Sexual Dimorphism in the Regulation of Liver Connexin32 Transcription in Hexachlorobenzene-Treated Rats

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Hexachlorobenzene (HCB), an epigenetic carcinogen, causes female rats to be more susceptible to liver tumor formation than males. HCB exposure in females downregulates the expression of Cx32, a gap junction protein, through the activation of Akt. The objectives of this study were to determine (1) the implication of different regions of the hepatic Cx32 promoter in the observed sexual dimorphism in the expression of Cx32, (2) the implication of different regions of the hepatic Cx32 promoter in the HCB-induced downregulation of Cx32 in female rat liver, and (3) if HCB exposure modulates the binding of transcription factors on the Cx32 promoter through Akt activation. Male and female rats were exposed to HCB during 5 consecutive days and sampled 45 days later. Electrophoresis mobility shift assays showed that the intensity of only one nuclear protein–DNA complex differed between males and females. The formation of this complex requires two binding sites to be intact in a fragment of the basal promoter (Fr26). Following HCB exposure, the intensity of two complexes (Fr26 and Fr110) was decreased in females, but not in males, consistent with the decrease in Cx32 expression observed only in HCB-treated females. In vitro studies using a rat hepatocyte cell line (MH1C1) showed that the formation of the Fr110 protein–DNA complex appears to be controlled by Akt and requires the integrity of a Myb site. Overall, results suggest that both the sexual dimorphism and the downregulation of Cx32 in HCB-treated female rats are mediated by a reduction in the binding of activating transcription factors on the Cx32 promoter.

Key Words: hexachlorobenzene; connexin32; gap junctions; Akt; integrin-linked kinase; promoter; EMSA.

Gap junctions are composed of intercellular channels, or connexons, formed by a family of proteins termed connexins (Sosinsky and Nicholson, 2005). Adult rat hepatocytes express only two connexins: Cx32 and Cx26. These channels permit bidirectional communication between cells by selectively allowing the passage of ions, metabolites, and secondary messengers. Gap junctional intercellular communication (GJIC) is essential for the regulation of homeostasis. A downregulation of Cxs and GJIC in tumor cells has been well documented (Cottrell and Burt, 2005). Many epigenetic carcinogens have been shown to promote tumor formation by disturbing GJIC (Ruch and Trosko, 2001).

Hexachlorobenzene (HCB), a widespread environmental pollutant, is an epigenetic carcinogen. We and others have reported that HCB exposure renders females more susceptible than males to chemically induced liver carcinogenesis (Krishnan et al., 1991; Lambrecht et al., 1983; Larouche et al., 1993; Pereira et al., 1982; Smith et al., 1985). We have reported that a 5-day exposure to HCB results in a decrease in GJIC in females (Plante et al., 2002). Hepatic Cx32 and Cx26 mRNA and protein levels were significantly lower at day 50 in livers of HCB-treated females when compared to controls, whereas in males there were no differences between controls and HCB-treated rats. These changes are consistent with the occurrence of sexually dimorphic tumor formation (Erturk et al., 1986; Smith et al., 1985; Wainstok de Calmanovici et al., 1991). Our studies have shown that HCB-induced tumor promotion in females is associated with the activation of the integrin-linked kinase pathway (ILK) in which Akt, a downstream target of ILK, regulates Cx32 expression (Plante et al., 2005, 2006). Furthermore, a marked sexual dimorphism was observed in Cx32 and Cx26 basal mRNA levels, since they were eightfold lower and threefold higher, respectively, in females as compared to males (Plante et al., 2002).

Cx32 knockout mice are more susceptible to chemically induced liver tumor formation, suggesting an important role of Cx32 in carcinogenesis (King and Lampe, 2004; Omori et al., 2001; Temme et al., 1997). The mechanisms by which Cx32 transcription is regulated as well as the transcription factors implicated in the decrease of Cx32 during hepatocarcinogenesis are poorly understood. Various transcription factors have been associated with Cx32 regulation. In the liver, few studies have looked at the promoter of Cx32, and studies suggest that different transcription factors may be implicated in Cx32 regulation in vivo and in vitro. Three
promoters have been identified that are involved in regulating Cx32 expression. The first is located upstream of the first exon. This promoter lacks a TATA box and contains a CCAAT box and Sp1 elements, which stimulate Cx32 expression in adult rat liver (Field et al., 2003; Neuhaus et al., 1996; Fig. 1). Two additional promoter regions located within the intron have been shown to regulate Cx32 expression in neural and embryonic tissues, but are inactive in adult rat liver. Several response elements have been identified in the Cx32 promoter, including hepatocyte nuclear factor-1 (HNF-1), Sp1, YY1, and NF-1 (Fig. 1). However, their role in carcinogenesis remains to be elucidated (Bai et al., 1993, 1995; Field et al., 2003; Koffler et al., 2002; Morsi et al., 2003; Piechocki et al., 2000).

The aim of this study was to evaluate the binding of nuclear proteins to specific regions of the hepatic Cx32 promoter and assess whether or not these are associated with Cx32 downregulation. More precisely, the objectives were to (1) determine if the sexual dimorphism in the expression of Cx32 is associated with a gender-specific binding of transcription factors on the Cx32 promoter; (2) identify regions of the Cx32 promoter implicated in the HCB-induced downregulation of Cx32 in female rat liver; and (3) determine if HCB exposure modulates the binding of transcription factors on the Cx32 promoter through Akt activation.

MATERIAL AND METHODS

Animals. Male and female Sprague-Dawley rats (180–200 g) were purchased from Charles River Canada (St. Constant, Quebec, Canada). Rats were maintained under a constant photoperiod of 12 h light:12 h dark and received food and water ad libitum. All animal protocols used in this study were approved by the University Animal Care Committee.

Animal treatments. Rats were administered HCB (100 mg/kg) or vehicle (corn oil, controls) by gavage for 5 consecutive days and killed 45 days after the last HCB dose. At this time point (day 50), a significant decrease in hepatic Cx32 and Cx26, both at the mRNA and protein levels, and an overexpression of the ILK pathway in female rats has been previously observed (Plante et al., 2002, 2005, 2006). HCB administered to rats using this experimental protocol does not cause liver injury as determined by the absence of morphological changes and normal plasma levels of alanine aminotransferase. At the time of sampling, rats were anesthetized by isoflurane inhalation and the liver was removed, frozen in liquid nitrogen, and stored at −80°C for subsequent analyses.

Cell cultures and transfection. MH1C1 rat liver cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. Cells grown at 95% confluence were transiently transfected using 40 μg/ml lipofectamine 2000 (InVitrogen Life Technologies, Invitrogen Canada, Burlington, Ontario, Canada) in DMEM without FBS, according to the manufacturer’s instructions. Cells were lysed 24 h later for extraction of nuclear proteins. The constitutively active myr-Akt1 expression vector pUSEamp(+) was purchased from UpState Biosciences. PCL-neo (Promega, Madison, WI)

FIG. 1. Schematic map of the Cx32 promoter sequence identifying main regulatory sites. Transcription factor–binding sites are identified by boxes. NF-1–binding site and three CCAAT are underlined (adapted from Piechocki et al., 2000). Sequences used in EMSA are in italic (Morsi et al., 2003)
was used as a negative control vector; no differences were observed between PCL-neo transfected cells and untransfected cells. These transfections did not affect cell viability as determined by trypan blue exclusion assays and methylthiazol tetrazolium colorimetric analysis.

**Nuclear protein extraction.** Nuclear proteins from liver and MH1C1 rat hepatocytes were extracted using the Nuclear and Cytoplasmic NE-PER protein extraction kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were assessed using Bio-Rad Protein Assay reagents (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

**Electrophoresis mobility shift assay.** Electrophoresis mobility shift assay (EMSA) were done according to Morsi et al. (2003). Briefly, Cx32 promoter fragments (10 pmol of sense oligonucleotides) were incubated with T4 Polynucleotide Kinase (InVitrogen Life Technologies) and [γ-32P]ATP (3000 Ci/mmol), for 30 min at 37°C, and subsequently purified on a NICK Sephadex column (Amersham Biosciences, Baie d’Urfe, Quebec, Canada). The labeled DNA probe was then incubated with the corresponding antisense fragment. Radioactive double-stranded DNA (20 fmol) was incubated with 4 μg of nuclear protein in EMSA incubation buffer (40% glycerol, 10mM MgCl2, 5mM ethylenediaminetetraacetic acid (EDTA), 100mM Tris, pH 7.5, 500mM NaCl, 5mM dithiothreitol (DTT), 2 μg/ml of poly d-l-dc) for 30 min at room temperature. When appropriate, samples were incubated for 30 min at room temperature with either antibody (Sp1 (PEP2) SC-59; Sp3 (D-20) SC-644; ER alpha (C-311) SC-787; ER beta (H-150) SC-8974; B-myb (C-20) SC-725; GR) at 5°C for 1 h with either antibody. The complexes were then separated on a 4% nonradioactive polyacrylamide, dried, exposed to a phosphorus screen, and analyzed using a phosphorimager (PhosphorImager SI Molecular Dynamics). Intensity of the complexes was measured using the Phosphorimager SI Molecular Dynamics software as the number of pixels representing the promoter was generously donated by Dr S. Minchin (The InVitrogen Life Technologies). The sequence Fr110 was obtained by digesting Fragments (Fr26, Fr53, Fr70, Fr26 mA, Fr26 mB, Fr110A, Fr110B, Fr110C, Fr110A mA, Fr110B mA, Fr110C mA, and Fr110 mA) used for EMSA were synthesized by InVitrogen (InVitrogen Life Technologies). The sequence Fr110 was obtained by digesting the full-length promoter of Cx32 with specific restriction enzymes. The vector containing the promoter was generously donated by Dr S. Minchin (The University of Birmingham, United Kingdom) (Field et al., 2003; Morsi et al., 2003). Briefly, after being extracted from the vector, the promoter was sequentially cut in appropriate buffer with Alu I for 1 h and Mbo I for 1 h. The fragments were separated on a 2% agarose gel and the Fr110 fragment was extracted from the gel using QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario, Canada). The sequence of each fragment is indicated in Table 1.

**Southwestern blots.** Southwestern blots were done according to Bai et al. (1995), with minor modifications. Briefly, nuclear proteins were separated on a 12% polyacrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature in blocking buffer (5% dry milk, 4% glycerol, 1mM MgCl2, 0.5mM EDTA, 10mM Tris, pH 7.4, 50mM NaCl, 0.5mM DTT, 0.05% Tween) and then incubated for 2 h at room temperature in blocking solution containing 7.5 × 106 counts per minute of radioactively labeled double-stranded DNA (Fr26 and Fr110A probes). After hybridization, membranes were washed twice (15 min each) in blocking solution and rinsed in washing buffer (100mM NaCl, 10mM Tris pH 7.6, 1mM EDTA), and then analyzed using a phosphorimager (PhosphorImager SI Molecular Dynamics).

**Statistical analysis.** Statistical differences between groups, that is, control and HCB-treated groups or between MH1C1 and MH1C1-Akt+, were determined using either a Student’s t-test when there were only two experimental groups or an ANOVA followed a posteriori by a Tukey’s test for multiple comparisons between multiple experimental groups. Each experiment was repeated three times using 3 or 4 independent samples per group. Significance was set at p < 0.05. All statistical analyses were done using the SigmaStat computer software (Jandel Scientific Software, San Rafael, CA).

### RESULTS

**The Sexual Dimorphism in Cx32 Expression**

The first objective of the study was to determine if the sexual dimorphism observed previously for hepatic Cx32 expression was due to differential binding of transcription factors to the promoter region. In these experiments, we used four different fragments of the Cx32 promoter to conduct EMSA (Fig. 1 and Table 1, Fr26, Fr53, Fr70, and Fr110). These fragments

### TABLE 1

Sequences of the Promoter Fragments Used as DNA Probes for EMSA

<table>
<thead>
<tr>
<th>Name of the fragment and mapping point</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Fr26 (mp-152 to mp-127)</td>
<td>5'-AAG CTC CGG TGCT TGGC TTTG CG-3'</td>
</tr>
<tr>
<td>Fr53 (mp-687 to mp-635)</td>
<td>5'-GAT CAA CTG CCT TAG CCC TAA GAG TAG AAG ACA TTT TTT AAG TAA GTT TTA TG-3'</td>
</tr>
<tr>
<td>Fr70 (mp-757 to mp-688)</td>
<td>5'-GAT CCT TCA GTT AAT GGT TGA CAT TCT GTT TAC CTA AAT GAT GTC CAA GAG TTT CTT CTG CTT ACA TAT GAC A-3'</td>
</tr>
<tr>
<td>Fr110 (mp-373 to mp-264)</td>
<td>5'-CTG AGA TGT GTT AAG CAG CCA AAT CCA GTT CAC ATG ACA ATG TGG CAT CAA TAT GAC AAA TGA TGC TTT TCT TCT TCC TCT TGG ACA GGG CAA GAA CTT TAG A-3'</td>
</tr>
<tr>
<td>Fr110A (mp-373 to mp-335)</td>
<td>5'-CTG AGA TGT GTT AAG CAG CCA AAT CCA GTT CAC ATG ACA ATG TGG CAT CAA TAT GAC AAA TGA TGC TTT TCT TCT TCC TCT TGG ACA GGG CAA GAA CTT TAG A-3'</td>
</tr>
<tr>
<td>Fr110B (mp-340 to mp-299)</td>
<td>5'-TAC CCC CAC ATO GTT GCA ATA TGG CAT TTT AAT GAC AAA TGA T-3'</td>
</tr>
<tr>
<td>Fr110C (mp-304 to mp-264)</td>
<td>5'-AAA TGA TGC TTT TCT TCC TCT TCC TGC CCT GCA AAG GAG GCA GAA CTT TAG A-3'</td>
</tr>
<tr>
<td>Fr26 mA (mp-152 to mp-127)</td>
<td>5'-AAG CTC CGG TCC TCT AAT GGT TGA CAT TCT CCT TGG ACA GAG GAA CTA TGG CAT A-3'</td>
</tr>
<tr>
<td>Fr26 mB (mp-152 to mp-127)</td>
<td>5'-AAG CTC CGG TCC TCT AAT GGT TGA CAT TCT CCT TGG ACA GAG GAA CTA TGG CAT A-3'</td>
</tr>
<tr>
<td>Fr110A mA (mp-373 to mp-335)</td>
<td>5'-CTG AGA TGT GTT AAG CAG TGG AAT CCA GTT CAC ATG ACA ATG TGG CAT CAA TAT GAC AAA TGA TGC TTT TCT TCT TCC TCT TGG ACA GGG CAA GAA CTT TAG A-3'</td>
</tr>
<tr>
<td>Fr110A mB (mp-373 to mp-335)</td>
<td>5'-CTG AGA TGT GTT AAG CAG CCA AAT CCA AAG TGG CAC TAC ATG ACA ATG TGG CAT CAA TAT GAC AAA TGA TGC TTT TCT TCT TCC TCT TGG ACA GGG CAA GAA CTT TAG A-3'</td>
</tr>
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**Note:** Letters in bold and italic represent a binding site sequence, whereas underlined letters in bold represent a mutation site. For Fr 26, the boxes indicate overlapping binding sites.
represent critical regions implicated in the hepatic regulation of Cx32 as well as in the downregulation of Cx32 in primary cultures of hepatocytes (Morsi et al., 2003). Fr26 is located in the basal promoter, Fr53 and Fr70 are both located in positive regulatory regions, and Fr110 partially located in a negative regulatory region and partially in a positive region (Bai et al., 1993, 1995; Morsi et al., 2003). The gender-specific Cx32 regulation in rat liver was assessed using EMSA by looking at the formation of complexes between these fragments and nuclear protein extract from male and female rats.

Using Fr26, only one specific protein-DNA complex was formed when incubated with liver nuclear proteins. In male rats, the Fr26 complex was more abundant by twofold than those from female (Fig. 2A). Using Fr53, two specific complexes were formed; neither of them showed significant differences between males and females (Fig. 2B). For Fr110, only one complex was formed and its abundance did not differ between the two groups (Fig. 2C). For Fr70, no specific complexes were formed when incubated with hepatic nuclear proteins (data not shown). These results suggest that a transcription factor binding to the Cx32 promoter between mp-153 and mp-127 is likely implicated in the sex-specific regulation of Cx32.

Modulation of Transcription Factors on Cx32
Promoter by HCB

In previous studies, we have shown that Cx32 expression was decreased in the liver of HCB-treated female rats as compared to controls, while there were no differences between HCB-treated and control males (Plante et al., 2002). Changes in binding complexes formed between nuclear proteins and the different fragments of the Cx32 promoter were assessed in control and HCB-treated animals of both sexes. For Fr26, the binding complex formed with liver nuclear proteins showed a significant fivefold decrease in HCB-treated female livers as compared to controls (Fig. 3A, left side). In males, there were no differences between HCB-treated and control animals (Fig. 3A, right side). For fragment Fr53, two different binding complexes were formed. There were no differences in hepatic DNA-protein complexes with Fr53 between the control and HCB-treated groups for either males or females (Fig. 3B). Finally, using Fr110 we observed the formation of a single binding complex. This complex was decreased threefold in HCB-treated females as compared to controls (Fig. 3C, left side). In males, there were no differences between HCB-treated and control animals (Fig. 3C, right side). Taken together, the data indicate that HCB exposure resulted in a decrease in two binding complexes in the Fr26 and Fr110 regions of the Cx32 promoter of female rat liver.

Modulation of Transcription Factors Binding to Cx32 Promoter by Akt

We have previously shown that HCB exposure leads to the activation of the ILK pathway in female rat livers and that the decreased Cx32 expression appears to result from the activation of Akt by ILK (Plante et al., 2005, 2006). In order to assess whether or not Akt activation alters the transcription factors that bind to the Cx32 promoter, nuclear proteins were extracted from MH1C1 cells transfected with a constitutively active Akt vector (MH1C1-Akt+) as described previously (Plante et al., 2006). Transfection with this vector induces a marked increase in exogenous Akt in both cytoplasmic/membrane and nuclear fractions of MH1C1 cells, as well as a 40% decrease in Cx32 at both mRNA and protein levels (Plante et al., 2006). EMSA were conducted with the Fr110 and the Fr26 DNA fragments, which were shown to be modulated by HCB exposure in females (Fig. 3). For both MH1C1 and MH1C1-Akt+, the nuclear protein–DNA complexes formed (Fig. 4) were similar to those observed using rat liver extracts (Fig. 2). No differences were observed between MH1C1 and MH1C1-Akt+ cells for Fr26, suggesting that the Akt-driven downregulation of Cx32 is not exerted via transcription factors binding to this region of the promoter (Fig. 4A). However, a significant 20% decrease was observed in complex formation resulting from binding to Fr110 (Fig. 4B). This suggests that one of the two transcription factors implicated in the HCB-induced Cx32 decrease in rat liver may be modulated via Akt.

Identification of Transcription Factors in the Fr26 and Fr110 Complexes

The Fr26 fragment contains various consensus binding sequences, identified either in silico (TESS [Transcription Element Search Software] on the WWW, Jonathan Schug and G. Christian Overton, Technical Report CBIL-TR-1997-1001-v0.0, Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997, URL: http://www.cbil.upenn.edu/cgi-bin/tess/tess) and TFSEARCH program (Parallel Application TRC Laboratory, Japan) or in the literature, including a Sp site and a site similar to an androgen response element (AR) and an estrogen response element (ER) (Bai et al., 1993). In order to determine if these sites were important for Fr26 complex formation, two different mutated fragments were synthesized: Fr26 mA contains a mutation in the Sp site and Fr26 mB contains a mutation in the AR/ER site (Table 1). Surprisingly, both unlabeled mutated probes failed to compete with the Fr26 complex (Fig. 5A, lanes 4–7), suggesting that these sequences are necessary for the binding of nuclear proteins to the Cx32 promoter. In order to confirm these results, mutated probes were labeled and incubated with nuclear extracts. No specific complexes were observed with either probe, confirming the necessity of both sites to be intact for proper Fr26 complex formation (Fig. 5A, lanes 8–11). To determine if the complex observed was the result of a member of the Sp family binding to this site, Sp1 and Sp3 antibodies were used for supershift assays. These analyses suggested, however, that the complex formed between Fr26 and nuclear proteins does not involve either Sp1 or Sp3 (Fig. 5B, lanes 3–8). Surprisingly, AR, ERα, and ERβ antibodies also failed to supershift the Fr26 complex (Fig. 5B, lanes 9–17).
FIG. 2.  Gender-specific binding of transcription factors from liver nuclear extracts on rat Cx32 promoter fragments in female and male rats. Radiolabeled promoter fragments Fr26 (A), Fr53 (B), and Fr110 (C) were incubated with nuclear protein extract, separated by nondenaturating PAGE, and analyzed using a phosphorimager. For every panel, lane 1 represents nonspecific binding and lane 10 competition assays using 100-fold molar excess of unlabeled probe. Lanes 2–5 represent nuclear extracts from different females, and lanes 6–9 represent nuclear extracts from different males. Data are expressed as the mean±SEM. *Significant difference from females (p < 0.05).
Fr110 fragment was first cut into three overlapping fragments (Fr110A, Fr110B, and Fr110C) to determine which specific region is involved in the Fr110 complex formation (Table 1). Only EMSA using the Fr110A labeled probe resulted in the formation of a specific DNA-protein complex (Fr110A), suggesting that the nuclear proteins bind Fr110 between mp-373 and mp-335 (Fig. 6A). Fr110A is located partially in both a negative and a positive regulatory region and contains a HNF-1 site and a Myb-binding site (TESS and TFSEARCH programs; Bai et al., 1993). In order to determine the implication of these binding sites in Fr110/Fr110A complex formation, two mutated probes were synthesized: Fr110A mA contains a mutation in HNF-1 site and Fr110A mB contains a mutation in the Myb-binding site (Table 1). While competition assays using Fr110A mA unlabelled probe abolished the radioactive Fr110/Fr110A complex (Fig. 6B, lanes 4 and 5), the Fr110A mB failed to compete with the Fr110/Fr110A complex (Fig. 6B, lanes 6 and 7). These results were confirmed using radiolabeled Fr110 mA and Fr110 mB. A Fr110/Fr110A complex was formed even though the HNF-1 site was mutated (Fig. 6B, lanes 8 and 9) suggesting that the nuclear protein do not bind to this specific site. However, when the Myb-binding site was mutated, the Fr110/Fr110A complex was abolished (Fig. 6B, lanes 10 and 11). Together, these results suggest that a HNF-1 intact site is not required for Fr110/Fr110A complex formation, while the Myb site must be intact. Supershift analysis did not confirm, however, that the complex formed between Fr110/Fr110A and nuclear extracts involves B-myb (Fig. 6C, lanes 3–5). Since the B-myb site is similar to the glucocorticoid receptor (GR) response element, supershift assays using a GR antibody were done; GR antibody also failed to supershift the Fr110/Fr110A complex (Fig. 6C, lanes 6–8).

In order to better characterize the proteins that bind to Fr26 and Fr110, Southwestern blots were performed. Using a Fr26-labeled probe, a single band was observed, of approximately 60 kDa (Fig. 7A). Using Fr110A as a probe, two bands were observed of approximately 60 and 110 kDa (Fig. 7B).


discussion

HCB causes females to be more susceptible to develop hepatic tumors. We have previously hypothesized that this may be due in part to the fact that females normally express lower levels of Cx32 than males and that these levels are further decreased by HCB exposure (Plante et al., 2002). Little is known about the cellular mechanisms implicated in both the HCB response and the regulation of Cx32 expression. Our previous results have shown that HCB exposure stimulates the ILK pathway, leading to the activation of Akt (Plante et al., 2005). Once activated, Akt translocates into the nucleus, resulting in the downregulation of Cx32 levels (Plante et al., 2006). Since HCB causes a further decrease in Cx32, we wanted to know whether or not the HCB effect was mediated via the same mechanism that appears implicated in the gender-specific difference in Cx32 levels.

Our results indicate that it is partially the case, with the respect to the binding of specific nuclear proteins to the Cx32 promoter. Indeed, our data indicate that one nuclear protein–DNA complex formed in the basal region of Cx32 promoter (Fr26, mp-152 to mp-127) is less intense in females as compared to males. This binding of the protein is further decreased in HCB-treated female as compared to untreated females. These results suggest that the Fr26 fragment is associated with an activating transcription factor, since the intensities of these protein-DNA complexes in males, females, and HCB-treated females are well correlated with Cx32 mRNA levels observed previously (Plante et al., 2002). Moreover, in addition to the decrease in gender-associated Fr26 protein-DNA complex, HCB and Akt act on another protein-DNA complex, Fr110, formed between mp-373 and mp-353. These results confirm that both the gender-specific and the HCB-induced decreases of Cx32 mRNA levels are mediated at the transcription levels by the differential binding of proteins on two sites of Cx32 promoter. Furthermore, these results are consistent with the gender-specific effects of HCB as these changes were not observed in livers from male rats.

Southwestern analysis suggested that the transcription factor involved in the formation of the Fr26 complex is a protein of approximately 60 kDa. This is in agreement with a report by Bai and coworkers using a fragment comprising most of the basal promoter region of Cx32 (mp-177 to mp-106) in which they reported three distinct binding complexes in HuH7 human hepatoma cells and in FAO-1 rat hepatoma cells (Bai et al., 1995). However, using rat liver tissue, only one complex was formed, located between mp-152 and mp-127, and which involved the binding of a 60-kDa protein. This suggests that only this region of the basal promoter is important for the in vivo regulation of Cx32. In contrast, using the same experimental conditions for the EMSA, three binding complexes with Fr26 have been shown in liver nuclear extracts from male Wistar rats (Morsi et al., 2003). Whether or not strain-specific

FIG. 3. Effect of HCB on protein-DNA complexes formed from rat Cx32 promoter fragments (A) Fr26 (mp-152 to mp-127), (B) Fr53 (mp-687 to mp-635), and (C) Fr110 (mp-373 to mp-264) and liver nuclear extracts from control and HCB-treated animals. Radiolabeled promoter fragments were incubated with nuclear protein extract separated by nondenaturating PAGE and analyzed using a phosphorImager. For all panels, left side shows female and right side shows male results; lane 1 represents nonspecific binding and lane 10 competition assays using 100-fold molar excess of unlabeled probe. Lanes 2–5 represent nuclear extracts from different control animals, and lanes 6–9 represent nuclear extracts from different HCB-treated animals. Data are expressed as the mean ± SEM. *Significant difference from controls (p < 0.05).
differences in binding are responsible for this discrepancy remains unclear.

Our results suggest that two binding sites are important for the formation of the Fr26 complex: an Sp site, located at mp-139, and an ER/AR site, located around -147. Indeed, mutations on either site abrogate the formation of the complex. The Fr26 complex was not the result of Sp1 or Sp3 binding, as shown by the absence of a supershift when liver nuclear proteins were preincubated with antibodies against either Sp1 or Sp3. Using a longer sequence (mp-179 to mp-33) than our Fr26 (mp-152 to mp-127), Piechocki et al. (2000) reported the presence of three binding complexes using MH1C1, all of which competed with unlabeled oligonucleotides for Sp1. These observations are consistent with our results and suggest that the transcription factor binding to the basal promoter interacts with the Sp DNA-binding site; however, the binding protein is not Sp1 or Sp3. Other members of the Sp family have also been shown to bind to this site (Suske, 1999); whether or not the Fr26 complex is formed by the binding of another member of this family to this promoter region remains to be elucidated.

Our studies showed that another binding site is also important for the formation of the Fr26 DNA-protein–binding complex, since mutations in the ER/AR-binding site inhibit the formation of this complex. However, neither ER nor AR appears to bind to this site, as shown by the absence of supershift using ERα-, ERβ-, or AR-specific antibodies. Interestingly, both Sp and ER/AR sites have to be intact to allow the formation of the Fr26 complex. These data thus suggest that this complex is formed by either a single transcription factor binding simultaneously to both sites or by multiple interacting transcription factors.

Data showed that HCB also induced the decrease of another protein-DNA complex, Fr110, in HCB-treated females as compared to untreated females. Moreover, HCB apparently acts at least in part through Akt since there was a 20% decrease in the intensity of the Fr110 complex in MH1C1-Akt+ cells as compared to wild-type MH1C1 cells. However, other regulatory factors may be responsible for the in vivo effect since the HCB treatment induced a threefold decrease in the Fr110 complex level in female rats. Using overlapping shorter sequences, we demonstrated that the Fr110 complex is formed between mp-373 and mp-335 (Fr110A). Moreover, using mutation assays, we showed that a Myb-binding site located around mp-347 has to be intact to allow the formation of the complex, whereas a mutation in the HNF-1 site located at -356 had no effect. This
was unexpected since HNF-1 has been suggested to be a regulator of Cx32 expression in hepatic cells (Koffler et al., 2002). However, the promoter region used in previous studies was shorter than the sequence used in the present study (mp-244 to mp-33) and did not include the Fr110A sequence, which is located upstream from this site. Piechocki et al. (2000) also showed an increased promoter activity correlated with the binding of HNF-1 to two HNF-1 consensus sites. Binding to these elements was only observed with MH1C1 cells, suggesting that this regulation may be different between cell types and between in vivo and in vitro conditions (Piechocki et al., 2000). However, preincubation of the complex using either B-myb– or GR-specific antibodies failed to supershift the complex. Southwestern analyses using Fr110A probe demonstrated that the Fr110 complex was formed either by two different transcription factors, one of approximately 60 and one of approximately 110 kDa. It is also possible that this represents a single transcription factor of approximately 60 kDa, which can dimerize, resulting in a 110-kDa complex. Further studies will be needed to identify this transcription factor.

Interestingly, when MH1C1 and MH1C1-Akt+ cells were subjected to EMSA using Fr26 and Fr110 fragments, the binding complex formed was similar as those observed in vivo using liver nuclear extract. In other studies, the regulation of Cx32 appears to differ between in vivo and in vitro systems using cultured cell lines. Using primary cultures of hepatocytes, in which Cx32 expression is low relative to liver extracts, three or five DNA-protein complexes were formed with the Fr26 fragment, depending upon the condition of the cells (Morsi et al., 2003). As previously described, three binding complexes were formed using either the HuH7 human hepatoma cell line or the FAO-1 rat hepatoma cell line (Bai et al., 1995). However, only one complex was observed for MH1C1 nuclear extracts, which is similar to whole rat liver. MH1C1 is a differentiated rat hepatoma cell line that expresses Cx32. These results suggest that in the more differentiated

![FIG. 5. Analyses of binding sites on fragment Fr26 (mp-152 to mp-127). Radiolabeled promoter fragments were incubated with nuclear protein extract and separated by non-denaturing PAGE exposed to a phosphorus screen and analyzed using a phosphorimager. (A) Lane 1 represents nonspecific binding, lane 2 represents liver nuclear extracts with labeled Fr26 probe, and in lane 3, the radioactive complex was competed with 100-fold molar excess of unlabeled probe. Lanes 4 and 5 (Fr26 mA) and lanes 6 and 7 (Fr26 mB) represent competition assays using either 100- or 200-fold molar excess of unlabeled mutated probes. Lanes 8 and 10 represents liver nuclear extracts with the labeled mutated probes and lanes 9 and 11 competition assays using 100-fold molar excess of unlabeled mutated probes. (B) Lane 1 represents nonspecific binding (no antibody), and lane 2 represents liver nuclear extracts with labeled Fr26 probe. Sp1 (lanes 3–5), Sp3 (lanes 6–8), AR (lanes 9–11), ERα (lanes 12–14), and ERβ (lanes 15–17) antibodies were preincubated with nuclear protein extract for 30 min. Radiolabeled fragment Fr26 was then incubated with nuclear protein extract and separated by nondenaturing PAGE.](https://academic.oup.com/toxsci/article-abstract/96/1/47/1656844)
MH1C1 cell line, Cx32 regulation appears similar to in vivo conditions.

Finally, results obtained for the Fr53 (mp-687 to mp-635) and Fr70 (mp-757 to mp-688) probes differed from previously reported observations. Using Fr70, we observed a band at the bottom of the gel; however, this band appears nonspecific, since it was not competed by unlabeled probe. The use of Fr53 resulted in the formation of two binding complexes rather than a single complex, which had been reported by Morsi and coworkers in the liver (Morsi et al., 2003). Whether strain-specific differences are responsible for these discrepancies is not known. Since there were no variation between males and females and no HCB-induced effects observed for these binding complexes using the Fr53 fragments, it suggests that transcription factors binding to this region are not implicated in the decrease in Cx32 mRNA levels following HCB exposure or in the sex-specific regulation of Cx32.

FIG. 6. Analyses of binding sites on fragment Fr110 (mp-373 to mp-264). Radiolabeled promoter fragments were incubated with nuclear protein extract and resolved by nondenaturating PAGE and exposed to a phosphorus screen, and the radioactive bands were detected with a phosphorimager. The Fr110 was divided into three overlapping probes (Fr110A, Fr110B, and Fr110C). (A) Lanes 1, 4, and 7 represent nonspecific binding; lanes 2, 5, and 8 are liver nuclear extract incubated with the labeled probes, and lanes 3, 6, and 9 were competed with 100-fold molar excess of unlabeled probe. (B) Lane 1 represents unspecific binding, lane 2 are liver nuclear extracts with the labeled Fr110A probe, and in lane 3 the radioactive complex was competed with 100-fold molar excess of unlabeled probe. Lanes 4–7 represent competition assays using either 100- or 200-fold molar excess of unlabeled mutated probes (Fr110A mA or Fr110A mB, respectively). Lanes 8 and 10 represent liver nuclear extracts with the labeled mutated probes, and lanes 9 and 11, competition assays using 100-fold molar excess of unlabeled mutated probes. (C) Lane 1 represents unspecific binding, and lane 2 represents liver nuclear extracts with the labeled

FIG. 7. Characterization of Fr26 and Fr110/Fr110A complexes using Southwestern analysis. Nuclear protein extracts were resolved on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, membranes were incubated 2 h at room temperature with $7.5 \times 10^6$ counts per minute of radiolabeled Fr26 (A) or Fr110A (B) fragments, exposed to a phosphorimager, and analyzed using a phosphorimager.

Fr110A probe. B-myb (lanes 3–5) and GR (lanes 6–8) antibodies were preincubated with nuclear protein extract for 30 min. Radiolabeled fragment Fr110A was then incubated with nuclear protein extract and resolved by nondenaturating PAGE.
In the present study, we have shown that the sexual dimorphism observed in Cx32 mRNA levels in female rats as compared to males resulted, at least in part, from decreased binding of a transcription factor in the basal promoter region of Cx32 (Fr26). Moreover, this nuclear protein–DNA complex is also implicated in the female-specific HCB-induced decrease of Cx32 as well as another complex, Fr110. *In vitro* experiments suggested that Fr110-binding complex appears to be regulated by Akt. Together, our results suggest that HCB renders females more susceptible to hepatocarcinogenesis by decreasing the binding of transcription factors implicated in the expression of Cx32.

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