Mitogenic Up-regulation of the PRL-1 Protein-tyrosine Phosphatase Gene by Egr-1

Egr-1 ACTIVATION IS AN EARLY EVENT IN LIVER REGENERATION*

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The cellular signals that initiate cell growth are incompletely understood. Insight could be provided by understanding the signals regulating the transcriptional induction of immediate-early genes which occurs within minutes of the growth stimulus. The expression of the PRL-1 gene, which encodes a unique nuclear protein-tyrosine phosphatase, is rapidly induced in regenerating liver and mitogen-treated cells. Transcription of the PRL-1 gene increased in the rat liver remnant within a few minutes after partial hepatectomy and largely explained the increase in steady-state PRL-1 mRNA in the first few hours posthepatectomy. Egr-1 (early growth response factor) specifically bound a region of the proximal PRL-1 promoter P1 (−99). Egr-1 binding activity was more rapidly induced in regenerating liver than mitogen-treated H35 and NIH 3T3 cells, remained elevated through 4 h posthepatectomy, and appeared to be dependent not only on new Egr-1 protein synthesis but on post-translational regulation of Egr-1. Egr-1 efficiently transactivated a PRL-1 promoter reporter construct containing an intact but non-mutant Egr-1 site, and the Egr-1 site largely accounted for PRL-1 gene up-regulation in response to mitogen stimulation. These data predict that Egr-1 activation is an early event in liver regeneration and mitogen-activated cells that provides a regulatory stimulus for a subset of immediate-early genes.

The phosphatase of regenerating liver-1 (PRL-1)
 encodes an evolutionarily conserved nuclear protein-tyrosine phosphatase, and its expression is highly induced in liver regeneration and mitogen-stimulated fibroblasts (1–5). Overexpression of PRL-1 in transfected NIH 3T3 cells modifies cell growth (3). PRL-1 may play an important role in cell growth via regulation of protein tyrosine phosphorylation and dephosphorylation of specific substrates that remain unknown (4). Expression of the PRL-1 gene is positively associated not only with cellular growth during liver regeneration, development, and oncogenesis but also with differentiation in intestine and other tissues (5).

Following a partial hepatectomy or toxic liver injury, remnant liver cells that are normally quiescent rapidly reenter the cell cycle within minutes followed by DNA replication and restoration of liver mass within a few days. The factors regulating this process are incompletely understood, but a number of growth factors and cellular signals have been implicated (1). We showed that interleukin-6 (IL-6) which is increased in the liver posthepatectomy is required for normal liver regeneration (2). We found that IL-6 specifically regulates the expression of a subset of genes that are transcriptionally activated in the regenerating liver, but other genes such as PRL-1 are induced normally posthepatectomy even in the absence of IL-6. We reasoned that examination of the regulation of PRL-1 gene expression during liver regeneration would help us better understand the IL-6 independent signals that are critical for liver regeneration.

Functional promoter analysis demonstrated the presence of two promoters, P1 and P2, in the human PRL-1 gene, P1 directed by a TATA box upstream of the non-coding first exon (6). A non-canonical internal promoter, P2, was found in the first intron that results in a PRL-1 transcript beginning 8 bp downstream of the 5′ end of exon 2 and causes no alteration in the encoded protein. The first 200-bp region upstream of promoter P1 and P2 confers high basal transcriptional activity. An enhancer that binds a developmentally regulated factor was localized to the first intron of the human PRL-1 gene.

Promoter P1 of the PRL-1 gene contained two potential Egr-1 DNA-binding elements. Early growth response gene 1 (Egr-1, also named NGFI-A, krox-24, zif268, Cef5, and Tis8) is an immediate-early gene and encodes a transcription factor with three zinc fingers recognizing a GC-rich sequence, 5′-CGC-C CCCGC-3′, which has been identified in the promoter regions of a number of genes (7–12). Egr-1 expression is rapidly and transiently induced during the transition of cells from the G0 to G1 phase in response to various mitogens such as growth factors, cytokines, injury, and partial hepatectomy (7, 9, 13–19).

In this study, we demonstrated that the expression of PRL-1 gene was dramatically induced at a transcriptional level in the remnant liver following partial hepatectomy. Egr-1 protein expression and Egr-1 binding activity increased in concert with PRL-1 gene transcription in regenerating liver. Egr-1 transactivated the PRL-1 promoter in the presence of an intact but non-mutant Egr-1 site, and the Egr-1 site at −99 was responsible for mitogen stimulation of PRL-1 promoter activity.

MATERIALS AND METHODS

Rat Tissue Preparation—For regenerating liver, female Fisher rats (160–220 g, Charles River) were anesthetized with metofane (Pitman-Moore, Inc.) and subjected to midventral laparotomy with approximately 70% liver (left lateral and median lobes) (20). For cycloheximidem
treated samples, rats were pretreated 15 min prior to the laparotomy with 50 mg of cycloheximide per kg of body weight (5% solution in phosphate-buffered saline) intraperitoneally. Sham surgeries were performed by subjecting rats to midventral laparotomy and closure. Animals were allowed to recover for the times indicated in the figure legend. Isolation of the nucleus and nuclear protein extracts.

RNA and RNA Analysis—Regenerating liver RNA was prepared exactly as reported (21). To make the RNA probe for the RNase protection assay, EcoRI-linearized Bluescript KS plasmid template containing the EcoRI/PstI fragment of rat PRL-1 cDNA was transcribed in vitro with T7 RNA polymerase in the presence of [α-32P]UTP using an in vitro transcription kit (Stratagene, La Jolla, CA). The probe used contained 32 bp of exon 1 and 180 bp of exon 2 (see Fig. 1). 50–70 μg of total regenerating liver RNA was used for the assay. After overnight hybridization of 0.5–1 × 10^6 cpm of urea-denatured 5% polyacrylamide gel-purified probe to the RNA, the hybrids were digested according to the described protocol (22). The protected fragments were then analyzed on 6% polyacrylamide gel containing 8 M urea.

Nuclear Run-on Assays—Nuclei preparation and nuclear run-on assays were carried out by following the previously described procedure (21, 23). Frozen nuclei were quickly thawed and pelleted, resuspended in 100 μl of nucleic acid binding buffer (50 mM Tris HCl, pH 7.9, 5 mM MnCl2, 50 mM NaCl, 0.35 M (NH4)2SO4, 2 mM MgCl2, 50 mM EDTA) plus 0.5% Triton X-100, 100 μg/ml heparin, 100 μg/ml heparinase II (specific activity, 600 U/mg, NEN Life Sciences, Boston, MA), 1 μM of each ATP, GTP, TTP, and CTP. The resulting solution was allowed to proceed at 30 °C for 30 min with shaking. The reaction was stopped by the addition of 400 μl of a solution containing 10 mM Tris-HCl, pH 7.4, 0.5 μl of NaCl, 50 mM MgCl2, 2 mM CaCl2, and 50 μg/ml RNase-free DNase I was incubated at 30 °C for 10 min with shaking. Following proteinase K digestion and phenol/chloroform extraction, the in vitro synthesized RNA was precipitated with ethanol-sodium acetate and redissolved in H2O. The same amount of the radio-labeled RNA (approximately 1–5 × 10^5 cpm) was hybridized to nitrocellulose membrane applied with 2 μg of linearized insert-bearing plasmids using a slot blot apparatus in the exactly same way as reported (22). We used ATP synthase and β2-microglobulin as positive controls and Bluescript SK+ as negative control.

Cell Culture and DNA Transfections—Human hepatoma cell line HepG2 cells, mouse embryonic carcinoma cell line F9 cells, and mouse fibroblast NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM l-glutamine, 100 units of penicillin, and 50 units of streptomycin (Flow Laboratories) as described previously (6, 23). Rat hepatic cell line H35 cells, a rat hepatoma cell line derived from newborn rats, were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units of penicillin, and 50 units of streptomycin. To produce quiescent H35 and NIH 3T3 cells, the medium was changed to 0.5% serum/DMEM for 72 h. Following serum deprivation, NIH 3T3 and H35 cells were treated with 20% serum and insulin (10 μg/ml), resuspended at divided time points, and assayed for protein synthesis.

Preparation of Nuclear Protein Extracts—Nuclei were isolated by subjecting rats to midventral laparotomy and closure. Animals were allowed to recover for the times indicated in the figure legend. Isolation of the nucleus and nuclear protein extracts.

Preparation of Nuclear Protein Extracts—All steps were performed at 4 °C, and 2 μg/ml protease inhibitors antipain, aprotinin, bestatin, and leupeptin were added into all buffers except phosphate-buffered saline, pH 7.4. Nuclei from regenerating liver and from cell lines 3T3-C2 (3T3-C2) and NIH 3T3 were prepared by using the described procedure (6, 24, 25). Nuclei were lysed by the directse volume of 4.2 μl NaCl and shaken for 30 min before being centrifuged at 50,000 × g for 50 min. The supernatant was dialyzed against Buffer D (20 mM Hepes, pH 7.6, 0.2 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol) for at least 6 h. Finally, the extract was centrifuged at 12,000 × g for 10 min, and aliquots were quick-frozen in dry ice, 95% ethanol mixture and stored at −70 °C. Protein concentration was determined using Bio-Rad protein assay reagent.

Electrophoresis Mobility Shift Assays (EMSA)—End labeling of oligonucleotides was carried out with T4 polynucleotide kinase and [γ-32P]ATP and purified by Sephadex G-25 spin column. Binding reactions were performed essentially as reported (6, 24). Nuclear protein extracts (2 μg) were incubated with 2 μg of a 32P-labeled probe containing 0.5 μl of binding buffer containing 20 mM Hepes, 7.9 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol for at least 6 h. Finally, the extract was centrifuged at 12,000 × g for 10 min, and aliquots were quick-frozen in dry ice, 95% ethanol mixture and stored at −70 °C. Protein concentration was determined using Bio-Rad protein assay reagent.

RESULTS

Transcriptional Regulation of the PRL-1 Gene during Liver Regeneration—PRL-1 is expressed as an immediate-early response gene in regenerating liver and mitogen-stimulated 3T3 fibroblasts. To assess the relative induction of the two rat PRL-1 gene promoters in liver regeneration, an RNase protection assay was performed. Like the human gene in which two promoters were identified (6), two protected fragments of ap-
aproximately 212 and 172 bp (Fig. 1A), which respectively represented the products transcribed from PRL-1 promoter (P1) and internal promoter (P2) located in the first intron, were increased during liver regeneration. Both PRL-1 transcripts were rapidly induced, reached a peak by 4 h, with 17.9- and 6.3-fold induction for P1 and P2, respectively, and remained elevated through 8 h posthepatectomy.

Because the regulation of gene expression can be both transcriptional and post-transcriptional, we tested whether the induction of PRL-1 gene expression was transcriptionally regulated. Nuclear run-on assays indicated that PRL-1 gene transcription, which was virtually absent in normal liver, increased by 14.8-fold at 30 min posthepatectomy and remained elevated through 8 h posthepatectomy (Fig. 1B). As a control, there was no induction of the constitutively expressed genes for ATP synthase and β2-microglobulin and no signal detected for vector Bluescript SK. This indicated that PRL-1 was transcriptionally up-regulated during liver regeneration and that an increase in transcription could largely explain the induced expression of PRL-1 mRNA following partial hepatectomy.

**Nuclear Egr-1 Activity during Liver Regeneration**—Because of its larger transcriptional induction, we focused on determining the regions upstream of promoter P1 responsible for transcriptional activation posthepatectomy. Transient transfection experiments had revealed that virtually all of the basal trans-acting elements are located within the first 200 bp upstream of the human P1 promoter in both human hepatoma HepG2 and mouse NIH 3T3 cell lines (6). A number of potential DNA-binding sites were identified within this region. Gel mobility shift experiments were performed to characterize the various DNA-binding proteins that might contribute to PRL-1 gene transcription during liver regeneration. EMSA experiments with nuclear extracts from normal and regenerating liver confirmed the binding of transcription factors including CRE (PRL/A, −123 to −116), Sp1, Egr-1 (PRL/B, −118 to −87), and E2 (28) (PRL/C, −68 to −48) proteins to the consensus sequences (not shown). Most of these DNA binding activities were constitutive in normal and regenerating liver. The AP1 complex that bound the CRE is known to increase in liver regeneration (14). However, transfection with c-Jun and c-Fos expression vectors did not have a major effect on the PRL-1 promoter P1 activity in NIH 3T3 cells or HepG2 cells (data not shown) nor did deletion of this region reduce mitogenic stimulation of the promoter (see Fig. 7), so no further analyses of PRL/A were performed.

**PRL/B** bound Egr-1 and Sp1, and Egr-1 activity increased posthepatectomy indicating a possible correlation with the transcriptional induction of the PRL-1 gene posthepatectomy. Two potential overlapping Egr/Sp1 sites were found in region PRL/B. Gel mobility shift assays were employed to examine Egr-1 and Sp1 binding activities with nuclear extract from regenerating rat liver at 30 min posthepatectomy. Egr-1 and Sp1 specifically bound to oligonucleotide PRL/B (−118 to −87) containing the two potential overlapping Egr/Sp1 sites (Fig. 2, A and B). PRL/B oligonucleotide specifically competed Sp1 binding sequence oligonucleotides. As indicated in the figure, unlabeled oligonucleotides and antibodies against Sp1 and Egr-1 were employed to characterize protein binding specificity. B, EMSA assay of Egr-1 binding specificity with 200-fold molar excess of the unlabeled Sp1 oligonucleotides in all of the reactions. Lane 1, probe alone; lane 2, no competition; lane 3, the unlabeled PRL/B; lane 4, preimmune serum (negative control); lane 5, supershift, antibody specific for Egr-1. The unlabeled competitor oligonucleotides were included as indicated: lane 6, Ap1; lane 7, Sp1; lane 8, Sp1; lane 9, cAMP response element (CRE).
and Egr-1 binding to the consensus Sp1 and Egr-1 oligonucleotides (Fig. 2A, lanes 8 and 12).

Sp1 is a ubiquitous protein that is constitutively expressed at very high levels in normal and regenerating liver (see Fig. 4), and its DNA binding profile is well characterized. Therefore, we focused on Egr-1 interactions with the PRL-1 promoter region PRL/B. 200-fold molar excess of unlabeled Sp1 oligonucleotides was routinely included in EMSA experiments to prevent Sp1 from binding to the overlapping Sp1 sites in PRL/B. As shown (Fig. 2B), Egr-1 binding to PRL/B was specific because excess unlabeled PRL/B oligonucleotides (lane 3) and antibody against Egr-1 (lane 5) inhibited complex formation, whereas preimmune serum (lane 4) or a large excess of unrelated oligonucleotides Ap1 (lane 6), Sp1 (lane 7), Ap2 (lane 8), and CRE (lane 9) did not.

To assess the role of two potential Egr-1 binding motifs in PRL-1 promoter, we carried out in vitro binding studies to compare Egr-1 binding affinity to the two Egr-1-binding sites. Nuclear extracts from mitogen-stimulated NIH 3T3 cells were used in gel mobility shift assays. As shown (Fig. 2A), mA oligonucleotide bound Egr-1 much less efficiently than either wild type or mA (16- versus 72-h exposure) (Fig. 3A). The unlabeled wild type oligonucleotide and mA oligonucleotide efficiently competed Egr-1 binding (Fig. 3A, lanes 2, 3, 7, and 8), whereas mA competed Egr-1 binding weakly (Fig. 3A, lane 4). Titration of increasing amounts of unlabeled competitor oligonucleotide confirmed the relative affinities of mA and mB (Fig. 3B). Addition of as little as a 50-fold molar excess of wild type or mA-mutated oligonucleotides resulted in a complete inhibition (Fig. 3B, lanes 3 and 9). In contrast, over a 250-fold molar excess of mB oligonucleotides was necessary to effectively compete Egr-1 binding to the wild type oligonucleotides (Fig. 3B, lane 8). The results demonstrated that Egr-1 has an estimated binding affinity for the site B which is over 5-fold higher than for the site A. On the other hand, Sp1 binding activity was well competed in the presence of mB and wild type oligonucleotides but not mA or mAB, indicating that Sp1 binds most strongly to site A (Fig. 3C). Mutation of the −99 site does not have a major impact on total Sp1 binding to the region (Fig. 3C, and data not shown).

![Fig. 3.](image)

**FIG. 3.** Effects of selective mutagenesis of Egr-1 consensus motifs on Egr-1 binding abilities. A, left, the effects of selective mutations to Egr-1 sites were analyzed by gel mobility shift assays. Films were exposed at room temperature for 16 h (left panel) and 72 h at −70 °C (right panel); right, sequences of oligonucleotides used in gel mobility shift assays. Sp1 and Egr-1 motifs are underlined and the mutated nucleotides are represented with lowercase letters. B, the competitive (Comp.) gel mobility shift assays were performed to determine Egr-1 binding affinity by using increasing molar excess of the indicated competitors (25 times for lanes 2 and 4; 50 times for lanes 3, 5, and 9; 100 times for lane 6; 150 times for lane 7; and 250 times for lane 8). C, impact of oligonucleotide mutagenesis on Sp1 binding to consensus Sp1 binding motif. The wild type and mutated PRL-1 Egr-1 binding motifs were employed to compete Sp1 binding to consensus Sp1 binding motif with increasing molar excess (10 times for lanes 2, 5, 8, and 11; 20 times for lanes 3, 6, 9, and 12; and 50 times for lanes 4, 7, 10, and 13).

**Rapid Activation of Egr-1 DNA Binding in Regenerating Liver and Mitogen-stimulated H35 and NIH 3T3 Cells**—We then examined the time course of induction of Egr-1 binding activity following hepatectomy and mitogen-stimulated NIH 3T3 and H35 cells. As shown (Fig. 4A), some Egr-1 binding activity was present in normal rat liver, and Egr-1 binding activity was rapidly induced 30 min posthepatectomy with a 5–7-fold induction at the 1-h peak. The induction of Egr-1 binding activity was specific to regenerating liver and not simply due to the stress of surgery or induction of an acute-phase response, as sham operation did not significantly increase Egr-1 binding during this time (Fig. 4A, lane 6 and 7). Cycloheximide, a protein synthesis inhibitor, did not block the increase in Egr-1 binding activity at 30 min posthepatectomy (Fig. 4A, lane 8) but inhibited Egr-1 retardation complex formation at 3 h posthepatectomy (Fig. 4A, lane 9).

In contrast, no Egr-1 binding activity was detected in quiescent NIH 3T3 cells and H35 cells, and there was little or no induction during the first 30 min after serum or insulin (10−8 m) stimulation, respectively (Fig. 4, B and C, lanes 1–3). Egr-1 retardation complexes were induced by serum or insulin at 1 and 2 h (Fig. 4, B and C, lanes 4 and 5) and were completely abolished in the presence of cycloheximide (Fig. 4, B and C, lanes 6–9). These results are in agreement with those previously reported in which de novo synthesized Egr-1 protein fully accounts for the induced Egr-1 binding activities in mitogen-induced NIH 3T3 cells (29).

Immunoblot analysis was used to further examine Egr-1 protein expression in the early phases of liver regeneration. As shown (Fig. 5A), Egr-1 protein was present at a very low level in normal liver nuclear extracts and increased dramatically over 30-fold at 30 min posthepatectomy (Fig. 5A, lanes 1 and 2). However, gel mobility shift assays showed only a 5-fold increase in Egr-1 DNA binding during this same period (Fig. 4A). Cycloheximide treatment inhibited 80% of the increase in Egr-1 protein at 30 min posthepatectomy (Fig. 5A, lanes 2 and 4). On the other hand, a similar amount of Egr-1 DNA binding activity was present at 30 min posthepatectomy with or without cycloheximide treatment (Fig. 4A, compare lanes 2 and 8). These results suggested that the increase in Egr-1 DNA bind-
Lane 2

EMSAs were performed with the PRL/B oligonucleotide using 5 μg of nuclear extracts from rat regenerating liver at the indicated time points after hepatectomy (A). The lower panel shows constitutive SP1 binding in the absence of excess cold SP1 oligonucleotide. B, insulin (10−8 M)-treated H35 cells; C, 20% serum-treated NIH 3T3 cells in the presence or absence of cycloheximide (CHX) at indicated times after insulin or serum treatment. 200-fold molar excess of the unlabeled Sp1 oligonucleotides was routinely included to keep Sp1 from binding to the overlapping Sp1 site. Quantification was by densitometry.

Protein phosphorylation and dephosphorylation are the most common post-translational modifications of protein function and play an important role in regulation of gene expression, cell proliferation, and differentiation (4). We therefore examined the effect of phosphorylation on Egr-1 DNA binding by treating nuclear extracts with potato acid phosphatase. We found that potato acid phosphatase abolished most Egr-1 binding (Fig. 5B, lane 4). This effect was largely blocked (Fig. 5B, lane 4) by preincubation with phosphatase inhibitor Na2MoO4, indicating that the effect of potato acid phosphatase was specific. These data suggested that phosphorylation of Egr-1 protein was required for its DNA binding activity.

Egr-1 Activation of PRL-1 Promoter P1—Egr-1 DNA binding activity increased in regenerating liver and mitogen-treated NIH 3T3 cells at the same time PRL-1 gene expression and/or transcription increased. Because it is difficult to assess transcriptional activity of liver in vivo, we ascertained whether Egr-1 could regulate the PRL-1 P1 promoter in cell culture systems. First, we further delineated the sequences within the first 200 bp 5′ of the promoter essential for transcription of the human PRL-1 gene in cell culture systems, and we then assessed the relative amount of Egr-1 DNA binding in HepG2 and NIH 3T3 cells under normal growth conditions. Detailed deletions of the first 205 bp of the 5′-flanking region with exonuclease Bal-31 were performed (Fig. 6A). Transfection and luciferase assays demonstrated the presence of several potential regulatory sequences in regions −198 to −110, −105 to −6, and −65 to −58 as the luciferase activity fell more than 20-fold following serial deletions along this region. Loss of the strong Egr-1-binding site at −99 did not have a major effect on the transcriptional activity of the PRL-1 promoter in either HepG2 or NIH 3T3 cells. EMSA analyses confirmed that little Egr-1 binding activity is present in these cells under conditions used to assess PRL-1 reporter activity (Fig. 6B), consistent with the lack of impact of deletion of the −99 Egr-1 site. Therefore these cells provided an appropriate background in which the role of Egr-1 in PRL-1 promoter activity could be assessed.

As shown (Fig. 7A), cotransfection of PRL-1 P1 promoter plasmid pSma I or p-D1 containing the −99 Egr-1 binding element with Egr-1 expression plasmid pCMV-Egr-1 resulted in 5–7-fold induction in luciferase activity. No obvious induction was observed when the Egr-1 site-deleted plasmid p-D3 was cotransfected with pCMV-Egr-1. Mutation of the Egr-1 site at −99 (mB in Fig. 3) abolished most Egr-1-mediated induction.

The importance of the Egr-1 binding motifs in mitogen-induced transcription of the PRL-1 gene was assessed following serum induction of serum-deprived NIH 3T3 cells when phys-
Egr-1 Activation Is an Early Event in Liver Regeneration

Fig. 6. Promoter activity of the P1 region of the PRL-1 gene and Egr-1 binding activity in growing HepG2 and 3T3 cells. A, left, the schematic representation of the pGL2-Basic luciferase constructs used for transfections. Right, graphical presentation of relative luciferase activity after normalization for β-galactosidase. HepG2 cells and NIH 3T3 cells were transfected using the calcium phosphate method. 5 μg of luciferase reporter constructs were transiently co-transfected with 1 μg of pSV-β-galactosidase expression plasmid as internal control to normalize transfection efficiency. Relative luciferase activity was reported after normalization for transfection efficiency. Six independent determinations were made for each construct by performing duplicate analyses in three separate experiments. Standard deviations were determined from the duplicate values in a single experiment and were representative of the deviation in all six determinations. B, EMSAs showing Egr-1 binding activity from HepG2 and NIH 3T3 cells. EMSAs were performed with the PRL/B oligonucleotide using 5 μg of nuclear extracts from growing NIH 3T3 cells (lanes 1–3), HepG2 cells (lanes 4–6), and regenerating liver (RL) 30 min posthepatectomy (lanes 7–9). As indicated, 50-fold excess of the unlabeled consensus Sp1 and Egr-1 oligonucleotides were used as cold competitors.

Discussion

We demonstrated that PRL-1 mRNA expression is up-regulated at the transcriptional level during the first few hours posthepatectomy. PRL-1 run-on transcripts, which are virtually absent in normal liver, were increased by 14.8-fold at 30 min posthepatectomy and returned to base line by 6 h. However, Northern blot and RNase protection assays indicate that PRL-1 mRNA expression remains elevated through 24 h posthepatectomy (3). This suggests that PRL-1 gene expression may be regulated at the post-transcriptional level at later times posthepatectomy. RNA stabilization could account for the maintenance of PRL-1 mRNA at later times posthepatectomy as has been seen for other induced transcripts (30).

Egr-1 is transiently and rapidly expressed during the transition of cell from the G0 to G1 phase in response to various mitogens such as growth factors, cytokines, injury, and partial hepatectomy (7, 9, 13, 14). Egr-1 bound to an oligonucleotide containing the consensus site at −99 with 5-fold greater affinity than for the −114 site of PRL-1 promoter P1. Egr-1 activated the PRL-1 promoter primarily via the −99 site. The Egr-1 binding motif was necessary for transcriptional induction of the PRL-1 gene following mitogen stimulation of NIH 3T3 cells (5). This result supports the involvement of Egr-1 in PRL-1 gene transcription during liver regeneration as well when Egr-1 is even more rapidly induced than in mitogen-treated 3T3 cells.

The region of the PRL-1 promoter with which Egr-1 interacts is complicated by the presence of overlapping Sp1 binding motifs. Overlapping Egr-1/Sp1-binding sites have been found in many Egr-1-regulated genes such as the genes for tumor necrosis factor α, basic fibroblast growth factor, the human IL-2 and human platelet-derived growth factors A-chain and B-chain and others (9, 13, 31, 32). Competition between Egr-1 and Sp1 for the site plays a role in regulation of some of these genes (33). We have not formally addressed competition between these factors for the motifs in PRL-1 promoter as Sp1 is constitutively expressed in normal liver, and its binding activity does not change during liver regeneration. Although these results do not support a role for Sp1 in the inducible expression of PRL-1 gene in regenerating liver, it is possible that Sp1 is important for basal expression of the PRL-1 gene and Sp1, resident on the promoter in normal liver, may be displaced by increasing amounts of Egr-1, which confers higher transcriptional activation.

Egr-1 binding activity was rapidly induced during the early phase of rat liver regeneration at 30 min with peak at 1 h, a good correlation with the temporal increase in PRL-1 gene transcription. The initial increase in Egr-1 DNA binding activity in liver regeneration may be caused at least in part by post-translational activation of Egr-1, because cycloheximide, a protein synthesis inhibitor, blocked most of the increase in the absolute level of nuclear Egr-1 protein but did not affect the increase in Egr-1 binding activity at 30 min posthepatectomy. Post-translational activation of transcription factors existing in a latent state in the normal liver is characteristic of signals that initiate liver regeneration. Some examples of transcription factors that are activated by post-translational mechanisms in
Egr-1 Activation Is an Early Event in Liver Regeneration

4519

Fig. 7. Egr-1 activation of PRL-1 promoter P1. A, left, the schematic representation of the pGL2- Basic luciferase constructs used for transfections; right, graphical presentation of relative luciferase activity after normalization for β-galactosidase. 2 μg of luciferase reporter constructs were transiently transfected into growing NIH 3T3 cells with 7 μg of either parent expression plasmid pCMV (vector) or Egr-1 expression plasmid pCMV-Egr-1 and 1 μg of pSV-β-galactosidase expression plasmid totaling 10-μg plasmids in each transfection. B, serum induction of PRL-1 promoter. Left, the schematic representation of the pGL2- Basic luciferase constructs used for transfections; right, graphical presentation of relative luciferase activity after normalization for β-galactosidase. NIH 3T3 cells were transfected with 2 μg of luciferase reporter constructs, 1 μg of pSV-β-galactosidase expression plasmid, and 7 μg of either pCMV-Egr-1 or pCMV vector totaling 10-μg plasmids in each transfection. At 16–18 h after CaPO₄ transfection, cells were serum-deprived (0.5% fetal calf serum) for 24 h. Serum stimulation was accomplished by treating serum-deprived cells with 20% serum in DMEM for 4 h. Cells were then harvested for luciferase and β-galactosidase assays. Relative luciferase activity was reported after normalization for transfection efficiency. Six independent determinations were made for each construct by performing duplicate analyses in three separate experiments. Standard deviations were determined from the duplicate values in a single experiment and were representative of the deviation in all six determinations.

the remnant liver immediately posthepatectomy include NF-κB (24), STAT3 (33), and c-Jun (34) among others. In addition to post-translational activation, many of these transcription factors (NF-κB, STAT3, and c-Jun) are also induced at the level of gene expression and de novo translation as is Egr-1.

Potato acid phosphatase, a general phosphatase, abolished Egr-1 binding, and this effect was largely reversed by the phosphatase inhibitor Na₂MoO₄. Further studies with more specific phosphatases have not allowed us to define further the specific nature of the phosphorylation-dependent DNA binding (not shown). Previous studies indicate that the regulation of Egr-1 DNA binding activity is under the control of protein phosphatases and kinases. Binding of cellular Egr-1 to its consensus sequence is transiently stimulated by phorbol ester, which stimulates protein kinase C, okadaic acid (35), and calycin (36) which are inhibitors of serine/threonine protein phosphatases I and 2A. Egr-1 is phosphorylated by the protein kinase casein kinase II, which has a negative effect on its DNA binding and transcriptional activities (37). Casein kinase II may be one of several kinases that regulate Egr-1 function, since the casein kinase II phosphorylation pattern of Egr-1 does not exactly coincide with that of serum-induced Egr-1. As another example, hyperphosphorylation of Egr-1 on tyrosine residues is seen in v-Sis-induced transformation of NIH 3T3 cells (38). Thus multiple activities of Egr-1 may be determined by site-specific phosphorylation. Further research is necessary to determine critical regulatory sites that are phosphorylated in Egr-1, and their delineation may ultimately provide insight into early signals in liver regeneration.

Although Egr-1 may play an important role in PRL-1 gene transcription in regenerating liver and mitogen-treated fibroblasts, there is no obvious correlation between Egr-1 expression and/or activation and high expression of PRL-1 gene in normal tissues such as brain and muscle or tumor cell lines like HeLa cells, where PRL-1 expression is constitutive, HepG2 cells, HeLa cells and CV1 cells (3). For example, in intestinal epithelia that contain both terminal differentiated and proliferating cells, PRL-1 is expressed in the terminally differentiated villus but not proliferating crypt enteroctyes (5). As consensus CdxA sites are present in PRL-1 promoter P1, Cdx homeodomain proteins, which control intestinal differentiation, may contribute to this differentiation related expression of the PRL-1 gene (6, 39). Likewise in other tissues, distinct transcriptional regulatory factors are predicted to be major regulators of PRL-1 gene expression.

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