Identification of an intronic enhancer that nullifies upstream repression of SPARC gene expression

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Received February 13, 1997; Revised and Accepted June 2, 1997

ABSTRACT

The SPARC gene 5′ flanking sequence has been shown to contain enhancer elements, but also negative control elements immediately upstream of the enhancer elements. Although these 5′ enhancer elements are active in F9 and PYS-2 cells, their activities are nullified by the 5′ repressor activity. In the present study we have identified within intron 1 between nucleotides (nt) +5000 and +5150 of the SPARC gene an enhancer element that bound to two transcription factors of 48 and 52 kDa and between nt +5000 and +5523 a DNase I hypersensitive site. Furthermore, a region containing the 3′ intron 1 enhancer element, together with the 5′ enhancer elements, neutralized the 5′ repressor activity and stimulated efficient transcription. The resulting SPARC promoter activity is about equal in F9, differentiated F9 and PYS-2 cells. We consistently found that the rate of SPARC transcription is nearly the same in F9 and PYS-2 cells. Association of the 3′ enhancer element in intron 1 with the DNase I hypersensitive site suggests that both play a role in regulating SPARC expression in vivo.

INTRODUCTION

SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40) is an extracellular matrix-associated glycoprotein expressed in many embryonic tissues and organs and in adult gut and bone (1), indicating that SPARC might be involved in cell proliferation and migration and tissue remodeling. Increased SPARC expression is also associated with wound repair and angiogenesis (1–4) and thus the protein is implicated in these processes.

We showed previously that SPARC expression is low in inner cell masses of 4.5 day mouse blastocysts and becomes activated as these cells differentiate into parietal endoderm (5). This in vivo regulation is closely mimicked by the in vitro regulation seen during differentiation of the mouse embryonal carcinoma F9 cell line. F9 cells resemble early embryonic cells and upon treatment with retinoic acid and dibutyryl cAMP differentiate into cells that resemble parietal endoderm (6). SPARC mRNA levels were shown to be ~20-fold higher in differentiated F9 cells than in undifferentiated F9 cells (7). SPARC mRNA is also very abundant in a murine parietal endoderm cell line, PYS-2 (7,8). Despite this large difference in the steady-state SPARC mRNA levels in F9 and PYS-2 cells, activity of the SPARC promoter extending between nucleotides (nt) 449 and +11 (+1 being the transcription initiation site) is only slightly higher in the latter than in the former, as measured by transient transfection assays (9).

In addition to the enhancer elements located between nt –449 and +11, the 5′ flanking region of the murine SPARC promoter contains negative regulatory elements (between nt –449 and –638) (9) located immediately upstream of the enhancer elements. The repressor elements are dominant, such that the 5′ flanking sequence containing both enhancer and repressor elements yields only basal promoter activity in F9 and PYS-2 cells (9). Inclusion of up to 2.2 kb of the flanking sequence only partially restores promoter activity in these cells (9). Thus it appears that additional control elements located outside the 2.2 kb flanking sequence are required for efficient expression of the murine SPARC gene.

Similar results were found with the bovine SPARC promoter. Transient transfection assays have identified a positive element between nt –504 and +11 and a dominant negative element between nt –900 and –504. The positive element of the promoter confers preferential expression of the SPARC gene in fetal bovine bone cells and skin cells (10). In addition, exon 1 and intron 1 of the gene may also play a role in modulating bovine SPARC promoter activity (10). In humans an enhancer and a repressor element for the promoter have been mapped between nt –120 and –51 and –131 and –120 respectively. However, the repressor element is not dominant over the enhancer element (11).

In the present investigation of promoter activity in F9 and PYS-2 cells we have confirmed the results of the previous study that the 5′ flanking sequence contains a dominant repressor element. We have also located an enhancer element within intron 1 between nt +5000 and +5150 and shown that a region containing this 3′ intron 1 enhancer element acts cooperatively with the 5′ regulatory elements to counteract negative regulation by the 5′ repressor and stimulate equally efficient transcription in both F9 and PYS-2 cells.

MATERIALS AND METHODS

Isolation of cosmid clones

A mouse cosmid library with 129/sv mouse liver DNA partially digested with Sau3A1 and inserted into pcosEMBL2 (12; originally from Dr Ann-Maria Frischauf, Imperial Cancer Research Fund, London, and kindly provided by Dr Maja Bucan, Research Fund, London, and kindly provided by Dr Maja Bucan,...
University of Pennsylvania) was first screened with a partial SPARC cDNA (8). A genomic fragment containing part of the first intron and second exon of the SPARC gene was isolated from SPARC cDNA (8). A genomic fragment containing part of the first intron and second exon of the SPARC gene was isolated from SPARC cDNA (8). A genomic fragment containing part of the first intron and second exon of the SPARC gene was isolated from SPARC cDNA (8). A genomic fragment containing part of the first intron and second exon of the SPARC gene was isolated from SPARC cDNA (8).

Plasmid constructs

A DNA fragment containing nt –1200 to +110 was obtained from SPcos7 by HindIII digestion (see Fig. 1) and inserted upstream of the promoterless pBLCA T3 (13). 5′ Deletion constructs were generated by digestion of the –1200/+110 fragment with appropriate restriction enzymes followed by cloning the subfragments in pBLCA T3. The enhancer from the LTR of the intracisternal A particle (IAP) gene was shown by us to be active in both F9 and PYS-2 cells (14,15). A +5000/+9000 CAT or +5000/+9000 TK promoter in a CAT reporter gene (pBLCA T2) and analyzed on a 6% polyacrylamide/urea gel.

RESULTS

Deletion analysis of the 5′ flanking region of the SPARC gene

A series of 5′ deletion fragments from the 5′ flanking region were generated and linked to the promoterless CAT reporter gene (pBLCA T3). Analysis of these constructs in F9 and PYS-2 cells (Fig. 2A) indicated that the sequence from nt –1200 to +110 had no transcriptional activity and that limited deletion from nt –1200 to –473 did not abolish the repression. However, additional deletion of nt –473 to –405 resulted in transcriptional activation and this enhancer activity was maintained even when the deletion was increased to nt –272. Further deletion to –80 abolished enhancer activity. The results indicate the presence of a repressor element(s) within nt –473 to –405 and an enhancer element(s) between nt –405 and –80. Activity of the repressor element is dominant over that of the enhancer element and the enhancer activities are similar in PYS-2 and F9 cells, consistent with results reported previously (9).

The sequence from nt –40 to +110 contains basal promoter activity, since this region was able to support heterologous IAP enhancer activity in an orientation-dependent manner (Fig. 2B). The SPARC promoter appeared to be comparable with the HSV TK promoter in its ability to increase IAP enhancer activity.

Identification of DNase I hypersensitive sites in the first intron

DNase I hypersensitive sites often reflect discrete regions of open chromatin which are accessible to regulatory proteins that participate in transcription. To identify additional regulatory elements outside the 5′ flanking sequence, we examined DNase I hypersensitive sites (Fig. 3). We located a hypersensitive site between nt +5000 and +5523 within the first intron which was slightly more hypersensitive in PYS-2 cells than in F9 cells (Fig. 3). A weak hypersensitive site was also found upstream of +5000 within the first intron (Fig. 3).

Characterization of an intronic enhancer element

The presence of a DNase I hypersensitive site between nt +5000 and +5523 in the first intron led us to locate transcriptional regulatory elements around this region. A 4 kb BamHI fragment containing nt +5000 to +9000 and its various deletion fragments were inserted, in forward and reverse orientation, upstream of the TK promoter in a CAT reporter gene (pBLCA T2) and analyzed for their transcriptional activity in F9 and PYS-2 cells (Fig. 4). An activity was found between nt +5000 and +5888 and within it an
Figure 2. Analysis of the 5′-flanking sequence. (A) Transcriptional activity of deletion mutants. Various deletion constructs were transfected into either PYS-2 or F9 cells. CAT activity is expressed relative to the activity of the TK promoter-driven CAT reporter gene (pBLCA T2 = TKCA T). (B) Identification of the basal promoter. A DNA fragment −40 to +110 was cloned in pBLCA T3. The IAP enhancer, which is active in F9 and PYS-2 cells, was used as a heterologous enhancer.

enhancer element was found between nt +5000 and +5150. No enhancer activity was detectable between nt +5243 and +5536. One can also infer from the data that little or no activity exists between nt +5150 and +5243. The region between nt +5536 and +5888 was not examined. The intronic enhancer elements (+5000/+5150) exhibited about equivalent activity in F9 and PYS-2 cells.

To determine whether the 3′ intronic element and the 5′ flanking elements act cooperatively, DNA fragments were cloned into promoterless pBLCAT3 and assayed for CAT activity (Fig. 5). Insertion of the +5000 to +5888 sequence in the 3′-end of the −405/+110 CAT plasmid cooperatively enhanced CAT reporter gene expression to a similar extent in F9, differentiated F9 and PYS-2 cells. Incorporation of the +5000 to +5888 sequence in the 3′- or 5′-end of the −1200/+110 CAT plasmid eliminated repressor activity located within nt −1200 to +110 in a position-independent manner and produced similar positive transcriptional activity in F9, differentiated F9 and PYS-2 cells.
Figure 5. Cooperativity between the 5′-flanking sequence and the intronic regulatory sequence. The indicated DNA fragments were cloned into promoter-less pBLCAT3 and assayed as described in the Figure 2 legend.

To further substantiate equivalent promoter activity in F9 and PYS-2 cells, we measured the rate of SPARC mRNA synthesis in these cells using nuclear run-off transcription assays as described previously (17). The transcription rates of SPARC mRNA in PYS-2 and F9 cells were about the same (results not shown), indicating that the rate of SPARC transcription correlates with its promoter activity in these two cell types.

Protein factors binding to the intronic enhancer element

Band shift analysis (Fig. 6A) indicated that transcription factors bound to the enhancer element located within nt +5000 to +5150 to yield faster and slower migrating complexes. Binding of the factors to the element was specific, as indicated by efficient competition by the homologous sequence but not by an irrelevant sequence. Co-migration of the F9 and PYS-2 complexes suggests that they contain the same binding factors. Indeed, UV crosslinking analysis of the slower (Fig. 6B) and the faster (not shown) migrating complexes showed that both F9 and PYS-2 complexes contained 48 and 52 kDa proteins. A search of the database revealed a number of candidate DNA elements within the nt +5000 to +5150 sequence (Fig. 7). Some of their binding factors, e.g. TCF-1 and GA TA-1 (19), with a molecular size of 53–57 kDa and 51 kDa respectively, might be candidate binding factors.

Identification and characterization of an intronic promoter

We also inserted the 4 kb BamHI fragment containing nt +5000 to +9000, in forward and reverse orientation, upstream of a promoterless CAT reporter gene (pBLCAT3) and analyzed it in F9 and PYS-2 cells (Fig. 8). The sequence failed to yield transcriptional activity in either cell type. However, the sequence from nt +5000 to +5888, which contained an enhancer element and a DNase I hypersensitive site, yielded an orientation-dependent promoter activity. The promoter was about equally active in PYS-2 and F9 cells, with the basal promoter residing within nt +5536 to +5888, which supported heterologous IAP enhancer activity. The basal promoter contained a candidate TATA box at nt +5620, but no obvious translation initiation site (Fig. 7).

RNase protection assays (Fig. 9A) showed that mRNA with a sequence from nt +5536 to +5888 was protected from RNase A and T1 digestion, indicating that this intronic promoter is not cryptic, but is instead functional. Northern hybridization (Fig. 9B) using a probe containing the sequence between nt +5536 and +5888 of intron 1 detected a transcript of ~9.4 kb. This transcript was abundant in PYS-2 cells but undetectable in F9 cells. In tissues it was high in the thymus but undetectable in the spleen, brain and skin, in contrast to SPARC expression, which is high in the spleen, brain and skin and low in the thymus. A search of the databases did not reveal any homologous gene. Together, these results suggest that the intronic promoter belongs to a novel gene.

DISCUSSION

In the present study we have located the SPARC minimal promoter between nt –40 and +110, an enhancer element(s) between nt –405 and –80 and a repressor between nt –473 and –405, which inactivated the 5′ SPARC promoter in F9 and PYS-2 cells. We also identified an enhancer element and a DNase I hypersensitive site within the intron 1 sequence between nt +5000 and +5150 and nt +5000 and +5523 respectively. The enhancer sequence bound to two factors of molecular size 48 and 52 kDa. Most importantly, this intron 1 enhancer element, alone or possibly with other unidentified elements within nt +5150 to +5888, provided enhancer activity in a position-independent manner and contributed to elimination of the repressed activity of the 5′ promoter and transcriptional activation of the SPARC gene. Thus an active chromatin structure and binding of transcription factors within intron 1 immediately downstream of nt +5000 is...
crucial to active transcription observed in vivo in F9, differentiated F9 and PYS-2 cells.

SPARC promoter activity was about the same in F9, differentiated F9 and PYS-2 cells, with the 5' flanking and the 3' intron 1 elements having similar enhancer activities in these three cell types. The DNase I hypersensitive site in intron 1 was also only slightly more hypersensitive in PYS-2 than in F9 cells. Consistently, nuclear run-off assays showed that SPARC was transcribed at approximately the same rate in F9 and PYS-2 cells. Thus our findings are in accord with previous studies (9) in which the SPARC promoter was reported to be only slightly more active in PYS-2 than in F9 cells. The results also indicate that the relevant transcription factors, including those binding to the region

![Figure 7](image1.png)

**Figure 7.** Partial restriction map and DNA sequence between nt +5000 and +5888. Possible cis elements within the positive-acting fragment between nt +5000 and +5150 are indicated.

![Figure 8](image2.png)

**Figure 8.** Identification of an intronic 1 promoter. DNA fragments as indicated in the constructs were cloned in promoterless pBLCAT3 and assayed as described in the Figure 2 legend.

![Figure 9](image3.png)

**Figure 9.** Analysis of the intronic promoter. (A) RNase protection assays. A DNA fragment (nt +5536 to +5888) was used to prepare the antisense RNA probe. The probe hybridized to a transcript to yield a protected fragment of 460 bases, indicating that +5536/+5888 represents a portion of an mRNA. (B) Northern hybridization using the [32P]dCTP-labeled probes: (a) SPARC intron 1 sequence between nt +5536 and +5888 containing an intronic promoter; (b) SPARC cDNA sequence between nt +795 and +1015 (8); (c) mouse β-actin cDNA. 28S rRNA was visualized with ethidium bromide. The blots were washed at 60°C with a buffer containing 0.5× SSC and 0.1% SDS for 15 min with two changes (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate). The source of RNA and the size of the detected transcript are indicated. Hybridized transcripts were quantified by phosphorimaging. The intron:SPARC ratio shows the relative abundance of intron 1 promoter-directed transcript compared with SPARC mRNA.
between nt +5000 and +5150, do not contribute to cell type-specific or differentiation-specific expression and suggest that the high mRNA level in differentiated F9 and PYS-2 cells as compared with F9 cell is the result of post-transcriptional processes.

We also identified a novel promoter activity within intron 1 between nt +5536 and +5888 which directed expression of a transcript of ~9.4 kb. The nt +5536 to +5888 sequence did not match the SPARC-related genes SC1, QR1 and testican (20–22) or other genes in the database. Immediately upstream of the promoter is the +5000/+5150 enhancer, which was effective not only for this promoter but also for the TK promoter. The presence of a promoter within an intron of another gene has been described in a number of cases, e.g. the WTI promoter in the WTI gene (23), the N-cym promoter within the N-myocyte gene (24) and an unrelated transcript in an intron of the human factor VIII gene (25). Similarly, the presence of regulatory elements in the first intron has been identified in many genes in addition to the murine SPARC gene, such as the genes for human ß-enolase (26), mouse α-1 type I collagen (27), murine mts1 (28) and bovine SPARC (10). The bovine SPARC promoter activity in bone cells has been shown to be repressed by a 1.2 kb fragment of intron 1 located between nt +141 and +1381 (10). We detected no enhancer activity in this region (results not shown), but did not examine it for repressor activity.

In the Drosophila heat shock gene hsp26 a (CT)₉ sequence located near a DNase I hypersensitive site contributes to formation of chromatin structure active in transcription (29). In the SPARC gene a (CT)₉ sequence is located between nt +5624 and +5672. Analysis of the possible role of the (CT)₉ repeat in formation of the DNase I hypersensitive site near nt +5000 will be interesting, especially in view of the fact that the hypersensitive site and the neighboring enhancer elements play a critical role in determining promoter activity.

ACKNOWLEDGEMENTS

We thank Dr L. Showe for critical reading of the manuscript. This work was supported by Public Health Service grants GM37762 and GM47421 from the National Institute of General Medical Sciences (C.C.H.), by grants CA10815 from the National Cancer Institute and BIR-9318027 (to The Wistar Institute).

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