Identification and Characterization of the Chicken Transforming Growth Factor-β3 Promoter

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The promoter regions of the three mammalian transforming growth factor-β genes (TGF-βs 1, 2, and 3) have been recently cloned and characterized. The sequences show little similarity, suggesting different mechanisms of transcriptional control of these genes. To study differences in transcriptional regulation of mammalian and avian TGF-β, we have cloned and sequenced the 5′-flanking region of chicken TGF-β3. Characterization of this region showed a TATA box and cAMP-responsive element (CRE) and AP-2 binding site consensus sequences starting at 12 and 28 base pairs, respectively, upstream from the TATA box. Moreover, four additional AP-2-like sites, 10 binding sites for the transcription factor Sp1, as well as two AP-1-like sites were also identified. Except for 32 base pairs of identity centered around the TATA box and CRE site and four other relatively small regions of identity, the chicken TGF-β3 promoter was found to be structurally very different from the human TGF-β3 promoter. Promoter fragments were cloned into a chloramphenicol acetyltransferase reporter plasmid to study functional activity. Basal transcriptional activity of the promoter was regulated in quail fibrosarcoma QM7 cells and in human adenocarcinoma A375 cells by multiple upstream elements including the TATA, CRE, and AP-2 sites. As in the human TGF-β3 promoter, the CRE site showed activation by forskolin, an effect which could be shown by expression of TGF-β3 mRNA in cultured chicken and quail cells as well. Our results indicate a complex pattern of transcriptional regulation of the chicken TGF-β3 gene and suggest that differences in the regulation of expression of the genes for mammalian and avian TGF-β3 may result in part from the unique structure of their 5′-flanking regions. (Molecular Endocrinology 6: 1285–1298, 1992)

INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide growth factor and has been shown to regulate the growth and proliferation of numerous cell types (for reviews, see 1–3). The TGF-β super gene family contains five closely related members, TGF-βs 1, 2, 3, 4, and 5, and an increasing number of structurally related, but functionally distinct, polypeptides. The multiple isoforms of TGF-β expressed in vertebrate cells show 64–82% identity with one another and share similar biological activities which seem to be mediated through the same set of receptors (4–7). The high degree of conservation of the mature TGF-β isoforms suggests the existence of evolutionary pressure to conserve distinct biological properties in each isoform.

TGF-β has been demonstrated to influence the growth and differentiation of many different cell types, suggesting that it may play an important role in such processes as myogenesis, chondrogenesis, osteogenesis, hematopoiesis, epithelial cell differentiation, and adipogenesis, as well as in wound healing and embryogenesis (reviewed in Ref. 1). Recent studies comparing developmental expression of TGF-βs 1, 2, and 3 by immunohistochemical and *in situ* hybridization analyses indicate a complex pattern of expression for each isoform in developing mammals (8–15). Although these three TGF-β isoforms are sometimes coexpressed, the temporal pattern of specific cell types involved are often distinct. Because the TGF-βs can act on nearly all cells, many mechanisms may exist for precise control of their activity. Based on immunohistochemical and *in situ* hybridization analyses, it has been hypothesized that the differential expression of these genes may be of greater importance than the functional specificity of each isoform. To investigate the mechanistic basis for the differential regulation of expression of the TGF-β isoforms, the 5′-flanking regions of the human genes for TGF-β1, 2, and 3 have been cloned, and the sequence analyses have identified significant differences in the regulatory regions of these genes (16–20).

The majority of research dealing with the structure, function, and regulation of TGF-β has focused on mammalian TGF-βs. In order to be able to study the role of the TGF-βs in the avian embryo, we have recently cloned and characterized avian homologs of TGF-β to facilitate more detailed analysis of their developmental

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roles (21–24). We have recently reported coordinate expression of TGF-βs 2, 3, and 4 in chicken embryo chondrocytes and myocytes, both *in vitro* and *in vivo* (25). The level of expression of TGF-β3 mRNA was shown to be higher than that of the mRNAs for TGF-βs 2 or 4 in both the chicken embryo heart and sternum, as well as in other embryonic tissues, suggesting that TGF-β3 may have a prominent role during chicken embryogenesis.

To understand the mechanisms underlying the differential regulation of expression of one of the TGF-β isoforms, TGF-β3, in avian and mammalian species, we have characterized the 5′-flanking region of the chicken TGF-β3 gene. We now report the cloning, sequence, structure, and basal activity of the chicken TGF-β3 promoter. We show that although the chicken and human TGF-β3 5′-flanking regions share some common structural features, there are substantial differences which may be responsible for the observed unique expression patterns of this gene in mammalian and avian species.

RESULTS

Isolation of Genomic Clones Containing the 5′-Flanking Region of the Chicken TGF-β3 Gene

Screening of a chicken liver genomic library with a full-length chicken TGF-β3 cDNA resulted in the isolation of four clones. These clones, which might contain 5′-flanking sequences, were also chosen by hybridization to the first exon and rescreened with an oligonucleotide from the 5′-untranslated region of TGF-β3 cDNA. Based on these multiple hybridization procedures, a 3.7-kilobase (kb) *Pst*I fragment of one of the clones called pC4.24 was selected for further analysis and subcloned into pBluescript II (Fig. 1A). Preliminary restriction enzyme analysis of this clone showed it contained a 1.7-kb *Xho*I fragment that hybridized strongly with an oligonucleotide from the 5′-untranslated region of TGF-β3 cDNA using Southern blot analysis (data not shown). Sequence analysis of pC4.24 showed that it extended into the first intron and contained 342 nucleotides of the first exon of TGF-β3, 39 nucleotides of the 5′-untranslated region that has been previously reported (22), and, in addition, 1430 nucleotides 5′ to that previously reported (Fig. 1B).

Transcriptional Start Site and Analysis of the 5′-Flanking Region

The transcriptional start site of the chicken TGF-β3 gene was determined by S1 nuclease protection analysis. The start site was located using two different fragments of TGF-β3 DNA as probes. Using these two probes, a single S1 nuclease-resistant fragment was observed after hybridization with RNA isolated from either cultured primary chicken embryo chondrocytes or myocytes (Fig. 2); the S1 nuclease-resistant fragment was about eight nucleotides smaller when protected with the *Xho*I-*Sty*I fragment compared to the *Xho*I fragment. Using this approach, the transcriptional start site could be mapped to within five nucleotides. S1 nuclease protection analysis using fragments 3′ to the *Xho*I site at nucleotide +227 gave full-length protection of the probe, confirming our observation that the TGF-β3 gene has no additional transcriptional start sites 3′ to the one identified (data not shown).

Examination of the chicken TGF-β3 gene sequence 5′ to the translational initiation codon revealed several notable features. Whereas the 132-base pair (bp) 5′-untranslated region has a G + C content of 89%, the remainder of the sequence has a G + C content of 77%. Another region of high G + C content was noted further upstream in the sequence between nucleotides −47 and −344. Potential regulatory elements in the 5′-flanking region were identified by computer analysis (Fig. 1B). An AP-1-like binding site which differed from the AP-1 consensus sequence (TGAC/GTCA) by only one nucleotide was identified 10 nucleotides upstream from the transcriptional start site. Another AP-1-like sequence, which differed from the AP-1 consensus sequence by two nucleotides, was located at nucleotide −23. A consensus TATA box was identified 359 bp upstream of the transcriptional start site. Eighteen base pairs further upstream of the TATA box, a consensus sequence for a cAMP-responsive element/activating transcription factor (CRE/ATF) binding site was located. A sequence for an AP-2-like binding site was identified 17 bp upstream of the CRE/ATF binding site. Furthermore, 17 additional AP-2-like binding sites were located further upstream at nucleotides −780, −835, −849, −858, −869, −907, −934, −938, −971, −983, −988, −1002, −1029, and −1229, as well as three AP-2-like binding sites downstream of the CRE/ATF binding site at −201, +16, and +76. The AP-2-like sequences at −1229, −1001, −780, and +16 differed from the AP-2 consensus sequence (CCCCAGGC) by only one nucleotide, while the other potential AP-2-like sequences differed by two or three nucleotides. The sequence GGGCGG and its reverse complement, corresponding to sequences identified as binding sites for the transcription factor Sp1, are found 10 times in the sequence at nucleotides +119, +86, +55, −115, −217, −475, −522, −632, −997, and −1104. A region of seven GGA repeats was located between nucleotides −506 and −486 flanked by two GGC repeats immediately downstream and by four GGC repeats within two nucleotides upstream. Furthermore, three additional GGA repeats were identified between nucleotides −1039 and −1031.

Comparison of Chicken and Human TGF-β3 5′-Flanking Region DNA

A computer search for similarity between the chicken and human TGF-β3 genes was performed using the programs COMPARE and DOTPLOT (26) and is presented in Fig. 3A. Dot-plotting is the best way to see all the structures in common between two sequences.

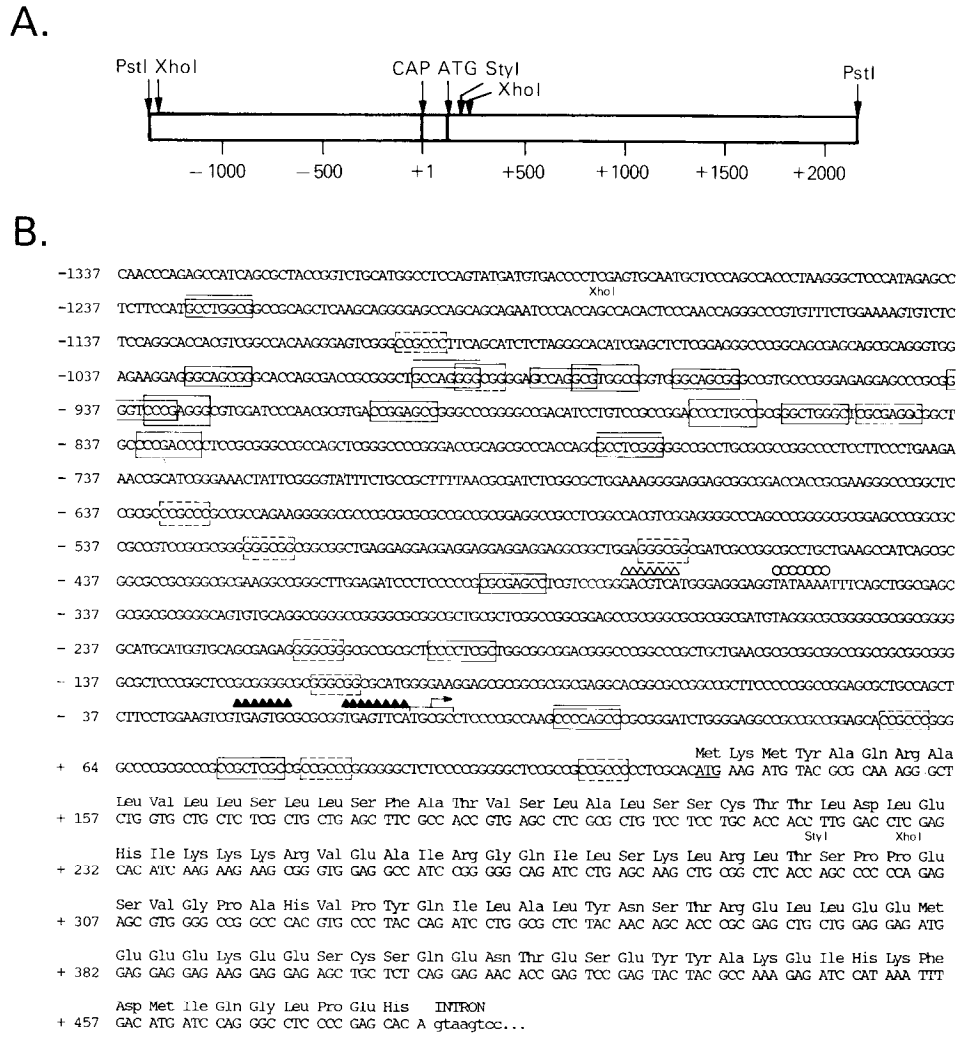

Fig. 1. Restriction Enzyme Map and Nucleotide Sequence of the 5′-Flanking Region of the Chicken TGF-β3 Gene

A, The plasmid pC4.24 containing the 3.7-kb *PstI-PstI* fragment of clone C4 is diagrammed showing the *XhoI* and *StyI* restriction enzyme sites, the transcriptional initiation (CAP) site (*arrow*), and the translational initiation (ATG) site (*underlined*). B, The nucleotide sequence of the chicken TGF-β3 promoter region containing 1337 nucleotides 5′ of the transcriptional initiation site was determined by the dideoxy chain termination method. Shown also is the sequence of the 5′-untranslated region, a portion of which has been previously reported (22), the initial 117 amino acids of the coding region, and the initial eight nucleotides of the first intron. The TATA box is indicated by *open circles*. The AP-1, AP-2, CRE, and Sp1 sites are indicated by *closed triangles*, *closed boxes*, *open triangles*, and *dashed boxes*, respectively. The AP-2 sites that differ from the AP-2 consensus sequence by one nucleotide are indicated by *raised lines* above closed boxes.

The program COMPARE analyzes two sequences in every register, searching for all the places (represented as dots) where a given number of matches (stringency) occurs within a given range (window) (27). The region containing a relatively high density of dots in Fig. 3A between nucleotides −1 and −1000 in the chicken TGF-β3 5′-flanking region and nucleotides −300 and −900 in the human TGF-β3 5′-flanking region corresponds to regions that have high G + C contents in these genes, thereby giving similarity that is statistically random. The *diagonal line* in the upper right corner of Fig. 3A represents similarity starting 108 nucleotides 5′ of the transcriptional start site of the chicken TGF-β3 gene and extending to the translational initiation codon and into the amino terminus of the encoded protein. Regions of identity in this diagonal are shown in detail in Fig. 3B. In addition, a small region of identity was found in the 5′-flanking region centered around the TATA box and CRE consensus sequences between nucleotides −377 and −346 in the chicken and nucleotides −45 and −14 in the human. A comparison of the nucleotide sequence of this region of the two genes in Fig. 3B shows 32 uninterrupted identical nucleotides starting at the CRE sequence and extending seven nucleotides beyond the TATA box in each gene. Four additional smaller regions of identity were located immediately downstream of the

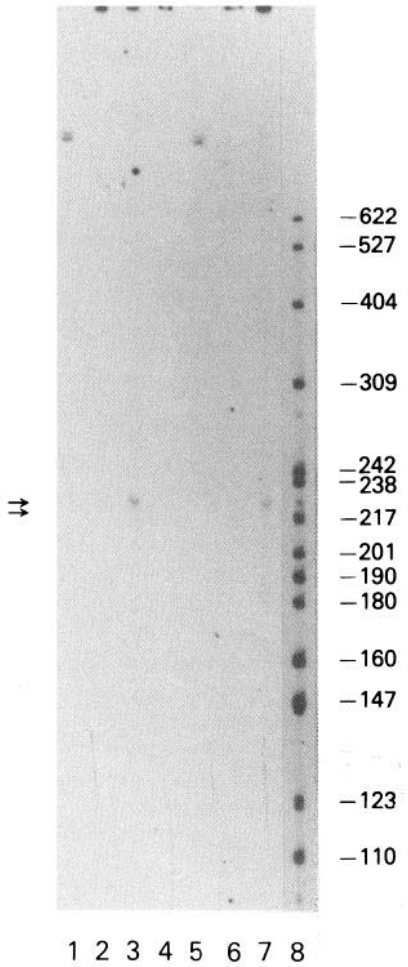

Fig. 2. S1 Nuclease Mapping of the TGF-β3 Transcriptional Start Site

The 1508-bp *Xho*I DNA fragment (lane 1) was used as a hybridization probe for yeast tRNA (lane 2), RNA isolated from cultured primary chicken embryo chondrocytes (lane 3), and RNA isolated from cultured primary chicken embryo cardiac myocytes (lane 4). The 1500-bp *Xho*I-*Sty*I DNA fragment (lane 5) was used as a hybridization probe for yeast tRNA (lane 6) and RNA isolated from primary chicken embryo chondrocytes (lane 7). A *Msp*I digest of pBR322 DNA (lane 8) was used as a size marker. Fragments of approximately 228 and 220 nucleotides indicated by the *arrows* were protected by using the *Xho*I and *Xho*I-*Sty*I fragments, respectively.

Sp1 site at nucleotide −114 and extending to the translational initiation site which showed about 60% identity with the human TGF-β3 gene (Fig. 3B).

The structure of the 5′-flanking region of the chicken and human TGF-β3 genes is represented diagrammatically in Fig. 3C. It is notable that while the human TGF-β3 gene has a relatively long 5′-untranslated region (1104 bp), the 5′-untranslated region in the chicken TGF-β3 gene is relatively short (132 bp). Also, unlike the human TGF-β3 5′-untranslated region, which contains 11 open reading frames (28), there are no open reading frames in the 5′-untranslated region of the chicken TGF-β3 gene. Also notable is the significantly larger number of potential AP-2-like sequences in the chicken TGF-β3 gene compared to the human TGF-β3 gene. In addition, while the chicken TGF-β3 5′-flanking region contains 10 potential Sp1 binding sites, three of which are located in the 5′-untranslated region, the human TGF-β3 5′-flanking region contains three Sp1 binding sites, and none of these are located in the 5′-untranslated region. One of the two AP-1-like sequences of the chicken TGF-β3 gene is also present in the human TGF-β3 gene.

Construction of Chimeric TGF-β3/Chloramphenicol Acetyltransferase Reporter Plasmids

Chimeric TGF-β3 5′-flanking region/chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed in order to identify important regulatory regions within the 5′-flanking sequence. Two sets of 5′-flanking fragments were generated; the first by amplification using the polymerase chain reaction (PCR) and the second by Restriction enzyme digestion (Fig. 4). Using PCR, several different fragments were amplified that contained specific elements of the 5′-flanking region. For example, a 5′-oligonucleotide was synthesized terminating at nucleotide −370 so that the resulting amplified fragment contained only the TATA box (Fig. 4). In similar fashion, other 5′-oligonucleotides were synthesized so that upon amplification by PCR, the resulting fragments contained only the TATA box and CRE consensus sequences (oligonucleotide terminating at nucleotide −384) and the TATA, CRE, and AP-2 consensus sequences (oligonucleotide terminating at nucleotide −406). In addition, in order to investigate the potential Sp1 consensus sequences, a series of 5′-oligonucleotides were synthesized that contained increasing numbers of Sp1 binding sites (oligonucleotides terminating at nucleotides −406, −497, −577, and −859 containing 0, 1, 2, and 3 Sp1 binding sites, respectively).

Because of the high G + C content in the 5′-flanking region of chicken TGF-β3 between nucleotides −317 and +132, which includes the transcriptional start site, it was not possible to amplify fragments using PCR in this region. Instead, a series of fragments was generated by restriction enzyme digestion that shared the same *Sma*I site at their 3′-ends at nucleotide +60 and terminated at the 5′-end at nucleotides −285, −346, and −379 so that the resulting fragments contained the transcriptional start site, 3 Sp1, 2 AP-1-like, and 2 AP-2-like consensus sequences (terminating at nucleotides −285 and −346) and, in addition to these consensus sequences, the TATA and CRE consensus sequences (terminating at nucleotide −379).

Activity of the TGF-β3 Promoter

Three different cell types, including quail QM7, chicken embryo chondrocytes, and human A375 cells, were selected for analysis of promoter activity based on their expression of high levels of TGF-β3 mRNA (data not shown). The TGF-β3-CAT chimeric plasmids described

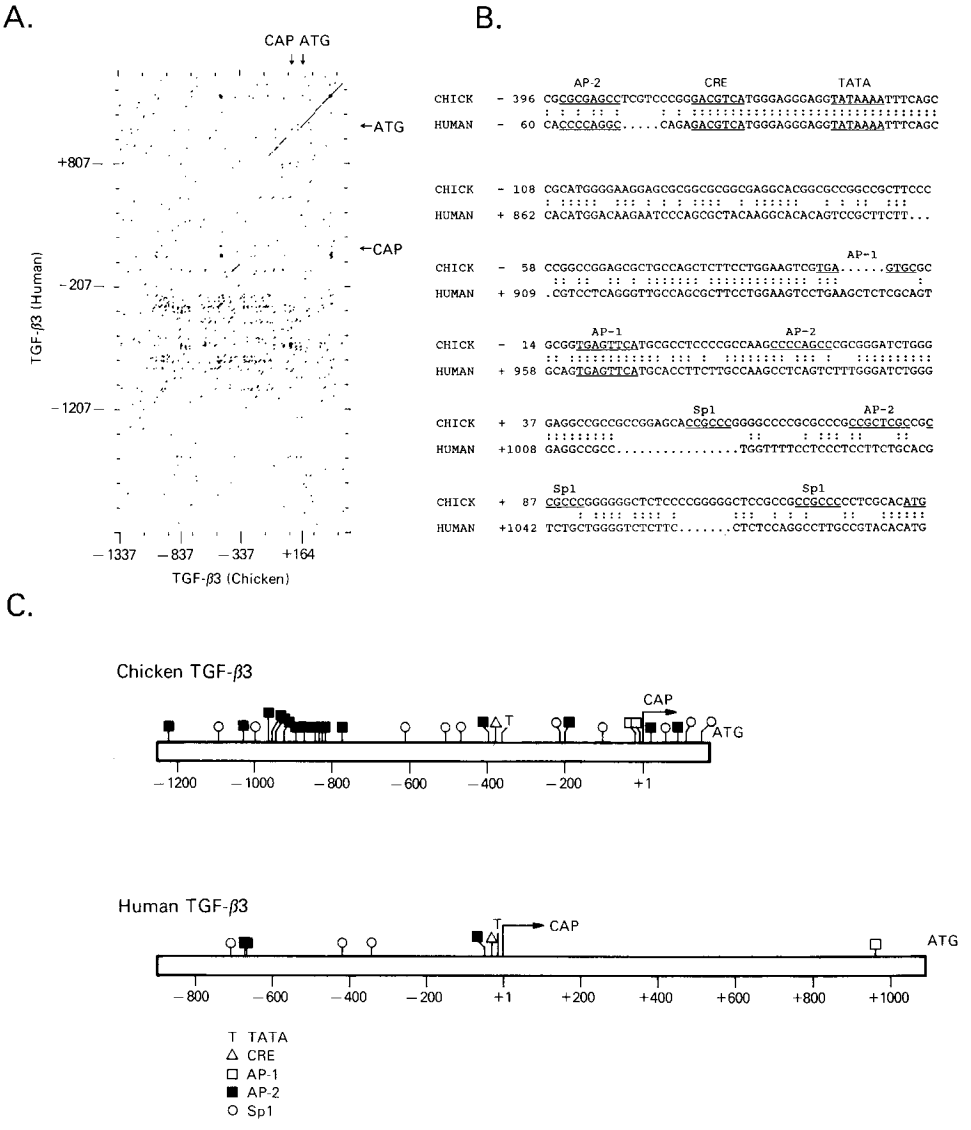

Fig. 3. Comparison of the Chicken and Human TGF-β3 Promoters

A, The chicken and human TGF-β3 promoters are compared using dot matrix analysis. The sequence comparison (COMPARE program from the Genetics Computer Group sequence analysis package) includes 349 nucleotides upstream of the translational initiation codon for the chicken sequence (X axis) and 349 nucleotides upstream of the translational initiation codon for the human sequence (Y axis). Each *dot* represents a match of 14 nucleotides (stringency) within a 21-nucleotide stretch (window size). The transcriptional initiation site (CAP) and translational initiation site (ATG) are indicated. B, Sequence identity between the chicken and human TGF-β3 proximal promoter regions. Alignment was determined by the BESTFIT program from the Genetics Computer Group sequence analysis program (gap wt, 5.0; length wt, 0.3). The chicken sequence is shown *above* the human sequence. Potential transcription factor binding sites are indicated. C, A diagrammatic representation of the consensus sequences for DNA binding proteins in the chicken and human TGF-β3 5′-flanking regions. The translational (ATG) and transcriptional (CAP) initiation sites are indicated along with the TATA, CRE, AP-1, AP-2, and Sp1 consensus sequences.

above were transfected into QM7 and A375 cells, and after 48 h the cells were harvested and the CAT activities were determined. As shown in Fig. 5, A and B, in QM7 cells, the plasmids containing the transcriptional start site, pβ3–285 (3 Sp1, 2 AP-1-like, and 2 AP-2-like sequences) and pβ3–346 (3 Sp1, 2 AP-1-like, and 2 AP-2-like sequences) showed minimal CAT activity, while pβ3–379 (3 Sp1, 2 AP-1-like, 2 AP-2-like, TATA, and CRE sequences) showed a higher level of CAT activity. Similar results were obtained upon transfection of A375 cells (data not shown). These results suggest that sequences downstream of nucleotide −347 in the chicken TGF-β3 gene, which includes the transcriptional start site, do not contribute significantly to promoter activity in these cells. QM7 cells were also transfected with the plasmid pβ3–499, a chimeric human TGF-β3 promoter/CAT plasmid containing TATA, CRE, AP-2-like, and 2 Sp1 sequences, which has been demonstrated to include important regulatory elements of the human TGF-β3 promoter (19). The plasmid pβ3–499,

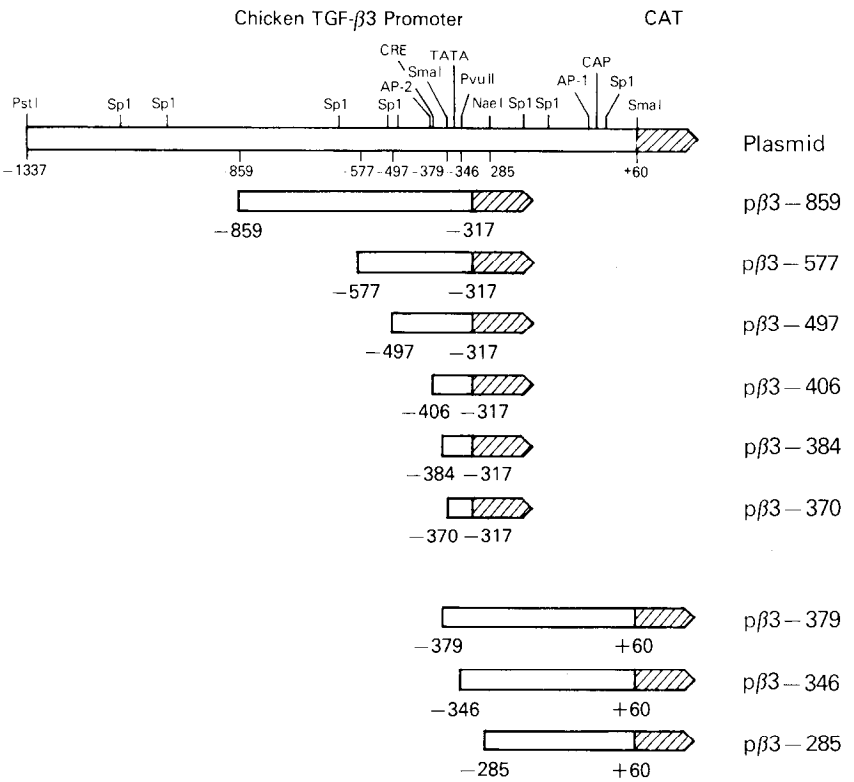

Fig. 4. Chimeric Chicken TGF-β3 Promoter/CAT Reporter Plasmids

The promoter constructs generated for construction of TGF-β3/CAT reporter plasmids are shown. The first set of constructs (pβ3–859, pβ3–577, pβ3–497, pβ3–406, pβ3–384, and pβ3–370) was generated by PCR using pC4.24 as a template and oligonucleotides from different upstream regions to establish different 5′-extensions along with a common oligonucleotide at the 3′-end. The 3′-oligonucleotide extends from nucleotides −338 to −318. The second set of constructs (pβ3–379, pβ3–346, and pβ3–285) was generated from restriction fragments (*Sma*I, *Pvu*II, and *Nae*I) with a common 3′-end and includes the transcriptional start site.

like pβ3–379, showed a high level of CAT activity (Fig. 5, A and B), demonstrating that the human TGF-β3 promoter can also function in avian cells.

To determine whether the two 2 AP-1-like sequences at nucleotides −23 and −10 might be involved in regulation of the chicken TGF-β3 gene by phorbol esters, QM7, A375, and CCL-64 cells were transfected with pβ3–285, pβ3–346, and pβ3–379, and incubated with either 12-*O*-tetradecanoylphorbol 13-acetate or dimethylsulfoxide (DMSO). No change was observed in the level of CAT activity of all three chimeric plasmids whether the cells were incubated with or without 12-*O*-tetradecanoylphorbol 13-acetate, suggesting that these two AP-1-like sites do not confer phorbol ester responsiveness (data not shown).

The TGF-β3-CAT chimeric plasmids corresponding to regions further upstream in the chicken TGF-β3 gene were also transfected into QM7 and A375 cells. As shown in Fig. 6A, the plasmids pβ3–370, pβ3–384, and pβ3–406, containing just the TATA sequence, the TATA and CRE sequences, and the TATA, CRE, and AP-2-like sequences, respectively, showed increasing levels of activity. A significant increase in the level of CAT activity was also found in the plasmid pβ3–497, which contains, in addition to the TATA, CRE and AP-2-like sequences, and an additional AP-2-like and Sp1 consensus sequence. In contrast, addition of the region between nucleotides −497 and −859 in plasmids pβ3–497 and pβ3–859 caused a decrease in CAT activity compared to pβ3–497. These plasmids contained additional AP-2-like and Sp1 consensus sequences and high G + C content in addition to the TATA, CRE, and AP-2-like sequences. All of these constructs showed similar effects in both QM7 and A375 cells, except that the level of CAT activity was consistently lower in QM7 cells compared to A375 cells. All activity of pβ3–384 was lost upon mutation of two nucleotides in the CRE sequence of pβ3–384 when transfected into both A375 and QM7 cells (Fig. 6, B and C).

Sp1 Binding Sites in the TGF-β3 Promoter

In order to examine potential Sp1 binding sites in the TGF-β3 5′-flanking region, a cell line derived from *Drosophila* embryos (Schneider line 2) was used as recipient cells for transfection of the chicken TGF-β3 5′-flanking region/CAT plasmids including pβ3–406, pβ3–497, pβ3–577, and pβ3–859, containing zero, one, two,

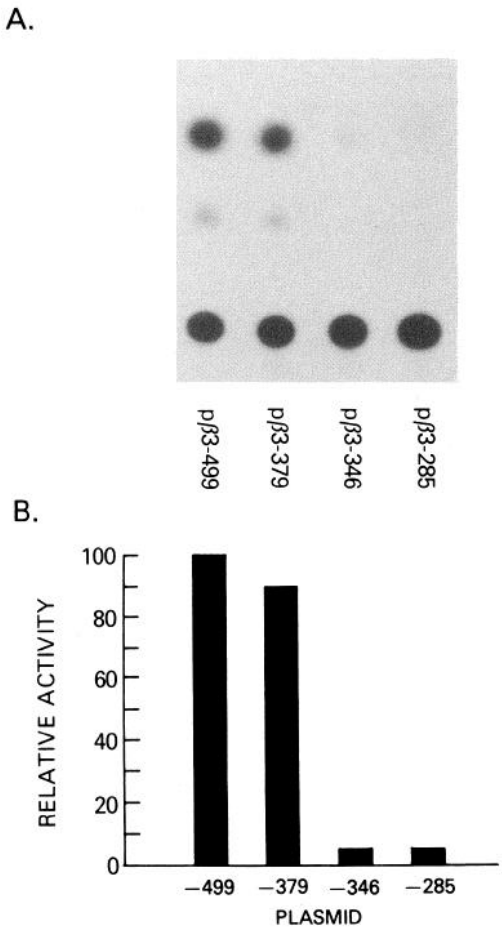

Fig. 5. Activity of the TGF-β3 Promoter

A, Raw data; B, graphical presentation of CAT activity in cell lysates of QM7 cells 48 h after transfection with the TGF-β3-CAT deletion mutant plasmids indicated. Cells were transfected with the plasmids pβ3–285, pβ3–346, and pβ3–379. (All plasmids contain the transcriptional initiation site, and only pβ3–379 contains TATA and CRE sequences.) The plasmid pβ3–499 is a chimeric human TGF-β3 promoter/CAT plasmid containing TATA and CRE sequences. All cells were cotransfected with the plasmid pXGH5, and cell lysates were normalized to GH levels before CAT activity analysis. The values represent percentages of the activity expressed by the strongest (100%) and are the averages of three independent assays.

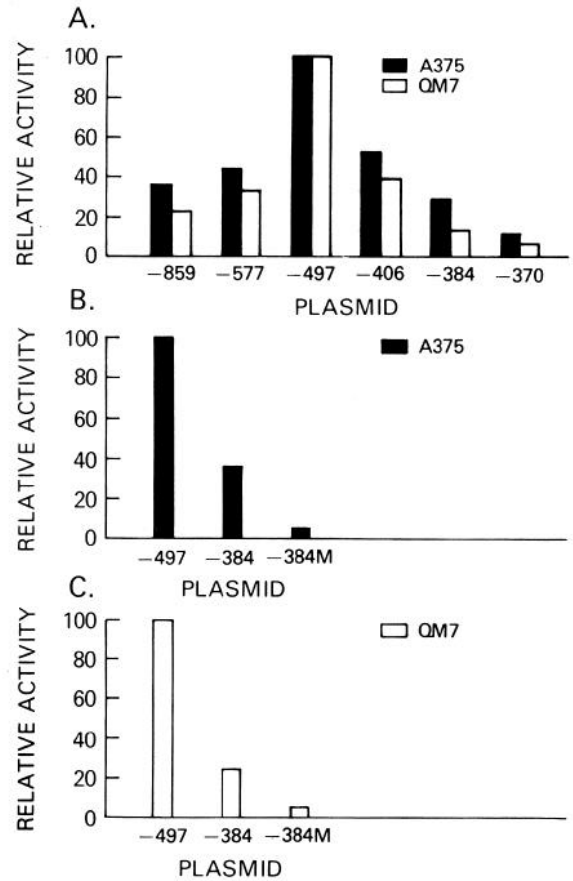

Fig. 6. Activity of the TGF-β3 Promoter

A, Graphical presentation of CAT activity in cell lysates of QM7 and A375 cells 48 h after transfection with the TGF-β3-CAT deletion mutant plasmids indicated. Cells were transfected with pβ3–370 (containing the TATA box), pβ3–384 (containing the TATA and CRE), pβ3–406 (containing the TATA, CRE, and AP-2-like), pβ3–497 (containing the TATA, CRE, AP-2-like, and Sp1), pβ3–577 (containing the TATA, CRE, AP-2-like, and 2 Sp1), and pβ3–859 (containing the TATA, CRE, 5 AP-2-like, and 3 Sp1). All plasmids were cotransfected with the plasmid pXGH5, and cell lysates were normalized to GH levels before CAT activity analysis. Values are represented as in Fig. 5. A375 (B) and QM7 (C) cells were transfected with two versions of the plasmid pβ3–384 containing either a normal (pβ3–384) or mutated (pβ3–384M) CRE in separate experiments. In addition, cells were transfected with pβ3–499 and used to express relative activity. Cell lysates were normalized to GH, and CAT activity was determined 48 h later.

and three Sp1 consensus sequences, respectively (29). These plasmids were contransfected with the plasmid $pP_{ac}Sp1$ or with the same expression plasmid without Sp1 coding sequences (pP_{ac}) to determine relative induction of each by Sp1 (Fig. 7). Sp1 was found to stimulate transcription as measured by CAT activity with increasing effectiveness, as the 5′-flanking region/CAT plasmids extended in the 5′ direction and included increasing numbers of Sp1 consensus sequences.

CRE in the TGF-β3 Promoter

To study the involvement of the CRE consensus sequence in cAMP regulation of the TGF-β3 gene, QM7 cells were transfected with pβ3–370, pβ3–384, pβ3–406, and pβ3–859 and incubated with either forskolin or DMSO (Fig. 8A). The plasmid pβ3–370 showed no increase in CAT activity after incubation with forskolin. However, the plasmids pβ3–384, pβ3–406, and pβ3–859, which all contained the CRE sequence, showed a significant increase in CAT activity after incubation with forskolin. Similar results were obtained upon transfection of A375 cells and incubation with forskolin (data not shown).

To further study the involvement of the CRE se-

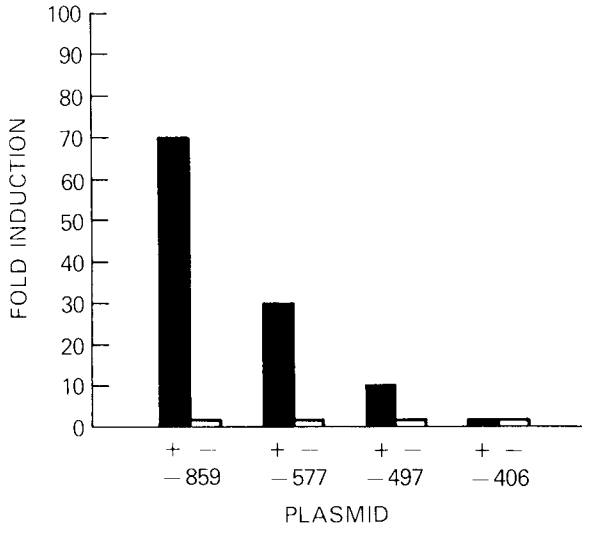

Fig. 7. Sp1 Stimulation of Chimeric TGF-β3 Promoter/CAT Plasmids

Drosophila Schneider line 2 cells were transfected with the indicated chimeric TGF-β3 promoter/CAT plasmids. The plasmids were either cotransfected with 100 ng $pP_{ac}0$ (−) or 100 ng $pP_{ac}Sp1$ (+). Fold induction refers to the amount of CAT activity produced from the chimeric TGF-β3/CAT plasmids cotransfected with the plasmids expressing Sp1 compared to the CAT activity from cotransfecting control plasmids. The data shown are from averaged values of three experiments.

quence in regulation of the TGF-β3 gene, QM7 cells were transfected with chimeric promoter/CAT plasmids corresponding to either the chicken TGF-β3 CRE sequence (pβ3CRE-pβ2–68) or rat somatostatin CRE sequence (pSOMCRE-pβ2–68), both individually ligated to the region of the human TGF-β2 promoter between nucleotides −68 and +63, which contains the TATA sequence but not the TGF-β2 CRE sequence as shown in Fig. 8B (20). In addition, these cells were also transfected with a chimeric plasmid consisting of the region of the human TGF-β2 promoter between nucleotides −40 and +63 which does not contain a CRE sequence (pβ2–40) as a control. Upon incubation with forskolin, both pβ3CRE-pβ2–68 and pSOMCRE-pβ2–68 showed an increase in CAT activity compared to incubation with DMSO (Fig. 8C). In contrast, no increase in CAT activity was observed when pβ2–40 was incubated with forskolin. In addition, the level of expression of TGF-β3 mRNA increased in both QM7 cells and primary chicken embryo chondrocytes after incubation with forskolin as demonstrated by RNA Northern blot analysis (Fig. 9).

DISCUSSION

We have described the sequence and structure of the chicken TGF-β3 promoter and found only a limited degree of nucleotide identity with the human TGF-β3 promoter despite the high degree of conservation of the amino acid sequence of chicken and human TGF-β3, the two proteins differing only in the substitution of a phenylalanine for a tyrosine residue at amino acid position 340. Sequence analysis of a 3.7-kb chicken TGF-β3 subclone identified 39 nucleotides of 5′-untranslated region identical to the sequence of a chicken TGF-3 cDNA (22) as well as an additional 1.4 kb 5′-flanking DNA. Comparison of the 5′-flanking regions of the chicken and human TGF-β3 genes revealed only limited sequence identity including conserved TATA, CRE, AP-1, and AP-2 consensus sequences. A similar pattern of limited sequence identity which includes these same consensus sequence elements has also been demonstrated in the chicken and human TGF-β2 5′-flanking regions (30), despite the similar high degree of conservation of the amino acid sequences of chicken and human TGF-β2 (24).

Sequence analysis of the 5′-flanking DNA of chicken TGF-β3 identified a consensus TATA box. However, unlike many other eukaryotic genes in which the TATA box is located approximately 30 nucleotides upstream from the transcriptional initiation site, our S1 nuclease protection analysis clearly shows that the TATA box in the chicken TGF-β3 gene is located 359 nucleotides upstream from the transcriptional initiation site. This is in contrast to a recent report by Burt *et al.* (31), in which the transcriptional start site of the chicken TGF-β3 gene was proposed to be located 28 nucleotides downstream of the TATA box based on the structural identity of this region of the chicken TGF-β3 gene with the human TGF-β3 gene (19). However, it is clear that except for relatively small regions of identity, the 5′-flanking regions of the chicken and human TGF-β3 genes are structurally quite different. Similarly, the 5′-flanking regions of the chicken and human TGF-β2 genes are also poorly conserved except for only a relatively small region of identity that also contains TATA, CRE, and AP-2 consensus sequences (30); the major transcriptional start site in the chicken TGF-β2 gene was shown to be 15 nucleotides upstream relative to the transcriptional start site in the human TGF-β2 gene. Moreover, although the human and mouse TGF-β1 promoters have been shown to be very conserved in their sequences, the first transcriptional start site in the mouse TGF-β1 gene was identified to be 42 nucleotides upstream relative to that of the human TGF-β1 transcriptional start site (32).

In many respects, the structure of the 5′-flanking region of the chicken TGF-β3 gene more closely resembles that of the human TGF-β1 gene than that of the human TGF-β3 gene. For example, the sequence between the TATA box and translational initiation site in the chicken TGF-β3 gene has a G + C content of 85%, while that of the similar region in the human TGF-β1 and TGF-β3 genes is 72% and 50%, respectively. Moreover, the sequence between nucleotides −859 and −1, which contains the putative promoter of the chicken TGF-β3 gene, has a G + C content of 77%, while the sequence between nucleotides −262 and −1 of the human TGF-β1 gene, which also contains the putative promoter, has a G + C content of 80%. In contrast, the

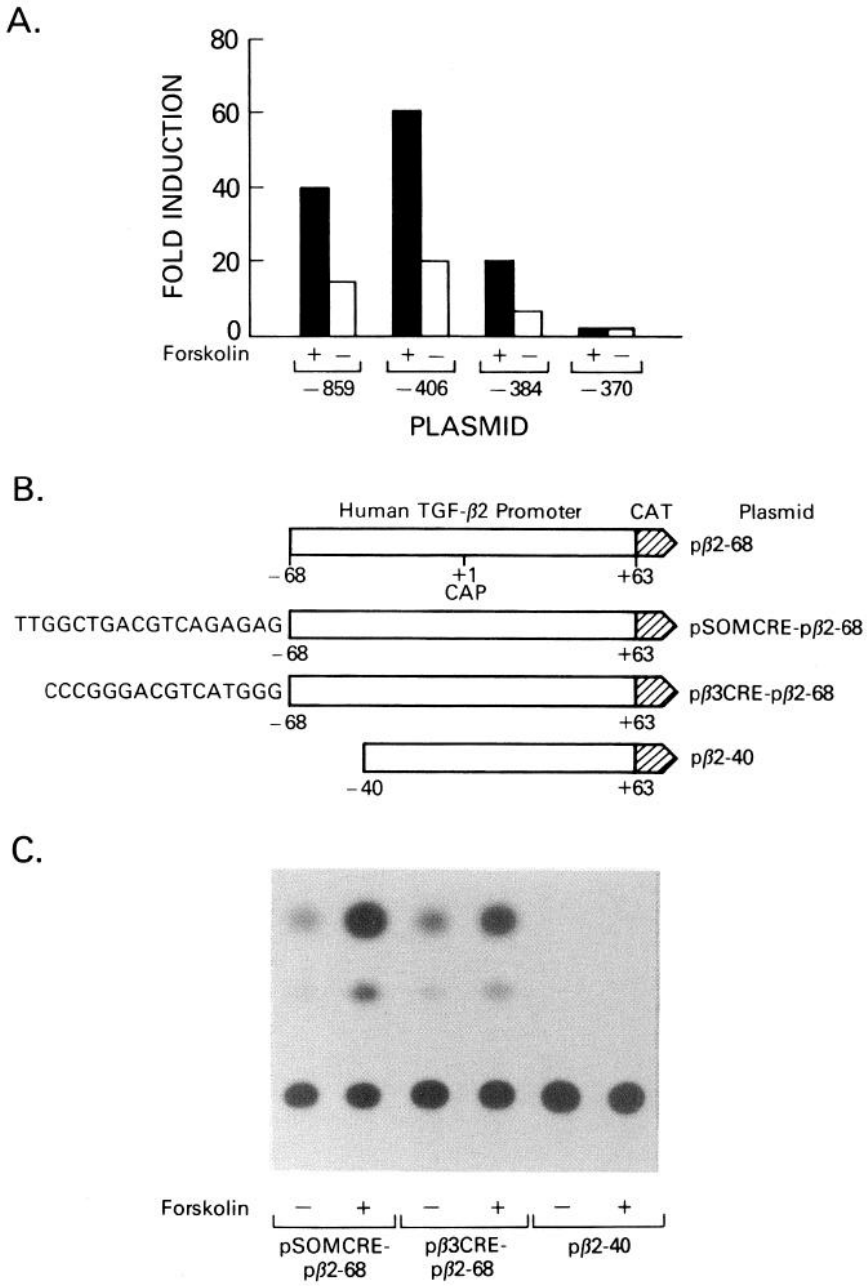

Fig. 8. CAT Activity of the CRE

A, Graphical presentation of CAT activity in cell lysates of QM7 cells after transfection with the TGF-β3-CAT plasmids pβ3–370 (containing the TATA box), pβ3–384 (containing the TATA and CRE), pβ3–406 (containing the TATA, CRE, and AP-2-like), and pβ3–859 (containing the TATA, CRE, 5 AP-2-like, and 3 Sp1) and incubated with forskolin (25 μm; +) or DMSO (−). B, The promoter constructs generated for promoter/CAT reporter plasmids are shown. The plasmids pSOMCRE-pβ2–68 and pβ3CRE-pβ2–68 were generated by PCR amplification using the CRE-containing oligonucleotides and pβ2–68 as a template. The plasmid pβ2–40 was also generated by PCR using pβ2–68 as a template. C, QM7 cells were transfected with the plasmids pSOMCRE-pβ2–68, pβ3CRE-pβ2–68, and pβ2–40 and incubated with either 25 μm forskolin (+) or DMSO (−). CAT activity was determined 48 h later.

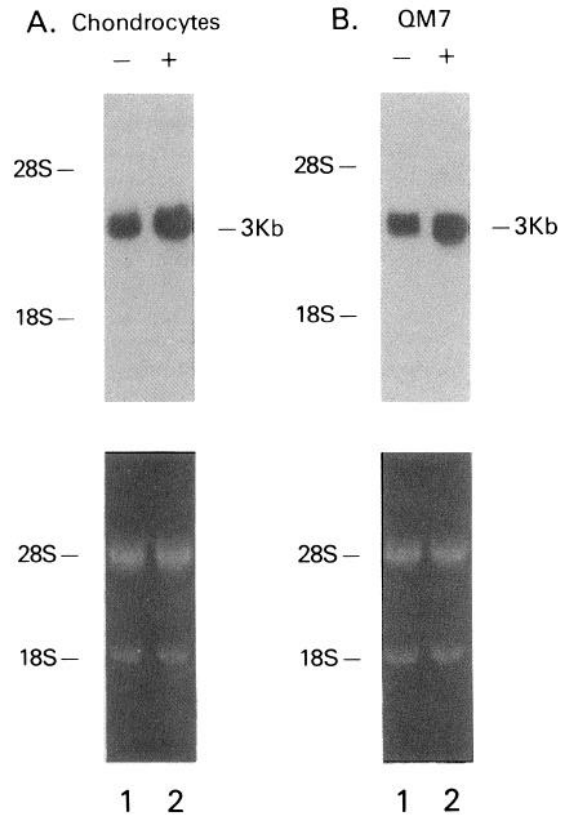

Fig. 9. Effect of Addition of Forskolin on Expression of TGF-β3 mRNA in Chicken Embryo Chondrocytes and Quail Fibrosarcoma QM7 Cells

Total RNA (15 μg) isolated from exponentially growing subconfluent primary chondrocytes (A) or QM7 cells (B) cultured for 24 h in MEM containing 0.1% bovine platelet-derived serum with DMSO (lane 1) and 25 μM forskolin (lane 2) was electrophoresed on a 1% agarose-formaldehyde gel and transferred to a Nytran filter as described in *Materials and Methods*. Hybridization was performed with ^{32}P-labeled chicken TGF-β3 cDNA. The position of TGF-β3 mRNA is shown as 3 kb. The ethidium bromide staining pattern of the gel showing 18S and 28S rRNA is shown below the blots. The blots were exposed for 12 h.

corresponding promoter region of the human TGF-β3 gene between nucleotides −700 and −1 has a G + C content of only 65%. Moreover, there are 10 consensus sequences for the transcription factor Sp1 in the chicken TGF-β3 gene and nine sites in the human TGF-β1 gene; three of these Sp1 binding sites are located in the 5′-untranslated region of the chicken TGF-β3 gene and one in this corresponding region of the human TGF-β1 gene. In contrast, only three potential Sp1 binding sites have been identified in the 5′-untranslated region of the human TGF-β3 gene, and none of these is located in the 5′-flanking region. Based on the structural similarities of the chicken TGF-β3 gene to the human TGF-β1 gene, it is possible that transcriptional initiation in the chicken TGF-β3 gene may be controlled by factors other than the TATA sequence.

Although the TATA box acts to orient the location of the transcription start site in many eukaryotic promoters, in several other eukaryotic promoters, including the human immunodeficiency virus and adenovirus 2 promoters, interaction with the TATA box does not appear to specify the start site of transcription, but rather to control the efficiency of transcription from a downstream initiation site (33–35). Moreover, in the rat brain creatine kinase gene, a nonconsensus TTAA sequence at nucleotide −28 appears to provide the principal TATA box function for its promoter, even though there is a consensus TATAAA sequence at nucleotide −66 relative to the transcriptional start site (36, 37); in this gene the upstream TATAAA sequence is required for efficient transcription from the downstream TTAA element (37). Within the chicken TGF-β3 promoter, besides the consensus TATA sequence at nucleotide −353, the sequences TGGAA, TTTTAA, TATTT, TATT, and TGGAAAA at nucleotides −32, −700, −712, −721, and −1151 are the other closest approximations to the consensus TATA box. It is possible that an upstream consensus TATA box, such as the one at nucleotide −353, may control the activity of a downstream element such as the sequence TGGAA at nucleotide −32 to specify initiation of transcription in the chicken TGF-β3 gene. This is suggested by the increase in basal activity of the chimeric CAT plasmids containing both the TATA and TTAA sequences compared to the CAT plasmids containing just the TTAA sequence.

Sequence analysis of the chicken TGF-β3 promoter region showed it to be extremely G + C rich and to contain 10 Sp1 consensus binding site sequences (38, 39). Sp1, originally identified as a protein from HeLa cell extracts that could bind to the sequence motif GGGCGG in the 21-bp repeat elements of the SV40 early promoter and activate transcription, has been shown to activate many other viral and mammalian promoters. Sp1 is generally considered to be a proximal promoter element that affects transcription when bound close to the transcriptional start site, in contrast to enhancer binding factors, which may exert transcriptional influence from thousands of base pairs away from the start site (40). Although little is known about how the Sp1 binding protein promotes transcription, recent studies have shown that Sp1 does not activate transcription by enhancing the DNA binding activity of the TATA box (TFIID) factor (41). Furthermore, it has been shown that multiple Sp1 binding sites spaced throughout a gene can allow synergistic activation of a promoter by Sp1 (42, 43). The chicken TGF-β3 contains three consensus Sp1 sites located within 300 nucleotides upstream of the TATA box which are spaced between 50–100 apart. Using the Schneider cell line of *Drosophila* cells, we were able to show increasing levels of CAT activity with increasing numbers of Sp1 sites. It is possible that these Sp1 consensus binding sites may also have an effect on influencing transcription of the chicken TGF-β3 gene *in vivo*.

Both the chicken and human TGF-β3 promoters also contain a CRE consensus sequence located 18 bp upstream of the TATA box and just downstream of the AP-2 site. Most of the identity between the chicken and

human TGF-β3 promoters is centered around the TATA, CRE, and AP-2 consensus sequences; both genes have 32 uninterrupted identical nucleotides beginning at the CRE sequence and extending seven nucleotides downstream of the TATA sequence. As with the human TGF-β3 promoter, the CRE was found to mediate both basal and cAMP-induced activity of the chicken TGF-β3 promoter. Furthermore, we have demonstrated that the chicken TGF-β3 CRE and the rat somatostatin CRE can both effectively mediate increased expression of human TGF-β2 activity by forskolin. The close proximity of the CRE to the TATA box in both the chicken and human TGF-β3 promoters and the complete absence of promoter activity when this CRE sequence is mutated in both these genes suggest that this promoter element is important in the function of the TGF-β3 gene. Also, the CRE is relatively close to the AP-2 binding site in both the chicken and human TGF-β3 promoters. It has been determined that the sequences surrounding the CRE are important in determining the level of CRE-mediated promoter activity in such genes as choriogonadotropin and vasoactive intestinal peptide (44, 45). Since the chicken and human TGF-β3 genes show more identity downstream of the CRE rather than upstream, this would suggest that the identity downstream of the CRE may be important for the function of the CRE in these two genes.

The present characterization of the structure and basal activity of the 5′-flanking region of the chicken TGF-β3 gene, as well as our demonstration of the control of regulation by specific sequence elements in avian as well as mammalian cells, provides a basis for further study of the transcriptional regulation of this gene. Further study will be needed to understand developmental and tissue-specific expression of the chicken TGF-β3 gene. In addition, it is hoped that future studies with the TGF-β3 promoter in combination with the promoters of the chicken TGF-β 1, 2, and 4 genes will enable us to understand more about the differential regulation of these genes both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Genomic Cloning and Sequence Analysis

A chicken leukocyte genomic library packaged in phage EMBL 3 (Clontech, Palo Alto, CA) was screened by plaque hybridization using a full-length chicken TGF-β3 cDNA (22). The cDNA was labeled by random priming and used to screen 6×10^6 plaques. Filters were hybridized overnight in 50% formamide, 5× SSC (1× SSC = 150 mM sodium chloride/15 mM sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 50 μg/ml calf thymus DNA at 42 C. Filters were washed three times in 10× SSC, 0.1% SDS at room temperature and then three times in 1× SSC, 0.1% SDS at 37 C. Positive clones were further screened by Southern blot analysis using the same chicken TGF-β3 cDNA. Four clones gave positive signals, and the 3.7-kb *Pst*I-*Pst*I fragment of the longest one was subcloned into the *Pst*I site of the vector pBluescript II (Stratagene, La Jolla, CA). This clone (pC4.24) was sequenced in both directions using the Sequenase dideoxy chain termination sequencing kit (U.S. Biochemical Corp., Cleveland, OH) (46).

Cell Culture

The human melanoma A375, mink lung epithelial CCL-64, and *Drosophila* Schneider line 2 cells were obtained from the American Type Culture Collection (Rockville, MD). The A375 and CCL-64 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) at 37 C. The Schneider cells were cultured in Schneider's *Drosophila* medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum in the presence of antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 27 C. The quail fibrosarcoma QM7 cells were a kind gift from Dr. Parker Antin (University of California, San Francisco) and cultured in M199 medium supplemented with 10% fetal calf serum and 10% tryptose phosphate in the presence of antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37 C. For differentiation of QM7 cells, cells were grown to confluency and then cultured in M199 medium for 3 days in the presence of antibiotics.

Preparation of Primary Chicken Embryo Chondrocytes and Myocytes

Primary chicken embryo chondrocytes and myocytes were prepared from the sterna and hearts, respectively, of 15- and 7-day-old white Leghorn chicken embryos and cultured as previously described (25).

RNA Extraction and RNA Northern Blot Analysis

Total RNA was extracted from chicken embryo chondrocytes, chicken embryo cardiac myocytes, QM7 cells, and A375 cells according to the LiCl-urea procedure described by Auffray and Rougeon (47). For RNA Northern blot analysis, equal amounts of total RNA (15 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred to Nytran filters (Schleicher and Schuell, Keene, NH). Ethidium bromide (33 μg/ml) was included in both the gels and running buffers in order to visualize the positions of ribosomal RNAs by UV illumination after electrophoresis. Blots were hybridized using ^{32}P-labeled (3000 Ci/mmole, New England Nuclear, Boston, MA) random-primed probes at 65 C and washed at 65 C according to Church and Gilbert (48) and exposed for various times at −70 C using an intensifying screen.

cDNA Probes

Hybridization was performed using the following cDNA probes: 2.2-kb *Eco*RI-*Hin*dIII fragment of the plasmid pTGFB-ChX17 (chicken TGF-β3); nucleotides −137 to +262 of the plasmid pC4.24, which includes 155 nucleotides of the coding region and 269 nucleotides of the 5′-flanking region of the chicken TGF-β3 gene; and nucleotides +839 to +1259 of the plasmid ph17.4, which includes 155 nucleotides of the coding region and 265 nucleotides of the 5′-flanking region of the human TGF-β3 gene.

S1 Nuclease Protection Analysis

pC4.24 DNA was digested with either *Xho*I or *Xho*I and *Sty*I, extracted with phenol/chloroform, and precipitated with ethanol. These digested fragments were end-labeled with [^{32}P] ATP as described (49). The resulting labeled 1508-bp *Xho*I-*Xho*I fragment (−1281 to +227) and 1500 *Xho*I-*Sty*I fragment (−1281 to +219) were isolated from an agarose gel and purified with GeneClean (Bio 101, La Jolla, CA). Then, 5.0×10^4 cpm of each probe were coprecipitated with 60 μg total RNA from primary chicken embryo chondrocytes and myocytes. The precipitate was dissolved in 40 μl hybridization buffer (80% formamide, 20 mM Tris-HCl, pH 7.4, 400 mM NaCl, and 1 mM EDTA), heated to 75 C for 15 min, and incubated overnight at 55 C. Unhybridized probe was digested by the

addition of 360 μl S1 nuclease buffer (10 mM NaCl, 0.1 mM $ZnSO_4$, 2 mM sodium acetate, pH 4.5, and 2 μg/ml denatured salmon sperm DNA) containing 200 U S1 nuclease for 1 h at 37 C. The protected fragments were recovered by ethanol precipitation, denatured, and analyzed on a 6% sequencing gel.

Construction of Plasmids

Chimeric promoter/CAT plasmids were constructed by ligation of TGF-β3 5′-flanking region fragments of pC4.24 produced by PCR amplification into the promoterless CAT-containing plasmid pGEM4-SVOCAT (16). A similar plasmid containing the SV40 early promoter (pSV2CAT) was used as a control (50). The 3′-oligonucleotides used in all amplifications corresponded to the 24-bp sequence between nucleotides −341 and −318 of pC4.24 to which an *Xba*I site was added. The 5′-oligonucleotides consisted of a series of 24-bp oligonucleotides to regions between nucleotides −370 and −859 of the TGF-β3 5′-flanking region to which a *Hin*dIII site was added. Fragments were amplified according to the standard protocol of the GeneAmp kit (Perkin Elmer-Cetus, San Francisco, CA) with a modification of including 4% formamide in the reaction. Also included was an oligonucleotide synthesized to the CRE that was mutated by changing the A at nucleotide −376 to a C and the T at nucleotide −373 to a G to which a *Hin*dIII site was added. These fragments were digested with *Hin*dIII and *Xba*I, gel-purified, and ligated into the multiple cloning site of pGEM4-SV0CAT. Correct orientation of the inserts with respect to the CAT coding sequence was verified by restriction enzyme analysis and then by DNA sequence analysis.

Another series of chimeric promoter/CAT plasmids was constructed by ligation of TGF-β3 5′-flanking region fragments of pC4.24 produced by restriction enzyme digestion into pGEM4-SV0CAT. The 3′-end of each fragment was generated using *Sma*I at nucleotide +60. A series of enzymes including *Sma*I, *Pvu*II, and *Nae*I was used to generate the 5′-ends of the fragments between nucleotides −379 and −285. The fragments were gel purified and ligated into the *Sma*I site of the multiple cloning site of pGEM4-SVOCAT. Correct orientation of the inserts with respect to the CAT coding sequence was verified as before.

Two additional chimeric promoter/CAT plasmids were constructed by ligation of the CRE sites of the chicken TGF-β3 promoter and the rat somatostatin promoter, both produced by PCR amplification, to the region of the human TGF-β2 promoter corresponding to the 131-bp sequence between nucleotides −68 and +63, and then into the *Sma*I site of pGEM4-SV0CAT and verified as before.

DNA Transfection and CAT Assays

Recipient cells were seeded 24 h before transfection at a density of 10^7 cells per 100-mm-diameter culture dish. Plasmids were prepared for transfection by purification in two sequential CsCl banding steps (49). Ten micrograms of plasmid DNA from each TGF-β3 promoter/CAT chimeric plasmid were cotransfected with either 1 μg pXGH5 (an expression plasmid containing the human GH, obtained from Nichols Institute Diagnostics, San Juan Capistrano, CA and used as internal controls to allow for normalization of transfection efficiency) into A375 or QM7 cells or 0.1 μg P_{ac}Sp1 (Sp1 expression plasmid) or P_{ac}O (no insert) [expression plasmids designed for expression in *Drosophila* Schneider line 2 cells as described (29)]. DNA was transfected by calcium phosphate coprecipitation (51). After incubation of the cells with the DNA solution for 12–16 h, the A375 and QM7 cells were washed three times with PBS, nutrient medium was added, and the cells were incubated for an additional 48–72 h. The Schneider cells were left without washing for 48 h. The cells were then scraped from the culture dishes, and protein extracts were assayed for CAT activity either according to Gorman *et al.* (51) or by a method that uses [^{3}H]acetyl coenzyme A (52). The amount of extract used (250 μg) was determined by protein quantitation (Bio-Rad protein assay, Bio-Rad, Richmond, CA) of each sample, and the reactions were allowed to proceed for 24 h at 37 C before scintillation counting of acetylated chloramphenicol.

Computer Analysis

The 5′-flanking region of the chicken TGF-β3 gene was analyzed for homologies to known DNA binding sites (34), using IBI PUSTELL sequence analysis software (IBI, New Haven, CT). The 5′-flanking regions of chicken and human TGF-β3 genes were compared using the University of Wisconsin (Madison, WI) Genetics Computer Group sequence analysis package using the COMPARE and DOTPLOT programs, with a window of 21 and a stringency of 14 (26).

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