Growth Factor Regulation of the Promoter for Calcyclin, a Growth-regulated Gene*

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The steady-state levels of calcyclin mRNA are regulated by growth factors. Using deletion mutants of the 5'-flanking region and a linked reporter (the bacterial chloroamphenicol transferase gene), we have investigated the elements of the calcyclin gene's promoter that respond to growth factors. By a transient expression assay after transfection in BALB/c/3T3 cells, we have been able to show that the serum-inducible sequences are contained in a 164-base pair fragment just upstream of the cap site. This fragment also contains an enhancer, and responds to platelet-derived growth factor as well as to serum. The sequences from −1371 to −1194 upstream of the cap site contain an element which is negatively regulated by epidermal growth factor. These findings have been confirmed in hamster cell lines in which the deletion mutants of the calcyclin promoter controlled the expression of the cDNA for human thymidine kinase. These results indicate that, like in other growth-regulated genes the activity of the calcyclin promoter is modulated by both positive and negative elements. Even more intriguing, though, is the finding that some of these negative elements may be influenced by growth factors in the environment.

The calcyclin gene (1) belongs to a gene family which also includes the α and β subunits of the S100 protein (2, 3), the cystic fibrosis antigen (4), the pll subunit of calpactin I (5, 6), and others (7). Calcyclin was originally identified as a cDNA clone whose cognate RNA was growth-regulated (8). It includes the 3' untranslated region of the calcyclin mRNA and the cDNA clone whose cognate RNA was growth-regulated (8). It was subsequently shown that the steady-state levels of calcyclin mRNA are increased in certain forms of human acute myeloid leukemia (9) and when quiescent cells are stimulated by serum or platelet-derived growth factor. More recently, calcyclin promoter activity appears to be influenced by growth factors in the environment.

The bulk of the experiments were carried out by a transient expression assay in transfected BALB/c/3T3 cells that are strictly regulated by growth factors (11). However, in some cases, we have extended our studies to established cell lines carrying the desired constructs.

MATERIALS AND METHODS

Cell Lines—BALB/c/3T3 stocks have been passed in our laboratory for several years as previously described (10). TK ts13 Syrian hamster fibroblasts (12), a thymidine kinase (TK)-deficient cell line, derived from ts13 cells were transfected in suspension (12) using the ScaI-BamHI or the SmaI-BamHI deletion mutants of the calcyclin promoter driving the cDNA of the human TK gene, as described below. After selection in 10% calf serum and gHAT medium the resistant cells were expanded to obtain two different lines: one producing TK under the control of the ScaI-BamHI promoter (Sc6TKE cells) and the other under the SmaI-BamHI promoter, Sm4TKE cells. To study the effect of EGF on these lines, the cells were plated onto 16-mm diameter plastic wells in 5% horse plasma (HP) plus gHAT medium with or without EGF; 4 days later the medium was changed. After 7 days the cultures were terminated and the number of clones and cells were counted.

Plasmids—The construction and characterization of the calcyclin promoter and its mutants driving a linked reporter have been previously described (1). The SmaI-BamHI deletion mutant (Fig. 1), with filled ends, has been cloned in the two orientations in the HindIII-digested pUC9-CAT plasmid after filling the ends. The calcyclin promoter-TK constructs have also been described previously (1). A new plasmid is the TqI-TqI mutant which was made by isolating the TqI fragment (see Fig. 1) and subsequent subcloning into the Ascl-BamHI site of pUC18T. This plasmid has lost the enhancer-like structure between −164 and −144. To reinsert this latter fragment into the TqI promoter mutant, we simply recloned it 300 bp upstream of the TqI restriction site.

Transient Expression Assay—70-80% confluent BALB/c/3T3 mouse fibroblasts were transfected following the protocol of Shen et al. (12). 20 μg of plasmid DNA were used for 1.5 × 10^6 cells and 20% polyethyleneglycol was employed for the osmotic shock. Cells were replated at a concentration of 1.5 × 10^6 cells/100-mm dish. During transfection, 5% horse plasma was employed. Growth factors (unless otherwise noted) were added after 24 h and the cultures were usually terminated 48 h after transfection. The fibroblasts were then detached by scraping and CAT activity of cell extracts measured as percentage of acetylated chloroamphenicol by standard techniques (13).

RNA Extraction and RNA Blots—Total cytoplasmic RNA was extracted from cells by the method of Chirgwin et al. (14), and RNA blots were carried out by standard procedures (15). A radioactive probe was prepared from plasmid pTK11, which contains the human TK cDNA (16) by the random priming method (17). Growth Factors—Calf serum, PDGF, and EGF (Collaborative Research) were added to cell cultures at concentrations of 10%, 2 ng/ml, and 40 ng/ml respectively. Horse plasma (Hyclone) is actually plasma-derived horse serum. Since it behaves like platelet-poor...
plasma, to avoid confusion, we shall call it horse plasma. The growth promoting activity of HP varies from batch to batch; the data in this paper were all obtained with a batch that did not support growth of BALB/c/3T3 cells.

RESULTS

For convenience, the restriction sites in the calcyclin promoter that have been used in making the various constructs are shown in Fig. 1, which is derived from the previously reported sequence of the calcyclin gene (1). All mutants, with one exception, have as a 5' boundary the indicated restriction sites and extend 134 base pairs into the 5' untranslated region of the calcyclin gene, where they are linked to the coding sequence of the CAT gene. The exception is the TaqI mutant, in which the CAT gene is driven by a TaqI-TaqI promoter fragment that extends from −144 to +8 (the nucleotide numbers are the same as in the paper by Ferrari et al.; Ref. 1).

Transfection in Horse Plasma—BALB/c/3T3 cells do not grow appreciably in HP or in platelet-poor plasma (PPP), unless PDGF is also added (11, 18). In early experiments, we used PPP instead of HP. Since they gave similar results, subsequent experiments were done with HP, which is commercially available. Subconfluent monolayers of BALB/c/3T3 cells were transfected in suspension by the method of Shen et al. (12) and, after shock, they were incubated in either 10% calf serum (CS) or 5% HP. Fig. 2 shows a typical experiment in which BALB/c/3T3 cells were transfected with the Scal-CAT plasmid (Fig. 1). At all times between 24 and 72 h after transfection, CAT activity was considerably higher in cells incubated in CS than in cells incubated in HP. The difference was not due to variations in transfection efficiency, because one single pool of cells was transfected and subsequently divided into aliquots. If cells incubated for 24 h in HP were then stimulated with 10% CS, CAT activity increased to approximately the same level as in cells left in CS for 48 h (Fig. 2). This indicated that cells in HP were still in good condition and had not lost the capacity of expressing the transfected CAT gene. Indeed, BALB/c/3T3 cells in HP look as healthy as in CS.

Fig. 2 therefore indicates that our assay in HP is valid and can be used to determine the effect of growth factors on the activity of the calcyclin promoter and its mutants.

Effects of Growth Factors on the Activity of the Calcyclin Promoters and Its Mutants—Figs. 3–5 show some representative experiments, in which CAT activity was assayed in cells transfected with various constructs and treated with different combinations of growth factors. Transfection efficiency varied (and sometimes considerably) from one experiment to another, but we emphasize that, in each experiment, one single pool was transfected with a construct and subsequently divided into aliquots, one in CS, one in HP, and others in HP for 24 h, followed by stimulation with growth factors. The percentage increase in CAT activity caused by serum or growth factors is therefore real and can be compared in separate experiments.

Fig. 3 shows that calf serum, PDGF, and EGF increase CAT activity (in respect to HP only) in BALB/c/3T3 cells transfected with the Neol-CAT plasmid. Fig. 4 shows the same CAT assay in cells transfected with the Sma-CAT plasmids. CS, added to cells incubated in HP, increases CAT activity (lanes A and C). Lanes D–F show that if the Sma promoter is cloned in the reverse orientation, no CAT activity is detectable in transfected cells. Fig. 5 compares cells transfected with either the Sma-CAT or the Taq-CAT constructs (refer to Fig. 1). The TaqI promoter has lost the enhancer-like element located around −160 (1). This enhancer-like element has a strong similarity to the core of the SV40 enhancer (19, 20). Loss of this element dramatically reduces promoter activity (compare lanes A and E); however, the stimulation by growth factors is not lost, as lanes E–H clearly show.

The results of several such experiments are summarized in Tables I and II. Each experiment was repeated at least twice, but the majority were repeated several times.

In Table I, we compare the activities of the calcyclin promoter and its mutants, transfected into BALB/c/3T3 cells and incubated in 10% CS. In these particular experiments,
Calcyclin and Growth Factors

FIG. 4. CAT assay of BALB/c/3T3 cells transfected with the SmaI-CAT plasmid (Fig. 1). All cultures were terminated at 48 h. Lanes: A, horse plasma only; B, calf serum only; C, horse plasma (24 h) followed by calf serum (24 h). D-F, same three treatments, except that the SmaI promoter had been cloned in the reverse orientation.

FIG. 5. CAT assay of transfected BALB/c/3T3 cells. Cultures were terminated 48 h after transfection. A-D, cells transfected with the SmaI-CAT plasmid; E-H, cells transfected with the TaqI-CAT plasmid promoter (see Fig. 1). A and E, horse plasma only; B and F, calf serum only; C and G, horse plasma followed by calf serum; D and H, horse plasma followed by PDGF.

we used an internal standard to monitor the efficiency of transfection with the different constructs and the results are normalized to the internal standard. As reported previously with ts13 cells (1), even the SmaI promoter is active in directing transcription of a linked reporter. Again, the NcoI promoter is the most efficient and sequences from -164 to +134 (no TATAA box, 1). The reverse SmaI is the SmaI promoter cloned in the reverse orientation. Elimination of the enhancer-like structure (TaqI) greatly reduces activity.

Table II summarizes the effect of growth factors on the activity of the calcyclin promoter and its mutants

<table>
<thead>
<tr>
<th>Promoter</th>
<th>CAT activity</th>
</tr>
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<tbody>
<tr>
<td>ScaI</td>
<td>119</td>
</tr>
<tr>
<td>NcoI</td>
<td>190</td>
</tr>
<tr>
<td>SphI</td>
<td>142</td>
</tr>
<tr>
<td>XhoI</td>
<td>62</td>
</tr>
<tr>
<td>SmaI</td>
<td>100</td>
</tr>
<tr>
<td>TaqI</td>
<td>26</td>
</tr>
<tr>
<td>Sma vector</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Reverse Sma</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The promoters are described in the legend to Fig. 1. The ScaI vector is the ScaI promoter without the sequences from -164 to +134 (no TATAA box, 1). The reverse SmaI is the SmaI promoter cloned in the reverse orientation. The efficiency of the transfection technique was monitored for each plasmid by cotransfecting the cells with pRSVneo (21) and selection of an aliquot of cells in G418. After 14 days, the number of colonies was counted, and used to normalize the CAT activities.

The stimulation of the calcyclin promoter by growth factors is not simply due to the fact that cells in serum grow while they do not grow in HP. When the CAT gene is driven by an
SV40 promoter, serum does not stimulate CAT expression. In a typical experiment, CAT activity in serum was 55 and in HP it was 63. In addition, EGF also stimulates most of the promoter mutants, but by itself (plus HP) EGF does not sustain growth of BALB/c/3T3 cells.

Although the XhoI promoter is also weaker than the SmaI promoter, we have extended our studies only to the ScaI promoter, because it seems to be down-regulated by EGF. First, though, we confirmed that the enhancer-like structure has actually enhancer activity.

The SmaI-TaqI Sequence Has Enhancer Activity—Fig. 5 shows that removal of the sequences between -164 and -144 of the SmaI promoter greatly reduces its activity, while not eliminating its response to serum. To determine whether this sequence has enhancer-like activity, we simply recloned it 300 bp upstream of the promoter in the TaqI plasmid. Fig. 6 shows that the reinsertion of this fragment at a distance of 300 bp restores activity to the TaqI promoter.

Effect of EGF on the Growth of Cells Carrying Different Promoters—To further test the negative effect of EGF on the ScaI promoter, we took advantage of the fact that baby hamster kidney cells and their derivatives tK-ts13 cells grow poorly in HP, but grow much better if EGF is added (22). We made two cell lines by transfecting tK-ts13 cells with constructs in which the human TK coding sequence is driven by either the ScaI or the SmaI promoter of the calcyclin gene. Appropriate clones were selected in gHAT plus 10% CS. Two clones were eventually expanded, Sc3TKE, carrying the plasmid with the ScaI promoter (plus the human TK coding sequence), and Sm4TKE, carrying the SmaI-TK construct. To understand this experiment, the following premises are necessary: 1) in regular medium (Dulbecco’s modified Eagle’s medium) tK-ts13 cells and their derivatives Sc3TKE and Sm4TKE grow very well in 10% CS, poorly in HP, and again well if EGF is added to HP; 2) tK-ts13 cells cannot grow in gHAT (regardless of which growth factors are used), while Sc3TKE and Sm4TKE can. We reasoned that, for growth in gHAT, besides the growth factors the calcyclin promoters must also be active, otherwise TK is not produced. If we added EGF to HP, the cells could grow in normal medium where TK activity is not necessary, but not in gHAT (where TK activity is necessary) unless the respective calcyclin promoters are activated. We therefore seeded Sc3TKE and Sm4TKE in 5% HP plus EGF, in gHAT medium. The number of cells were counted 7 days later. A typical result is shown in Table III. Sc3TKE do not grow in EGF + gHAT, while Sm4TKE do, the number of cells increasing 6-fold in 7 days. In 10% serum, of course, both cell lines rapidly grow to confluence, even in gHAT (not shown). This experiment was repeated twice with similar results, indicating again that the ScaI promoter is not active in the presence of EGF (the number of cells actually decreased), while the SmaI promoter is sufficiently activated by EGF to allow growth of cells in gHAT.

Activation of the ScaI Promoter by Serum Results in Increased Steady-state Levels of mRNA—We have shown in a previous paper (10) that growth factors, like serum and PDGF, increase the steady-state levels of calcyclin mRNA in a variety of cells. We show here that the effect of serum on the ScaI promoter also results in an increase in mRNA levels. For this purpose, we have selected the Sc3TKE cells, in which the ScaI promoter controls the expression of the human TK gene. We have shown in a previous paper (10) that growth factors, like serum and PDGF, increase the steady-state levels of calcyclin mRNA in a variety of cells. We show here that the effect of serum on the ScaI promoter also results in an increase in mRNA levels. For this purpose, we have selected the Sc3TKE cells, in which the ScaI promoter controls the expression of the human TK gene.

### Table III

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Promoter</th>
<th>No. of clones</th>
<th>No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc3TKE</td>
<td>ScaI</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Sm4TKE</td>
<td>SmaI</td>
<td>36</td>
<td>641</td>
</tr>
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Fig. 6. Enhancer activity of the -164 to -144 sequence of the calcyclin promoter. BALB/c/3T3 cells were transfected with the pUC9 plasmid containing the Taq-Taq-CAT plasmid (A) or a plasmid with the Taq-Taq promoter sequences (B), plus other sequences so that the putative enhancer is about 300 bp upstream of the TaqI site. Cells were transfected in horse plasma containing medium and grown in 10% calf serum for 48 h before CAT assay.

Fig. 7. Serum-dependent expression of the TK cDNA directed by the SmaI promoter. RNA extracted from: A, nontransfected tK-ts13 cells; B, Sc3TKE cells after 4 days of incubation in gHAT + 5% HP; C, Sc3TKE cells after 4 days of incubation in gHAT + 10% calf serum.
cDNA (1). Sc3TKE cells were incubated in 5% HP or 10% CS for 4 days, after which RNA was extracted. The RNA blots (Fig. 7) indicate that the levels of TK mRNA are higher in serum-stimulated (lane C) than in HP-treated (lane B) cells. Two bands are recognized by the human TK probe, one probably due to read-through transcription. This interpretation is supported by the fact that our probe does not detect any RNA band in the parent cell line, tk-ts13 (lane A).

DISCUSSION

In this paper, we have examined the regulation of the calcyclin promoter by growth factors, specifically calf serum, PDGF, and EGF. For most experiments, we have chosen BALB/c/3T3 cells because their regulation by growth factors is well documented (11, 18). These cells do not grow in PPP or HP, but require the addition of PDGF for stimulation of cellular DNA synthesis. Since calcyclin is a growth-regulated gene (8) inducible by PDGF and EGF, but not by PPP (10), we set out to identify the sequences in the 5'-flanking region that are regulated by growth factors. We have obtained three novel and interesting findings.

1) All mutants tested respond to serum or PDGF. The PDGF-inducible sequences must therefore be located in the SmaI fragment, which includes 164 base pairs upstream of the cap site. These 164 base pairs include a TATAA homology, GC boxes (25), and an enhancer-like sequence (1). It does not contain appreciable similarities to other sequences that have been described as serum-inducible, such as the serum-regulated sequence GGAAAAGC of the promoter of the 70-kDa heat shock protein (24), and that of the c-fos promoter (25, 26). The closest similarity is in the enhancer-like structure, but removal of this sequence from the calcyclin promoter does not abolish response to serum, although the basal activity is greatly reduced (see Fig. 5 and Table II).

All mutant promoters also respond to EGF, except the ScaI promoter, whose activity is actually decreased by EGF (Tables I and III). Even with the promoters inducible by EGF, the response is weak, reasonably so, since BALB/c/3T3 do not respond too vigorously to EGF only (11). We can conclude, at any rate, that the SmaI promoter fragment and, indeed, the ScaI promoter contain the necessary information for inducibility by growth factors. Site-directed mutagenesis should eventually pinpoint the precise serum-regulated sequences in this region.

2) We have identified a negative element in the ScaI promoter, which is downstream-regulated by EGF (see Tables I and III). The sequence from ScaI to NcoI is 178 base pairs long: this sequence may be decreased by EGF but not by PDGF or serum. This was evident in transient expression assays and also in the ability of Sc3TKE and Sm4TKE cells to grow in G418 medium supplemented with EGF and HP. In these cells, derived from a TK-deficient cell line, growth in G418 depends both on EGF as a growth factor and on EGF as an activator of the calcyclin promoters from which the human TK cDNA is transcribed. Sm4TKE cells can grow in G418 + HP + EGF because the SmaI promoter is somewhat activated by EGF. The fact that Sc3TKE cells cannot grow under the same conditions confirms that the ScaI promoter is inhibited EGF, suggesting the presence of a negative regulatory element. Incidentally, this model (of a promoter driving a TK coding sequence in a TK-negative cell line) could be conveniently used to detect and study negative regulatory elements in other promoters and to identify the relevant factors.

It is known that EGF can exert a negative effect on cell proliferation: for instance A431 cells, that express high concentrations of EGF receptors (27) stop proliferating when treated with EGF (28–30). However, the endogenous calcyclin gene is inducible, albeit weakly, by EGF in BALB/c/3T3 cells (10). There must therefore be, upstream of the Sca1 restriction site, other sequences that override the negative regulatory element, a situation that we have already observed in the human vimentin promoter (31). We are presently subcloning a 3-kilobase fragment upstream of the cap site, searching for other regulatory elements.

3) We have confirmed, functionally, the presence of an enhancer, that was previously suggested by the sequence (1). Incidentally, besides this enhancer at −150, there is another enhancer-like structure at −935 with an 11/13 nucleotide similarity to the SV40 enhancer (1). This putative second enhancer may account for the fact that the NcoI promoter is consistently more active than the others.

In conclusion, we have completed a partial analysis of the 5'-flanking region of the calcyclin gene, a growth-regulated gene. We have shown the presence of a PDGF-inducible element (contained in the ScaI promoter), of an enhancer (between the SmaI and the ScaI restriction sites), and of a negative regulatory element, whose activity is decreased by EGF (between −1371 and −1194). Several reports have already appeared indicating that the promoters of some growth-regulated genes contain both positive and negative regulatory elements (31–33). Most notable, in the calcyclin promoter, is that a negative regulatory element is actually down-regulated by a growth factor, EGF. This opens the intriguing possibility that growth factors may exert both positive and negative effects on the regulatory regions of growth-regulated genes.

REFERENCES