Identification and characterization of the promoter for the cytotactin gene

(gene expression/regulatory elements/extracellular matrix/embryogenesis)

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ABSTRACT The extracellular glycoprotein cytotactin is expressed in a characteristic and complex spatiotemporal sequence during development of the chicken embryo. To identify the various control elements underlying its expression, the promoter region of the cytotactin gene has been isolated and characterized. Clones were isolated from genomic libraries by using a fragment near the 5' end of the cDNA sequence. The sequence of this cDNA fragment was found to be distributed over two exons separated by a large first intron. The site of transcription initiation was determined by S1 nuclease and primer-extension mapping. Sequencing of a 4.3-kilobase (kb) genomic DNA clone that contains 3986 base pairs (bp) upstream of the RNA start site, the first exon, and part of the first intron revealed a number of sequence motifs implicated in the regulation and expression of eukaryotic genes. These included CCAAT boxes, phorbol ester-responsive elements, enhancer elements, and a consensus TATA sequence located 24 bp upstream of the major RNA cap site. The flanking sequence also contained a number of regions of dyad symmetry and direct repeats unique to cytotactin, as well as an array of A+T-rich sequences that resemble engrailed elements. Constructs containing fragments of the upstream region of the cytotactin gene fused to a promoterless gene for chloramphenicol acetyltransferase were transiently transfected into chicken embryo fibroblasts to define functional promoter sequences. Although sequences from -721 to +121 exhibited minimal promoter activity, the entire region between -3986 to +374 was required to yield maximal expression in chicken embryo fibroblasts. Transfection of the -3986/+374 chloramphenicol acetyltransferase plasmid into the human U251MG astrocytoma cells but not HT1080 fibrosarcoma cells resulted in chloramphenicol acetyltransferase expression, consistent with the observed synthesis of cytotactin protein only by the U251MG cell line. These data indicate that the chicken cytotactin promoter can control expression in a cell type-specific fashion within cells of another species. These studies provide a basis for the dissection of cis elements and trans factors that govern the developmental expression of the cytotactin gene.

Cytotactin/tenascin is an extracellular matrix glycoprotein that plays a regulatory role in cell migration and tissue patterning during embryogenesis and regeneration (1–3). The molecule is composed of multiple polypeptides assembled in a complex hexameric structure, or hexabrachion (4). Based on recent cDNA cloning studies (5–7) showing that cytotactin is composed of four structural domains, a model of the molecule (5, 7) has been proposed. The amino-terminal region contains the cysteine residues involved in the inter-chain linking of the six polypeptide chains at the core of the hexabrachion. The proximal portions of the radiating arms are composed of compact epidermal growth factor-like re-

peats; the outer, thicker segments of the arms are composed of fibronectin type III repeats, and the terminal knob is made of a fibrinogen-like distal domain.

Cytotactin contributes in a complex fashion to important functions of embryonic extracellular matrices, including those that regulate cell migration, process extension, and attachment to basement membranes. Cytotactin affects neuron–glia adhesion (1), and synthesis of cytotactin by glia is a necessary requirement for granule cell migration (8). In certain cases, however, cytotactin inhibits cell (9) and neurite (10) migration. The biological significance of these “repulsin” effects has been suggested by recent studies in the somatosensory cortex of the early postnatal mouse, in which cytotactin and its proteoglycan ligand delineate barriers to neurite outgrowth (11, 12).

The expression patterns of cytotactin, as observed by immunohistochemistry (13) and more recently by *in situ* hybridization (14), are dynamic during development and can be transient. Cytotactin appears first at gastrulation and then during neurulation and somite formation in a cephalocaudal pattern. Later, cytotactin is partitioned into the rostral half of the sclerotome, a region into which neural crest cells migrate (9). At later periods of development, cytotactin mRNA and protein continue to be produced in characteristic and complex patterns at inductive sites and during morphogenesis of a number of organs (14).

These properties suggest that the control of cytotactin gene expression is complex and that it may be governed at many levels depending on cell type, cell state, and place-dependent interactions in the developing embryo. To understand better the factors that might direct the patterns of gene expression, we have isolated and characterized the promoter region of the chicken cytotactin gene.*

MATERIALS AND METHODS

An EMBL3 bacteriophage library of chicken genomic DNA (Clontech) was screened with an *EcoRI*–*Sph* I DNA probe containing nucleotides 1–368 of the cytotactin cDNA sequence (5). Sequencing of the 4.3-kilobase (kb) *Sal* I–*Cla* I genomic DNA fragment was performed on Bluescript subclones with oligonucleotide primers synthesized by The Rockefeller University Protein Sequencing Facility. S1 nuclease mapping was carried out using 5 μ g of poly(A)⁺ RNA (15). DNA probes were end-labeled at the *Bss*HII site. Hybridizations were performed for 12 hr at 45°C and were treated with 300 units of S1 nuclease in the presence of 5 μ g of salmon sperm DNA. Primer extension analysis was performed as described (15). Chloramphenicol acetyltransferase (CAT) gene plasmids were constructed from the 4.3-kb *Sal* I–*Cla* I cytotactin genomic DNA fragment or subfragments

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Abbreviations: CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35369).

thereof by insertion into promoterless pCAT basic or pCAT enhancer vectors (Promega).

U251MG and HT1080 (American Type Culture Collection) cells were grown in DMEM supplemented with 10% fetal bovine serum. Fibroblasts were prepared from 11-day-old chicken embryo body walls (16). Cultures were transfected in duplicate using CaPO_4 (15) (20 μg of plasmid per 100-mm tissue-culture dish), harvested 60 hr later, and assayed for CAT activity (15 μg of lysate) (17). CAT activities were standardized by comparison to that of a pCAT control plasmid (Promega) that contains simian virus 40 (SV40) promoter/enhancer sequences.

RESULTS

Isolation of Cytotactin Genomic Clones. A 368-base pair (bp) *EcoRI-Sph I* cDNA probe, which included a 5' untranslated segment, signal sequence, and amino-terminal segments of the mature protein (5), was used to screen chicken genomic libraries for clones containing potential promoter sequences. Three clones, CTG1, CTG2, and CTG3, were isolated and characterized by restriction mapping and DNA sequencing. The probe sequence was distributed into two separate exons that were not contained in any one clone (Fig. 1). The 5' half of the cDNA probe resides in an exon common to clones CTG1 and CTG2, whereas the 3' half of the probe is represented in an exon found in CTG3. The CTG1 and CTG2 clones had no sequence in common with the CTG3 clone, and restriction mapping indicated that at least 12 kb of DNA separate the two exons. The cDNA sequence, however, is continuous over the two exons indicating that, barring the existence of new cytotactin splice variants with an additional 5'-untranslated segment, the two exons represent contiguous cytotactin exons. These data and the localization of the mRNA cap site (see below) established the identity and order of the two exons (Fig. 1). Exon 1 contains 177 bp and encodes 5' untranslated mRNA sequences. Exon 2 contains 593 bp and encodes 5' untranslated sequences as well as the first 154 amino acids of cytotactin, including the signal peptide and six of eight cysteine residues of the amino-terminal interchain disulfide linking domain (5).

Determination of the Transcription Initiation Site. The start of transcription for cytotactin mRNA was determined by a combination of S1 nuclease mapping and primer-extension analysis. A small segment of DNA surrounding the first exon (Fig. 2) was used to make two independent S1 nuclease probes. DNA was linearized and end-labeled with ^{32}P at the *Bss*HII site inside the first exon. S1 nuclease probes of 248 and 630 bp were prepared by digesting DNA with *Sph I* and *Pvu II*, respectively; the DNA was hybridized to RNA and digested with S1 nuclease. The protected labeled fragments

FIG. 1. Restriction map of genomic DNA segments that flank exons 1 and 2 of the chicken cytotactin gene. Most of intron 1 is not represented; thus the map appears discontinuous between exons 1 and 2. The 5' termini of the three genomic clones are demarcated above. Black boxes represent exons 1 and 2; the lengths, border sequences, and donor splice signals of these exons are listed below the map.

FIG. 2. S1 nuclease and primer extension mapping of the cytotactin mRNA cap site. (Upper) Restriction map of genomic DNA surrounding the first exon. The 248- and 630-bp S1 nuclease probes are drawn beneath the map. ---, Unprotected portions of probes digested by S1 nuclease; —, protected regions. The arrow represents the position, direction of synthesis, and length of extension cDNA products from an antisense 42-mer oligonucleotide primer. (Lower) Lanes 1 and 2. ^{32}P -labeled 42-mer primer was annealed to either 5 μg of poly(A)⁺ RNA from embryonic day 14 chicken brain (lane 1) or liver (lane 2) RNA and extended with reverse transcriptase. The arrow demarcates major extension product of 126 nucleotides. Lanes 3–5, S1 mapping. Five micrograms of poly(A)⁺ liver (lane 3) or brain (lanes 4 and 5) RNA from embryonic day 15 chickens was hybridized with either ^{32}P -labeled *Bss*HII-*Sph I* probe (lanes 3 and 4) or *Bss*HII-*Pvu II* probe (lane 5) and treated with 300 units of S1 nuclease. Arrows indicate major protected fragments of 45 and 46 bp.

were analyzed on sequencing gels. Two major fragments of 45 and 46 bp were protected from S1 digestion when either the 248- or 630-bp probe was hybridized to poly(A)⁺ RNA prepared from embryonic day 15 chicken brain (Fig. 2, lanes 4 and 5, respectively). However, no protected fragments were observed when the 248-bp probe was hybridized to poly(A)⁺ RNA from embryonic day 15 chicken liver (Fig. 2, lane 3), a tissue in which cytotactin mRNA was not detected (18). An end-labeled synthetic oligonucleotide complementary to bp +125–+84 of exon 1 was hybridized to either poly(A)⁺ brain (Fig. 2, lane 1) or liver (Fig. 2, lane 2) RNA and was extended upstream using reverse transcriptase. Primer extension products of 126 nucleotides appeared when brain RNA was used but not when liver RNA was used, suggesting that the products arise from specifically primed cytotactin mRNAs. Because the 46-bp S1-protected fragment and the 126 nucleotide extension product have termini at exactly the same upstream base pair, the nucleotide at this position was designated +1, the start of transcription initiation. Similar results were obtained for both experiments with gizzard RNA, another tissue actively transcribing the cytotactin gene, suggesting that the start site is the same in both brain and gizzard (data not shown). Despite the agreement of these data, the reported cDNA sequences (5) and the genomic sequence (see Fig. 3) each contain the same 6-bp

FIG. 3. DNA sequence of a 4.3-kb *Sal I*–*Cla I* genomic DNA fragment that contains 3986 bp of 5'-flanking sequence, the first exon (shadowed in gray), and part of the first intron of the chicken cytotactin gene. Restriction sites used in the preparation of constructs and probes are shown. The site of transcription initiation is indicated by a downward arrow and labeled +1. The additional 6 bp found in a cDNA clone for cytotactin are enclosed in a dotted box. Tandem direct repeats are represented by flat arrows appearing in twos; extended dyad symmetrical sequences are delineated with facing arrows → ←. All other sequences are boxed and labeled with an appropriate symbol: ●, CCAAT box; en, engrailed-like sequence; T, phorbol ester-responsive element; c, calcium-responsive element; TAA, TAA repeat; TG, TAATGAT repeat; CB, CAAT box-associated repeat; G, GAGA box; GC, GC box; R, gastrin negative repressor element; O, octamer; S, SV40 core element; TATA, TATA box. The 5' termini of genomic clones CTG1 and CTG2 are marked with vertical line and arrow.

sequence (GCTGAT) 5' of the predicted start site, suggesting that these base pairs can be included in some mRNAs.

Sequence of the 5' End of the Chicken Cytotactin Gene. The DNA sequence of the 4.3-kb *Sal* I-*Cla* I DNA fragment from CTG2, which includes 3986 bp of DNA upstream of the RNA start site, the first exon, and part of the first intron, was determined. In Northern (RNA) blot analysis, restriction fragments of the *Sal* I-*Cla* I fragment hybridized with cytotactin mRNAs and not with any other transcripts (data not shown). The sequence of the *Sal* I-*Cla* I fragment (Fig. 3) displays a remarkable array of elements and motifs, some of which are characteristic of eukaryotic promoters and regulatory regions found in other genes and some of which appear unique to the cytotactin gene. The 24 bp upstream of the transcription start site include the sequence TATATAAA, which is homologous to TATA-like sequences in many eukaryotic promoters (19). A computer-assisted search revealed a number of potential regulatory elements, including a viral core enhancer (-60) (20), an octamer (-200) (21), two GAGA boxes (-123 and -641) (22), two phorbol ester-responsive elements (-279 and -2561) (23), a GASNE repressor element (-289) (24), a GC box (-439) (25), a CBAR element (-702) (26), a calcium response (CaRE) element (-1565) (27), six CCAAT boxes (19), and six engrailed-like elements (28, 29). The sequence also displays an array of unique regions of dyad symmetry, direct repeats, and a recurring TAATGAT sequence that is imbedded in a region of TAA repeats (30).

Analysis of Upstream Cytotactin Genomic Segments for Promoter Activity. Chimeric CAT expression plasmids were constructed in which the 4.3-kb *Sal* I-*Cla* I fragment and subfragments of it were cloned in front of a promoterless CAT gene. Each plasmid construct was transfected into chicken embryo fibroblasts and assayed 60 hr later for functional CAT activity. Construct 9, containing sequences -3986 to +121 (Fig. 4), gave the highest levels of relative CAT expression (lane 9). Constructs 2 and 5 (sequences -1477/+121 and -2027/+121, respectively) showed similar

FIG. 4. Structure of cytotactin upstream sequence CAT gene constructs and corresponding CAT activity in extracts of transfected chicken embryo fibroblasts. The number of each construct corresponds to the appropriate lane in the CAT assay. No linear representation is shown for constructs 6 and 8 because they are vector controls. Constructs: 1, -721/+121 CAT; 2, -1477/+121 CAT; 3, -1477/-201 CAT; 4, -2766/-1190 CAT; 5, -2027/+121 CAT; 6, pCAT enhancer; 7, -1477/+121 CAT enhancer (SV40 EN); 8, pCAT basic; and 9, -3986/+374 CAT. The smaller cytotactin genomic constructs, derived from the segment in construct 9, are drawn to scale and positioned appropriately.

FIG. 5. Chicken cytotactin promoter drives CAT gene expression in human astrocytoma line U251MG but not in human tumor line HT1080. (A) CAT assay of transient transfection of HT1080 (lanes 1, 3, and 5) or U251MG (lanes 2, 4, and 6) cells with pCAT controls (lanes 1 and 2), pCAT basic (lanes 3 and 4), and construct 9 (-3986/+374 CAT) (lanes 5 and 6) plasmids. (B) Immunoblot analysis of culture supernatants from HT1080 (lane 1) and U251MG (lane 2) cells with polyclonal rabbit antiserum prepared against mouse cytotactin. The migration of molecular weight standards $\times 10^{-3}$ is indicated at right.

levels of CAT activity, approximately 2-fold less than construct 9 (compare lanes 2 and 5 to lane 9). From these data, the cis sequences responsible for most of upstream promoter activity lie between -1477 and +121. Furthermore, when an SV40 enhancer was added to these sequences (construct 7) CAT expression increased dramatically (compare lane 7 to lane 2). Deletion of the 5' end from -1477 to -721 (construct 1) reduced CAT expression levels (compare lanes 2 and 1). A deletion of the 3' end from +121 to -201, in which the TATA, SV40 core, GAGA, and octamer elements upstream of the predicted start site were eliminated, significantly reduced but did not eliminate CAT activity (compare lanes 3 and 2). Construct 4 (sequences -2766 to -1190, lane 4), promoterless CAT (lane 8), and promoterless CAT with SV40 enhancer (lane 6) plasmids all exhibited no detectable CAT activity.

Chicken Cytotactin Promoter Constructs Exhibit Cell-Type-Specific Expression in Human Cells. Construct 9 (Fig. 4), which exhibited the highest CAT activity in transfection experiments of chicken embryo fibroblasts, was transfected into human tumor cell lines to determine whether the promoter could function in cells from other species. Two cell lines were used so that we could also determine whether the promoter could confer regulatory specificity—i.e., to be active in a cell type that constitutively expressed cytotactin and to be inactive in a cell type that did not. The two cell lines were selected by immunoblot analysis of culture supernatants with cytotactin antibody. A fibroblastic human astrocytoma, U251MG, expressed cytotactin constitutively (Fig. 5B, lane 2), whereas in epithelioid human fibrosarcoma, HT1080, did not (Fig. 5B, lane 1). SV40 promoter/enhancer CAT constructs were efficiently expressed in both HT1080 and U251MG cells (Fig. 5A, lanes 1 and 2, respectively) and promoterless CAT constructs exhibited no detectable CAT activity in either cell type (lanes 3 and 4). The chicken cytotactin promoter (construct 9) exhibited a significant level of activity in U251MG cells (Fig. 5A, lane 6) but showed no detectable CAT activity in HT1080 cells (Fig. 5A, lane 5) that do not normally make cytotactin. Thus, the chicken cytotactin promoter can function in human cells, and the upstream regulatory sequences include regions that regulate expression appropriately in specific cell types.

DISCUSSION

We have isolated the promoter region of the cytotactin gene and have shown that genomic DNA fragments upstream of the RNA cap site can drive the expression of the CAT gene in chicken embryo fibroblasts and in a human astrocytoma line, U251MG. The promoter does not function in HT1080

fibrosarcoma cells, indicating that these cells express repressors that silence cytotactin expression or that they fail to express one or more factors required to initiate transcription.

Analysis of the structure of the 5'-most portion of the cytotactin gene indicates that there is an intron of at least 12 kb between exons 1 and 2. Additional sequences governing expression may exist in this intron, as have been reported in comparable introns of genes specifying other extracellular matrix molecules—for example, $\alpha 1$ (IV) collagen (31) and thrombospondin (32). The first intron for $\alpha 1$ (IV) collagen is >30 kb in length and contains an enhancer region that boosts expression 10-fold in cells that actively transcribe the gene (31).

The segment of DNA upstream of the cytotactin gene needed to produce the highest levels of CAT activity in fibroblasts was a 4.3-kb fragment. A 1.4-kb fragment gave modest levels of expression, and a number of smaller constructs yielded minimal expression. These data suggest that the cis sequences governing cytotactin expression in fibroblasts are dispersed, rather than confined to a small region a few hundred base pairs upstream of the mRNA cap site, as found in a number of genes including the gene for another matrix protein, fibronectin (33).

Surprisingly, construct 3 (−1477/−201) exhibited small but significant promoter activity (Fig. 4, lane 3), indicating that deletion of the upstream sequence close to the start site did not abrogate expression. This region includes the TATA sequence, and it has been suggested (19) that the TATA box is important for the precise positioning of the RNA start site. Perhaps the TATA sequence in cytotactin is less critical, and sequences upstream of −201 are sufficient to initiate transcription. Alternatively, one or more of the many T+A-rich sequences in the construct could substitute for the normal TATA sequence (34). Another possibility, which cannot yet be ruled out, is that the upstream region of cytotactin contains more than one promoter, each of which could be used selectively by different cell types.

A remarkable number of sequences previously shown to function as regulatory elements in other systems are found upstream of the cytotactin gene. Some or all may participate in the expression of the gene, and various combinations of elements may be used in different cell types. Gel-mobility shift and DNase-footprinting assays using nuclear extracts from specific cell types will be required to determine more precisely the particular cis and trans components dictating the promotion or repression of cytotactin gene expression in specific cell or tissue types.

It is an attractive hypothesis that homeobox-containing transcription factors may be involved in the spatiotemporal expression of cytotactin. The array of engrailed-like elements clustered far upstream from the site of transcription initiation provides potential targets for factors that may participate in the graded cephalocaudal expression of cytotactin seen during segmentation and in the highly patterned condensation of mesoderm in the somites. Analysis of the elements involved in the developmental expression of the cytotactin gene can be best studied by introducing various cytotactin cis sequence-bearing constructs into animals. As shown here, the chicken cytotactin promoter drives the expression of the CAT reporter gene in human cells. We have also recently found that constructs containing the cytotactin promoter driving a β -galactosidase reporter gene are expressed when injected into *Xenopus laevis* embryos (F.S.J., D. A. Williamson, and G.M.E., unpublished data). It may therefore be feasible to study the factors governing developmental expression of cytotactin by introducing the chicken promoter into the germ line of other species.

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