Molecular and Cellular Biology, July 1997, p. 4096–4104 Vol. 17, No. 7
0270-7306/97/$04.00+0
Copyright © 1997, American Society for Microbiology

HRS/SRp40-Mediated Inclusion of the Fibronectin EIIIB Exon, a Possible Cause of Increased EIIIB Expression in Proliferating Liver

KEYONG DU,[1] YONG PENG,[1] LINDA E. GREENBAUM,[1,2] BARBARA A. HABER,[3] and REBECCA TAUB[1]*

Department of Genetics,[1] Division of Gastroenterology, Department of Medicine,[2] and Division of Gastroenterology and Nutrition, Children's Hospital of Philadelphia,[3] University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received 23 December 1996/Returned for modification 24 February 1997/Accepted 2 April 1997

Serine-arginine (SR)-rich proteins are believed to be important in mediating alternative pre-mRNA splicing. HRS/SRp40 expression is elevated in liver cell proliferation during development, regeneration, and oncogenesis. We tested whether HRS expression correlates with the appearance of alternatively spliced *fibronectin* transcripts during liver growth. HRS was highly expressed during the proliferative phase of liver development, correlating with expression of the *fibronectin* EIIIB alternative exon. In regenerating liver, HRS protein was induced in a time course consistent with the observed increase in *fibronectin* transcripts containing the EIIIB exon, particularly in nonparenchymal liver cells. Furthermore, in an in vivo assay, HRS, and not other SR proteins, directly mediated EIIIB exon inclusion in the *fibronectin* transcript. This alternative splicing was dependent on a purine-rich region within the EIIIB exon to which HRS specifically bound. We have established that HRS has the potential to contribute to the regulation of *fibronectin* pre-mRNA splicing during liver growth. Changes in fibronectin forms may be important in modifying liver architecture during the proliferative response, thus providing a potential mechanism by which SR proteins may participate in cellular growth control.

The splicing factor HRS (hepatic arginine-serine protein) is expressed in proliferating liver cells. *HRS* is induced as an immediate-early gene in insulin-treated, mitogen-activated rat hepatoma H35 cells and in our study represented 12% of all insulin-induced cDNAs (7). *HRS* is a delayed-early gene in regenerating liver and is expressed at a very high level during liver development when the liver is rapidly proliferating (7, 46; reviewed in reference 9). HRS belongs to the serine-arginine (SR) protein family, which is comprised of at least nine structurally related proteins, including SRp20, SRp30a (SF2/ASF), SRp30b (SC35), SRp40, SRp55, and SRp75 (11, 12, 27, 31, 39, 52–54). Comparing the partial sequence of human SRp40 and HRS sequence revealed that HRS is rat SRp40 (7, 52). Recently, the human homolog of HRS was identified and shown to have properties of other splicing factors in both in vitro and in vivo splicing assays (39).

Pre-mRNA alternative splicing provides an important mechanism of gene and protein expression regulation (19, 29, 31, 40). Through alternative splicing, one primary transcript can generate multiple proteins which may have different functions. Alternative splicing occurs via several pathways, including skipped exons, included introns, alternative 5′ splice sites, alternative 3′ splice sites, and mutually exclusive exons. SR proteins have been shown to play essential roles in both constitutive pre-mRNA splicing and regulation of pre-mRNA alternative splicing. In vitro and in vivo splicing assays indicate that SR proteins regulate alternative splicing by promoting the use of proximal 5′ splice sites (10, 23, 26, 55), and different SR proteins have distinct abilities to regulate alternative splicing of various model pre-mRNAs (2, 31, 53). Specificity may be mediated by differential binding to purine-rich sequences or splicing enhancers that have been identified within several alternatively spliced exons (31). Tissue-specific variations in total and relative amounts of SR proteins or their mRNA have been described (39, 52). The genetics and control of specific splicing pathways that are important in mammalian cell growth and development are largely unknown. It is likely that the relative amount of individual SR proteins in particular cells or tissues may determine the level of various spliced forms of different pre-mRNAs, thereby reflecting the specific role of each SR protein. The finding of uncompensated growth defects in *ASF* gene-targeted cells suggests that each of these SR proteins plays a unique role in cellular processes (50).

Because *HRS* is induced at a high level in hepatic regeneration, development, and oncogenesis, we reasoned that pre-mRNAs that are regulated by HRS would be alternatively spliced in all three types of hepatic proliferation. Based on the literature, *fibronectin* pre-mRNA has this property. During hepatic proliferation in development, regeneration, and oncogenesis, changes in hepatocyte polarity occur (42). Among adhesion molecules, *fibronectin* mRNA has several spliced forms which change in proliferative states of the liver (3, 8, 33–37, 47). During liver development, EIIIB exon-containing ($EIIIB^+$) transcripts represent a significant percentage of *fibronectin* transcripts (37). In the adult liver, nonproliferative hepatocytes produce almost all of the plasma forms of fibronectin (24) which do not contain the EIIIA (EDA and ED1) and EIIIB (EDB) exons. The *fibronectin* gene is induced as a delayed-early gene, and its elevated expression is prolonged up to 48 h posthepatectomy during liver regeneration (13, 32). EIIIA and EIIIB exon inclusion in the *fibronectin* transcript has not been examined before 12 h posthepatectomy

* Corresponding author. Mailing address: Department of Genetics, University of Pennsylvania School of Medicine, 422 Curie Blvd., 705A Stellar Chance, Philadelphia, PA 19104. Phone: (215) 898-9131. Fax: (215) 573-2195. E-mail: taubra@mail.med.upenn.edu.

(3), when many of the early G_1 events have already occurred. At 24 h posthepatectomy, elevated levels of the EIIIA exon are detected. In a form of liver injury caused by bile duct ligation, an increase in EIIIA and to some extent EIIIB occurs particularly in hepatic fat-storing cells at 12 to 24 h after the stimulus (24). The function of the alternative forms of fibronectin that occur in proliferating liver and other cell types is not known, but it is postulated that they play a role in organ structure (4).

Here, we assessed temporal and cell-type-specific changes in HRS/SRp40 protein levels and *fibronectin* pre-mRNA splicing forms in developing and regenerating liver and found that inclusion of the EIIIB exon in *fibronectin* mRNA correlated with HRS expression. In in vivo splicing assays, HRS mediated the inclusion of the EIIIB exon in the mature *fibronectin* transcript. This splicing depended on the presence of a purine-rich sequence within the EIIIB exon to which HRS specifically bound. These findings suggest that HRS is at least in part responsible for increased EIIIB containing *fibronectin* transcripts during liver growth.

MATERIALS AND METHODS

Animals and isolation of hepatocytes and nonparenchymal cells. For partial hepatectomy, female Fisher rats (160 to 200 g) were ether anesthetized and subjected to midventral laparotomy with approximately 70% liver resection (32). To isolate hepatocyte and nonparenchymal cells either in the absence of or following 70% partial hepatectomy, female Fisher rats were anesthetized and subjected to ventral laparotomy, and the portal vein was cannulated. The liver was perfused for 15 min with solution A (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES) and 15 min with solution C (same as solution A but with 5 mM $CaCl_2$ and 0.5 mg of collagenase A per ml) (17). All solutions were pH 7.4 and prewarmed to 37°C. After perfusion, the liver was removed, minced, and filtered through 16-ply gauze. The hepatocytes were pelleted at $170 \times g$ for 1 min. The supernatant was spun at $2{,}000 \times g$ for 10 min to pellet the nonparenchymal cells. The purity of both cell populations was about 90 to 95% as determined by microscopy.

Preparation of whole nuclei. Whole liver nuclei were prepared from liver as described previously (15). Briefly, at indicated times following 70% partial hepatectomy, the regenerating liver or whole liver (time zero) was removed into homogenization buffer (10 mM HEPES [pH 7.6], 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0], 0.1 mM EGTA [pH 8.0], 0.1 mM spermine, 0.1 mM spermidine, 0.3 M sucrose). After homogenization, the whole liver suspension was layered on cushion buffer (homogenization buffer plus 2.2 M sucrose), and nuclei were pelleted by centrifuging at 26,000 rpm for 50 min. The pelleted nuclei were resuspended in nucleus suspension buffer (100 mM NaCl, 10 mM Tris [pH 7.6], 1 mM EDTA). Following protein concentration determination (Bio-Rad), an equal volume of 2× Laemmli buffer was added. Then the nuclei were boiled for 10 min, sheared three times by passage through a 23-gauge needle, and stored at −70°C. Whole H35 nuclei were prepared as described previously (1). Briefly, after insulin treatment, the cells were harvested in 200 μl of phosphate-buffered saline (PBS) at indicated times. The cells were lysed by adding 200 μl of 2× lysis buffer, and the nuclei were pelleted and resuspended in nucleus suspension buffer.

Expression of HRS in bacteria. HRS proteins were expressed as full-length or RNA binding domains only. To express the full-length HRS, the *HRS* coding region was amplified with primers 5′-CTAGCCATGGGGATCATGAGTGGC-3′ and 5′-AGCTGGATCCTTCAATTCATTGATTT-3′ by PCR and cloned into the *Nco*I/*Bam*HI sites of the pET expression vector. The pET-*HRS* insert sequence was confirmed by dideoxy sequencing using Sequenase (U.S. Biochemical). The resultant HRS protein was first purified over a nickel-agarose column (Qiagen, Inc.) into 8 M urea. To make native protein, HRS in urea was dialyzed against a urea gradient (6, 3, 1, and 0.5 M; 4 h each) and finally dialyzed into buffer D (20 mM HEPES [pH 7.6], 0.1 M KCl, 2 mM EDTA, 1.5 mM $MgCl_2$, 1.5 mM dithiothreitol [DTT], 20% glycerol). To express the *HRS* RNA binding domain (amino acids [aa] 1 to 165), the cDNA fragment was amplified with 5′-CTAGCCATGGGGATCATGAGTGGC-3′ and 5′-CCTTGGTGGATCCACATCTACAAG-3′ by PCR and cloned into the *Nco*I/*Bam*HI sites of the pET expression vector. The pET-*HRS* RNA binding domain insert sequence was confirmed by dideoxy sequencing using Sequenase (U.S. Biochemical). The resultant 19-kDa protein was purified over a nickel-agarose column (Qiagen).

Antibody production and immunoblots. Bacterially expressed HRS RNA binding domain was used as an antigen to produce anti-HRS antibodies. Rabbit polyclonal antibodies to the purified protein were prepared by Cocalico Biologicals (Reamstown, Pa.). To affinity purify the anti-HRS antiserum, 2 mg of purified 19-kDa HRS peptide (pI = 4.3) was cross-linked on an Affi-15 gel resin (Bio-Rad). Then the anti-HRS antiserum, precipitated with 80% $(NH_4)_2SO_4$ from 10 ml of anti-HRS antiserum and resuspended in 3 ml of PBS, was passed through the HRS column eight times and washed three times with PBS. The anti-HRS antibodies were eluted four times with 2 ml of 0.05 M glycine · HCl (pH 2.3). The elution was monitored by measuring the optical density at 280 nm (OD_{280}). The fractions with an OD_{280} of >0.08 were pooled and concentrated with a Centricon 30 column. The final concentration of affinity-purified antibodies was 0.9 mg/ml. The mouse monoclonal antibody M104 was a gift from M. Roth (52).

Immunoblots made from either liver or H35 nuclei were incubated with 1:750 anti-HRS antiserum or 1:10 M104. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (for anti-HRS antiserum), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (for M104) secondary antibody, and a chemiluminescence detection system (Amersham Corp.) were used.

Immunohistochemistry. Immunohistochemistry for detection of HRS was performed as described previously (15). Tissue sections were rehydrated in xylene following graded ethanol. The Vectastain Elite ABC (Vector Laboratories) avidin-biotin-horseradish peroxidase detection system was used according to the manufacturer's instructions, with some modifications. After the blocking step in 1.5% goat serum in PBS (pH 7.4), avidin and biotin blocking steps were performed with the Vector Laboratories avidin-biotin blocking kit. Affinity-purified anti-HRS antibody was diluted 1:250 in 1.5% goat serum, and tissue sections were incubated for 30 min at room temperature in a humidified chamber. After secondary antibody incubation, the sections were incubated with 0.3% hydrogen peroxide in methanol for 40 min to quench endogenous peroxidase activity. The sections were then dehydrated in ethanol and xylene and mounted with Permount.

Plasmids. The *fibronectin* minigene pBAGHsv-7iBi89 (7iBi89) was a gift from Richard Hynes (Massachusetts Institute of Technology) (21, 22). Plasmid pBA was constructed by deletion of a portion of the EIIIB 3′ intron with *Bgl*II and *Afl*II in pBAGHsv-7iBi89. To overexpress HRS protein in eucaryotic cells, the *HRS* cDNA was cloned into *Eco*RI/*Hin*dIII sites in a pCMV expression vector (6).

To construct deletion 7iBi89d128-187, pBAGHsv-7iBi89 was digested with *Not*I (in the actin promoter) and *Bam*HI. The resultant *Not*I-*Bam*HI and *Bam*HI-*Bam*HI fragments were cloned into pBluescript-SK. The *Bam*HI-*Bam*HI fragment was subjected to *Bal* 31 nuclease digestion. The deleted fragment, which contained only one *Bam*HI site, was ligated into *Not*I-*Bam*HI fragments in 7iBi89 after the *Bam*HI site was blunted. To construct the deletions 7iBi89d95-147 and d95-160, the sequence between *Kpn*I and *Bam*HI was deleted from the *Not*I-*Bam*HI fragment, blunted, and ligated to nuclease-digested *Bam*HI-*Bam*HI fragment. To delete the purine-rich region from the EIIIB exon, the overlapping extension PCR method (49) was used. The primers were FibI (5′-AGCTCACTGACCTAAGCTTT-3′), FibII (5′-GAACAGCTCAAAGATCTAGA-3′), FibIII (5′-ACTACAGTAGTTGCGTCCCCATTTTTGAAG-3′), and FibIV (5′-CTTCAAAAATGGGGACGCAACTACTGTGAT-3′). 7iBi89 was first amplified with primers FibI and -III or FibII and -IV. The resultant PCR products were purified, mixed, and further amplified with FibI and -II. The final PCR product was digested with *Hin*dIII/*Bgl*II and cloned into pBAGHsv-7iBi89 between *Hin*dIII and *Bgl*II sites. All deletion mutants were verified by DNA sequence analysis.

To construct pKS-FN520 to examine the expression of $EIIIB^+$ and $EIIIB^-$ *fibronectin* mRNA by RNase protection assay, the cDNA fragment which contains exons EIIIB and 8IIIa,b was amplified from fetal liver RNA by reverse transcription (RT)-PCR with primer FibI and 5′-GTACACGCTGGAGACACTGAC-3′ (in exon 8b), filled in with T4 DNA polymerase, and digested with *Hin*dIII. After digestion, the fragment was cloned into pBluescript-KS between the *Hin*dIII and *Eco*RV sites. Plasmid pGEM-GA was prepared as follows: the synthetic oligonucleotides 5′-AGCTTGCGGCAGGAGAAGG-3′ and 5′-GATCCCTTCTCCTGCCGCA-3′, which correspond to the purine-rich enhancer in the EIIIB exon, were annealed and cloned into pGEM4 between the *Bam*HI and *Hin*dIII sites. Plasmid pHRS350 for determining *HRS* expression by RNase protection was constructed as follows: the amplified cDNA fragment for encoding the unique *HRS* RNA binding domain was digested with *Xho*I and *Bam*HI and cloned into pBluescript-KS between the *Xho*I and *Bam*HI sites.

Cell culture and transfections. H35 cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 5% calf serum (Life Technologies) and 5% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Flow), and 100 U of penicillin and 50 U of streptomycin (Flow) as previously reported (7). To produce quiescence, the medium was changed to serum-free DMEM for 72 h, at which time the cells were between 40 and 60% confluent. Following serum deprivation, cells were treated for the indicated times with insulin (10^{-8} M). For transient transfection, NIH 3T3 cells were grown in high-glucose DMEM supplemented with 5% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Flow), and 100 U of penicillin and 50 U of streptomycin (Flow). Then 40 to 60% confluent NIH 3T3 cells in 100-mm-diameter dishes were transiently transfected by the calcium phosphate method (20, 30) with 5 μg each of pCMV-*HRS* (or pCG-SC35 or pCG-SRp55) or pCMV (control) and the 7iBi89 minigene. After 16 h of incubation, the precipitate was removed, and the medium was changed to fresh medium; 20 to 24 h later, the cells were harvested for RNA preparation.

RNA and RNA analysis. The RNAs from fetal liver and regenerating liver were prepared and run on Northern blots as previously reported (18). The RNA from isolated hepatocytes, nonparenchymal cells, and transfected NIH 3T3 cells was prepared essentially as described previously (5). For isolated hepatocytes

and nonparenchymal cells, the pelleted cells were directly dissolved into solution D (4 mM guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 100 mM 2-mercaptoethanol [pH 7.0]) followed by phenol-chloroform extraction and isopropanol precipitation. For transfected NIH 3T3 cells, the cells were washed twice with cold PBS and lysed directly in solution D. Contaminating DNA was removed by incubating with RNase-free DNase I (Promega Co.) at 37°C for 30 min. The RNA concentration was determined by measuring the OD_{260}.

To make the RNA probe for the RNase protection assay, *Hin*dIII-linearized pKS-FN520 or *Xho*I-linearized pHRS350 was in vitro transcribed with T7 DNA polymerase by adding [^{32}P]UTP. After in vitro transcription, the radiolabeled probe was purified on a urea-denatured 5% polyacrylamide gel. The RNase protection assay was performed by using an Ambion RPA II kit as instructed by the manufacturer. Briefly, 10 μg of RNA was hybridized with 10^4 cpm of RNA probe at 45°C overnight. After RNase A and T_1 digestion, the protected RNA was precipitated and separated on a denaturing 5% polyacrylamide gel. The gel was dried and exposed to Kodak X-ray film.

RT-PCR was performed as follows (16). A 2.5-μg aliquot of total RNA and 125 ng of random hexanucleotides in 20 μl of H_2O were denatured at 70°C. The total volume was brought to 50 μl containing 50 mM Tris (pH 8.3), 65 mM KCl, 15 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 20 U of RNasin (Promega), and 5 U of Superscript RNase H^- reverse transcriptase (Gibco BRL). The reaction mixture was incubated at 37°C for 60 min and then heat inactivated. For PCR, 2.5 μl of the RT mixture was used as a template in 25-μl reaction volume containing 0.2 mM deoxynucleoside triphosphate, 2 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 150 ng of upstream primer, 150 ng of downstream primer, 0.25 μl of [α-^{32}P]dCTP, and 2 U of *Taq* DNA polymerase. The reaction was run for 24 cycles (94°C, 45 s; 55°C, 30 s; 72°C, 30 s). Two microliters of the final product was separated on a denaturing 5% polyacrylamide gel. The gel was dried and exposed to Kodak X-ray film. The primers for PCR were as follows: *fibronectin* $EIIIB^-$ 5′ primer, 5′-CCTATTCTCTGAATACCGTCAT-3′ (in exon 7b); *fibronectin* $EIIIB^+$ 5′ primer, 5′-GACTATGACATCAGGTTATC-3′ (in exon EIIIB); and 7iBi89 minigene 5′ primer, 5′-GAGCACAGAGCCTCGCCTTTG-3′ (actin exon). All amplifications of *fibronectin* mRNA used the same 3′ primer, 5′-GTACACGCTGGAGACACTGAC-3′ (in exon 8b).

RNA binding and UV cross-linking. To prepare the RNA probe, *Eco*RI-linearized pGEM-GA and pGEM empty vector were in vitro transcribed with T7 DNA polymerase by adding [^{32}P]UTP. After in vitro transcription, the radiolabeled probe was purified on a urea-denatured 15% polyacrylamide gel. For RNA binding and UV cross-linking (55), either 1 μg of bacterially expressed full-length HRS or 10 μg of H35 nuclear extract was incubated with the 10 μg of tRNA in RNA binding buffer (10 mM HEPES [pH 7.6], 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 0.25 mM DTT) in 25 μl at room temperature for 20 min. Then 5×10^3 cpm of RNA probe was added, and the mixture was incubated for another 20 min. UV cross-linking was carried out at 4°C at 254 nm for 20 min. After UV cross-linking, 5 μg of RNase A was added, and the mixture was incubated at 37°C for 30 min. Proteins were separated on a sodium dodecyl sulfate–12% polyacrylamide gel. The gel was dried and exposed to Kodak X-ray film.

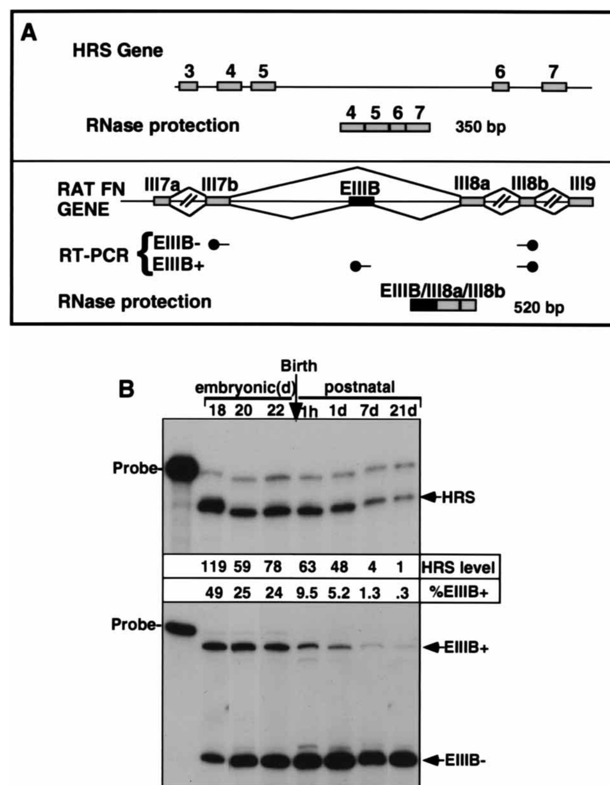

FIG. 1. Expression of *HRS* and $EIIIB^+$ mRNAs during liver development. (A) Schematic diagram of the relevant portion of the *HRS* gene along with the RNase protection probe and of the *fibronectin* (FN) gene, used for detection of the $EIIIB^+$ and $EIIIB^-$ forms of *fibronectin* mRNA by RT-PCR (Fig. 2B) and RNase protection assays (panel B and Fig. 2A). (B) RNase protection assay of developing liver RNAs to detect *HRS* mRNA (top) and $EIIIB^+$ and $EIIIB^-$ mRNAs (bottom) (10 μg of total RNA per lane). The quantitation is indicated for each transcript (*HRS*, relative to 21-day [21d] liver; $EIIIB^+$, as a percentage of total *fibronectin* transcripts.

RESULTS

Correlation of *HRS* expression with the level of $EIIIB^+$ *fibronectin* RNA in developing and regenerating liver. Preliminary data from Northern blot analyses indicated that *HRS* mRNA expression is high in developing liver (data not shown), particularly in the prenatal and immediately postnatal time periods, when liver proliferation is maximal. We confirmed these observations by using an RNase protection assay which specifically detects the protein-encoding *HRS* transcript (*HRS* Short Form in reference 7) (Fig. 1A). *HRS* mRNA was extremely high just prior to birth, 100-fold higher than in normal adult liver (21 days postbirth). The level of *HRS* mRNA declined rapidly in concert with other growth-regulated genes (18) and the diminishing growth rate of the liver. Previous reports indicate that the EIIIB exon is included in a high proportion of *fibronectin* transcripts in developing liver (37). In agreement with these findings, we found that the level of *fibronectin* transcripts containing the EIIIB exon as detected by an RNase protection assay represented a large fraction of total *fibronectin* transcripts (up to 50% at embryonic day 18) and rapidly declined to the adult, nearly undetectable level at 21 days after birth (Fig. 1).

Using Northern blot analyses, we had determined that *HRS* mRNA increases approximately fivefold in the early phases of liver regeneration (7). This was confirmed by using the RNase protection assay for *HRS* mRNA (Fig. 2A). Although the relative increase and ultimate level of *HRS* transcripts are much less in the regenerating than in the developing liver, we assessed whether there was any coordinate increase in the level of $EIIIB^+$ *fibronectin* transcripts in the posthepatectomy liver. Total *fibronectin* mRNA increased threefold in the first few hours posthepatectomy. $EIIIB^+$ *fibronectin* transcripts increased to 2% of *fibronectin* transcripts, an increase of approximately 20-fold compared to normal liver. The relative increase was higher in nonparenchymal liver cells than in hepatocytes (Fig. 2B). In nonparenchymal liver cells, which include Kupffer, endothelial, and other sinusoidal cells, the increase in $EIIIB^+$ transcripts posthepatectomy was approximately 35-fold, and in hepatocytes it was 2-fold. Using similar approaches, we did not detect an increase in EIIIA *fibronectin* transcripts in this time frame in regenerating liver (data not shown).

Induction of HRS protein in regenerating liver, particularly in nonparenchymal liver cells. By Northern blot and RNase protection assays, we observed an approximately fivefold induction of *HRS* mRNA in regenerating liver and insulin-treated H35 hepatoma cells (7) (Fig. 2A). To determine if protein levels increased as well, we prepared antibodies against the HRS RNA binding domain (Fig. 3A). Immunoblots of regenerating liver and insulin-treated H35 cell extracts were probed with both anti-HRS and anti-SR antibodies. Anti-HRS

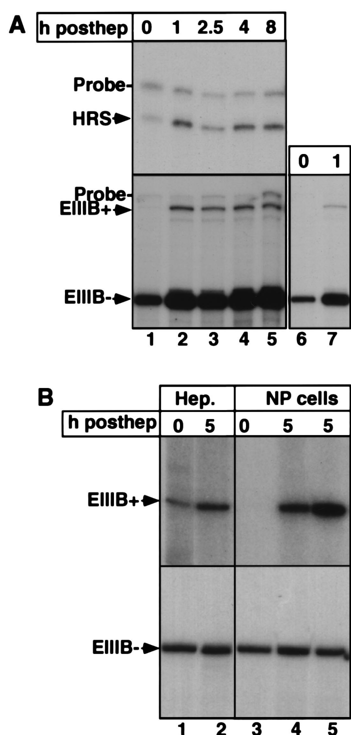

FIG. 2. Induction of EIIIB$^+$ *fibronectin* mRNA in liver regeneration. (A) Detection of *HRS* (top) and EIIIB$^+$ and EIIIB$^-$ *fibronectin* mRNAs (bottom) in regenerating liver mRNA at the indicated times (hours posthepatectomy [posthep]), using an RNase protection assay as for Fig. 1. Lanes 6 and 7 represent shorter exposures of lanes 1 and 2 and indicate that EIIIB$^+$ mRNA is detectable in regenerating liver RNA when normalized for the level of EIIIB$^-$ mRNA. (B) Induction of EIIIB$^+$ *fibronectin* mRNA in nonparenchymal (NP) cell and hepatocyte (Hep.) RNA during liver regeneration. An RT-PCR assay was used to detect *fibronectin* EIIIB$^+$ and EIIIB$^-$ forms. The reactions for EIIIB$^+$ and EIIIB$^-$ forms were performed in separate tubes on equal aliquots from the same RT reaction. For locations of primers, see Materials and Methods and Fig. 1A. Although the upstream primer used to detect EIIIB$^-$ mRNA could have detected both EIIIB$^+$ and EIIIB$^-$ mRNAs, the detection of EIIIB$^-$ mRNA, which is a smaller product, was highly favored. EIIIB$^+$ transcripts were not detected with these EIIIB$^-$ primers even in fetal liver, where EIIIB$^+$ transcripts represent 50% of *fibronectin* transcripts.

detects total HRS protein, and anti-SR detects specific phosphorylated forms of the SR proteins which may not coincide with absolute level or activity (31). As shown (Fig. 3B), consistent with mRNA induction, HRS protein was induced in insulin-treated H35 cells, and the relative level of phosphorylated HRS increased in concert. Interestingly, the level of phosphorylated SRp30 decreased, the level of SRp75 increased, and the level of SRp55 was unchanged throughout the time course. In regenerating liver nuclei (Fig. 3C), like *HRS* mRNA, HRS protein increased as early as 1 h posthepatectomy to a peak of 2.5-fold at 3 to 5 h posthepatectomy). The increase in HRS protein posthepatectomy was significant relative to normal liver ($P < 0.02$). During this time there was a small (less than twofold) and somewhat inconsistent increase in the detectable level of other SR proteins (SRp75 and SRp30), particularly at the 1-h time point.

If the increase in HRS is coordinate with an increase in EIIIB$^+$ *fibronectin* transcripts, the relative increase in HRS protein should be greater in nonparenchymal liver cells posthepatectomy than in hepatocytes. To assess the relative change in HRS protein in individual liver cell types, we used immunohistochemistry with normal and regenerating liver sections. For these studies, affinity-purified HRS antibodies with enhanced specificity were used (Fig. 3A, lane Af). In normal liver, some hepatocyte nuclei expressed the HRS protein, and at 3 and 16 h posthepatectomy, about twice as many hepatocytes expressed HRS (Fig. 4A to F). The numbers of HRS-positive hepatocytes per 20× field were 96 ± 13 at time zero, 202 ± 23 at 3 h, and 190.5 ± 17.6 at 16 h, and the twofold increase at 3 and 16 h posthepatectomy was highly significant relative to the level at time zero ($P < 0.001$). Hepatocytes represent the majority of liver cell protein. Thus, a 2-fold increase in hepatocyte staining during regeneration is in good agreement with the 2.5-fold increase in HRS protein detected by immunoblotting (Fig. 3C) and also correlates well with the 2-fold increase in EIIIB$^+$ *fibronectin* transcripts in hepatocytes (Fig. 2B). Unlike hepatocytes, no HRS protein was detected in sinusoidal nonparenchymal liver cells in normal liver (Fig. 4G). At 3 h posthepatectomy, sinusoidal nonparenchymal cells had high levels of HRS (Fig. 4H), and this expression was no longer detected at 16 h posthepatectomy except in a few perivascular nonparenchymal cells (Fig. 4I). The numbers of HRS-positive nonparenchymal cells at each time point within the liver parenchyma per 20× field were 0.8 ± 0.8 at time zero, 12.4 ± 3.5 at 3 h posthepatectomy, and 0.8 ± 0.8 at 16 h posthepatectomy. These results indicate that there is a highly significant increase in nonparenchymal cell labeling at 3 h posthepatectomy ($P < 0.001$). Thus, like EIIIB$^+$ transcripts, the relative increase in HRS protein in nonparenchymal liver cells was greater than in hepatocytes.

HRS-mediated EIIIB exon inclusion. Taken together, data from assays of regenerating and developing liver suggest that HRS expression is correlated with increased inclusion of the EIIIB exon in the *fibronectin* transcript. To determine whether HRS can directly regulate EIIIB exon inclusion, we cotransfected a *fibronectin* minigene (Fig. 5A) (21) and the *HRS* cDNA cloned into a pCMV5 expression vector into NIH 3T3 mouse fibroblasts. Immunoblots (Fig. 5B) demonstrated that pCMV-*HRS* transfection resulted in overexpression of HRS protein in NIH 3T3 cells. In untransfected cells, SRp30 is the predominant SR protein, and HRS is virtually undetected. In these cotransfection experiments (Fig. 5C [RNase protection] and D [RT-PCR]), HRS clearly activated EIIIB inclusion (Fig. 5C, lane 4; Fig. 5D, lane 4). In the absence of HRS but presence of endogenous SR proteins, little or no EIIIB$^+$ transcript was detected (Fig. 5C, lane 3; Fig. 5D, lane 3).

Previous studies suggested that several six-nucleotide repeat elements located in the *Afl*II/*Bgl*II fragment of the *fibronectin* gene may be important for the splicing reaction (22). We wondered if HRS-mediated regulation of EIIIB inclusion is dependent on this region. Therefore, we constructed the deletion plasmid pBA, in which all six-nucleotide *cis*-acting elements were deleted. Surprisingly, in the presence of the deleted minigene, HRS still directed EIIIB exon inclusion, suggesting that HRS regulates EIIIB exon splicing through different *cis*-acting element(s).

Studies indicate that purine-rich motifs within an alternative exon may be required for inclusion of the alternative exon in the spliced transcript (31, 38). This alternative splicing may be mediated in vitro by specific SR proteins, including HRS/SRp40. Exon EIIIB of *fibronectin* contains a purine-rich sequence that is similar though not identical to previously described sequences (Fig. 6A). To determine if this sequence is important in HRS-mediated EIIIB inclusion, we made several deletion mutants within the exonic region of EIIIB and assessed the impact on EIIIB inclusion in a transient-transfection assay. Deletion of the purine-rich sequence alone was enough to render the exon refractory to most though not all HRS-mediated inclusion (Fig. 6B, lanes 9 and 10). In this experi-

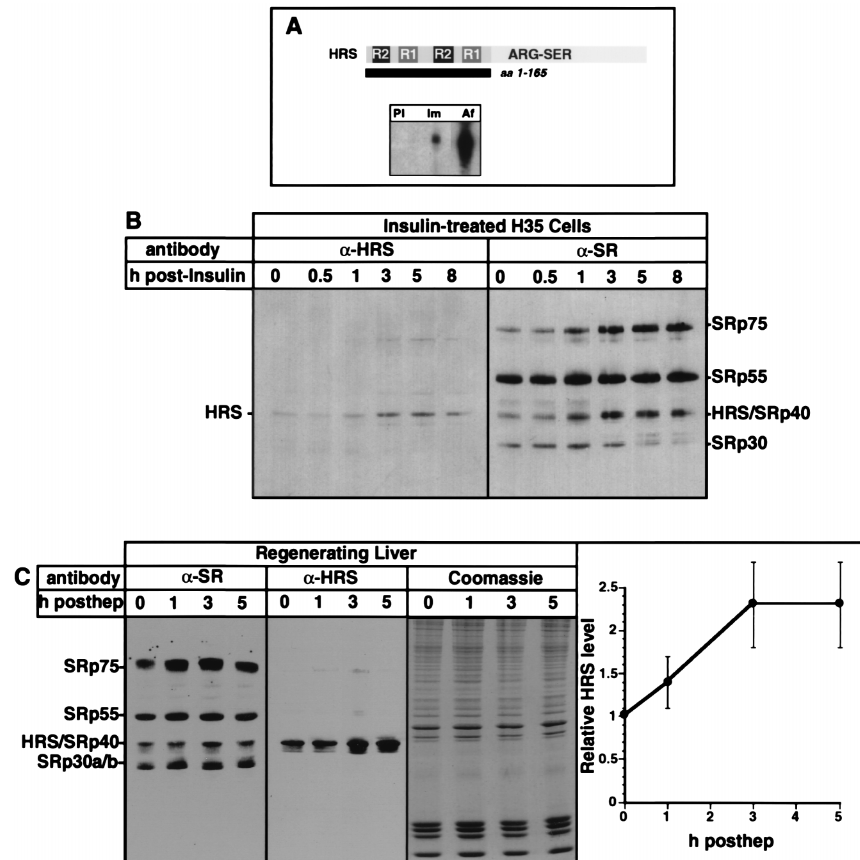

FIG. 3. Relative increase in HRS in insulin-treated hepatoma cells and regenerating liver. (A) Top, Schematic diagram of the HRS protein showing RNA binding domains (R1 and R2), an arginine-serine-rich region (ARG-SER), and the region (aa 1 to 165) against which antibodies are directed. Bottom, immunoblot on which 0.05 μg of purified bacterial HRS (aa 1 to 165)/lane was blotted with preimmune serum (PI), immune serum (Im), or affinity-purified serum (Af) against the HRS peptide. (B) Immunoblot of insulin-treated H35 cell nuclear extracts with anti-HRS serum (α-HRS) or anti-SR (anti-SR monoclonal antibody M104) (α-SR). (C) Immunoblot of regenerating liver nuclei at indicated times posthepatectomy (posthep). Left, anti-SR antibody M104; middle, anti-HRS; right, Coomassie blue stain of the middle panel. The graph shows levels of HRS as detected by anti-HRS antibodies based on three separate determinations, three animals for each time point. Standard deviations were calculated by using Student's *t* test (StatWorks software for the Macintosh).

ment, which was confirmed by two similar experiments, HRS mediated a 51-fold increase in EIIIB inclusion in the presence of the intact minigene (lanes 1 and 2) that was reduced by 80 to 90% when the purine-rich sequence was deleted (5.2- to 10.9-fold HRS-mediated inclusion [lanes 5 to 10]). When the region of EIIIB exon just downstream of the purine sequence was deleted (Fig. 6B, lanes 3 and 4, d128-187), EIIIB exon inclusion was high even in the absence of HRS. The level of basal splicing of the *fibronectin* minigene was unchanged for all of these constructs, as demonstrated by the consistently high level of total spliced products ($EIIIB^+$ plus $EIIIB^-$).

These experiments indicate that HRS is able to direct inclusion of EIIIB in the *fibronectin* transcript and that this splicing regulation depends on a *cis*-acting sequence that resides within exon EIIIB. However, the relative ability of HRS to direct EIIIB inclusion compared to other SR proteins was not directly assessed. To begin to address this issue, pCG-SC35 and pCG-SRp55 (pCMV based), which had been previously used to direct alternative splicing in vivo (39), were cotransfected with the *fibronectin* minigene (Fig. 7A). In three separate transfections, neither SC35 nor SRp55 increased the level of $EIIIB^+$ transcripts significantly above the control level.

Finally, an RNA binding assay was used to show that HRS can directly bind to the purine-rich region in exon EIIIB (Fig. 7B). Bacterially expressed HRS, which migrates at 29 kDa, bound to the purine-rich region containing RNA (pGEM-GA) but not pGEM control RNA (lanes 1 and 2). A protein of 40 kDa, the molecular size of HRS, was the only protein that significantly bound pGEM-GA RNA (lane 4) when growing H35 cell nuclear extracts which contain abundant HRS (Fig. 3B) were used. In a separate experiment in which the binding reaction was allowed to proceed for twice as long, a small amount of binding to 55- and 75-kDa proteins in addition to the 40-kDa protein was detected (data not shown). Perhaps because the HRS antibodies are directed to the RNA binding domain which interacts with the RNA, it was not possible to effectively immunoprecipitate even the bacterial HRS-RNA product (lane 3) by using anti-HRS antibodies (not shown).

DISCUSSION

Our initial studies resulted in the cloning of *HRS/SRp40* cDNA because *HRS* mRNA is strongly induced during proliferation of hepatic cells. Here we have shown that the expression of HRS correlates with inclusion of the *fibronectin* EIIIB exon in developing and regenerating liver. Based on this correlation, we predicted that *fibronectin* pre-mRNA may be one of the biological targets of HRS. Using an in vivo splicing assay, we showed that HRS mediates EIIIB exon inclusion and

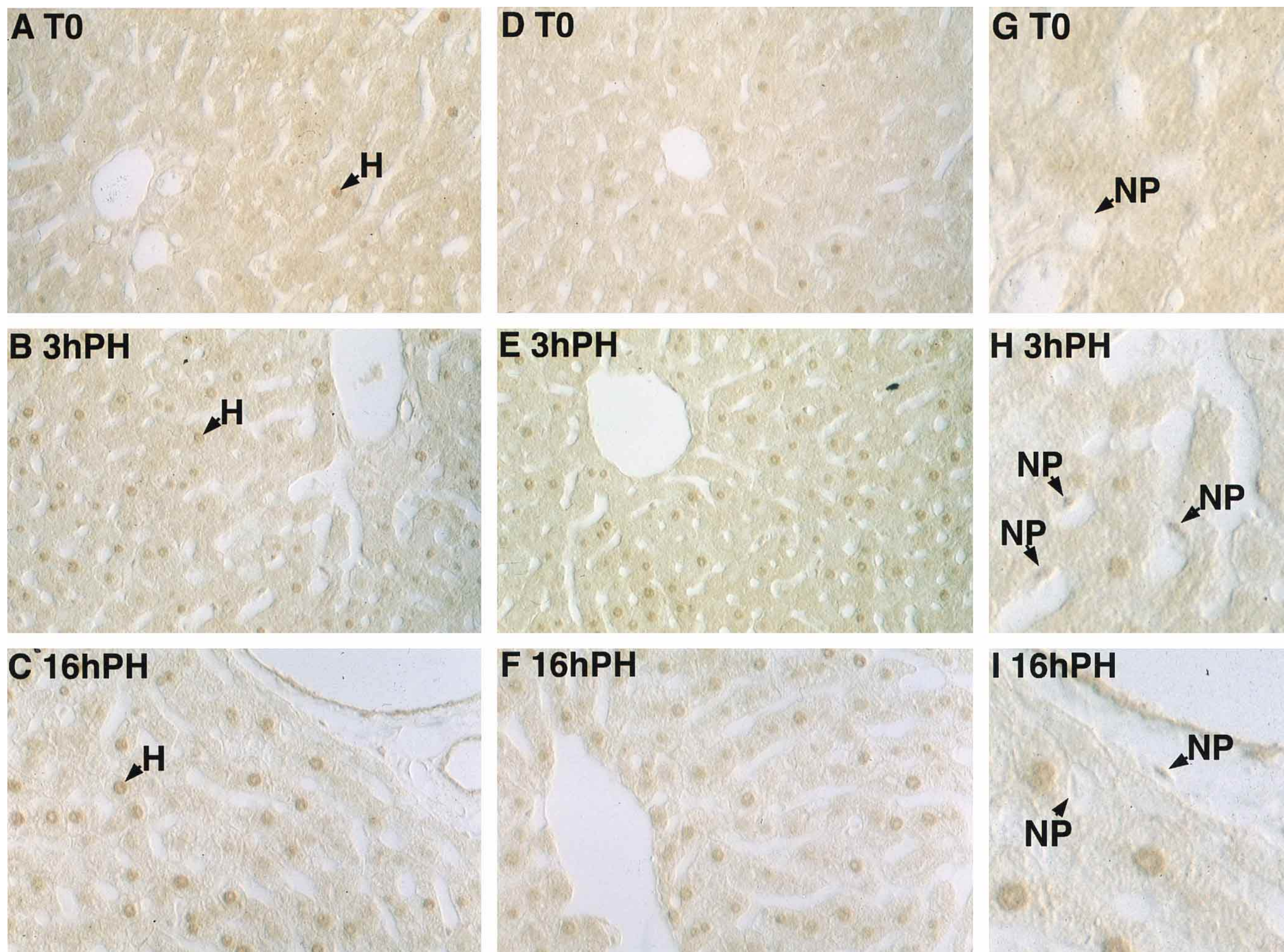

FIG. 4. Increase of HRS in hepatocytes and nonparenchymal cells in regenerating liver. Immunohistochemistry with anti-HRS antibodies demonstrates nuclear HRS levels in nonparenchymal cells (NP) and hepatocytes (H) at indicated times posthepatectomy (PH). (A to C) Portal triad; (D to F and G to I) central vein at magnifications of ×380 and ×760. (Nomarsky optics). This experiment was repeated twice. Quantitation of nonparenchymal cell number was done by counting positively stained cells in five 40× fields for each time point and hepatocytes in four 20× fields.

that this activity depends on a purine-rich region within the EIIIB exon to which HRS binds.

Coordinate induction of HRS protein and *fibronectin* EIIIB$^+$ transcripts. In developing liver, *HRS* mRNA correlates with the presence of the EIIIB$^+$ transcripts, as both are elevated during the major proliferative phase which ends about 1 month after birth. In developing liver, the level of *HRS* transcripts is approximately 100-fold higher than in adult liver and correlates well with the level of EIIIB$^+$ *fibronectin* transcripts, which represent approximately 50% of *fibronectin* transcripts, compared to an almost undetectable level in the adult liver. The level of induction of HRS in the regenerating liver is much lower than in the developing liver. The level of EIIIB$^+$ transcripts is also much lower in regenerating liver, representing only 2% of the *fibronectin* transcripts but nonetheless a significant increase relative to normal liver. The greatest relative increase in EIIIB$^+$ transcripts in regenerating liver is seen in nonparenchymal liver cells, which, unlike hepatocytes, contain detectable HRS only during regeneration. Thus, a reasonable correlation is seen between EIIIB$^+$ *fibronectin* mRNA and HRS protein levels posthepatectomy, particularly in nonparenchymal cells.

Inclusion of *fibronectin* EIIIB exon is mediated by HRS in transfected cells. Studies in which the expression of HRS and that of exon EIIIB are correlated provided a rationale for determining if HRS can direct the inclusion of the EIIIB exon in *fibronectin* mRNA. Using an in vivo splicing assay, in which *fibronectin* minigenes were transfected in the presence or absence of HRS, we demonstrated that HRS can mediate *fibronectin* EIIIB exon inclusion. This transfection assay worked very well, perhaps because NIH 3T3 cells contain little endogenous HRS. The increase in HRS relative to other SR proteins was substantial following the transfection and sufficient to change the ratio of EIIIB$^+$ transcripts from undetectable to approximately one-half of the spliced products (Fig. 5C). When other SR proteins (SRp55 and SC35) were tested, no significant increase in EIIIB$^+$ transcripts was observed. However, we have not established whether SR proteins which were not tested (e.g., SRp75) can direct the inclusion of the EIIIB exon. Moreover, the relative ratio of other SR factors in specific cells may have an impact on the relative efficiency of HRS in mediating EIIIB inclusion.

HRS-directed EIIIB inclusion does not depend on a *cis*-acting sequence within an intron of the *fibronectin* gene (22)

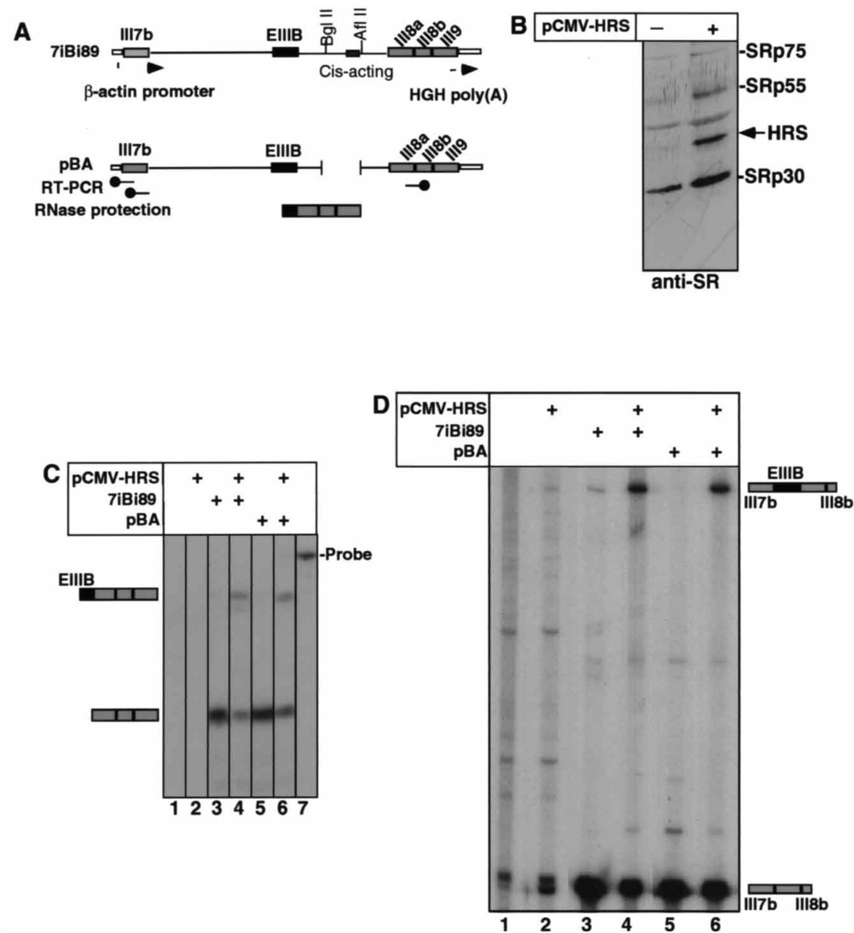

FIG. 5. Inclusion of the EIIIB exon in *fibronectin* mRNA mediated by HRS. (A) Schematic diagram of *fibronectin* minigene and deletion and the methods of detection by RT-PCR and RNase protection. HGH, human growth hormone. (B) Immunoblot with anti-M104 (detects SR proteins) of NIH 3T3 cells transfected with 5 μg of pCMV or pCMV-*HRS* (one plate of cells per lane; less protein loaded in lane 1). (C) Cotransfection of 5 μg of pCMV-*HRS* (+) or 5 μg of pCMV with the indicated minigene (5 μg). Lanes 1 and 2, pCMV and pCMV-*HRS*, respectively, in the absence of minigene showing that no endogenous fibronectin is detected. RNase protection analysis used a probe extending from EIIIB to III9 as shown (10 μg of total RNA/reaction). Reactions were run on the same gel, but spaces between sample lanes were eliminated in the figure. (D) Cotransfection as for panel C except that analysis was done by RT-PCR. Lanes 1 and 2 are as in panel C, in the absence of minigene, showing that some endogenous fibronectin is detected in this assay. The relative amount of $EIIIB^+$ spliced product was best estimated by the RNase protection assay, as RT-PCR underestimated the amount of the larger $EIIIB^+$ PCR product. The 5′ primer was the same as that used for the assay shown in Fig. 2B. Experiments with the intact minigene with or without pCMV-*HRS* were repeated several times with the same results.

but clearly depends on a purine-rich sequence within the EIIIB exon to which HRS binds. In the absence of the purine-rich sequence (Fig. 6), there is about 10 to 20% residual HRS-mediated EIIIB inclusion, which could be due to another purine-rich sequence near the 3′ end of the exon; alternatively, HRS may interact with the 5′ or 3′ site to promote exon inclusion. The sequence of the purine-rich stretch to which HRS binds is very similar though not identical to sequences of exonic splicing enhancers that have been found in the alternative exons of troponin T, EIIIA, and other mRNAs (23, 38, 43, 45, 48). It is believed that SR proteins, through binding to exonic splicing enhancers, recruit the basal splicing factors such as U2, U1, and other accessory splicing factors (26, 51). In one model, SR proteins that bind to specific purine-rich sequences allow for exon inclusion by facilitating the formation of a bridge between the splice acceptor proteins (U2AF) at the 5′ end of the exon and the splice donor proteins at the 3′ end of the exon (31). In studies of troponin T, it was found that SRp40, SRp55, SRp30a, and SRp75 bind to the purine-rich region in exon 5, whereas other SR proteins (e.g., SRp30b) do not (38). Binding correlates with the ability of these proteins to direct alternative splicing. A similar sequence within the bovine growth hormone gene binds to a different set of SR proteins (43). Based on the data accumulated so far, SR proteins bind to purine-rich sequences but may demonstrate specificity for certain pre-mRNAs, thus determining the set of alternative exons that can be regulated by each SR protein (44).

In our studies, removal of the sequence just downstream of the purine-rich sequence resulted in constitutive EIIIB inclusion that was not enhanced much by the presence of HRS. Potential explanations for this effect could be the loss of adverse secondary structure or removal of a splicing repressor protein which normally binds to this downstream sequence. Our data do not directly address these two possibilities.

Significance of HRS to liver growth: other potential targets. It is clear that changes in hepatocyte polarity occur during hepatic proliferation, and adhesion proteins may help mediate these changes (42). Alternative forms of fibronectin produced in nonparenchymal liver cells like Ito and endothelial cells may be deposited in the space of Disse. This may aid the proliferating and postproliferative hepatocyte in moving into its new position within the liver lobule, thereby ultimately restoring

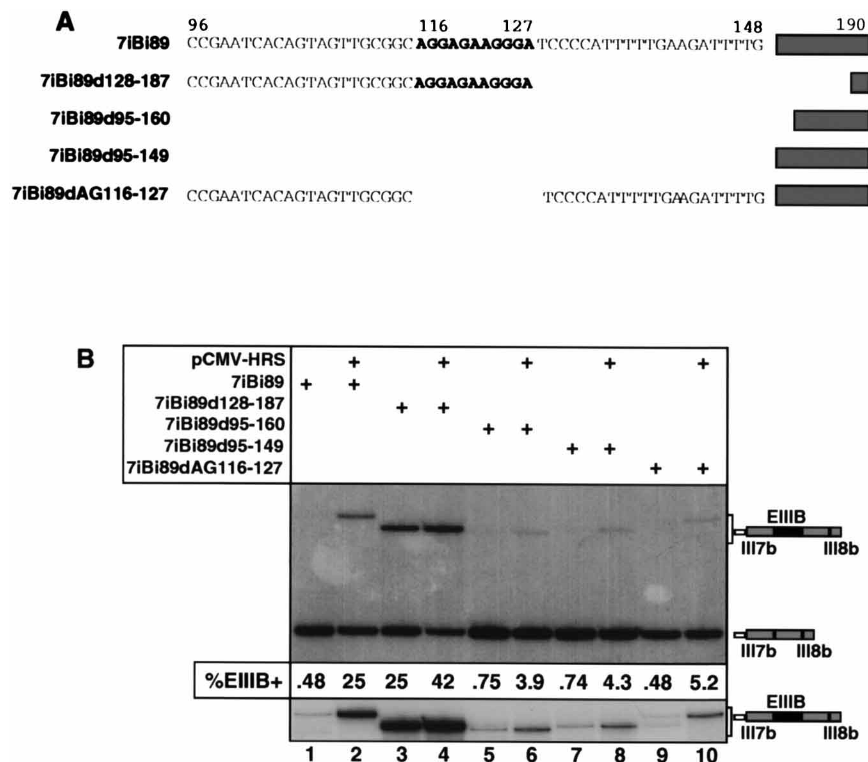

FIG. 6. Dependence of HRS-mediated inclusion on a purine-rich sequence in exon EIIIB. (A) Schematic diagram of exon EIIIB showing the location of the purine-rich sequence and indicating the deletions of the original 7iBi89 construct that were used in the transfection experiment in panel B. The first base pair of exon EIIIB is designated 1. (B) Cotransfection of the various EIIIB deletion constructs in the presence or absence of 5 μg of pCMV-*HRS*. RT-PCR was used to detect the spliced products indicative of EIIIB inclusion (upper band) or exclusion (lower band). The different-size upper band corresponds to the size of the EIIIB deletion (2.5 μg of total RNA/reaction). In the lower panel, a longer exposure of the EIIIB$^+$ portion of the gel is shown, and percentages of EIIIB$^+$ transcripts relative to total (EIIIB$^+$ plus EIIIB$^-$) transcripts are indicated. Similar experiments were performed three times with the same results.

normal polarity. However, as no specific function has been ascribed to different forms of fibronectin, the contribution of alternatively spliced forms to restoration of hepatic architecture is not known (4). Our report does not attempt to address the biological function of exon EIIIB. However, the fact that EIIIA does not appear in the early phases of normal liver regeneration lends support to the idea that exon EIIIA and exon EIIIB alternative splicing are regulated by different mechanisms and may have different functions.

Although we presented evidence that *fibronectin* pre-mRNA is a potential target of HRS, a number of other messages may be spliced alternatively during liver proliferation and could be targets of HRS and other SR proteins that increase in proliferative states of the liver. These mRNAs include those for protein tyrosine phosphatases, cathepsin B, and cytochrome P450 as well as other liver-specific genes that are known to have different spliced forms in normal liver (14, 25, 41). In addition to new RNA synthesis, which increases dramatically during cellular proliferation, alternative splicing provides a mechanism by which proliferation-specific protein forms may be produced.

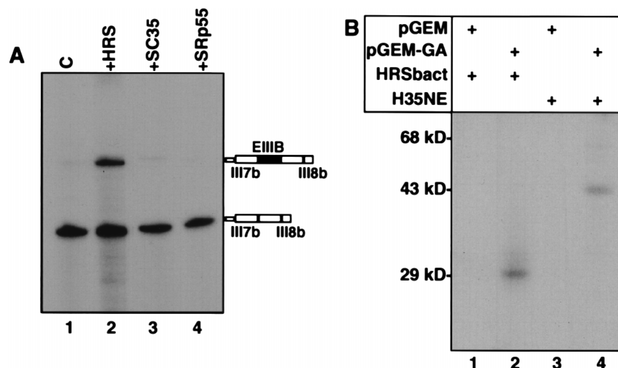

FIG. 7. Specific interactions of HRS with the EIIIB purine-rich sequence. (A) Cotransfection of the 7iBi89 minigene with equal amounts of the control plasmid pCMV (C; lane 1), pCMV-*HRS* (lane 2), pCG-SC35 (lane 3), and pCG-SRp55 (lane 4). Spliced products were detected by the RT-PCR (Fig. 5 and 6). (B) Binding of HRS to the purine-rich sequence. Labeled RNA containing only pGEM sequences (pGEM) or pGEM plus the purine-rich sequence (pGEM-GA) were allowed to interact with bacterially expressed HRS (HRSbact) or nuclear extract from growing H35 cells (H35NE) as indicated. After UV cross-linking, the products were electrophoresed and exposed to autoradiography as described in Materials and Methods.

ACKNOWLEDGMENTS

We thank Mark Roth for M104 and Richard Hynes for *fibronectin* constructs.

We thank Adrian Krainer for the SC35 and SRp55 expression plasmids. This work was in part supported by a Juvenile Diabetes Foundation Award, NIH grants R01 DK44237 and P50 DK49210 to R.T., and NRSA NIH K08 to L.E.G.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology, vol. 1, p. 4.10.1–4.10.5. John Wiley & Sons, Inc., New York, N.Y.
2. **Caceres, J. F., S. Stamm, D. M. Helfman, and A. R. Krainer.** 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science **265:**1706–1709.

3. **Caputi, M., C. A. Mela, and F. E. Baralle.** 1995. Regulation of fibronectin expression in rat regenerating liver. Nucleic Acids Res. **23:**238–243.
4. **Carnemolla, B., A. Leprini, G. Allemanni, M. Saginati, and L. Zardi.** 1992. The inclusion of the type III repeat ED-B in the fibronectin molecule generates conformational modifications that unmask a cryptic sequence. J. Biol. Chem. **267:**24689–24692.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Diamond, R. H., D. E. Cressman, T. M. Laz, C. S. Abrams, and R. Taub.** 1994. PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. Mol. Cell. Biol. **14:**3752–3762.
7. **Diamond, R. H., K. Du, V. M. Lee, K. L. Mohn, B. A. Haber, D. S. Tewari, and R. Taub.** 1993. Novel delayed-early and highly insulin-induced growth response genes. J. Biol. Chem. **268:**15185–15192.
8. **Enrich, C., W. H. Evans, and C. G. Gahmberg.** 1988. Fibronectin isoforms in plasma membrane domains of normal and regenerating rat liver. FEBS Lett. **228:**135–138.
9. **Fausto, N., and E. M. Webber.** 1994. Liver regeneration, p. 1059–1084. *In* I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jacoby, D. Schachter, and D. A. Shafritz (ed.), The liver: biology and pathobiology. Raven Press, New York, N.Y.
10. **Fu, X.-D.** 1993. Specific commitment of different pre-mRNAs to splicing by single RS proteins. Nature **365:**82–85.
11. **Fu, X.-D., and T. Maniatis.** 1992. The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3′ splicing site. Proc. Natl. Acad. Sci. USA **89:**1725–1729.
12. **Ge, H., P. Zuo, and J. L. Manley.** 1991. Primary structure of the human splicing factor ASF reveals similarities with Drosophila regulators. Cell **66:** 373–382.
13. **Gluck, U., L. R. Fernandez, R. Pankov, and A. Ben-Ze'ev.** 1992. Regulation of adherens junction protein expression in growth-activated 3T3 cells and in regenerating liver. Exp. Cell Res. **202:**477–486.
14. **Gong, Q., S. J. Chan, A. S. Bajkowski, D. F. Steiner, and A. Frankfater.** 1993. Characterization of the cathepsin b gene and multiple mRNAs in human tissues: evidence for alternative splicing of cathepsin b pre-mRNA. DNA Cell Biol. **12:**299–309.
15. **Greenbaum, L. E., D. E. Cressman, B. A. Haber, and R. Taub.** 1995. Coexistence of C/EBP α,β growth-induced proteins and DNA synthesis in hepatocytes during liver regeneration: implications for maintenance of the differentiated state during liver growth. J. Clin. Invest. **96:**1351–1365.
16. **Haber, B. A., S. Chin, E. Chuang, W. Buikhuisen, A. Naji, and R. Taub.** 1995. High levels of glucose-6-phosphatase gene and protein expression in proliferating liver and diabetes. J. Clin. Invest. **95:**832–841.
17. **Haber, B. A., K. L. Mohn, R. H. Diamond, and R. Taub.** 1993. Induction patterns of 70 genes during nine days after hepatectomy define the temporal course of liver regeneration. J. Clin. Invest. **91:**1319–1326.
18. **Haber, B. A., L. Naji, D. E. Cressman, and R. Taub.** 1995. Coexpression of liver-specific and growth-induced genes in perinatal and regenerating liver: attainment and maintenance of the differentiated state during rapid proliferation. Hepatology **22:**906–914.
19. **Hodges, D., and S. I. Bernstein.** 1994. Genetic and biochemical analysis of alternative RNA splicing. Adv. Genet. **31:**207–281.
20. **Hsu, J.-C., R. Bravo, and R. Taub.** 1992. Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G_1 phase of liver regeneration. Mol. Cell. Biol. **12:**4654–4665.
21. **Huh, G. S., and R. O. Hynes.** 1993. Elements regulating an alternatively spliced exon of the rat fibronectin gene. Mol. Cell. Biol. **13:**5301–5314.
22. **Huh, G. S., and R. O. Hynes.** 1994. Regulation of alternative pre-mRNA splicing by a novel repeated hexanucleotide element. Genes Dev. **8:**1561–1574.
23. **Humphrey, M. B., J. Bryan, T. A. Cooper, and S. M. Berget.** 1995. A 32-nucleotide exon-splicing enhancer regulates usage of competing 5′ splice sites in a differential internal exon. Mol. Cell. Biol. **15:**3979–3998.
24. **Jarnagin, W. R., D. C. Rockey, V. E. Koteliansky, S.-S. Wang, and D. M. Bissell.** 1994. Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. J. Cell Biol. **127:**2037–2048.
25. **Kimura, H., K. Sogawa, Y. Sakai, and Y. Fujii-Kuriyama.** 1989. Alternative splicing mechanism in a cytochrome P-450 (P-450PB-1) gene generates the two mRNAs coding for proteins of different functions. J. Biol. Chem. **264:** 2238–2342.
26. **Kohtz, J., S. F. Jamison, C. L. Will, P. Zuo, R. Luhrmann, M. A. Garcia-Blanco, and J. L. Manley.** 1994. Protein-protein interactions and 5′-splicing-site recognition in mammalian mRNA precursors. Nature **368:**119–124.
27. **Krainer, A. R., A. Mayeda, D. Kozak, and G. Binns.** 1991. Functional expression of cloned human splicing factor SF2: homology to RNA-binding proteins, U1 70K, and Drosophila splicing regulators. Cell **66:**383–394.
28. **Lavigueur, A., H. La Branche, A. R. Kornblihtt, and B. Chabot.** 1993. A splicing enhancer in the human *fibronectin* alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. Genes Dev. **7:**2405–2417.
29. **Legrain, P., and G. Chanfreau.** 1994. Pre-mRNA splicing: from intron recognition to catalysis. Bull. Inst. Pasteur **92:**153–179.
30. **Malim, M. H., and B. R. Cullen.** 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. Mol. Cell. Biol. **13:**6180–6189.
31. **Manley, J. L., and R. Tacke.** 1996. SR proteins and splicing. Genes Dev. **10:**1569–1579.
32. **Mohn, K. L., T. M. Laz, J.-C. Hsu, A. E. Melby, R. Bravo, and R. Taub.** 1991. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison to serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. Mol. Cell. Biol. **11:**381–390.
33. **Norton, P. A., and R. O. Hynes.** 1987. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. Mol. Cell. Biol. **7:**4297–4307.
34. **Oyama, F., S. Hirohashi, M. Sakamoto, K. Titani, and K. Sekiguchi.** 1993. Coordinate oncodevelopmental modulation of alternative splicing of *fibronectin* pre-messenger RNA at ED-A, ED-B, and CS1 regions in human liver tumors. Cancer Res. **53:**2005–2011.
35. **Oyama, F., S. Hirohashi, Y. Shimosato, K. Titani, and K. Sekiguchi.** 1989. Deregulation of alternative splicing of *fibronectin* pre-mRNA in malignant human liver tumors. J. Biol. Chem. **264:**10331–10334.
36. **Oyama, F., Y. Murata, N. Suganuma, T. Kimura, K. Titani, and K. Sekiguchi.** 1989. Patterns of alternative splicing of *fibronectin* pre-mRNA in human adult and fetal tissues. Biochemistry **28:**1428–1434.
37. **Pagani, F., L. Zagato, C. Verganii, G. Casari, A. Sidoli, and F. E. Baralle.** 1991. Tissue-specific splicing pattern of fibronectin messenger RNA precursor during development and aging in rat. J. Cell Biol. **113:**1223–1229.
38. **Ramchatesingh, J., A. M. Zahler, K. M. Neugebauer, M. B. Roth, and T. A. Cooper.** 1995. A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer. Mol. Cell. Biol. **15:**4898–4907.
39. **Screaton, G. R., J. F. Caceres, A. Mayeda, M. V. Bell, M. Plebanski, D. Jackson, J. Bell, and A. R. Krainer.** 1995. Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. EMBO J. **14:**4336–4349.
40. **Sharp, P. A.** 1994. Split genes and RNA splicing. Cell **77:**805–815.
41. **Shifrin, V. I., and B. G. Neel.** 1993. Growth factor-inducible alternative splicing of nontransmembrane phosphotyrosine phosphatase PTP-1B pre-mRNA. J. Biol. Chem. **268:**25476–25484.
42. **Stamatoglou, S. C., and R. C. Hughes.** 1994. Cell adhesion molecules in liver function and pattern formation. FASEB J. **8:**420–427.
43. **Sun, Q., A. Mayeda, R. K. Hampson, A. R. Krainer, and R. M. Rottman.** 1993. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. Genes Dev. **7:**2598–2608.
44. **Tacke, R., and J. L. Manley.** 1995. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. EMBO J. **14:**3540–3551.
45. **Tanaka, K., A. Watakabe, and Y. Shimura.** 1994. Polypurine sequences within a downstream exon function as a splicing enhancer. Mol. Cell. Biol. **14:**1347–1354.
46. **Taub, R.** 1995. Transcriptional control of liver regeneration. 1996. FASEB J. **10:**413–427.
47. **Tavian, D., G. DePetro, M. Colombi, N. Portalani, S. M. Giulini, R. Gardella, and S. Barlati.** 1994. RT-PCR detection of fibronectin EIIIA and EIIIB isoforms; molecular markers for hepatocellular carcinoma. Int. J. Cancer **56:**820–825.
48. **Tian, M., and T. Maniatis.** 1993. A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. Cell **74:**105–114.
49. **Vallejo, A. N., R. J. Pogulis, and L. R. Pease.** 1995. Mutagenesis and synthesis of novel recombinant genes using PCR, PCR primer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Wang, J., Y. Takagaki, and J. L. Manley.** 1996. Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. Genes Dev. **10:**2588–2599.
51. **Wu, J. Y., and T. Maniatis.** 1993. Specific interactions between proteins implicated in splicing site selection and regulated alternative splicing. Cell **75:**1061–1070.
52. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev. **6:**837–847.
53. **Zahler, A. M., K. M. Neugebauer, W. S. Lane, and M. B. Roth.** 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. Science **260:**219–222.
54. **Zahler, A. M., K. M. Neugebauer, J. A. Stolk, and M. B. Roth.** 1993. Human SR proteins and isolation of a cDNA encoding SRp75. Mol. Cell. Biol. **13:**4023–4028.
55. **Zuo, P., and J. L. Manley.** 1994. The human splicing factor ASF/SF2 can specifically recognize pre-mRNA 5′ splice sites. Proc. Natl. Acad. Sci. USA **91:**3363–3367.