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 immediate-early gene in insulin-treated, mitogen-activated rat hepatocytes. HRS/SRp40 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 composed 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.
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(3), when many of the early G1 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/Rp40 protein levels and fibronectin pre-mRNA splicing forms in developing and regenerating liver and found that inclusion of the EIIIA exon in fibronectin mRNA correlated with HRS expression. In vivo splicing assays, HRS mediated the inclusion of the EIIIA exon in the mature fibronectin transcript. This splicing depended on the presence of a purine-rich sequence within the EIIIA exon to which HRS specifically bound. These findings suggest that HRS is at least in part responsible for increased EIIIA 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 anesthetized and subjected to ventral laparotomy with approximately 70% liver resection. 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, pH 7.4) and 15 min with solution C (same as solution A but with 5 mM CaCl2 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 170,000 × g for 1 min. The supernatant was spun at 2,000 × g for 10 min to pellet the nonparenchymal cells. The purity of both cell populations was about 90 to 95% as determined by microscopic observation.

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 MgCl2, 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 30 min. The pellet nuclei were resuspended in nucleus suspension buffer (100 mM NaCl, 10 mM Tris [pH 7.6], 1 mM EDTA) (17). Following protein concentration determination (Bio-Rad), an equal volume of 2× Laemmli buffer was added. Then the nuclei were boiled for 10 min in a water bath at 90°C. The nuclei were centrifuged at 10,000 × g for 5 min at 4°C and analyzed by SDS-PAGE.

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'-CTAGGAGTTGGAGATCATGCGG-3' and 5'-AGCTGTCTCCTCTATCCTATTGTTT-3' using Ex-Taq DNA polymerase (Takara) and cloned into the NcoI/BamHI sites of the PET expression vector. The PET-HRS insert sequence was confirmed by digest with restriction enzymes [Ex-5 murine leukemia virus (Qiangen, Inc.) into 8 M urea. The final PCR product was purified, mixed, and further amplified with FbiI and -II. The primary PCR products were purified, digested, cloned into the NcoI/BamHI fragment of pET20b (Novagen) into the PET-HRS expression vector. The cloned vector was transformed into E. coli strain BL21 (DE3) and grown in LB broth. The expression of HRS in E. coli was confirmed by SDSPAGE and Western blotting with antibodies specific for HRS.

Preparation of cellular extracts. After overnight growth at 37°C, E. coli cells were harvested by centrifugation, washed with PBS, and resuspended in 1× SDs-PAGE loading buffer. The cell suspension was boiled for 5 min and centrifuged at 13,000 × g for 10 min. The supernatant was removed and frozen at −70°C until analyzed. The protein concentration was determined with the Bio-Rad assay.

Affinity-purified anti-HRS antiserum was incubated with 2 mg of 19-kDa HRS peptide (pI 5.9) for 1 h on ice. The mixture was transferred to a 1-ml column and washed with 2 column volumes of 50 mM Tris-HCl, pH 7.4, and 2 column volumes of 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4. Bound proteins were eluted from 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 (OD280). The fractions with an OD280 > 0.08 were pooled and concentrated with a Centricon 100 concentrator. The final concentration of affinity-purified antibodies was 0.9 mg/ml. The mouse monoclonal antibody M104 was a gift from M. Roth (52).

Plasmids. The fibronectin minigene pBAGH57b1-890 (7B8b9) was a gift from Richard Hynes (Massachusetts Institute of Technology) (21, 22). Plasmid pBmA-HRS was constructed by deletion of a portion of the EIIIB 3' intron with Bgl II and ApaI by digesting pBAGH57b1-890. To clone the HRS cDNA was cloned into EcoRl/HindIII sites in a pcMV expression vector (6).

For 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 MgCl2, 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 30 min. The pellet nuclei were resuspended in nucleus suspension buffer (100 mM NaCl, 10 mM Tris [pH 7.6], 1 mM EDTA) (17). Following protein concentration determination (Bio-Rad), an equal volume of 2× Laemmli buffer was added. Then the nuclei were boiled for 10 min in a water bath at 90°C. The nuclei were centrifuged at 10,000 × g for 5 min at 4°C and analyzed by SDS-PAGE.
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 OD260.

To make the RNA probe for the RNase protection assay, HindIII-linearized pHRS350 was in vitro transcribed with T7 DNA polymerase by adding [32P]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 109 cpm of RNA probe at 45°C overnight. After RNase A and T1 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.

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 and the diminishing growth rate of the liver. Previous studies demonstrated the level of EIIIB+ fibronectin transcripts in developing liver (21 days postbirth). The level of 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). Immunoblotting 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 H3S 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 A). 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. 4C).

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 mini-gene (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 A/H/B/G/B 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-
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 (EIIIB1 plus EIIIB2).

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 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 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).
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 were 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. 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.
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. 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.
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 architec-
ture 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 prolif-
erative 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.

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