Biglycan Gene Expression in the Human Leiomyosarcoma Cell Line SK-UT-1

BASAL AND PROTEIN KINASE A-INDUCED TRANSCRIPTION INVOLVES BINDING OF Sp1-LIKE/Sp3 PROTEINS IN THE PROXIMAL PROMOTER REGION

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In this study we demonstrate that the gene encoding the small leucine-rich proteoglycan biglycan is expressed in human myometrial tissue and in the human leiomyosarcoma cell line SK-UT-1. Treatment of SK-UT-1 cells with forskolin or 8-bromo-cAMP strongly increased biglycan mRNA and this effect was transcriptional as shown by transient transfection experiments with biglycan promoter-luciferase reporter fusion genes. The cAMP-mediated induction of the transfected biglycan promoter in SK-UT-1 cells was abolished by coexpression of a specific protein kinase A inhibitor, and was mimicked by overexpression of the catalytic subunit (Cβ) of protein kinase A. By 5’ deletion analysis, part of the cAMP response was localized to the segment from residues −78 to −46 of the biglycan promoter. This region conferred strong cAMP responsiveness to a heterologous promoter. Electrophoretic mobility shift and antibody supershift assays identified two specific complexes that contained nuclear proteins antigenically related to the ubiquitous transcription factors Sp1 and Sp3, respectively. The binding site of these proteins was mapped to a CT-rich sequence extending from −59 to −49 in the biglycan promoter. Mutating this sequence eliminated complex formation and markedly reduced basal and cAMP-dependent promoter activity of transfected reporter genes. In vitro binding studies using recombinant Sp1 revealed that the nuclear factor binding to the CT element was not Sp1 but a Sp1-like protein(s). Western blot analysis of SK-UT-1 nuclear proteins confirmed expression of Sp3, Sp1 and nuclear proteins that crossreacted with Sp1 antibody but according to their molecular weight were not Sp1. These results indicate that all cAMP-dependent as well as some basal biglycan transcription in SK-UT-1 cells is mediated through activated protein kinase A and that both functions are conferred at the promoter level through the interaction of Sp1-like/Sp3 factors with the CT element at −59 in the biglycan promoter.

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¶¶ The abbreviations used are: BGN, biglycan; bp, base pair(s); 8-Br-cAMP, 8-bromo-adenosine 3’-5’-cyclic monophosphate; CRE(B), cAMP responsive element (binding protein); ds, double-stranded; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Luc, luciferase (reporter); mSMC, myometrial smooth muscle cell(s); nt, nucleotide(s); PG, proteoglycan; PKA, protein kinase A; PKI, heat-stable inhibitor of PKA; PMA, phorbol 12-myristate 13-acetate; wt, wild type; PRL, prolactin; rPRL, rat prolactin; PCR, polymerase chain reaction.

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activity. For instance, the presence of several interleukin-6 response elements correlated with transcriptional activation by interleukin-6 in the breast cancer cell line T47D (19). Recently, a short region was identified in the BGN promoter that was differentially regulated in individuals with sex chromosome anomalies and that bound to the transcription factor c-Krox in vitro (20). Clustered in the proximal BGN promoter are putative binding sites for the ubiquitous transcription factors Sp1 and AP2, both of which can function as enhancer-binding proteins and can stimulate basal transcription through direct interaction with components of the basal transcription machinery. Both proteins are also targeted by certain signaling pathways through protein-protein interactions or post-translational modifications. For instance, AP2 activity is stimulated by phorbol 12-myristate 13-acetate (PMA) (21), and both AP2 and Sp1 have been shown to increase transcription in response to the second messenger cyclic AMP (cAMP) (21, 22).

In the course of studies that investigated the role of cAMP in the regulation of the prolactin (PRL) gene in myometrial smooth muscle cells (mSMC) (23), it was discovered that BGN mRNA expression was strongly induced in long term cultures of myometrial tissue explants; since after several days in culture mSMC spontaneously increase the formation of cAMP (23), it was conceivable that up-regulation of the BGN gene occurred in response to cAMP in one or more myometrial cell types. Prompted by this observation in conjunction with recent results from our laboratory showing that the human BGN gene is transcriptionally regulated by cAMP in osteosarcoma cells (24), we decided to investigate in greater detail the molecular basis of basal and cAMP-regulated transcription in myometrial cells. By using the leiomysarcoma cell line SK-UT-1, we demonstrate that 1) BGN mRNA expression is strongly up-regulated by forskolin and that this up-regulation is, at least in part, transcriptional, 2) induction of BGN gene expression by cAMP involves activation of the cAMP-dependent protein kinase A (PKA), and 3) basal and cAMP-induced transcription from the BGN gene is partially mediated through Sp1-like/Sp3

**Experimental Procedures**

**Tissues, Cell Lines, and Cell Culture—Human uterine tissues were obtained from either cycling or menopausal nonpregnant women at routine hysterectomy. Tissues were dissected post-surgically into endometrium and myometrium and rapidly frozen in liquid nitrogen (for in vivo experiments) or in RNA Clean, frozen pieces of human myometrial tissue were finely minced, whereas cultured cells were lysed directly in the wells. Poly(A)^+ enriched RNA was prepared by chromatography on oligo(T)-cellulose (25). Ribonuclease protection assay were carried out with the RPA II kit from Austin, TX as described elsewhere (19). The probe used was an 181-bp cDNA containing 158 bases of BGN sequence that had been transcribed in vitro with T7 RNA polymerase from nt +1267 to the Ncol site at +1110 of the human BGN cDNA (15). Northern blotting and hybridization were carried out as described in detail elsewhere (18). The Northern blots were probed with a 1144-bp human BGN cDNA fragment (nt +124 to +1267) generated by PCR as described previously (18). The probe DNAs were labeled with [α-32P]dCTP by the random priming procedure to a specific activity of 1 × 10^9 cpm/µg (26).

**Reverse Transcription-PCR Analysis of BGN mRNA in SK-UT-1 Cells and Southern Blotting—Total RNA (5 µg) per sample was transcribed using oligo(dT)15 and SuperScript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. After termination of the reaction, the reaction volume was diluted 5-fold with water, and 1/50th of first strand cDNA was used for PCR amplification.**

For BGN, a stringent hot-start-touch-down program was designed that comprised 35 cycles using oligonucleotide primers P + 124 and P + 135 that were derived from a parallel reaction of identical amounts of cDNA. Amplification of BGN cDNA for 16 cycles with primers GAPDH 5′-GGGGTTACCAAGTTGACGAAATG-3′ and GAPDH 3′-GAATTTCATGGATGAGCAGCAAGA-3′ resulted in a 206-bp fragment (nt +358 to +565). The BGN amplification products were blotted onto Hybond N membrane (Amer sham-Buchler, Braunschweig, Germany) and hybridized with an internal 32P-labeled cDNA fragment (nt +150 to +538 of the human BGN cDNA).

**Plasmid Construction**—A detailed description of the generation of the BGN promoter 5′ deletion luciferase fusion genes designated BGNc-A-Luc, BGNBam-Luc, BGNBgl-Luc, BGN-A-Luc, and BGN-1-Luc has been given previously (19). The plasmid –36rPRL-Luc containing the minimal promoter from the rat prolactin (rPRL) gene (nt -36 to -34) was a generous gift from Dr. M. G. Fedorko, Howard Hughes Medical Institute, San Diego, CA. Inserting the –78 to –35 and –46 to –1 regions of the BGN gene in sense orientation into the Smal site in the polylinker of –36rPRL-Luc gave rise to the plasmids –78hBGN-36rPRL-Luc and –46hBGN-36rPRL-Luc, respectively. These fragments had previously been amplified by PCR using Pfu polymerase (Stratagene) and the following oligonucleotide primers: 5′-CCCGCTCTCCCCGCTGTC-3′ (nt –78 to –59) and 5′-GGGCAACGCAGGGAGGGACAG-3′ (antisense to nt –64 to –35), or 5′-CCCGCTCGCCAGCTTTGAC-3′ (nt –66 to –26) and 5′-GGGAGCACGGGCCCCG-3′ (antisense to nt –19 to –1), respectively. The plasmid –78hBGNrev-36rPRL-Luc contains the –78 to –35 fragment from the human BGN gene promoter in reverse orientation in the Smal site of –36rPRL-Luc. The plasmid CRE-36rPRL-Luc contains two cAMP-responsive elements (CREs, bold) 5′-TTCGCGGGCCCAGGCGGAGACG-3′ linked to the RPL promoter and the luciferase gene in the vector pGL3-Basic (Promega). Its construction is described elsewhere (27). A derivative of the plasmid BGN-78-Luc containing point mutations (BGN-78mut-Luc) was synthesized as follows: an upstream interleukin-6 CRE mutation was generated by PCR as described previously (18). The probe DNAs were labeled with [α-32P]dCTP (26). A stringent hot-start-touch-down program was designed that comprised 35 cycles using oligonucleotide primers P + 124 and P + 135 that were derived from a parallel reaction of identical amounts of cDNA. Amplification of BGN cDNA for 16 cycles with primers GAPDH 5′-GGGGTTACCAAGTTGACGAAATG-3′ and GAPDH 3′-GAATTTCATGGATGAGCAGCAAGA-3′ resulted in a 206-bp fragment (nt +358 to +565). The BGN amplification products were blotted onto Hybond N membrane (Amer sham-Buchler, Braunschweig, Germany) and hybridized with an internal 32P-labeled cDNA fragment (nt +150 to +538 of the human BGN cDNA).


**RESULTS**

**Expression of the BGN Gene in Normal Human Myometrium and Its Regulation by Forskolin in the Leiomyosarcoma Cell Line SK-UT-1**—To investigate if the BGN gene is expressed in human myometrium, RNA was extracted from non-pregnant human myometrium and hybridized with a BGN cRNA probe in a ribonuclease protection assay. Significant expression was noted in myometrial tissue from all three patients analyzed (Fig. 1A). BGN expression in the myometrium was confirmed by Northern blot hybridization of total RNA from monolayer cultures of mSMC (Fig. 1B) and in poly(A)⁺-enriched RNA from cultured tissue explants (Fig. 1C). Interestingly, BGN mRNA was more abundant in myometrial tissue from long-term culture as compared with uncultured tissue (Fig. 1C); it had been shown previously that cultured mSMC cells spontaneously increase the formation of cAMP, eventually leading to changes in the expression of cAMP-responsive genes (23). We therefore sought to determine whether treatment of myometrial cells with agents known to increase intracellular cAMP, such as forskolin and stable cAMP analogs, would affect BGN gene expression. Since primary isolates of mSMC were not available for subsequent experimentation, we employed the leiomyosarcoma cell line SK-UT-1 which was previously shown to express the endogenous BGN gene and transfected BGN promoter-reporter fusion genes (19). As depicted in Fig. 1D, SK-UT-1 cells responded to a 24-h treatment with forskolin (50 μM) or 8-Br-cAMP (1 mM, data not shown) with a strong up-regulation of BGN mRNA.

**The Transfected Human BGN Promoter Is Responsive to 8-Br-cAMP in SK-UT-1 Cells**—To investigate whether the molecular basis for the up-regulation of BGN mRNA levels in SK-UT-1 cells is an enhanced transcription, we performed transient transfection assays with various BGN promoter-luciferase reporter constructs (Fig. 2). Transfection of the full-length promoter construct BGNSac-Luc (1218) into SK-UT-1 cells and treatment with 0.5 mM 8-Br-cAMP resulted in a strong induction of promoter activity. Upon removal of 5'
flanking sequences the cAMP inducibility increased and even
the shortest construct with appreciable basal promoter activity
(BGN-78-Luc) (19) maintained high responsiveness. Under the
same transfection conditions the plasmid −36rPRL-Luc har-
boring the minimal rat prolactin promoter in the same plasmid
backbone was only weakly inducible. Very similar results were
obtained with 10 μM forskolin (data not shown). These results
show that 1) cAMP inducibility was conferred to the plasmids
by the BGN promoter sequences and 2) the sequence(s) medi-
ating the cAMP response of the human BGN gene reside in the
proximal promoter region and/or the first 42 bp of exon 1.

To address the question whether regulation by cAMP is
direct or indirect (e.g. that a nuclear protein is initially (de
novo) synthesized which then directs efficient BGN gene ex-
pression), we followed the time course of BGN promoter activ-
iation. As shown in Fig. 2B, BGN promoter activation was
rapid with a 3-fold induction over unstimulated controls after
3 h and was essentially complete by 6 h. Although being of
lower magnitude, the overall activation kinetics of the BGN
promoter were similar to those of the CRE-containing control
construct known to be induced transcriptionally via phospho-
ylated cAMP response element-binding protein.

cAMP Responsiveness of the BGN Promoter in SK-UT-1 Cells
Is Mediated by PKA—Cyclic AMP almost exclusively exerts its
effects via activation of PKA and subsequent phosphorylation
of nuclear proteins (34). To test the role of PKA in mediating
the transcriptional activation of BGN by cAMP, the high affinity
peptide inhibitor of the catalytic subunit(s) of PKA, PKI, 2
was used to block the actions of PKA. Expression vectors en-
coding either PKI or a mutant, biologically inactive form
thereof, PKImut, were cotransfected with BGN-78-Luc into SK-
UT-1 cells (Fig. 3A). Cotransfection of PKImut had no effect on
the stimulation of BGN promoter activity by 8-Br-cAMP (5.5-
fold stimulation). In contrast, transfection of PKI diminished
the basal level of BGN-78-Luc promoter activity by 38% and
inhibited 8-Br-cAMP-induction by 73.1%. To confirm that the
effect of 8-Br-cAMP was mediated through activation of PKA,
cotransfection experiments were carried out with an expres-
sion vector containing the coding sequence for the catalytic subunit(s) of PKA, PKI, in
BGN-78-Luc. In these experiments, expression of the PKA
catalytic subunit alone (without the addition of exogenous
cAMP) increased BGN promoter activity at least to a similar
level as the 8-Br-cAMP stimulation and could not be increased
further by addition of this cAMP analog (Fig. 3B).

Mapping and Functional Analysis of the cAMP-responsive
Regions within the Proximal Promoter Region of the BGN
Gene—Results from previous transfection experiments sug-
ggested the presence of (at least) one element between posi-
tions −78 and −46 of the BGN gene that dramatically increased
basal transcription in a cell type-independent manner (19). As
shown in Fig. 4, removal of this region also partially reduced
cAMP responsiveness, since BGN-46-Luc was only inducible
3.7-fold (versus 5.5-fold in BGN-78-Luc). Further truncation
(BGN-1-Luc) largely abolished promoter activity.

In an attempt to dissect further the relative importance of
the regions between −78 and −46 and −46 and −1, respec-
tively, for BGN transcription, we tested their ability to confer
basal activation and cAMP responsiveness on a heterologous
promoter. SK-UT-1 cells were cotransfected with pRSV-Cβ or
pRSV-Cβmut and fusion plasmids in which two overlapping
fragments comprising nt −78 to −35 and −46 to −1 of the BGN
promoter, respectively, were cloned upstream of the minimal
rPRL promoter in the plasmid −36rPRL-Luc (Fig. 5A). As
shown in Fig. 5B, the plasmid −78bGN-36rPRL-Luc dis-
played a basal luciferase activity that was only 1.8-fold higher
than that for the control vector −36rPRL-Luc, whereas cAMP

inducibility was increased dramatically (31.6-fold over basal
levels). When the −78 to −35 region was inserted in the re-
verse orientation (clone −78hBGNrev-36rPRL-Luc), cAMP re-
ponsiveness was still increased over the parent vector albeit to

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**FIG. 2. Effect of 8-Br-cAMP on BGN promoter activity in SK-
UT-1 cells.** A, SK-UT-1 cultures were transfected with the full-length
BGN promoter/Luc construct BGNSac-Luc (containing 1218 bp of the
BGN promoter linked to the Luc reporter), various 5′ deletions thereof
(5′ ends as indicated), or the plasmid −36rPRL-Luc along with the
plasmid pCH110 as described under “Experimental Procedures.” After
transfection, the cultures were incubated for 18 h in normal growth
medium containing 0.5 mM 8-Br-cAMP followed by harvesting and
measurement of luciferase and β-galactosidase activities in the cell
extracts. Luciferase activities, reflecting relative promoter activities,
are shown as -fold induction over unstimulated controls. Three inde-
dependent experiments were performed in triplicate of which one repre-
sentative is depicted (mean ± S.D.). B, kinetics of 8-Br-cAMP-mediated
activation of the BGN-78-Luc construct in SK-UT-1 cells. The positive
control vector for cAMP induction, CRE-36rPRL-Luc3, contains two
CREs linked to a minimal promoter as described under “Experimental
Procedures.” The promoterless vector pGL2-Basic served as negative
control. Cells were lysed for reporter gene assay 12 h post-transfection.
Triplicate dishes had been exposed to 0.5 mM 8-Br-cAMP for the last 3,
6, 9, or 12 h; controls had received no cAMP. Luciferase gene activation
by 8-Br-cAMP is expressed as -fold induction compared with unstimu-
lated control values (mean, S.D. <15%).
a lower extent (11.5- versus 5.5-fold over controls), thus indicating orientation independence. In contrast, the clone 246hBGN-36rPRL-Luc was only slightly more cAMP-inducible than the minimal rPRL promoter alone (6.3- versus 5.5-fold over controls). These experiments clearly show that the sequence from 278 to 235, independently from downstream sequences, can function as a cAMP-inducible module.

Specific Protein-DNA Complexes Are Formed between Positions 282 and 235 of the BGN Promoter—Deletion of the region from 278 to 246 of the BGN promoter resulted in a substantial loss of basal promoter activity and cAMP responsiveness. In turn, this sequence could increase cAMP responsiveness when cloned upstream of a heterologous promoter. Therefore, we sought to define the trans-acting factors that interact with this sequence. A PCR-generated fragment encompassing the region from 278 to 235 (Fig. 6A) was used as a probe in EMSAs with nuclear extracts from 8-Br-cAMP and forskolin-treated, PMA-treated, and untreated SK-UT-1 cells. As shown in Fig. 6B, multiple shifted bands were obtained, but there was no apparent effect of any of the treatments on the relative proportions of the factors bound to the 278/235 probe, except for a band of fast electrophoretic mobility that likely represented a degradation product. We (19) and others (15) have previously identified a consensus binding site in the BGN promoter for the ubiquitous transcription factor Sp1 being present in reverse complement form at 278 (5'-CCGCCC-3') and a sequence 5'-CCCTCCCC-3' at 259 that is identical to a previously described functional Sp1-binding site in the rat ornithine decarboxylase promoter (35). Of note, both sequences are conserved between the human (19) and the mouse (36, 20) BGN gene (Fig. 6A). The presence of Sp1-like binding activity in SK-UT-1 nuclear extracts was confirmed using a specific Sp1 antibody in supershift reactions (Fig. 6B, lanes 3, 5, 7, and 9). Again, no major qualitative differences were seen among the various extracts in the supershift patterns. To more precisely map the nuclear protein-binding sites, two overlapping ds oligonucleotides were designed (~82~57 and ~64~39, Fig. 6A) and analyzed in EMSAs. As shown in Fig. 6C, lane 1, weak binding of nuclear proteins from 8-Br-cAMP-treated SK-UT-1 cells could be detected when the sequence

![Fig. 3. The cAMP response of the human BGN promoter in SK-UT-1 cells is mediated by PKA. A, effect of PKI on the activity of the BGN promoter in the presence or absence of 8-Br-cAMP. SK-UT-1 cells were cotransfected with the BGN promoter construct BGN-78-Luc and expression vectors for either PKI (pRSV-PKI ver2) or an inactive mutant thereof (pRSV-PKImut ver2). Following removal of the transfection solution, one-half of the cells was treated with 0.5 mM 8-Br-cAMP for 18 h, after which cells were lysed and luciferase activities determined. B, effect of the PKA catalytic subunit on the activity of the BGN promoter. SK-UT-1 cells were cotransfected with BGN-78-Luc and expression plasmids for either PKA-Cβ (pRSV-Cβ) or an inactive mutant thereof (pRSV-Cβmut). Following removal of the transfection solution, cells received normal growth medium with or without 8-Br-cAMP for 18 h. The data represent means ± S.D. of three independent experiments run in triplicate.](image-url)
extending from −82 to −57 was used as a probe. The DNA-protein complex observed most likely involved the Sp1 site at −78 as its formation was effectively competed for by a 50-fold molar excess of unlabeled Sp1 consensus oligonucleotide (Fig. 6C, lane 3). The presence of Sp1 or an Sp1-like protein in this complex was confirmed with an Sp1-specific antibody, the addition of which resulted in a supershift of this complex (Fig. 6C, lane 2). Much stronger sequence-specific gel shifts, however, were noted with the −64/−39 probe (Fig. 6C, lane 4). Three complexes were visible of which complexes I and II were specific since their formation could be strongly decreased by the addition of a 200-fold (lane 5), 100-fold (lane 6), or 50-fold (lane 7) molar excess of homologous unlabeled oligonucleotide but not by a 100-fold molar excess of subfragments extending downstream to only nt −52 (lane 10) or lacking the nucleotides upstream of nt −56 (lane 11). Complexes I and II were specifically competed for by a shorter version of the probe sequence (−64/−47, lane 8) as well as by an Sp1 consensus oligonucleotide (lane 12) suggesting that the involved proteins bind to the same or a very similar site within the −64/−47 region. In contrast, a third complex of faster electrophoretic mobility could not or only marginally be reduced by adding an up to 200-fold molar excess of unlabeled homologous DNA (lane 5) and was thus considered nonspecific.

The results from the initial supershift experiments shown in Fig. 6B together with data from the competition experiments with oligonucleotides containing known Sp1-binding sites strongly suggest that complexes I and II contain Sp1-related proteins. In order to identify which Sp1 family members are involved, we performed supershift assays with antibodies to Sp1, Sp2, Sp3, and Sp4. Whereas antisera to Sp2 (Fig. 6C, lane 14), Sp4 (lane 16), and AP2 (lane 17) failed to shift the electrophoretic mobility of any band, formation of the specific complex of slowest mobility (I) was completely supershifted by antiserum to Sp1 (lane 13). Likewise, incubation with an antiserum to Sp3 resulted in the disappearance of complex II and the occurrence of a weak supershift (lane 15).

Another set of EMSA competition experiments was performed to identify the coordinates and the critical nucleotides of the putative Sp1/Sp3-binding site (Fig. 6D). Changing the C residues at −58/−57 and/or −55/−54 to a purine abolished the ability of these oligonucleotides to compete for binding with the −64/−39 probe in complexes I and II (lanes 3, 4, 6, and 7). However, an oligonucleotide containing point mutations of nts
FIG. 6. Binding of nuclear proteins from SK-UT-1 cells to DNA fragments spanning the region from −82 to −35 of the BGN promoter. A series of short dsDNA fragments spanning the region between −82 and −35 of the human BGN gene was generated and analyzed in EMSAs for their ability to bind transcription factors. A, comparison of the human (h) BGN promoter sequence from −82 to −35 with the corresponding murine (m) sequence and nucleotide sequences of the various dsDNA fragments used as probes or competitor DNAs in EMSAs. Sequences that resemble binding sites for the transcription factor Sp1 are boxed. Mutant nucleotides are in lowercase letters. B, mapping of nuclear protein binding to the −78/−35 fragment by EMSA. The nuclear extracts (nuc. extr., 8 μg) from SK-UT-1 cells (untreated control, or treated for 2.5 h with PMA, or 6.5 h with 8-Br-cAMP or forskolin) were incubated with the labeled probe for 20 min at room temperature followed by 4% PAGE.
−62 through −60 partially retained its ability to compete (lane 2) indicating that these nts are less critical for the interaction of these factors with the BGN promoter thereby mapping the 5′ end of the binding site to −59. Its 3′ end was localized to somewhere between nt −52 and −49 as the −74/−49 oligonucleotide (lane 5) unlike the −64/−52 oligonucleotide (Fig. 6C, lane 10) competed with the −64/−39 probe for binding. As suggested from the corresponding competition experiment (compare Fig. 6C, lane 8) complexes I and II (but not the nonspecific complex) were also formed with a shorter version of the probe sequence (−64/−47, Fig. 6D, lane 12) but were absent from mutant versions of this oligonucleotide containing C to G (mut1) or C to A (mut2) transversions in positions −58/−57 and −55/−54 (lanes 8–11).

A Mutation in the Pyrimidine-rich Sequence between −59 and −49 in the BGN Promoter Reduces Both Basal and cAMP-induced Promoter Activity—The mutant oligonucleotide −64/−47mut1 which was shown above to be unable to bind nuclear proteins from SK-UT-1 cells was used to replace the corresponding wt sequence in the construct BGN-78-Luc. BGN-78mut-Luc was then functionally compared with the wt construct for its ability to confer basal and cAMP-dependent transcriptional induction in the natural context of the BGN promoter (Fig. 7). Basal promoter activity of BGN-78mut-Luc was only 31% that of the wt construct. Moreover, cAMP-dependent transcription was reduced to 20.8%. These data further attest to the dual role of the CT-rich sequence between nt −59 and −49 in the promoter in modulating BGN gene expression.

Further Characterization of the Nuclear Protein from Complex I—The competition and supershift data presented in Fig. 6, B–D, suggested that complex I contained an Sp1-like protein. We then addressed the question whether this protein was indeed Sp1 by investigating if recombinant Sp1 was capable of binding in vitro to the −64 to −39 region in the BGN promoter (Fig. 8). Surprisingly, no binding could be detected (lanes 1 and 2). This was not due to degradation of the Sp1 protein since it was able, like the endogenous Sp1 from SK-UT-1 cells, to produce a strong gel shift with the Sp1 consensus oligonucleotide (compare lanes 6 and 8–10) which could be supershifted using the same Sp1 antibody as above (lanes 5 and 9, respectively). The presence in SK-UT-1 cells of Sp1 as well as proteins antigenically related to Sp1 was further suggested by results from immunoblot analysis (Fig. 9). The Sp1 antibody specifically recognized several proteins in the size range of 60–120 kDa in addition to the p106/p95 doublet characteristic of Sp1 in many cell types (Fig. 9, lanes 1–2). In contrast, the Sp3 antibody detected two doublets of ∼120 and ∼80 kDa (Fig. 9, lanes 4–5) which have been reported to represent the full-length Sp3 protein and Sp3 isoforms that arise from internal translational initiation, respectively (37). Immunoreactivity of the Sp1 and Sp3 antibodies was fully abolished by preincubation with the respective blocking peptides (lanes 3 and 6, respectively). No qualitative or quantitative differences in Sp1-like and Sp3 protein expression were evident between 8-Br-cAMP-treated and control cells (compare lanes 1 and 2 for Sp1 and lanes 4 and 5 for Sp3, respectively). Taken together, we conclude that Sp3 and an Sp1-like protein other than Sp1 bind to the sequence 5′-CCCTCCCCGTC-3′ in the proximal BGN promoter and that this interaction can enhance both basal transcriptional activity and cAMP responsiveness.

DISCUSSION

In this study we have analyzed the molecular components involved in basal and cAMP-dependent transcription of the BGN gene in the human leiomyosarcoma cell line SK-UT-1. The increase in BGN mRNA levels observed upon treatment of these cells with 8-Br-cAMP or forskolin was primarily transcriptional as judged from up-regulation of transiently transfected BGN promoter-luciferase reporter constructs and was mediated by PKA. Partial mapping of the cAMP-responsive regions in a homologous and a heterologous promoter context in combination with DNA-nuclear protein binding, competition, and supershift studies led to the identification of a short pyrimidine-rich sequence in the proximal promoter (nts −59 to −49) that served as a binding site for Sp1-like and Sp3 transcription factors. A mutation of this element that disrupted binding of the Sp1-like/Sp3 proteins dramatically decreased both basal and cAMP-stimulated transcription from transfected human BGN promoter-reporter constructs.

Transcriptional regulation by cAMP typically involves nuclear translocation of the free catalytic subunits of PKA which then phosphorylate and thereby increase the transactivation potential of CRE-binding proteins (38). However, since no CRE-
like sequences are present in the proximal BGN promoter, a participation of this class of transcription factors seems unlikely. Nevertheless, the rapid rise in cAMP-induced promoter activity and the fact that we could not observe any qualitative differences in specific protein binding to the −78 to −35 region between 8-Br-cAMP/forskolin-treated and untreated cells favors a direct mode of regulation (see below).

Interestingly, we found that PKI decreased BGN transcriptional activity in SK-UT-1 cells abundantly express Sp1 and Sp3-like proteins. Nuclear proteins (20 μg) from SK-UT-1 cells were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibody against Sp1 (anti-Sp1) or Sp3 (anti-Sp3) and subjected to ECL detection. Specificity controls were performed by incubating the primary antibodies with an excess of the respective blocking peptide prior to antigen detection. Molecular size markers are indicated on the right. The large arrowheads in the anti-Sp1-treated blot point to specific bands that likely represent Sp1-related nuclear proteins.

Regulation of Basal and cAMP-induced Biglycan Transcription

The nuclear protein of complex I is not Sp1 itself. Recombinant (rec.) Sp1 (1–2 footprinting units) or nuclear extract (nucl. extr.) from 8-Br-cAMP-treated SK-UT-1 cells were used for EMSA on BGN −64/−39 fragment or an Sp1 consensus (cs) element. The position of complex I in lane 4 is indicated by an arrowhead. Due to partial degradation complex II is hardly visible here.

The precise functional role of each of these factors upon interaction with the proximal BGN promoter remains speculative at this stage. Whereas Sp1 in most cellular systems stimulates transcription, Sp3 has been shown to be a bifunctional regulator that can act either as a transcriptional repressor or activator of Sp1-mediated transactivation depending on cell type and promoter context (reviewed in Ref. 44). Repression may occur in an active fashion due to the presence of a putative repressor domain in the Sp3 protein (43, 44) by antibody supershift and subsequent Western blot analysis. This was given support by results from immunoblots with SK-UT-1 nuclear proteins showing that the same Sp1 antibody detected several specific bands of which only two (106 and 95 kDa) are known to represent Sp1 itself. Studies are now underway to purify, characterize, and clone this Sp1-related factor. The protein from complex II was identified as Sp3 (42, 43) by antibody supershift and subsequent Western blot analysis. This was not surprising as Sp1 and Sp3 bind to the same or very similar DNA-binding sites in gene promoters (44) eventually resulting in a gel shift pattern of striking similarity to that in the BGN promoter (45). Consistent with this notion, in vitro binding studies with various mutant versions of the −59/−52 sequence resulted in a simultaneous disappearance of both proteins suggesting a competition between them for binding to the CT element.

The following table shows the positions of the probes and competitors used in the EMSA experiments:

| probe | -64/−39 wt | Sp1 cs |
|-------|----------------|---------|
| protein | rec. Sp1 | nucl. extr. | SK-UT-1 + 8-Br-cAMP | rec. Sp1 |
| competitor | - | Sp1 cs | - |
| antibody | Sp1 | - | Sp1 | - | Sp1 | - |

FIG. 8. The nuclear protein of complex I is not Sp1 itself. Recombinant (rec.) Sp1 (1–2 footprinting units) or nuclear extract (nucl. extr.) from 8-Br-cAMP-treated SK-UT-1 cells were used for EMSA on BGN −64/−39 fragment or an Sp1 consensus (cs) element. The position of complex I in lane 4 is indicated by an arrowhead. Due to partial degradation complex II is hardly visible here.

FIG. 9. SK-UT-1 cells abundantly express Sp1 and Sp3-like proteins. Nuclear proteins (20 μg) from SK-UT-1 cells were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibody against Sp1 (anti-Sp1) or Sp3 (anti-Sp3) and subjected to ECL detection. Specificity controls were performed by incubating the primary antibodies with an excess of the respective blocking peptide prior to antigen detection. Molecular size markers are indicated on the right. The large arrowheads in the anti-Sp1-treated blot point to specific bands that likely represent Sp1-related nuclear proteins.
found that the 78–80-kDa isoforms of Sp3 that arise via internal translational initiation function as potent inhibitors of Sp1/Sp3-mediated transcription. Strikingly, we detected this doublet in immunoblots of SK-UT-1 nuclear proteins at an equal intensity as the full-length protein. However, it remains to be tested which of these different Sp3 isoforms bind to the CT element in the BGM promoter in vivo.

The mutation of the Sp1/Sp3-binding site in BGN-78-Luc reduced CAMP inducibility to the level seen with BGN-46-Luc, whereas basal promoter activity, although strongly diminished, was still significantly higher than in BGN-46-Luc. This indicates that additional cis-acting sequences within the 78 to 46 region contribute to basal transcription. A likely candidate is the Sp1 consensus sequence at 78 which was shown to bind an Sp1(-like) factor, most likely Sp1 itself. In fibroblasts, Sp1 binding has recently also been detected at an Sp1 consensus site located further upstream (~216) (20).

Members of the Sp1 family of transcription factors (others than Sp1) play a role in the transcriptional regulation of a large and diverse array of genes, among which are other extracellular matrix genes. In the mouse syndecan-1 gene whose promoter shares several features with the BGN gene (TATA-less, GC-rich 5'-flanking region with several putative Sp1 sites), Sp1-like factors were shown to be responsible for the constitutive expression in epithelial cells (46). In the human α2(I) collagen gene an Sp1/Sp3-binding site (TCTCC) mediates both basal transcriptional activity and oncostatin M stimulation (41). Whereas the involvement of Sp1-like proteins in conferring high levels of constitutive and cytokine/hormone-stimulated transcriptional activity to many genes is well established, their participation in CAMP-regulation is less well documented and has so far only been clearly demonstrated for the bovine CYP1A gene (22) and the rhesus monkey growth hormone-variant gene (45). If one postulates a direct mode of regulation for the CAMP-PKA signal transduction pathway in inducing BGN gene expression (see above), then the question arises how it is mechanistically linked to the Sp1-like/Sp3 proteins. Rather than recruitment of new proteins to DNA-binding sites, the effects of CAMP may involve modulation of protein-protein interactions, transcriptional complex assembly, or post-translational modifications of a trans-acting protein. Post-translational modifications by the protein kinase A pathway have been described (47–52). Specifically, Sp1 can be phosphorylated (53), but it does not have a PKA phosphorylation site. However, this may not be true for the Sp1-like protein binding to the BGN promoter, and it is thus conceivable that its transactivation function is increased upon phosphorylation by PKA. Alternatively, the Sp1-like/Sp3 factors bound to the BGN promoter in SK-UT-1 cells may interact with a cell-specific coactivator that itself is not a DNA-binding protein but can serve as a target for PKA and subsequently activate the Sp1-like/Sp3 proteins.

Preliminary data from our laboratory suggest that CAMP regulation of BGN gene expression is also operating in mSMC, the predominant cell type in myometrial tissue. What could be the physiological significance of CAMP-mediated up-regulation of BGN expression in myometrial cells? Adenylate cyclase activity has been reported to increase in the human myometrium during pregnancy (54), reflecting stimulation by hormones like gonadotropins, PGE2, relaxin, and/or corticotropin-releasing factor that act through elevation of intracellular CAMP. This could result in chronic activation of BGN synthesis and may indicate a greater need for BGN for uterine growth during pregnancy and/or the preparation process of the uterus for parturition. A relevant reproduction-associated participation is well documented in the uterine cervix, a tissue that undergoes...
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