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Interaction between the Human Nuclear Cap-Binding Protein Complex and hnRNP F

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hnRNP F was identified in a screen for proteins that interact with human CBP80 and CBP20, the components of the nuclear cap-binding complex (CBC). In vitro interaction studies showed that hnRNP F can bind to both CBP20 and CBP80 individually. hnRNP F and CBC bind independently to RNA, but hnRNP F binds preferentially to CBC-RNA complexes rather than to naked RNA. The hnRNP H protein, which is 78% identical to hnRNP F and also interacts with both CBP80 and CBP20 in vitro, does not discriminate between naked RNA and CBC-RNA complexes, showing that this effect is specific. Depletion of hnRNP F from HeLa cell nuclear extract decreases the efficiency of pre-mRNA splicing, a defect which can be partially compensated by addition of recombinant hnRNP F. Thus, hnRNP F is required for efficient pre-mRNA splicing in vitro and may participate in the effect of CBC on pre-mRNA splicing.

The splicing of mRNA precursors takes place in multicomponent complexes called spliceosomes. The major class of spliceosome contains five essential U snRNAs and a large number of both snRNA-associated and free proteins (32, 49, 55). Spliceosome assembly in vitro occurs as an ordered stepwise process. The earliest step appears to be relatively conserved between *Saccharomyces cerevisiae* and humans. In yeast it involves the ATP-independent recognition of the 5′ splice site by the U1 snRNP and of the branch point region of the intron by a factor whose identity is not yet established (1, 34, 60, 61). This complex commits yeast pre-mRNAs to splicing and was thus named the commitment complex. In humans, the apparently homologous complex is the E complex, which forms in the absence of ATP and involves U1 snRNP binding at the 5′ splice site and U2AF binding to the polypyrimidine tract (2, 47). A further component of both complexes is the nuclear cap-binding protein complex (CBC) (8, 35, 36).

CBC is composed of two subunits, CBP80 and CBP20 (23, 24, 28, 29). It binds either to an RNA cap dinucleotide, m^7GpppN, or to capped RNAs (26, 50). CBC is bifunctional. In addition to its stimulatory role in the formation of E or commitment complex, described above, vertebrate CBC has been shown to play an important role in the export of U snRNAs from the cell nucleus in the first stage of U snRNP assembly (23; see reference 25 for a review). The precise role of CBC in RNA export is not defined, but CBC is translocated through the nuclear pore complex together with the RNA (67). Removal of CBC from the RNA in the cytoplasm appears to involve binding of importin, the nuclear protein import receptor (17, 18), to the CBC-RNA complex. It is unclear at present if the transport function of CBC is conserved; however, both vertebrate and yeast CBCs are found in abundant nuclear complexes together with importin-α, the nuclear localization signal-binding subunit of the protein import receptor. Dissociation of both yeast and human CBCs from capped RNA requires the interaction of this complex with importin-β (17), providing circumstantial evidence of a conserved transport function.

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hnRNP proteins are a diverse family of highly abundant (45) nuclear proteins whose common property is their association in the nucleus with poly(A)-containing RNA, i.e., pre-mRNA and mRNA (11). As a family, these proteins share with CBC the characteristic of having been functionally implicated in both pre-mRNA splicing and RNA export from the nucleus. The involvement of mammalian hnRNP proteins in splicing was first suggested by the fact that monoclonal antibodies directed against the hnRNP C proteins inhibited splicing either when added to mammalian cell nuclear extracts or when used to deplete the extracts (7). Similarly, monoclonal antibodies against hnRNP M inhibit splicing upon their addition to HeLa cell nuclear extracts in vitro (16). More specific roles for vertebrate hnRNP proteins in splicing regulation have also been discovered. A role for the hnRNP F protein as one factor required for regulation of neuron-specific splicing of the c-*src* N1 exon has been defined (48). A second mammalian hnRNP protein, hnRNP A1, has been shown to specifically affect splice site choice by influencing discrimination between alternative 5′ splice sites (5, 44). The *Drosophila melanogaster* hnRNP protein, hrp48 (40, 42), has a role in ensuring the germ cell specificity of splicing of a P element intron (62). Mutation of other members of the *Drosophila* family, like hrp40 (30, 39) and Rb97D (27), has specific effects on dorsoventral axis formation and spermatogenesis, respectively. Although the molecular basis for these defects is not clear, they may also result from alterations in pre-mRNA splicing.

There is also good evidence for a role for mammalian hnRNP A1 in the nuclear export of mRNA. hnRNP A1, like several other hnRNP proteins, shuttles continuously between the nucleus and cytoplasm, and it is found in both compartments in association with poly(A)-containing RNA (52, 53). A 38-amino-acid region of hnRNP A1, called M9, is capable of directing active export out of, as well as active import into, the nucleus (45, 63, 68). Further, microinjection of saturating amounts of hnRNP A1, but not of a mutant protein lacking the M9 domain, into *Xenopus laevis* oocyte nuclei results in specific competitive inhibition of the mRNA export pathway (22). Taken together, these data suggest that hnRNP A1 is likely to have an active role in the transport of mRNA to the cytoplasm.

hnRNP A1 is unlikely to be the only member of the family involved in this function. hnRNP L, for example, has been proposed to have a stimulatory effect on the nuclear export of

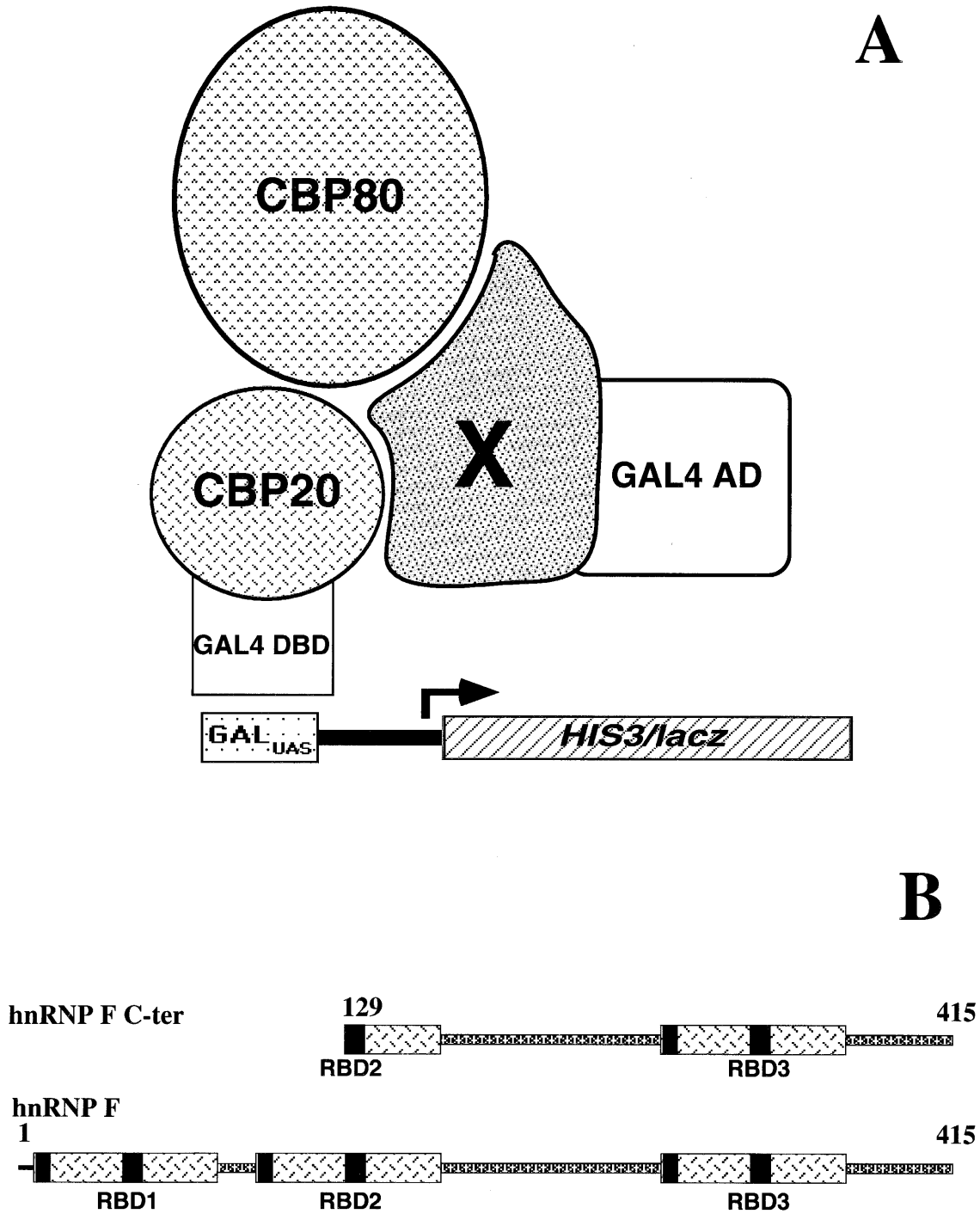

FIG. 1. A three-hybrid screen with CBC selects a C-terminal fragment of hnRNP F. (A) Cartoon showing the principle of the CBC three-hybrid interaction screen described in the text. The human CBP80 protein was coexpressed in *S. cerevisiae* with the GAL4DBD-CBP20 fusion. A human GAL4 activation domain-tagged cDNA library was screened for proteins interacting with the CBC proteins. The interaction between one or both of the CBC proteins and X activates the transcription of the two reporter genes (*HIS3* and *lacZ*) whose structure is schematically shown at the bottom. Positive clones were selected on the basis of the expression of both reporter genes. (B) Clone 3 encodes the C-terminal two-thirds of the hnRNP F protein. Diagramatic structure of the partial cDNA (hnRNP F c-ter) rescued from a positive clone in the three-hybrid screen. The three RNA-binding domains (RBDs) of hnRNP F (20, 43) are shown as stippled boxes, and the RNP1 and RNP2 consensus sequences are shown as black bars. The other regions of the protein are shown as thick, patterned lines. The amino acid positions at the termini of hnRNP F c-ter and full-length hnRNP F are indicated.

mRNAs transcribed from genes lacking introns (37). Several other mammalian hnRNP proteins show shuttling behavior similar to that of A1 (12) and may also be involved in mRNA export. There is also accumulating evidence that Npl3p/Nop3p, a candidate yeast hnRNP protein, also has a role in mRNA export (33).

In an attempt to deepen our understanding of the mode of action of CBC, we carried out a screen for proteins with which

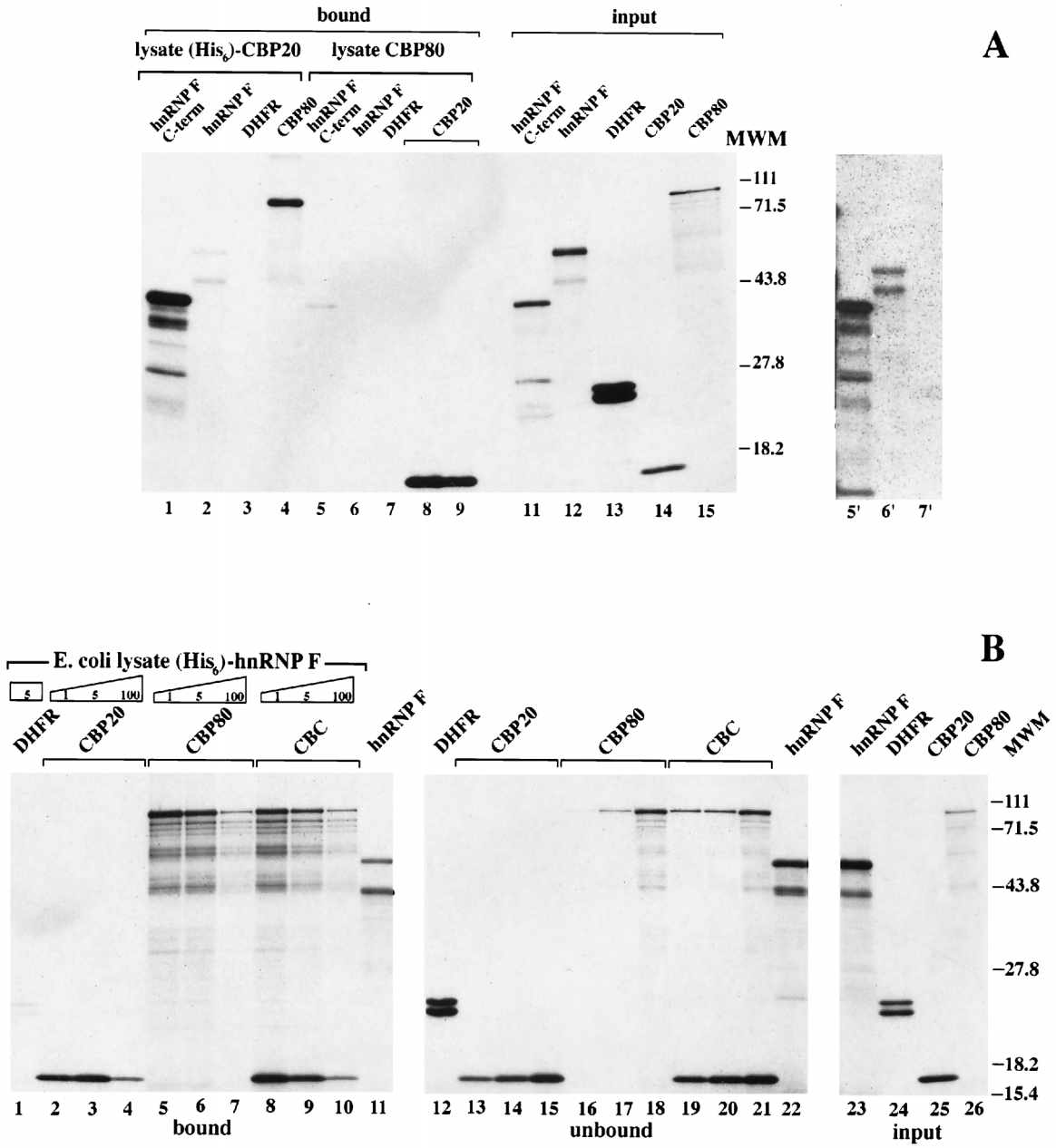

FIG. 2. CBC and hnRNP F interact in vitro. (A) In vitro-translated ^{35}S-labelled hnRNP F c-ter (C-term) and full-length hnRNP F were incubated with *E. coli* lysates from strains expressing His-tagged human CBP20 (lanes 1 to 4); the bound fractions were precipitated via Ni-NTA agarose resin, washed with buffer containing 25 mM imidazole, and eluted by boiling in protein sample buffer. The same in vitro-translated polypeptides were mixed and incubated with *E. coli* lysates expressing untagged human CBP80 (lanes 5 to 9), anti-CBP80 immune serum, and protein A-Sepharose. The bound fractions were recovered after washing in low-salt buffer and eluted as described above. One-fifth of the eluted fraction was resolved by SDS-PAGE. Lanes 11 to 15 contain the input polypeptides (1/50 of the amount in the binding reaction mixture). Lanes 3 and 7 show negative controls (DHFR), and lanes 4, 8, and 9 are positive controls (CBP80 and CBP20, respectively, for the CBP20 and CBP80 lysates). Lane 9 shows a binding reaction mixture identical to that in lane 8 except for the use of twice as much anti-CBP80 antiserum, a control to show that the amount of anti-CBP80 antibody used was not limiting. The migration positions of the protein molecular weight marker (MWM) are shown. On the right, lanes 5′ to 7′ show a five-times-longer exposure of lanes 5 to 7, respectively. (B) In vitro-translated CBP20 and CBP80 were incubated with increasing amounts of a lysate of *E. coli* expressing His-tagged hnRNP F and treated as described for panel A. One-fifth of each of the bound fractions was resolved by SDS-PAGE. Lanes 2 to 4, CBP20 alone; lanes 5 to 7, CBP80 alone; lanes 8 to 10, a mixture of CBP20 and CBP80; lane 1, DHFR (negative control). Lane 11 shows a positive control: in vitro-translated hnRNP F selected from a (His_6)-CBP20 lysate. One fiftieth of each unbound fraction was loaded to assay for protein degradation (lanes 12 to 22). Lanes 23 to 26 contain 1/50 of the input polypeptides. The migration positions of the protein molecular weight marker (MWM) are on the right.

it interacts. Among several candidate proteins identified was hnRNP F. Given the known functions of other hnRNP proteins, we examined the interaction between hnRNP F and CBC in some detail. We show that hnRNP F can interact separately with both CBP80 and CBP20. hnRNP F binds preferentially to CBC-RNA complexes compared to naked RNA, providing evidence that the hnRNP F-CBC interaction has functional consequences. We also show that, in addition to its previously defined specific role in splicing (48), depletion of hnRNP F has a more general effect on splicing efficiency in vitro.

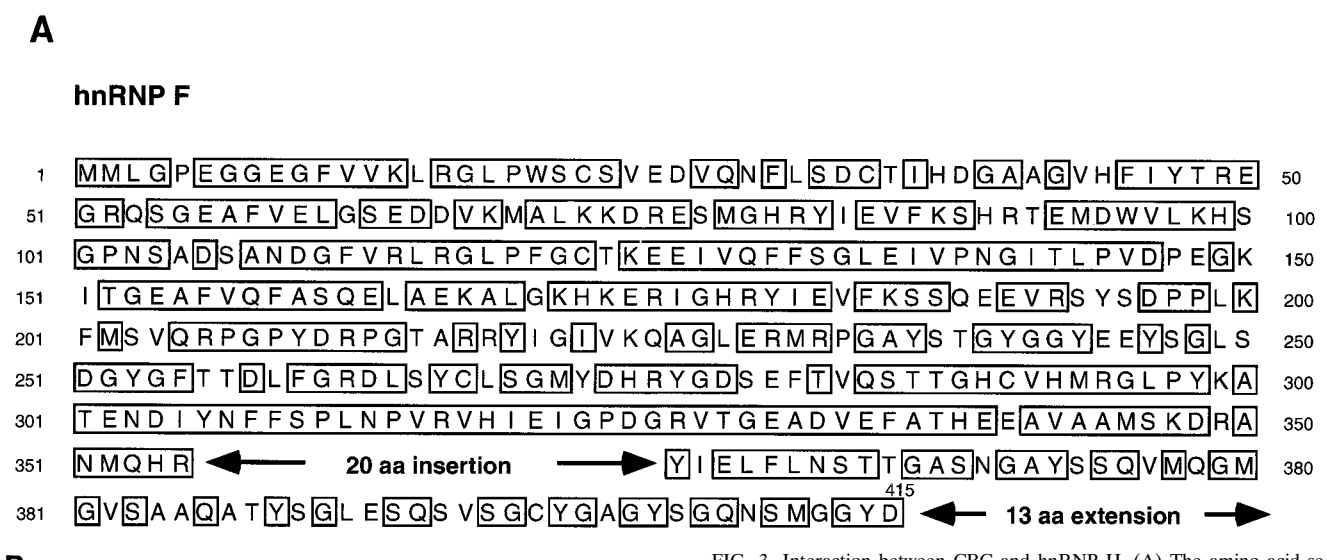

FIG. 3. Interaction between CBC and hnRNP H. (A) The amino acid sequence of hnRNP F. Residues that are identical in hnRNP F and hnRNP H are boxed. Positions of insertion of amino acids in hnRNP H compared to hnRNP F are also indicated. (B) CBC and hnRNP H interact in vitro. In vitro-translated, [^{35}S]methionine-labelled hnRNP H was tested for binding to His-tagged CBP20 or untagged CBP80 as described in the legend to Fig. 2. Lanes 1 to 3 show the proteins selected by $(His)_6$-CBP20 (hnRNP H, CBP80, and DHFR, respectively). Lanes 4 to 6 show the proteins selected by CBP80 and the anti-CBP80 antiserum (hnRNP H, CBP20, and DHFR, respectively). Lanes 7 to 10 contain the reference input polypeptides, hnRNP H, CBP80, CBP20, and DHFR, respectively. The migration positions of the protein molecular weight markers (MWM) are on the right.

MATERIALS AND METHODS

All restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were purchased from New England BioLabs. The DNA manipulations and cloning procedures were done as previously described (57). T7, T3, and SP6 RNA polymerases were from Promega, and AmpliTaq DNA polymerase was from Perkin-Elmer Cetus. The cap analog m^7GpppG (9) was a gift from E. Darzynkiewicz. The Protein A Sepharose Fast Flow was purchased from Pharmacia.

RNA probes were labelled according to standard protocols (57) with ^{32}P-labelled nucleotide triphosphates from Amersham International. DNA sequences were determined by the dideoxynucleotide chain termination method (58) with a kit purchased from Pharmacia.

Two- and three-hybrid screens. The bait plasmids were constructed as follows: the CBP20 open reading frame was amplified with AmpliTaq DNA polymerase and the oligonucleotides 5′CTCGAATTCATGTCGGGTGGCCTCCTG3′ and 5′CACCTCGAGCTGGTTCTGTGCCAGTTTTCC3′, the fragment was then cleaved with *Eco*RI and *Xho*I, and the end was repaired with T4 DNA polymerase and cloned in the plasmid pAS2 (13) at the end-repaired *Nde*I site to give pAS2-CBP20. The nucleotide sequence was checked. The cDNA for CBP80, as a *Bam*HI-*Xho*I fragment, was first inserted between the yeast alcohol dehydrogenase promoter and terminator cassettes in the pVT102U plasmid (65), and the resulting transcription unit was excised with *Sph*I, end repaired with T4 DNA polymerase, and cloned in the pAS2-CBP20 plasmid at the end-repaired *Sac*I site to give pAS2-CBC.

The *S. cerevisiae* Y190 strain (13) was first transformed with the pAS2-CBC bait plasmid according to a standard lithium acetate protocol (21) to give the 190-CBC strain. Expression of the bait proteins was checked by Western blotting. The strain was then transformed with a human cDNA library in the vector pACTII (13) according to a modified protocol (15a).

Transformants (6×10^6) were selected on SD plates lacking histidine, leucine, and tryptophan and containing 25 or 50 mM 3-aminotriazole (Sigma) and 65 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml.

Bait plasmids were cured from the positive cells by active selection on cycloheximide-containing media and were then mated to Y187 cells transformed with unrelated baits (CDK2, SNF1, and p53; the gift of S. Elledge), with the empty pAS2 plasmid or with the original bait plasmid. Library plasmids were extracted from positive yeast cells as previously described (54) and transformed into *Escherichia coli* cells by electroporation.

Cloning and production of recombinant hnRNP F and hnRNP H proteins. The hnRNP F cDNA was amplified by PCR from human cDNA with the oligonucleotides 5′GCCAGATCTCGCCATGGATGATGCTGGGCCCTGAG GGAG3′ and 5′CTCTAGATCTGGTACCGTCATAGCCACCCATGCTGTT C3′ as primers, digested with *Nco*I and *Bgl*II, and cloned in the vector pQE60 (Qiagen). The resulting C-terminal His-tagged protein was expressed in *E. coli* M15(pREP4) (66). The bacterial cells were grown in Luria broth containing 100 μg of ampicillin per ml; the optimal induction time was determined by checking the amount of protein produced in a time course experiment. The pelleted cells were then lysed in PGK buffer (50 mM sodium phosphate [pH 7.2], 100 mM KCl, 10% glycerol, 0.5% Triton X-100) containing 6 M guanidine–HCl and lysed with a French press. The recombinant protein was then purified by binding to nickel nitriloacetate agarose (Ni-NTA; Qiagen) as previously described (23). The activity of each eluted fraction was then tested for RNA binding by gel retardation assays. Only fractions displaying maximal RNA binding were capable of restoring splicing activity to extracts depleted of hnRNP F ("depleted extracts"), even if these fractions contained less hnRNP F protein. The hnRNP F cDNA was then cloned as a *Bam*HI-*Eco*RI fragment in the pGEX-2T plasmid, and the protein was expressed and purified from *E. coli* cells according to standard procedures (64). The hnRNP F cDNA was also cloned as a *Bgl*II fragment in the pBluescript II SK(+) vector (Stratagene) to give the plasmid pBS-hnRNPFT3 that was used to transcribe and translate hnRNP F in vitro. The hnRNP F c-ter protein was produced by PCR amplification of the insert contained in the clone originally isolated in the screen with the primers 5′TAATACGACTCACTATAGGGAG ACCACATGGATGATGTATATAACTATCATTTC3′ and 5′CTACCAGAAT TCGGCATGCCGGTAGAGGTGTGGTCA3′. The hnRNP H cDNA was amplified with AmpliTaq polymerase from human cDNA with the oligonucleotides

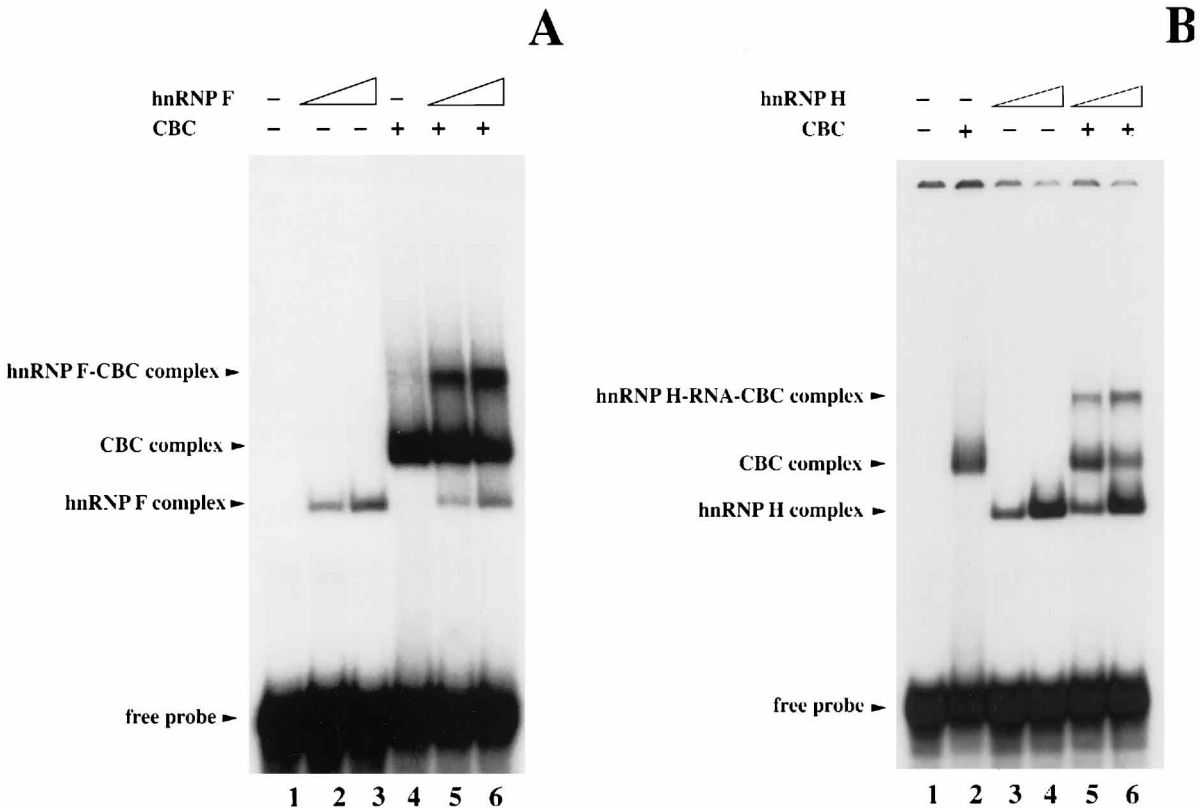

FIG. 4. hnRNP F binds preferentially to a CBC-RNA complex. (A) Recombinant hnRNP F or recombinant human CBC was incubated with a capped RNA substrate and analyzed by native gel electrophoresis in order to resolve the different RNA and RNA-protein complexes: free RNA (lane 1), hnRNP F-RNA (lanes 2, 3, 5, and 6), and CBC-RNA complexes (lanes 4, 5, and 6). In the presence of both hnRNP F and CBC, an additional slower-migrating complex was formed (lanes 5 and 6). (B) hnRNP H does not bind CBC-RNA complexes preferentially. The experiment is analogous to that used for panel A, except that recombinant hnRNP H was used instead of hnRNP F. Free RNA (lane 1), CBC-RNA complexes (lanes 5 and 6), hnRNP H-RNA complexes (lanes 3, 4, 5, and 6), or complexes formed by incubation of RNA with both CBC and hnRNP H (lanes 5 and 6) are indicated.

5′GCAGGATCCATGATGTTGGGCACGGAAGG3′ and 5′CGAGCCATGG TTACCTATGCAATGTTTGATTG3′ and cloned as a *Bam*HI-*Nco*I fragment in the pRSETA (Invitrogen) vector to give the plasmid pRSET-RNPH. The hnRNP H cDNA was also cloned as a *Bam*HI-*Xho*I fragment in the pBluescript II SK(+) vector to give the plasmid pBS-hnRNPHT3 that was used to transcribe and translate hnRNP H in vitro.

Production of antibodies against hnRNP F. Polyclonal antibodies were raised against an N-terminal histidine-tagged recombinant protein containing amino acids 283 to 415 of hnRNP F according to standard protocols (19). The antibodies were purified over an affinity column made by coupling a glutathione *S*-transferase-hnRNP F fusion, encoding the same amino acids, to Affigel 10 beads (Bio-Rad).

Electrophoretic mobility retardation assay. The 77-nucleotide RNA used as a probe was obtained by in vitro transcription of U1 SII− (59). Binding reactions were done in a 10-μl volume with 10% glycerol, 20 mM Tris-HCl (pH 7.4), 0.125 mM EDTA, 60 mM KCl, 5 mM dithiothreitol, 5 μg of yeast tRNA, 2 U of RNasin per μl, and approximately 5×10^4 cpm of labelled RNA. Samples were incubated at 30°C for 30 min and then loaded on 6% native polyacrylamide (60:1) gels in 1× Tris-borate-EDTA. The gels were run in 0.5× Tris-borate-EDTA at 10 V/cm and at room temperature.

Pull down assays and immunoprecipitations. The lysates from *E. coli* strains expressing recombinant proteins were prepared in PGK buffer (see above) at a ratio of 1 g (wet weight) of cells to 25 ml of buffer, and the cell suspension was sonicated extensively and centrifuged for 15 min at 12,000 × *g* at 4°C. The cleared supernatant was then used for the binding assays.

The amount of lysate used in each interaction assay was variable, depending on the expression level of the different proteins, and was determined empirically. When the lysates had to be diluted more than twofold for the reaction, a blank lysate, prepared from bacteria not expressing recombinant proteins, was used as a diluent.

The proteins were in vitro translated either from RNAs transcribed in vitro according to standard procedures (57) or from plasmids by using the TNT coupled transcription-translation system (Promega) according to the manufacturer's recommendations.

The assays were performed in a 500-μl volume by slow rotation of the mixtures for 2 h at room temperature. The bound fraction was washed with PGK buffer containing 25 mM imidazole four times for 5 min each time and then eluted by boiling in 2× protein sample buffer (19).

Immunoprecipitations were carried out in IPP150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Nonidet P-40) from a mixture including bacterial lysate containing rCBP80, in vitro-translated proteins, anti-CBP80 immune serum (24), and protein A-Sepharose (Pharmacia); samples were incubated with slow rotation for 2 h at room temperature. Beads with bound proteins were washed with IPP150 buffer as described above and eluted by boiling in 2× protein sample buffer. One-fifth of the bound fractions was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures (19), and the gels were treated with Entensify (Dupont) according to the recommendations of the manufacturer.

Immunodepletion and in vitro splicing assays. The immunoaffinity matrix was prepared as previously described (24), except that a different ratio of serum to Protein A Sepharose Fast Flow was used (1:6.5). The beads for the mock depletion were prepared by coupling immunoglobulins G from the serum of a nonimmune rabbit to the resin. HeLa splicing extracts were prepared as described previously (10) and were brought to 500 mM KCl prior to depletion. Depletions were carried out at 500 mM KCl as described previously (24), except that a 1:2.5 ratio of beads to extract was used. Extracts were then dialyzed against buffer D containing 100 mM KCl.

The levels of depletion were estimated by Western blotting with anti-hnRNP F monoclonal antibody 7C2 (43) or with anti-CBP80 immune serum used as described previously (6, 24).

Splicing assays were carried out essentially as previously described (24), except that the optimal $MgCl_2$ concentration was determined empirically for the extract. The spliced products were resolved on 10% polyacrylamide–urea gels (40:1). The pre-mRNA splicing probes for the adenovirus major late and the chicken δ-crystallin mRNAs were transcribed from linearized plasmids (pBSAd1 [31] and pSP14-15 [56], respectively), using T3 and SP6 RNA polymerases.

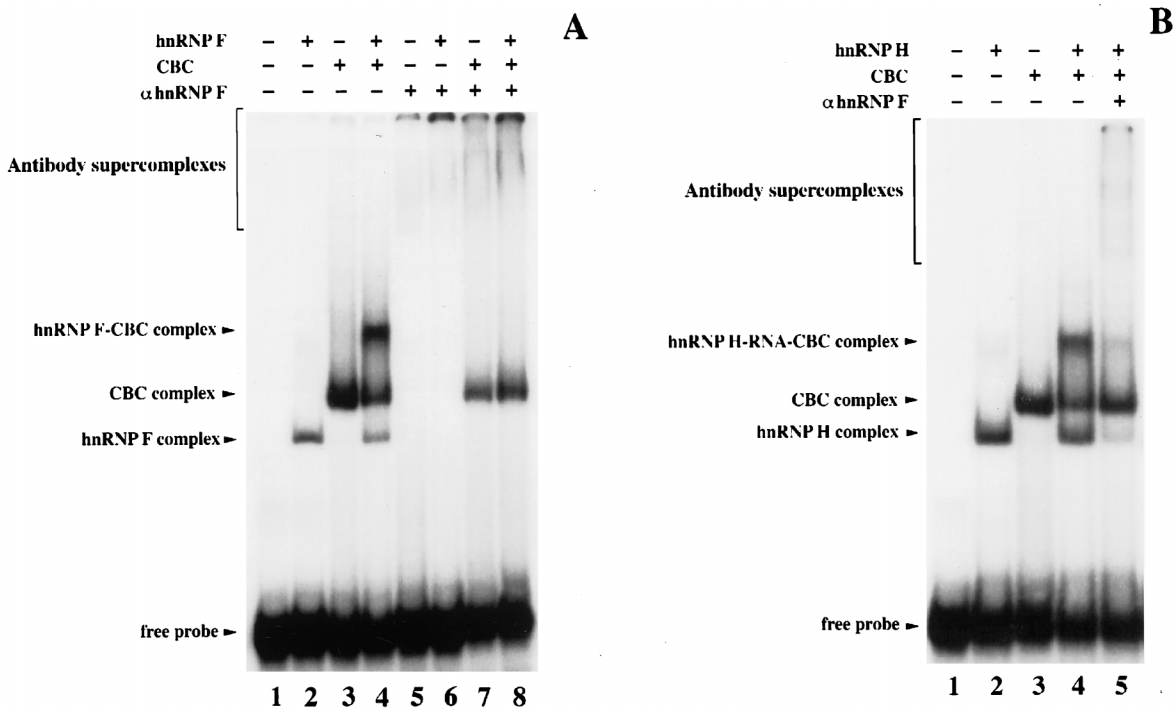

FIG. 5. Identification of hnRNP F- and H-containing RNA complexes. (A) Analysis of hnRNP F-containing complexes. The RNP complexes indicated on the left were formed as described in the legend to Fig. 4. Identical reactions were performed either in the absence (lanes 1 to 4) or in the presence (lanes 5 to 8) of affinity-purified polyclonal anti-hnRNP F antibody. (B) Analysis of hnRNP H-containing complexes. The RNP complexes indicated on the left were formed as described in the legend to Fig. 4. Lane 1, free RNA; lane 2, hnRNP H and RNA; lane 3, CBC and RNA; lane 4, hnRNP H, CBC, and RNA; lane 5, hnRNP H, CBC, RNA, and affinity-purified anti-hnRNP F antibody.

RESULTS

The three-hybrid screen. The active form of CBC requires the presence of both subunits (23, 24). In order to identify factors able to interact with the assembled human CBC, we coexpressed the large subunit, CBP80, in an untagged form in the GAL4-CBP20-expressing strain (Fig. 1A). This CBC-expressing strain was then transformed with a human lymphocyte cDNA library (13), and a total of 6×10^6 transformants was plated on selective medium. Five blue colonies were picked, and the plasmids were rescued in *E. coli* and retransformed into the CBC-expressing strain or in Y190 strains containing other, unrelated baits or the empty vector. Three clones were identified as being positive in the second screen, and these plasmids were subjected to further analysis. Sequencing of the inserted cDNAs revealed that two of them are novel clones; they will be described in more detail elsewhere. The third clone encoded the C-terminal two-thirds of the hnRNP F protein (43) (hnRNP F c-ter) (Fig. 1B).

hnRNP F interacts with CBP80 and CBP20. The abilities of hnRNP F c-ter and of full-length hnRNP F to bind either CBC or CBP20 or CBP80 individually in vitro were next tested. An *E. coli* lysate from a strain expressing (His_6)-CBP20 was incubated with in vitro-translated hnRNP F and hnRNP F c-ter. Dihydrofolate reductase (DHFR) and CBP80 were used as a negative control and a positive control, respectively. Proteins bound to CBP20 were selected on Ni-NTA agarose. None of the proteins bound to Ni-NTA agarose in the absence of (His_6)-CBP20 (data not shown). The bound fraction was recovered and separated by SDS-PAGE (Fig. 2A, lanes 1 to 4). As expected, CBP80 was found to bind to CBP20, while DHFR was not detectably associated (Fig. 2A, lanes 3 and 4). Both hnRNP F c-ter and, although with reduced efficiency, full-length hnRNP F were coprecipitated with (His_6)-CBP20 (Fig. 2A, lanes 1 and 2). To test the interaction of hnRNP F with CBP80, an *E. coli* lysate from a strain expressing untagged human CBP80 was incubated with in vitro-translated hnRNP F c-ter, hnRNP F, DHFR, or CBP20 and anti-CBP80 immune serum. The bound fractions were recovered by binding to protein A-Sepharose and then separated electrophoretically (Fig. 2A, lanes 5 to 9). CBP20 was efficiently coprecipitated, and antibody was in excess (Fig. 2A, lanes 8 and 9). Small quantities of both hnRNP F c-ter and hnRNP F were specifically precipitated (Fig. 2A, lanes 5 and 6 and 5′ to 7′).

Thus, both hnRNP F and hnRNP F c-ter can interact with CBP20 and CBP80. It was difficult to assess the relative affinities of hnRNP F for CBP20 and CBP80, due to the differences in the assay conditions. The weaker signal obtained for the binding to CBP80 could, for example, be a reflection of interference of bound antibodies with the interaction between CBP80 and hnRNP F.

To confirm the binding interactions detected as described above, we next expressed a (His_6)-hnRNP F protein in *E. coli* and used the corresponding lysate to examine binding to in vitro-translated CBP20 and CBP80 by means of Ni-NTA agarose. CBP20 was bound in the presence of various amounts of hnRNP F-containing bacterial lysate (Fig. 2B, lanes 2 to 4 and 13 to 15). The bound fractions in the case of either CBP80 alone or the mixture of CBP20 and CBP80 are shown (Fig. 2B, lanes 5 to 7 and 16 to 18 and 8 to 10 and 19 to 21, respectively.) The negative control, DHFR, did not bind to hnRNP F (Fig. 2B, lane 1). As a positive control, in vitro-translated hnRNP F binding to (His_6)-CBP20 was included (Fig. 2B, lanes 11 and 22). hnRNP F bound CBP20 and CBP80 at comparable levels,

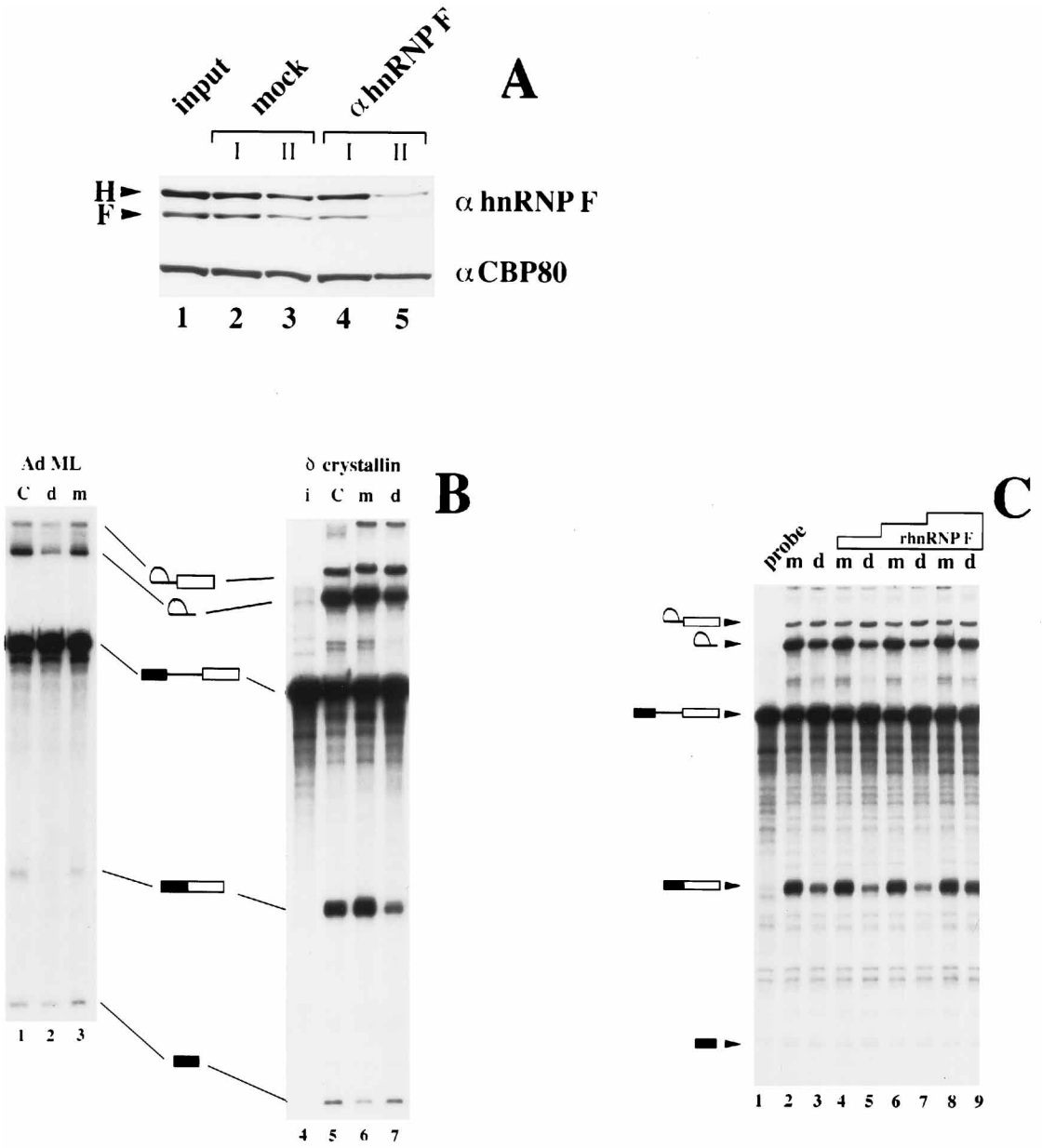

FIG. 6. Depletion of HeLa cell extract with anti-hnRNP F antibodies reduces splicing efficiency. (A) Depletion with anti-hnRNP F antibodies. HeLa cell nuclear extract was passed twice over beads to which affinity-purified antibodies from either nonimmune or immune serum raised against recombinant hnRNP F had been coupled. Depleted or control extracts were fractionated by SDS-PAGE and analyzed by Western blotting with either monoclonal antibody 7C2, which interacts with both hnRNP F and hnRNP H (43), or polyclonal anti-CBP80 antiserum (24). Lane 1, nondepleted extract; lanes 2 and 3, mock-depleted extract after one (lane 2) or two (lane 3) rounds of depletion; lanes 4 and 5, hnRNP F-depleted extract after one (lane 4) or two (lane 5) rounds of depletion. (B) Splicing in hnRNP F-depleted HeLa nuclear extracts. The splicing of an adenovirus major late (lanes 1 to 3) or a δ-crystallin (lanes 4 to 7) pre-mRNA were analyzed in mock-depleted or depleted extracts. Lanes 1 and 5, splicing in untreated extract; lanes 3 and 6, splicing in mock-depleted extract; lanes 2 and 7, splicing in hnRNP F-depleted extract. Lane 4 contains the unspliced precursor δ-crystallin RNA. The positions of pre-mRNA, intermediates, and products of the reactions are indicated. (C) Addition of recombinant hnRNP F (rhnRNP F) increases splicing in a depleted extract. Reactions were performed as described for panel B, using δ-crystallin pre-mRNA as the splicing substrate. Lane 1, pre-mRNA; lanes 2 and 3, splicing in mock-depleted (m) or depleted (d) extract as indicated; lanes 4 to 9; splicing in mock-depleted (m) or depleted (d) extracts to which increasing amounts of rhnRNP F protein were added. The splicing reactions were all performed in the same final volume; to achieve identical reaction volumes, buffer D (10) was added to compensate for the different amounts of recombinant protein added.

whether they were present alone or in combination in the binding mixture.

The addition of increasing amounts of hnRNP F lysate led to a decreased recovery of both CBPs (Fig. 2B, lanes 4, 7, and 10). This result is probably explained by the ability of hnRNP F to dimerize or multimerize, as indicated by the fact that ^{35}S-labelled hnRNP F binds to immobilized (His_6)-hnRNP F (data not shown). In order to exclude the possibility that the interaction between CBP20 or CBP80 and hnRNP F was dependent on binding to RNA, we performed the same assays in the presence of excess RNase A. Under these conditions, we did not detect any reduction in the interactions (data not shown).

hnRNP F is very closely related (with identities of 78 and 75%, respectively) to at least two other hnRNP proteins,

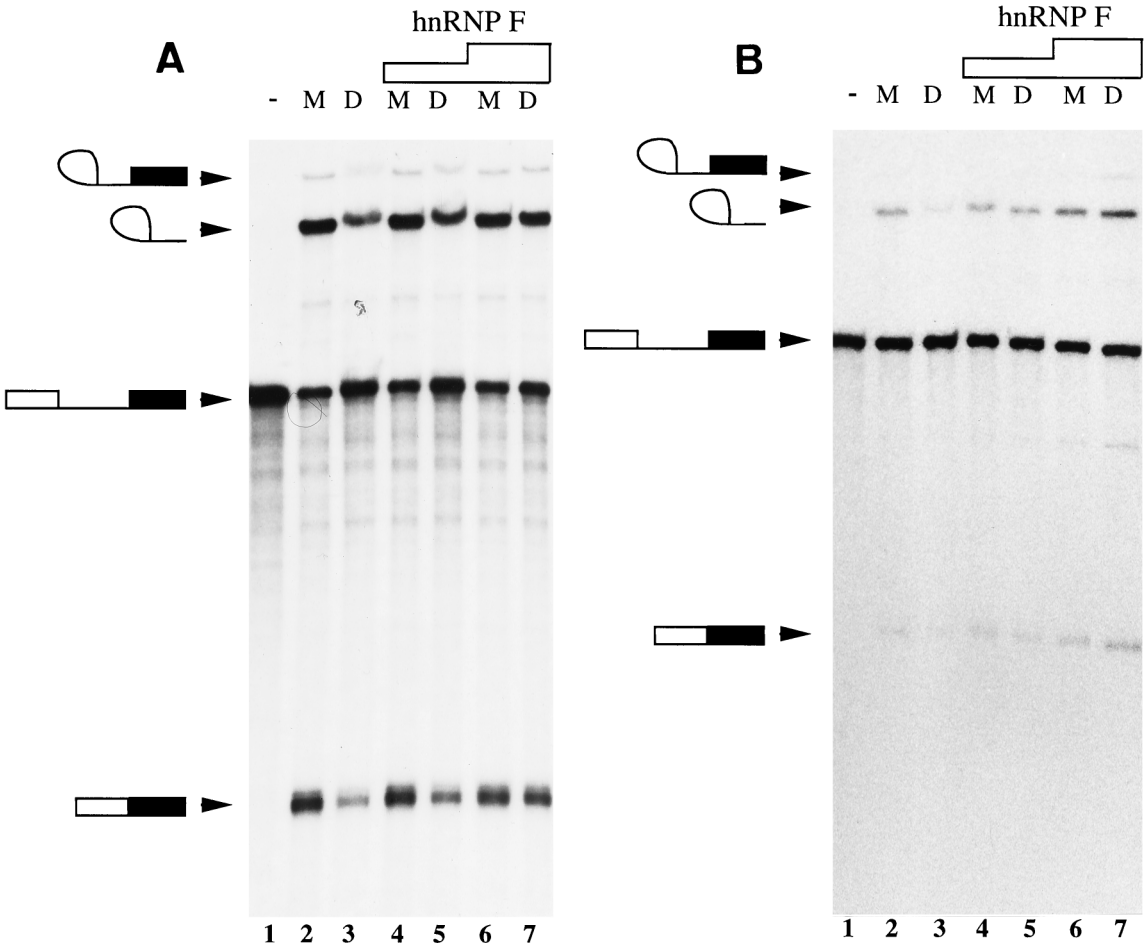

FIG. 7. Splicing in hnRNP F-depleted extract. (A) Splicing of a δ-crystallin pre-mRNA (lane 1) in mock-depleted (M) or anti-hnRNP F-depleted (D) HeLa cell nuclear extract in the absence (lanes 2 and 3) or in the presence of increasing quantities (lanes 4 to 7) of purified recombinant hnRNP F. (B) Splicing of an adenovirus major late pre-mRNA (lane 1) in mock-depleted (M) or anti-hnRNP F-depleted (D) HeLa cell nuclear extract in the absence (lanes 2 and 3) or in the presence of increasing quantities (lanes 4 to 7) of purified recombinant hnRNP F.

hnRNP H and hnRNP H′ (20, 43) (Fig. 3A). hnRNP H interaction with the CBC proteins was therefore assayed by the methods used in the experiment whose results are shown in Fig. 2A. hnRNP H was found to interact with both His-tagged CBP20 (Fig. 3B, lane 1) and nontagged CBP80 (Fig. 3B, lane 4). Again, DHFR (Fig. 3B, lanes 3 and 6) and CBP80 or CBP20 (Fig. 3B, lanes 2 and 5, respectively) served as negative and positive controls, respectively. No DHFR binding was seen even after longer exposure of the autoradiograph.

CBC affects hnRNP F RNA binding. As an initial test of the possible functional consequences of interaction between CBC and hnRNPs F and H, the ability of the proteins to affect each other's RNA binding in vitro was tested. Recombinant hnRNP F and CBC purified from *E. coli* both bind to a capped RNA probe (Fig. 4A, lanes 1 to 4). In the presence of CBC, a significantly larger amount of hnRNP F appeared to bind to RNA than in its absence (Fig. 4A, lanes 5 and 6). Quantitation of the effect of CBC on the formation of hnRNP F-containing complexes in several experiments showed that in the presence of CBC, between three- and fourfold more hnRNP F complexes were detected. To prove that the additional complex migrating more slowly than the CBC-RNA complex in lanes 5 and 6, Fig. 4A, was indeed due to hnRNP F binding, the effect of adding affinity-purified polyclonal antibodies directed against hnRNP F to the reaction mixture was examined. The complexes proposed to contain either hnRNP F alone or hnRNP F plus CBC were either disrupted or further retarded by the anti-hnRNP F antibodies (Fig. 5A, compare lanes 2 to 4 with lanes 6 to 8), whereas the CBC-RNA complexes were not greatly affected. In conclusion, hnRNP F binds preferentially to a CBC-RNA complex rather than to RNA alone.

We next determined whether this effect would also be observed with hnRNP H. In contrast to the result obtained with hnRNP F, the presence of CBC had little or no effect on the total amount of recombinant hnRNP H-containing RNA complexes (Fig. 4B, lanes 3 to 6). Although some hnRNP H did bind to CBC-RNA complexes, most was still found in the hnRNP H-RNA form. The increase in hnRNP H-containing complexes detectable in the presence of CBC was measured to be only 1.2-fold. Since the polyclonal antibody raised against hnRNP F cross-reacts with hnRNP H (data not shown), we could use this antibody to show that the putative hnRNP H complexes indeed contained the protein (Fig. 5B, lanes 4 and 5). Thus, even if both hnRNP proteins can interact with the CBC proteins in solution, only hnRNP F shows preferential binding to a CBC-RNA complex rather than to naked RNA.

Depletion of hnRNP F affects in vitro splicing efficiency. CBC has two known functions, in pre-mRNA splicing and U

snRNA nuclear export. While hnRNP proteins in general may affect many aspects of RNA metabolism, the only defined role for hnRNP F is in a particular example of neuron-specific pre-mRNA splicing (48). Since the role of CBC in pre-mRNA splicing seems to be general, it was of interest to determine whether hnRNP F might also play a more general role. HeLa cell nuclear extract was therefore depleted of hnRNP F by repeated passage over beads to which polyclonal antibodies raised against hnRNP F were bound. This procedure resulted in a significant depletion of hnRNP F protein as measured by Western blotting with a monoclonal antibody, 7C2, that interacts with both hnRNP F and hnRNP H (43) (Fig. 6A, lane 5). As expected from the cross-reactivity mentioned above, hnRNP H levels in the depleted extract were also reduced. No detectable reduction in CBP80 (Fig. 6A) was seen, indicating that the depletion was specific and that no significant fraction of the CBC in a HeLa cell nuclear extract is tightly associated with hnRNP F.

The effect of depletion on splicing was initially tested with two different pre-mRNA substrates, one derived from adenovirus (31) and the other derived from the δ-crystallin gene (51, 56). Although mock depletion had some effect on splicing of both the pre-mRNAs (Fig. 6B, lanes 1, 3, 5, and 6), this effect was less than that caused by hnRNP F depletion (Fig. 6B, lanes 2 and 7). In neither case was splicing completely inhibited. It should, however, be noted that hnRNP F was not entirely removed by depletion (Fig. 6A). Further reduction in hnRNP F levels could not be achieved under conditions in which pre-mRNA splicing activity was retained in extracts which had undergone mock depletion ("mock-depleted extracts") (data not shown). The splicing of additional substrates tested, derived from the *Xenopus* ribosomal protein LI gene or from the *Drosophila transformer* or *fushi tarazu* genes, was also reduced by the immunodepletion.

If the effect of depletion is specific, then it should be reversed by the readdition of hnRNP F protein to the depleted extract. The splicing of δ-crystallin pre-mRNA was therefore monitored in mock-depleted or depleted extract to which increasing amounts of recombinant hnRNP F were added. hnRNP F addition partially restored splicing activity (Fig. 6C, lanes 2 to 9). Addition of larger quantities of hnRNP F did not lead to greater recovery of activity but, rather, to increased inhibition of splicing (data not shown). In view of the incompleteness of both the depletion of activity and of its restoration by addition of hnRNP F, it was important to document the reproducibility of the effects seen. In Fig. 7, the effect on splicing of a δ-crystallin (Fig. 7A) and an adenovirus major late (Fig. 7B) pre-mRNA are shown. The extracts and preparations of recombinant hnRNP F used in these experiments are different from those used for Fig. 6, and these experiments demonstrate the reproducibility of the inhibition of hnRNP F activity on immunodepletion (Fig. 7 lanes 1 and 3 [both panels]) and the generally partial restoration of activity after addition of recombinant hnRNP F (Fig. 7 lanes 4 to 7 [both panels]). When recombinant hnRNP H, which was codepleted with hnRNP F (Fig. 6A), was added to depleted extract either alone or in combination with hnRNP F, no effect on splicing activity was detected (data not shown). In spite of this negative result, it is possible that additional factors, including members of the hnRNP F-H subfamily, might be codepleted with hnRNP F and be responsible for the lack of complete restoration of activity we saw in most add back experiments. Examination of the reduction in accumulation of splicing intermediates and products (see particularly Fig. 6B and C) indicated that both steps of splicing were affected by hnRNP F depletion. Accumulation of the products of the first step of splicing were not reduced in parallel with products of the second step, indicating a more severe effect on the second step than on the first step. On the other hand, in all of the experiments carried out in depleted extracts, the amount of first-step products accumulating was insufficient to account for the reduction in second-step products. The quantities of first-step products observed were usually virtually identical in mock-depleted and depleted extracts (Fig. 6B and C). This indicates that the first step in splicing is also affected by hnRNP F depletion.

DISCUSSION

Using a variation of the two-hybrid screening method (14), we have selected several human cDNAs encoding proteins that interact either with CBP20 or with CBC. One of the proteins that interacted with CBC was the previously characterized hnRNP F protein (43). Because of the dual role of CBC in pre-mRNA splicing and U snRNA nuclear export (8, 24, 35, 36) and since hnRNP proteins have been implicated in both of these aspects of cellular RNA metabolism (11, 46), we have characterized this interaction in detail. Interestingly, hnRNP F can interact with either CBP80 or CBP20 individually. In addition, hnRNP F appears to interact with CBC as an RNA-bound heterodimer, as demonstrated by the preferential binding of hnRNP F to CBC-RNA complexes in vitro.

hnRNP F is a member of a subfamily of hnRNP proteins which includes at least the F, H, and H′ proteins (20, 43). These proteins are highly related; hnRNPs H and H′ are 96% identical to each other and 78 and 75% identical to hnRNP F, respectively. In spite of this similarity and in spite of the fact that hnRNP H interacts with both CBP20 and CBP80 individually, we did not detect interaction between CBC and hnRNP H in a more functional context, as measured by the lack of effect of CBC on hnRNP H binding to RNA. The least similar regions of the three proteins are located at their C termini, but further study will be required to define the regions of hnRNPs F and H required for interaction with the individual CBC proteins and with RNA-bound CBC.

hnRNP F has previously been shown to be a component of a complex that is responsible for activation of a splicing event that results in the inclusion of a neuron-specific exon in the c-*src* gene (3, 4, 48). We show here that hnRNP F also plays a more general role in splicing, since depletion of the protein results in a reduction of the in vitro splicing efficiency of several introns tested. In their study of the role of hnRNP F in c-*src* splicing, Min et al. (48) failed to see inhibition of splicing of an adenovirus intron similar to the one used here on addition of a monoclonal antibody against hnRNP F to HeLa nuclear extract. Presumably, the different results obtained reflect the considerable differences in experimental design between the two studies. In the one case, a specific monoclonal antibody was added to the splicing extract, and in the other, hnRNP F protein was immunodepleted from the extract with polyclonal antibodies. The specificity of at least part of the reduction in splicing observed on depletion with anti-hnRNP F antibodies in our study was proven by the partial restoration of splicing activity observed upon readdition of recombinant hnRNP F. Since we are unable to totally remove hnRNP F from extracts under conditions which are consistent with splicing activity in control extracts, we cannot say whether or not hnRNP F is essential for in vitro splicing. Previous work has suggested a general role for hnRNP proteins in pre-mRNA splicing (7, 16), but hnRNP F is the first individual hnRNP protein for which such a role has been demonstrated by depletion and adding back of the protein. In contrast to the lack of compelling previous evidence for a general role in splicing of a specific

hnRNP protein, it is clear that hnRNP F, hnRNP A1, *Drosophila* hrp40, and probably other *Drosophila* hnRNP proteins are involved in the regulation of specific pre-mRNA splicing events (see Introduction). The existence of both a general (this study) and at least one intron-specific (48) function for hnRNP F is reminiscent of the dual role played by some SR splicing factors, which are required for both constitutive splicing and the regulation of particular alternative splicing events (reviewed in references 15 and 38). Both SR proteins and hnRNP proteins are families of highly abundant nuclear RNA-binding proteins, and our data indicate that they may be more functionally similar than has been generally believed.

CBC is required for efficient removal of cap-proximal introns in vitro and in vivo (24, 36). CBC acts to allow efficient commitment or E complex assembly and, more directly, to allow efficient binding of U1 snRNP to the 5′ splice site (8, 35, 36). It is, however, unclear how this is achieved. CBC does not appear to interact directly with U1 snRNP, suggesting the involvement of one or more proteins that mediate its function in pre-mRNA splicing (36). hnRNP F is an attractive candidate for such a protein. The fact that hnRNP F binds preferentially to CBC-RNA complexes rather than to RNA suggests a model by which CBC might act to seed the formation of a specific hnRNP–pre-mRNA complex, beginning by CBC favoring the binding of some hnRNP proteins, like hnRNP F, over others, like hnRNP H. The RNA-bound hnRNP F would then influence the binding of subsequent hnRNP proteins, contributing to the established variability in hnRNP composition on different nascent transcripts and pre-mRNAs (2, 41). Further study will be required to examine the accuracy of this model and its possible contribution to CBC function. The fact that hnRNP F depletion affects both steps of splicing whereas the effect of CBC is restricted to the first step means that, even if hnRNP F is involved in mediating CBC function, this is unlikely to be its only function in splicing.

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