The **Pax-5** Gene Is Alternately Spliced during B-cell Development*

(Received for publication, October 16, 1996, and in revised form, January 15, 1997)

Patty Zwollo§§*, Hector Arrieta‡, Kaleo Ede‡, Karen Molinder‡, Stephen Desiderio‡, and Roberta Pollock‡

From the §Department of Biology, Occidental College, Los Angeles, California 90041, the ¶Department of Molecular and Cellular Biology, University of California, Berkeley, California 94720, and the ‡Department of Molecular Biology and Genetics and the Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

*This work was supported by a research in undergraduate institutions grant from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Molecular and Cellular Biology, Life Sciences Addition 435, University of California, Berkeley, CA 94720. Tel.: 510-642-2436; Fax: 510-643-6791; E-mail: Zwollop@bengal.oxy.edu.

The transcription factor **Pax-5** is expressed during the early stages of B-cell differentiation and influences the expression of several B-cell-specific genes. In addition to the existing isoform (**Pax-5**), which we have named **Pax-5a**, we have isolated three new isoforms, **Pax-5b, Pax-5d**, and **Pax-5e**, from murine spleen and B-lymphoid cell lines using library screenings and polymerase chain reaction amplification. Isoforms **Pax-5b** and **Pax-5e** have spliced out their second exon, resulting in proteins with only a partial DNA-binding domain. Isoforms **Pax-5d** and **Pax-5e** have deleted the 3′-region, which encodes the transactivating domain, and replaced it with a novel sequence. The existence of alternative **Pax-5** transcripts was confirmed using RNase protection assays. Furthermore, **Pax-5a** and **Pax-5b** proteins were detected using Western blot analysis. **Pax-5a** was detectable in pro-, pre-, and mature B-cell lines, but not in two plasmacytomas; **Pax-5b** was shown to be present at low levels in mature B-cell lines and, unexpectedly, in one plasma cell line, but not in pro-B-cell or T-cell lines. Mobility shift assays showed that *in vitro* translated **Pax-5a** and **Pax-5d**, but not **Pax-5b** or **Pax-5e**, could interact with a B-cell-specific activator protein-binding site on the *bkl* promoter. Using this assay, we also showed that Pax-5d was present in nuclear extracts of some (but not all) B-lymphoid lines and interacts with the B-cell-specific activator protein-binding site. The pattern of differential expression of alternatively spliced **Pax-5** isoforms suggests that they may be important regulators of transcription during B-cell maturation.

Development of multicellular organisms involves highly regulated expression of specific transcription factors that direct cells into their proper differentiation pathways through interactions with specific target genes. Typically, transcription factors are divided into groups on the basis of the structural and functional similarity of their DNA-binding domains, such as the basic leucine zipper, basic helix-loop-helix, zinc finger, and homeobox families. One small family of transcription factors involved in development is the paired box (**Pax**) gene family, containing at least nine members, **Pax-1** through **Pax-9** (1, 2). All members contain a conserved DNA-binding domain encoding the paired box, which consists of a 128-amino acid sequence at the amino terminal side of the protein. Other conserved regions in Pax-proteins include an octamer and a homeodomain sequence in the center of the protein and a transactivating domain that spans 100 amino acids in the Ser/Thr/Pro-rich region at the carboxyl-terminal end (3, 4).

**Pax** genes are grouped into four classes based on structural similarity (5). Class I contains **Pax-1** and **Pax-9**, which lack a homeodomain. Two Class II members, **Pax-3** and **Pax-7**, encode both an octamer and a complete homeodomain. Class III, which includes **Pax-2**, **Pax-5**, and **Pax-8**, represents the class of **Pax** genes containing an octamer sequence as well as an incomplete homeodomain. Finally, Class IV contains **Pax-4** and **Pax-6**, both of which have a full homeodomain, but no octamer sequence.

**Pax-5** is one of the best studied members of the **Pax** family. In mice, **Pax-5** expression is first detected in developing embryos in the mesencephalon and spinal chord; the spatial and temporal expression pattern of **Pax-5** is distinct from other **Pax** genes in the developing central nervous system (4). Later, **Pax-5** is detected in fetal liver, where it correlates with the onset of B-cell development (4). Within the B-cell lineage, **Pax-5** transcripts are found in the early stages of B-cell development, including the pro-, pre-, and mature B-cell stage, but this expression ceases when the B-cell reaches the plasma cell stage (4). To date, the only other adult tissue where **Pax-5** expression has been observed is adult mouse testis (4). This expression pattern is in agreement with the **Pax-5** null mice phenotype. Mice lacking **Pax-5** through targeted deletion of their second exon show a complete block at the pro-B-cell stage of B-cell differentiation as well as an altered pattern of the posterior midbrain, but appear to undergo normal embryonic development and spermatogenesis (6).

The **Pax-5** gene encodes the B-cell-specific activator protein (BSAP). In *in vitro* experiments using mobility shift assays have shown that several B-cell-specific genes contain BSAP-binding sites, including the *CD19* (4, 7), *CD20* (8), *V_{pre,B}*, λ5 (9), and *blk* (10) promoters, as well as IgH chain switch regions (11) and the Ig 3′α (12) enhancer. These results are somewhat in contrast with *in vivo* data from Pax-5 null mice (which lack BSAP) because, although pro-B-cells isolated from these mice no longer express *CD19* (as would be expected), *blk*, *V_{pre,B}*, and *A5* genes are still expressed at wild-type levels (13). These latter results could suggest that their regulation is not strictly dependent on BSAP during early B-cell development. Alternatively, this expression pattern may be caused by redundancy of transcription factors similar to **Pax-5**.

The alternative splicing of RNA transcripts encoding transcription factors is likely to play an important role during cell...
Alternative Splicing of the Pax-5 Gene

Development and differentiation. This mechanism provides the cell with rapid and efficient ways to dictate a variety of functionally related activities from a single gene locus. Alternative splicing of transcription factors as a means of regulating their behavior has been observed in at least four members of the Pax family, and it is likely that some other members of this family will use this mechanism as well. Alternative splicing of a conserved 83-nucleotide segment in the 3′-portion of the human PAX-2 gene results in a frameshift mutation (14). Four different isoforms of the human PAX-8 and mouse Pax-8 genes are present in kidney tissue, and resulting proteins contain different C termini, which affects their transactivating properties (3). Two PAX-3 isoforms have been detected in humans through the alternate use of exons 4A and 4B, both of which contain the octapeptide; each resulting isoform has a distinct tissue distribution (15). The human PAX-6 and murine Pax-6 genes have one alternative isoform, in which a 14-amino acid sequence is inserted in the paired domain, resulting in a profound change in its interaction with DNA (16).

Within the pathways of lymphocyte development, alternative splicing has recently been shown to occur in two different genes, Ikaros and Tcf-1. The Ikaros gene is a lymphocyte-restricted zinc finger gene encoding a DNA-binding protein expressed in early lymphocytes and mature T-cells (17, 18). At least five distinct Ikaros transcripts that may encode functionally diverse proteins have been detected. Tcf-1 is a high mobility group box-containing transcription factor expressed in T-cells that has at least eight different isoforms, either as a result of alternative use of exons and/or the use of a second reading frame (19).

Here we describe the isolation of murine Pax-5 cDNAs by library screenings and by PCR and show that at least four different isoforms of Pax-5 mRNAs are expressed in several B-cell lines as well as in spleen, testis, and thymus tissue. Using RNase protection assays, Western analysis, and mobility shift assays, we show that at least three of these isoforms are differentially expressed in cells of the B-lymphoid lineage. This suggests that they may play important roles during B-cell development.

MATERIALS AND METHODS

Isolation of Multiple Pax-5 cDNAs—Pax-5 cDNAs were initially isolated by PCR amplification using cDNA generated from total RNA, isolated from murine spleen or thymus or the pro-B-cell line 2D6.5. First strand cDNA was generated from total RNA using a reverse transcription-PCR kit (Perkin-Elmer). PCR primers were as follows: PP8, 5′-gtgaggggagccaaATGTTAGAGAAAAATAC-3′ (sense primer); SD664, 5′-ccaggggagccaATGTTAGAGAAAAATATCCG-3′; and SD663, 5′-ccaggggagccgtGAGCTGATATGGACTGATGGC-3′ (antisense primer) (upper-case letters correspond to nucleotides −2 to 21, nucleotides −2 to 24, and nucleotides 1173 to 1153 of murine Pax-5a cDNA sequence, respectively, with nucleotides 1–3 (underlined) representing the first translational start site). Primers PP8 and SD663 were designed to have a unique restriction site with flanking nucleotides (I-KpnI) was made from poly(A) RNA from an 8-week-old B-cell line A20/2J. The murine spleen cDNA library (Lambda ZAP II; Stratagene) was made from poly(A) RNA by ligating with random priming; the B-cell cDNA library (Lambda uni-ZAP; Stratagene) was made from poly(A) RNA using random and oligo(dT) priming. For the PCRs, 1 μl (~10⁶ plaque-forming units) of λ phage stock was used per 100-μl PCR volume, and Pax-5 coding sequence was amplified using primers PP8 and SD663. The resulting PCR-amplified products were cloned into pBluescript as described above.

To isolate alternative isoforms and to confirm the authenticity of PCR-amplified isoforms, the murine spleen cDNA library (see above) was screened with a total of 10⁶ plaques using 5′-I-KpnI restriction enzyme digestion to remove the BSAP sequence. The following primers: PS-1, 5′-CCCTACCTCCTTGGCG-3′ (anti-sense); PS-2, 5′-CCAGTGGCAGCAACCAAC-3′ (sense); PP13, GATTCCTACCTATTGTCC (sense); PP14, GACCCCTGATGTTC (sense); and PP15, 5′-TTTTTACCCACACTTGGGAGG-3′ (antisense), as well as primers PP8 and SD663 (see above) and PP15 (see below). The positions of these primers on Pax-5a cDNA are indicated in Fig. 1. PP15 (5′-gaagggagcctTCCCTCGTCTGGAGAAGG-3′; antisense; corresponding to nucleotides 775 to 736 of sequence unique to isoforms Pax-5d and Pax-5e) was used in combination with PP8 to PCR amplify isoforms Pax-5d and Pax-5e and forideoxy sequencing.

Cell Lines—All cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum (Hyclone Laboratories), 2 mm glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol. DNA isolation and RNase Protection assay—Total cellular RNA was isolated by the lithium chloride/urea method (21) or by the guanidine isothiocyanate/cesium method (22). Antisense radiolabeled Pax-5 probes were prepared as described (23, 24). To analyze the sizes of Pax-5 transcripts at the 5′-end (detecting the deletion of nucleotides 47–212), the plasmid pBS-20.2, containing a 184-nucleotide NotI-Styl fragment of Pax-5a cDNA, was linearized using the restriction enzyme NotI and transcribed in vitro with T7 RNA polymerase in the presence of [α-32P]CTP, resulting in a 262-nucleotide “probe 20.2,” as shown in Fig. 3. After transcription, DNA was removed by digestion with 400 units/ml RNase-free DNase I.

The Pax-5d and Pax-5e isoforms were analyzed using the plasmid pBS-10.1, containing the complete Pax-5d sequence (nucleotides 1–607 identical to Pax-5e and nucleotides 608–735 unique to Pax-5d and Pax-5e). pBS-10.1 was linearized using the restriction enzyme BsrFI and transcribed using T7 RNA polymerase, resulting in an antisense probe of 392 nucleotides, covering nucleotides 447–607 of Pax-5a and nucleotides 608–735 of sequence unique to Pax-5d and Pax-5e. A 5′-tubulin-specific probe was similarly synthesized from the Bow/HI-linearized form of the plasmid pa5, which contains a 396-base pair Bow/HI-Styl cDNA fragment. Antisense RNA probe design is shown in Fig. 3.

RNase protection assays were performed as described (23) with modifications. For each cell line assayed, the RNA probe (5 × 10⁵ cpm) was annealed to 20 μg of total cellular RNA for 16 h at 55 °C in a reaction volume of 30 μl. After digestion with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 30 min at 37 °C, 100 μl of RNase-free water were added to 150 μg/ml and 1%, respectively, and the reactions were incubated for an additional 15 min at 37 °C. Products were extracted with phenol/chloroform, precipitated with ethanol in the presence of 10 μg of RNA, and resuspended in sample buffer containing 80% formamide. Products were fractionated by electrophoresis through a 5% polyacrylamide gel containing 7 m urea and detected by autoradiography. Radioactive RNA size markers were prepared using linearized recombinant pBluescript vectors that were available in the laboratory as templates and T3 and T7 RNA polymerases, resulting in labeled RNA transcripts ranging in size from 50 to 291 nucleotides.

In Vitro Transcription and Translation of Pax-5 Isoforms—The plasmids (pBluescript) containing the various Pax-5 isoforms were transcribed in sense and antisense directions with T3 and T7 RNA polymerases. Translation was carried out using a rabbit reticulocyte lysate (Promega) in the presence or absence of [35S]methionine according to the supplier’s directions. Products were analyzed by SDS-polyacrylamide gel electrophoresis and radiography.

Anti-Pax-5 Antibodies—Anti-Pax-5 antibodies were directed against a synthetic peptide covering residues 234–245 of Pax-5 protein, conjugated to keyhole limpet hemocyanin (25). New Zealand White rabbits were immunized intradermally with 1 mg of coupled peptide, followed by five booster immunizations (250 μg each) that were administered every other week beginning 21 days after the initial immunization (horseradish peroxidase). Antiserum (named OC-1) was obtained at 12 weeks, analyzed by enzyme-linked immunosorbent assay, and used at a 1:250 dilution in Western blot analysis.
Western Blot Analysis—Nuclear and cytoplasmic extracts from the pro-B-cell lines HAFTL-1–14.5 and 2.1AJP4, the pre-B-cell line 703Z, the mature B-cell lines 2PK3 and A20/2J, the plasmacytoma lines SP2/0 and RPC5.4, and the T-cell line EL-4 were prepared as described (10). Extracts were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose filters (Schleicher & Schuell) as described (10). Membranes were first incubated for 2 h in blocking solution (Kirkegaard & Perry Laboratories, Inc), followed by a 1-h incubation with rabbit anti-Pax-5 antiserum OC-1 in blocking solution. Next, membranes were incubated with biotin-conjugated anti-rabbit antibody (1:20,000; Zymed Laboratories, Inc.) for 1 h, followed by a 20-min incubation in the presence of horseradish peroxidase-conjugated streptavidin at 0.5 μg/ml (Kirkegaard & Perry Laboratories, Inc). Between each incubation, membranes were washed three times for 10 min in 1 × wash solution (Kirkegaard & Perry Laboratories, Inc). The blots were developed with an enhanced chemiluminescence kit (Kirkegaard & Perry Laboratories, Inc), and bands were visualized on Eastman Kodak XAR-5 film.

Electrophoretic Mobility Shift Assays—Binding assays were carried out for 30 min at 30 °C in 10-μl reactions containing 60 mM KCl, 12 mM HEPES, pH 7.9, 4 mM Tris-Cl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 30 ng of bovine serum albumin, 12% glycerol, 1–5 μl of nuclear extract, 0.1 ng of 32P-labeled DNA probe, and 1–5 μl of poly(dIdC). The DNA probe P3 was identical to the one used in an earlier study (10) and spans nucleotides 70 to 136 of the murine blk promoter. Products were separated by electrophoresis on a 5% polyacrylamide gel in a buffer containing 30 mM Tris-Cl, pH 7.5, 29.3 mM boric acid, and 0.66 mM EDTA. Gels were dried and exposed to Eastman Kodak XAR-5 film.

RESULTS

Isolation of Alternatively Spliced Pax-5 Sequences—The presence of alternatively spliced forms of the Pax-5 gene was initially investigated using PCR amplification. Pax-5 mRNA from the pro-B-cell line 22D6.5 or from mouse tissue was reverse-transcribed into cDNA (Stratagene) and PCR-amplified (Fig. 1). Several amplified DNA fragments ranging from 1.4 to 0.7 kilobase pairs were isolated individually by gel purification and cloned into pBluescript. Dideoxy sequence analysis of different clones resulted in the identification of two Pax-5 isoforms with sizes of 1.2 and 1.1 kilobase pairs. These were named Pax-5a and Pax-5b, respectively.

From a total of 17 spleen amplified clones containing Pax-5 sequence, 11 were isoform Pax-5a, and three were Pax-5b. Of 39 analyzed Pax-5 clones from 22D6.5 cDNA, isoform Pax-5a again was most abundant (32 clones), whereas four clones were isoform Pax-5b. Some remaining clones containing Pax-5 sequence were either hybrid or incomplete clones. In addition, we found a number of identical clones, which we named Pax-5c, that were similar to Pax-5b, but had an additional internal deletion at their 3′-ends. The structure of Pax-5c is still under investigation.

The sequence of Pax-5a matches that of the published full-length mouse Pax-5 cDNA (GenBankTM/EMBL Data Bank accession no. M97913; Ref. 4) and is 1176 nucleotides long (391 amino acids). Isoform Pax-5b contains DNA sequence identical to that of Pax-5a with the exception of one deletion, covering nucleotides 47–212, which corresponds to its second exon, as indicated in Fig. 2A.

Both Pax-5a and Pax-5b contain two in-frame translational start sites, one at nucleotide 1 (proximal ATG codon) and one at nucleotide 325 (distal ATG codon). Use of the proximal ATG codon in isoform Pax-5a results in a protein of ~53 kDa. Pax-5b also contains this proximal ATG codon. However, use of this start codon creates a termination codon (in exon 3) at nucleotide 245, 12 codons downstream from the deletion, resulting in a peptide of 27 amino acids (3.2 kDa). When the distal ATG codon is used (for either Pax-5a or Pax-5b), which results in an open reading frame of 849 nucleotides, a 41-kDa protein will be expressed.

The paired domain of Pax-5a is encoded by nucleotides 45–428. Because of the presence of two ATG codons, one Pax-5a transcript could theoretically give rise to two translated proteins, one containing a complete and one containing a partial DNA-binding domain. In this situation, it is likely that the most upstream (proximal) ATG codon will be used most frequently. In contrast, translation of the Pax-5b transcript (from the downstream ATG codon) will result in Pax-5 protein with an incomplete DNA-binding domain.

To rule out the possibility of PCR artifacts and to find additional isoforms, two λ phage cDNA libraries, one from spleen and one from the mature B-cell line A20/2J, were screened with a full-length Pax-5a probe. PCR analysis was also used on these libraries to search for additional alternatively spliced forms of Pax-5.
From the spleen library, 45 Pax-5-containing clones were analyzed by dideoxy sequencing. Of these clones, 35 represented isoform Pax-5a, and two clones were Pax-5b. In addition, two new isoforms were identified, Pax-5d and Pax-5e. These two isoforms had both replaced the 3′-half of their transcript (nucleotides 607–1176 of Pax-5a) by novel sequence (nucleotides 607–735 of Pax-5d). Seven Pax-5d clones and one Pax-5e clone were identified.

The breakpoint at nucleotide 607 corresponds to the boundary between exons 5 and 6 of the Pax-5 gene (26). Thus, both isoforms Pax-5d and Pax-5e contain exons 3–5, but not exons 6–10. Pax-5e differs from Pax-5d because it has deleted its second exon (nucleotides 47–212), which is identical to the deletion present in isoform Pax-5b. The structures of these two new isoforms are indicated in Fig. 2A. The new sequence shared by Pax-5d and Pax-5e is given in Fig. 2B and C. Comparison of this sequence with the peptide data bases did not reveal any obvious homology to existing sequences, and therefore, its function remains to be determined.

To investigate the possibility that this new sequence contains a transactivating domain, the percentage of Ser/Thr/Pro residues was determined. The transactivating domain of the Pax-5a protein (residues 283–382) contains 38% of these three residues (10), as compared with an average occurrence of ~19% in mammals. The novel sequence in Pax-5d and Pax-5e had 26% Ser/Thr/Pro residues, which is considerably lower than the transactivating domain of Pax-5a. This makes it less likely that the novel Pax-5d sequence encodes a transactivating domain.

Use of primer set PP8 and SD663 for PCR amplification of the spleen cDNA library (see Fig. 1) resulted in the detection of isoforms Pax-5a and Pax-5b. When a different primer set was used in these experiments (PP8 and PP15), isoforms Pax-5d and Pax-5e were detected. (The Pax-5d- and Pax-5e-specific primer PP15 anneals immediately downstream from their stop codon.) PCR amplification (using either primer set PP8 and SD663 or primer set PP8 and PP15) and library screening of the A20/2J library (using a full-length Pax-5a probe) did not yield any additional isoforms, although all four isoforms, Pax-5a, Pax-5b, Pax-5d, and Pax-5e, were isolated from this library.

It should be noted that for some of the PCR experiments, we used primer PP9 (nucleotides 1152–1176) as the antisense primer in the place of SD663 (see Fig. 1). Its sequence was based on the published mouse Pax-5 sequence (GenBank™/EMBL Data Bank accession no. M97013; Ref. 4), but this primer did not amplify Pax-5 sequences efficiently. Later experiments using conventional library screening followed by dideoxy sequencing indicated that all murine Pax-5 sequences isolated by our group contained 4 nucleotides in the area covered by primer PP9 that differed from the published murine Pax-5 sequence: nucleotides 1158 (C to T), 1161 (C to G), 1164 (T to C), and 1170 (T to C) (our results to published sequence), all of which are third or “wobble” positions of the codons. This discrepancy explains the inability of PP9 to anneal efficiently to Pax-5 sequence in this region. The antisense primer SD663, although based on human Pax-5 sequence, has only one mismatch (out of 21 nucleotides) with mouse Pax-5 in this region and resulted in highly efficient PCR amplifications.

Fig. 3. Overview of the plasmid templates pBS-20.2 (Pax-5a) and pBS-10.1 (Pax-5d), riboprobes 20.2 and 10.1, and expected protected regions on Pax-5 mRNA, as used in the RNase protection assays. See “Materials and Methods” for details. The position of exon 2 as well as the novel sequence unique to Pax-5d and Pax-5e are indicated. nts, nucleotides.
Detection of Heterologous Pax-5 Transcripts in B-cell Lines and Tissues—To obtain additional evidence for the existence of differentially spliced Pax-5 transcripts in B-cell lines as well as to investigate the Pax-5 distribution pattern in mouse tissues, RNase protection assays were used. The following antisense Pax-5 RNA probes were used to analyze the sizes of Pax-5 transcripts: 1) the 5′-probe 20.2, which detects the 5′-deletion present in Pax-5b and Pax-5e; 2) the internal probe 10.1, which detects the novel sequences present in isoforms Pax-5d and Pax-5e; and 3) a “control” tubulin probe (p\(\mu\)), which detects β-tubulin transcripts. P.C., plasmacytoma.

Detection of Heterologous Pax-5 Transcripts in B-cell Lines and Tissues—To obtain additional evidence for the existence of differentially spliced Pax-5 transcripts in B-cell lines as well as to investigate the Pax-5 distribution pattern in mouse tissues, RNase protection assays were used.

The following antisense Pax-5 RNA probes were used to analyze the sizes of Pax-5 transcripts: 1) the 5′-probe 20.2, which detects the 5′-deletion present in Pax-5b and Pax-5e; 2) the internal probe 10.1, which detects the novel sequences present in isoforms Pax-5d and Pax-5e; and 3) a “control” tubulin probe (p\(\mu\)), which detects overall levels and quality of RNA. Probe design and the “expected” protected RNA fragments are indicated in Fig. 3.

Because it was important to determine accurately the sizes of the protected fragments, an array of RNA markers were run in parallel, using several recombinant pBluescript vectors as DNA templates. Using T3 and T7 RNA polymerases, these produced labeled RNA molecular mass markers ranging in size between 48 and 291 nucleotides.

Results from the 5′-probe 20.2 are shown in Fig. 4A. As expected, a 184-nucleotide band, corresponding to Pax-5 transcripts that contain exon 2, was present in all pro-, pre-, and mature B-cell lines tested. This band was not detectable in the plasmacytoma lines or in the T-cell line tested. The levels of the 184-nucleotide band were relatively high in most B-cell lines, except for the pre-B-cell line 18-8 and the mature B-cell line A20/2J. The only tissue that had detectable levels of the 184-nucleotide band was the spleen.

In isoforms Pax-5b and Pax-5e, where the second exon has been spliced out, a 46-nucleotide fragment (covering nucleotides 1–46) would be expected (Fig. 4A). This fragment was indeed present in all pro-, pre-, and mature B-cells tested (Fig. 4A), although at a much lower intensity in all cases. This is not only a function of the lower level of this transcript, but also of
the relative amount of label incorporated into this smaller fragment. Following extended exposure to x-ray film, very low levels of the 46-nucleotide fragment could also be detected in the plasmacytoma lines SP2/0 and XS63 and in the plasmacytoma line S194 (data not shown), but not in the T-cell line EL-4. The only tissue that clearly showed this 46-nucleotide fragment was the spleen.

An additional protected band of ~135 nucleotides was detected in all pro-, pre-, and mature B-cell lines, but not in the plasmacytoma or T-cell lines (Fig. 4A). This band was also clearly detected in spleen tissue. The size of this band corresponds to a transcript that does not contain exon 1 (see Fig. 3). Recently, Busslinger et al. (26) reported the presence of a second promoter downstream from exon 1. This is in agreement with the presence of the 135-nucleotide fragment because both promoters are active in B-cells (26).

Interestingly, the ratio of the 184- to 135-nucleotide bands varied among the different cell lines. This was most strikingly visible in the mature B-cell line A20/2J. In this cell line, the 184-nucleotide band was relatively weak, whereas the 135-nucleotide band was very strong. For the 2.1AJP4 pro-B-cell line, both bands had similar intensity. Most other B-cell lines had a much stronger 184-nucleotide band relative to the 135-nucleotide band. This pattern was not the result of degraded RNA (for example, in A20/2J), as the quality of RNA was tested with the presence of the 135-nucleotide fragment because both promoters are active in B-cells (26).

An additional protected band of ~135 nucleotides was detected in all pro-, pre-, and mature B-cell lines, but not in the plasmacytoma or T-cell lines (Fig. 4A). A band was also detected in spleen tissue. The size of this band corresponds to a transcript that does not contain exon 1 (see Fig. 3). Recently, Busslinger et al. (26) reported the presence of a second promoter downstream from exon 1. This is in agreement with the presence of the 135-nucleotide fragment because both promoters are active in B-cells (26).

Interestingly, the ratio of the 184- to 135-nucleotide bands varied among the different cell lines. This was most strikingly visible in the mature B-cell line A20/2J. In this cell line, the 184-nucleotide band was relatively weak, whereas the 135-nucleotide band was very strong. For the 2.1AJP4 pro-B-cell line, both bands had similar intensity. Most other B-cell lines had a much stronger 184-nucleotide band relative to the 135-nucleotide band. This pattern was not the result of degraded RNA (for example, in A20/2J), as the quality of RNA was tested for all samples using the tubulin (p1B) probe (Fig. 4C). This would imply that the activity of the two promoters varies among certain B-cell lines, most clearly in A20/2J.

One additional protected band was clearly present in the cell line A20/2J, but not in any other samples. This band is ~108 nucleotides long, and its origin is unclear.

RNase protection assays were particularly important to show that isoforms Pax-5d and Pax-5e were real and not the result of artifacts during the creation of the cDNA libraries, which can sometimes lead to hybrid clones. A 392-nucleotide RNA probe (10.1) was used, covering nucleotides 447–735 of Pax-5d and Pax-5e, resulting in a 288-nucleotide protected fragment. This probe should also protect nucleotides 447–607 of the Pax-5a and Pax-5b isoforms, resulting in a 160-nucleotide fragment.

Fig. 4B shows the results of this experiment. Relatively high levels of the 288-nucleotide band, unique to isoforms Pax-5d and Pax-5e, were detected in all pro-, pre-, and mature B-cell lines, but not in two plasmacytomas or in the T-cell line EL-4. The 288-nucleotide band is also present at relatively high levels in spleen tissue and clearly in testis. As expected, a 160-nucleotide band, corresponding to isoforms Pax-5a and Pax-5b, was found in the same tissues and cell lines as the 288-nucleotide band, although the intensity of the 160-nucleotide band is ~10–50-fold higher than that of the 288-nucleotide band. No obvious variation in relative intensity of the 288-nucleotide versus the 160-nucleotide band was noted in each individual B-cell line or tissue tested.

It should be noted that in testis, the 10.1 probe detects a strong 160-nucleotide band, in contrast to a nondetectable signal using the 5′-probe (20.2) (compare testis samples in Fig. 4, A versus B). This is likely the result of the presence of a second downstream promoter that is active in this tissue, as reported by others (6). Some additional bands are detected using the 10.1 probe, including a 270-nucleotide and a 105-nucleotide protected band, with a B-cell and tissue distribution identical to that of the 288-nucleotide band, although with a lower intensity. The origin of these bands is unclear, but may be the result of additional alternatively spliced isoforms of Pax-5.

Finally, a strong band of ~120 nucleotides was detected in the cell line 2PK3 only. Its size could suggest that it may represent a new Pax-5 isoform that contains the novel sequence, but has deleted a region immediately upstream of nucleotide 607; such an isoform would appear as a band with a theoretical size of 128 nucleotides (Fig. 3).

Presence of Alternative Forms of Pax-5 Protein in B-cell Lines—To investigate the presence of alternative isoforms of Pax-5 protein, nuclear extracts from various B-cell lines as well as from one T-cell line were separated by size on SDS-polyacrylamide gels, and the proteins were transferred onto nitrocellulose paper. Western analysis was performed using polyclonal rabbit antiserum OC-1 (specific to isoforms Pax-5a and Pax-5b). Unfortunately, we had no antisera available to detect Pax-5d and Pax-5e.

To identify bands corresponding to Pax-5a and Pax-5b, a plasmid containing full-length Pax-5a cDNA sequence was transcribed and translated in vitro in the presence of radiolabeled [35S]methionine. The resulting in vitro translated protein products, containing both Pax-5a and Pax-5b, were used in parallel with the nuclear extracts during gel electrophoresis and nitrocellulose filter transfer and were visualized by exposure to x-ray film.

One strong 53-kDa band, identical in size to the in vitro translated isoform Pax-5a, was observed in all nuclear extracts derived from pro- and mature B-cell lines, but not in two plasma cell lines or in the T-cell line, as shown in Fig. 5. This band was also detected in the pre-B-cell line 70Z/3 (data on this cell line not shown). A weak 41-kDa band, corresponding to the Pax-5b isoform, was detected in pre- and mature B-cell lines and in one of the two plasma cell lines tested (SP2/0). No Pax-5b was detected in the second plasmacytoma cell line (RPC5.4), in two pro-B-cell lines, or in the T-cell line EL-4 (Fig. 5). An additional band of ~38 kDa with an expression pattern similar to that of Pax-5b was also detected (Fig. 5). The origin of this latter band is unclear, but its expression pattern suggests that it may represent an additional isoform of Pax-5.

Interactions of Pax-5 Isoforms with a BSAP-binding Site in Vitro—To investigate the DNA binding properties of the various Pax-5 isoforms, electrophoretic mobility shift assays were performed using the DNA fragment F3 from the murine blk promoter (nucleotides −70 to 136), which contains a BSAP-binding site at nucleotides −68 to −38 (10). In vitro translated Pax-5 isoforms were used in parallel with the extracts to enable identification of retarded DNA-Pax-5 complexes on the gel.
Initially, the four in vitro translated Pax-5 isoforms were incubated with the F3 DNA probe and analyzed for the presence of retarded bands. As expected, in vitro translated Pax-5a and Pax-5d, both containing intact DNA-binding domains, were able to bind to the F3 probe (Fig. 6A, tenth and second lanes, respectively), whereas Pax-5b and Pax-5e did not (data not shown).

To test the relative affinity of Pax-5a and Pax-5d for the BSAP-binding site on the blk promoter, both were translated in vitro in the presence of [35S]methionine. The fact that both isoforms contain two methionine residues was used to determine the relative amount of the two isoforms. The translated products were separated by SDS-polyacrylamide gel electrophoresis; labeled Pax-5a and Pax-5d were cut out; and [35S]methionine incorporation of each was counted in a scintillation counter. Various relative molar ratios of the two in vitro translated proteins were then incubated with the F3 probe. The resulting mobility shift pattern indicates that Pax-5a and Pax-5d proteins had a very similar affinity for the BSAP-binding site.

Next, we investigated the presence of Pax-5d in B-lymphoid nuclear extracts. As a control, we used in vitro translated Pax-5d bound to the F3 probe. A retarded band corresponding in position to the F3 probe-Pax-5d complex was detectable at low levels in three of the six cell lines tested (Fig. 6B, second through fourth lanes), including a plasmacytoma, a pro-B-cell, and a mature B-cell line (S107.3.4, 2.1APJ4, and 2PK3, respectively). Competition shifts using an excess of unlabeled oligonucleotide F38 (which contains the blk BSAP-binding site; see “Materials and Methods”) and nuclear extract from the mature B-cell line 2PK3 indicated that protein binding occurs specifically at the BSAP-binding site. (Fig. 6B, fifth lane). In addition, competition shift assays in which excess in vitro translated Pax-5a was added to the 2PK3 nuclear extract resulted in the disappearance of the F3 probe-Pax-5d complex (data not shown).

A possible cytoplasmic localization of the various isoforms was investigated by mobility shift assay using cytoplasmic membranes (S-100) fractions collected during the preparation of the nuclear extracts (10). From these experiments, it was clear that none of the four Pax-5 isoforms are present at detectable levels in the cytoplasm of the B-cell lines (data not shown).

**DISCUSSION**

Pax-5, a member of the Pax family of transcription factors, encodes the B-cell-specific transcription factor BSAP, which plays important roles in transcriptional regulation during B-lymphoid development. Here we describe experiments demonstrating the presence of alternative isoforms of the Pax-5 gene as well as interactions between its encoded products and the BSAP-binding site in vitro. In addition to the published Pax-5 sequence, which we have named Pax-5a, three alternative isoforms of Pax-5 transcripts were detected, Pax-5b, Pax-5d, and Pax-5e. Two types of splice variants were found: Pax-5b and Pax-5e have spliced out their second exon, resulting in an incomplete DNA-binding domain, whereas Pax-5d and Pax-5e have replaced a region containing the transactivating domain with a novel sequence.

Pax-5b Is Present in the Nucleus of B-lymphoid Cells, but Does Not Interact with the BSAP-binding Site—The presence of Pax-5b protein appears to correlate with the differentiation stage of the B-cell lines. For example, pro-B-cells had no detectable levels of Pax-5b, but this protein becomes detectable during later stages of B-cell development. Because the levels of Pax-5a are relatively high during all stages of development until the plasma cell stage, at which point Pax-5a expression ceases, the relative level of Pax-5b increases during B-cell development. This pattern became even more pronounced with comparison of Pax-5b levels between mature B-cell and plasma cell lines. One highly differentiated mature B-cell line (A20/2J) had decreased levels of Pax-5a and slightly increased levels of Pax-5b. Even more striking was the situation in the plasma cell line SP2/0, which had undetectable levels of Pax-5a, whereas Pax-5b was clearly detectable. This expression pattern may indicate an important regulatory role for this alternatively spliced protein in transcriptional regulation.

The affinity of Pax-5b for the BSAP-binding site on the blk promoter was tested by mobility shift assay. No retarded bands were detected using in vitro translated Pax-5 under conditions where isoform Pax-5a bound strongly. This is in agreement with a report by Czerny et al. (27), who showed that the region on Pax-5 encoded by exon 2 (which is absent in Pax-5b) is essential for DNA binding. From our data, we hypothesize that Pax-5b, although it may no longer be able to interact with the BSAP site, will still be able to interact with other regulatory proteins. Possible functions for Pax-5b include that of a corepressor, by competing with Pax-5a for binding to as yet unidentified regulatory molecules, or, alternatively, a coactivator, by interacting with other transcription factors. We cannot rule out, however, the possibility that Pax-5b will still bind...
BSAP sites of selected target genes, albeit with much lower affinity.

Whether or not the alternatively spliced isoform Pax-5b will have significant effects on B-cell development in vivo is not known. In this regard, such a function has been shown to exist for the pou-2 gene, a member of the Pou domain-containing genes, which are important regulators of early development and differentiation (28). In this case, the alternative form, t-pou-2, lacked DNA binding activity because its DNA-binding domain was incomplete. Although this alternative form had no binding activity in vitro (using mobility shift assays), overexpression of t-pou-2 in embryos caused severe developmental retardation. It was hypothesized that t-pou-2 is involved in negative regulation of a set of genes required for further embryonic development, probably through interactions with other regulatory proteins (28).

Alternative Splicing of Pax-5 Results in Two Isoforms with a Novel 3’-Sequence (Pax-5d and Pax-5e)—The splice site used in isoforms Pax-5d and Pax-5e (between nucleotides 606 and 607) represents the intron-exon boundary between exons 5 and 6 (26). Interestingly, the intron-exon boundary at nucleotide 606 falls between two conserved sequences shared by the majority of the Pax family members: the octamer sequence encoded by exon 5, which is present in all four Pax-5 isoforms (nucleotides 535–558), and the homeodomain homology box encoded by exon 6, which is present in isoforms Pax-5a and Pax-5b (nucleotides 685–753), but absent in Pax-5d and Pax-5e. As the functions of these two conserved domains are unknown, the significance of this alternative splice site remains to be determined.

In addition to the absence of the homeobox homology sequence, Pax-5d and Pax-5e have also deleted the region encoding the transactivating domain. The genomic position of the novel sequence in the Pax-5 gene is not known. It is possible that the novel Pax-5d sequence is an as yet unidentified alternative exon, comparable to Pax-3, which contains alternative exons 4A and 4B (15). Alternatively, this sequence may be an exon downstream from the exons present in Pax-5a and Pax-5b. In any case, the replacement of the 3’-segment of Pax-5 by this novel sequence may result in profound changes in its activator function. This idea is supported by co-transfection studies using Pax-8 isoforms that vary at their C termini. These isoforms showed significant differences in their transactivating potential as measured in transcriptional activation of a reporter gene, depending on the structure of their C termini (3).

Pax-5d Is Detectable in Nuclear Extracts of Certain B-cell Lines, where It Interacts with the BSAP-binding Site—Unfortunately, we could not detect isoforms Pax-5d and Pax-5e by Western blot analysis because we were not able to produce polyclonal anti-Pax-5 antibodies in rabbits that would recognize Pax-5d and Pax-5e (attempts were made with three different Pax-5 peptides). To investigate the presence of Pax-5 protein in B-lymphoid cells, we used the mobility shift assay instead. Using both in vitro translated Pax-5d and B-cell nuclear extracts in the same experiment, we could show that Pax-5d 1) is present at low levels in some B-lymphoid lines, 2) can interact specifically with the BSAP site, and 3) has equal affinity for this site compared with Pax-5a. The expression pattern of Pax-5d is clearly different from that of Pax-5b, although both are expressed at low levels.

As a direct result of the competition between Pax-5a and Pax-5d for binding to the BSAP site, we hypothesize that only little Pax-5d will be bound to its BSAP target site in situations where Pax-5a levels are high (relative to Pax-5d). Under conditions (as yet unknown) where the Pax-5a/Pax-5d ratio decreases, relatively more Pax-5d can bind to its target sequence (as compared with Pax-5a). We hypothesize that in this latter situation, it has a dominant-negative function, down-regulating expression of Pax-5 target genes. Transcriptional repression of Pax-5 target genes could thus be controlled, at least to some extent, at the level of RNA splicing of the Pax-5 transcript.

What Are the Functions of Alternatively Spliced Pax-5 Products?—Several studies have pointed toward a role for Pax-5 in cell growth and proliferation. For example, overexpression of Pax-5 by transient transfection induces proliferation of splenic B-cells or plasmacytomas (29), whereas injection of cells overexpressing various Pax proteins in mice results in tumor formation (30). Pax-5 has also been shown to interact directly with a sequence on the basal promoter of the tumor suppressor gene p53, where it suppresses its expression (31). Based on these and our own observations, it is possible that alternatively spliced Pax-5 products are involved in the specific suppression of Pax-5 target genes by direct interaction with the BSAP-binding site on these genes (e.g. Pax-5d) and/or through interactions with other regulatory factors necessary for initiation of transcription (e.g. Pax-5b and Pax-5e). (Temporary) down-regulation of Pax-5 target genes could be particularly important during the differentiation phases the cell encounters during its development from a pro-B-cell to a plasma cell. Alternatively, it is still possible that Pax-5b, Pax-5d, and Pax-5e can serve as positive regulators of transcription, depending on the presence of other transcription factors in the cell at a particular stage. Future studies to address these questions will include the generation of transgenic mice that overexpress one of the alternative isoforms of Pax-5.

In conclusion, we have identified three new isoforms of the Pax-5 gene, Pax-5b, Pax-5d, and Pax-5e. The structure of these isoforms, their expression pattern in B-cell lines, and their DNA binding characteristics in vitro suggest that they may serve important functions in the regulation of expression of Pax-5 target genes during B-cell development.

Acknowledgments—We thank Dr. Suzanna Horvath and the Beckman Institute at the California Institute of Technology for synthesis of the peptides and helpful advice and Dr. Steve Smale, Hans Clevers, Susan Dymerci, and Marian Koshland for discussions and advice.

REFERENCES
1. Walther, C., Guenet, J. L., Simon, D., Deuthich, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R., and Gruss, P. (1991) Genomics 11, 424–434
2. Stapleton, P., Weith, A., Urbanek, P., Koziak, Z., and Busslinger, M. (1993) Nat. Genet. 3, 424–427
3. Kriznik, Z., Kurzrauer, R., Dörrler, P., and Busslinger, M. (1993) Mol. Cell. Biol. 13, 6024–6035
4. Adams, B., Dörrler, P., Aguzi, A., Koziak, Z., Urbanek, Z., Maurer-Fogly, I., and Busslinger, M. (1992) Genes Dev. 6, 1589–1607
5. Suster, E. T., and Gruss, P. (1995) Hum. Mol. Genet. 4, 1717–1720
6. Urbanek, P., Wang, Z.-Q., Fetka, I., Wagner, E. F., and Busslinger, M. (1994) Cell 79, 901–912
7. Kriznik, Z., van der, P., Dörrler, P., Adams, B., and Busslinger, M. (1992) Mol. Cell. Biol. 12, 2662–2672
8. Thorsen, C., Wilson, G. L., and Kehrli, J. H. (1994) FASEB J. 8, 221 (Abstr. 4570)
9. Oake, T., Watanabe, T., and Kudo, A. (1992) Eur. J. Immunol. 22, 37–43
10. Zevolo, P., and Desiderio, S. (1994) J. Biol. Chem. 269, 15310–15317
11. Liao, P., Giannini, S. L., and Birnstein, B. (1992) J. Immunol. 148, 2909–2913
12. Singh, M., and Birshtein, B. (1993) Mol. Cell. Biol. 13, 3611–3622
13. Busslinger, M., and Urbanek, P. (1985) Curr. Opin. Genet. & Dev. 5, 595–601
14. Ward, T. A., Nebel, A., Steever, E. E., and Kedes, M. R. (1994) Cell Growth & Differ. 5, 1051–1021
15. Tsukamoto, K., Nakamura, Y., and Nikiawa, N. (1994) Hum. Genet. 93, 270–274
16. Epstein, J. A., Glaser, T., Cai, J., Patel, L., Walton, D. S., and Maas, R. L. (1994) Genes Dev. 8, 2022–2034
17. Hahm, K., Ernst, P., Lo, K., Kim, G. S., Turck, C., and Smale, S. T. (1994) Mol. Cell. Biol. 14, 7111–7123
18. Molnar, A., and Georgopoulos, K. (1994) Mol. Cell. Biol. 14, 8292–8305
19. van de Wetering, M., Castrop, J., Körner, V., and Clevers, H. (1996) Mol. Cell. Biol. 16, 745–752
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A 74, 5463–5467
21. Auffrey, C. (1979) Eur. J. Biochem. 107, 303–311
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
23. Dymecki, S. M., Izvollo, P., Zeller, K., Kuhajda, F. P., and Desiderio, S. V. (1992) J. Biol. Chem. 267, 4815–4823
24. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acids Res. 12, 7035–7056
25. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 9.3.1–9.4.8, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Busslinger, M., Klix, N., Peper, P., Graninger, P., and Kozmik, Z. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6129–6134
27. Czerny, T., Schaffner, G., and Busslinger, M. (1993) Genes Dev. 7, 2048–2061
28. Takeda, H., Matsuzaki, T., Oki, T., Miyagawa, T., and Amanuma, H. (1994) Genes Dev. 8, 45–53
29. Wakatsuki, Y., Neurath, M. F., Max, E. E., and Strober, W. (1994) J. Exp. Med. 179, 1099–1108
30. Mailheber, C. C., and Gruss, P. (1993) EMBO J. 12, 2361–2367
31. Stuart, E. T., Haffner, R., Oren, M., and Gruss, P. (1995) EMBO J. 14, 5638–5645
The Pax-5 Gene Is Alternatively Spliced during B-cell Development
Patty Zwollo, Hector Arrieta, Kaleo Ede, Karen Molinder, Stephen Desiderio and Roberta Pollock

J. Biol. Chem. 1997, 272:10160-10168.
doi: 10.1074/jbc.272.15.10160

Access the most updated version of this article at http://www.jbc.org/content/272/15/10160

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 17 of which can be accessed free at http://www.jbc.org/content/272/15/10160.full.html#ref-list-1