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# Amplification of a DEAD box protein gene in retinoblastoma cell lines

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ABSTRACT **DEAD box proteins, characterized by the conserved motif Asp-Glu-Ala-Asp, are putative RNA helicases implicated in a number of cellular processes involving alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division. Here, we report that the mRNA encoding a DEAD box protein, designated HuDBP-RB, is present at elevated levels in two of six retinoblastoma (RB) cell lines tested and is preferentially expressed in fetal tissues of neuroectodermal origin. It is not possible to classify HuDBP-RB as a member of any of the DEAD box protein subgroups identified to date since the regions of amino acid similarity between HuDBP-RB and other DEAD box proteins are restricted to the conserved motifs found in all members of this family. The HuDBP-RB gene, which has been mapped to chromosome band 2p24, is amplified in the RB cell lines that overexpress HuDBP-RB RNA. Furthermore, the *MYCN* gene is also present in multiple copies in these two cell lines, suggesting coamplification of the two genes.**

Retinoblastoma (RB) tumors arise from retinal precursor cells that have undergone two mutational events resulting in the complete inactivation or absence of the RB protein (pRB) (1–3). Recent data indicate that pRB can bind to E2F, a transcription factor thought to regulate a number of cellular genes that encode products with putative functions in the cell cycle (4–6). By binding to E2F, pRB may normally repress the function of this transcription factor, thereby playing a role in regulation of the cell cycle by constraining cellular proliferation (7, 8). Also, pRB has been shown to positively regulate transcription from the fourth promoter of the insulin-like growth factor II gene and the type β2 transforming growth factor promoter (9, 10) and to negatively regulate transcription from the promoters of c-*fos* and c-*myc* (11, 12). This diversity of pRB functions suggests that a number of gene products may be altered in RB cells as a direct consequence of pRB inactivation. Additional alterations may be a consequence of the tumorigenic process and may be only indirectly related to the primary transforming events. To identify transcripts preferentially expressed in RB cell lines compared to other tumorigenic cell lines and normal tissue, a subtracted library was prepared by hybridizing single-strand cDNA from two RB cell lines to excess poly(A)$^+$ RNA derived from a colon carcinoma cell line and fetal tissue and selecting the nonhybridized material for construction of the library. Differential screening of this library resulted in identification of a transcript that is preferentially expressed in a subset of RB cell lines and that represents a member of the Asp-Glu-Ala-Asp (DEAD) box protein family.§

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## MATERIALS AND METHODS

**Cell Lines.** Five of the six RB cell lines (Y79, RB412, RB429, RB522A, RB544) were obtained from Brenda Gallie (Hospital for Sick Children, Toronto). The sixth RB cell line, WERI-Rb-1, was obtained from the American Type Culture Collection. The RB cell lines were cultured in suspension in Dulbecco's modified Eagle's medium plus 15% fetal calf serum, antibiotics, 50 μM 2-mercaptoethanol, and insulin (10 μg/ml). The malignant glioma cell lines and the two simian virus 40 (SV40)-transformed human fibroblast strains have been described (13). GM38 represents a normal human fibroblast strain obtained from Malcolm Paterson (Cross Cancer Institute, Edmonton, Alberta). The five bladder carcinoma (HTB-1, HTB-3, HTB-5, HTB-9, T24), four colon/duodenum carcinoma (HuTu, CaCo-2, LoVo, HT29), hepatoma (Hep3B), osteosarcoma (Saos-2), and neuroblastoma (IMR32) cell lines were from the American Type Culture Collection.

**Construction and Screening of cDNA Libraries.** Poly(A)$^+$ RNA was extracted from Y79, RB522A, LoVo, and human fetal tissue (head region without brain and eyes) as described (14). Poly(A)$^+$ RNA (5 μg) from Y79 and RB522A, respectively, served as a template for first-strand cDNA synthesis using oligo(dT) as a primer and Moloney leukemia virus reverse transcriptase (BRL). [$^{32}$P]dCTP (20 μCi; 1 Ci = 37 GBq)/50 μl of reaction mixture was included to serve as a tracer in subsequent steps. Poly(A)$^+$ RNA (12 μg) isolated from the LoVo colon carcinoma cell line and human tissue, respectively, was added to an estimated 2 μg of single-strand cDNA and the RNA and DNA were hybridized in 1 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA for 20 hr at 65°C. Single-strand cDNA was separated from double-stranded hybrids using hydroxylapatite (Bio-Rad). The second strand was synthesized by using Klenow polymerase. The double-stranded cDNA was treated with T4 DNA polymerase and ligated to a 50-fold excess of *Eco*RI linkers. After digestion with *Eco*RI, the cDNA was size-selected on a 5% acrylamide gel (≥400 bp) and ligated to *Eco*RI-digested Lambda ZAP II (Stratagene) to generate a cDNA library of $10^5$ independent phages. The library was amplified once by plate lysis (titer, $2 \times 10^{10}$ plaque-forming units/ml). Replicate filters containing a total of 20,000 plaques were hybridized with $^{32}$P-labeled single-strand cDNA derived from Y79/RB522A RNA (probe A) or LoVo/fetal tissue RNA (probe B). Plaques hybridizing to probe A but not to probe B underwent secondary and tertiary rounds of screening. Phagemids containing inserts of interest were excised from Lambda ZAP II following the supplier's directions. Construction of the RB522A cDNA library has been described (14).

Abbreviations: RB, retinoblastoma; SV40, simian virus 40; FISH, fluorescence *in situ* hybridization; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; cl., clone.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X70649).

**Northern and Southern Blot Analyses.** Procedures for extraction of poly(A)$^+$ RNA and genomic DNA and conditions for probe hybridization, washing filters, and stripping filters were as described (14).

**DNA Sequencing.** Sequencing was by the dideoxynucleotide chain-termination method with T7 DNA polymerase (Pharmacia) as modified for double-stranded DNA templates (15). A sequential deletion strategy was used to obtain overlapping sequences (16). Searches for amino acid homology were performed at the National Center for Biotechnology Information using the BLAST network service.

**Fluorescence *in Situ* Hybridization (FISH) Mapping.** Conventional chromosome spreads were prepared from normal human lymphocytes and the human neuroblastoma cell line IMR32. The FISH method used was based on published procedures (17). The biotinylated genomic probe was detected with avidin-fluorescein isothiocyanate. The chromosome preparations were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) or propidium iodide. Separate images of counterstained chromosomes and of genomic DNA hybridization signals were merged using image analysis software (courtesy of Tim Rand and David Ward, Yale University) (18).

## RESULTS

**Isolation of cDNA Clones and Expression of Clone (cl.) 1042 in Transformed Cell Lines.** A RB-enriched cDNA library was generated by hybridizing a mixture of single-strand cDNAs derived from the poly(A)$^+$ RNA of two RB cell lines (Y79 and RB522A) to a 12-fold excess of a mixture of poly(A)$^+$ RNAs derived from the LoVo colon carcinoma cell line and human fetal tissue. The LoVo cell line was selected because it has normal levels of RB RNA and it represents a class of tumors in which the RB gene has not been implicated to date. Hybridization with LoVo and fetal tissue mRNAs should result in the depletion of transcripts commonly associated with cell proliferation, thereby allowing selective enrichment of transcripts specifically expressed in RB cells. Twenty thousand plaques were screened with radioactively labeled single-strand cDNA from Y79/RB522A (+ screen) and LoVo/fetal tissue (− screen). Twenty-one of the 26 selected bacteriophages contained cross-hybridizing inserts. A representative of the 21 clones (cl. 1042) containing a 1.6-kb *Eco*RI insert was selected for further analysis. Screening of 48 cell lines including 6 RB, 29 malignant gliomas, 5 bladder carcinomas, 4 colon/duodenum carcinomas, 1 hepatoma, 1 osteosarcoma, and 2 SV40-transformed human fibroblast cultures revealed elevated cl. 1042 RNA levels in two RB cell lines (Y79 and RB522A) but not in the other lines tested (see Fig. 1*a* for a subset of the cell lines analyzed). Gross abnormalities in size or amount of the RB transcript were found in 5/6 RB, 2/5 bladder carcinoma, 1/1 osteosarcoma, and 9/29 malignant glioma cell lines (examples are shown in Fig. 1*b*). Probing with actin cDNA ensured that similar amounts of RNA had been loaded in each lane (Fig. 1*c*). Based on the electrophoretic mobility of the cl. 1042 transcript relative to the actin and RB transcripts and the 28S and 18S rRNA markers, the size of the cl. 1042 mRNA was estimated to be ≈2800 nt.

**Expression of cl. 1042 RNA in Fetal Tissues.** Elevated RNA levels in a subset of RB tumor cell lines could reflect expression in the cell of origin of the tumor rather than represent a property unique to the tumor cells. To analyze the tissue distribution of cl. 1042 RNA, the cDNA was hybridized to human fetal tissues including the retina at 9–11 weeks of gestation. At this stage, the fetal retina is already differentiated to some extent, although cellular proliferation is still important (21). Interestingly, cl. 1042 RNA was expressed at higher levels in tissues of neuroectodermal origin, including retina, brain, and spinal cord, than in other tissues (Fig. 2*a*). However, cl. 1042 RNA levels in the fetal retina were markedly lower than that found in the two RB cell lines Y79 and RB522A. A ribosomal protein probe, rpL32/4A (22), rather than actin served as an internal standard in these experiments because of the difficulty in quantitating actin RNA levels in tissues of different lineages (Fig. 2*b*).

**Identification of cl. 1042.** The cDNA sequence was obtained from cl. 1042 as well as an additional 20 independent cDNAs isolated from the RB-enriched cDNA library and a second library prepared from total RB522A poly(A)$^+$ RNA. The sequence was obtained from both strands and was verified by using at least two independent cDNA clones except for 34 bp at the 5' end of the cDNA, which was found in only one of the clones. The 2.4-kb cDNA contains an open reading frame of 739 amino acids spanning nt 1–2217. Three in-frame termination codons are followed by a consensus polyadenylylation signal (AATAAA) at nt 2381 and a stretch

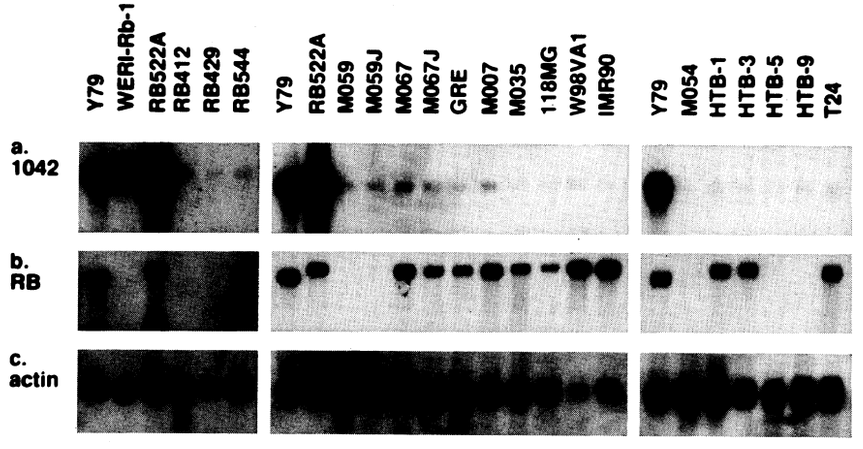


FIG. 1. Northern blot analysis of cl. 1042 in transformed cell lines. The following cell lines were analyzed: 6 RB (Y79, WERI-Rb-1, RB522A, RB412, RB429, RB544), 29 malignant glioma (A172, T98, U87MG, SAN, RIC, GRE, P4, MIL, CLA, H4, A1235, 118MG, U251MG, U138MG, M002, M006, M007, M010, M012, M016, M021, M027, M035, M040, M054, M059, M059J, M067, M067J, M071, and M072, where J differentiates between two cell lines derived from the same tumor but by different research groups), 5 bladder carcinoma (HTB-1, HTB-3, HTB-5, HTB-9, T24), 4 colon/duodenum carcinoma (HuTu, CaCo-2, LoVo, HT29), 1 hepatoma (Hep3B), 1 osteosarcoma (Saos-2), and 2 SV40-transformed human fibroblast strains (W98VA1 and IMR90-830). A subset of the cell lines analyzed is shown. Poly(A)$^+$ RNA (2 μg) was loaded in each lane. Filters were sequentially hybridized with the 1.6-kb *Eco*RI fragment from cl. 1042 (*a*), the 3.8-kb *Eco*RI fragment from RB cDNA (19) (*b*), and the 1.2-kb *Pst* I insert from mouse α-actin cDNA (20) (*c*).

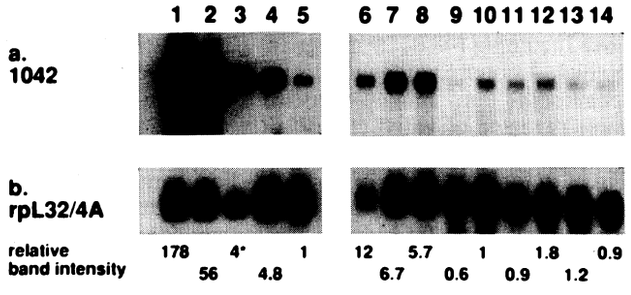


FIG. 2. Human fetal tissue distribution of cl. 1042 RNA. Samples were loaded as follows: 1 $\mu$g of poly(A)$^+$ RNA in lanes 1 (RB522A), 2 (Y79), 3 (WERI-Rb-1), 4 (fetal brain), and 5 (fetal lung); $\approx$6 $\mu$g of total RNA in lane 6 (retina); and 20 $\mu$g of total RNA in lanes 7 (brain), 8 (spinal cord), 9 (heart), 10 (lung), 11 (liver), 12 (kidney), 13 (gut), and 14 (head region without brain and eyes). Filters were sequentially hybridized with cl. 1042 (a) and a 1.3-kb *Hin*dIII fragment from rpL32/4A, a ribosomal protein gene (22) (*b*). RNA concentration was estimated from spectrophotometric readings except for retina RNA, which was roughly quantitated by pellet size. To calculate relative band intensities, autoradiograms at different exposures were scanned with the Ultroscan XL laser densitometer and the Gel Scan XL V2.1 program (Pharmacia). To account for differences in RNA loaded in each lane, areas under peaks obtained for cl. 1042 ($area_{1042}$) were measured and divided by areas under peaks of the rpL32/4A standard ($area_{rpL32/4A}$). The number generated for lanes 1–14 (with the exception of lane 3) were then compared to ($area_{1042}/area_{rpL32/4A}$) using fetal lung as a reference (lanes 5 and 10). Relative band intensity therefore represents $(area_{1042}/area_{rpL32/4A})_{\text{lanes 1, 2, 4–14}}/(area_{1042}/area_{rpL32/4A})_{\text{reference lane}}$. It was not possible to use rpL32/4A as the internal standard in the case of WERI-Rb-1 (lane 3) since the rpL32/4A RNA levels were consistently 3 times lower in this cell line than in other RB cell lines. For this reason, we reprobed the filter with actin and obtained very similar levels of RNA in all three RB cell lines. The relative band intensity listed for WERI-Rb-1 (4*) was therefore obtained by using actin as an internal standard.

of 13 A residues is found 13 nt downstream of the polyadenylylation signal.

A search of the GenBank and EMBL DNA data bases for similar sequences revealed significant homology with members of the DEAD box protein family, including the mouse translation initiation factors eIF-4AI and eIF-4AII, *Drosophila melanogaster vasa* gene, and mouse PL10 protein (23–26). Approximately 30 members of the DEAD box protein family have been isolated to date in organisms ranging from bacteria to humans (27). Eight conserved amino acid motifs characterize each member of the family, including the DEAD box, which is an integral part of one of the eight motifs [(V/I)LDEADX(M/L)LXXGF] and which is unique to this protein family. As shown in Fig. 3, all eight motifs are present in the deduced amino acid sequence of cl. 1042. The protein encoded by cl. 1042 RNA has been named HuDBP-RB since it represents a human DEAD box protein identified in RB cells.

To determine whether a functional homologue of HuDBP-RB had previously been cloned, the predicted amino acid sequence of HuDBP-RB was compared to that of other DEAD box proteins (some of the sequences compared are shown in Fig. 3). Little variation in the number of identical residues was observed, with HuDBP-RB demonstrating as high a level of identity with the yeast SPP81/DED1 protein associated with RNA splicing (30) (117 of 636 aa compared) as with the murine eIF-4AI translation initiation factor (23) (114/636).

**Southern Blot Analysis of the HuDBP-RB Gene.** Elevated transcript levels could be due to an increased transcription rate, an increase in the concentration of a transcription factor(s) present in limiting amounts, an increase in transcript stability, and/or an increase in gene copy number. To test the latter possibility, genomic DNA was extracted from the six RB cell lines as well as from normal fetal tissue and a normal human fibroblast strain (GM38) and then hybridized to cl. 1042 cDNA (Fig. 4*a*). Gene amplification was observed in the two cell lines shown to have elevated levels of cl. 1042—Y79 (15–20 times) and RB522A (100–150 times). All the bands hybridizing to cl. 1042 showed a similar level of amplification, suggesting that there is only one cl. 1042 gene. Probing with human $\alpha$-fetoprotein cDNA ensured that a similar amount of DNA was loaded in each lane (Fig. 4*b*). Since *MYCN* is amplified in a subset of RB cell lines (31, 32), the filter was stripped and rehybridized with *MYCN* cDNA (Fig. 4*c*). *MYCN* was found to be amplified in both Y79 and RB522A.

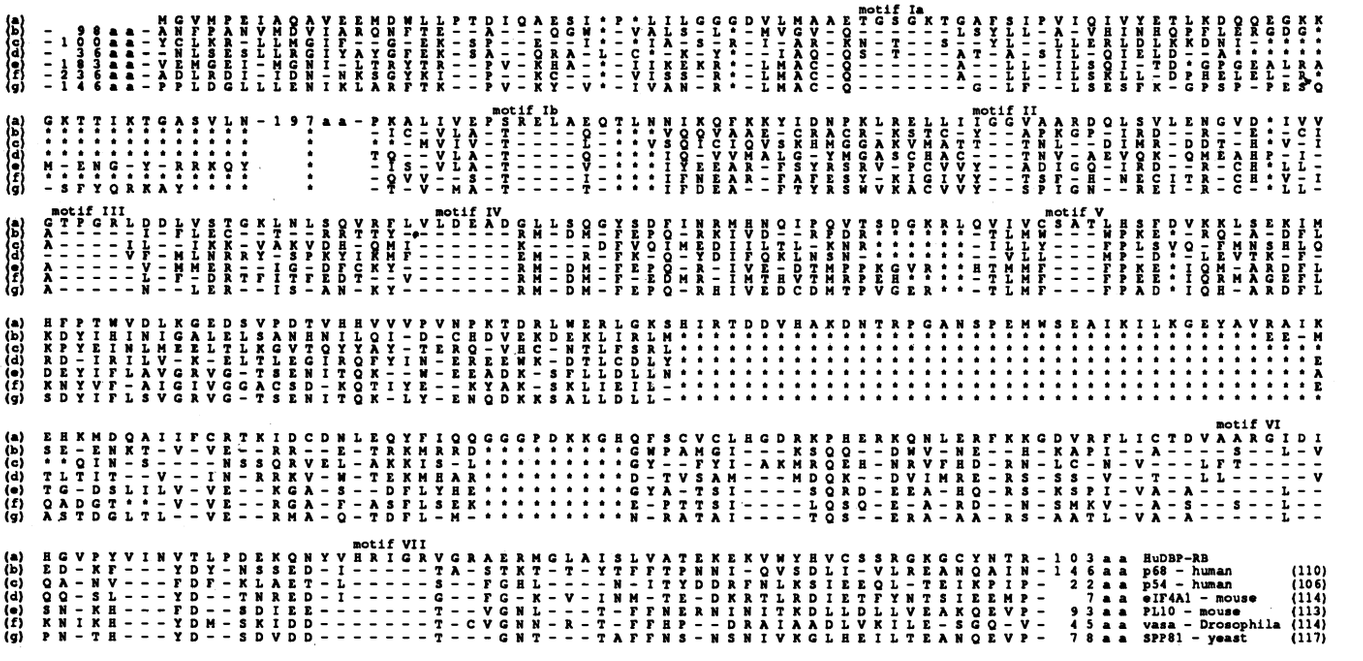


FIG. 3. Alignment of HuDBP-RB and other DEAD box proteins. Order of comparison from top to bottom is HuDBP-RB (*a*), p68 (28) (*b*), p54 (29) (*c*), eIF-4AI (23) (*d*), PL10 (26) (*e*), *vasa* (25) (*f*), SPP81/DED1 (30) (*g*). Identical residues are indicated by a dash; asterisk indicates the absence of an amino acid(s). Gaps have been introduced to optimize alignment.

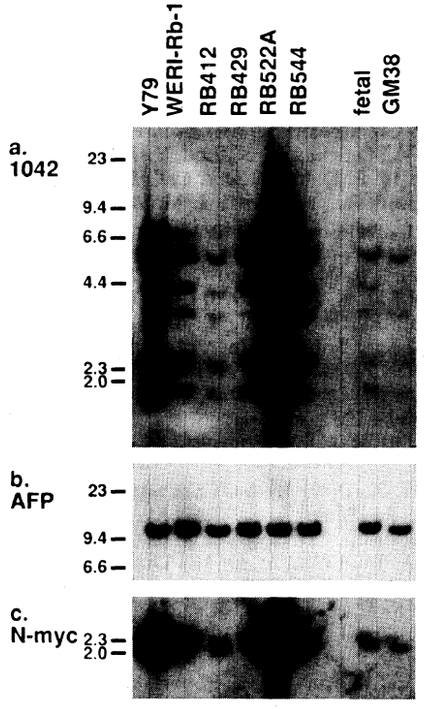


FIG. 4. Amplification of the gene encoding HuDBP-RB in RB cell lines. Ten micrograms of genomic DNA extracted from six RB cell lines (Y79, WERI-Rb-1, RB412, RB429, RB522A, RB544), normal fetal tissue, and GM38 normal fibroblasts was digested with *Eco*RI and hybridized to $^{32}$P-labeled cl. 1042 cDNA (*a*), human α-fetoprotein (AFP) cDNA (*b*), and human *MYCN* cDNA (*c*). Markers (in kb) are indicated on the left.

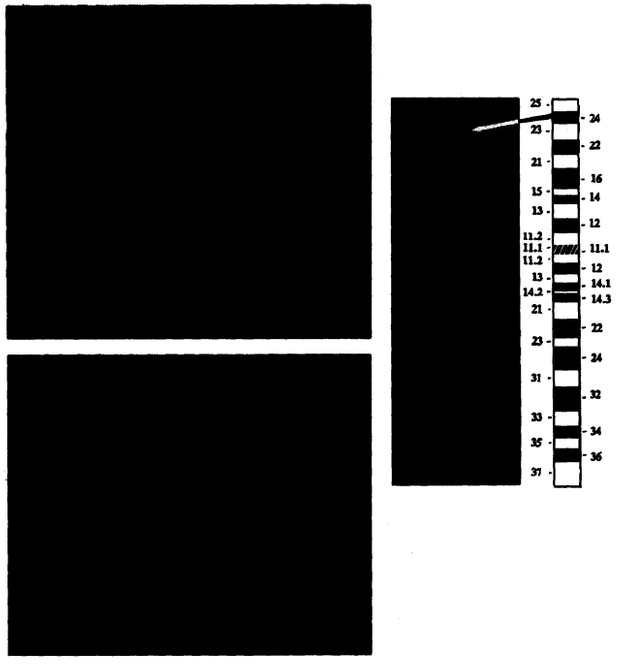

FIG. 5. Regional mapping of HuDBP-RB gene by FISH. (*Upper Left*) FISH to normal human lymphocyte chromosomes counterstained with DAPI using a genomic DNA clone that hybridizes to the 5′ end of cl. 1042 cDNA. (*Right*) DAPI banded chromosome 2 together with schematic diagram indicating that the HuDBP-RB gene hybridizes to band 2p24. (*Lower Left*) FISH to IMR32 metaphase chromosomes counterstained with propidium iodide. Clusters of yellow fluorescein isothiocyanate fluorescence on the two homogeneously staining regions of IMR32 located on chromosome 1 are clearly visible.

The level of amplification was similar to that observed for the HuDBP-RB gene (Y79, 30 times; RB522A, 150 times).

**Regional Mapping of the HuDBP-RB Gene.** The regional assignment of the HuDBP-RB gene was determined by FISH with a λ2001 genomic clone that hybridized to the 5′ end of cl. 1042 cDNA. Approximately 13 kb of the 14-kb genomic DNA insert has been sequenced and found to represent 10 exons corresponding to ≈1 kb at the 5′ end of HuDBP-RB cDNA. Analysis of 20 mitoses from normal human lymphocytes revealed a positive hybridization signal on chromosome 2p24 on all four chromatids in 17 cells and on three chromatids in 3 cells (Fig. 5 *Upper Left*). The chromosome band assignment was determined by measuring the fractional chromosomal length and by analyzing the banding pattern generated by the DAPI-counterstained image. A representative metaphase chromosome indicating the position of HuDBP-RB at 2p24 is shown in Fig. 5 *Right*.

Amplified copies of the *MYCN* gene have been located to two homogeneously staining regions on chromosome 1 in the neuroblastoma cell line IMR32 (33, 34). Analysis of metaphase preparations of the IMR32 cell line revealed multiple copies of the HuDBP-RB gene at these two sites (Fig. 5 *Lower Left*).

## DISCUSSION

A DEAD box protein, HuDBP-RB, was identified by differential screening of a RB-enriched cDNA library. Northern blot analysis revealed that HuDBP-RB RNA levels were elevated in RB cell lines Y79 and RB522A but not in other RB cell lines or in cell lines derived from malignant gliomas, colon carcinomas, bladder carcinomas, one hepatoma, one osteosarcoma, and SV40-transformed fibroblasts. Since the RB gene product is absent or inactive in all RB tumors and abnormal or absent RB transcripts were observed in a number of the transformed cell lines included in this analysis, it is clear that there is not a direct relationship between inactivation of the RB protein and overexpression of HuDBP-RB, at least at the RNA level. However, it will be necessary to analyze the product encoded by the HuDBP-RB gene to determine the extent to which this protein is implicated in RB and other tumors since different mechanisms can lead to overproduction of a protein.

The sequences of two human DEAD box proteins, p68 and p54 [also designated RCK (35)], have previously been published. p68 is an evolutionarily conserved protein expressed in dividing cells (28) and p54 is encoded by a gene located at the breakpoint of the translocation involving chromosomes 11 and 14 in a cell line derived from a B-cell lymphoma (29). It has been postulated that fusion of a strong enhancer/promoter region of p54 to a truncated Igγ2 gene may provide a proliferative advantage to these lymphoma cells (29). Based on the demonstrated *in vitro* RNA helicase and RNA-dependent ATPase activities of eIF-4A and p68, it has been postulated that all members of this family have similar activities (36–39). Some DEAD box proteins are ubiquitous and appear to be involved in general cellular processes such as RNA splicing and translation initiation. Other members, such as the male germ-line-restricted PL10 and the maternally expressed *vasa*, ME31B, and An3 genes, are cell-, tissue-, or stage-specific. These may play regulatory roles in the cell, perhaps as translation initiation factors with affinity for selected transcripts or as factors mediating cell- or tissue-specific functions involving the modulation of RNA secondary structure (25, 26, 40, 41). It is logical to postulate that HuDBP-RB can also modify RNA secondary structure. Increased production of this protein could therefore result in significantly altered levels of different RNAs and proteins in the cell as the result of alterations in RNA stability, RNA

splicing, or accessibility of RNA for translation. Whether specific categories of RNAs are targeted by this DEAD box protein remains to be determined.

The HuDBP-RB gene was amplified in the two RB cell lines that demonstrated elevated levels of HuDBP-RB RNA. Furthermore, the *MYCN* gene, also amplified in some RB tumors and cell lines (31, 32), was present in multiple copies in these two cell lines (Y79 and RB522A), suggesting coamplification of the two genes. The fact that the *MYCN* and HuDBP-RB genes colocalize to chromosome band 2p24 strongly supports this possibility. It is generally believed that overexpression of genes through gene amplification confers a selective advantage to the cells. For example, amplification of the *MYCN* gene correlates with an aggressive phenotype in neuroblastoma tumors and a poor clinical prognosis (42, 43). However, since hundreds to thousands of kilobase pairs of genomic DNA are included in one amplification unit (44), it can be difficult to prove that a given gene (such as *MYCN*) is solely responsible for conferring a growth advantage to the tumor cells. The discovery that the *MYCN* and the HuDBP-RB genes are coamplified in two RB cell lines as well as in the neuroblastoma cell line IMR32 suggests the possible involvement of the HuDBP-RB gene in these two categories of tumors.

Complete inactivation of the RB gene is necessary for initiation of tumorigenicity in RB tumors, but additional genetic events may enhance the tumorigenic potential of the cells. By studying transcripts that are preferentially expressed in RB cells compared to other tumors and normal cells, a gene has been identified that may have a role in the posttranscriptional control of gene expression. Furthermore, this gene may have a role particular to cells of neuroectodermal origin and, in conjunction with *MYCN*, may be involved in RB (and neuroblastoma) tumor formation or progression when overexpressed.

**Note Added in Proof.** The Committee on Human Gene Mapping has designated the HuDBP-RB gene as *DDX1*.

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