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Promoter Activity of the Rat Connexin 43 Gene in NRK Cells

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Abstract Cellular communication mediated by gap junctions plays a major role in organ function. Gap junction channels are formed by the organization of polypeptide subunits, termed connexins (Cx), on the cell surface of adjacent cells. One mechanism to regulate gap-junctional communication is by change in Cx expression. In the present study, the promoter region of the rat *Cx43* gene was characterized. Nested deletions of the 5′ flanking region of the first *Cx43* exon were coupled to the human growth hormone gene and transfected into normal rat kidney (NRK) cells, that express this gene constitutively. The minimal region of the *Cx43* gene that showed maximal promoter activity was localized within 110 bp upstream of the transcriptional initiation site. One particular subregion that contains a Sp-1 binding site (located within 98–93 bp from the transcriptional initiation site) was found to sustain *Cx43* promoter activity to the same extent as that of the 110 bp promoter region. Mutations of this Sp-1 binding site abolished transcriptional activity and DNA–protein interactions. These observations suggest that the Sp-1 binding site plays a major role in the basal transcriptional activity of *Cx43* gene in NRK cells. J. Cell. Biochem. 81:514–522, 2001. © 2001 Wiley-Liss, Inc.

Key words: gap junctions; transcription; gene expression; Sp-1

Gap junctions are low resistance channels that allow the passage of low molecular weight metabolites (cAMP, inositol 1,4,5-trisphosphate) and ions (Ca^{2+}, H^+, Cl^-) between neighboring cells [Bennett et al., 1991]. Gap-junctional cellular communication plays an important role in smooth muscle contraction, propagation of electrical signals between neurons, and metabolic regulation [Bennett and Goodenough, 1978; Hooper and Subak-Sharpe, 1981; Page and Manjunath, 1986]. Gap junctions are formed by the association of two hemichannels, or connexons, on the plasma membrane of opposite cells. Each connexon is formed by six identical protein subunits, termed connexins (Cx), which are integral plasma membrane proteins [Bennett et al., 1991]. The expression of Cx genes is tissue- and cell-type-specific [Kumar and Gilula, 1996]. Cx43 is also the major Cx in cardiac gap junctions [Beyer et al., 1988].

Although gap-junctional cellular communication is mainly modulated by gating mechanisms [Spray et al., 1985; Bennett et al., 1991], changes in Cx gene expression also play an important role in the regulation of this type of cellular communication [Bennett et al., 1991]. The importance of gene expression in the regulation of gap-junctional communication has been illustrated in different disease conditions. For example, reduction of Cx32 mRNA and polypeptide has been observed in the liver during an acute inflammatory state induced by the administration of bacterial lipopolysaccharide (LPS) [Gingalewski et al., 1996; De Maio et al., 2000]. The disappearance of hepatic Cx32 has also been observed after regional hepatic ischemia/reperfusion [Gingalewski and De Maio, 1997], partial hepatectomy [Traub et al., 1983; Kren et al., 1993], and hepatic cholestasis [Traub et al., 1983]. Alterations in cardiac gap junctions have been observed in ischemic heart disease and have been associated with the

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development of cardiac arrhythmias [Luke and Saffitz, 1991; Green and Servers, 1993]. A decrease in cardiac gap-junctional communication has been detected in post-ischemic myocardium [Kieval et al., 1992]. The density of gap junctions in ventricular myocardium has also been found to be reduced in hearts subjected to ischemia or chronic hypertrophy [Peters et al., 1993]. Moreover, inflammatory conditions triggered by the injection of bacterial LPS or hepatic ischemia/reperfusion resulted in a decrease in the expression of Cx43 within the heart [Fernandez-Cobo et al., 1999]. In contrast, the same inflammatory conditions resulted in an increase of Cx43 expression in the kidney and lung [Fernandez-Cobo et al., 1998].

An increase in myometrial gap junctions, which are composed of Cx43, has been reported during parturition [Garfield et al., 1977; Dahl and Berger, 1978]. Addition of estrogen was found to upregulate gap junctions in the myometrium, whereas progesterone had the opposite effect [Dahl and Berger, 1978; Burghardt et al., 1984]. Analysis of the rat Cx43 promoter has revealed a putative progesterone/glucocorticoid recognition element that has been postulated to function as a negative regulatory element, since the uterus lacks gap junctions under progesterone control [Yu et al., 1994]. Basic fibroblast growth factor has also been shown to stimulate Cx43 expression in cardiac fibroblasts [Doble and Kardami, 1995]. Inflammatory mediators such as tumor necrosis factor α and interleukin 1β have been observed to alter the trascriptional activity of the *Cx43* gene [Fernandez-Cobo et al., 1998, 1999]. In the present study, the rat *Cx43* promoter has been characterized in normal rat kidney (NRK) cells which have been extensively used to study Cx43 biosynthesis and regulation [Musil and Goodenough, 1991]. We have found that a Sp-1 binding site located within 98–93 bp from the transcriptional initiation site (TIS) of the *Cx43* gene is a major element involved in the transcriptional regulation of this gene in these cells during basal conditions.

METHODS

Transfection and Reporter Gene Analysis of Rat *Cx43* Promoter

A region containing the 5′ flanking region of the rat Cx43 first exon was generated by PCR amplification and subcloned in front of the human growth hormone (hGH) gene (pϕGH) as previously described [Fernandez-Cobo et al., 1998]. Nested deletions of the promoter were performed by digestion with Exonuclease III (Erase a base system, Promega). An internal deletion, Δ80, was obtained by inverse PCR amplification (denaturation, 1 min, 94°C; annealing, 1 min, 55°C; elongation, 5 min, 72°C; 20 cycles and 5 min of final elongation at 72°C) using Taq extender (Stratagene) and primers: GAGGAGGTCGACGAACGTG and AGTTGAGTCGACGGCTTGAAAC. These constructs were transfected into 50% confluent monolayers of NRK cells maintained in D-MEM suplemented with 5% fetal bovine serum. The levels of hGH in the extracellular medium were measured using a commercial radioimmunoassay (Nichols Institute Diagnostics). In addition to the Cx43 constructs, NRK cells were cotransfected with the vector pOPRSVI-CAT containing the RSV promoter and the chloramphenicol acetyl transferase (CAT) gene which allows the constitutive expression of CAT. Expression of CAT was analyzed by ELISA ($5' \rightarrow 3'$, INC). Expression of CAT was used to evaluate the efficiency of transfection. Consequently, the activity of the *Cx43* promoter (hGH) was normalized by the level of CAT expression and the protein content in the sample. Protein was determined by the BCA assay (Pierce).

Electrophoretic Gel Shift Analysis

Protein extracts were prepared as described [Andrews and Faller, 1991]. Briefly, NRK cells were scraped with cold phosphate-buffered saline (3 ml) and centrifuged for 15 min at $3{,}000 \times g$. The cell pellet was resuspended in 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, incubated on ice for 10 min, and vortexed for 10 s. The lysate was centrifuged for 10 s at $13{,}000 \times g$, the pellet was resuspended in 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol, and incubated on ice for 20 min, centrifuged for 5 min at $13{,}000 \times g$, and the supernatant stored at −70°C. Protein concentration in the cell extract was measured by the BCA assay.

The DNA probes (PCR products or oligonucleotides) were radiolabeled by a filling reaction using [α^{32}P]dCTP and [α^{32}P]dATP. The radiolabeled probes (0.1 pmol) were incubated with

protein extracts (12–16 μg) in 10 mM Tris–HCl pH 7.5, 25 mM KCl, 0.5 mM EDTA, 6% glycerol, 5 μg BSA, and 2 μg poly(dI-dC) · poly(dI-dC) at 25°C for 20–30 min. The reaction mixture was separated by polyacrylamide gel electrophoresis (5% polyacrylamide, 0.5 × TBE buffer) at 5 W for 1 h. The gel was dried and exposed to X-ray films. To determine the specificity of the reaction, a 150–200-fold excess of the unlabeled probe was added; nonspecific competitors were used at a 150–250-fold molar excess concentration with respect to the labeled probe.

RESULTS

Deletions of *Cx43* Promoter Region

Previous studies have shown that a fragment containing 725 bp upstream of transcriptional initiation site (TIS) of the rat *Cx43* gene showed transcriptional activity as measured by a reporter gene analysis after transfection of NRK cells [Fernandez-Cobo et al., 1998]. The transcriptional activity of this fragment (pCx43/725/hGH) was characterized by nested deletions of the 5′ end generated by digestion with exonuclease III. These deletions as well as the 725 fragment were transfected into NRK cells. Removal of 140 bp from the 5′ end of the *Cx43* original clone (pCx43/585/hGH) increased promoter activity by 30%. Further deletions up to 263 bp did not change *Cx43* promoter activity significantly. The minimal construct that showed maximal *Cx43* promoter activity contains 110 bp upstream of the TIS (pCx43/110/hGH). A deletion of 69 bp from this construct (pCx43/TAT/hGH), which contains only the putative TATA box of the gene, showed significantly decreased promoter activity. These data suggest that the *Cx43* basal promoter activity is located within 110 bp from the TIS (Fig. 1). Transfection with a construct containing an internal deletion of 80 bp (Δ80) between nucleotides −116 and −42 of the promoter, which removed the majority of the 110 bp region (pCx43/134/Δ80/hGH), resulted in 70–80% lower promoter activity than the 110 construct (not shown).

Protein Binding Pattern of the *Cx43* Promoter

If indeed the 110 bp region upstream of the TIS of *Cx43* gene contains the element(s) involved in the transcriptional regulation of this gene, it would be expected that transcriptional factors present in NRK cells would bind to this region. The 110 bp promoter region was end-labeled by a filling reaction and incubated with protein extracts of NRK cells. Protein binding to this region was detected by the

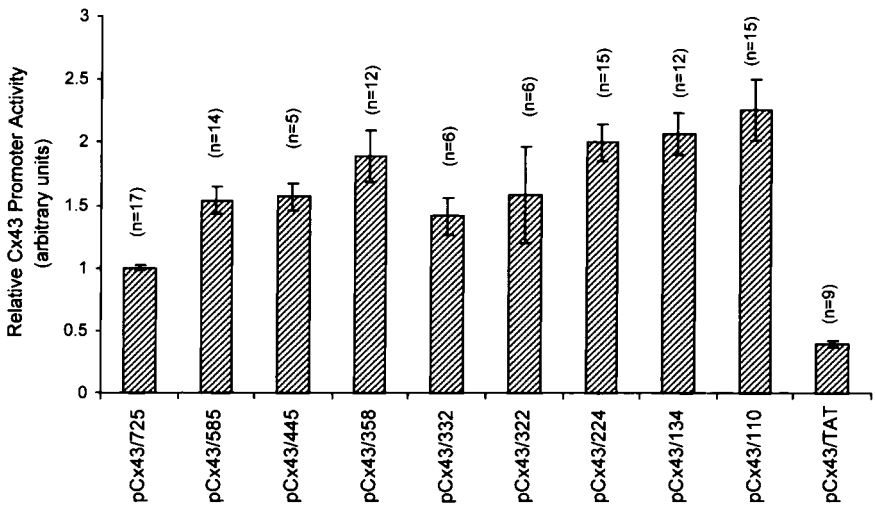

Fig. 1. Activity of *Cx43* promoter containing nested deletions and transfected in NRK cells. A series of deletions was performed in the Cx43 promoter by incubation with exonuclease III. NRK cells were transfected with different constructs containing whole (pCx43/hGH) or deleted *Cx43* promoter and hGH as a reporter gene. Cells were also co-transfected with pOPRSVI-CAT to measure the efficiency of the transfection. Expression of hGH was measured in the extracellular medium using a radioimmunoassay 48 h after the transfection. Expression of CAT was measured by an ELISA performed on cell lysates harvested at the same time that the extracellular medium was collected. The levels of hGH expression by the different deletions of *Cx43* promoter were normalized to the whole Cx43/hGH construct (pCx43/725/hGH). The results are presented as average ± standard error. The number of independent determinations (n) for each of the different constructs is indicated in the figure.

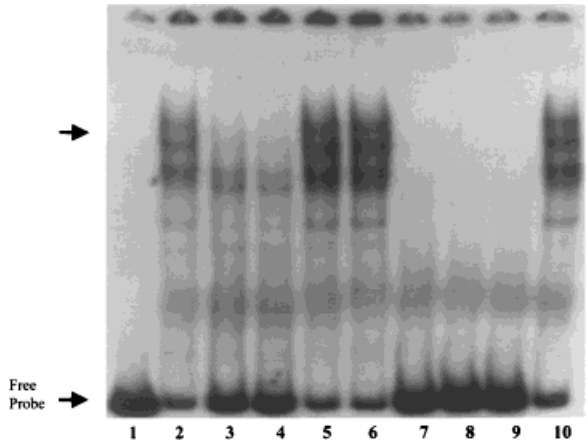

Fig. 2. Electrophoretic mobility shift (EMS) assay of the promoter region of *Cx43* gene. The radiolabeled 110 bp probe (PCR product) of Cx43 was incubated with NRK cell extracts (16 μg of protein, **lanes 2–10**), or in the absence of cell extracts (**lane 1**). The reaction was carried out in the absence of inhibitors (lane 2), or in the presence of a 150-fold excess of nonradiolabeled 134 probe (lane 3), 110 probe (lane 4), 134Δ80 probe (lane 5), or nonlabeled oligonucleotides (250-fold excess) corresponding to SR I (lane 6), SR II (lane 7), SR III (lane 8), SR IV (lane 9), and TATA box (lane 10). The reaction mixtures were separated in a 5% polyacrylamide gel, dried, and exposed to X-ray films for 18 h.

electrophoretic mobility shift assay (EMSA). Incubation of the 110 bp probe with protein extracts resulted in the detection of several DNA–protein complexes (Fig. 2; lane 2). Addition of a 150-fold molar excess of nonradioactive 134 or 110 bp fragments resulted in the disappearance of some protein–DNA complexes (Fig. 2; lanes 3 and 4, see arrows). These results suggest that the protein binding site responsible for this EMS is common to the 110 and 134 bp regions. This assumption was confirmed by two different experiments. First, an EMS assay using the radiolabeled 134 bp fragment resulted in protein binding, which could be competed by addition of the nonradioactive 110 bp region (not shown). Second, addition of nonradioactive 134Δ80 fragment, which showed decreased promoter activity, did not inhibit protein binding to the 110 bp probe (Fig. 2, lane 5). These observations suggest that elements responsible for the observed EMS pattern reside within the Δ80 region of the *Cx43* gene. Competition experiments using a fragment of 164 bp corresponding to the 5′ upstream region of the 725 promoter region generated by PCR also failed to block protein binding to the 110 bp probe (data not shown). Sequence analysis of the 110 promoter region revealed the presence of several binding sites for transcriptional factors. Operationally, this region was divided into four subregions (SR): (see Fig. 3) SR I (−58 to −35 bp) containing an AP-1 binding site, SR II (−75 to −56 bp) with no known protein binding sites, SR III (−90 to −71 bp) with an AP-2-like binding site, and SR IV (−106 to −86 bp) containing an Sp-1 binding site. In order to identify which of these regions contains the protein binding site responsible for the observed EMS pattern of the 110 bp probe, oligonucleotides corresponding to each of the SRs were used in competition experiments with the radiolabeled 110 bp probe. In addition, an oligonucleotide containing the sequence of the putative TATA box was used as a control. SR II–IV were all found to compete for the protein binding to the 110 probe (Fig. 2; lanes 7–9), whereas SR I (AP-1) or TATA box did not compete for protein binding (Fig. 2; lanes 6 and 10, respectively). These observations indicate that elements within the 110 bp promoter region are specifically recognized by proteins in the NRK cell extract.

To further characterize the 110 promoter region, each subregion was radiolabeled and analyzed for protein binding by EMSA. The EMSA pattern obtained by using radiolabeled SR IV (Sp-1) was completely abolished by the addition of nonradiolabeled SR IV indicating the specificity of the interaction. Addition of nonradiolabeled SR II and III partially competed for protein binding to SR IV probe, whereas no competition was observed by the addition of SR I (Fig. 4). When the SR II and III were used as probes for the EMSA, the addition of the corresponding nonradiolabeled probes competed off for protein binding, indicating again the specificity of the interaction. Addition of nonradiolabeled SR IV also completely blocked protein binding to these probes, whereas addition of SR I did not affect protein binding to the DNA (Fig. 4). These observations suggest the presence of at least two distinct protein binding sites on the 110 *Cx43* promoter region: one corresponding to SR II–IV and another to SR I.

Functional Contribution of the *Cx43* Promoter Subregions

To study the independent contribution of each *Cx43* promoter subregion, constructs were prepared using the plasmid that contains a 80 bp deletion of the promoter (pCx43/134/Δ80/

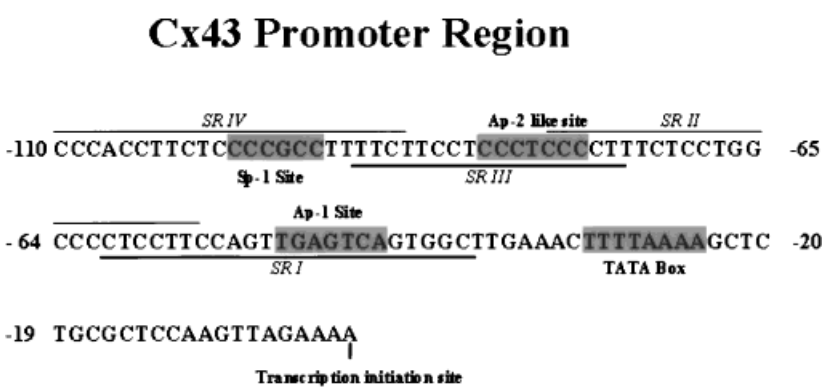

Fig. 3. Subregions of the *Cx43* promoter. The minimal promoter region of *Cx43* gene (110 bp) was operationally divided into four subregions. Sequence according to Yu et al. [1994].

hGH). This construct, which lacks SR I–IV, showed reduced promoter activity. This construct was modified by the addition again of SR IV, which contains the Sp-1 binding site. An additional construct was made in which the Sp-1 binding site (CCCGCC) was mutated (CCCTCC). Two other different constructs were used, one with SR II added to the pCx43/134/Δ80/hGH plasmid and a second that contains SR I (AP-1) and the authentic TATA box of *Cx43* gene. These plasmids were transfected into NRK cells and the level of transcriptional activity (reporter gene expression) was compared with cells transfected with the original 110 *Cx43* promoter region (pCx43/110/hGH). Transfection with the construct containing SR IV (Sp-1 binding site) resulted in transcriptional activity identical to that observed with the 110 bp construct (pCx43/110/hGH). A single mutation on the Sp-1 binding site, which generated a Ap-2-like binding site similar to the one observed in SB III, resulted in a 70% decrease of transcriptional activity. Constructs with SR II or SR I (AP-1) showed low levels of

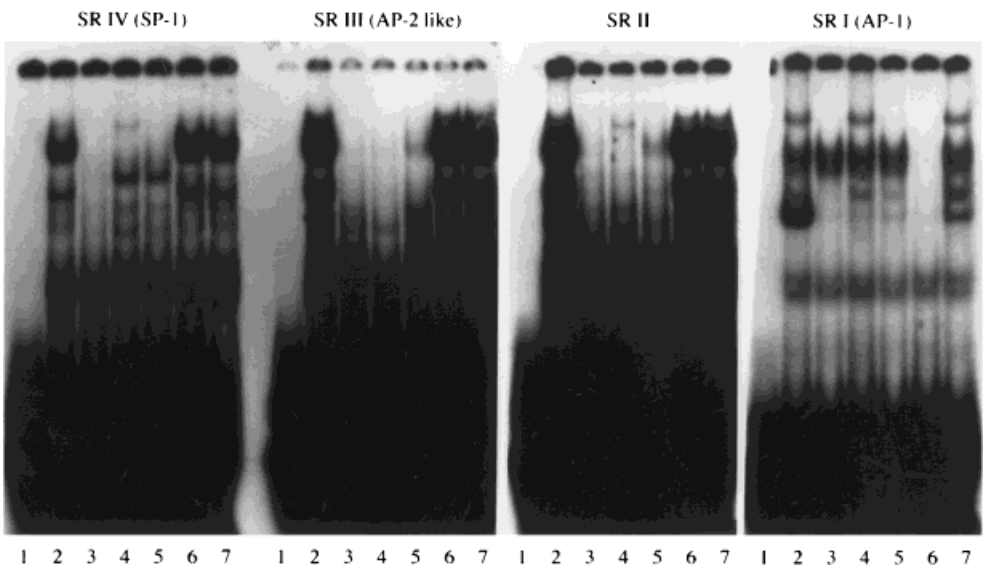

Fig. 4. EMS assay of *Cx43* promoter subregions. The radiolabeled SR I–IV bp probes (oligonucleotides) were individually incubated with NRK cell extracts (16 μg of protein, **lanes 2–7**), or without cell extract (**lane 1**). The reaction was carried out in the absence of inhibitors (**lanes 2** and 7), or in the presence of a 200-fold excess of nonradiolabeled SR IV (**lane 3**), SR III (**lane 4**), SR II (**lane 5**), and SR I (**lane 6**) for each probe. The reaction mixtures were separated in a 5% polyacrylamide gel, dried, and exposed to X-ray films for 18 h. Notice the inhibition in protein binding by addition of probes 110, 134, and SR II, III (AP-2-like), and IV (Sp-1).

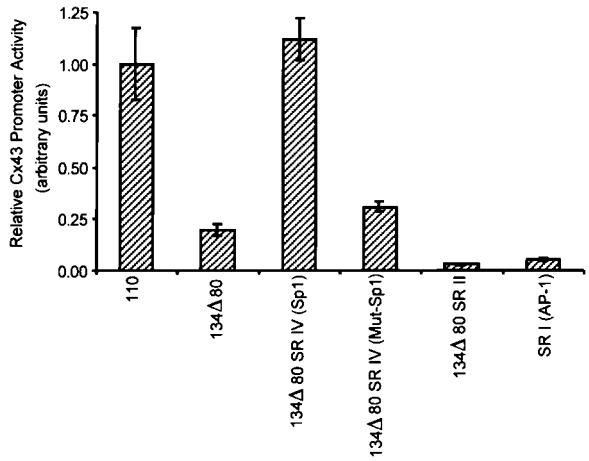

Fig. 5. Effect of the Sp-1 binding site on the *Cx43* promoter activity in NRK cells. NRK cells were transfected with each of the following constructs: pCx43/725/hGH, pCx43/110/hGH, pCx43/134Δ80/hGH (containing an internal deletion of 80 bp), pCx43/134Δ80/SR IV (Sp-1)/hGH, pCx43/134Δ80/SR II/hGH, or pCx43/SR I (AP-1)/TATA/hGH. In addition, a single mutation was introduced in pCx43/134Δ80/SR IV (Sp-1)/hGH. The mutation was CCCGCC (wild-type) for CCCTCC. The expression of hGH/mg protein is presented as the average of 6 independent determinations ± standard error using the construct pCx43/725/hGH as standard.

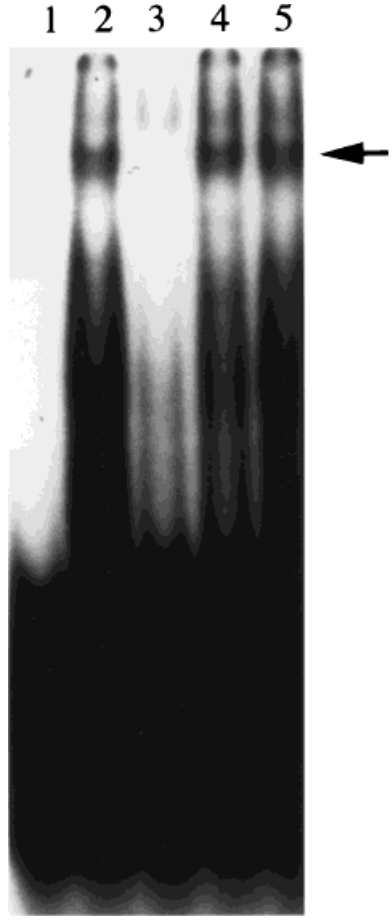

Fig. 6. Competition of protein binding to SR IV (Sp-1) by mutations on the region. A radiolabeled oligonucleotide probe containing the Sp-1 binding site was incubated NRK cell extracts (6 μg of protein, **lanes 2–5**) or in the absence of protein extract (**lane 1**). Competition was performed by addition of a 200-fold excess of nonradiolabeled wild-type Sp-1 site (CCCGCC), or a single mutation (CCCTCC, lane 4) or triple mutation (ACACAC, lane 5) of Sp-1 sites. The reaction mixtures were separated in a 5% polyacrylamide gel, dried, and exposed to X-ray films for 18 h. Notice the lack of inhibition in protein binding by addition of mutated Sp-1 binding sites.

Cx43 promoter activity (Fig. 5). An EMSA was performed using the SR IV (Sp-1 binding site) as a probe and competed with nonradiolabeled oligos containing either a wild-type or mutated Sp-1 binding site. Neither of the two different mutations in the Sp-1 binding element was capable of competing off for protein binding to the SR IV probe (Fig. 6). These observations together suggest that the Sp-1 binding site plays a major role in the basal transcriptional activity of the *Cx43* gene in NRK cells.

DISCUSSION

Changes in the level of Cx expression play an important role in controlling gap-junctional cellular communication [Bennett et al., 1991]. However, the mechanisms that modulate the expression of the different Cx genes are not well known. The genomic organization of the majority of the Cx genes is very similar. These genes contain two exons, one short with part of the 5′ untranslated region of the message and a second with the rest of the untranslated region and encoding sequence, separated by a large intron [Miller et al., 1988; Hennemann et al., 1992; Yu et al., 1994; Chen et al., 1995]. In spite of this similarity at the level of genomic organization, the expression of the different Cx genes is regulated at different levels. For example, two different Cx32 transcripts have been detected. Their expression is also cell-specific. The longest transcript is expressed in the liver and the promoter activity has been localized in the 5′ flanking region of the first exon. The shorter transcript lacks the first exon and the promoter activity is comprised within the two exons [Hennemann et al., 1992; Bai et al., 1993, 1995; Neuhaus et al., 1995]. Changes in Cx32 mRNA stability also play a role in the expres-

sion regulation of this gene [Gingalewski et al., 1996; Gingalewski and De Maio, 1997; Theodorakis and De Maio, 1999].

In the case of *Cx43*, promoter activity is localized within the 5′ upstream region of the first exon [De Leon et al., 1994; Yu et al., 1994; Chen et al., 1995; Fernandez-Cobo et al., 1998, 1999]. A single transcript has been observed in all tissues and cell types tested. In the present study, the promoter activity of *Cx43* has been evaluated in NRK cells. These cells express Cx43 at large levels and have been the subject of extensive studies on the biosynthesis of gap junctions [Musil and Goodenough, 1991]. The minimum DNA region of the *Cx43* gene that showed maximal promoter activity was located within 110 bp of the TIS. This region also showed a specific protein binding pattern as demonstrated by EMSA. The upstream region of the 110 bp minimal *Cx43* promoter, which contains a Sp-1 binding site (SR IV), was found to play a major role in the basal transcriptional activity of this gene in NRK cells. This assumption is based on the observation that this region alone is capable of maintaining a promoter activity identical to the 110 bp fragment. Furthermore, mutations of the Sp-1 binding site, which result in the appearance of a Ap-2-like site similar to the one present in SR III, decreased the promoter activity of this region. Sp-1 binds to GC-rich motifs. This binding specificity is shared by other factors such as AP-2 and NF-1 [Courtois et al., 1990; Saffer et al., 1991; Williams and Tjian, 1991]. Since the *Cx43* promoter region is very rich in these GC motifs, it is possible that proteins with various affinities for the GC-rich regions are responsible for the complex protein-binding pattern that we have observed. Sp-1 has also been found involved in the transcriptional regulation of Cx26 in rat mammary gland and uterus [Jin et al., 1998]. Sp-1 has been reported to be a ubiquitous transcriptional factor [Comb and Goodman, 1990]. Thus, it is possible that other elements within the *Cx43* promoter different from the Sp-1 binding site may contribute to the tissue-specific expression of the *Cx43* gene. Indeed, an internal deletion of the promoter region removing the Sp-1 binding site did not decrease *Cx43* promoter activity completely. Thus, other sites within the promoter may act as alternate elements in the transcriptional regulation of this gene. These results do not discard the possibility that other elements within the *Cx43* promoter may act in conjunction with the Sp-1 binding site to regulate the expression of this gene. One potential element is the AP-1 site. Our data do not show that the AP-1 site can sustain promoter activity by itself. However, it may require other promoter regions to modulate the activity. The AP-1 site may be important in the regulation of Cx43 expression during inflammatory conditions. We have previously shown that Cx43 expression is altered during inflammation induced by LPS or hepatic ischemia/reperfusion [Fernandez-Cobo et al., 1998, 1999].

The 5′ flanking region of the *Cx43* first exon has been previously cloned in rat [Yu et al., 1994], mouse [Chen et al., 1995], and humans [De Leon et al., 1994]. There is a high degree of sequence similarity [Chen et al., 1995]. The *Cx43* promoter activity in human myocytes was localized within 175−100 bp of the TIS [De Leon et al., 1994]. Studies in rat H9c2 myoblasts showed that basal transcriptional activity is localized with 224 bp upstream of the TIS [Fernandez-Cobo et al., 1999]. In mouse uterine smooth muscle and human myometrial cells, *Cx43* promoter activity was located in a different region. A positive regulatory element between −72 to −62 with respect to the TIS was reported in these cells [Chen et al., 1995]. We did not observe any positive transcriptional activity in this region (SR II) in NRK cells. A negative element has also been proposed within the *Cx43* promoter region in myometrial cells. This negative element is located between −102 and −92 region of the gene [Chen et al., 1995]. This region contains the Sp-1 site, which we found to be important in the transcriptional regulation of the *Cx43* gene in NRK cells. The discrepancy between our results and those previously reported by Chen et al. [1995] could be explained by the difference in cell types that have been used. Our studies were performed in NRK cells, which are of renal origin, whereas myometrial cells were used by Chen et al. [1995]. This discordance in the expression of Cx43 in various cell lines is not surprising since it has been well established that the expression of different Cx genes is tissue-specific [Beyer et al., 1988; Kumar and Gilula, 1996]. Thus, the transcription of the *Cx43* gene may be modulated by different elements depending on the cell type. In the case of NRK cells, the transcriptional activity of this gene seems to be regulated by a Sp-1 binding site.

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