MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1439–1445 Vol. 15, No. 3
0270-7306/95/$04.00+0


# Basal Promoter of the Rat Connexin 32 Gene: Identification and Characterization of an Essential Element and Its DNA-Binding Protein

SHUANG BAI,[1,2,3] ALAN SCHOENFELD,[1,4] ANTONELLO PIETRANGELO,[1,2] AND ROBERT D. BURK[1,4,5]*

*Marion Bessin Liver Research Center[1] and Departments of Medicine,[2] Neuroscience,[3] Pediatrics,[5] and Microbiology and Immunology,[4] Albert Einstein College of Medicine, Bronx, New York 10461*

Received 11 July 1994/Returned for modification 17 August 1994/Accepted 19 December 1994

**The connexin 32 (Cx32) gene, a member of a multigene family, is expressed preferentially in the liver. The basal promoter complex of the rat Cx32 gene was previously localized to a 146-bp region (map positions [mp] −179 to −34) immediately upstream of the first exon. To investigate the biochemical factors contributing to the basal promoter activity, nuclear protein-DNA complexes within this region (mp −177 to −106) were investigated by using a DNA mobility shift assay. Three DNA-protein binding activities, termed Cx32-B1, Cx32-B2, and Cx32-B3, were identified with nuclear protein extracts from hepatoma cell lines, HuH7 and FAO-1. However, only Cx32-B2 binding activity was detected in nuclear protein extract from normal rat liver tissue. This activity was significantly more abundant in rat liver tissue than in hepatoma cell lines and tissues from various other organs. By using methylation interference footprinting, the Cx32-B2 complex was localized to the region between mp −152 and −127 and a DNA probe containing this region bound to a 60-kDa protein in rat liver nuclear extracts. Mutation of two nucleotides in the Cx32-B2 binding site abrogated the formation of the Cx32-B2 protein-DNA complex and significantly reduced the transcriptional activity of the Cx32 promoter. These results indicate that the Cx32-B2 complex is an essential component of the rat Cx32 basal promoter and is likely a major factor in the preferential expression of this gene in the liver.**

The major protein components of gap junctions are connexins, which in each cell form hexameric assemblies that extend across the cell membrane to provide direct communication between neighboring cells. Sequence-specific antibodies and cDNA probes for specific connexins have demonstrated that individual connexins are expressed in a complex overlapping tissue distribution, such that individual cells can express more than one type of connexin (for reviews, see references 7, 31, and 34). In addition to the tissue-specific distribution of the various connexins, their expression has also been shown to change during development (9, 12, 14) and in response to various signals (28, 32). Moreover, accumulating data indicate that expression of one connexin can be inhibited while expression of another connexin may increase during tumorigenesis (3, 13, 23, 24, 29). The relative tissue specificity of connexin gene expression and the modulation of their expression by physiological and pathological stimuli indicate that connexin protein levels are regulated at both the transcriptional and posttranscriptional levels (18, 27).

The connexin 32 (Cx32) gene is the predominant connexin gene expressed at both the mRNA and protein levels in rat liver (35). Nuclear run-on analysis has demonstrated that the transcription rate of Cx32 in rat liver is significantly higher than that of Cx26 (18), indicating that the predominance of Cx32 in rat liver corresponds to its rate of transcription. In addition to being identified in the liver, Cx32 mRNA has been identified in kidney, stomach, intestine, brain, uterus, and pancreas tissue (8, 25, 35), and low levels of Cx32 mRNA have been detected in lung, spleen, and testis tissue (35). Moreover, mutations in human Cx32 have recently been shown to cause the X-linked form of Charcot-Marie-Tooth disease (4).

The expression of eukaryotic genes is governed to a large extent by sequence-specific interactions between a promoter and specific DNA-binding proteins which facilitate the activity of polymerase II (for a review, see reference 33). Most promoters of class II genes have canonical DNA elements, such as the TATA and CAT motifs, which bind transcription factors essential for mRNA initiation (for a review, see reference 6). However, other class II promoters lack TATA elements and initiate transcription by using alternative mechanisms to associate with the TFIID complex (26). As previously reported, the promoter of the Cx32 gene falls into this latter class (22).

In a previous study, we investigated the genetic basis of the transcriptional expression of Cx32 (2). Through serial deletions of the rat Cx32 promoter in a reporter construct, the regulatory elements were identified. When an approximately 800-bp sequence upstream of Cx32 with strong promoter activity was deleted up to map position (mp) −358 (where mp 1 of the cDNA is A in the ATG translation start codon), promoter activity dramatically decreased, indicating a potential enhancer in the region deleted. Further deletion up to mp −179 caused a fivefold increase in promoter activity, suggesting the presence of a negative regulatory element in this region. Finally, when the Cx32 promoter was deleted up to mp −134, activity diminished to levels seen with a promoterless reporter construct. Thus, the region between mp −179 and −134 was defined as the basal promoter. In the present study, we present data demonstrating that the rat Cx32 basal promoter active in liver cells contains two major DNA-protein binding sites. Characterization of one binding site identified interaction with a protein enriched in liver tissue. These findings indicate that differential DNA-protein interactions asso-

* Corresponding author. Mailing address: Marion Bessin Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-3720. Fax: (718) 918-0857. Electronic mail address: burk@aecom.yu.edu.

ciated with the rat Cx32 promoter contribute to its tissue-specific expression.

## MATERIALS AND METHODS

**Northern (RNA) blot assay of Cx32 transcript.** Total RNA was isolated as previously described (2). RNAs were separated by electrophoresis on a formaldehyde-agarose gel and transferred to a GeneScreen membrane (Dupont, Wilmington, Del.). The blot was first hybridized with a $^{32}$P-labeled Cx32 cDNA probe, washed, and exposed to Kodak XAR film. Subsequently, the blot was stripped and rehybridized with a $^{32}$P-labeled β-actin probe to verify lane loading.

**DNA mobility shift assay.** Nuclear extracts were prepared from freshly isolated rat liver tissue and cultured tumor cells (HuH7 and FAO-1) as previously described (11, 15). For comparison of various tissues, nuclear extracts were prepared by a slight modification of a method described by Deryckere and Gannon (10). Double-stranded (ds) oligonucleotide probes were labeled with $^{32}$P either by filling in a 5′ overlapping end with Klenow DNA polymerase or by end labeling with T4 polynucleotide kinase. The labeled oligonucleotides were purified by Sephadex G-25 column chromatography. DNA-protein binding reactions were carried out in a solution containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 100 μg of poly(dI-dC) · (dI-dC) per ml. A 2-μg amount of nuclear protein extracts was added to each reaction mix. Where appropriate, an unlabeled ds-DNA oligonucleotide competitor, at 200- to 500-fold molar excess, was added to each reaction mixture. The reaction mixtures were incubated for 10 min at room temperature (RT), after which $1 \times 10^4$ to $2 \times 10^4$ cpm (0.1 to 0.4 ng) of end-labeled DNA probe was added to each tube and the tubes were incubated for an additional 20 min at RT. One microliter of 1% bromophenol blue was added, and the resultant protein-DNA complexes were resolved by electrophoresis through 4% native polyacrylamide gels in 44.5 mM Tris base (pH 8.0)–44.5 mM boric acid–1 mM EDTA. Gels were prerun at 50 mA for 30 min, after which the samples were run at the same current for 1.5 to 2 h, dried on Whatman 3MM paper, and autoradiographed to Kodak XAR film.

**Methylation interference footprinting assay.** The method used for the methylation interference footprinting assay was as described elsewhere (1a). Briefly, a 72-bp ds oligonucleotide, mp −177 to −106, was radiolabeled with [γ-$^{32}$P]ATP on a single strand by using T4 polynucleotide kinase, annealed with the unlabeled complementary strand, and purified on a Sephadex G-25 column. Approximately $10^6$ cpm of probe was exposed to dimethyl sulfate in 50 mM sodium cacodylate–1 mM EDTA (pH 8.0) for 3 min at RT. The partially dimethyl sulfate-methylated probe was then used in a DNA mobility shift assay with nuclear extract from rat liver. Both the free probe and the protein-DNA complex bands were localized by autoradiography, excised from the gel, and recovered by electrophoresis onto DEAE paper. The DNA was purified and cleaved with 10% piperidine, dried under a vacuum, and resuspended in a formamide loading buffer. A $3 \times 10^3$ cpm amount of each sample was run on a 12% polyacrylamide sequencing gel. A G+A ladder was generated from the 72-bp sequence by the Maxam-Gilbert method (21) and run in parallel.

**Southwestern (protein-DNA) blotting.** Rat liver nuclear extract was partially purified on a heparin-agarose column, and the eluate was further purified by affinity binding to ds DNA (corresponding to mp −152 to −127 within the Cx32 promoter) attached to magnetic beads (Dynal). Samples from both purification steps were electrophoresed on a sodium dodecyl sulfate–8% polyacrylamide gel with the Laemmli buffer system (19). Proteins were transferred to a polyvinylidene difluoride membrane, and the blot was incubated for 2 h in a binding buffer containing 5% nonfat dry milk, 4% glycerol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM DTT and then incubated for 3 h at RT in 6 ml of binding buffer containing $7.5 \times 10^6$ cpm of a $^{32}$P-end-labeled ds oligonucleotide corresponding to mp −152 to −127 of the Cx32 promoter. The blot was washed four times for 30 min each time at RT in binding buffer and rinsed with a solution of 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA. The blot was air dried and exposed to Kodak XAR film for 3 days.

**PCR-directed mutagenesis and luciferase plasmid construction.** Plasmid pCx32-146-LUC was previously reported as plasmid pCx32-179/-33-LUC (2). The pCx32-146Δ-LUC plasmid was constructed as follows. The forward primer (5′-gcaaGCTTCCTCTTGGGCTGTGGGCCATGTCAAGCTCC**TT**CCTGG GC-3′), corresponding to mp −179 to −137, was synthesized with modified oligonucleotides at mp −144 and mp −145 (i.e., two guanines [G] were changed to two thymidines [T] [boldface]), and a *Hin*dIII restriction enzyme site (underlined) was introduced into the 5′ end. The reverse primer (5′-gcggtACCTGGT TGCAACTGCTTT-3′), corresponding to mp −34 to −53, was synthesized, and a *Kpn*I restriction enzyme site (underlined) was introduced into its 5′ end. PCR was then carried out with the above-described primers and plasmid pCx32-146-LUC, which contains the native rat Cx32 promoter fragment, as template DNA. The PCR-generated mutated DNA was digested with *Hin*dIII and *Kpn*I, purified, and cloned into the upstream region of the luciferase coding sequence in the promoterless vector p19LUC (Promega, Madison, Wis.). The composition of the new construct, pCx32-146Δ-LUC, was confirmed by sequence analysis.

**Cell culture and transient expression assay.** A highly differentiated human hepatoma cell line, HuH7, was maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, Md.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transient gene expression assays were performed by DNA transfection, by using a calcium phosphate precipitation technique as described elsewhere (2). Briefly, cells were seeded on 35-mm-diameter plates 24 h prior to transfection. The DNA mixture for transfection included 2 μg of the test plasmid DNA and 0.1 μg of pXGH5 DNA, a human growth hormone (hGH) expression vector which served as an internal control to monitor transfection efficiency (Nichols Diagnostic, Geneva, Switzerland). Each transfection experiment was performed in triplicate at least three times. Cell extracts were prepared 48 h after transfection by lysing cells in 25 mM glycylglycine–15 mM $MgSO_4$–4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–15 mM $KH_2PO_4$–1 mM DTT. Luciferase activity was determined as previously described (5). Light output was measured for 20 s at RT in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.). Each luciferase assay was performed in duplicate. Luciferase activity was expressed as relative light units per μl of cell extract per ml of hGH.

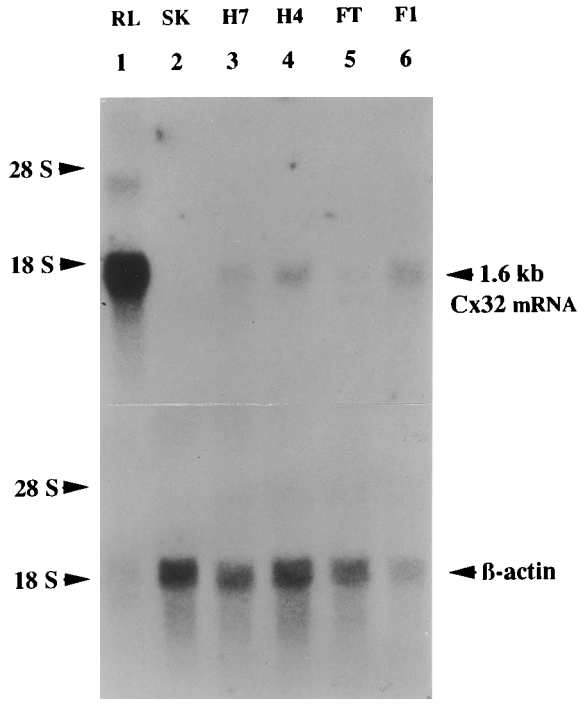


FIG. 1. Northern blot analysis of Cx32 transcripts from hepatoma cell lines and liver tissue. A 20-μg amount of total RNA from normal rat liver tissue (RL) and cell lines SkHep1 (SK), HuH7 (H7), H4AzC2 (H4), FTO-2B (FT), and FAO-1 (F1) was resolved on a 1.2% formaldehyde-agarose gel, transferred to a GeneScreen membrane (Dupont), and hybridized with a $^{32}$P-labeled rat Cx32 cDNA probe. The migrations of 28S and 18S rRNAs are shown on the left, and the Cx32 hybridizing band is indicated by an arrow on the right (upper panel). The blot was subsequently stripped and rehybridized with a $^{32}$P-labeled β-actin probe to verify lane loading (lower panel).

## RESULTS

**Cell- and tissue-specific expression of Cx32 mRNA.** The expression of Cx32 mRNA was determined by Northern blot analysis with total RNA isolated from a variety of human (SkHep1 and HuH7) and rat (H4AzC2, FTO-2B, and FAO-1) hepatoma cell lines and compared with expression of Cx32 RNA isolated from normal rat liver tissue. As shown in Fig. 1 (upper panel), an abundant 1.6-kb Cx32 transcript was present in the normal rat liver tissue (lane 1). In contrast, SkHep1 (Fig. 1, lane 2) had no detectable Cx32 message and HuH7, H4AzC2, FTO-2B, and FAO-1 (lanes 3, 4, 5, and 6, respectively) had dramatically reduced levels in comparison with those in rat liver. To validate the integrity of the mRNA, the blot was stripped and reprobed with a β-actin probe (Fig. 1, lower panel). The hepatoma cell lines all had abundant β-actin

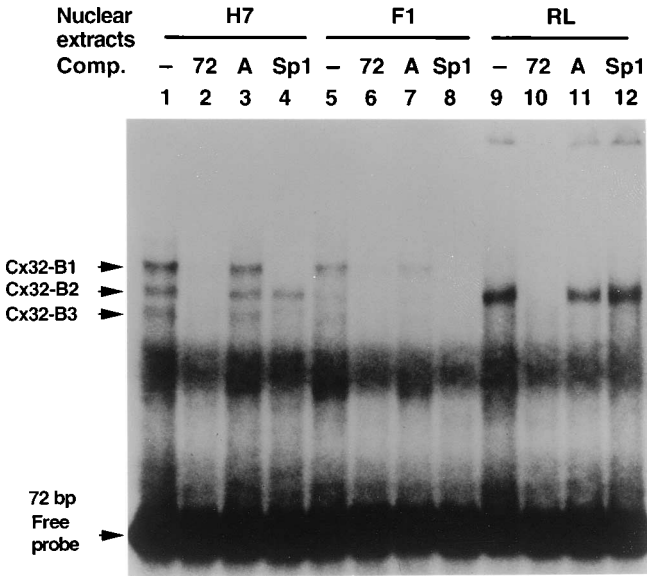


FIG. 2. DNA mobility shift assay of the rat Cx32 basal promoter region. Nuclear extracts from human hepatoma cell line HuH7 (H7), rat hepatoma cell line FAO-1 (F1), and rat liver tissue (RL) were prepared as described in Materials and Methods. A 2-μg amount of nuclear proteins was incubated with $1 \times 10^4$ to $2 \times 10^4$ cpm of the $^{32}$P-end-labeled 72-bp fragment (mp −177 to −106) corresponding to the basal promoter region (2). Protein-DNA complexes were resolved by electrophoresis through a 4% native polyacrylamide gel and autoradiographed to Kodak film. Specific DNA-protein complexes are indicated by arrows on the left. ds competitor DNAs (Comp.) are indicated as follows above the autoradiogram: −, no competitor (lanes 1, 5, and 9); 72, unlabeled 72-bp fragment (lanes 2, 6, and 10); A, DNA corresponding to mp −176 to −153 of the rat Cx32 basal promoter (lanes 3, 7, and 11); Sp1, oligonucleotide containing an Sp1 consensus binding site (lanes 4, 8, and 12). In lane 8, a band with the same migration as Cx32-B2 was more apparent upon longer exposure of the autoradiogram (data not shown).

transcripts, confirming the presence of intact mRNAs. The abundant level of Cx32 mRNA in liver tissue corresponds to the previously reported high rate of transcription detected by a nuclear run-on assay in liver tissue (18).

**Characterization of DNA-protein complexes associated with the basal promoter region of the Cx32 gene.** The sequences between mp −179 and −134 of the rat Cx32 gene were previously shown to be required for the basal promoter activity by transient transfection assays (2). To investigate the genetic basis of and identify the potential transcription factor(s) responsible for Cx32 gene expression, DNA-protein interaction assays were carried out with nuclear extracts from rat liver tissue and two hepatoma cell lines (previously shown to have low levels of Cx32 transcripts) by using a $^{32}$P-labeled 72-bp (mp −177 to −106) ds oligonucleotide probe containing the basal promoter region of the rat Cx32 gene. As shown in Fig. 2, three DNA-protein complexes, termed Cx32-B1, Cx32-B2, and Cx32-B3, were reproducibly identified when nuclear extracts from hepatoma cell lines HuH7 (lane 1) and FAO-1 (lane 5) were used. However, only one DNA-protein complex (Fig. 2, lane 9), which comigrated with the Cx32-B2 from tumor cells HuH7 and FAO-1, was identified with the nuclear extract obtained from rat liver tissue. Cx32-B2 activity was significantly more abundant in the liver tissue than in the tumor cell lines. After preincubation of the nuclear extracts with the unlabeled ds 72-bp competitor, the $^{32}$P-labeled 72-bp probe no longer formed DNA-protein complexes with nuclear extracts from HuH7 (Fig. 2, lane 2) or FAO-1 (lane 6) cell lines or rat liver tissue (lane 10).

The probe sequence used in this study contains four GGGCTG motifs which are clustered between mp −170 and −110 and are highly homologous to the consensus Sp1 binding site (GGGCGG) (for a review, see reference 17). Therefore, we sought to determine whether the three DNA-protein complexes associated with the basal promoter might contain Sp1. Nuclear extracts from HuH7 (Fig. 2, lane 4), FAO-1 (lane 8), and rat liver (lane 12) were preincubated with a 200-fold molar excess of cold ds oligonucleotide containing the Sp1 consensus binding site (5′-GATCGATCGGGGCGGGGCGATC-3′; Promega), and a gel shift experiment was performed with the 72-bp probe. Cx32-B1 and Cx32-B3 complexes were no longer detected, whereas the Cx32-B2 shift remained intact in HuH7 (Fig. 2, lane 4), FAO-1 (lane 8; can be seen on longer exposure [data not shown]), and rat liver tissue (lane 12) extracts. To test whether the GGGCGT motif may be responsible for the Sp1 binding in Cx32-B1 and Cx32-B3 complexes, a ds oligonucleotide fragment, fragment A (mp −177 to −153), containing one of the GGGCTG motifs was synthesized and used as a competitor. No change in the DNA-protein complexes was seen (Fig. 2, lanes 3, 7, and 11), indicating the GGGCTG motif was not responsible for the binding of Cx32-B1 and Cx32-B3. An additional experiment using the ds fragment A oligonucleotide indicated that it neither bound Sp1 nor competed with the labeled Sp1 oligonucleotide probe for Sp1 binding (data not shown). However, when another unlabeled DNA fragment (mp −135 to −106), which contains a CCCTCCCC sequence, was used as the competitor, the Cx32-B1 and Cx32-B3 complexes were eliminated, indicating that Sp1 binds in this region (data not shown).

**Identification of the Cx32-B2 binding motif.** The Cx32-B2 DNA-protein complex was singularly associated with the rat Cx32 basal promoter region by DNA mobility shift assay using nuclear protein from rat liver. This protein-DNA complex was found to be more abundant in the rat liver nuclear extract than in the hepatoma cell lines. In order to localize the nucleotide sequence responsible for the formation of the Cx32-B2 complex, nuclear extract from rat liver was footprinted with the 72-bp probe by using a methylation interference assay. As shown in Fig. 3, methylation of several guanine residues decreased the binding affinity of the liver nuclear protein for the probe (see arrows; compare lane B [bound] with lane F [free]). On the upper strand, methylation of guanines at positions −145, −144, −139, and −134 and on the lower strand, methylation of guanines at positions −147, −142, −141, −136, and −133 reduced protein-binding activity. Thus, Cx32-B2 associated with the sequence between positions −152 and −127 (called the E fragment).

**Cx32-B2 complexes identified in rat liver tissue and hepatoma cells have similar sequence requirements.** Although the major protein-DNA complex identified in nuclear extracts from rat liver tissue migrated with the same mobility as did the Cx32-B2 complex found with nuclear extracts from the human hepatoma cell lines FAO-1 and HuH7 (Fig. 2), we sought to determine whether these Cx32-B2 activities were identical. To examine this possibility, Cx32-B2 complexes present in both nuclear extracts from rat liver and the human hepatoma cells were subjected to competition with the unlabeled E fragment by using the $^{32}$P-labeled 72-bp ds-DNA probe (Fig. 4). Cx32-B2 binding was eliminated in the presence of cold E fragment competition with both extracts. This result demonstrated that Cx32-B2 complexes formed with nuclear proteins from rat liver and human hepatoma cells not only have similar migration patterns but also have the same or similar sequence specificities and supports the notion that they contain the same DNA-binding protein.

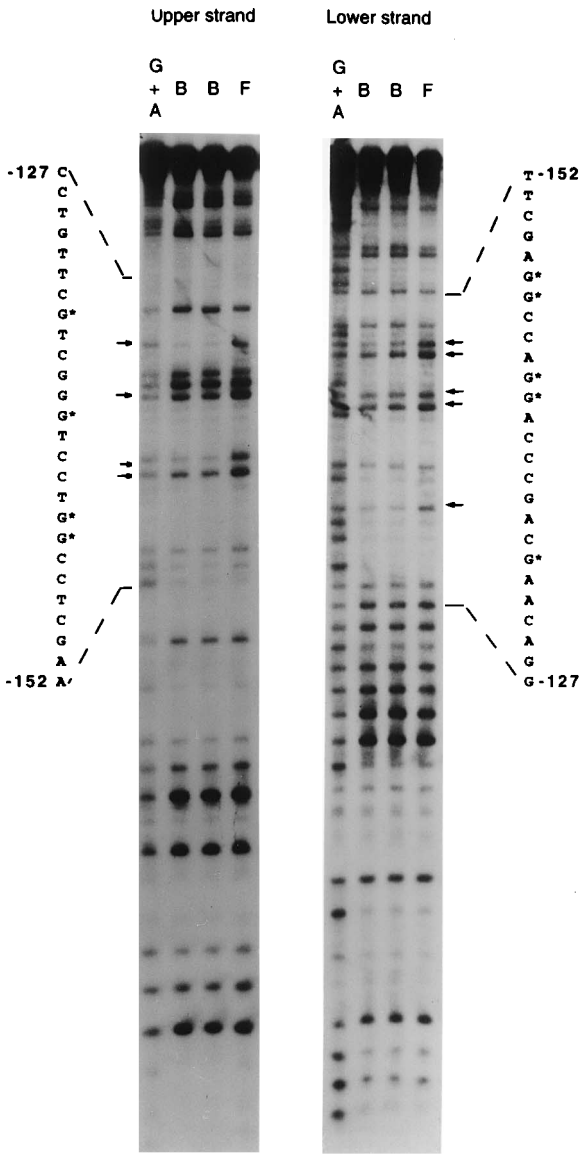


FIG. 3. Methylation interference footprinting. The 72-bp DNA fragment (mp −177 to −106) was end labeled with [γ-$^{32}$P]ATP, and approximately $10^6$ cpm was exposed to dimethyl sulfate prior to incubation with rat liver nuclear extract. Free probe (F) and the protein-DNA binding complex (B) were separated in a DNA mobility shift assay as described in the legend to Fig. 2. The free (F) and bound (B) complexes were isolated and cleaved with piperidine, and the cleavage products were resolved on a 12% polyacrylamide sequencing gel. The region affected by methylation interference (mp −152 to −127) corresponds to the nucleotide sequences shown to the left and right of the gel. The guanine nucleotide sequences affected by methylation interference are indicated by small arrows to the left and right of the gel and by asterisks in the corresponding sequences. The lanes labeled G+A represent Maxam-Gilbert sequencing ladders, indicating the locations of guanine and adenine residues.

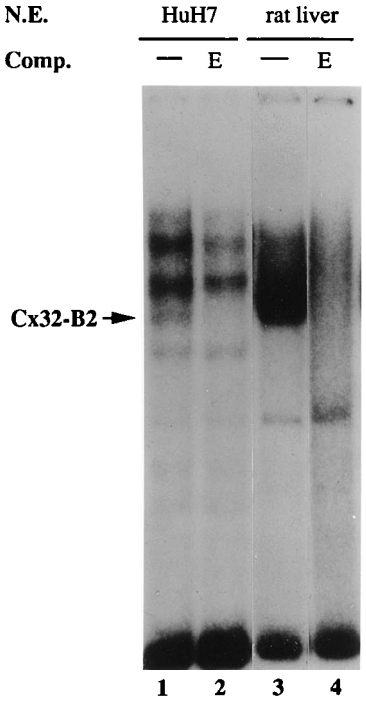


FIG. 4. DNA mobility shift assay comparing rat liver and human hepatoma cell nuclear extracts. Nuclear proteins from rat liver and HuH7 cells were incubated with the $^{32}$P-labeled 72-bp DNA (mp −177 to −106) probe in the absence (−) (lanes 1 and 3) or presence (lanes 2 and 4) of the unlabeled Cx32-B2 sequence (E, mp −152 to −127). The reaction mixtures were resolved on a 4% native polyacrylamide gel. The migration of the Cx32-B2 complex is indicated on the left. N.E., nuclear extract; Comp., competitor DNA.

**Cx32-B2 complex is enriched in rat liver tissue.** To further determine the tissue specificity of the Cx32-B2 complex, nuclear extracts from various rat tissues (liver, heart, brain, lung, and testis) were prepared as described in Materials and Methods and subjected to a DNA mobility shift assay. The overall DNA-binding activity of each extract was verified by its ability to bind to an AP1 consensus oligonucleotide probe (Fig. 5, lower panel). Each extract was then tested for its ability to form the Cx32-B2 complex by mobility shift assay with an E fragment probe. As shown in Fig. 5, upper panel, the Cx32-B2 complex is enriched in nuclear extracts from rat liver tissue. Both the Cx32-B2 and AP1 mobility shift assays were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and the ratio of Cx32-B2 binding to AP1 binding was calculated as a more accurate measure of Cx32-B2 binding activity. The ability to form the Cx32-B2 complex with nuclear extract from liver was approximately 3-fold greater than that with extract from testis, 5-fold more than that with extract from brain, and over 500-fold greater than that with extract from heart or lung tissue. Similar results were obtained when NF1 replaced AP1 as the control for binding activity (data not shown).

**Cx32-B2 protein characterization.** To identify the molecular weight of the proteins responsible for the formation of the Cx32-B2 complex, a Southwestern blot was performed with extracts that were enriched for proteins capable of complexing with the Cx32-B2 binding site (E fragment). Rat liver nuclear extract was subjected to heparin-agarose column chromatography to enrich for DNA-binding proteins. A portion of the eluate from this column was further purified by affinity binding to three tandem repeats of the E fragment linked to magnetic beads. A DNA mobility shift assay with a $^{32}$P-labeled E probe confirmed that the proteins involved in the Cx32-B2 complex were present in both the heparin-agarose-purified and affinity-purified extracts (data not shown). A Southwestern blot with samples from both purification steps identified a protein with an apparent molecular mass of approximately 60 kDa which was capable of binding to the E fragment probe (Fig. 6).

To characterize the heat stability of the Cx32-B2 activity, rat liver nuclear extracts were heated at 30 and 50°C prior to DNA

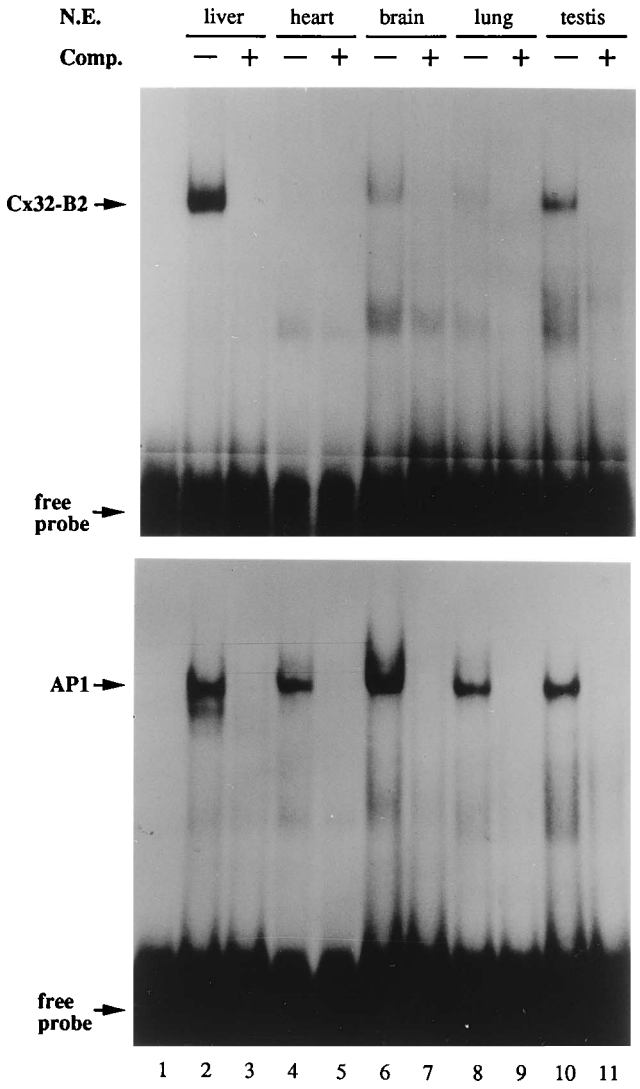


FIG. 5. DNA mobility shift assay comparing nuclear extracts from rat liver and various other tissues. Nuclear proteins were prepared as described in Materials and Methods. (Upper panel) Nuclear proteins from rat liver (lanes 2 and 3), heart (lanes 4 and 5), brain (lanes 6 and 7), lung (lanes 8 and 9), and testis (lanes 10 and 11) tissues were incubated with the $^{32}$P-labeled Cx32-B2 binding site (E fragment) in the absence (−, lanes 2, 4, 6, 8, and 10) or presence (+, lanes 3, 5, 7, 9, and 11) of unlabeled E fragment. Resulting complexes were analyzed on a 4% native polyacrylamide gel. Lane 1 contains unreacted probe. (Lower panel) The experiment shown in the upper panel was repeated to verify the activity of the nuclear extracts. Extracts were incubated with a $^{32}$P-labeled AP1 consensus binding site oligonucleotide as probe in the absence (−, lanes 2, 4, 6, 8, and 10) or presence (+, lanes 3, 5, 7, 9, and 11) of unlabeled AP1 oligonucleotide. The migrations of the Cx32-B2 and AP1 complexes and free probe are indicated on the left. N.E., nuclear extract; Comp., competitor DNA.

binding. Incubation of extracts at 50°C for 10 min abolished the formation of the Cx32-B2 complex, whereas a smaller decrease in complex formation was seen at 30°C, demonstrating that this binding activity was heat labile (data not shown).

**Mutation analysis of the Cx32-B2 binding site.** To further investigate the nucleotides required for the formation of the Cx32-B2 complex, a set of ds oligonucleotide probes containing the native binding site, mp −152 to −127 (E fragment), and various mutations at sites previously shown to reduce binding when methylated (refer to Fig. 3) were synthesized and used in gel shift analyses with rat liver nuclear extract (Fig. 7A). As shown in Fig. 7, the $^{32}$P-labeled probes EΔ1 and EΔ2

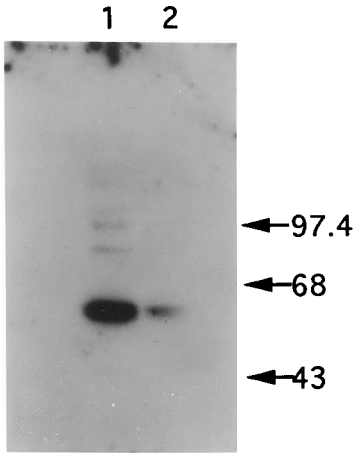


FIG. 6. Southwestern blot of the Cx32-B2 binding protein in rat liver nuclear extract. Rat liver nuclear extract was purified on a heparin-agarose column (lane 1), and the eluate was further purified by affinity binding to the E fragment (lane 2). A 10-μl volume of each sample was run in each lane, and a $^{32}$P-labeled E fragment probe was used to detect DNA-binding proteins. The positions of the molecular mass markers (in kilodaltons) are shown to the right.

containing single nucleotide mutations, G to T at mp −145 and C to A at mp −142, respectively, did not change Cx32-B2 complex formation (compare lanes 1, 2, and 3 in panel B). However, EΔ3, containing a GG-to-TT mutation at mp −144 and −145, completely abrogated binding (Fig. 7B, lane 4). Furthermore, unlabeled EΔ3 was unable to inhibit Cx32-B2 binding to the E probe even at a 1:500 molar excess (Fig. 7B, lanes 6 to 8), indicating that nucleotides at positions −144 and −145 were essential for the formation of the Cx32-B2 complex.

**Cx32-B2 binding is required for the efficient expression of rat Cx32 promoter.** To study the functional contribution of the Cx32-B2 binding on the basal promoter activity, the mutation previously shown to abolish DNA-protein interaction, EΔ3, was introduced into the basal promoter fragment (mp −179 to −34) by PCR. The mutated promoter fragment was cloned into a luciferase expression vector and analyzed by a transient expression assay with HuH7 cells. As shown in Fig. 8, the luciferase plasmid pCx32-146Δ-LUC, containing the mutated rat Cx32-B2 site, directed significantly decreased amounts of luciferase activity in comparison with those directed by the native promoter, pCx32-146-LUC (2). This result indicates that binding of the Cx32-B2 complex within the basal promoter fragment was required for the efficient expression of the Cx32 gene from the basal promoter.

## DISCUSSION

This report describes the characterization of protein-DNA interactions in the basal promoter of the rat Cx32 gene. Three protein-DNA complexes, Cx32-B1, Cx32-B2, and Cx32-B3, were formed with a basal promoter DNA fragment by using nuclear extracts from a human hepatoma cell line, HuH7, and a rat hepatoma cell line, FAO-1. However, only one DNA-protein complex, Cx32-B2, was formed when the same promoter fragment was used with nuclear extract from normal rat liver. The Cx32-B2 complex was shown to contain a 60-kDa heat-labile protein which bound to a region between mp −152 and −127. Two nucleotides at mp −144 and −145 were critical for the formation of the complex. Moreover, using a transient expression system, we demonstrated that a promoter fragment unable to form a Cx32-B2 complex had a dramatically decreased amount of activity in comparison with the native promoter. These data argue that the Cx32-B2 binding site and

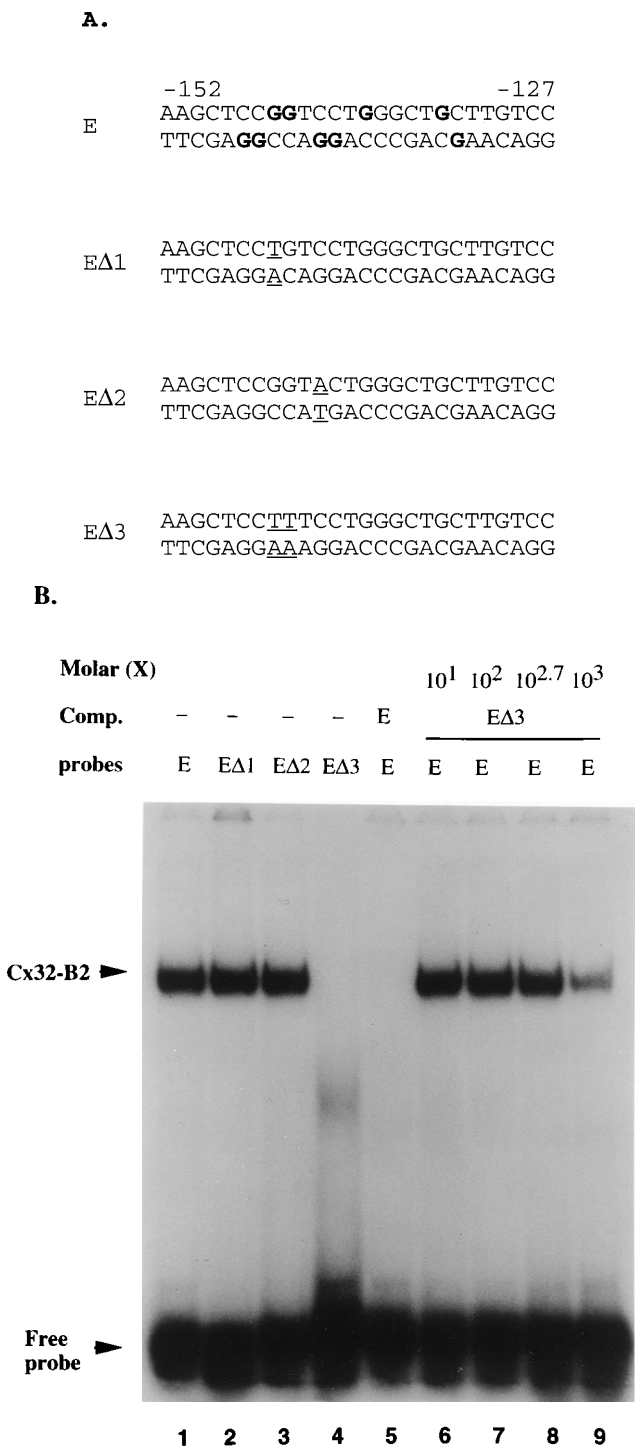


FIG. 7. Mutational analysis of the Cx32-B2 binding site. (A) Sequence content of a set of ds oligonucleotides used in DNA mobility shift experiments. The sequence of the E oligonucleotide corresponds to the region identified in Fig. 3. The residues affected by methylation interference are indicated by bold letters in the nucleotide sequence. Three 26-bp ds oligonucleotides containing either one (EΔ1 and EΔ2) or two (EΔ3) nucleotide mutations are shown with the mutation position(s) underlined. (B) Results of a gel mobility shift assay. Lanes 1 to 4 show the binding activity of rat liver nuclear protein to each of the $^{32}$P-labeled ds probes shown in panel A. Lane 5 indicates the self competition of E fragment. Lanes 6 to 9 show the Cx32-B2 binding activity with the E probe in the presence of increasing amounts of unlabeled competitor EΔ3. The migrations of the Cx32-B2 protein-DNA complex and free probe are indicated on the left. –, no competitor DNA; Comp., competitor DNA.

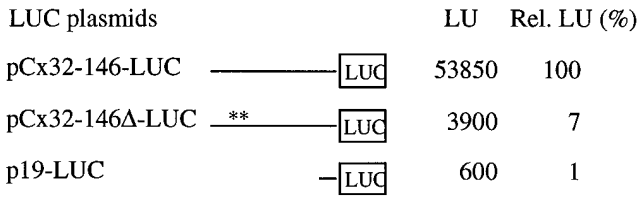


FIG. 8. Transient transfection analysis of mutations in the Cx32-B2 motif. The transcriptional activity of the mutated and the native basal promoter of Cx32 was analyzed by quantitating luciferase activity after transfection into HuH7 cells. pCx32-146-LUC contains the native promoter (mp −179 to mp −34). pCx32-146Δ-LUC contains the 2-bp mutation (GG to TT, mp −145 and −144) which was previously shown to eliminate Cx32-B2 binding (Fig. 7). Luciferase activities are expressed as light units (LU) and percent relative light units (Rel. LU) in relationship to the native promoter construct (pCx32-146-LUC).

formation of the Cx32-B2 complex are an essential component of the basal promoter of the rat Cx32 gene.

Numerous studies have shown that Cx32 expression can be diminished at both the mRNA and protein levels during tumorigenesis (3, 13, 23, 24). Our Northern blot results are consistent with the results of these studies. The levels of Cx32 mRNA were shown to be highest in normal rat liver tissue and considerably reduced in the less differentiated human and rat hepatoma cell lines. Decrease in Cx32 mRNA levels during dedifferentiation has been suggested to be controlled at both transcriptional and posttranscriptional levels (27). The rate of transcription measured by a nuclear run-on assay has been shown to be considerably higher for Cx32 than for Cx26 (18), indicating that the mRNA levels are determined, at least in part, by the transcription rate.

Cx32-B1 and Cx32-B3 appear to be related to the transcription factor Sp1, since unlabeled ds oligonucleotides containing the consensus sequence for Sp1 prevented these protein-DNA complexes from forming in the promoter region. The unlabeled DNA fragment (mp −135 to −106), which contained a CCCTCCCC sequence, also eliminated by competition the Cx32-B1 and Cx32-B3 complexes, indicating that Sp1 or an Sp1-related factor binds in this region (data not shown). Sp1 is a DNA-binding protein required for the efficient expression of a wide group of genes (17). In addition, Sp1 has the ability to direct transcription from promoters lacking the TATA motif (26) by interacting via its activation domain with a component of the TFIID complex, TAF110 (16). It is not clear whether Sp1 functions analogously with the TATA-less Cx32 promoter, since only a single, nonconsensus Sp1 motif is present. We (29a) and others (1) have observed that Sp1 from rat liver nuclear extracts displays a decreased ability to bind to its cognate DNA site. This may explain the finding that the Cx32-B1 and Cx32-B3 complexes, which are formed when extracts from hepatoma cell lines are used, are absent when extracts from rat liver tissue are used.

The Cx32-B2 complex associated with the rat Cx32 promoter was highly enriched in nuclear extracts from liver tissue in comparison with the amount in cell lines and other tissues. Numerous liver-specific transcription factors have been identified and characterized, and these include the C/EBP gene family, HNF-1a and HNF-1b, the HNF-3 gene family, and HNF-4 (for a review, see reference 30). Preincubation of the Cx32 promoter and liver nuclear proteins in the presence of ds oligonucleotides corresponding to the consensus sequences for the liver-specific factors mentioned above did not affect the binding activity of Cx32-B2, nor did preincubation with the A, B, C, D, E, and F elements in the albumin gene promoter (20) (data not shown). Therefore, we presume that Cx32-B2 is a less

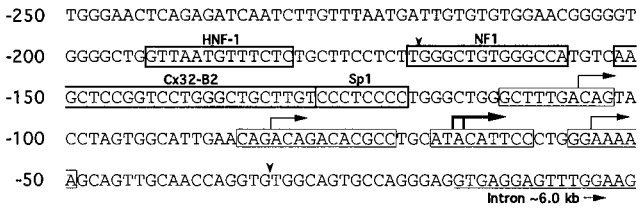


FIG. 9. Basal promoter of the rat Cx32 gene. The DNA sequence of the rat Cx32 gene first exon and upstream region is shown (22). The basal promoter, localized by previous deletion analyses (2), is demarcated by carats (▼). The binding sites for HNF-1, NF1, Cx32-B2, and Sp1 are shown in bold boxes; DNA sequences with homology to initiator elements (34a) are shown in plain boxes; and transcription start sites are indicated by arrows. The 5′ region of the intron is underlined.

well-characterized, hepatic tissue-enriched transcription factor involved in regulating the expression of the Cx32 gene and, potentially, other liver-specific genes.

The sequence of the rat Cx32 basal promoter region and the binding sites of the factors characterized in this paper are shown in Fig. 9. In addition, a nuclear factor 1 (NF1) consensus site present between mp −171 and −158 and an HNF-1 site present between mp −193 and −181 are shown. Transcription start site domains mapped with RNA isolated from rat liver tissue which contain sequences with homology to consensus initiator (Inr) elements (for a review, see reference 34a) are also shown (Fig. 9). However, the potential role that these Inr elements play in the regulation and control of cell- and tissue-specific expression of the Cx32 gene remains to be investigated. Since the formation of the Cx32-B2 complex was shown to be necessary for activity of the basal promoter, the mechanism by which the factors involved in this complex regulate Cx32 transcription is currently under investigation.

## ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants DK-41918 and DK-41296.

We thank Allan Wolkoff and David C. Spray for continued support and encouragement and R. Werner, University of Miami, for his generous donation of the rat Cx32 genomic clone. R. Burk was the recipient of a faculty award from the American Cancer Society.

DNA oligonucleotides were synthesized in the oligonucleotide synthesis facility of the Cancer Center at the Albert Einstein College of Medicine (5P30CA13330) and in the Molecular Biology Core of the Liver Research Center.

## REFERENCES

1. **Ammendola, R., M. Mesuraca, T. Russo, and F. Cimino.** 1992. Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues. J. Biol. Chem. **267:**17944–17948.

1a. **Ausubel, F., R. Brent, R. F. Kingston, D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.

2. **Bai, S., D. C. Spray, and R. D. Burk.** 1993. Identification of proximal and distal regulatory elements of the rat connexin32 gene. Biochim. Biophys. Acta **1216:**197–204.

3. **Beer, D. G., M. J. Neveu, D. Paul, U. R. Rapp, and H. C. Pitot.** 1988. Expression of the c-*raf* protooncogene, r-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. Cancer Res. **48:**1610–1617.

4. **Bergoffen, J., S. S. Scherer, S. Wang, M. O. Scott, L. J. Bone, D. L. Paul, K. Chen, M. W. Lensch, P. F. Chance, and K. H. Fischbeck.** 1993. Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science **262:**2039–2042.

5. **Brasier, A. R., J. E. Tate, and J. F. Habener.** 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques **7:**1116–1122.

6. **Buratowski, S.** 1994. The basics of basal transcription by RNA polymerase II. Cell **77:**1–3.

7. **Dermietzel, R., T. K. Hwang, and D. C. Spray.** 1990. The gap junction family: structure, function and chemistry. Anat. Embryol. **182:**517–528.

8. **Dermietzel, R., and D. C. Spray.** 1993. Gap junctions in the brain: where, what type, how many and why? Trends Neurosci. **16:**186–192.

9. **Dermietzel, R., O. Traub, T. K. Hwang, E. Beyer, M. V. L. Bennett, D. C. Spray, and K. Willecke.** 1989. Differential expression of three gap-junction proteins in developing and mature brain tissues. Proc. Natl. Acad. Sci. USA **86:**10148–10152.

10. **Deryckere, F., and F. Gannon.** 1994. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. BioTechniques **16:**405.

11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.

12. **Fishman, G. I., R. Eddy, T. Shows, L. Rosenthal, and L. A. Leinwand.** 1991. The human connexin gene family of gap junction proteins: distinct chromosomal location but similar structure. Genomics **10:**250–256.

13. **Fitzgerald, D. J., M. Mesnil, M. Oyamada, H. Tsuda, N. Ito, and H. Yamasaki.** 1989. Changes in gap-junction protein (connexin32) gene expression during rat liver carcinogenesis. J. Cell. Biochem. **41:**97–102.

14. **Gimlich, R. L., N. M. Kumar, and N. B. Gilula.** 1990. Differential regulation of the levels of three gap junction mRNAs in Xenopus embryos. J. Cell Biol. **110:**597–605.

15. **Hattori, M., A. Tugores, L. Veloz, M. Karin, and D. A. Brenner.** 1990. Laboratory methods: a simplified method for the preparation of transcriptionally active liver nuclear extracts. DNA Cell Biol. **9:**777–781.

16. **Hoey, T., R. O. J. Weinzierl, G. Gill, J.-L. Chen, B. D. Dynacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72:**247–260.

17. **Kadonaga, J. T., K. A. Jones, and R. Tjian.** 1986. Promoter-specific activation of RNA polymerase II transcription by Sp1. Trend Biochem. Sci. **11:**20–23.

18. **Kren, B. T., N. M. Kumar, S. H. Wang, N. B. Gilula, and C. J. Steer.** 1993. Differential regulation of multiple gap junction transcripts and protein during rat liver regeneration. J. Cell Biol. **123:**707–718.

19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

20. **Lichtsteiner, S., J. Wuarin, and U. Schibler.** 1987. The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. Cell **51:**963–973.

21. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.

22. **Miller, T., G. Dahl, and R. Werner.** 1988. Structure of a gap-junction gene: connexin32. Biosci. Rep. **8:**455–464.

23. **Neveu, M. J., J. R. Hully, K. L. Babcock, E. L. Hertzberg, B. J. Nicholson, D. L. Paul, and H. C. Pitot.** 1994. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. J. Cell Sci. **107:**83–95.

24. **Neveu, M. J., J. R. Hully, D. L. Paul, and H. C. Pitot.** 1990. Reversible alteration in the expression of the gap-junctional protein connexin 32 during tumor promotion in the rat liver and its role during cell proliferation. Cancer Commun. **2:**21–31.

25. **Paul, D. L.** 1986. Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. **103:**123–134.

26. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell **61:**1187–1197.

27. **Rosenberg, E., D. C. Spray, and L. M. Reid.** 1992. Transcriptional and posttranscriptional control of connexin mRNAs in periportal and pericentral rat hepatocytes. Eur. J. Cell Biol. **59:**21–26.

28. **Saez, J. C., W. A. Gregory, T. Watanabe, R. Dermietzel, E. L. Hertzberg, L. Reid, M. V. L. Bennett, and D. C. Spray.** 1989. cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. Am. J. Physiol. **257:**C1–C11.

29. **Sakamoto, H., M. Oyamada, K. Enomoto, and M. Mori.** 1992. Differential changes in expression of gap junction proteins connexin 26 and 32 during hepatocarcinogenesis in rats. Jpn. J. Cancer Res. **83:**1210–1215.

29a. **Schoenfeld, A.** Unpublished data.

30. **Simone, V. D., and R. Cortese.** 1992. Transcription factors and liver-specific genes. Biochim. Biophys. Acta **1132:**119–126.

31. **Spray, D. C., M. Chanson, A. P. Moreno, R. Dermietzel, and P. Meda.** 1991. Distinctive gap junction channel types connect WB cells, a clonal cell line derived from rat liver. Am. J. Physiol. **260:**C513–C527.

32. **Spray, D. C., M. Fujita, J. C. Saez, H. Choi, T. Watanabe, E. Hertzberg, L. C. Rosenberg, and L. M. Reid.** 1987. Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. J. Cell Biol. **108:**541–551.

33. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–8.

34. **Traub, O., J. Look, D. Paul, and K. Willecke.** 1987. Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. Eur. J. Cell Biol. **43:**48–54.

34a. **Weis, L., and D. Reinberg.** 1992. Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. FASEB J. **6:**3300–3309.

35. **Zhang, J. T., and B. Nicholson.** 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J. Cell Biol. **109:**3391–3401.