of a gene from this family being cloned. Therefore, it is unknown whether this evolutionary loss of introns in the ras GTPases family is conserved in the Rho sub family or if it only applies to Rho B. Since the 3 Rho proteins, Rho A, B and C, have over 85% sequence homology at the level of protein sequence and close to 90% at the nucleotide sequence, it is quite conceivable that this evolutionary trait of Rho B is also present in the other members of this subfamily.

This loss of introns during evolution could have a risen from separate intron deletion events or from reverse transcription of a processed cDNA intermediate that has been integrated into the genome. The latter process gives rise to intronless pseudogenes. The loss of introns from the Rho B gene by this process would be likely if the pseudogene has retained its functional
capability. In this way it could be assumed that maybe the pseudogene was made by reverse transcription event of either Rho A or C mRNA and its incorporation into a locus downstream of the Rho B regulatory elements. This hypothesis does seem likely since the expression of Rho B gene is under a different set of regulatory elements than that of Rho A or C genes. An alternative mechanism could be responsible for this loss of intron and that is whatever number of introns were eliminated simultaneously by an homologous conversion event between a processed cDNA and the Rho B gene, thus preserving the position of the gene in relation to its upstream regulatory sequences. Evidence for such a mechanism in higher organisms comes from the intronless mt NADH dehydrogenase subunit 4-encoding gene of lettuce, which is thought to have lost its introns by homologous recombination with a cDNA copy (37).

As mentioned previously the conclusion as to the Rho B gene being intronless was conceived by only restriction digest analysis and in theory there could be introns present in the Rho B gene which cannot be detected by the accuracy of restriction digests. Therefore; further DNA sequencing of the genomic coding sequences of Rho B are essential to prove that this is an intronless functional gene.

2- THE Rho B PROMOTER APPEARS TO LACK A TATA BOX.

My ribonuclease protection assay results show that the transcription start site of the Rho B gene is 74 nucleotides upstream of the 5' end of the cDNA which was previously cloned (54) (Figure 7). Analysis of the proximal promoter sequences indicate that there is no evidence for the existence of a consensus TATA box, although a sequence somewhat similar to a TATA box is present at -42 to -35 (Figure 20). The Rho B promoter has the characteristics of a TATA-less promoter in that it does not contain a consensus TATA box and at the same time it contains a perfect consensus sequence for an initiator element which spans its transcription start site. The initiator element of Rho B is -3 CGCAGTCC +5 which agrees perfectly with the reported initiator element consensus of (R)2 CAN^T/A (R)2 in TATA-less promoters (56). In almost all of the TATA-less promoters studied so far the A nucleotide acts as the major transcription start site and this is also true for the Rho B promoter.
The Rho B gene also has 3 minor start sites: transcription can also initiate at a much lower frequency from the C, G, and C nucleotides positioned from -3 to -1 (Figure 7). This also is consistent with what is known about TATA-less promoters; transcription from these promoters usually initiates from a cluster of nucleotides (119). Nevertheless, Rho B cannot be identified as a true TATA-less promoter since there is a weak TATA box consensus site of TAAATA at approximately 30 nucleotides upstream of the start site. It has been reported that in some promoters which lack a TATA box and contain an initiator element, a weak TATA consensus site helps in the correct positioning of RNA polymerase II (21, 119). Mutational analysis of this TATA like box and subsequent study of Rho B promoter activity would be necessary in order to make a conclusion as to the importance of this sequence in the context of Rho B transcription.

A computer search of the 200 bp fragment spanning from -96 to +123 identified two putative protein binding sites. The first one which is in close proximity of the start site is a core consensus binding sequence for a helix-loop-helix (bHLH) transcription factor (Figure 20). These proteins are called bHLH because their DNA binding domains consists of a helix-loop-helix domain (58). Interestingly, the 43 kDa USF (upstream stimulatory factor) which is a member of helix-loop-helix DNA binding proteins has been identified as a sequence specific initiator binding protein (31, 98). Initiator binding proteins are factors which have been implicated in facilitating formation of the transcription preinitiation complexes. Therefore it is possible that a bHLH DNA binding protein such as USF is acting as an initiator binding protein for the Rho B promoter.

The second putative protein binding site is 30 nucleotides downstream of the transcription start site; this is a putative binding site for the factor LBP-1. This factor has been shown to interact with two sites that surround the transcriptional initiation site of the human immunodeficiency virus type 1 (HIV-1) promoter (59, 86). It has a binding site upstream of the start site and one downstream of the start site. The binding site downstream of the start site has been shown to act as an enhancer of transcription (59). This protein has also been identified as an initiator binding protein in the major histocompatibility complex class II Ea promoter (10). Binding of this protein
near the initiator element may augment the strength of the initiator. It would be interesting to make a Rho B promoter/CAT plasmid which contains upstream sequences of the Rho B promoter only up the transcription start site and hence deleting the LBP-1 consensus sequence and study the promoter activity of this construct.

3- CONSTITUTIVE PROMOTER ACTIVITY OF Rho B PROMOTER IN TRANSIENT TRANSFECTIONS.

Deletion mutants of the 3.2 kb promoter fused to the CAT gene were constructed and transiently transfected into fibroblast cells; the basal promoter activity of the mutants was studied. Plasmid pOCrbS which contains 1 kb of the 5'-flanking sequences of the Rho B gene showed promoter activity comparable to the levels of the full 3.2 kb fragment and its transcription is not stimulated by EGF treatment. Hence it is assumed that this 1 kb region contains all the basal promoter elements responsible for the constitutive expression of the Rho B promoter. In order to elucidate the functional elements responsible for the constitutive expression of Rho B promoter, nested 5' deletions of the pOCrbS were made.

Table 2 compiles these putative protein binding sites in each deleted region with the effect of each deletion event on the basal promoter activity of Rho B in both transiently transfected Rat-2 and NIH3T3 cells.

3.1- Region -932 to -833.

Deletion of the first 77 bp from the original pOCrbS plasmid causes a 2 fold increase in the transcriptional activity from the promoter in both transiently transfected cell lines. This suggests that a suppressive element may reside in this region (Figure 18). The only putative protein binding sequence in this region is the CACGTG hexanucleotide, a consensus binding site for a group of transcription factors which are classified based on their DNA binding and dimerization motif, a basic helix-loop-helix structure (bHLH) (58). These proteins are involved in differentiation and proliferation processes in the cell (88). Members of this family can be ubiquitous or cell-specific factors. Protein-protein interactions can occur between various
member of this family; the ability to form heterodimers and the large number of these factors gives rise to numerous possibilities on how these factors could regulate transcription from a promoter. In some cases these factors function as positive regulators of transcription (eg E2A) and in other cases they act as negative regulatory factors (7, 65, 78). The binding site present in the 77 bp deleted region appears to be acting as an repressive element of basal activity, therefore the specific bHLH protein which binds to this motif is likely one which has a repressive function. A good candidate for such a factor would be the bHLH protein USF. In a previous section this protein was described as an initiator binding protein for TATA-less proteins; however, it has also been shown that this protein has antiproliferative properties which are inherent to its DNA binding ability (70).

3.2- Region -833 to -622.

In the second deletion mutant, pOCrb622-, 233 bp have been deleted. The effect of this deletion on the transcriptional activity of the Rho B promoter is different between transiently transfected Rat-2 and NIH-3T3 cells in that transcription is reduced in NIH-3T3 cells but unaffected in Rat-2 cells (Figure 19). Putative binding sites for 5 DNA binding proteins reside in this region (Table 2). Since the size of this deleted region is large, it is difficult to make assumptions as to the role of each factor in Rho B transcription. In the case of NIH-3T3 cells, the decrease observed in the promoter activity of the Rho B promoter is sum of all 5 binding sites being removed. Therefore, each binding site could be acting as an repressor or enhancer of basal transcription and no conclusion as to the effect of individual binding sites can be made. There are 3 putative LBP-1 binding sites in this region. LBP-1 is a ubiquitous factor which in some cases acts as an enhancer of transcription and in others as a repressor of transcription (59). In some cases it has also been considered an initiator binding protein (10). The exact function of this protein is not known however it may have a distinctive function in the Rho B transcription since there are 3 binding sites present in the a span of 233 nucleotides and they are positioned very close to each other. The first LBP-1 site is in close proximity with a bHLH binding motif, raising the possibility of an bHLH protein binding to this region in cooperation with LBP-1.
The most interesting putative protein binding sequence in this region is the CArG box and the overlapping site for the factor CF-1. The CArG motif is the binding site for the Serum Response Factor. SRF was first identified in the context of the c-fos promoter. A CArG box in conjunction with a closeby ets protein binding site (positioned 5 bp upstream of CArG) is referred to as a SRE (Serum Response Element) (110). Through the mitogen-induced DNA binding activity of the ets protein p62^TCF, SRF can respond to various mitogenic signals. However, in the absence of the ets binding motif it has been observed that the SRF protein can only respond to the signaling pathways initiated by serum (49). DNA binding activity of SRF is constitutive in that it is not induced as the result of signaling pathways (47).

Recently it was observed that another ubiquitous DNA binding protein, YY1 (Ying-Yang 1), is involved in positioning of SRF on its binding motif. It was shown that in the presence of YY1, SRF can occupy the CArG sequences with much faster kinetics (82). YY1 exerts its functions through its ability to bend DNA (81). Therefore, depending on where and how it exerts its function it can be an activator or a suppresser of transcription. A suppressive effect of DNA bending of YY1 is observed in the c-fos promoter; this factor can induce bends in the DNA at a site between the CRE element and the TATA box resulting in repression of the promoter activity. Based on biochemical properties and molecular weight it has been shown that the ubiquitously expressed protein Common Factor 1 (CF-1) is in fact YY1 (94, 95).

As mentioned before, the CArG box in the Rho B promoter at position -789 is overlapped by the CF-1 site (Figure 20). Therefore in the Rho B promoter context, CF-1 or YY1 may behave the same way as it has been shown in the context of the c-fos promoter, assisting in the nucleation of the CArG box by the factor SRF. As stated previously, SRF alone is unable to respond to various mitogenic signals such as EGF but it is able to respond to serum; hence, it would be interesting to see if this promoter region of Rho B can respond to specific signaling pathways induced by serum alone.

There is a putative binding site for the protein E2A at -729. This DNA binding protein, also a helix-loop-helix protein, has been shown to be involved in regulation of progression of mammalian cells through the G1
stage of the cell cycle (88). This factor has an intrinsic repressive effect on transcriptional activity when bound to DNA; therefore, it actually has a growth-suppressive activity. The up-regulation of the expression of this protein causes an inhibition of G1 progression. Interestingly, this repression can be alleviated by another HLH protein family named Id (7). These proteins are able to form hetero-dimers with other bHLH proteins but they are not able to bind DNA. As a result Id proteins can interact with E2A protein and form a hetero-dimer which is inactive. As a result, E2A cannot exhibit its G1 suppressive activity and the cell progresses through the cycle. In line with this, Id proteins are encoded by immediate early genes and their mRNA production is induced as the result of mitogenic stimulation (80). Therefore it is conceivable that after the mitogenic stimulation, the newly synthesized Id protein exerts its effect during the G1 stage of the cell cycle by interacting with E2A and in turn inhibiting it from binding to its site on the promoter, relieving the promoter of the repressive effects of E2A. However, this function of E2A does not agree with the observation that Rho B is an immediate early gene, its mRNA reaches its highest levels during the G0 to G1 transition and this does not depend on protein synthesis.

The next putative protein binding site (at -655) is FSE2 (Fat-Specific Element). This sequence was first identified in the promoter of genes whose expression is regulated during adipocyte differentiation. For example, the adipocyte P2 gene, has 3 FSE2 motifs. It was shown that this element binds to Fos-Jun complexes binds to this element inhibiting the transcriptional activation of genes needed for adipocyte differentiation (30). A similar sequence was found in the promoter region of the tyrosine hydroxylase gene in PC12 cells. In this promoter it was also shown that the sequence bound to Fos-Jun heterodimers but that this binding was positively regulates the expression of tyrosine hydroxylase and it is induced as the result of signaling pathways triggered by NGF (41). In agreement with the finding that Rho B transcription is also positively regulated in response to NGF in PC12 cells (122), it would be interesting to test the inducibility of this region of the Rho B promoter in response to NGF in PC12 cells.

3.3- Region -622 to -433.
Deletion of the -622 to -433 region causes an increase in the promoter activity of Rho B. The only putative protein binding site in this region is the PEA1 site at -558. The factor which binds to this consensus site is homologous to AP1, Fos-Jun heterodimer (33). The activity of PEA1 is stimulated by serum components, TPA and the oncoprotein v-src (117). This site is likely not functional in the context of the Rho B promoter since the fragment containing this site is not responsive to mitogenic stimulations, as was evident from the CAT assays performed from transiently transfected cells. Moreover, PEA1 has very low DNA binding activity in resting fibroblast cells such as those used in my experiments. As a result it is possible that other unidentified protein binding motifs are contained within this 190 bp region and these factors may act as repressors of transcription in resting cells.

3.4- Region -433 to -337.

Deletion of the -433 to -337 region from the Rho B promoter results in a decrease of its activity in both cell lines, NIH-3T3 and Rat-2 fibroblasts. There are 2 putative protein binding sites in this region; GATA-1 and AP-2.

AP-2 was originally characterized by its ability to bind to sequences on the human metallothionein and SV40 genes that function as both basal and second messenger inducible enhancers (53). AP-2 is responsive to second messengers such as cAMP and also to PKC activation. AP-2 DNA binding activity is stimulated as the result of these signalling pathways and this causes an induction in the expression of numerous genes which have an AP-2 binding site in their 5'-flanking region (76, 92, 121). In addition, this factor has also been shown to be very important for basal level expression of a number of genes. For instance in the case of the Dopamine β-Hydroxylase gene, this protein seems to be important for the basal expression of the gene (42). If the consensus sequence for this protein is removed, the basal transcription is decreased by 7-fold whereas stimulation of the promoter by second messengers is retained. Also AP-2 can only respond to TPA levels if its consensus site has Sp-1 and AP-1 sites in its close proximity.

Therefore, it is likely that in the case of Rho B promoter this factor is acting as an activator of basal level expression of the gene since deletion of the
region containing the AP-2 consensus site causes a decrease in the level of Rho B transcription. This decrease is 2 fold in the case of NIH-3T3 cells and 10 fold in Rat-2 fibroblasts.

The GATA-1 consensus site in this region is unlikely to be important for Rho B transcription since this factor is involved exclusively in transcriptional regulation of tissue specific genes. GATA-1 is present in erythroid cells, megakaryocytes and mast cells and is an essential factor for regulation of erythroid differention and megakaryocyte-specific gene expression (67, 84, 111).

3.5- Region -337 to -305.

Deletion of this region in both NIH-3T3 and Rat-2 fibroblasts causes an increase in the promoter activity of Rho B. This 32 bp fragment does not contain any known putative protein binding sites; hence, an un-identified repressive factor may bind to this region. EMSA studies can be performed on this fragment to see if there is any DNA binding activity in nuclear extracts of fibroblast cells.

3.6- Region -305 to -241.

Deletion of this 64 nucleotide region causes an decrease in the promoter activity of Rho B in both cell types tested here. Similar to the previous deleted region, this region does not contain any putative protein binding sites, however, preliminary foot printing analysis of this region using NIH-3T3 nuclear extracts shows a protected region from -302 to -297 (data not shown). The sequence which is protected is CTGCCT. No known DNA binding protein has been identified with binding affinity for this sequence.

3.7- Region -241 to -156.

Deletion of this 85 bp region causes a further reduction in the Rho B transcription levels. There are 2 putative protein binding sites in this region; AP-2 and E4F1 binding sites. Deletion of the AP-2 binding site and its effect on Rho B transcription was discussed in previous sections. The E4F1 site is
the consensus site for the cellular transcription factor E4F. This factor binds to sites which are very similar to ATF binding sites (97). It is not known to what stimuli E4F responds to; however, ATF responds to signaling pathways initiated by cyclic AMP (33). Since Rho B transcription is non-responsive to levels of cAMP in the cell, this putative protein binding site in the Rho B promoter is assumed to be nonfunctional.

3.8- Region -156 to -92.

Deletion of this 64 bp region results in an additional decrease of Rho B transcriptional activity. This effect seems to be more pronounced in NIH-3T3 cells than in Rat-2 fibroblasts. No putative protein binding sites are found in this region of the promoter.

3.9- The putative protein binding sites in the minimal promoter region.

There are four putative protein binding sites present in the 214 bp fragment from -92 to +122. These include an NF-IL6 site, a bHLH site, a GCF site and a LBP-1 site. The importance of the bHLH and LBP-1 site were discussed in a previous section.

NF-IL6 is a transcription factor of the CCAAT/enhancer-binding protein family (C/EBP) (3, 33). Synthesis of the transcription factor is stimulated by cytokines such as IL-1, IL6 and TNF-α (2, 52). This protein can be activated as the result of signaling pathways induced by the same cytokines and TPA (61). In fact, NF-IL6 is translocated from the cytoplasm to the nucleus upon stimulation of the cells with TPA (75). This protein is phosphorylated near or within its DNA binding region and this phosphorylation plays an enhancing effect on the activity of the factor (118). Interestingly the modulation of the ratio of activator to inhibitor isoforms of NF-IL6 regulates transcription of the target gene. NF-IL6 has different reading frames and depending on which AUG is used the protein can either be an inhibitor of transcription or activator of transcription (27). The abundance and ratio of the activator to inhibitor NF-IL6 isoforms vary according to cell type and can be altered by cytokine stimulation (52). In any event, the importance of this site in the context of Rho B promoter is unknown since
the effect of IL-1, IL-6 and TNF-α on the transcriptional regulation of Rho B have not been studied.

The second putative protein binding site (GCF) in this region is at the +30 position. GCF is a transcriptional regulator that is known to repress transcription of the epidermal growth factor receptor and several other genes (9). The expression of this factor is inversely proportional to that of the EGF receptor. It is unlikely that this factor can be a repressive element in the context of the Rho B transcription because recent evidence shows that the expression of Rho B is also inversely proportional to that of EGF receptor (25).

4- RESPONSIVENESS OF Rho B PROMOTER TO MITOGENIC STIMULI.

All of the transcribed sequences for the Rho B gene are contained within the isolated 11.5 kb genomic fragment. In addition to the transcribed sequences, this genomic fragment has sequences 4.8 kb upstream and approximately 5 kb downstream of the Rho B transcribed region. The promoter activity of the 3.2 kb 5'-flanking sequences were studied by constructing CAT fusion vectors, transfections and subsequent CAT assays. CAT assays of transiently transfected fibroblast cells show that this 3.2 kb fragment exhibits promoter activity and this activity is observed only in the sense orientation displaying the specificity of the sequences for the Rho B gene (Figure 9). Further deletion of the 3.2 kb fragment shows that the minimal promoter elements are contained within the 90 bp 5'-flanking region. This agrees with the data describing the transcription start site and the putative initiator sequences residing in this region.

Mitogenic stimulation of the fibroblast cells which are transiently transfected with the promoter constructs does not indicate an increase in the level of CAT protein production. Temperature shift experiments using the transiently transfected CNA7 fibroblast cell line (expressing the ts v-fps mutant) does not indicate an elevation in the transcription activity of the 3.2 kb Rho B promoter fragment; the same result is observed in EGF stimulated transiently transfected Rat-2 fibroblast line. These results are in disagreement with the response of the endogenous Rho B gene to mitogenic stimuli such as v-fps tyrosine kinase activity and EGF treatment (54). Induction of Rho B
expression in response to proliferative and differentiative signals has been shown by several studies to be at the level of transcription (122). Nuclear run on experiments were used as the assay for the rate of transcription initiation and although Rho B transcripts are observed in the resting quiescent cells, the level of transcription initiation is elevated 3 to 4 fold as the cells are treated with mitogens.

In order to resolve the discrepancies observed between the previously published results and my transfection results, several possibilities were examined. All the results which show induction of Rho B RNA expression were performed using RNA isolated from cells which were rendered quiescent and subsequently treated with mitogens. Expression of Rho B both at the mRNA level and protein level coincides with the progression of the cells through the cell cycle and this expression is periodic through the cell cycle as described in the introduction section (122). The induction at the RNA level is only observed as the cells progress from the resting state to the proliferative state. Therefore, transiently transfected cells were rendered quiescent and then treated with EGF. However, I was unable to observe any induction of the Rho B promoter transcriptional activity. Figures 12 and 13 show a time course of induction of the transiently transfected cells which were rendered quiescent by serum starvation and stimulated with v-fps kinase activity (Figure 13) or EGF treatment (Figure 12). Stimulation of cells up to 2 hours does not cause an induction of CAT expression driven by Rho B promoter fragments.

It has been reported that expression of a number of genes including c-fos can be induced at the level of transcription by CaPO4-DNA precipitates and DEAE dextran during the transfection procedure (71, 89). Since a high basal level of CAT activity is observed in the transfected/starved fibroblast cells it was thought that the Rho B gene might also be a target of whatever signaling pathway the CaPO4-DNA precipitates induce. In turn Rho B promoter sequences could already be stimulated before the mitogenic signal is given to the cells and as a result no further stimulation is observable. This possibility was tested by transiently transfecting cells with plasmid DNA which is unrelated to Rho B, starving the cells and stimulating them with EGF. The levels of total Rho B RNA was measured by ribonuclease protection. In this
experiment transcription of the endogenous Rho B gene is not affected by the CaPO₄-DNA complexes as shown by the low level of endogenous Rho B RNA in resting cells.

The effect of chromosomal integration of Rho B on the transcriptional induction was also considered. It has been reported that chromatin structure plays a crucial role in the regulation of eukaryotic gene transcription (34, 79). Nucleosomes and higher orders of chromatin structure repress promiscuous gene expression by increasing its dependence on the function of activator proteins that regulate gene transcription in eukaryotic cells (43, 85). In some cases the presence of chromatin structure on the promoter region could inhibit binding of factors to that region and hence result in reduction in the transcription of the gene. Treatment of cells with mitogenic factors could alleviate this repression. This can happen upon activation of proteins which interact with nucleosomes and either cause their removal from the core promoter elements or alleviate the repressive effects that they have by relaxing their conformation (5). When cells are transiently transfected, the majority of the DNA which is introduced into the cell does not integrate into the genome and therefore does not have any nucleosomes or histones bound to it. The deregulation of the Rho B transcription which is observed here could be due to the fact that most of the plasmid DNA introduced into the cells transiently, remains as an autonomous unit and does not integrate into the genome. In the case of Rho B gene this possibility is supported by the observation that the transcriptional activity of the Rho B promoter as measured by CAT activity in the resting transfected cells was somewhat higher in comparison to the endogenous Rho B transcription in resting cells.

In order to see whether chromosomal integration affects the transcription of Rho B, stable transfected fibroblast cells were isolated using Rho B promoter/ CAT fusion constructs. These cells were rendered quiescent by starvation and then stimulated by EGF treatment. As can be seen in Figure 16, transcription of the stably transfected Rho B promoter is also not stimulated by treatment of cells with EGF. Hence, nucleosomal structures likely does not play a role in Rho B transcription.
Sequences downstream of the coding region of some genes have been shown to contain protein binding motifs which influence the transcription of the gene either positively or negatively (87). The existence of such a motif in the 3' region of the Rho B gene was also considered. A vector containing approximately 2 kb of the 5'-flanking Rho B sequences fused to the CAT coding sequences and carrying 3' sequences of the Rho B gene fused to the end of the CAT coding sequences was constructed for this purpose (constructed by I. Sadowski). Transient transfection of fibroblast cells with this vector and their subsequent stimulation with EGF did not result in induction of Rho B promoter activity upon stimulation of the cells (data not shown).

In the studies mentioned above, the promoter activity of 3.2 kb of 5' flanking sequences of Rho B coding sequences in addition to 350 bp downstream of the start site was studied. This 350 bp region is transcribed but not translated, therefore it contains sequences from the transcription start site up to the translation start codon ATG. Although the genomic fragments exhibit promoter activity as measured by CAT enzyme activity, this activity is not stimulatable by EGF treatment or v-fps tyrosine kinase activity. It must be concluded that the cis-elements responsive to these two signaling pathways which take part in the transcriptional induction of the endogenous Rho B gene are not located in the 3.2 kb upstream of the start site nor in the 350 bp of non-translated region downstream of the start site. Moreover, the 3' sequences of the Rho B gene are also devoid of cis-elements solely responsive to EGF treatment.

The borders of the Rho B gene have not been defined, hence, it is highly possible that the regulatory elements which are responsible for transcriptional induction are located further upstream of the region that I have studied here. In eukaryotic gene regulation there are no guidelines as to where cis-elements should reside in order to be effective activators or repressors of transcriptional induction. For instance, in the case of nitric oxide synthase (NOS2), the first 3.8 kb upstream of the gene demonstrates basal promoter activity and no cytokine inducibility. In fact, cytokine responsive elements have been found to be located 16 kb upstream of transcription start site (26). It seems highly probable that the EGF responsive element of the Rho B gene is located outside the 3.2 kb region that I had
focused on. If that is the case then the most 5' region of the 11.5 kb genomic clone isolated here should be used for further cloning in order to isolate sequences further upstream of the *Rho B* gene.

There is also the possibility that the responsive elements are positioned in the coding sequences of *Rho B* gene. As mentioned previously, my constructs contained 350 bp of the non-translated cDNA; however, it is possible that the EGF and *v-fps* responsive element(s) are located inside the coding region. Moreover, it is possible that the responsive element(s) are located in the regions that I had studied but that they are functional only in cooperation with accessory elements present in the coding region of the *Rho B* cDNA. Therefore, it is crucial to study the transcriptional activity of the intact 11.5 kb genomic fragment in transient transfections. This could be achieved by inserting sequences in the coding sequence which can make it possible to distinguish between the endogenous gene and the transfected one. In this way total RNA from the transfected cells can be isolated and the transcription of the transfected genomic sequences can be monitored.

It is important to note that *Rho B* transcription is induced via treatment of cells with a variety of mitogenic and stress related signals. Therefore, although responsive elements to EGF and *v-fps* tyrosine kinase activity do not solely reside in this region it is possible that responsive elements to the pathways induced by U.V. light treatment or the TPA responsive elements specific to Hela cells may be positioned in the 3.2 kb *Rho B* promoter fragment studied here.
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