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Sequence specific protein binding to and activation of the TGF-β3 promoter through a repeated TCCC motif

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ABSTRACT

We have previously characterized the TGF-β3 promoter and shown that the activity of this promoter is highly variable in different cell types. Although the promoter contains a proximal cAMP responsive element, which is critical to basal and forskolin-induced promoter activity, this element is not responsible for the variable, cell-specific regulation of the promoter. In this paper, we identify a 25 base pair sequence in the proximal region of the TGF-β3 promoter that binds a novel DNA-binding protein. This region includes the sequence T-CCCTCCCTCCC, (3 x TCCC), and mutation of these T-CCC repeats inhibits protein binding. Further, we show that in the cell line A375, which we have previously shown expresses high levels of TGF-β3 mRNA, this region is responsible for mediating high level TGF-β3 promoter activity. Immediately 3′ to the 3 x TCCC sequence is a consensus AP-2 binding site, however, we show that this region does not bind AP-2, and AP-2 does not transactivate the TGF-β3 promoter. Therefore, we provide strong evidence that high level expression of TGF-β3 in A375 cells results from transactivation of the TGF-β3 promoter by a protein that binds to a repeated TCCC motif in the promoter and suggest that this DNA-binding protein likely also regulates aspects of developmental and tissue-specific expression of this cytokine

INTRODUCTION

The TGF-βs are a family of highly homologous proteins that regulate cell growth, transformation, and differentiation in a wide variety of cell types (1, 2, 3). Three TGF-βs have been cloned from mammals, TGF-b1, 2, and 3 (4, 5, 6, 7, 8, 9). Although the activated peptides of each of the TGF-βs share similar biologic activities (10), the pattern of expression in developing and adult tissues is quite distinct. For example, TGF-b1 is expressed most highly in mouse spleen, and to a lesser degree in the heart and lung, however, TGF-β3 mRNA is expressed highly in mouse heart and lung, but minimally in the spleen (7, 11, 12, 13, 14). This differential tissue expression suggests distinct roles for the various TGF-b isotypes in various tissues and highlights the likely importance of regulation of TGF-b mRNA expression during embryogenesis and in the regulation of both physiological and pathophysiological cellular functions in adult tissues.

We have recently characterized the 5′ flanking region of the TGF-β3 gene (15). This gene has a long 5′ untranslated region (1104 base pairs, bp), and a single major transcriptional start site 21 bp downstream from a consensus TATA box . It also contains a consensus cAMP responsive element (CRE) and a consensus sequence for AP-2 binding; these elements are located 12 and 23 bp, respectively, upstream from the TATA box. The CRE regulates both basal and forskolin induced activity of the gene, whereas, deletion of the AP-2 consensus binding site is associated with a 2–3 fold decrease in TGF-β3 promoter activity in all cell lines studied. Since other studies have noted increased expression of AP-2 in retinoic acid-induced differentiation of human embryonal carcinoma cells (16), we previously have suggested that AP-2 might regulate TGF-β3 promoter activity during differentiation (15).

Other regions further upstream are also associated with further increases in TGF-β3 promoter activity. In general, promoter activity correlates closely with mRNA levels, indicating that promoter regulation is an important feature in the regulation of TGF-β3 mRNA expression (15). In this regard we have noted that the A375 melanoma cell line expresses over 10-fold greater levels of TGF-β3 mRNA than other tumor cell lines studied (15). This increased mRNA expression is associated with a marked increase in promoter activity which is lost upon deletion of the region between −221 and −60. These data strongly suggest the presence of a regulatory element in this region which has no previously described DNA binding protein consensus sequence.

In this report, we describe a more detailed analysis of the TGF-β3 promoter. We show that the AP-2 consensus binding site we described previously does not bind AP-2 under the condition tested, and that expression of AP-2 in Drosophila cells does not regulate TGF-β3 promoter activity. A protein(s) extracted from A375 cells does, however, bind to the region immediately upstream from this site which we show is also the region

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responsible for the markedly increased expression of TGF-β3 in A375 cells. This region is notable for containing three repeats of the motif TCCC. Mutations in this motif both inhibit protein binding to this region and dramatically decrease promoter activity.

MATERIALS AND METHODS

Cell culture

The A375 cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, in the presence of antibiotics (50 units/ml penicillin, 50 mg/ml streptomycin). Cells were grown in a humidified incubator at 37°C in 5% CO_2.

Drosophilia Schneider cells were cultured in Schneider's Insect medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, and antibiotics (50 units/ml penicillin, 50 mg/ml streptomycin). Cells were grown in room air at 27°C.

Plasmid Construction

Chimeric TGF-β3/CAT plasmids were constructed by ligating fragments of the TGF-β3 5′ flanking region generated by polymerase chain reaction (PCR) into pGEM4-SV0CAT as previously described (15). The 3′ oligonucleotides used in all amplifications corresponded to the 20-bp sequence with a 3′ terminus at +110 of the TGF-β3 5′ untranslated region to which an XbaI site and four random nucleotides were added. The 5′ oligonucleotide used in each amplification consisted of a 20-bp sequence of the TGF-β3 promoter with a 5′ terminus at the nucleotide of the desired 5′ fragment terminus, to which a HindIII site and four random nucleotides were added. For construction of the mutated TGF-β3 promoter/CAT plasmids, pB3-75Δ1−pB3-75Δ7, the 5′ oligonucleotides were synthesized to contain the mutated nucleotide that was desired in the completed CAT plasmid. Using these oligonucleotides, TGF-β3 promoter fragments were amplified according to the standard protocol included in a GeneAmp kit (Perkin Elmer Cetus Instruments). Each fragment was digested with XbaI and HindIII, separated on and purified from an agarose gel, and ligated into the multiple cloning sit of pGEM4-pSV0CAT (17). This method was used to construct the plasmids pB3-200, pB3-180, pB3-160, pB3-140, pB3-120, pB3-75, and pB3-75Δ1−pB3-75Δ7.

Construction of the plasmid, pB3-499ΔA required amplification of two fragments followed by a tripartate ligation into pSV0CAT as previously described for the construction of pB3-499Δ (15). The amplified fragments spanned from −499 to −75, and −75 to +110. The second fragment contained two point mutations in the putative AP-2 binding site (G to A at −52 and C to T at −56). These fragments were then ligated into pSV0CAT. After contruction of each reporter plasmid, approximately one hundred base pairs of the inserted fragment of each construct was sequenced to acertain fragment orientation and sequence of the mutated inserts.

DNA Transfection and CAT assay

Plasmids were prepared for transfection by double CsCl banding. For transfection of A375 cells, 10 μg of plasmid DNA from each TGF-β3 promoter/CAT chimeric plasmid was cotransfected with 1 μg of pXGH5 (an expression plasmid containing the human growth hormone gene under regulation of the mouse metallothionein I promoter, obtained from Nichols Institute Diagnostics, San Juan Capistrano, CA) as an internal control for transfection efficiency. For transfection of Schneider cells, the TGF-β3 promoter/CAT chimeric plasmid, pB3−120, or the plasmid pSV_2CAT (obtained from American Type Cultire Collection Rockville, MD), was cotransfected with either an AP-2 expression vector under regulation of the Drosophila alcohol dehydrogenase promoter or the vector alone (without an AP-2 insert; see (16)). In addition these cells were cotransfected with 1 μg of pMK18 (an expression plasmid containing the beta-galactosidase gene under control of the Drosophila β-actin gene, kindly provided by M. Horner) as an internal control. In both A375 cells and Schneider cells, DNA was transfected by calcium phosphate coprecipitation as described (18). The cells were exposed to the precipitate for 8−12 hours, washed until all precipitate was removed, and fed with fresh media. Cells were harvested two days later and CAT activity determined in the cell lysates. Cells transfections were repeated at least three times in all experiments shown with similar results.

Gel Retardation

Preparation of AP-2 and whole cell extracts. Purified AP-2 protein was prepared from HeLa cells as previously described (16). Whole cell extracts were prepared from A375 cells. Cultured A375 cells were scraped into phosphate buffered saline, centrifuged (1000×g for 5 minutes), the cell pellet washed with phosphate buffered saline, frozen on dry ice, and thawed/resuspended in a 5×volume of extraction buffer (10 mM Hepes, pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5.0% glycerol and 0.5 mM PMSF). The lysate was transfered to a precooled ultracentrifuge tube and centrifuged at 100,000×g for 5 minutes. The supernatant was then aliquoted, frozen, and stored at −70° C until use.

Gel retardation. Double stranded oligonucleotides (DS-oligos) were prepared from single stranded oligos by heating complementary oligonucleotides in a 50 mM NaCl solution at 95°C for 5 minutes and then allowing the oligonucleotides to cool slowly to room temperature. Alternatively, the TGF-β3 promoter fragment from −120 to −35 (120/35) was prepared by PCR using the plasmid pH17.4 (containing the TGF-β3 5′ flanking region) as a template, and separated on and purified from an agarose gel using the Genclean kit (Bio 101). For gel retardation, The DS-oligo containing an AP-2 binding site from the metallothionein II_A promoter, AP2, (supplied with the Gelshift kit, Stratagene, La Jolla, CA), or the TGF-β3 promoter fragment, 120/35, were labeled with ^{32}P using T_4 polynucleotide kinase as described (19). Labeled DNA, competitor DS-oligos, and cell extract were incubated 30 minutes in incubation buffer supplied with the Stratagene Gelshift kit and the resulting DNA/protein complexes analyzed on a non-denaturing polyacrylamide gel according to the protocol of the manufacturer.

RESULTS

Investigation of the role of the putative AP-2 consensus binding region in the TGF-β3 promoter

Mutations in the putative AP-2 binding region decrease promoter activity. In our previous description of the TGF-β3 promoter we noted the presence of an 8 bp sequence between −50 and −58 with complete homology to a previously described AP-2 binding site in the SV40 promoter ((15), see Figure 1A for a diagram of the proximal region of the TGF-β3 promoter). Deletion of

this region reduces promoter activity by 63% (compare pB3-60 and pB3-47 in Figure 4, and (15)). In order to further characterize the importance of this region in promoter activity, two mutations were introduced in this region (changing the G at −52 to A, and the C at −56 to T) using mutated PCR primers and cloned into the promoterless CAT vector pGEM4-pSV0CAT as described in the materials and methods. The resulting promoter/CAT chimeric plasmid, pB3-499ΔA, contains the same TGF-β3 promoter fragment as in an otherwise intact promoter fragment spanning from +110 to −499 except for these two

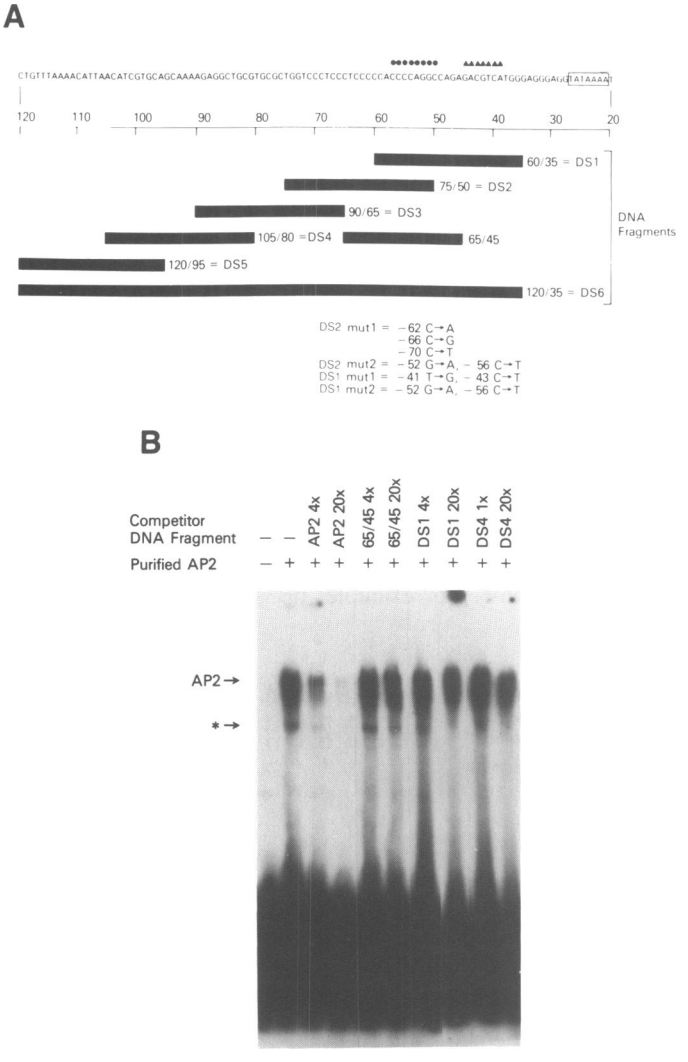

Figure 1. Gel retardation of AP-2 consensus sequence (AP2) by purified AP-2 protein and competition by TGF-β3 promoter fragments. **A.** Diagram of the proximal region of the TGF-β3 promoter and DNA double-stranded promoter fragments. The proximal region of the TGF-β3 promoter spanning the region from −120 to −20 is shown. The TATA box is boxed; the CRE and consensus AP-2 site are marked by *filled triangles* and *filled circles*, respectively. The DNA fragments of the promoter used in gel retardation studies are diagrammed and named below the promoter sequence, including the mutated fragments DS2 mut1, DS2 mut2, DS1 mut1, and DS1 mut2. **B.** ^{32}P labeled AP-2 consensus DNA (AP2) was incubated with purified AP-2 protein without or with the addition of the unlabeled competitor DNA fragments AP2 (the same AP2 consensus sequence as the labeled AP2 sequence), 65/45, DS1, or DS4. After incubation at room temperature for 30 minutes, the protein/DNA complexes were analyzed by non-denaturing polyacrylamide gel electrophoresis. The consensus AP2 DNA/purified AP-2 protein complex is marked (AP2) as well as a non-specific band (*). Labeled AP2 consensus sequence DNA fragment incubated without the addition of purified AP-2 protein is shown in the first lane (no retarded bands).

mutations in the putative AP-2 binding site. Mutation of these two nucleotides reduced CAT activity to 32% of the unmutated promoter/CAT plasmid (data not shown). Although mutations in the putative AP-2 binding site significantly reduced promoter activity, they did not render the promoter inactive as we had previously shown mutations in the more proximal CRE site do. We have previously suggested that a protein binding to the CRE might play a role in forming a preinitiation complex for the TGF-β3 promoter. While the putative AP-2 binding region does not appear to be as critical to promoter function, mutations in this region do significantly affect its activity.

Purified AP-2 does not bind to the TGF-β3 promoter. The potential for AP-2 to bind to the TGF-β3 promoter was studied by gel retardation. A double-stranded olgonucleotide (DS-oligo) containing an AP-2 binding site from the metallothionein promoter (designated AP2) was end-labeled with ^{32}P, and the binding of purified AP-2 protein to this fragment analyzed on a non-denaturing polyacrylamide gel. Competition of binding by unlabeled AP2 DS-oligo, or TGF-β3 promoter DS-oligos was then determined; a diagram of the proximal TGF-β3 promoter region and the promoter fragments used in these studies is presented in Figure 1A. Two retarded bands could be identified as the result of binding of purified AP-2 protein to the AP2 DS-oligo (Figure 1B, second lane). The lower of these two bands was identified in this and other experiments as binding of a contaminating protein nonspecifically to DNA. Binding to this band was competetively blocked by either excess unlabeled AP2 DS-oligo, or the TGF-β3 promoter fragments DS1 (containing the putative AP-2 binding site), and DS4 (not containing the putative AP-2 binding site). In contrast, the upper band reflected specific binding by AP-2 to the consensus AP-2 DS-oligo; this binding was competively blocked by excess unlabeled AP2 consensus DS-oligo, but not by the TGF-β3 promoter DS-oligos, DS1, 65/45 (both containing the putative AP-2 binding site), or

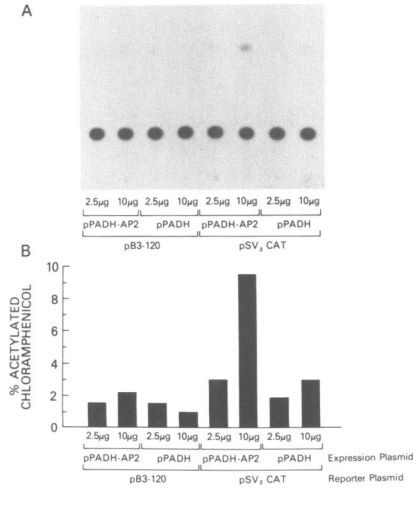

Figure 2. Failure of trans-activation of the TGF-β3 promoter by AP-2. Drosophila Schneider cells were transfected with either the TGF-β3 promoter/CAT construct, pB3-120, or the SV40 virus early promoter/CAT construct, pSV_2CAT. The cells were cotransfected with either the AP-2 expression plasmid, pPADH-AP2, or the same expression vector without insert, pPADH. In addition all the cells were cotransfected with the control plasmid pMK18. **A**, raw data, and **B**, graphical presentation of CAT activity from lysates after normalization to beta-galactosidase.

DS4 (Figure 1B). In other experiments, the purified AP-2 protein was allowed to bind to a labeled DS1 DNA fragment. This fragment gave only a single gel retarded band which comigrated with the lower band, indicating non-specific binding (data not shown). The non-specific binding of this protein could be competed with excess AP2, DS1, or DS4 fragment DNAs. We, therefore, consistently observed a lack of binding of purified AP-2 to fragments of DNA from the region of the TGF-β3 promoter containing the putative AP-2 binding site.

Lack of trans-activation of the TGF-β3 promoter by AP-2. In order to confirm the above results using a functional assay, the TGF-β3 promoter/CAT chimeric reporter plasmid (pB3-120) was co-transfected into Drosophila Schneider cells with either a Drosophila expression plasmid containing the AP-2 cDNA under regulation of the Drosophila alcohol dehydrogenase promoter, (pADH-AP2) or the same expression plasmid without the AP-2 cDNA insert. As a positive control, in parallel, a chimeric SV40 early promoter/CAT (pSV_2CAT) plasmid was co-transfected with either pPADH-AP2, or pPADH. The SV40 early promoter has previously been characterized as responsive to AP-2 (20). Although the AP-2 expression plasmid increased expression of CAT by SV_2CAT, it had no effect on expression of CAT by pB3-120 (Figure 2). These studies show that despite the presence of a consensus element for AP-2 binding, AP-2 protein neither binds, nor transactivates the TGF-β3 promoter.

The above series of experiments, therefore, show that the region of the previously identified consensus AP-2 binding site does regulate TGF-β3 promoter activity, but not through binding of AP-2 protein.

Deletion mapping of the region responsible for increased TGF-β3 activity in A375 cells

We have previously shown that A375 cells express and transcribe much higher levels of TGF-β3 mRNA than other tumor cell lines including HT1080, A549, A673, A498, and other cell lines (15). This increased transcriptional activity is mediated by a region between −221 and −60 of the TGF-β3 promoter (15). In order to more precisely define the promoter region responsible for mediating the enhanced transcription of TGF-β3 in A375 cells, a series of deletion mutant/CAT chimeric plasmids were constructed with deletions spaced throughout this region of the promoter.

We have previously described the TGF-β3 promoter/CAT chimeric plasmids, pB3-1387, pB3-499, pB3-301, pB3-220, pB3-91, pB3-60, pB3-47, and pB3-38. They consist of a series of plasmids each containing fragments of the TGF-β3 5′ flanking region starting 110 nucleotides 3′ of the TGF-β3 transcription start site (+110) and extending 5′ to the number indicated in the plasmid name. For example, pB3-499 contains a TGF-β3

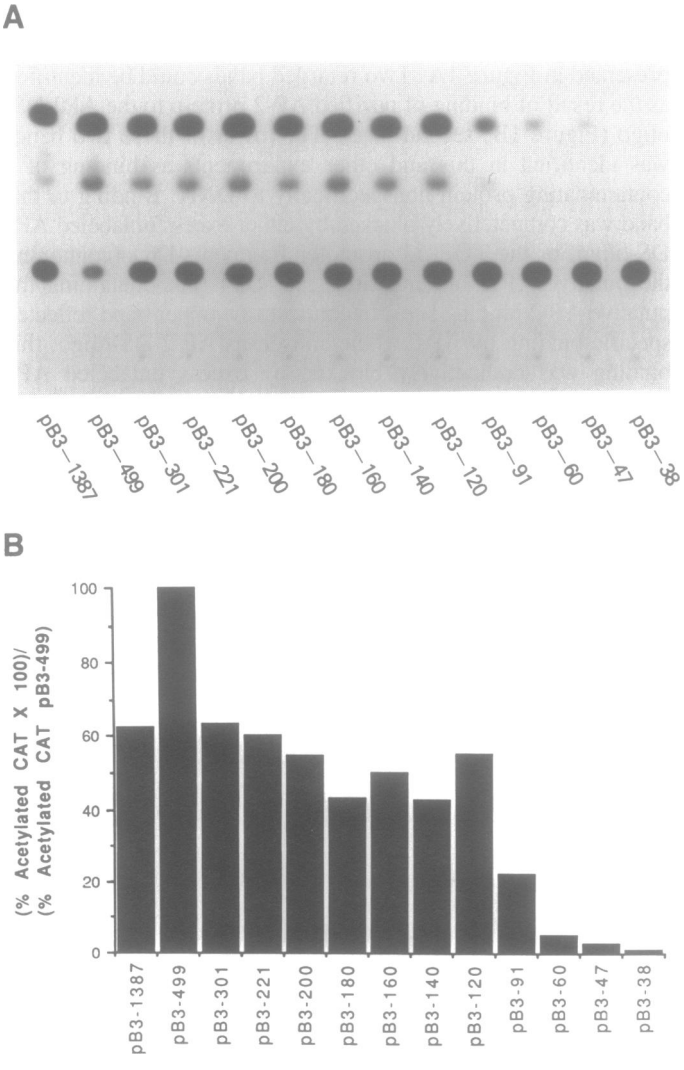

Figure 3. TGF-β3 promoter activity upon deletion of the promoter region between −221 and −120 A375 cells were transfected with TGF-β3 promoter constructs as labeled in the figure. **A.** shows raw data from a typical transfection, lysates were normalized to total protein prior to assay of CAT activity. **B.** shows the average CAT activity from two experiments after normalization to growth hormone expressed by the cotransfected control plasmid, pXGH5.

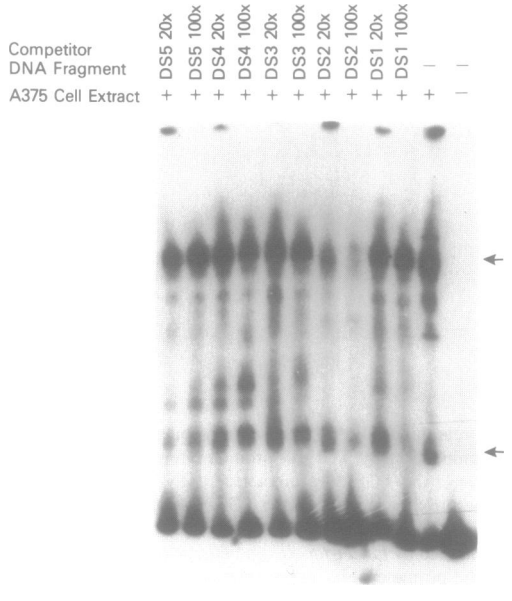

Figure 4. Gel retardation of the −120/−35 TGF-β3 promoter region by A375 whole cell extract and competition by TGF-β3 promoter fragments. A PCR generated, ^{32}P labeled TGF-β3 promoter fragment from −120 to −35 (120−35/DS6) was incubated with A375 whole cell extract without or with the addition of 20X and 100X unlabeled competitor DNA fragments DS1, DS2, DS3, DS4, or DS5 (TGF-β3 promoter fragments, see Figure 2A). The protein/DNA complexes were analyzed by non-denaturing polyacrylamide gel electrophoresis. Major retarded protein/DNA complexes are labeled with arrows.

promoter fragment spanning −499 to +110 of the transcriptional start site ligated to a CAT gene. To allow more precise mapping of the TGF-β3 promoter region between −91 and −220, TGF-β3 fragments with 5′ termini at −200, −180, −160, −140, or −120, and 3′ termini each at +110 were amplified by PCR and ligated into the promoterless CAT vector pGEM4-pSV0CAT as described in the materials and methods section. Keeping with the naming convention noted above, each plasmid was named according to its 5′ terminus; i.e., pB3-200 contains the TGF-β3 5′ flanking region between −200 and +110 ligated upstream from the CAT gene in pGEM4-pSV0CAT.

Each of these TGF-β3 promoter/CAT plasmids were transfected into A375 cells and CAT activity assayed 48 hours later (Figures 3A and B). Promoter activity was nearly constant upon deletion of each of 20 base pair fragment until the region between −120 and −91. These results indicate that virtually all of the promoter activity previously ascribed to the region between −220 and −91 was actually the result of promoter activation in the region between −120 and −91. These results in combination with our previous results (15), which demonstrated enhanced promomter activity mediated by the region between −91 and −60, show that the enhanced activity of the TGF-β3 promoter in A375 cells is attributable to the region betweeen −120 and −60.

Analysis of A375 cell proteins binding to the region between −120 and −35 of the TGF-β3 promoter

A375 cell extract binds to a region between −75 and −50 of the TGF-β3 promoter. Our work above shows that most of the promoter activity in the TGF-β3 promoter is relatively proximal to the transcriptional start site (between −120 and −35), and also shows that this region, although containing an AP-2 consensus binding sequence, is not actually bound or transactivated by AP-2. Further, the data above, in combination with our initial characterization of this promoter (15) show that the region responsible for increased promoter activity in A375 cells lies between −120 and −60 of the TGF-β3 promoter. Therefore, in order to identify potential DNA-binding proteins that might bind to this region in the TGF-β3 promoter, we prepared whole cell extract from A375 cells and tested the ability of this extract to bind to a fragment of the TGF-β3 promoter spaning −120 to −35, designated 120−35 (or DS6, Figure 1A). This fragment was endlabeled with ^{32}P and binding of proteins determined by gel retardation (Figure 4). We were able to identify several DNA/protein complexes using this extract (see arrows, second lane from the right, Figure 4). To test more specifically which region of the promoter was responsible for binding of the extract protein(s), overlapping 25 base pair DNA DS-oligos of this region were used to attempt to compete binding (see Figure 1A for a diagram of these fragments). The fragment DS2 was found to inhibit binding of the extract proteins almost completely in a dose dependent manner, including both high and low molecular weight complexes (Figure 4). DS1, and DS5, also partially inhibited binding of the lower protein/DNA complex, however, this was not consistent (see lack of competition by DS1 competition, Figure 5, also note DS5 inhibition, although seen at 20× of oligo, was not seen at 100× concentration).

A375 cell extract binding depends on a region containing a repeated TCCC element. DS2, the TGF-β3 oligo that specifically inhibited binding of A375 extract protein(s), contains a portion of the AP-2 consensus binding site and a region of three repeats

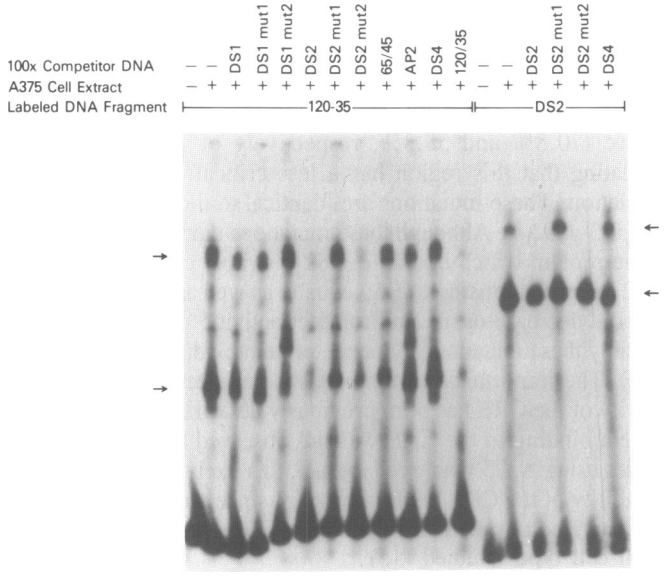

Figure 5. Gel retardation of the −120/−35 and DS2 TGF-β3 promoter fragments by A375 whole cell extract and competition by TGF-β3 promoter fragments. A PCR generated, ^{32}P labeled TGF-β3 promoter regions from −120 to −35 (120−35/DS6) and −75 to −50 (DS2) were incubated with A375 whole cell extract without or with the addition of 20X unlabeled competitor DNA fragments. The protein/DNA complexes were analyzed by non-denaturing polyacrylamide gel electrophoresis. Major retarded protein/DNA complexes are labeled with arrows.

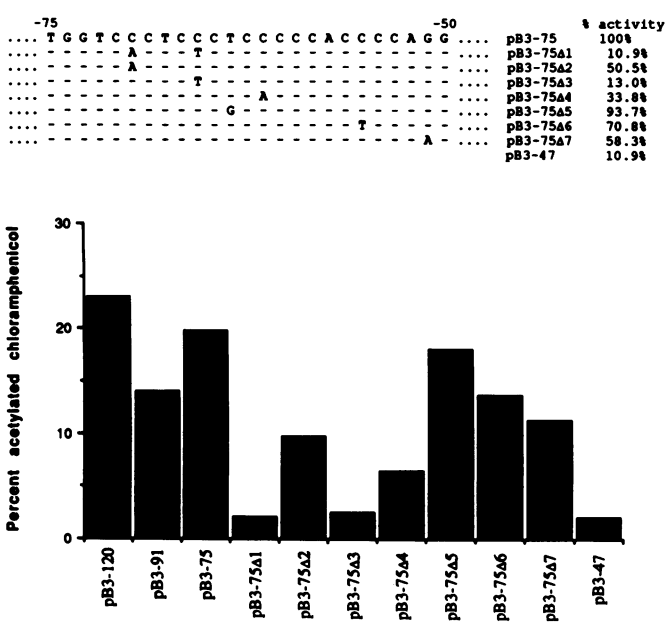

Figure 6. Effect of mutations in the region between −75 and −50 of the TGF-β3 promoter. A375 cells were transfected with the plasmids pB3-120, pB3-91, pB3-75, pB3-75Δ1−pB3-75Δ7, or pB3-47 along with the control plasmid pXGH5. 48 hours after transfection CAT activity was determined in cell lysates and normalized to growth hormone levels produced from the cotransfected pXGH5 plasmid. These results are presented graphically at the bottom of the figure. The mutations in each of the chimeric TGF-β3 promoter/CAT plasmids are diagrammed at the top of the figure. Adjacent to each deletion mutant is the percent of promoter activity compared to the native promoter construct, pB3-75.

of the sequence TCCC (see Figure 1A). Several additional oligos were also synthesized in order to investigate the potential importance of these two regions in binding protein(s) from the A375 cell extract. DS2 mut1 was mutated in each of the TCCC repeats, and DS2 mut2 was mutated in the previously identified consensus AP-2 site (see Figure 1A). Binding of the proteins to the TGF-β3 promoter fragment 120−35 (or DS6, Figure 1A) was again inhibited by unmutated DS2, and also DS2 mut2, but not DS2 mut1 (Figure 5). These results indicate that the TCCC repeat, but not the portion of the putative consensus AP-2 site in DS2, is important in mediating protein binding to the TGF-β3 promoter. Several mutated DS1 oligos were also synthesized including one mutated in the putative AP-2 biding site. None of these, including the native DS1, however, blocked protein binding (Figure 5). Also of note, the AP2 DNA fragment, a consensus AP-2 binding site from the metallothionein II_A promoter which we showed above does bind AP-2 protein, did not compete the binding of this protein. This result clearly shows that the protein(s) binding to the DNA fragment, 120−35/DS6, of the TGF-β3 promoter are distinct from AP-2.

DS2 was also labeled with ^{32}P and tested for its ability to specifically bind to protein(s) in the A375 cell extract. Two retarded complexes were identified (Figure 5). Only one of the protein-DNA complexes (the higher molecular weight complex) could be competed by unlabeled DS2; the other complex could not be competed by either DS2 or irrelevent oligonucleotide. This complex could also be competed by DS2 mut2, but not DS2 mut1 or DS4, indicating a sequence-specific DNA/protein interaction. These results are, therefore, consistent with the results using the longer DNA fragment (120−35/DS6, above) and show that a protein(s) in A375 cell extract binds specifically to a region in the TGF-β3 promoter between −75 and −50. The TCCC repeat in this region is critical for protein binding.

Analysis of mutations in the region between −75 and −50 of the TGF-β3 promoter

Deletion of the TGF-β3 promoter to −75 has similar promoter activity as deletion to −120. In order to correlate the binding of the protein we describe above with TGF-β3 promoter activity, a new deletion mutant was constructed, pB3-75, containing the TGF-β3 promoter region spanning from −75 to +110 ligated to the CAT gene (see materials and methods). This construct is deleted to contain a promoter fragment terminating 5′ of the T-CCC repeat motif (see Figure 1A), and upon transfection into A375 cells, expressed the CAT gene at a similar level as the TGF-β3 promoter/CAT plasmid containing −120 as its 5′ terminus (Figure 6). This is in contrast to the construct pB3-91 which expresses less activity than either pB3-120, or pB3-75 (Figures 6 and 3). The 9.2 fold increase in promoter activity associated with the region between −75 and −60, therefore, can account for all of the increased activity of the TGF-β3 promoter in A375 cells (versus other tumor cell lines) that we had originally attributed to the region between −221 and −120. Deletion of the regions between −120 and −75 has both positive (deletion from −91 to −75) and negative (deletion from −120 to −91) effects on promoter regulation; however, the magnitude of this regulation is small compared to the larger increase in promoter activity associated with the region between −75 and −60. This complex regulation might be the result of binding of several different proteins binding to these regions, although we did not see any evidence of such proteins binding from the gel retardation studies of whole cell extracts (Figures 4, and 5). Alternatively, this complex pattern of regulation might simply result from subtle changes in the binding affinity of the protein(s) that bind to the more proximal region of the promoter (−75 to −50) resulting from changes in the secondary or tertiary structure of the DNA in the various deletion constructs. Since we had previously shown that the increased promoter activity was 5′ to −60 (15), we have effectively defined the region critical for positive promoter activation in A375 cells to a 15 base pair sequence between −75 and −60.

Point mutations in the −75/−50 regulatory element that inhibit protein binding also inhibit promoter activity. Several mutated TGF-β3 promoter/CAT chimeric plasmids were constructed in order to understand the functional significance of mutations in the region between −75 and −50 in relation to the effect of these mutations on inhibition of protein binding as shown by gel retardation (fragment DS2, Figures 4, 5). Each of the constructs in this series span the TGF-β3 promoter region from −75 to +110, but each contains a different point mutation in the region between −75 and −50 as shown in Figure 6, top panel. These plasmids were transfected into A375 cells and the activity of the mutated plasmids compared to that of the unmutated construct, pB3-75, and the construct in which this entire region is deleted, pB3-47. Most notably, activity of the construct pB3-75Δ1, which has mutations in the middle C nucleotides of the two most 5′ TCCC repeats (two of the three nucleotides mutated in the DNA fragment DS2 mut1) was reduced to that of pB3-47 (Figure 6, 10.9% activity of pB3-75). A single mutation in the more 3′ of these two TCCC repeats (the middle of three TCCC repeats) was able to account for almost all of this decrease in activity (pB3-75Δ3). Single base pair mutations of the middle C nucleotide of the 5′ and 3′ TCCC repeats had moderate, but less dramatic effects (pB3-75Δ2, 50.5%, and pB3-75Δ4, 33.8% of pB3-75 activity) on promoter activity. In contrast, mutation of the T nucleotide in the most 3′ TCCC repeat had very little effect on promoter activity (pB3-75Δ5, 93.7% compared to pB3-75).

Mutations in the more proximal region corresponding to the previously identified putative AP-2 binding site (pB3-75Δ6 and pB3-75Δ7) also decreased activity of the promoter, but to a lesser degree (70.8% and 58.3%, respectively of pB3-75 activity), indicating that this region has a less critical role in promoter regulation. These mutations are identical to those in DS2 mut2, and pB3-499ΔA. Although these mutations did not affect binding of the protein which bound to DS2 in whole cell extracts (DS2 mut2 competed binding to DS2 equally as well as DS2, Figure 5), they did decrease upstream promoter activity (described above). Possibly these mutations influence protein binding to the adjacent CRE. The importance of the sequence adjacent to the CRE to activity of the CRE has been previously described (21). Although protein binding to the CRE was not observed in gel retardation studies using A375 whole cell extract, this likely reflects a relative instability of the CRE binding protein, since we have previously characterized this element to be critical in basal promoter activity in all cell types studied (15).

DISCUSSION

In this paper we identify a previously undescribed DNA binding protein which binds to a trimeric repeat of the sequence TCCC found in the region between −75 and −50 of the TGF-β3 promoter. Mutation of the 3xTCCC motif both dramatically decreased promoter activity (9.2-fold) and inhibited binding of

the sequence specific binding protein, indicating that the protein binding to this motif is likely responsible for the high level of expression of TGF-β3 mRNA in the A375 cell line. Although the new DNA binding protein we identify binds to a DNA fragment of the TGF-β3 promoter sequence that includes 7 of the 8 bases previously identified as a consensus AP-2 binding site, this protein does not bind to a DS-oligo that contains an AP-2 binding site from the metallothionein II_A promoter. This is consistent with our data showing sequence specific binding to the 3×TCCC motif, and further demonstrates that the protein binding to this region is a novel sequence specific DNA binding protein , apparently unrelated to AP-2.

In the absence of any structural information about this protein, we do not have any direct data indicating the nature of the DNA/protein interaction; however, the repetitive appearance of the TCCC sequence in the binding motif suggests that the DNA/DNA-binding protein interaction may be stabilized by multiple lower affinity interactions with the three TCCC repeats. We might, therefore, speculate either that multiple homologous sequences in one protein, or three similar, or identical proteins might bind cooperatively to this region. The nature of this interaction can be assessed only after cloning and expression of this novel DNA binding protein.

Immediately 3′ to the 3×TCCC motif is an AP-2 consensus sequence, previously suggested to be important in regulation of the TGF-β3 promoter. However, we now provide several independent lines of evidence showing that AP-2 does not directly regulate the TGF-β3 promoter. First, we show that purified AP-2 protein does not bind to the AP-2 consensus sequence in the TGF-β3 promoter, but does bind to a previously described AP-2 binding site in the metallothionein II_A promoter. The AP-2 binding site from the metallothionein II_A promoter contains the sequence, 5′-CCGCGGGC-3′; this is slightly different from the sequence, 5′-CCCCAGGC-3′, which is found in the SV40 early enhancer and is the basis for our original indication of a potential AP-2 binding site in the TGF-β3 promoter. Of note in this regard, we show that AP-2 expressed in Drosophilia cells, although able to transactivate the SV40 promoter (which has the identical AP-2 consensus binding site as the TGF-β3 promoter) was not able to trans-activate the TGF-β3 promoter. It, therefore, appears that promoter sequences outside of the previously identified core consensus AP-2 binding site of the SV40 enhancer can affect DNA transactivation by AP-2.

In summary, we have mapped the region of DNA responsible for high level expression of TGF-β3 in A375 cells to a 25 base pair element in the proximal region of the promoter. A sequence specific protein binds to this region, and it is likely that this protein regulates aspects of the developmental and tissue-specific expression of TGF-β3.

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