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A potential splicing factor is encoded by the opposite strand of the trans-spliced c-*myb* exon

(RNA binding/ribonucleoprotein 80/arginine/serine rich/trans-splicing/bidirectional transcription)

MICHEL VELLARD*, ALAIN SUREAU*, JOHANN SORET*, CÉCILE MARTINERIE*, AND BERNARD PERBAL*†‡

*Laboratoire d'Oncologie Virale et Moléculaire, Batiment 110, Institut Curie, Centre Universitaire, 91405 Orsay Cedex, France; and †Université Paris VII, Paris, France

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ABSTRACT **We previously established that the expression of a thymic c-myb mRNA species requires the intermolecular recombination of coding sequences expressed from transcriptional units localized on different chromosomes, in both chicken and human. We now report that a putative splicing factor (PR264), extremely well conserved in chicken and human, is encoded by the opposite strand of the c-*myb* trans-spliced exon. The PR264 polypeptide, which contains a typical ribonucleoprotein 80 and an arginine/serine-rich domain, is highly homologous to the *Drosophila* splicing regulators tra, tra-2, and su(w^a) and to the human alternative splicing factor ASF/SF2. Furthermore, we show that PR264-specific mRNAs are expressed in normal hematopoietic cells of chicken and human origin and that the relative proportion of the PR264 transcripts is developmentally regulated in chicken.**

In higher eukaryotes, alternative mRNA splicing pathways play a key role in controlling gene expression (1). Among the specific advantages inherent in these posttranscriptional processes, the most obvious one is that the regulated use of alternative splice sites or polyadenylylation signals can modulate gene expression at either a qualitative or a quantitative level (2). In the past few years, genetic studies have led to the identification of several trans-acting factors able to modulate alternative splicing pathways in a tissue-specific or developmentally regulated manner (2–4). Three *Drosophila* genes, sex lethal (*Sxl*), transformer (*tra*), and transformer 2 (*tra-2*) code for splicing regulators involved in sex determination (5–7), and a fourth gene, the suppressor of white apricot [*su(w^a)*], encodes a trans-acting factor that exerts an autoregulatory function through the production of nonfunctional *su(w^a)* mRNA (8). Recently, human cDNAs coding for a splicing regulator designated alternative splicing factor (ASF) (9) or SF2 (10) have been isolated. The ASF/SF2 factor plays a general role in the splicing reaction and influences the selection of splice sites *in vitro* (11).

During our studies on tissue-specific expression of the c-*myb* protooncogene, we had reported (12, 13) that, in thymic cells, c-myb RNA species result from the trans-splicing of coding sequences localized on different chromosomes, in both chicken and human. Recently, we established that the region encompassing the c-*myb* trans-spliced exon (ET) is subjected to a bidirectional transcription (14) and isolated cDNAs corresponding to antisense mRNA species. Here, we report that, in chicken and human, the opposite strand of the ET locus encodes an extremely well-conserved protein (PR264), which probably belongs to the splicing regulator family.§ We also show that three major PR264 mRNAs are expressed in chicken hematopoietic cells and that their relative abundance is developmentally regulated.

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MATERIALS AND METHODS

Screening of cDNA Libraries. Preparation of the chicken thymus cDNA library has been described (15). The human bone marrow cDNA library was purchased from Clontech. The chicken and human cDNA libraries were screened with the ^{32}P-labeled H521 and H230 genomic probes, respectively (12).

Nucleotide Sequencing. Dideoxynucleotide sequencing reactions were done with deoxyadenosine 5′-[α-[^{35}S]thio]-triphosphate and M13 single-stranded recombinant templates according to Pharmacia. G+C compressions were resolved by using the deaza-T7 sequencing kit (Pharmacia). Sequence data treatments were done by using computer facilities at the Centre Interuniversitaire de Traitement de l'Information in Paris (16).

RNA Purification and Analysis. Chicken tissues (White Leghorn, Edinburgh strain) were from the Institut National de la Recherche Agronomique, Nouzilly, France. The human thymic tissue was a surgery sample from a 7-month-old female. RNA preparation, selection of polyadenylylated species, and Northern (RNA) blotting were done as has been described (17).

Probes, Hybridization Procedures, and Blot Scanning Analyses. The chicken BE80 double-stranded probe (ref. 14) contains the entire coding sequence of the PR264CH (chicken) cDNA clone. The M552 single-stranded probe was obtained after subcloning of BE80 probe into vector M13mp19 (14). The C12 oligonucleotide (TCTCCTAGGGCTGCGGCTGC) is complementary to the chicken sequence delineated by nucleotides (nt) 389–408 in Fig. 1. The human H230 probe has been described elsewhere (12). The H1 oligonucleotide (CCGTCCATGGCATCCATAGC) is complementary to the human sequence delineated by nt 247–266 in Fig. 1. Labeling of probes and hybridization experiments were done according to Perbal (17). Autoradiograms were scanned with an LKB-Pharmacia Ultroscan XL densitometer.

***In Vitro* Transcription and Translation.** The 800-bp *Eco*RI–*Bam*HI fragment containing the entire coding sequence of the PR264CH cDNA (14) was inserted into the pBluescript II SK vector (Stratagene). After linearization at the *Bam*HI site, capped RNAs were transcribed by T7 RNA polymerase (Promega) under conditions described by the manufacturer. *In vitro* translation occurred in a 50-μl reaction mixture containing 70% nuclease-treated rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Proteins were resolved on a SDS/15% polyacrylamide gel and visualized by autoradiography.

Abbreviations: ET, c-*myb* trans-spliced exon; aa, amino acid(s); RNP, ribonucleoprotein; ASF, alternative splicing factor; snRNP, small nuclear RNP; nt, nucleotide(s).

‡To whom reprint requests should be addressed.

§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X62446 and X62447).

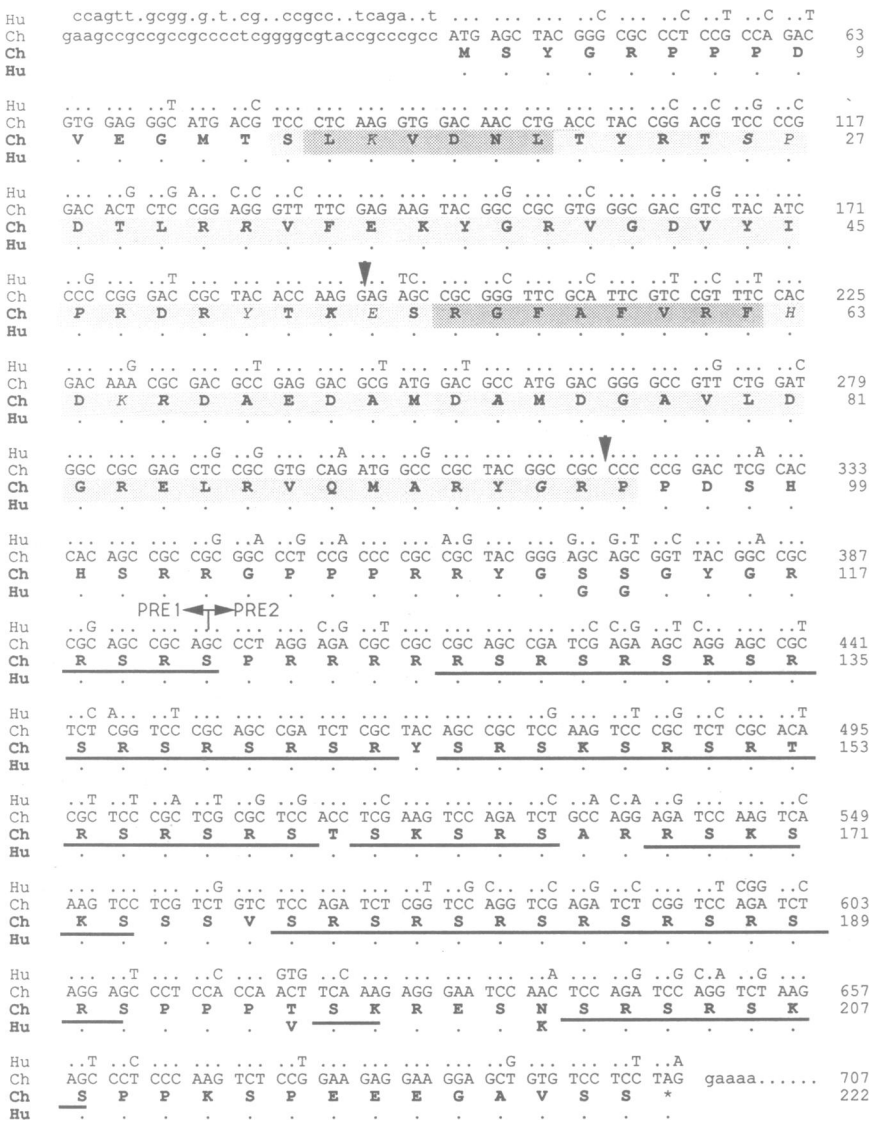

FIG. 1. Amino acid sequence and structural organization of chicken and human PR264 polypeptides. Coding sequences are indicated in capital letters. Chicken (Ch) and human (Hu) sequences were established from the 800-base-pair (bp) *Eco*RI–*Bam*HI fragment of PR264CH cDNA and from the 950-bp *Eco*RI–*Hin*dIII fragment of PR264HU cDNA, respectively. Only divergent nucleotides and amino acids found in human sequences are indicated. Position of ribonucleoprotein (RNP) 80 [amino acids (aa) 15–94] is indicated by light shaded box; darker shading within the box delineates RNP2 and RNP1 sequences. Amino acids diverging from the consensus are in italics; among them, those found at the same position in other RNP80 domains are in boldface italics. Arrowheads delineate sequences complementary to ET. The boundary between PRE1 and PRE2 is indicated by opposite arrows. Arginine/serine (RS) or lysine/serine (KS) repeats are underlined.

RESULTS

Antisense mRNA Species Complementary to ET Locus Encode a Potential Splicing Regulator in Chicken and Human. Screening of a chicken thymic cDNA library previously allowed us to isolate a 2.6-kilobase (kb) cDNA (PR264CH) corresponding to an antisense mRNA encoded by the opposite strand of the ET locus (14). In this cDNA, a 663-bp open reading frame, which overlaps—in the opposite orientation—the ET coding sequence expressed in thymic c-myb mRNA species (nt 195–319 in Fig. 1), encodes a putative 221-aa polypeptide. The PR264 open reading frame starts at nt 36 with an ATG initiation codon included in the CCGCCCGC-CATGA sequence, which agrees fairly well with the eukaryotic translation start point consensus motif (18).

After screening a human bone marrow cDNA library with the H230 genomic probe (12), we isolated a cDNA (PR264HU) homologous to PR264CH cDNA. Fig. 1 shows that human and chicken PR264 coding sequences are highly conserved (83% homology), whereas they diverge considerably in noncoding regions, especially upstream to the potential ATG initiation codon. The chicken and human PR264 polypeptides are even more conserved (98% homology), strongly suggesting an important biological role for them.

In both species, the PR264-coding sequence is split into two exons (PRE1 and PRE2), which interestingly define two distinct domains sharing a high degree of homology with two sets of consensus sequences previously described in RNA processing factors (see *Discussion*). The first domain extends from aa 15 to 94 and consists of a typical RNP80 or RNA-binding domain (19, 20), whereas the second domain, which broadly extends from aa 117 to 207, is a very basic region characterized by 26 repetitions of an arginine/serine (RS) motif and additional scattered arginine and lysine residues. In both PR264 proteins, the two domains are separated by a proline-rich stretch of 22 residues (aa 95–116) that may be involved in polypeptide folding, so as to allow proper protein–protein or protein–RNA interactions (Figs. 1 and 4*C*).

Expression of PR264 Proteins. After *in vitro* transcription of the pBluescript-PR264CH clone, capped RNAs were *in vitro*-translated in a rabbit reticulocyte lysate. As expected from studies of the 70-kDa small nuclear ribonucleoprotein (snRNP) U1 (21), ASF/SF2 (8, 9), tra (6), and tra-2 (7) arginine/serine-rich proteins, the PR264 polypeptide migrated more slowly (36 kDa) than predicted from its calculated (25.5 kDa) mass (Fig. 2). *In vitro* synthesis of a single product strongly suggested that PR264CH cDNA, indeed, corresponds to a mRNA species that, *in vivo*, is expressed in thymic cells. Interestingly, two human splicing factors (U2AF and SC-35) of similar molecular masses (35–36 kDa) have been described (22, 23).

Transcriptional Analysis of Chicken and Human PR264-Encoding Genes. Chicken RNA species expressed from the overlapping ET and PR264 transcriptional units were identified by Northern (RNA) blot hybridization analyses (Fig.

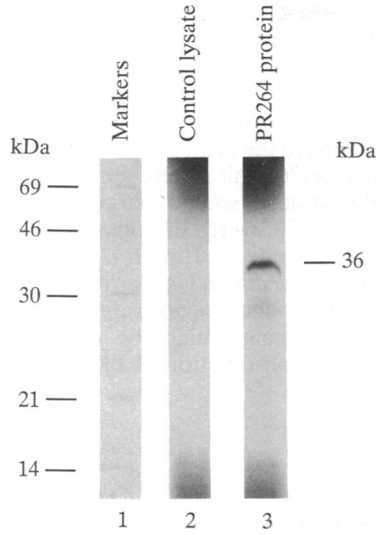

FIG. 2. *In vitro* translation of PR264CH cDNA. Nuclease-treated rabbit reticulocyte lysate was programmed as described with [^{35}S]methionine, and resulting proteins were visualized by autoradiography after SDS/PAGE. Lanes: 1, ^{14}C-labeled molecular mass markers (Amersham); 2, no RNA; 3, *in vitro*-synthesized PR264CH mRNA. Sizes of molecular mass markers and *in vitro*-translated product are indicated.

3*A*). Among the many transcripts (4.4, 3.2, 2.7, 2.2, 1.8, 1.5, and 0.5 kb) detected by the BE80 probe in total RNA purified from thymic or bursa cells, the three polyadenylylated species (3.2, 2.7, and 2.2 kb) corresponded to antisense PR264 mRNAs because they hybridized to the M552 and C12 probes.

Hybridization of human thymic polyadenylylated RNAs with the H230 probe revealed two major species (3.4 and 2.2 kb) that were also detected by the oligonucleotidic probe H1 and, therefore, corresponded to antisense PR264 mRNAs (Fig. 3*B*).

Expression Pattern of PR264-Encoding Gene in Chicken Cells. To determine whether the PR264 transcripts are expressed in a tissue-specific way, total RNA species purified from various day-18 embryonic chicken tissues were analyzed by Northern blot hybridization with the BE80 probe. Among the three PR264-specific mRNAs, the 3.2- and 2.2-kb species were expressed at different levels in the tissues tested, whereas the 2.7-kb transcript, previously detected in bursa and thymus, was abundant only in yolk sac cell preparations (Fig. 3*C*). These observations indicate that expression of the 2.7-kb mRNA species principally occurs in cells of hematopoietic origin. Similar results were obtained with adult chicken tissues (data not shown).

Expression of PR264-Encoding Gene Is Developmentally Regulated in Chicken. To gain more insight in the regulation of PR264 expression, thymic polyadenylylated mRNA species were purified at different stages of chicken development. Fig. 3*D* shows that Northern blot hybridization analyses done with the BE80 probe detected the three expected PR264-specific mRNAs (3.2, 2.7, and 2.2 kb) in all samples. However, the relative intensity of the signal corresponding to each species varied during development; densitometric analyses indicated that the relative proportion of the 2.2-kb mRNA, which is the most important in day-16 embryonic thymus, progressively decreased, reaching a lower level than that of the 3.2-kb mRNA in thymus from 14-day-old chickens. These observations indicate that expression of the different PR264-specific transcripts is differentially regulated throughout the developmental processes.

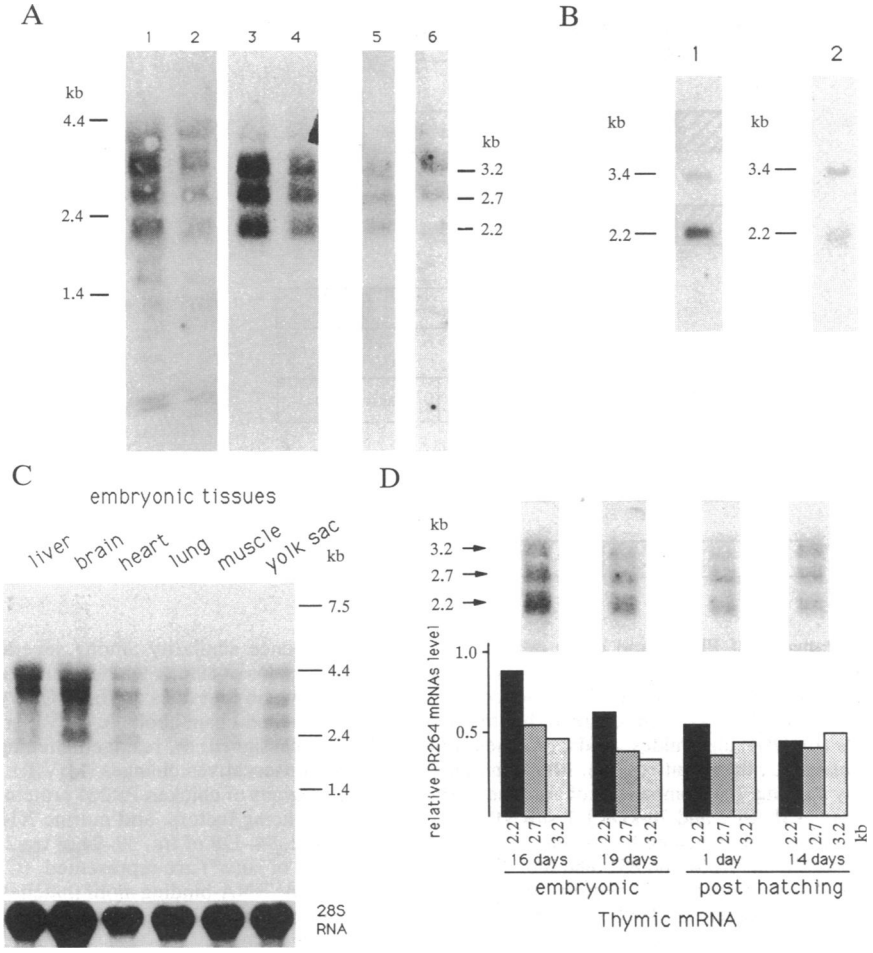

FIG. 3. (*A*) Northern blot analyses of ET- and PR264-specific RNAs expressed in either bursa (lanes 1 and 3) or thymic (lanes 2 and 4) hematopoietic cells from 14-day-old chicken. Ten-microgram samples of total RNA (lanes 1 and 2) and 2-μg samples of polyadenylylated RNA species (lanes 3 and 4) were hybridized with BE80 double-stranded probe. PR264-specific transcripts were identified after hybridization of 10-μg samples of total RNA with M552 (lane 5) or C12 (lane 6) single-stranded probes. (*B*) RNA blot analyses of PR264-specific RNAs expressed in human thymic cells. A 1-μg sample of polyadenylylated RNA (lane 1) and a 15-μg sample of total RNA (lane 2) were hybridized with the H230 double-stranded and H1 oligonucleotidic probes, respectively. (*C Upper*) Tissue distribution of PR264-specific RNAs. RNA blots of total chicken RNA (10 μg per lane) purified from various embryonic tissues were hybridized with BE80 probe. (*Lower*) Detection of 28S rRNA probe used as internal standard. (*D*) Expression of PR264-specific mRNAs during development. Samples of chicken polyadenylylated RNA species purified from day-16 and day-19 embryonic thymus and from 1- and 14-day-old chicken thymic cells were hybridized with BE80 probe. Relative amount of each PR264-specific mRNA species was estimated by densitometric analyses. Signal intensities were normalized on the basis of an identical amount of mRNA (5 μg) for each sample.

DISCUSSION

The chicken and human PR264 proteins encoded by the opposite strand of the ET locus (located on chicken chromosome 3 and at chromosome 17q25 in human) (12) almost exclusively each consists of two domains exhibiting the hallmarks of cellular factors involved in regulation of pre-mRNA splicing. As shown in Fig. 4*A*, the RNP80 domain of PR264 (aa 15–95) is one of the best fit to the current consensus (21), with 63 matching residues and 13 residues also represented in other RNA-binding proteins. Within this domain, we identified a RNP2 consensus sequence (aa 16–21) sharing homology with that of the yeast poly(A)-binding protein 4 (24) and the *Drosophila Sx1-2* gene product (5), and a RNP1 subdomain (aa 55–62) highly homologous to that of *Drosophila* fly p9#2 protein (heterogenous nuclear RNP A1) (25). A striking feature of the human and chicken PR264 RNP80 lies at aa 17 (within RNP2), where a lysine (K) replaces a conserved aromatic residue of the consensus motif (Fig. 4). According to the protein conformation predictions of Chou and Fasman (26), this difference and the presence of an aspartic residue at aa 19 should markedly affect the secondary structure of the PR264 RNP2 domain because this domain is predicted to be embedded in an α-helix region, whereas other RNP2s (such as human U1A or ASF/SF2, for example) are usually part of a β-sheet region referred to as β1 (27). Such a conformational change might be crucial for the RNA-binding specificity and function of PR264 protein.

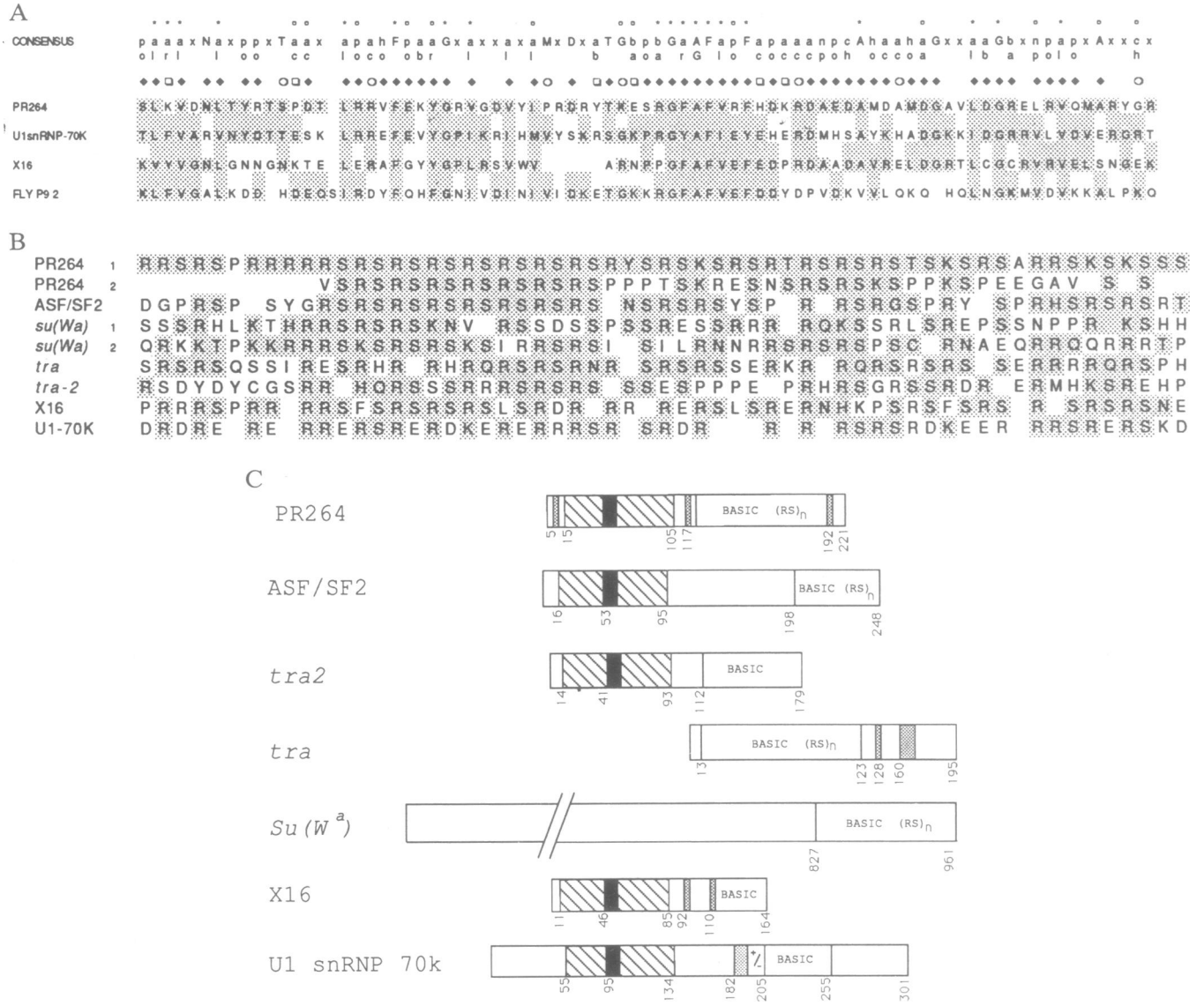

FIG. 4. (*A*) Conserved features between RNP80 domains of PR264 and other proteins. Regions of sequence similarity among several RNA-associated proteins and PR264 RNP80 are arranged for best alignment of conserved residues within subdomains of 80-aa regions (standard one-letter amino acid code). Consensus sequence and highly conserved positions (* more conserved than ◦) shown in first lines are from Query *et al.* (21). Abbreviations printed vertically: ac, acidic residue; ba, basic; ch, charged; ho, hydrophobic; al, branched-chain aliphatic (LIV); ar, aromatic; np, nonpolar; po, polar; aa, amides or acids (EQDN); ab, amides, acids, or bases (EQDNKR); x, unassigned; ◆, residue matching consensus; ○, residue also found in other RNP80 domains; □, divergent residue. Identical amino acids and conservatives changes (M:V:I:L, A:G, F:Y, S:T:Q:N, K:R, and E:D) are emphasized by shading. (*B*) Comparison of arginine/serine (RS)-rich regions of chicken PR264 protein, *Drosophila* tra, tra-2, su(w^a) splicing regulators, human ASF/SF2 and 70-kDa U1 snRNP (U1snRNP-70K) splicing factors, and murine X16 protein. Amino acid identities and semiconservative changes are indicated by shading. aa 195–248 of ASF/SF2, 74–128 of tra, 39–94 of tra-2, 234–283 of 70-kDa U1 snRNP, 117–175 (no. 1) and 176–221 of PR264 and 831–886 (no. 1) and 890–947 (no. 2) of su(w^a) are represented. (*C*) Overall structure of PR264 polypeptide and of several RNA-binding proteins. Various represented domains include RNA-binding motif (hatched box) with internal octamer RNP core (black box), proline-rich spacer (shaded box), domain containing alternate acidic and basic residues (+/−) and basic domain with [BASIC (RS)$_n$] or without (BASIC) arginine/serine (RS) repeats.

The basic domain of PR264 (aa 117–207) is similar to the highly charged regions of the human 70-kDa U1 snRNP (21), ASF/SF2 (9, 10), murine X16 protein (28), *Drosophila* su(w^a) (8), tra (6), tra-2 (7) proteins (Fig. 4*B*), and arginine-rich regions of human immunodeficiency virus tat (29) and rev (30) polypeptides. The PR264 arginine/serine-rich domain, which is the most extensive reported so far, could ensure several types of function, such as low-specificity RNA–protein interactions, as already documented for the 70-kDa U1 snRNP (21), interactions with other proteins containing identical domains (21), or nuclear targeting of the protein itself. This last hypothesis has recently been strengthened by results indicating that the arginine/serine-rich domains of *Drosophila* tra and su(w^a) factors are involved in specifically directing these products to a punctate subnuclear compartment enriched in several constitutive splicing components (31).

Of the two human splicing factors, which are from 35 to 36 kDa in mass (22, 23), only the SC-35 protein was unambiguously localized in this compartment. Whether any functional relationship exists between SC-35 and PR264 polypeptides would be interesting to determine.

Among the splicing regulators characterized thus far, ASF/SF2 is most closely related to PR264 protein (Fig. 4*C*). Both polypeptides share a RNP80 and arginine/serine-rich domain defined by two coding exons and possess, within their RNP80 domains, a specific RDAEDA sequence (aa 66–71 in Fig. 1), which does not occur in any other protein of the current data bases (GenPro and National Biomedical Research Foundation). This overall similar structure suggests that PR264 and ASF/SF2 polypeptides have related function(s).

Use of strand-specific probes indicated that three PR264-specific mRNAs are expressed in chicken hematopoietic cells, whereas only two of these mRNAs were clearly detected in the other tested tissues. Nucleotide sequencing of several cDNAs obtained from various chicken and human tissues revealed that the corresponding PR264 mRNAs only differ at the 3′ untranslated sequences as a result of alternative splicing and alternative polyadenylylation signal use (unpublished work). Interestingly, expression of one of the PR264 mRNAs principally occurred in hematopoietic tissues, suggesting that regulation of PR264 transcription is related to cellular differentiation. Such a developmental regulation related to processing events affecting noncoding regions of different RNA species has already been reported for the *Drosophila* 70-kDa U1 snRNP protein (32).

Although transcription of opposite DNA strands has been reported in a few cases (6, 33–38), a functional relationship between overlapping genes has been described only once (34).

We have previously reported that the ET encodes a regulatory domain of the c-*myb* transcriptional trans-activation activity in yeast (39) or human cell lines (unpublished work) and suggested that different c-*myb* products with distinct amino termini might be specifically expressed in the various hematopoietic cell lines and their related precursors (12). The bidirectional transcription of the ET/PR264 locus might be a key element in controlling the relative amounts of corresponding polypeptides expressed in hematopoietic cells. In this connection, the PR264 protein, which exhibits several expected features of a splicing regulator, could participate directly in the tissue-specific intermolecular recombination of the ET and c-myb RNA precursors.

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