A differentially expressed murine RNA encoding a protein with similarities to two types of nucleic acid binding motifs

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ABSTRACT

Using differential screening, a murine cDNA, termed X16, was isolated corresponding to an mRNA which is more strongly expressed in pre-B cell lines relative to mature B-cell lines. The complete coding sequence of the mRNA predicts a 19kD protein with two domains connected by a proline-rich spacer. The N-terminal domain of about 90 amino acids encodes an RNA binding motif including the ribonucleoprotein consensus octapeptide found in one class of RNA-binding proteins and highly conserved from yeast to man. Within the very basic C-terminal domain of about 60 amino acids, several copies of two different peptides are found which are also present in several proteins which bind DNA or RNA. The expression of X16 is not limited to the lymphoid lineage. In adult mice, although the strongest expression was seen in thymus, mRNA was also found in testis, brain, spleen, and very low in heart. X16 mRNA was not detected in liver and kidney. In tissue culture, the expression of X16 mRNA can be induced by serum. The conserved protein motifs and expression pattern suggest that X16 could be involved in RNA processing correlating with cellular proliferation.

INTRODUCTION

In order to better understand B-cell differentiation molecularly, we have looked for genes whose expression varies during B-cell ontogeny. A preB-cell cDNA library was differentially screened using radiolabelled cDNA probes from preB and hybridoma cell lines and clones were isolated which hybridized only with preB cDNA. One of these clones, designated X16, is a previously uncharacterized gene. Results are presented which suggest that X16 is an RNA binding protein not restricted to the lymphoid lineage and whose expression correlates with cellular proliferation.

METHODS

Cell culturing

Cell lines were grown in Iscove's DMEM (Gibco-BRL) with 10% fetal calf serum at 37°C and 7.5% CO_2 atmosphere. Lines used included preB; CB12, SCID1, SCID114 (all generous gifts from U.Fritzsche), 33.1.1+ (C.Paige and G.Köhler, unpublished), 33.1μ5 (M.Kopf and A.Iglesias, unpublished), 70Z/3 (1), B-lymphomas; NFS1 (2), M12 (3), A20 (3), plasmacytomas; SP6 (4), J558L (5), PC700 (6), VLF5, VLF7, VLF11 (all generous gifts from C.Paige), MOPC315 (7), MPC11 (8), the mastocytoma line P815 (9), the macrophage-like line P388D1 (10), the fibroblast line Ltk− (11), the proT line FTF1 (12), the erythro-leukemia line MEL1 (13), the T-lymphoma BW5147 (14) and the T-cell hybridoma 2B4 (called A1 in ref. 15). For serum stimulation of Ltk^- cells, 5×10^5 cells were plated per 13cm tissue culture dish and cultured for 2 days in Iscove's DMEM with 1% fetal calf serum. For serum stimulation, the medium was replaced with fresh medium containing 10% fetal calf serum and the cells were incubated for 2 or 11 hr. at 37°C. Cells were harvested and total cytoplasmic RNA prepared by standard proceedures (16).

cDNA library and screening

A cDNA library from the preB-cell line 33.1.1+ was constructed using standard procedures (16). The first strand cDNA was synthesized by oligo-dT priming of polyA+ mRNA and the second strand synthesis was by RNaseH/pol I. After methylation of EcoRI sites and addition of EcoRI linkers, the cDNA was ligated into EcoRI digested λgt11 and packaged by standard procedures (16). For differential cDNA screening, the cDNA library was plated on agar plates, transferred in replica to nitrocellulose filters (Schleicher and Schuell or Millipore). Radiolabelled, single-stranded cDNA probes from the preB line 33.1.1+ and the plasmacytoma X63Ag8.653 (17) were synthesized by incubating 1 μg polyA+ mRNA in 20 μl with 200U M-MLV reverse transcriptase (BRL),30 μCi ^{32}P-dATP (>3000Ci/mmol), 200μM dC,G and TTP, and 10μM dATP in a buffer containing 50mM Tris-HCl(pH 8.3), 75mM KCl, 10 mM dithiothreitol and 3 mM $MgCl_2$ for 1h at 37°C (18). After removing unincorporated radioactivity by column chromatography (16), the cDNA probes were hybridized to the replica filters at 62°C in 6×SSC, 10×Denhardt's with 0.1% SDS and 50 μg/ml denatured, sheared salmon sperm DNA for 16h. Filters were then washed twice at room temperature for 15 min 2×SSC and twice at 55°C in 0.1×SSC/0.1% SDS for 15m. After autoradiography, the phage plaques giving signals with the preB probe but not with the hybridoma probe were picked and the screening repeated until the phage were pure. cDNA inserts

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were removed from gt11 by EcoRI digestion, subcloned into pTZ18 or 19 (Pharmacia) and sequenced by the chain-termination method of Sanger et al (19) with modifications (20) using T7 DNA polymerase (USB or Pharmacia). Computer-aided sequence analysis was performed using the GCG program package (21) and Genbank and EMBL data bank releases 64 and 24 respectively running on a microVAX station.

Isolation of genomic DNA was by a modification of Blin and Stafford (16). Restriction enzyme digestions were as recommended by the suppliers (BRL, BioLabs, Boehringer). RNA was isolated either by total cell lysis in guanidinium or by homogenization in hypotonic buffer and phenol/chloroform extraction of the cytoplasmic fraction (16). Agarose gel electrophoresis and capillary transfer to nylon membranes (GeneScreen, NEN or Biodyne A, Pall) was by standard procedures (16). Transferred RNA and DNA was covalently fixed to the membrane by UV irradiation (Stratalinker, Stratagene) and hybridized by standard protocols (16).

RESULTS

Cloning of X16

In order to identify genes whose expression changes during B-cell development, a preB cDNA library in lambda gt11 was screened in replica with cDNA probes derived from preB and plasmacytoma cells. The preB cell was 33.1.1+, an Abelson-virus-transformed line derived from the bone marrow of a mouse transgenic for the immunoglobulin μ and κ chain genes both of which are expressed. The endogenous heavy chain locus has a non-productive VDJ rearrangement of one allele and a DJ rearrangement of the other allele (not shown). Although not known at the time the cDNA bank was screened, we have subsequently found that 33.1.1+ does not express the preB specific lambda 5 gene (22) nor the preB and preT specific recombination activating gene RAG1 (23). It also has no or a very low immunoglobulin gene recombinase activity when tested with an exogenous substrate (B.Bühler, unpublished). By these criteria we believe 33.1.1+ to be a late preB cell. The plasmacytoma used in the screening was a derivative of X63Ag8.653. After screening 1×10^5 recombinant phage we

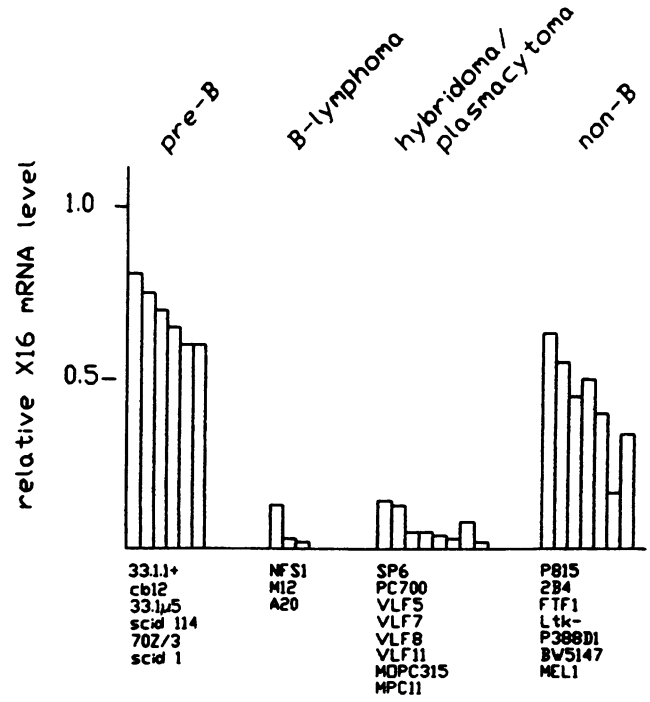

Figure 1. X16 mRNA levels in various cells of the B- as well as other lineages. After northern analysis, the hybridization signal for X16 mRNA was quantitated by laser-densitometry, corrected for loading using the hybridization signal for ribosomal protein S12, and plotted relative to each other. Each bar from left to right corresponds to a different cell line which is listed below the graph in order from top to bottom.

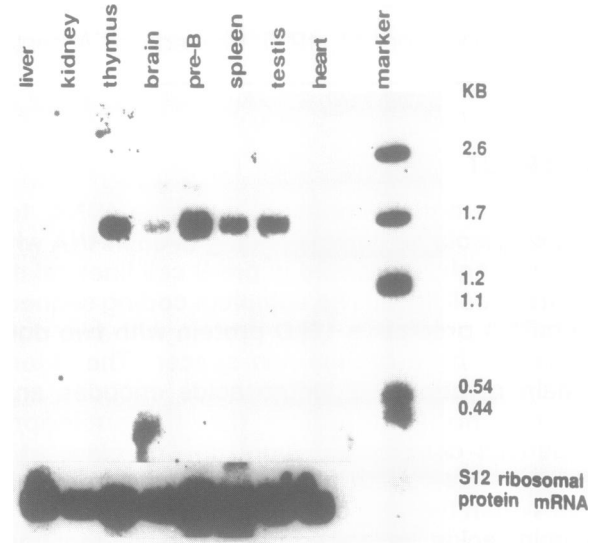

Figure 2. Tissue distribution of X16 mRNA. Total RNA (15μg) extracted from various mouse tissues was separated on denaturing agarose gels and hybridized with X16 cDNA. The lengths of co-electrophoresed DNA marker fragments is indicated in kilobase pairs to the right. To correct for variations in mRNA loading, a second hybridization of the same filter was performed using a cDNA probe for the murine ribosomal protein S12 (24) (inset at the bottom of the figure). The lane marked pre-B is RNA extracted from the Abelson-virus-transformed pre-B cell line 33.1.1+.

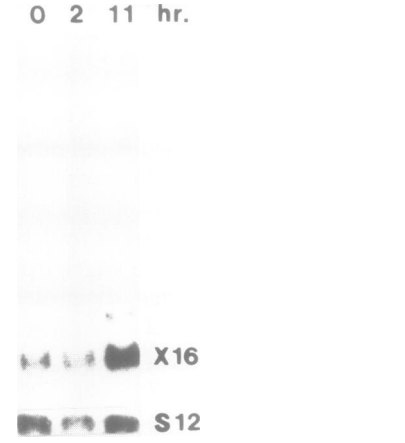

Figure 3. Serum induction of X16 mRNA expression. Total cytoplasmic RNA was extracted from the murine fibroblastic cell line Ltk$^-$ which had been cultured for 48 hr. in medium containing low (1%) serum and then stimulated with medium containing 10% serum for 2 hr. and 11 hr. RNA from unstimulated cells is also shown. After electrophoretic separation of 10μg RNA and blotting, the filter was probed with a radiolabelled X16 cDNA probe (nucleotides 1 to 1250 figure 4). The signal obtained following hybridization of the same blot with the ribosomal protein S12 probe (shown in the inset below the X16 signal) was used to correct for differences in sample loading.

picked 300 plaques with stronger hybridization signals for the preB relative to the hybridoma probe. The characterization of one of these clones, designated X16, is described below.

Expression of X16 mRNA

When measured in vitro, in transformed cell lines, the expression of X16 mRNA is highest in preB cells and about 5–10 fold lower in B-lymphoma and plasmacytoma cell lines (Fig. 1). Expression of X16 mRNA is also seen in other cell lines of the haematopoietic lineage such as the mastocytoma P815, the macrophage-like line P388D1, the thymoma BW5147, the T-cell hybridoma 2B4, and the proT line FTF1 (Fig. 1).

Northern analysis of X16 mRNA expression in various mouse tissues shows a major band of 1.6 Kb in several tissues (Fig. 2). When normalized for variations in the amount of mRNA applied to the gel using a probe for ribosomal protein S12 (24) and scanned with a laser densitometer, the highest expression was seen in thymus. When 15μg total RNA is loaded, considerable expression is seen in testis, brain,and spleen, very low in heart and essentially not detectable in liver and kidney.

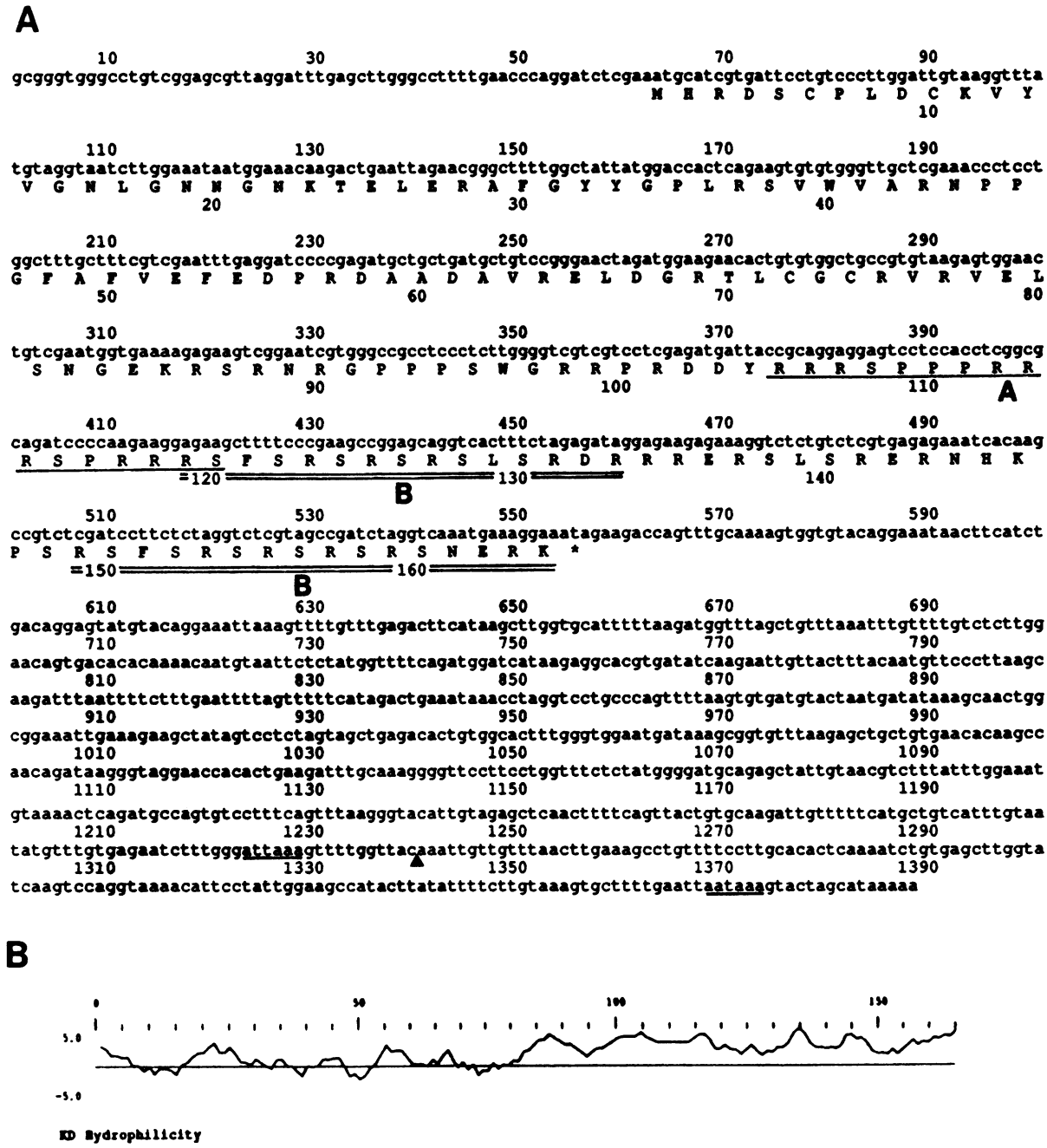

Figure 4. (A) The murine X16 cDNA and predicted amino acid sequences. The consensus cDNA sequence and derived amino acid sequence for the largest open reading frame is shown. The nucleotide sequence is numbered above the sequence and the amino acids below the sequence. The underlined regions A and B containing repeated peptides are also found in other nucleic acid-binding proteins (see text). The RNP-consensus binding domain encompasses amino acids 11 to 85. Polyadenylation signals in the 3'-untranslated region are underlined and the triangle at position 1239 marks the alternative polyadenylation site observed (see text). (B) Hydropathy plot of the predicted X16 amino acid sequence. Note the relatively neutral N-terminal half of the protein and the very hydrophillic C-terminal half contaning conserved nucleid acid-binding motifs discussed in the text.

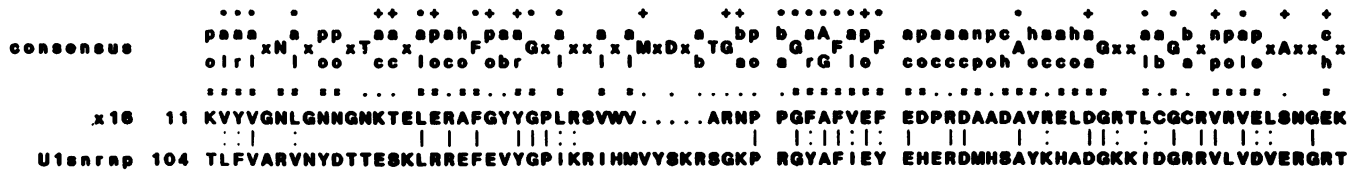

Figure 5. Alignment of the X16 N-terminal domain with an RNP-consensus binding motif and human U1snRNP 70K protein. The RNA.recognition motif derived by Query et al. (26) for one class of RNA-binding proteins is shown in the first line. The residue at each position i the consensus is abbreviated either by the upper case single letter code for amino acids, or by lower case double letters where more than one amino acid is permitted in the consensus. In this case, the amino acids are grouped according to Taylor (29) where: aa=amides or acids (E,Q,D,N); ab=amides, acids or bases (E,Q,D,N,K,R); ac=acidic (D,E); al=branched-chain aliphatic (L,I,V); ar=aromatic (F,Y,W,H); ba=basic (K,R,H); ch=charged (D,E,K,R,H); ho=hydrophobic (A,C,F,G,H,I,L,M,T,V,Y); np=nonpolar (A,F,G,I,L,M,V); po=polar (C,D,E,H,K,N,Q,R,S,T,W,Y); x=unspecified. The centrally located ribonucleoprotein consensus core octamer (RNP-CS, see text) is bounded by spaces. Positions marked with a dot immediately above the consensus line are more conserved than positions marked with a '+'. Below the consensus line, positions conserved between X16 and the consensus sequence are marked with a '=', those not conserved are marked '.'. The third sequence line is the human U1snRNP 70K protein (U1snRNP). Identical residues between X16 and U1snRNP are indicated by a line, conserved residues by a colon. The positions of the domains within the respective proteins are given by the numbers at each end of the sequences.

Interestingly, a second hybridizing band at about 1.9 Kb is seen with an intensity of about 10% that of the main band in all tissues except brain where they are equally strong. Several other minor bands are also seen in some cell types upon long exposure.

The serum inducibility of X16 mRNA was tested by culturing the murine fibroblastic cell line Ltk$^-$ for 2 days in low serum (see methods). After replacement of the medium with fresh medium containing 10% serum and incubation for 2 and 11 hr., total cytoplasmic RNA was extracted and analyzed by northern blotting for X16 mRNA levels (figure 3). Quantitation by scanning revealed 1.3 times higher X16 mRNA relative to unstimulated cells after 2 hr. and a 3.4 fold induction after 11 hr.

Nucleotide sequence

A full length cDNA sequence was compiled from overlapping cDNA's. The resulting sequence (Fig.4) is 1383 bp long and contains a 492 bp open reading frame beginning from the first AUG at nucleotide 63. There are 826 bp of 3' untranslated sequence with a canonical polyadenylation site 15 bp before the 3' poly A stretch. One X16 cDNA clone was isolated which presumably used an internal polyadenylation site. This clone terminated with 8 A's at nucleotide 1239. There is a potential, although less frequently used, polyadenylation signal ATTAAA located about 20 bp upstream. We believe this to be an infrequently used site based on the low frequency with which is was isolated (1 in 6 cDNAs from preB cells) and the weak hybridization signal on Northern blots.

From the sequence, the encoded protein is predicted to consist of 164 amino acids and have an unmodified size of 19.3 kD, one potential N-glycosylation site, and a pI of 12.2. X16 protein can be roughly divided into the N-terminal one-half which is fairly neutral with a pair of closely spaced cystein residues at each end. The C-terminal one-half of the protein is extremely basic and includes several copies of RRRS and alternating RS residues (labelled A and B in Fig.4). A computer comparison of X16 DNA and protein sequences with published data (EMBL/GenBank releases 24 and 64 respectively) revealed no identity with known genes. There were, however, similarities involving both the neutral N-terminal half and the basic C-terminal regions of X16 with several proteins known to interact with nucleic acid.

The N-terminal portion of X16 from amino acid 11 to 85 contains an RNA-binding domain. A large number of RNA-binding proteins have been shown to contain a (and often several) conserved amino acid motif of about 90 amino acids believed

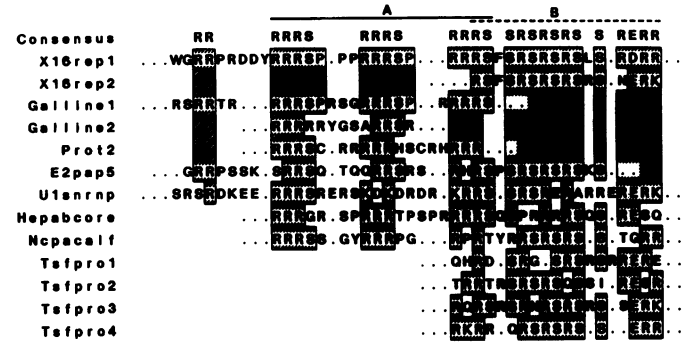

Figure 6. Alignment of the X16 C-terminal domain with several nucleic acid-binding proteins. A consensus sequence of the conserved amino acids is shown in the first row of the comparison. The A and B at the top of the figure refer to the repeat regions underlined in figure 4. The sequences shown (other than X16) were extracted from the computer data bank and include: X16 amino acids 96–134 (X16rep1), X16 amino acids 149–164 (X16rep2), two regions of a chicken protamine (galline1 and 2), mouse protamine 2 (prot2), the transcriptional-activator E2 protein of human papilloma virus type 5 (E2pap5), human U1snRNP 70K protein (U1snrnp), the core antigen of Hepatitis B virus subtype ayw (Hepabcore), the nucleocapsid protein of autographa californica multicapsid nuclear polyhedrosis virus (Ncpacalf), and four regions of the female-specific transformer protein from drosophila (Tsfpro1–4). In the alignment, the amino acid pairs Arg and Lys or Asp and Glu are treated as equivalent.

to be the RNA-binding domain (for review see 25). Within this domain is a highly conserved octapeptide core called the ribonucleoprotein consensus sequence (RNP-CS). Figure 5 shows an alignment of X16 with the consensus sequence for the RNA-binding domain. This consensus was derived by Query et al. (26) by comparing 27 RNA-binding motifs. Proteins containing this domain include: the U1snRNP 70K and U1snRNP A proteins, hnRNP C1/C2 protein, hamster nucleolin, yeast polyA binding protein, yeast single stranded nucleic acid binding protein, and T4 gene 32 protein. The RNP-CS is bounded by spaces in the figure. Seven of the 8 amino acids in the RNP-CS are conserved in X16. From the 19 positions previously shown (26) to be most strongly conserved in the overall consensus sequence (marked with dot above the consensus sequence in Figure 5), all but one are also conserved in X16. The strong conservation of the RNA binding motif in X16 is seen in a direct comparison with the human U1 snRNP 70K protein (Figure 5).

Figure 6 shows an alignment of the X16 basic A and B motifs underlined in Figure 3 with several nucleic acid binding proteins.

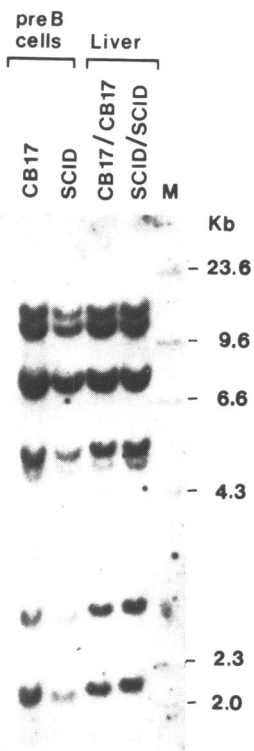

Figure 7. Genomic southern hybridization with X16 cDNA. Mouse liver and pre-B cell genomic DNA from CB17 and SCID strains of mice was digested with EcoRI, separated on agarose gels and hybridized with X16 cDNA spanning nucleotides 147 to 1245. The position of co-electrophoresed marker fragments is indicated on the right along with their approximate length in kilobase pairs.

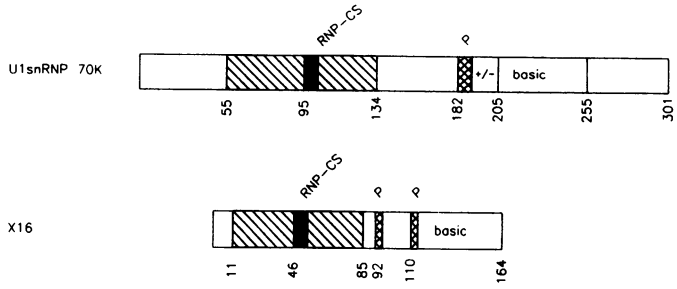

Figure 8. Schematic comparison of peptide domains between X16 and the human U1 snRNP 70K protein. The various protein domains depicted include the RNA-binding motif (diagonally striped) with the internal RNP-core octamer (see text, RNP.CS), proline rich spacer (cross-hatched, P), alternating acidic and basic residue domain (+/−), and the strongly basic domain containing the A and B motifs described in Figure 6. The numbers refer to the approximate amino acid position in each respective protein.

Like X16, most of the proteins have several copies of both RRRS and alternating RS motifs. The other proteins include the DNA-binding transcriptional activator E2 protein of papilloma virus, the human U1snRNP 70K protein, the core antigen of Hepatitis B virus, and the nucleocapsid protein of baculovirus. In addition, the RRRS motif can be found several times in histone-like proteins such as mouse protamine and a chicken protamine called galline. The alternating RS motif can also be found 4 times in the drosophila female-specific transformer protein. The redundancy of these motifs presumably reflects interaction with repetitive structures in DNA or RNA such as the sugar-phosphate backbone.

X16 also contains one copy of the sequence SPRR at the end of the A region (Figure 4). This tetra-peptide is similar to the SPRK and SPKK peptides found in many histones and which have recently been shown to bind DNA (27).

Genomic organization

Hybridization of radioactively labelled X16 cDNA (nucleotides 147−1245) with Eco RI-digested mouse genomic DNA resulted in 10 bands ranging from 2.2 to 15 Kb (Fig. 7). Preliminary experiments with somatic-cell hybrids show sequences hybridizing to X16 cDNA on several different mouse chromosomes (D.Schatz personal communication) suggesting the presence in the mouse genome of a family of X16-related sequences.

DISCUSSION

We report here the isolation of a novel mouse cDNA by virtue of its differential expression in an Abelson-transformed preB cell line and a plasmocytoma. Examination of several transformed preB, B-lymphocyte, and plasmacytoma cell lines maintained in vitro show approximately 5 to 10 fold higher expression in preB cells relative to B-lymphomas, hybridomas, and plasmacytomas.

Northern gel analysis of X16 mRNA expressed in various tissues and cell types shows several bands with a major signal at 1.6 Kb. The cDNA we describe here probably corresponds to the major band seen in pre-B cells. We isolated 5 independent cDNAs all of which terminate at the 5′ end within 12 bp of each other. This suggests either that they end very near the cap site of the mRNA or that there is a strong block of cDNA synthesis. The composite cDNA of 1380bp plus 100 nucleotides for the poly A tail predicts an mRNA of about 1500 nucleotides which roughly agrees with the estimated 1.6Kb major band seen on Northerns.

We isolated one cDNA which appears to have resulted from the use of an alternative polyadenylation signal at 1223−1228. This clone probably corresponds to a weak band seen on Northerns of 33.1.1+ mRNA at about 1400 nucleotides. We do not know the nature of the other mRNAs hybridizing with X16 probes.

Hybridization of X16 cDNA with restriction enzyme-digested genomic DNA results in numerous bands suggesting either a very large gene (>45 Kb) or a multigene family. Preliminary experiments with genomic DNA from mouse-hamster somatic cell hybrids suggests the presence of x16-hybridizing sequences on several different mouse chromosomes, consistent with a gene family (D.Schatz personal communication). We do not know how many of these potential X16 genes are expressed.

The unmodified protein encoded by X16 mRNA is predicted to be 164 amino acids long and 19kD. The protein consists of a proline rich spacer separating an N-terminal domain showing similarities to a RNA-binding motif and a C-terminal domain bearing similarities to DNA-binding proteins. The RNA binding motif found in X16 is present in one class of RNA binding proteins (25,26). Members of this class include proteins found in hnRNP, snRNP, pre-rRNP and mRNP particles, as well as several developmental control genes. Thus, for many of these proteins there is circumstantial evidence that they are involved in nuclear RNA processing.

The proteins which show similarity to the C-terminus of X16 can be divided functionally into 3 groups. The first is the drosophila transformer protein (tra) which is thought to regulate, either directly or indirectly, the alternative splicing of the double-sex gene transcript (28). However, it is has not been shown that tra, which contains 4 repeats of the alternating RS motif, binds directly to RNA or DNA. The second group of proteins with similarity to X16 are the histone and histone-like protamines from a variety of species. These relatively small proteins, which have several copies of the RRRS motif described above, all bind and play a role in the packaging of dsDNA. The third group of X16-similar proteins are those which have both RRRS and alternating RS motifs. These include the core or capsid proteins of two DNA viruses, the DNA-binding E2 transcription factor from papilloma virus, and the U1 snRNP 70K protein. The 70K protein has several structural similarities to X16 (see Figure 8). Both have RNA-binding consensus in the N-terminal part of the protein followed by a proline-rich stretch and then a basic region containing the RRRS and alternating RS motifs.(The 70K protein also contains regions of alternating acidic and basic residues which are not present in X16.) By deleting portions of the 70K protein it was shown that the in vitro binding to U1 RNA requires both the RNP-CN consensus domain and at least part of the basic region (26). In these studies it appeared that the specific U1 RNA binding was derived from the RNP-CN domain and that the basic region modified the strength of binding. Thus, one could speculate that the RRRS and RS motifs constitute a sequence-independent nucleic acid binding domain which can be coupled to other domains having protein-specific functions such as protein dimerization or RNA binding. We are currently testing whether X16 binds nucleic acid.

In adult mice, X16 is differentially expressed. The abundance of X16 mRNA is high in thymus, considerable in testis and very low or absent in liver and kidney. Since both the thymus and the testis contain high numbers of immature and proliferating cells while the liver and kidney do not, the strong expression of X16 suggests a role for X16 either in cell proliferation, or in cell maturation, or a combination of both. The expression in brain could be an exception. The correlation of X16 expression with immature or preB cells in vitro, where proliferation rates do not differ substantially, supports the cell maturation hypothesis. This is assuming that its expression has not become deregulated in the B-lineage tissue culture lines investigated, perhaps as a consequence of transformation or culturing in vitro. On the other hand, the stimulation of X16 expression observed in fibroblast cultures following serum addition argues for a role in cell proliferation. Further experiments are underway to determine whether X16 expresstion is correlated with differentiation or proliferation in other cell lineages.

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