Regulation of connexin32 and connexin43 gene expression by DNA methylation in rat liver cells

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Gap junction proteins (connexins) are expressed in a cell-specific manner and expression is often reduced in neoplastic cells. We investigated the mechanisms of connexin32 (Cx32) and connexin43 (Cx43) expression in hepatic cells using MH1C1 rat hepatoma cells and freshly isolated, adult rat hepatocytes that express Cx32 but not Cx43 and WB-F344 rat liver epithelial cells that express Cx43 but not Cx32. Southern blotting after DNA restriction with MspI and HpaII indicated that two MspI/HpaII restriction sites in the Cx32 promoter (positions –147 and –847) were methylated in WB-F344 cells, but not in MH1C1 cells or hepatocytes. In contrast, an MspI/HpaII restriction site in the Cx43 promoter (position –38) was methylated in MH1C1 cells, but not in WB-F344 cells or hepatocytes. Transient transfection of the cell lines with connexin promoter–luciferase constructs indicated that the Cx32 promoter was 7-fold more active in MH1C1 cells and the Cx43 promoter was 5-fold more active in WB-F344 cells. These results suggest that transcription of Cx32 and Cx43 in hepatic cells is controlled by promoter methylation and by cell-specific transcription factors. Similar mechanisms may be involved in the reduced expression of these genes frequently observed in neoplastic cells.

Introduction

Gap junctional intercellular communication functions in homeostasis, differentiation and growth control and is often defective in neoplastic cells (1,2). Gap junctions are comprised of protein subunits known as connexins; at least 13 connexins exist and they are expressed in a cell- and development-specific manner (3). In mammalian liver, hepatocytes express connexin32 (Cx32) predominantly and lesser amounts of connexin26 (Cx26) (4,5). Biliary epithelial cells, in contrast, express connexin43 (Cx43) as the major gap junction protein, much lower quantities of Cx26 and no Cx32 (6). Neoplastic cells, including hepatocellular and cholangiocellular carcinoma cells, often express less connexin, but the mechanisms are unknown (6–8).

In the present study, we investigated the roles of connexin promoter-specific DNA methylation and transcriptional activity in the liver cell-specific expression of Cx32 and Cx43. To do this, we utilized two rat liver cell lines and isolated rat hepatocytes that are distinct in their expression of these connexins. Rat hepatocytes and MH1C1 cells, a well-differentiated rat hepatoma cell line, express Cx32, but not Cx43 (9). WB-F344 is a normal rat liver epithelial cell line that expresses Cx43 but not Cx32 (9,10). We have reported that dexamethasone (DEX) increased Cx32 but not Cx43 mRNA content in MH1C1 cells and rat hepatocytes and had no effects on connexin expression in WB-F344 cells (9). Therefore, we also examined whether the steroid affected Cx32 and Cx43 promoter activity in these cells.

Materials and methods

Cell lines and culture conditions

MH1C1 cells were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum (FBS) and gentamycin sulfate (40 µg/ml); WB-F344 cells were cultured in Richter’s improved minimal essential medium supplemented as above. Both cell lines were cultured on standard plastic tissue culture dishes and incubated in an atmosphere of 95% air/5% CO2 at 37°C (9). Hepatocytes were isolated from adult male F344 rats (175–200 g) by two-stage collagenase perfusion of the liver as described (9). The viability of the isolated hepatocytes was >85%.

Northern blot analysis

Cells were cultured for 2 days to ~80% confluence then were treated with 10 µM DEX or 0.1% ethanol (solvent control) for 48 h prior to extraction of total RNA using TRI Reagent (Molecular Research Center, Cincinnati, OH). In some cases, WB-F344 cells were cultured in the presence of 2.5 µM 5-aza-2’-deoxycytidine (5-AZA) for 72 h prior to and during the 48 h treatment with DEX or ethanol. Northern blotting was performed using 20 µg total RNA as described (9). Probes were prepared from full-length cDNAs for rat Cx32 and Cx43 (4,11) by random primer labeling with [α-32P]dCTP (Life Sciences NEN, Boston, MA) using the DECAprime II Kit (Ambion, Austin, TX). After hybridization and washing as described (9), the membranes were exposed to Kodak BIOMAX MR or MS film with an intensifying screen for 1–2 days at ~80°C.

Southern blot analysis

High molecular weight genomic DNA was isolated from confluent untreated cultures of MH1C1 and WB-F344 cells, from WB-F344 cultures treated with 2.5 µM 5-AZA for 72 h and from freshly isolated rat hepatocytes as described (12). Genomic DNA (20 µg) was digested completely with restriction endonucleases [EcoRI and PstI (Gibco BRL, Grand Island, NY) and MspI and HpaII (New England Biolabs, Beverly, MA)] and fractionated in a 1% agarose gel. The gels were depurinated (0.25 M HCl for 15 min), denatured (0.5 M NaOH, 1.5 M NaCl twice for 20 min each) and neutralized (1 M Tris–HCl, 2 M NaCl, pH 7.0, twice for 15 min each). DNA was transferred by vacuum to Hybond N+ nylon membranes (Amer sham, Arlington Heights, IL) in 20X saline/sodium citrate (SSC). Membranes were hybridized with the radiolabeled probes indicated in the figures. Probe A corresponds to rat Cx32 promoter sequences –594 to –77 (13). It was isolated as an EcoRI fragment from pRJR12 which contains the fragment subcloned into pBSIIKS+ after its PCR amplification from rat liver DNA. Probe B corresponds to rat Cx32 promoter sequences –754 to –33 and was isolated as a BamHI–KpnI fragment from pKR25. This plasmid contains the fragment subcloned into pBSIKKS+ after isolation from pC32–800–33LUC (14). Probe C corresponds to rat Cx34 promoter sequences –644 to +87 (15) and was isolated as a HindIII fragment from pRJR19. This plasmid contains a 1610 bp SalI–MluI rat Cx43 genomic DNA fragment isolated from pGL2-cx43 (15) and subcloned into pBSIKKS+. Blots were washed at high stringency (0.1X SSC, 0.1% SDS at 65°C) and autoradiographed as described above.

Abbreviations: 5-AZA, 5-aza-2’-deoxycytidine; Cx26, connexin26; Cx32, connexin32; Cx43, connexin43; DEX, dexamethasone; FBS, fetal bovine serum; HBSS, Hank’s balanced salt solution; SSC, saline/sodium citrate.
Transient transfection assays

We transiently transfected MH1C1 and WB-F344 cells to determine whether unmethylated Cx32 and Cx43 promoters could be transcriptionally activated in both cell types and the relative strengths of the two promoters. The Cx32 promoter–luciferase construct (pCx32–800–33LUC) contained rat Cx32 promoter sequences from –754 to –33 inserted into the KpnI site of pGL2Luc (14). The Cx43 promoter–luciferase construct (pGL2-cx43) contained rat Cx43 genomic sequences (1250 bp of distal promoter, exon I and 87 bp of the intron) inserted into the pGL2-basic vector (15). Control cells were transfected with p19LUC. Cells were co-transfected with 5 µg of pSVluc (Promega, Madison, WI) in medium containing 5% NuSerum (Collaborative Biomedical Products, Bedford, MS) in place of FBS and 10 µM chloroquine using the DEAE–dextran method (16). Four hours later, the cells were treated with 10% dimethylsulfoxide in Hank’s balanced salt solution (HBSS) for 1 min to enhance entry of DNA precipitate into the cells. The cells were then washed twice with HBSS, refed with complete culture medium, cultured for 16 h to allow recovery and refed with complete medium containing 10 µM DEX or ethanol (0.1%, vehicle control). Forty-eight hours later, cell extracts were prepared and luciferase and β-galactosidase activities were determined as described (17,18). Luciferase values were corrected for transfection efficiency and normalized to the promoterless vector controls treated with DEX or ethanol.

Results

Cell-specific expression of Cx32 and Cx43

MH1C1 cells expressed Cx32, but not Cx43, transcripts, while the opposite pattern was observed for WB-F344 cells (Figure 1). Treatment of these cells with DEX increased Cx32 expression only in MH1C1 cells (Figure 1, lane 2) and did not affect Cx43 expression. These differences were also observed at the protein level and were not due to the different culture media (9).

Cx32 and Cx43 promoter structure

To investigate the mechanism(s) for this differential expression, we first investigated the structure of the Cx32 and Cx43 genes to ensure that the promoters were intact. The strategies for Southern blot analyses of the rat Cx32 and Cx43 promoters and the predicted fragments are depicted in Figure 2. The genomic organization of all connexin genes is strikingly similar (3). In the rat Cx32 and Cx43 genes, the uninterrupted reading frame is located in exon II and this is separated from a short non-coding exon I by a large intron of 6.1 or 8.5 kb, respectively. The Cx32 gene is transcribed from three distinct, tissue-specific promoters (14,19,20). The liver-specific promoter is located upstream of exon I while two neural-specific promoters are located within the intron upstream of exon II. The Cx32 promoter is also located upstream of exon I with major transcription start sites between –208 and –204 (15).
Methylation status of the Cx32 and Cx43 genes

Methylation of CpG sequences within promoters can inhibit binding of transcription factors and is a common mechanism of gene inactivation in neoplastic cells and in tissue-specific gene expression (21–25). Therefore, we hypothesized that MspI sites in the Cx32 and Cx43 promoters (Figure 2) would be methylated in non-expressing cells. To investigate this, we compared the restriction fragment patterns generated by digestion of the promoters with MspI and HpaII. Both enzymes recognize the sequence 5′-CCGG-3′. MspI will not cleave if the first cytosine is methylated, but will cleave if the second one is methylated; HpaII will not cleave if either base is methylated.

We first examined the Cx32 gene. When genomic DNA from MH1C1 cells was cleaved with MspI or HpaII and hybridized with Cx32 probes A or B, an ~0.7 kb fragment was obtained (Figure 3B and D, lanes 1 and 2). Using WB-F344 cell DNA, in contrast, digestion with MspI generated the ~0.7 kb fragment (Figure 3B and D, lane 3), but no such fragment was detected following HpaII restriction (Figure 3B and D, lane 4) even upon overexposure of the film. Instead, hybridization to a smear of high molecular weight DNA was seen (Figure 3B, lane 4). We also obtained a strong ~1.5 kb band with MspI-digested DNA from MH1C1 cells (Figure 3B, lane 1). This band was very faint with HpaII digestion and instead a prominent ~2.2 kb band was apparent (lane 2). These results suggest that in MH1C1 cells, the two MspI/HpaII sites in the Cx32 promoter were not methylated and the intronic site was methylated or only partially accessible to HpaII. In WB-F344 cells, the lack of any hybridizing band following HpaII digestion suggests that all three sites were methylated. This lack of HpaII cleavage was not the result of partial digestion because complete digestion of the Cx32 promoter was seen when these blots were rehybridized using Cx43 probe C (described below). Using DNA from freshly isolated rat hepatocytes, we observed that MspI and HpaII digestion generated identical 0.7 and 1.5 kb bands (Figure 3B, lanes 5 and 6). This result indicates that the Cx32 promoter was not methylated in rat hepatocytes.

The occurrence of a prominent ~2.2 kb band and weak ~1.5 kb band in HpaII-digested DNA from MH1C1 cells using probe B (Figure 3B, lane 2) also suggests that a second MspI/HpaII site exists ~0.7 kb downstream of the intronic site. Our inability to detect the ~2.2 kb band using Cx32 probe A (Figure 3D, lane 2) again may be due to the fact that probe A had less complementary sequence than probe B.

To confirm that the Cx32 promoter site at ~147 was not methylated in MH1C1 cells and was methylated in WB-F344 cells, genomic DNA was first digested with PstI then with MspI or HpaII. The ~5.7 kb PstI restriction fragment described above encompasses the promoter, exon I and part of the intron. If subsequent digestion of this fragment with MspI or HpaII is complete at the ~147 position, a 447 bp fragment will result (Figure 2A). This fragment was clearly observed following digestion of MH1C1 cell DNA with either enzyme (Figure 3A, lanes 5 and 6) and confirms that the ~147 site was not methylated in these cells. However, the ~2.2 kb band described above was seen following HpaII digestion (lane 6). This again suggests that the intronic site ~1.5 kb downstream of the ~147 site was methylated or inaccessible to HpaII and that a second non-methylated HpaII site exists ~0.7 kb further downstream. Following digestion of WB-F344 DNA with PstI and MspI, the 447 bp fragment was observed (Figure 3A, lane 2), but no fragment was seen following digestion with PstI and HpaII (lane 3). This result confirms that all sites within the PstI fragment were methylated in WB-F344 cells.

Role of DNA methylation in connexin expression

that two additional MspI sites are present within ~0.7 and ~1.5 kb of the published ~147 site (13). After an initial round of digestion with PstI which cleaves at ~594 (Figure 2A), the location of these fragments were mapped 5′ and 3′, respectively, of the site at ~147. This is because subsequent digestion with MspI had no effect on the migration of the ~1.5 kb fragment and reduced the size of the ~0.7 kb fragment to ~0.45 kb (Figure 3A, lanes 2 and 5). Therefore, we mapped the upstream site to a position at ~847 and the downstream site to a position extending ~1.35 kb into the intron (Figure 2A). This is consistent with the observation that hybridization to the ~1.5 kb fragment was dramatically reduced when Cx32 probe A was used (Figure 3D). With this smaller probe, the number of complementary residues was reduced from 114 to 70 and this may have hindered its hybridization.

Methylation of Cx32 and Cx43 genes

Methylation of Cx32 and Cx43 promoters was performed using the following probes: (Figure 3A and D) 1) Demethylation of Cx32 promoter sequences in WB-F344 cells, or hepatocytes (lanes 5 and 6) was digested to completion with MspI (lanes 1, 3 and 5) or HpaII (lanes 2, 4 and 6) and hybridized to Cx32 probe B. (C) Methylation patterns of the Cx32 promoter. Genomic DNA from MH1C1 (lanes 1 and 2) or WB-F344 (lanes 3 and 4) cells or hepatocytes (lanes 5 and 6) was digested to completion with MspI (lanes 1, 3 and 5) or HpaII (lanes 2, 4 and 6) and hybridized to Cx32 probe B. (D) Methylation patterns of the Cx43 promoter. Genomic DNA from MH1C1 (lanes 1–3) or WB-F344 (lanes 4–6) cells or hepatocytes (lanes 7–9) was digested to completion with EcoRI (lanes 1, 4 and 7) followed by digestion with MspI (lanes 2, 5 and 8) or HpaII (lanes 3, 6 and 9) and hybridized to probe C which spans sequences ~644 to ~87 of the rat Cx43 gene. (D) Methylation patterns of the Cx32 promoter. Genomic DNA from MH1C1 (lanes 1–2), WB-F344 (lanes 3–4) or WB-F344 cells treated with 5-AZA (lane 5–6) was digested to completion with MspI (lanes 1, 3 and 5) or HpaII (lanes 2, 4 and 6) and hybridized to probe A. This probe spans sequences ~594 to ~77 of the rat Cx32 gene.

To confirm that the Cx32 promoter site at ~147 was not methylated in MH1C1 cells and was methylated in WB-F344 cells, genomic DNA was first digested with PstI then with MspI or HpaII. The ~5.7 kb PstI restriction fragment described above encompasses the promoter, exon I and part of the intron. If subsequent digestion of this fragment with MspI or HpaII is complete at the ~147 position, a 447 bp fragment will result (Figure 2A). This fragment was clearly observed following digestion of MH1C1 cell DNA with either enzyme (Figure 3A, lanes 5 and 6) and confirms that the ~147 site was not methylated in these cells. However, the ~2.2 kb band described above was seen following HpaII digestion (lane 6). This again suggests that the intronic site ~1.5 kb downstream of the ~147 site was methylated or inaccessible to HpaII and that a second non-methylated HpaII site exists ~0.7 kb further downstream. Following digestion of WB-F344 DNA with PstI and MspI, the 447 bp fragment was observed (Figure 3A, lane 2), but no fragment was seen following digestion with PstI and HpaII (lane 3). This result confirms that all sites within the PstI fragment were methylated in WB-F344 cells.

Fig. 3. Southern blot analysis of Cx32 and Cx43 gene structure and methylation in MH1C1 and WB-F344 cells and freshly isolated adult male rat hepatocytes. (A) Mapping and resolution of three differentially methylated sites in the rat Cx32 promoter and intron. Genomic DNA from WB-F344 (lanes 1–3) or MH1C1 (lanes 4–6) cells were digested to completion with PstI followed by MspI (lanes 2 and 5) or HpaII (lanes 3 and 6) and hybridized to probe B which spans sequences ~754 to ~33 of the rat Cx32 gene. (B) Methylation patterns of the Cx32 liver-specific promoter. Genomic DNA from MH1C1 (lanes 1 and 2) or WB-F344 (lanes 3 and 4) cells or hepatocytes (lanes 5 and 6) was digested to completion with MspI (lanes 1, 3 and 5) or HpaII (lanes 2, 4 and 6) and hybridized to Cx32 probe B. (C) Methylation patterns of the Cx32 promoter. Genomic DNA from MH1C1 (lanes 1–3) or WB-F344 (lanes 4–6) cells or hepatocytes (lanes 7–9) was digested to completion with EcoRI (lanes 1, 4 and 7) followed by digestion with MspI (lanes 2, 5 and 8) or HpaII (lanes 3, 6 and 9) and hybridized to probe C which spans sequences ~644 to ~87 of the rat Cx43 gene. (D) Methylation patterns of the Cx32 promoter. Genomic DNA from MH1C1 (lanes 1–2), WB-F344 (lanes 3–4) or WB-F344 cells treated with 5-AZA (lane 5–6) was digested to completion with MspI (lanes 1, 3 and 5) or HpaII (lanes 2, 4 and 6) and hybridized to probe A. This probe spans sequences ~594 to ~77 of the rat Cx32 gene.
To assess Cx43 promoter methylation in these cells, genomic DNA was digested with EcoRI and then with MspI or HpaII. Cleavage of the ~4 kb EcoRI restriction fragment at the −38 MspI/HpaII position will result in a 0.95 kb fragment that hybridizes to Cx43 probe C (Figure 2B). This fragment was observed in both cell lines following digestion with MspI (Figure 3C, lanes 2 and 5), but was only seen in WB-F344 cells after HpaII digestion (lanes 3 and 6). This result indicates that the MspI/HpaII site at position −38 of the Cx43 promoter was methylated in MH1C1 cells, but not in WB-F344 cells. Interestingly, this site was also not methylated in rat hepatocytes. Cleavage of rat hepatocyte DNA with EcoRI followed by MspI or HpaII generated a 0.95 kb band, identical to that in WB-F344 cells (Figure 3C, lanes 7–9).

Activation of Cx32 expression by demethylation
Since Cx32 and Cx43 promoter methylation correlated with the lack of expression of these genes in MH1C1 and WB-F344 cells, we attempted to activate expression in these cells by treatment with the methyltransferase inhibitor, 5-AZA. This approach may lead to the reactivation of genes repressed by methylation (26,27). Treatment of WB-F344 cells with 5-AZA (2.5 µM for 72 h) rendered the Cx32 promoter sensitive to cleavage by both MspI and HpaII because both enzymes generated the ~0.7 kb fragment (Figure 3D, lanes 5 and 6). This result confirms that the Cx32 promoter was methylated in WB-F344 cells and could be demethylated with 5-AZA. However, northern blot analysis of total RNA from 5-AZA-treated cells indicated that Cx32 expression was not activated, even with dexamethasone treatment (Figure 1). Interestingly, 5-AZA treatment enhanced Cx43 transcript levels in these cells (Figure 1). This may be due to the demethylation of sites flanking the Cx43 gene locus or the enhanced expression through demethylation/derepression of genes that positively regulate Cx43 transcription and/or mRNA stability. We also attempted to demethylate the Cx43 gene in MH1C1 cells by 5-AZA treatment but found that the agent was very toxic in these cells and that demethylation could not be achieved with non-toxic concentrations (<0.5 µM; data not shown).

Activity of transfected Cx32 and Cx43 promoters
The expression of Cx32 and Cx43 in MH1C1 and WB-F344 cells, respectively, could also be affected by the presence and/or quantity of appropriate transcription factors. To test this hypothesis, transient expression assays were performed in each cell line using Cx32 and Cx43 promoter–luciferase reporter gene constructs. Following transfection of each promoter construct into WB-F344 and MH1C1 cells, cell-specific activity of the promoters was observed (Figure 4). The Cx32 promoter was active in both cell lines, but activity was 7-fold greater in MH1C1 cells. This activity was increased by DEX 2-fold in MH1C1 cells, but was not affected in WB-F344 cells. The Cx43 promoter was also active in both cell lines, but activity was 5-fold greater in WB-F344 cells and was not affected by DEX. These results suggest that regulation by transcription factors is also involved in the liver cell-specific expression of Cx32 and Cx43.

Discussion
Our data suggest that DNA methylation is one mechanism regulating the transcription of Cx32 and Cx43 in liver cells. We compared Cx32 and Cx43 promoter methylation in the well-differentiated, rat hepatoma cell line MH1C1 (which expresses Cx32 but not Cx43) with the diploid, non-transformed, rat liver epithelial cell line WB-F344 (which expresses Cx43 but not Cx32) (Figure 1). We identified several potential MspI/HpaII methylation sites in the Cx32 and Cx43 promoters (Figure 2) and found that the Cx32 sites were methylated in non-expressing WB-F344 cells, whereas the Cx32 promoter site was methylated in non-expressing MH1C1 cells (Figure 3). We also observed that the Cx32 promoter, when linked to a reporter gene, was active in both MH1C1 and WB-F344 cells, but that the activity was ~7-fold greater in the former cells and was enhanced by DEX only in these cells (Figure 4). Interestingly, both the Cx32 and Cx43 promoters were not methylated in rat hepatocytes (Figure 3). These data suggest that Cx43 silencing in liver cells involves promoter methylation, but that liver cell-specific Cx32 expression also depends upon the expression and activity of appropriate transcription factors. Our inability to detect Cx32 mRNA in WB-F344 cells after treatment with 5-AZA suggests that demethylation might be necessary but not sufficient for Cx32 expression in these cells. Alternatively, this result could also be due to instability of the Cx32 mRNA in these cells or to the activation by demethylation of a factor that represses Cx32 expression. The silencing of Cx43 expression in liver cells, on the other hand, does not appear to require promoter methylation (as noted in hepatocytes), but promoter methylation may play a role in some cases such as MH1C1 cells.

The Cx43 promoter methylation in MH1C1 cells and lack of such in hepatocytes suggests that this methylation might have occurred as a consequence of neoplastic transformation or adaptation to cell culture by MH1C1 cells and that these cells are not a representative model for Cx43 silencing in hepatic cells. To resolve whether or not Cx43 promoter methylation is a consequence of transformation or cell culture or is a common mechanism of Cx43 silencing, analyses of normal and neoplastic hepatic and non-hepatic cell lines will be necessary. The Cx43 gene, however, is frequently silenced in many types of neoplastic cells and this does not appear to be due to Cx43 mutation (reviewed in ref. 2), but might be due to DNA methylation. In that case, MH1C1 cells might be a useful model for Cx43 methylation. More importantly, perhaps, these cells do appear to be representative of hepatocytes in terms of Cx32 methylation and DEX response.
and, therefore, are an excellent model for Cx32 regulation in hepatic cells.

Gene hypermethylation, in particular within the promoter, is a common mechanism of tissue-specific gene silencing, inactivation of X chromosome-linked genes in females and inactivation of tumor suppressor genes such as p16\(^{ink4a}\) in neoplastic cells (21–26). Methylation of CpG sequences stabilizes chromatin, prevents binding of transcription factors and is the primary mechanism by which genes are turned on or off developmentally (21–23). DNA methyltransferase activity is also elevated in many types of neoplastic cells (28). In contrast, actively transcribed genes are frequently hypomethylated (29). Hypomethylation of oncogenes such as ras and myc may be an early event in liver carcinogenesis (30). Both Cx32 and Cx43 have demonstrated tumor suppressor activity and are expressed in a tissue-specific and development-specific manner (reviewed in ref. 2). Our data suggest that the expression of these genes may be controlled by DNA methylation similar to other tissue-specific and tumor suppressor genes. The contribution of methylation to connexin gene inactivation in neoplastic cells in general remains to be determined. It is noteworthy that the methylation we detected was not the most typical for silencing of genes involved in neoplasia, which usually involves densely methylated CpG islands (21–23,26). Rather, the low density of methylated sites we detected was more typical of tissue-specific gene silencing, such as that observed for keratin 18 in which methylation of a transcription factor (ETS) binding site was sufficient to prevent binding and suppress gene expression (31). It is interesting that the methylated MspI/HpaII site in the Cx32 promoter is located within the basal transcriptional element in close proximity to several Sp1 elements (14). This methylation may be sufficient to prevent efficient transactivation by Sp1. There may also be other methylated CpG sequences in the Cx32 and Cx43 promoters that we did not detect by MspI/HpaII digestion and that may repress expression.

Gene regulation by \textit{trans}-activating factors also appears to be a mechanism regulating the Cx32 and Cx43 gene promoters. The activities of the transfected Cx32 and Cx43 promoter–luciferase constructs in MH1C1 and WB-F344 cells correlated with expression of the endogenous genes. DEX also enhanced the activity of the transfected Cx32 promoter in MH1C1 cells only. These results suggest that, in addition to DNA methylation, the cell-specific expression of transcription factors and \textit{trans}-enhancer networks are also important in the regulation of Cx32 and Cx43 expression in hepatic cells. The nature of these transcription factors remains to be determined. Previous work indicates that a Cx32 promoter–specific complex designated B2 drives basal expression of this gene (14). We also have preliminary evidence that the liver-enriched transcription factor HNF-1 is a positively acting factor for Cx32 expression and is expressed in MH1C1 cells but not in WB-F344 cells (M.P. Piechocki et al., in preparation). The role of transcription factors in the control of Cx32 expression is also consistent with the ability of DEX to increase the transcriptional activity of the endogenous and transfected Cx32 promoters. A functional glucocorticoid response element does not appear to be present in the Cx32 promoter so that the DEX effect may involve other regulatory element(s) (M.P. Piechocki et al., in preparation). Other \textit{cis} elements and transcription factors contribute to the expression of Cx43. This gene is widely expressed in most epithelial and mesenchymal tissues, but is not expressed in parenchymal liver (11). Several functional \textit{cis} elements, including activator protein-1 and -2 response elements, cAMP response element and estrogen response elements, have been described in the human, rat and mouse Cx43 promoters (reviewed in ref. 2). The differential expression of appropriate transcription factors is undoubtedly also important in the liver cell-specific expression of Cx32 and Cx43.

Lastly, other mechanisms, including transcript stability, may be important in the liver cell-specific expression of Cx32 and Cx43. For instance, the half-life of the Cx32 mRNA is reduced in regenerating liver (32) and domains within the 3’-untranslated region of the Cx43 transcript have been identified that control its stability in a hormone-specific manner (33). In conclusion, we have identified DNA methylation and \textit{cis}/\textit{trans} regulation as mechanisms controlling the liver cell-specific expression of the rat Cx32 and Cx43 genes. These mechanisms may also be important in the cell-specific expression of other connexins and in the reduced connexin expression frequently observed in neoplastic tissues.

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