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Sp1 and Sp3 Activate the Rat Connexin40 Proximal Promoter

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The rat gap junction protein connexin40 (rCx40) has a characteristic developmental and regional expression pattern, for which the exact regulatory mechanisms are not known. To identify the molecular factors controlling Cx40 expression, its proximal promoter was characterized. The proximal rCx40 promoter is the most conserved noncoding region within the Cx40-gene known thus far and contains five potential binding sites for Sp-family transcription factors. The binding of both Sp1 and Sp3 to each of these DNA elements was demonstrated by EMSA. Luciferase assays of the natural rCx40 proximal promoter or mutated derivatives in Cx40-expressing (NCM, primary rat neonatal cardiomyocytes and A7r5, rat smooth muscle embryonic thoracic aorta cells) and -nonexpressing cells (N2A, mouse neuroblastoma cells) revealed that all sites are contributing to basal promoter activity. *Trans*-activation assays in *Drosophila* Schneider line 2 cells demonstrated that Sp1 and Sp3 activate the rCx40 proximal promoter in a dose-dependent and additive manner.

Key Words: **promoter; transcriptional regulation; connexin40; gap junctions; gene expression.**

Connexin40 (Cx40) is one of the gap junctions in the heart. In contrast to the abundantly expressed connexin43, Cx40 has a characteristic developmental and regional expression pattern (1), which suggests the presence of tight spatiotemporal control mechanisms. During development of the rat heart Cx40 mRNA expression becomes first apparent at embryonic day 13. Subsequently, the expression increases during the fetal period but it declines again towards birth (2). In the adult heart of most mammals, the endothelial layer of blood vessels, atrial myocytes (with the exception of the rat) and the proximal part of the conduction system display Cx40 expression. Ventricular myocytes, however, lack Cx40 (3).

Changes in the distribution and/or expression level of Cx40 in the heart have been demonstrated under several pathophysiological conditions, amongst which atrial fibrillation (4, 5), hypertrophy (6) and congestive heart failure (7). The molecular basis for these changes is unknown.

Interestingly, the relevance of Cx40-mediated intercellular communication for normal heart and endothelial function has been demonstrated by gene disruption studies. Compared to wild-type littermates, Cx40-deficient mice display cardiac conduction abnormalities correlated with increased incidence of arrhythmias (8–10). Level and cell type-specificity of Cx40 expression is probably determined at the transcriptional stage by regulatory sequences in the gene's promoter. The genomic organization for Cx40 has been determined (11, 12). The gene consists of two exons, separated by a large intron. A small part of the 5′-untranslated region, the complete coding sequence and the 3′-untranslated region are localized on the second exon. The first exon contains the remainder of the upstream 5′-untranslated region. We recently mapped the Cx40 proximal promoter and implicated two Sp-binding sites in transcriptional activation of the rat Cx40 (rCx40) gene (13). Our previous promoter/reporter analysis, however, suggested that additional regulatory regions distinct from those reported contribute to proximal rCx40 promoter activity as well.

In this study we focus on the conserved sequence of the proximal promoter to further elucidate regulatory sequences and transcription factors that regulate basal Cx40 expression. We have found that three additional Sp-binding sites are required for optimal proximal rCx40 promoter activity. The transcription factors Sp1 and Sp3 bind to these sites and activate the rCx40 promoter.

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MATERIALS AND METHODS

Cell culture. The origin and culture conditions for the rat thoracic aorta smooth muscle cell line A7r5 and mouse neuroblastoma N2A cells have been described previously (13). *Drosophila* Schneider line 2 (SL2) cells (14) were purchased from the American Type Culture Collection (ATCC, CRL-1963, Manassa, VA) and cultured in Schneider's *Drosophila* medium (Life Technologies, Rockville, MD) supplemented with 10% insect-qualified and heat-inactivated fetal bovine serum (Life Technologies), 50 U/ml penicillin and 50 μg/ml streptomycin at 24°C in ambient air.

Primary neonatal rat ventricular cardiomyocytes (NCM) were isolated from hearts of 1- to 2-day-old male Wistar rats (in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)). Hearts were excised aseptically and large vessels and both atria were removed. Ventricular tissue was cut into $\sim$2-mm^3 small pieces and remaining blood was washed out. Tissue fragments were transferred to a sterile glass container and shaken in 14 ml of a Hanks' saline solution, without Ca^{2+} and Mg^{2+}, supplemented with 0.13% trypsin and 20 μg/ml DNase (Roche, Mannheim, Germany) for 12 min at 37°C. Next, the suspension was gently triturated, and the first supernatant was discarded from the sedimented tissue and replaced by fresh enzyme solution. This was repeated for 5 cycles of 15 min, wherein now supernatants were collected and supplemented with 6 ml of routine culture medium composed of Ham's F10 with L-glutamine (Life Technologies) and 10% fetal bovine serum (Life Technologies). Cells were pelleted during 3 min by centrifugation. To enrich the population of myocytes a differential attachment protocol was used (15). Briefly, cell pellets were suspended in culture medium and plated for 2.5 h on uncoated culture dishes (Corning 430167, NY). In contrast to cardiomyocytes, non-myocytes rapidly adhere to the plastic and therefore can be removed from the cell preparation. The non-adhering cells were collected by centrifugation, resuspended in fresh culture medium, and plated on laminin (10 μg/ml, Boehringer, Mannheim, Germany) coated 24-wells plates (Costar 3542, Corning) in a density of 2.5–3 $\times$ 10^5 cells/well. NCM cultures were 80–90% pure.

Electrophoretic-mobility-shift assays (EMSA). Microscale preparations of nuclear extracts, (competitive) EMSA, and EMSA/supershift assays were essentially performed as described previously (13). The oligonucleotides, corresponding to selected regions in the rCx40 proximal promoter, and mutated derivatives thereof used for these assays are listed in Table 1. Antibodies specific for transcription factors of the Sp-family were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid construction, transfections, and reporter assays. Four constructs, differing in the length of the 5′-upstream sequence of the rCx40 promoter (map positions -1125, -455, -296, and -175 to $+85$ relative to the transcription initiation site (Fig. 2)) were placed in front of the *Firefly* luciferase reporter gene in the pGL3-Basic vector (Promega, Madison, WI) as described previously (13). The rCx40 promoter region in between map positions -175 and $+85$, named p(-175, $+85$), served as template for site-directed mutagenesis of Sp-binding site motifs performed according to the manufacturer's recommendations (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA). Briefly, synthetic oligonucleotides containing mutations in specific Sp-binding sites of the rCx40 promoter were used as primers to extend and amplify the desired mutated version of p(-175, $+85$). In order to exclude PCR-related mutations in the generated vector, the integrity of the mutated (-175, $+85$) region was identified by BigDye-cycle sequencing analysis in two directions on an ABI Prism 310 genetic analyzer (Perkin Elmer, Foster City, CA), excised by *Xho*I/*Hin*dIII digestion, and subsequently subcloned in the appropriate restriction sites of the original pGL3-Basic vector. For the generation of plasmids with mutations in more than one Sp-binding site this procedure was sequentially repeated with alternate primer pairs. The primers used in this regard are represented in Table 1 as mutated (mut) oligonucleotides.

pRL-CMV (Promega), in which the cytomegalovirus immediate-early enhancer/promoter directs *Renilla* luciferase expression, was used in luciferase reporter assays to normalize for differences in transfection efficiency in between individual transfections. The plasmids pPacSp1 and pPacUSp3 (16), respectively containing the transcription factors Sp1 and Sp3 under control of the *Drosophila* actin 5C promoter and pPac0, the empty control vector, where used to test for *trans*-activation in SL2 cells. The plasmids pPac0 and pPacSp1 were provided by Dr. Robert Tjian (Howard Hughes Medical Institute, University of California at Berkeley) and the pPacUSp3 plasmid was provided by Dr. G. Suske (Institut für Molekularbiologie und Tumorforschung, Klinikum der Philipps-Universität Marburg, Germany).

A7r5 and N2A cells were co-transfected by calcium phosphate precipitation with an experimental plasmid (i.e., p(-175, $+85$) or mutated derivatives thereof) and pRL-CMV essentially as described before (13). NCM were transfected using a modification of the calcium phosphate procedure (17). Essentially $\sim$2.5 $\times$ 10^5 cells/well in a 24-wells plate were transfected, overnight at 35°C and 3% CO_2, with 2 μg of pGL3-Basic or molecular equivalents of an experimental construct and 0.025 μg of pRL-CMV reporter vector. Cell lysates were prepared 24 to 48 h after transfection and assayed for luciferase activity on a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) according to the dual-luciferase reporter assay procedure (Promega). Variations in transfection efficiency in between individual transfections were normalized for by using the *Renilla* luciferase measurements. The results obtained were averaged from at least 6 individual transfections in which two different plasmid preparations for each construct were used, and are expressed as mean ± SEM.

To test for the *trans*-activation of the rCx40 proximal promoter by Sp1 and Sp3, 4.2 $\times$ 10^6 SL2 cells were plated in 60-mm-diameter culture dishes and transfected by calcium phosphate precipitation (18) using 4 μg of experimental plasmid p(-175, $+85$) and a total of 5/6 μg *Drosophila* expression plasmids. Different concentrations, 0.5 μg, 2 μg, and 5 μg, of pPacSp1 and pPacUSp3 were tested. Also a combination of pPacSp1 and pPacUSp3 was used to test for mutual effects. The pPac0 was applied as the negative control for *trans*-activation and used to adjust the amount of *Drosophila* expression plasmids to a total amount of 5 μg (when using different concentrations of a single expression plasmid) or 6 μg (when using a combination of expression plasmids). For normalizing variations in transfection efficiency 4 μg of a copia-LacZ (promoter-reporter) plasmid was co-transfected as well. Transfected cells were harvested after 24–48 h, lysed in passive lysis buffer (Promega) by 3 freeze-thaw cycles and assayed for luciferase activity according to the Luciferase Assay System procedure (Promega). Subsequently a β-galactosidase assay was performed, according to a standard protocol (19). The results obtained were averaged from at least 6 individual transfections in which two different plasmid preparations for each construct were used, and are expressed as mean ± SEM.

Sequence alignment. To search for conserved non-coding regions within the Cx40-gene, sequence alignments of the rat, mouse and human Cx40-gene sequence were performed. The mouse sequence is obtained from GenBank Accession Nos. AF375883 and AF023131. The human Cx40 sequence is extracted from the homo sapiens chromosome 1 working draft sequence segment, GenBank Accession No. NT008583. For obtaining the rat sequence we screened the λ DASH II rat genomic library (Stratagene, La Jolla, CA) with a probe against the (-1125, $+85$) sequence. We sequenced 1864 bp upstream and 3186 bp downstream of the transcription initiation site. This sequence has been deposited in the GenBank data base (Accession No. AY078389).

Alignments were performed using the mVISTA Web site (http://www-gsd.lbl.gov/vista/) at default settings (20, 21). Conserved non-

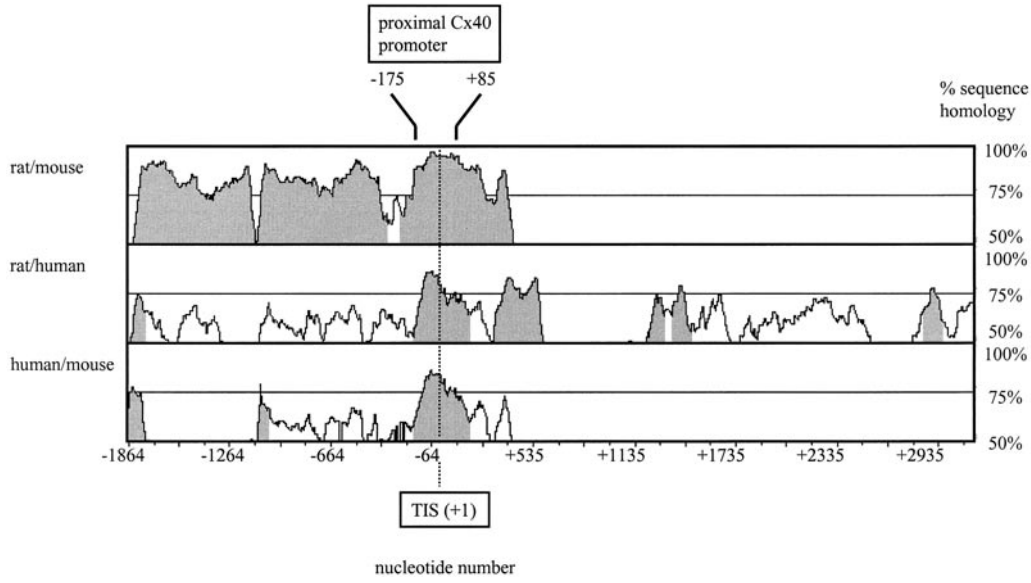


FIG. 1. Sequence alignment of the rat, human and mouse Cx40 genes. Rat, mouse, and human Cx40-gene sequences were aligned using mVISTA (20, 21). Dark areas indicate conserved regions. The criteria used were 75% identity over 100 bp. The *X* axis corresponds to the rat nucleotide sequence; the transcription initiation site (+1) is indicated by a dotted line. The proximal rCx40 promoter spans from −175 to +85.

coding sequences (CNS) were defined as regions of at least 75% identity over more than 100 bp.

RESULTS

Recently we demonstrated that the proximal rCx40 promoter is contained within the region extending from 175 nucleotides upstream towards 85 nucleotides downstream of the transcription initiation site (13). Alignment of the human, mouse and rat Cx40 sequences up- and downstream of the transcription initiation site revealed several stretches of homology, of which the proximal promoter is most conserved between the three species (Fig. 1). This conserved (−175, +85) region contains five potential binding sites for transcription factors of the Sp-family (22) (A–E in Fig. 2), of which only site D is not conserved between rat, mouse and human. For two of these elements (B and E, indicated with an asterisk in Fig. 2) Sp1 and Sp3 binding was demonstrated; deletion or mutations of these sites interfered with transcriptional activity and abrogated Sp1 and Sp3 binding. However, promoter/reporter assays with constructs in which the rCx40 promoter was serially deleted from the 5′-end clearly suggested the involvement of other Sp-binding elements (e.g., A, C and D in Fig. 2) in transcriptional activation as well (13). We now provide evidence that every single putative Sp-binding site can contribute to promoter activity in Cx40-expressing cells, including primary NCM, and -nonexpressing cells, via Sp1 and Sp3.

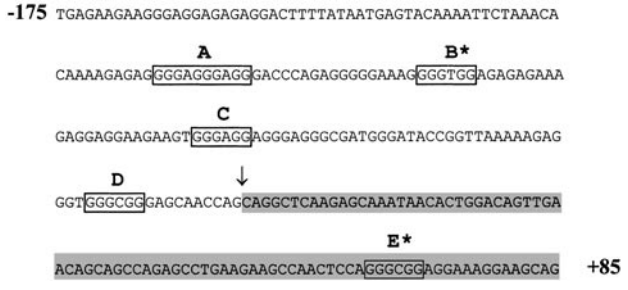


FIG. 2. Potential Sp-binding elements in the rCx40 proximal promoter. The core sequences of the Sp-binding motifs are boxed and the position of the transcription initiation site is indicated by an arrow (and defined as map position +1). The complete exon 1 sequence is shadowed (11). The Sp-binding elements previously implicated in rCx40 proximal promoter activation (13) are indicated with an asterisk.

rCx40 Promoter Activity in NCM

In order to map the rCx40 transcriptional regulatory region in NCM four different 5′-deletion constructs (p(−1125, +85); p(−455, +85); p(−296, +85); p(−175, +85)) were transfected in NCM. After an expression period of ~36 h cell lysates were prepared and analyzed for luciferase activity. As shown in Fig. 3, the results obtained with cardiomyocytes resembled those obtained with cell lines (A7r5, N2A) (13) in that the smallest construct tested (i.e., p(−175, +85)) provided for almost full promoter activity.

Sp1 and Sp3 Bind to DNA Elements A, C, and D

Double-stranded synthetic oligonucleotides encompassing the regions from (−125, −95), (−75, −45) and (−33, +2) (wildtype (wt) oligonucleotides in Table 1) were radiolabeled, incubated with nuclear extract from either A7r5 or N2A cells in the presence or absence of specific Sp-antibodies, and analyzed by non-denaturing polyacrylamide gelelectrophoresis. The results obtained with A7r5 nuclear extracts and the

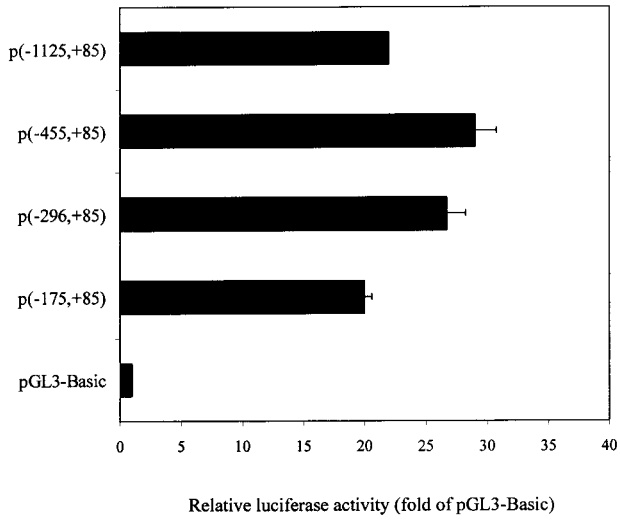


FIG. 3. Mapping of the rCx40 transcriptional regulatory region in NCM by luciferase expression analysis. Promoter-reporter constructs (13) were tested for transcriptional activity in NCM. The luciferase activity for the pGL3-basic vector, in which a functional promoter was absent, was set at 1.0 and all other luciferase activities were expressed as fold of the values obtained for pGL3-Basic.

(−125, −95), (−75, −45) and (−33, +2) regions are shown in Figs. 4A–4C, respectively; similar results were obtained with N2A extracts as well (data not shown). All three regions displayed very similar band-shift profiles. In the absence of specific antibody two prominent DNA-protein complexes were obtained (C1 and C2 in Fig. 4). In the presence of anti-Sp1 antibody a supershifted complex was obtained, that appeared to be primarily derived from the original complex C1. Similarly, when an anti-Sp3 antibody was added also a supershifted complex was obtained, that appeared to be primarily derived from complex C2. However, application of both anti-Sp1 and anti-Sp3 antibodies in the same binding reaction resulted in the almost complete shift of complexes C1 and C2, indicating that C1 consisted of both DNA-Sp1 and -Sp3 complexes. In contrast, when an anti-desmin control antibody was included the migrational behavior of the complexes C1 and C2 did not change.

To further confirm the involvement of the elements A, C, and D (Table 1) in Sp1/Sp3 binding, we performed competition experiments with wildtype and mutated regions. The effect of 20- and 100-fold molar excesses of non-labeled oligonucleotides wt(−125, −95), wt(−75, −45) and wt(−33, +2) or their non-labeled derivatives mut(−125, −95), mut(−75, −45) and mut(−33, +2) was investigated on Sp1/Sp3 binding to the radiolabeled (−125, −95) (Fig. 5A), (−75, −45) (Fig. 5B) and (−33, +2) (Fig. 5C) regions, respectively. In all instances, the competitors containing mutations in the binding site were unable to compete, whereas the wildtype oligonucleotides did compete for Sp1/Sp3 binding. Thus both Sp1 and Sp3 can bind to the rCx40-derived promoter regions A (−125, −95), C (−75, −45) and D (−33, +2).

Sp1/Sp3 Binding Sites A, C, and D Contribute to Transcriptional Activity

To investigate the contribution of the Sp-binding sites A, C and D to total rCx40 promoter activity luciferase reporter assays were performed in NCM, A7r5

TABLE 1

Oligonucleotides Used in the Characterization of rCx40 Promoter Regulation

Oligonucleotides	Sequence (5′ to 3′ direction)
	A
wt(−125, −95)	CACAAAAGAGAG GGGAGGGAGG GACCCAGAG
mut(−125, −95)	CACAAAAGAGAG G***TAT***G***TAT***GG GACCCAGAG
	B
wt(−98, −64)	AGAGGGGGAAAG GGGTGG AGAGAGAAAGAGGAGGA
mut(−98, −64)	AGAGGGGGAAAG GG***CA***GG AGAGAGAAAGAGGAGGA
	C
wt(−75, −45)	GAAAGAGGAGGAAGAAGT GGGAGG AGGGAGG
mut(−75, −45)	GAAAGAGGAGGAAGAAGT G***TAT***GG AGGGAGG
	D
wt(−33, +2)	CCGGTTAAAAAGAGGGT GGGCGG GAGCAACCAGCA
mut(−33, +2)	CCGGTTAAAAAGAGGGT G***TAT***GG GAGCAACCAGCA
	E
wt(+53, +87)	GAAGCCAACTCCA GGGCGG AGGAAAGGAAGCAGGT
mut(+53, +87)	GAAGCCAACTCCA G***TTA***GG AGGAAAGGAAGCAGGT

Note. Appropriate Sp-binding elements are boxed and named as in Fig. 2; substitutions in mutated (mut) oligonucleotides relative to the corresponding native sequence are indicated in bold italics.

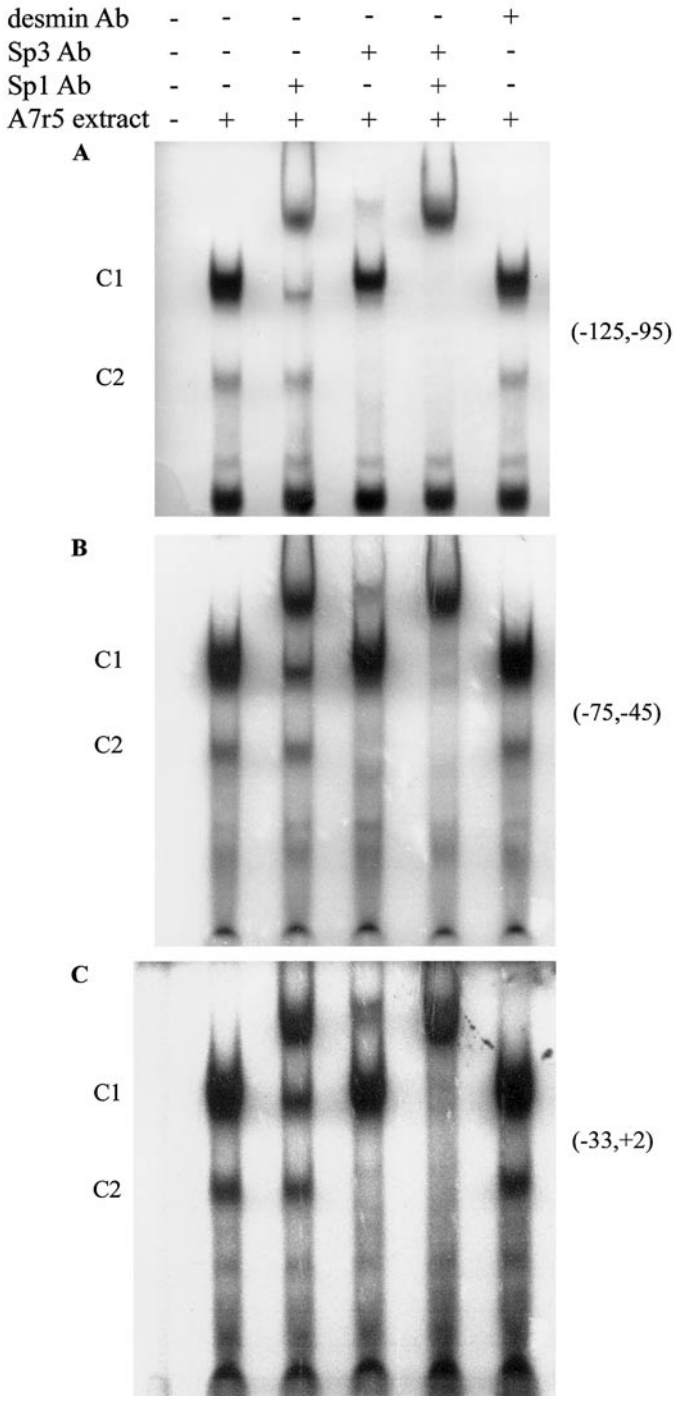


FIG. 4. EMSA of potential Sp-binding elements in the presence of Sp1 and/or Sp3 antibodies. The sequences of oligonucleotides used in these experiments are listed in Table 1. Nuclear extracts prepared from A7r5 cells were incubated with ^{32}P-end-labeled oligonucleotides wt(−125, −95) (A), wt(−75, −45) (B), and wt(−33, +2) (C) in the presence or absence of specific antibodies as indicated; the formed DNA–protein complexes were separated on a 4% non-denaturing polyacrylamide gel and visualized by autoradiography. Only bands representing DNA–protein complexes are shown; the bottom part of the gel, containing only the probe band, has been excluded from the figure. The position of the Sp1/Sp3 binding complexes (C1 and C2) is indicated at the left.

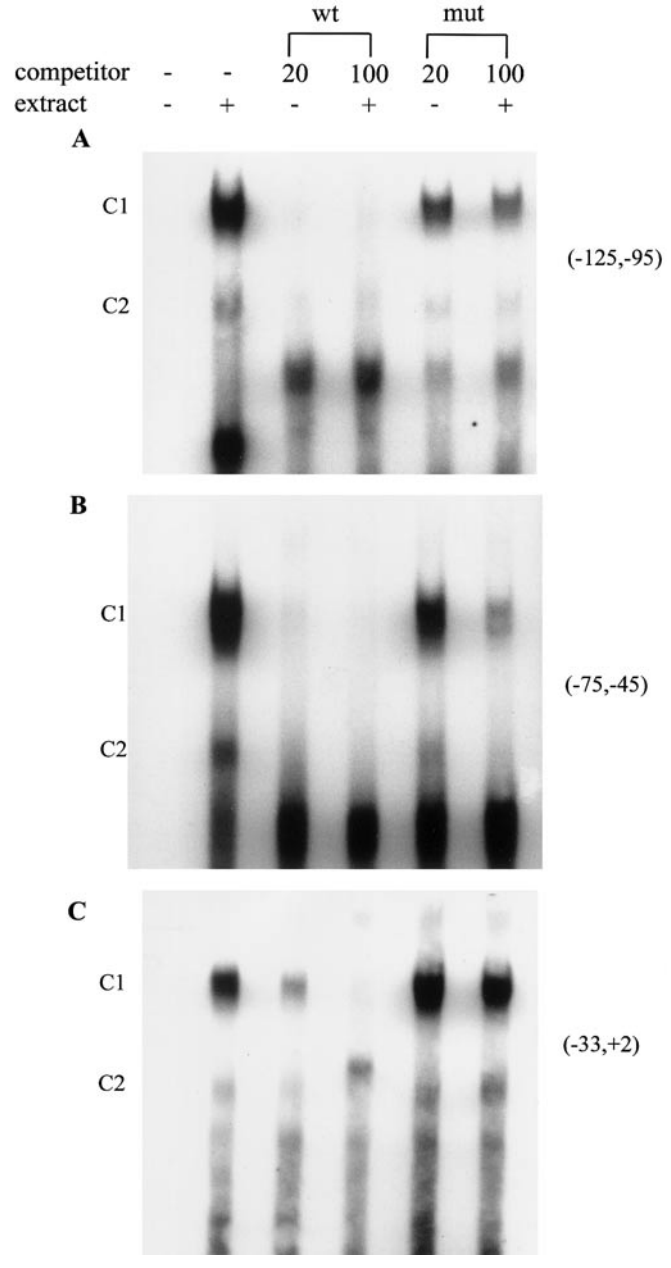
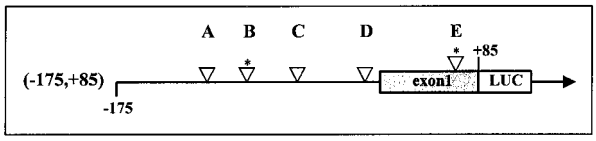


FIG. 5. Competitive EMSA of rCx40 promoter DNA. ^{32}P-end-labeled oligonucleotides wt(−125, −95) (A), wt(−75, −45) (B), and wt(−33, +2) (C) were incubated with A7r5 nuclear extract in the absence of presence of a 20- or 100-fold molar excess of the corresponding nonlabeled identical (wt) oligonucleotides or oligonucleotides containing specific mutations in the Sp-binding motif (mut) as indicated. The formation of DNA–protein complexes was analyzed by 4% non-denaturing polyacrylamide gel electrophoresis. Only bands representing DNA–protein complexes are shown; the bottom part of the gel, containing only the probe band, has been excluded from the figure. The positions of the Sp1/Sp3 binding complexes (C1 and C2) are indicated at the left.

and N2A cells. The plasmids used consisted of p(−175, +85) and mutated derivatives thereof containing nucleotide substitutions in one or more specific Sp1/Sp3



	Rel. luc. activity % (SEM)		
	NCM	A7r5	N2A
p(-175,+85)	100	100	100
mutA,C,D	6.7 (1.0)§	5.0 (0.3)§	9.9 (0.4)§
mutA	37.9 (2.4)§	88.2 (15.6)	66.9 (6.4)§
mutB	36.0 (2.4)§	52.7 (8.4)§	50.8 (4.0)§
mutC	37.5 (5.5)§	72.2 (14.8)	62.1 (4.8)§
mutD	35.4 (2.8)§	86.4 (14.6)	70.1 (5.6)§
mutE	56.3 (3.9)§	41.1 (3.2)§	58.2 (4.5)§
mutA,C	22.2 (1.7)§	13.8 (0.7)§	26.3 (0.3)§
mutA,D	7.1 (0.4)§	19.8 (1.6)§	18.8 (0.7)§
mutC,D	13.9 (2.7)§	26.9 (6.3)§	20.3 (0.9)§

§statistically significant difference ($p<0.05$) compared to p(-175,+85)

FIG. 6. Effect of mutations in Sp-binding sites on transcriptional activity of the rCx40 proximal promoter. The p(−175, +85) construct, in which the rCx40 proximal promoter directs luciferase (LUC) expression, is schematically represented at the top with the Sp-binding sites A–E indicated by open triangles. The Sp-binding elements previously implicated in rCx40 proximal promoter activation (13) are indicated with asterisks. Cells were transfected as indicated with this p(−175, +85) construct as well as with constructs containing mutations in one or more Sp-binding sites (indicated at the left) and assayed for luciferase activity. The luciferase activity obtained for p(−175, +85) was set at 100% and all other luciferase activities were represented relative to this value. Two independent experiments in triple were performed. The mean with standard error (SEM) is presented. Statistically significant differences ($P < 0.05$) compared to the p(−175, +85) are indicated with an §.

binding sites. Results of the reporter assays in the different cell types are presented in Fig. 6. The luciferase activity obtained for p(−175, +85) was set at 100% and all other luciferase activities were expressed relative to this value. The level of luciferase activity obtained with p(−175, +85) exceeded approximately 23-fold, 45-fold, and 8-fold that obtained with the promoterless pGL3-Basic vector in respectively NCM, A7r5 and N2A cells (data not shown). Mutations in all three binding sites A, C and D reduced transcriptional activity by approximately 90–95% in all three cell types, clearly indicating the importance of these sites for optimal promoter function.

To assess the individual contribution of each Sp-binding site to transcriptional activity, luciferase reporter assays were performed with constructs in which only one binding site was altered. In NCM, individual mutations of the Sp-binding sites A–D and E gave respectively a drop of approximately ~65% and ~45% in promoter activity, indicating that each of these Sp-binding sites is important for optimal promoter function. Also in N2A cells individually altering the core of the binding sites A–E led to a significant reduction in promoter activity (30–50%). In contrast, in A7r5 cells only mutations of site B and E significantly reduced the promoter activity, whereas individually mutating site A, C and D had no significant effect. Luciferase activity in NCM as well as A7r5 and N2A cell lines with double-mutants of sites A, C and D was significantly lower than the activity of a single mutant, indicating that these sites act in a cooperative fashion in providing full proximal promoter activity.

Sp1 and Sp3 Determine rCx40 Proximal Promoter Activation

Trans-activation studies of the rCx40 proximal promoter were performed in *Drosophila* SL2 cells, which lack the expression of endogenous Sp transcription factor family members (18), to investigate the role of Sp1 and Sp3 transcription factors in the control of rCx40 transcription. Therefore, p(−175, +85) was co-transfected with one or combinations of expression plasmid(s) for Sp1 and/or Sp3 into SL2 cells and cell lysates were assayed for luciferase activity. The luciferase activity obtained with p(−175, +85) without adding any Sp-factor was set at 1.0. Luciferase activities after co-transfection with a Sp-factor were expressed relative to this value. As shown in Fig. 7, addition of Sp1 or Sp3 expression plasmids led to a dose-dependent increase in activation of the (−175, +85) promoter. To determine whether the Cx40 promoter is influenced by a combination of Sp1 and Sp3, pPacSp1 and pPacUSp3 expression plasmids were co-transfected. As shown in Fig. 7, activation of the p(−175, +85) construct by Sp1 and Sp3 is clearly additive and significantly stronger than stimulation by either Sp1 or Sp3 alone.

DISCUSSION

In this report we characterized the highly conserved rCx40 proximal promoter, for the relative contribution of five potential Sp transcription factor binding sites to promoter activity. Our results demonstrate a role for the transcription factors Sp1 and Sp3 in rCx40 proximal promoter activation.

Sp-Binding Sites in rCx40 Proximal Promoter Activity

Previously we have reported the involvement of the Sp-binding sites B and E in rCx40 proximal promoter activation (13). However, promoter/luciferase assays in NCM, A7r5 and N2A cells with a construct containing

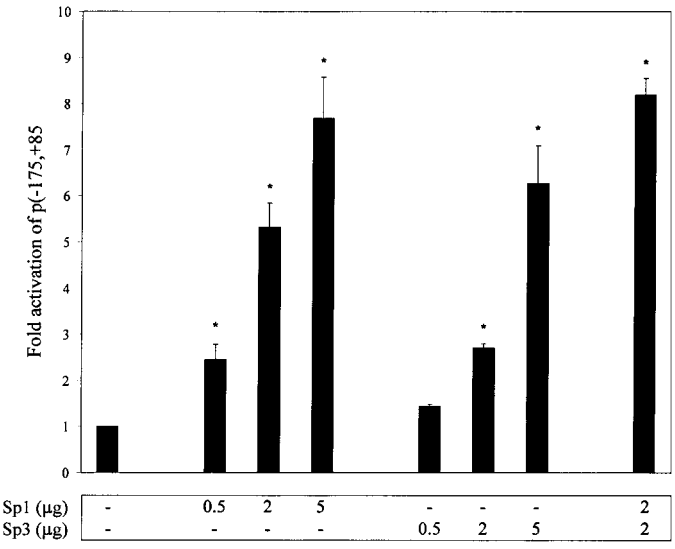


FIG. 7. Trans-activation of the proximal rCx40 promoter by Sp1 and/or Sp3 in *Drosophila* SL2 cells. SL2 cells were transfected with different concentrations of Sp1, and/or Sp3 expression plasmids (indicated at the bottom) in the presence of the p(−175, +85) construct, in which the rCx40 proximal promoter directs luciferase expression. To equalize for the amount of transfected DNA empty pPac expression vector was added. The luciferase activity obtained for p(−175, +85) alone was set at 1.0 and all other luciferase activities were represented relative to this value. Two independent experiments in triple were performed. Standard errors (SEM) are indicated. Statistically significant differences ($P < 0.05$) compared to p(−175, +85) without Sp factors are indicated with asterisks.

mutations in sites A, C and D demonstrated an almost complete abolishment of promoter activity, indicating the importance of these additional sites for promoter function as well. Similar experiments with constructs containing single mutations in Sp-binding sites A–E revealed that all sites contribute to promoter activity, without one site being significantly more important than the others. Application of constructs with two Sp-binding sites mutated (A + C, A + D, C + D) confirm these results in that the resulting promoter activity is intermediate relative to the activity obtained with the corresponding single and triple mutants. However Sp-binding site E is located in exon1, which recurs in the 5′-UTR of the mRNA, and therefore a translational effect of mutating this site can not be completely excluded, especially since the 5′-UTRs of Cx43 and Cx32 have been shown to contain an internal ribosomal entry site (IRES) (23, 24).

Involvement of Sp transcription factor family members in regulation of connexin gene expression appears to be a common phenomenon. In human mammary epithelial cells, for instance, the Cx26 gene is activated by both Sp1 and Sp3 through two upstream GC-boxes in the proximal promoter (25). Similarly, activation through Sp1-binding sites in the proximal promoter region was reported for Cx32 in rat hepatoma cells (26, 27), and for Cx43 in human primary myometrial (28) and rat kidney cells (29). Using *trans*-activation assays in *Drosophila* SL2 cells we extend on these findings by demonstrating that also the rCx40 proximal promoter is activated by Sp1 and Sp3 in a dose-dependent and additive manner.

Basal Promoter Activity versus Cell Type Specificity

Since both Sp1 and Sp3 are ubiquitously expressed in many mammalian cell types (22), it is likely that these transcription factors provide for a basal level of transcription of connexin genes in various cell types. However, Sp1-binding and activity may be affected by other transcription factors. For example, maximal Cx43 promoter activity in myometrial cells depends on both the transcription factors Sp1 and AP-1 (28). In addition it has been reported that hepatocyte nuclear factor-1 stimulates Sp1-driven Cx32 basal promoter activity in a cell type specific manner (27). Whether or not connexin genes are expressed in specific cell types most likely depends on cooperation of activators or repressors with Sp-family transcription factors and the basal transcription machinery. Similar mechanisms could be responsible for Cx40 gene expression as well. Recently, Bruneau *et al.* provided an explanation for cardiac specific Cx40 expression by showing that the transcription factors Tbx5 and Nkx2.5 interact with and activate the (mouse) Cx40 promoter (30). By characterizing the rCx40 proximal promoter, however, we obtained so far no indication for the involvement of transcription factors other than Sp1 and Sp3 in Cx40 gene regulation. This may also explain why we detect a significant but relatively low level of Cx40 proximal promoter activity in N2A cells, which do not endogenously express Cx40 protein or RNA. Alignment analysis of rat, mouse and human Cx40 noncoding sequences surrounding the transcription initiation site reveals several regions that are conserved between the three species. In addition, one region is identified which is conserved between human and mouse, but not between human and rat. It is expected that characterization of such regions will further elucidate the regulatory mechanisms determining the characteristic developmental and cell type specific expression pattern of Cx40.

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