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Reduction in DNA Binding Activity of the Transcription Factor Pax-5a in B Lymphocytes of Aged Mice[1]

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Aging has been associated with intrinsic changes of the humoral immune response, which may lead to an increased occurrence of autoimmune disorders and pathogenic susceptibility. The transcription factor Pax-5 is a key regulator of B cell development. Pax-5a/B cell-specific activator protein and an alternatively spliced isoform, Pax-5d, may have opposing functions in transcriptional regulation due to the lack of a transactivation domain in Pax-5d. To study B cell-specific changes that occur during the aging process, we investigated expression patterns of Pax-5a and 5d in mature B cells of young and aged mice. RNase protection assays showed a similar transcriptional pattern for both age groups that indicates that aging has no affect on transcription initiation or alternative splicing for either isoform. In contrast, a significant reduction in the DNA binding activity of Pax-5a but not Pax-5d protein was observed in aged B cells in vitro, while Western blot analyses showed that similar levels of Pax-5a and 5d proteins were present in both age groups. The observed decrease in Pax-5a binding activity correlated with changes in expression of two Pax-5 target genes in aged B cells. Expression of the Ig J chain and the secreted form of Ig μ, which are both known to be suppressed by Pax-5a in mature B cells, were increased in B cells of aged mice. Together, our studies suggest that changes associated with the aging phenotype cause posttranslational modification(s) of Pax-5a but not Pax-5d, which may lead to an abnormal B cell phenotype in aged mice, associated with elevated levels of J chain, and secretion of IgM. *The Journal of Immunology,* 2001, 166: 2617–2626.

As organisms age, changes in the immune system are of particular importance as they have been associated with increased occurrence of both autoimmune disorders and susceptibility to foreign pathogens. The impaired immune responses of aged organisms to vaccines and the greater susceptibility to infections are the result of changes in both cell-mediated and humoral response pathways. Because of the central role T cells play in cell-mediated immune responses, thymic involution has generally been viewed as the primary regulator event in immune senescence (1). Decreased numbers of naive CD4$^+$ T cells and reduced proliferation responses in aged rodents and humans result from a decreased ability of CD4$^+$ T cells to secrete cytokines, most notably IL-2 (2, 3).

Significant and intrinsic changes of the humoral immune response have been observed in aging rodents and humans. Aged mice show a shift in Ab repertoire from non-self- to self-recognition, and from high to low affinities (4, 5). Additionally, a decrease in class switching may explain the observed shift of Ig Abs from the IgG to IgM isotype (4), although this result has not been independently verified (6, 7). An increased number of B cells that spontaneously secrete Igs has also been observed (8). Lastly, a shift from Abs produced by CD5$^-$ to those produced by CD5$^+$ B lymphocytes has been found in the spleens of aged mice; whether this is the result of an increased number of CD5$^+$ cells, or a shift in Ig secretion activity from CD5$^-$ to CD5$^+$ cells or both, is unclear (4, 8).

One approach to studying the changes that take place in aging B lymphocytes is to examine expression patterns and functions of B cell-specific transcription factors. We have focused on the transcription factor *Pax-5*, a member of the paired-box gene family of transcription factors (9–11). In adult mice, Pax-5 expression is limited to testis and developing B lymphocytes (11). Within the B cell lineage, *Pax-5* is expressed in progenitor, precursor, and mature B cells, but expression is down-regulated in plasma cells (9, 10). *Pax-5* has been deleted in mice through targeted gene disruption and its absence in B cells from homozygous mutant mice results in a complete block in early B cell development, at the late pro-B cell stage (12).

Pax-5 or B cell specific activator protein (BSAP)[3] is now considered a "master regulator" of B cell development, as it plays a central role in both the developmental and activation pathways of these cells (10, 13, 14). Several studies suggest that Pax-5 is involved in B cell proliferation, but the exact mechanism is not well understood (14, 15). Putative B cell-specific target genes for Pax-5 include *Lambda 5, CD19, mb-1, blk, RAG-2, J-chain*, and *IgH* genes (15–23). Of those, *Lambda 5, CD19*, *mb-1, IgH*, and *blk* products are involved in activation and/or signaling events through the (pre-)B cell receptor. The J chain forms pentamer structures with secreted IgM molecules in activated B cells and plasma cells (24). *RAG-2* is first transcribed in progenitor B and precursor B cells, but reexpression has also been observed in immature B cells expressing an autoreactive B cell receptor, leading to receptor editing, and in germinal center B cells during immune responses (25, 26). Pax-5 also interacts with $3'\alpha$ enhancers and switch regions of *IgH* genes and is likely to affect both the amount and the isotype of secreted Ig (15, 22, 23, 27–29). Depending on the target gene

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[3] Abbreviations used in this paper: BSAP, B cell-specific activator protein; SRB, small resting (mature) B cells; PAB, partially activated B cells; ivt, in vitro translated; wt, wild type; mut, mutant; ROS, reactive oxygen species.

and/or the developmental stage of the B cell, Pax-5 can act either an activator, a repressor, or a docking protein (22, 23, 27–30).

In addition to the full-length *Pax-5a* (BSAP) isoform, mouse *Pax-5* transcripts can generate at least three additional splice variants, named *Pax-5b, 5d*, and *5e* (30). In resting, splenic B cells, the protein level of Pax-5b is very low (M. Lowen and P. Zwollo, unpublished observations) and Pax-5e is not present at detectable levels (30). In contrast, isoform Pax-5d is expressed at readily detectable levels in resting B cells and is of particular interest because the C-terminal region, which in Pax-5a contains the trans-activation, repression, and partial homeodomain homology regions, has been replaced by a novel sequence with unknown function (30). Based on the absence of a transactivating domain, we hypothesize that Pax-5d may have a regulatory function opposite that of Pax-5a and that relative levels of these two isoforms in the nucleus may, at least in part, determine their regulatory activities on target genes.

Given the potential involvement of Pax-5a in cell proliferation, Ig production, and isotype switching, changes in *Pax-5* expression are likely to affect humoral immune responses and B cell function in aging animals. The goals of this study were to compare the expression levels and DNA binding activities of Pax 5a and 5d in splenic B cell populations from young and aged mice and to determine whether levels correlated with the expression pattern of specific Pax-5 target genes. Pax-5a and 5d expression were determined both at the RNA and protein level in splenic, resting B cell populations. RNA transcript levels of both isoforms were shown to be unchanged in resting B cells of aged mice (18–23 mo old). In contrast, the amount of Pax-5a able to interact with specific DNA binding sites in vitro had decreased significantly in aged B cells, and this correlated with a relative increase in DNA binding activity of Pax-5d. Furthermore, Western blot analysis indicated that the protein levels of both isoforms remain constant as animals age. Lastly, the reduced Pax-5a DNA binding activity in aged B cells correlated with increased expression of two target genes that are normally repressed during this stage of development, namely the *J chain* gene and the *Igμ* gene.

Materials and Methods
Animals

Aged BALB/c mice 17–23 mo in age were obtained from the National Institute on Aging colony at Harlan Sprague-Dawley (Indianapolis, IN). Additional BALB/c mice between the ages of 12 and 23 mo were obtained from our own colonies. Young BALB/c mice 2–4 mo in age were either purchased from Harlan Sprague-Dawley or used from our own colonies. An outbred population of *Peromyscus leucopus* (white-footed mouse) with animals between 3–4 mo and 27–31 mo were a gift from Dr. Paul Heideman's colonies at the population laboratories at The College of William and Mary.

Generation of mAbs to isoform Pax-5d

Synthetic peptide 5d/e (residues 218–235 of the Pax-5d protein; see Ref. 30) was synthesized at the peptide facility in the Department of Cell and Molecular Biology at The University of California at Berkeley. Peptide was conjugated to keyhole limpet hemocyanin using maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL). Conjugate was dissolved in saline and emulsified with CFA. Next, 100 μg of Ag was injected i.p. and s.c. into BALB/c mice followed by a booster shot of the same Ag in saline both i.p. and s.c. after 6 wk. Four days after the booster shot, mice were sacrificed and spleens removed for the fusion with myeloma cell line NSO (31). Spleen cells were washed and RBC removed by lysis in 0.17 M ice-cold NH₄Cl for 10 min on ice. Myeloma cells and spleen cells were then mixed at a 1:4 ratio followed by drop-wise addition of 50% polyethylene glycol (Hybrimax; Sigma, St. Louis, MO) to induce cell fusion. Cells were plated on 96-well plates at 5×10^4 cells/well in DMEM containing 20% FBS (BioWhittaker, Walkersville, MD), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-ME containing hypoxanthine, aminopterin, and thymidine and incubated at 37°C with 5% CO_2.

Cell supernatants were tested by ELISA using Pax-5d peptide conjugated to BSA. A total of 28 strongly positive clones were cloned by limiting dilution and supernatants tested by ELISA and Western blot analysis. Ascites fluid was produced by i.p. injection of BALB/c mice with hybridoma cell suspensions using 1.5×10^7 cells per injection, filter sterilized, and stored as frozen aliquots at −80°C.

Isolation of cell fractions and preparation of nuclear extracts

Teased spleen cell suspensions from BALB/c mice were collected through a 40-μm nylon cell strainer in RPMI 1640 medium containing 10% FCS (BioWhittaker), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-ME, washed, and resuspended in HBSS. Percoll gradients (Amersham Pharmacia Biotech, Piscataway, NJ) were used to isolate small resting, (mature) B cells (SRBs): five different Percoll densities (70, 66, 63, 60, and 50%) were layered to maximize the isolation of pure resting cell populations (on the 70% layer) away from activated B cells and plasma cells (on the 50% layer). Similarly, partially activated B cells (PABs) were collected from the 66% Percoll layer in this system. The 70% Percoll-purified cell populations contain ~25% resting T cells as determined by flow cytometry, but because complement lysis reactions with anti-Thy.1, anti-CD4, and anti-CD8 to remove T cell populations resulted in partial activation of the SRBs, this step was omitted from our protocol. Comparison of 70% cell fractions from young and aged mice by flow cytometric analyses using anti-CD3 and anti-B220 Abs showed that the ratio of B and T cells is the same for each age group (P. Zwollo and Y. Deng, unpublished observations). The preparation of nuclear extracts is described elsewhere (32). The entire procedure for the generation of nuclear extracts was performed in a cold room at 6°C.

Transient transfections

COS-1 cells were cultured in DMEM containing 10% FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. One microgram of the expression constructs pcDNA.5d or pcDNA.5a (32) were transfected using Lipofectamine (Life Technologies, Grand Island, NY) according to the supplier's instructions. After 40 h incubation, cells were collected and nuclear extracts were prepared following a standard protocol described elsewhere (32).

Western blot analysis

Nuclear extracts from SRBs were separated on 12% SDS-polyacrylamide gels in a buffer containing 25 mM Tris-Cl, 0.2 M glycine, and 3.5 mM SDS. Equal amounts of nuclear protein were used in each sample as determined by Bradford assay (Bio-Rad, Richmond, CA). Samples were electrophoretically transferred onto nitrocellulose filters (Schleicher and Schuell, Keene, NH) in a buffer containing 48 mM Tris-Cl, 39 mM glycine, 1.3 mM SDS, and 20% methanol. For experiments using whole-cell lysates, B cells were purified from 70% layers of Percoll gradients and washed in PBS. Then 3×10^6 cells were lysed in sample buffer and boiled for 5 min and loaded onto a 12% SDS-PAGE gel. Membranes were incubated for 1 h in a blocking solution of PBS and 5% nonfat milk, followed by a 1- to 2-h incubation with a primary Ab diluted in blocking solution. Next, membranes were washed three times for 10 min in PBS, followed by a 1- to 2-h incubation with a secondary, HRP-conjugated Ab. Following three 10-min washes in PBS, filters were developed with an ECL kit (Amersham Pharmacia Biotech), and bands were visualized on Eastman Kodak XAR5 film (Rochester, NY).

Antibodies

ED-1, a polyclonal rabbit antiserum (32) that recognizes the paired domain sequence of Pax-5, was used at 32 μg/ml. Rabbit polyclonal antiserum to the transcription factor TFIID (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 2.5 μg/ml. Goat polyclonal anti-Pax-5 antiserum N-19 (against aa 2–20 of Pax-5a) was used at 3 μg/ml (Santa Cruz Biotechnology). Goat polyclonal NF-κB/p50 Ab was purchased from Santa Cruz Biotechnology and used at 0.2 μg/ml. Ascites fluid or cell supernatants containing the 6G11 mouse anti-Pax-5d/e mAb prepared in this laboratory was used at 34 μg/ml and detected using a HRP-conjugated goat anti-mouse IgG secondary Ab (Zymed, South San Francisco, CA) at a 4.8 μg/ml. Rabbit polyclonal anti-J chain serum (a gift from Dr. Marian Koshland, University of California, Berkeley, CA) was used at a 28 μg/ml. The ED-1, TFIID, and J chain Abs were detected with a HRP-conjugated donkey anti-rabbit IgG secondary Ab (Amersham Pharmacia Biotech) at 2.8 μg/ml. NF-κB/p50 and Pax-5/N-19 Abs were detected using a rabbit anti-goat IgG at 0.67 μg/ml (Zymed).

EMSAs

Equal amounts of nuclear protein were used in each sample as determined by Bradford assay (Bio-Rad). Binding assays were conducted for 20 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, 0.33 mM DTT, 300 ng BSA, 12% glycerol, 1–2 μg poly(dI · dC), and 0.5–1 μg nuclear extract (33), and 0.1 ng double-stranded oligonucleotide CD19/BSAP DNA (5′-CAG ACA CCC ATG GTT GAG TGC CCT CCA G-3′) labeled with [α-^{32}P]dCTP. The ds.mutCD19 oligonucleotide had a mutated Pax-5 binding site and was used for competition EMSAs (mutations are underlined): 5′-CTA GGA CAC CGG TGG TTT AGT GCC CTC C-3′. The ratio of nuclear extract to poly(dI · dC) (in micrograms) was kept constant at 2:1 in all experiments. Competition or supershift EMSAs containing Ab were preincubated in the presence of 1 μl (1:5 diluted) Ab without probe for 10 min at 30°C. Products were separated by electrophoresis through a 5% nondenaturing polyacrylamide gel (29:1, acrylamide to bisacrylamide) in buffer containing 33 mM Tris-Cl, 33 mM boric acid, and 0.74 mM EDTA. Gels were dried and exposed to Kodak XAR5 film.

In vitro transcription and translation of Pax-5 isoforms

The plasmids (pBluescript; Stratagene, La Jolla, CA) containing the isoform Pax-5a (pBS.1.2) or 5d (pBS.10.1) were transcribed in sense direction with T3 RNA polymerase as described previously (30). Translation was conducted using a rabbit reticulocyte lysate (TnT; Promega, Madison, WI) according to the suppliers' directions.

RNA isolation and RNase protection assay

Total cellular RNA was isolated from Percoll-purified SRBs using a RNeasy mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. From each spleen, ~0.5–1 × 10^7 resting lymphocytes could be purified from the 70% Percoll layer for RNA processing. The yields of the RNA extractions from SRBs were very low, ~2–4 μg RNA per spleen. Because multiple experiments needed to be performed on these RNAs to confirm obtained results, RNA from two to four mice was pooled for some samples. Antisense radiolabeled RNA probes were prepared as described (30). The 392-nt RNA probe p10.1 (30) protects nt 447–607 of *Pax-5* sequence resulting in a 160-nt fragment, as well as nt 447–735 of *Pax-5d/e* resulting in a 288-nt protected fragment. A β-tubulin-specific probe was synthesized from the *Bam*HI-linearized form of plasmid pμ.100, which contains nt 170–263 of the murine β-tubulin gene. The pμ.100 RNA probe is 152 nt and protects a 94-nt tubulin RNA fragment. RNase protection assays were performed as described (30) but only 1–5 μg of total cellular RNA was used. The two probes were incubated simultaneously with each RNA sample to obtain an optimal internal control.

The template for p(M&S) was generated using PCR amplification with sense primer cμ4.F (5′-ccgaattcACTGTGACAGAGGAGGAATGG; lower case is the *Eco*RI restriction site with flanking sequence) targeting exon cμ4 and antisense primer mIgM.B (5′cgggatccAAGCTGGAGGGC AACACAGGAAAG; lower case represents the *Bam*HI restriction site with flanking sequence) complementary to exon M2 of the Igμ gene (34). As the PCR target, a spleen cDNA library was used, which resulted in amplification of the membrane form of IgH containing exon cμ4, M1, and M2 sequence without the S segment at the 3′ side of cμ4 (34). The 251-nt PCR-amplified fragment was cloned into *Eco*RI and *Bam*HI restriction sites of pBluescript, and the DNA sequence was verified using dideoxy sequencing. The p(M&S) riboprobe was used to detect both a 251-nt membrane sequence of the IgH μ-chain (exon cμ4-S, M1, and M2 sequence) and a 111-nt sequence representing the Cμ4-S fragment only, indicative of the secreted form of the H chain (see Fig. 2A and Ref. 34).

Results

Generation of mAbs specific to isoform Pax-5d

Pax-5d/e-specific mAbs were generated to provide a tool to unequivocally identify the Pax-5d protein by gel electrophoresis, distinguishable from Pax-5a proteolytic degradation products. Using SDS-PAGE and Western blot analysis, we determined that the mAb 6G11 (see *Materials and Methods*) had the required specificity to recognize the 35-kDa Pax-5d protein, as shown in Fig. 1. Fig. 1A shows the reactivity of 6G11 to in vitro-translated (ivt) Pax-5d, but not ivt Pax-5a (Fig. 1A, *lanes 2* and *1,* respectively). Ivt Pax-5d runs at ~35 kDa on the gel. To verify the presence and location of both Pax-5a and 5d proteins, the same filter as used in Fig. 1A was stripped and reprobed with the polyclonal rabbit an-
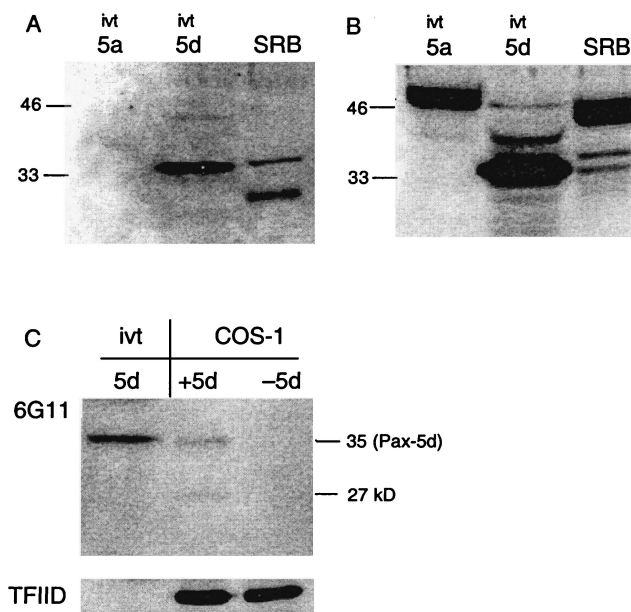
FIGURE 1. Monoclonal Ab 6G11 has the required specificity to recognize Pax-5d. *A,* Western blot analysis with the monoclonal anti-Pax-5d/5e Ab 6G11. *Lane 1,* Ivt Pax-5a (1 μl lysate); *lane 2,* ivt Pax-5d (1 μl lysate); *lane 3,* nuclear extract from SRBs (10 μg). Molecular mass markers (kDa) are indicated on the *left*. *B,* Filter from *A* was stripped and reprobed with anti-Pax-5 Ab ED-1, which recognizes all four Pax-5 isoforms. *C,* Western blot analysis with the monoclonal anti-Pax-5day/5e Ab 6G11 on nuclear extracts (10 μg) from COS-1 cells that had been transfected with the Pax-5d expression vector pcDNA.5d. *Lane 1,* Ivt Pax-5d, 1 μl lysate; *lane 2,* COS-1 nuclear extract transfected with Pax-5d expressing construct (+5d); *lane 3,* mock transfection (−5d). As a control, the same filters were stripped and reprobed with Abs to the basal transcription factor TFIID (*lower panel*).

tiserum ED-1, which recognizes the paired domain present on both Pax-5a and 5d (Fig. 1B). This experiment showed that 6G11 and ED-1 recognize the same 35-kDa protein that corresponds with ivt Pax-5d (*lane 2* in Fig. 1, *A* and *B*).

Using similar methods, the specificity of the 6G11 mAb was further tested on nuclear extracts derived from SRBs. Fig. 1A (*lane 3*) shows that 6G11 was able to recognize a 35-kDa band in SRBs, which migrates at the same position in the gel as ivt Pax-5d protein. This result provided the first evidence that Pax-5d protein is present (at readily detectable levels) in nuclear extracts from resting B cells. The mAb also detected a lower molecular mass band of ~27 kDa (Fig. 1A, *lane 3*), which could represent either a degradation product of Pax-5d, a modified form of Pax-5d, or a cross-reactive band. Given its position in the gel, the 27-kDa band seemed unlikely to represent Pax-5e. The 27-kDa band is also detectable in mature B cell lines but not plasma cell lines (M. Lowen and P. Zwollo, unpublished observations). Two bands in the 35-kDa range were detected in SRB using the Ab ED-1 (Fig. 1B, *lane 3*). Of those two, the position of the lower band corresponds to Pax-5d, whereas the top band may represent a degradation product of Pax-5a containing a paired domain sequence.

To confirm that 6G11 recognizes Pax-5d specifically, we performed Western blots on nuclear extracts from COS-1 (Fig. 1C) that had been transfected with the Pax-5d expression construct pcDNA.5d (32). Results in Fig. 1C show that both the 35-kDa and 27-kDa band are detected in COS cells transfected with pcDNA.5d (*lane 2*), but not in mock-transfected COS-1 cells (*lane 3*), showing that their presence is dependent upon expression of the Pax-5d

effector construct. The anti-TFIID antiserum that detects the basal transcription factor TFIID was used to verify that equal amounts of nuclear protein were present in both the COS-1 samples (Fig. 1*C*, *lower panel*).

*Transcript levels of Pax-5a and 5d are unchanged in aged mice*

To investigate whether levels of Pax-5a or Pax-5d RNA transcripts change in aged mice, RNase protection assays were performed on 1–5 μg of total RNA extracted from SRBs of young and aged mice. The yields of the RNA extractions from SRBs were very low (2–4 μg RNA per spleen), and RNA from multiple mice was pooled for some of the samples.

Anti-sense RNA probes were used to analyze the levels of Pax-5 isoform transcripts. Probe 10.1 (Fig. 2*A*) detects both Pax-5a/b and Pax-5d/e sequence (30), whereas a "control" tubulin probe, pμ.100, was used to detect levels and quality of each RNA sample. Both probes were incubated simultaneously in the presence of RNA, to ensure a proper internal control. A representative experiment is shown in Fig. 2*B*. Four additional independent samples (1–4 spleens each) were analyzed for each age group (data not

shown). A 160-nt sized band, corresponding to isoform *Pax-5a* (and *5b*), and a 288-nt band, which detects *Pax-5d* (and *5e*) transcripts, were detected in all mice. Both young and aged mice had similar relative amounts of the two isoforms, with *Pax-5a* levels being ~10 times more intense than *Pax-5d* in both aged groups. The level and ratio of the two isoforms was also similar to those observed in a number of B cells lines and spleen tissue as reported earlier (30). As had been established earlier (30), levels of Pax-5b and 5e are very low in B cell lines and splenic tissue, thus the 160- and 288-nt bands represent Pax-5a and 5d, respectively. Together, the data suggest that there are no changes in transcription initiation or alternative splicing of *Pax-5a* and *5d* in mature, resting B cells as animals age.

DNA binding activity of Pax-5a is decreased in B cells of aged mice

Next, we sought to test whether there was an age-associated change in the DNA binding activity of isoforms Pax-5a and 5d. A double-stranded radioactive oligonucleotide containing the high-affinity Pax-5 DNA binding site present on the CD19 promoter
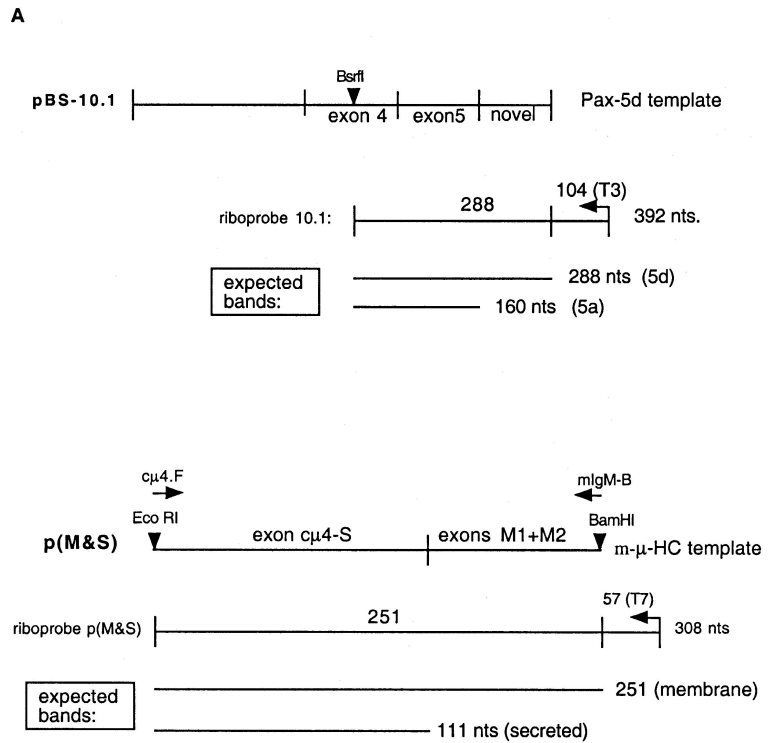
FIGURE 2. RNase protection assays on total RNA from young and old SRBs. *A,* Overview of riboprobes and expected sizes. *Top,* Plasmid template pBS-10.1(Pax-5d), riboprobe 10.1, and expected protected regions on *Pax-5* mRNA. The position of the novel sequence unique to 5d (nt 607–735) is indicated. *Bottom,* Plasmid template p(M&S) used to detect membrane and secreted Igμ chain mRNAs (see *Material and Methods* for details). *B,* Results from RNase protection assay using the p10.1 riboprobe. The 288-nt band shows *Pax-5d* levels, corresponding to *Pax-5* transcripts that contain sequence from exons 4 and 5 as well as the 5d/5e-specific sequence. The 160-nt band corresponds to *Pax-5a* transcripts. It contains transcripts that share the exons 4 and 5 sequence but not the novel sequence. The internal control RNA probe pμ.100 detects a 94-nt β-tubulin transcript.
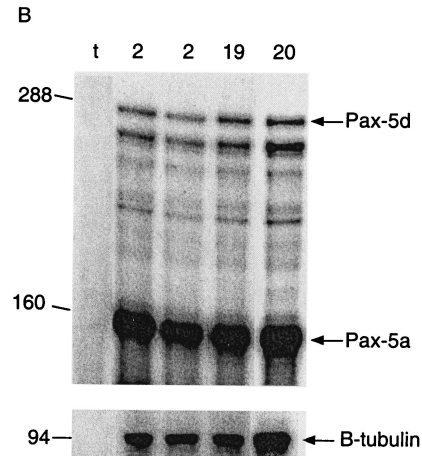
(CD19/Pax-5) was used as a probe (30). Previous studies had already shown that the CD19/Pax-5 probe binds to Pax-5a with high affinity (22). To unambiguously demonstrate that the observed protein-DNA complexes were Pax-5 specific, we performed competition EMSAs with excess double-stranded oligonucleotides containing wild-type (wtCD19) or mutant (mutCD19) Pax-5 binding sites using SRB nuclear extract from a young mouse. Results of this experiment showed that the complexes on the CD19 probe are Pax-5 specific, as excess wt unlabeled probe competed efficiently for binding, but mutated probe did not, as shown in Fig. 3A. Addition of the anti-paired domain antiserum ED-1 in Ab competition shifts resulted in removal of all complexes, confirming that all contained Pax-5 protein (Fig. 3A). Of interest, it was observed that ivt Pax-5d forms a much weaker complex as compared with ivt Pax-5a (Fig. 3A, lane 2), although Western blots (e.g., Fig. 1B) indicated that similar amounts of each Pax-5 isoform protein were present in samples. This may be the result of differential redox regulation or protein degradation of ivt Pax-5d during the EMSA incubation (M. Lowen and P. Zwollo, unpublished observations).

The mAb 6G11 could not be used for EMSAs as it did not recognize the native conformation of Pax-5d efficiently. To identify among the multiple Pax-5 complexes those that contained Pax-5d and Pax-5a species, we combined nuclear extract from young SRBs with increasing amounts of ivt Pax-5a or Pax-5d. A dose-dependent increase in intensity was observed for a single band corresponding to Pax-5a (Fig. 3B, lanes 3 and 4) and Pax-5d (Fig. 3B, lanes 5 and 6). When more Pax-5d was present, reduced amounts of Pax-5a bound to DNA (Fig. 3B, lane 2 compared with lanes 5 and 6), suggesting that Pax-5d replaced Pax-5a on DNA in a dose-dependent manner. No dose-dependent increase of lower molecular mass Pax-5a degradation products was detected (Fig. 3B, lanes 2–4). Lastly, two Pax-5a complexes were detected, named 5a.1 (slower) and 5a.2 (faster). Ivt Pax-5a protein runs in the slower migrating (5a.1) position on the gel (Fig. 3B). Both 5a.1 and 5a.2 complexes contained Pax-5a protein, as they were recognized by both a N-terminal-specific anti-Pax-5 Ab N-19 and paired domain Ab ED-1 (results not shown).
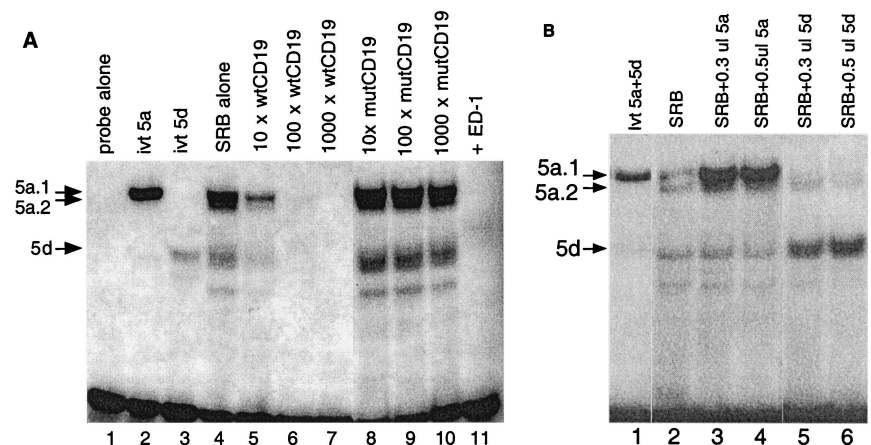
Once specific Pax-5d- and 5a-containing complexes had been defined, nuclear extracts from SRB of young (2–4 mo) and aged (18–22 mo) mice were compared using EMSAs. A representative experiment is shown in Fig. 4A. Results showed significant decreases in the amount of Pax-5a protein bound to the CD19/Pax-5 probe in aged mice, as compared with young mice. While both the 5a.1 (slower) and 5a.2 (faster) complexes were detected in the young mice, aged mice possessed only complex 5a.2, and at somewhat diminished levels as compared with young samples. In con-
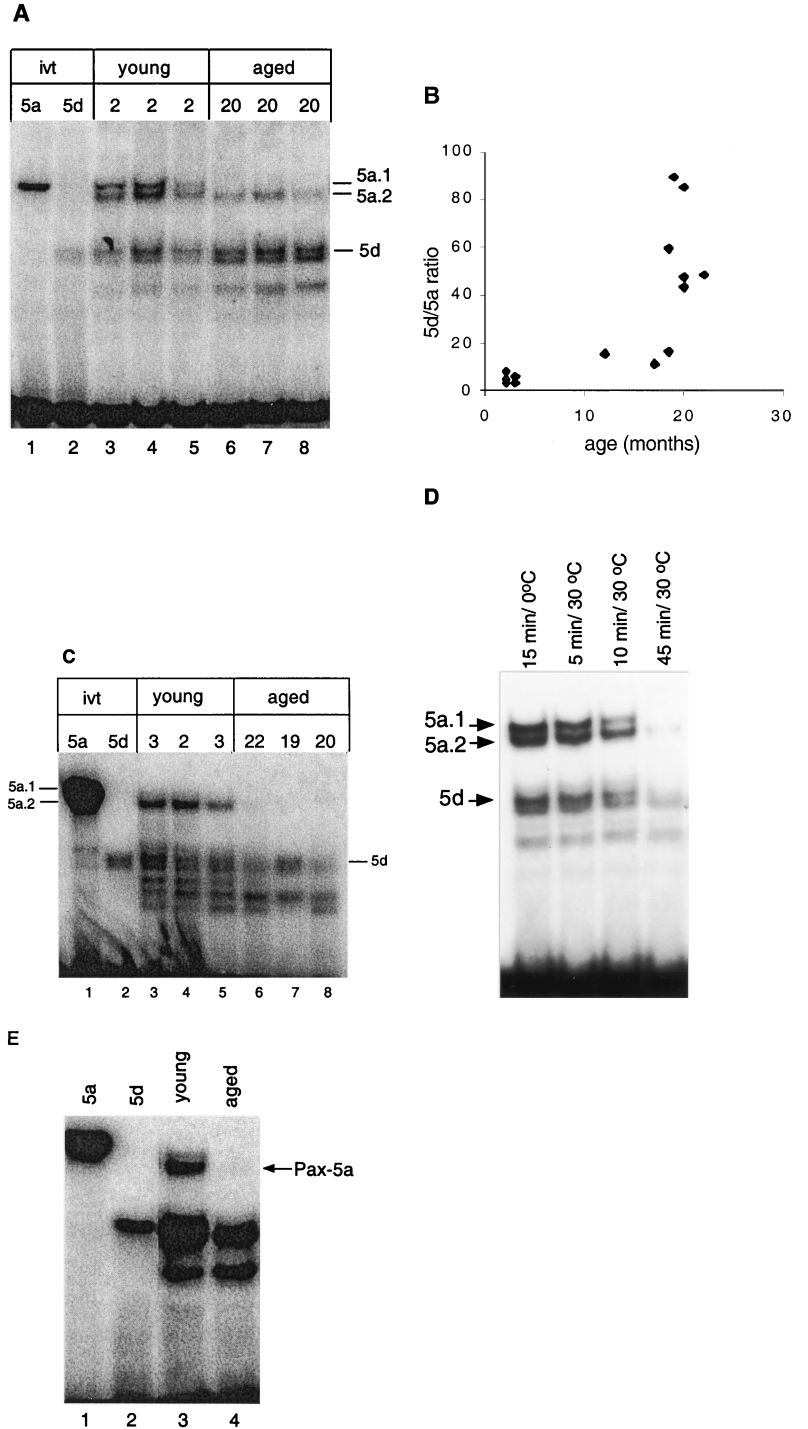
trast, isoform Pax-5d showed a relative increase in DNA binding in aged animals, resulting in a significant increase in the ratio of bound Pax-5d to Pax-5a. This pattern was similar to the control experiment in Fig. 3B, which showed that an increase of Pax-5d binding correlated with decreased binding of Pax-5a (Fig. 3B, compare lanes 2 and 6). A second, faster migrating band below Pax-5d may represent a degradation product of Pax-5a or 5d, as determined by competition shift using an anti-paired domain Ab (not shown). Eight additional mice of various ages were similarly analyzed by EMSA, and combined EMSA data of 14 mice were quantified using the NIH Image software analysis program (www: http://rsb.info.nih.gov/nih-image/). This analysis showed that the ratio of bound Pax-5d to 5a consistently increased as mice age, as shown in Fig. 4B (the Pax-5a value in this analysis represents the total of 5a.1 and 5a.2 in each sample).

To test whether a temperature-sensitive mechanism (involving an enzymatic activity) had caused the decreased DNA binding activity of Pax-5a in aged mice, SRB nuclear extracts from both age groups were preincubated for 15 min at 30°C in the absence of probe, before the EMSA. The results from this experiment (Fig. 4C) showed that under such conditions complex 5a.1 disappeared in both age groups, and that only young mice retained the 5a.2 complex. The effect of preincubation on Pax-5d levels was not as dramatic, but also resulted in a decrease in intensity of the Pax-5d complex in both age groups. An increased amount of Pax-5 degradation products as compared with Fig. 4A is evident in both age groups under those conditions.

To further compare Pax-5 banding patterns as a function of preincubation at 30°C, we exposed SRB nuclear extracts from young mice to 30°C temperatures for 5, 10, or 45 min before the EMSA (Fig. 4D). Importantly, the 5a.1 complex disappears more quickly as compared with the 5a.2 complex during the preincubation, suggesting either that 5a.1 represents a more labile protein conformation or, alternatively, that the 5a.2 complex has a protein conformation with higher affinity for DNA. (Fig. 4, C and D). Interestingly, the Pax-5a pattern in young mice after prolonged incubation at 30°C is similar to the pattern of aged mice before preincubation, including a relative decrease in the amount of Pax-5a.1 complex relative to 5a.2. Furthermore, the Pax-5d DNA binding activity in young mice also decreased with prolonged exposure to 30°C (Fig. 4D), and this was different from the Pax-5d pattern in aged mice, which showed a relative increase of Pax-5d binding (Fig. 4A). Together, the EMSA data suggests that the observed decrease in DNA binding activity of Pax-5a with age may be the result of an unknown differential enzymatic activity, possibly protease or redox regulated.

FIGURE 3. Identification of DNA binding activities of Pax-5 proteins using EMSAs. EMSA of nuclear extracts (1 μg) from SRBs of a young BALB/c mouse using the CD19/BSAP probe is shown. A, Competition shift assay using wild-type (lanes 5–7) and mutant (lanes 8–10) unlabeled ds.CD19/BSAP oligonucleotides. The amount of excess oligos is indicated on the top. Lane 1, Probe in the absence of protein; lane 2, ivt Pax-5a (1 μl lysate); lane 3, ivt Pax-5d (1 μl lysate); lane 4, SRB nuclear extract in absence of competitor DNA. Lane 11, SRB+ED-1. B, Identification of Pax-5a- and 5d-containing complexes. Lane 1, Ivt 5a and 5d, 0.5 μl lysate each; lane 2, nuclear extract from young SRB alone. Lanes 3–6, Nuclear extracts from young SRBs incubated with increasing amounts (0.3 μl or 0.5 μl lysate) of ivt Pax-5a (lanes 3 and 4) or ivt Pax-5d (lanes 5 and 6). Positions of Pax-5a.1, 5a.2, and Pax-5d complexes are shown on the left.

A

| ivt | young | aged |
| 5a 5d | 2 2 2 | 20 20 20 |

— 5a.1
— 5a.2

— 5d

1 2 3 4 5 6 7 8

B

C

| ivt | young | aged |
| 5a 5d | 3 2 3 | 22 19 20 |

5a.1
5a.2

— 5d

1 2 3 4 5 6 7 8

D

15 min/ 0°C   5 min/ 30 °C   10 min/ 30 °C   45 min/ 30 °C

5a.1 →
5a.2 →

5d →

E

5a   5d   young   aged

← Pax-5a

1 2 3 4

FIGURE 4. Comparison of Pax-5 DNA binding activity between young and aged mice. EMSA of nuclear extracts (1 μg) from SRBs using the CD19/BSAP probe. Position of 5a and 5d complexes are indicated. *A,* SRBs from three young (*lanes 3–5*) and three aged (*lanes 6–8*) BALB/c mice, using the CD19/Pax-5 probe, are shown. Ages of mice (in months) are shown above each lane. *Lane 1,* Ivt Pax-5a (1 μl lysate); *lane 2,* ivt Pax-5d (1 μl lysate). *B,* Quantitative analysis. The ratio of bound Pax-5d to bound Pax-5a (the sum of 5a.1 and 5a.2) was measured for each mouse sample using NIH Image analysis software. A total of 14 samples in the range of 2–23 mo were analyzed. *C,* EMSA similar to Fig. 3*B* but nuclear extracts were incubated for 15 min at 30°C before addition of the probe. Prolonged x-ray exposure. *D,* Correlation between increased exposure to 30°C conditions and decrease in Pax-5 DNA binding activities. Young SRB nuclear extracts. Incubation times in absence of probe are shown above each lane. *Left,* 15 min on ice. *E,* EMSA similar to Fig. 3*B* but using nuclear extracts of SRBs from *P. leucopus* mice. *Lane 1,* Ivt Pax-5a (1 μl lysate); *lane 2,* ivt Pax-5d (1 μl lysate); *lane 3,* 11 pooled spleens from young (3–4 mo); *lane 4,* 11 pooled spleens from aged (27–31 mo) mice.

To determine whether the observed change in DNA binding activity of Pax-5a was conserved in other species, we performed similar experiments on SRBs of an outbred mouse strain, *P. leucopus* (white-footed mouse). Because of the highly conserved nature of the paired domain, we were able to use our reagents for this species. Using EMSAs with the CD19/Pax-5 probe, nuclear extracts from *Peromyscus* SRBs showed a specific DNA-protein complex that was removed by anti-paired domain antiserum ED-1 (data not shown). Spleens were pooled for each age group because of the limiting amount of splenic sample (*Peromyscus* spleens are much smaller as compared with BALB/c). Comparison of a SRB nuclear extract prepared from 11 young (3–4 mo) *Peromyscus* with that prepared from 11 aged mice (27–31 mo) by EMSA gave similar results as was found

for the BALB/c mice (shown in Fig. 4*E*). This experiment was repeated twice with independent samples, with identical results. Most strikingly, the 5a.1 and 5a.2 complexes were absent from aged mice, but 5a.2 species was strong in young mice. Additionally, lower molecular mass Pax-5 species, possibly including Pax-5d as well as Pax-5a degradation products, were detected in both age groups. As the *Pax-5* gene has not been sequenced in this species, we had no information on either the expression or structure of alternative Pax-5 isoforms in *P. leucopus*. We conclude that the reduced DNA binding activity of Pax-5a observed in aged B cells from BALB/c is also detectable in a population of outbred mice, suggesting the presence a more general aging mechanism in B cells that is conserved among (mammalian) species.
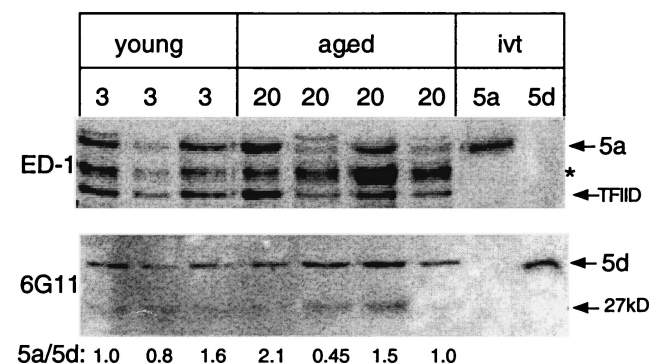
Nuclear Pax-5a protein levels are similar between young and aged mice

To investigate whether the observed decrease in DNA binding activity of Pax-5a in aged mice was caused by increased Pax-5a protein degradation, Western blot analysis was performed on nuclear extracts from young and aged (BALB/c) mice. (The remainder of this study was done using BALB/c mice only.) First, the mouse mAb 6G11 was used to determine the levels of Pax-5d. This filter was then stripped and reprobed with the anti-paired domain Ab ED-1 and the basal factor TFIID. The ED-1 antiserum detects Pax-5a as well as any proteolytic fragments of Pax-5a that contain at least a partial paired domain. An Ab to the basal transcription factor TFIID was used to control for equal loading and quality of extracts.

Using the ED-1 Ab (Fig. 5, *upper panel*), we found that both age groups had significant, but variable, levels of degradation of Pax-5a protein, as indicated by the presence of an ~45-kDa proteolytic Pax-5a fragment (indicated by an asterisk in Fig. 5), but this pattern was similar for both age groups. The 45-kDa fragment did not represent Pax-5b as this isoform runs at around the 40-kDa position in SDS-PAGE (30). The analysis was repeated using an additional eight young and nine aged SRB samples. Together, the data show that: 1) there is significant variation in the extend of Pax-5 degradation within both age groups, and 2) there are no significant differences in Pax-5 protein degradation between the two age groups. We conclude that the reduced DNA binding activity of Pax-5a is not the result of increased protein degradation in aged samples. Using the Ab 6G11, we showed that the levels of Pax-5d protein were similar in young and aged samples (Fig. 5, *lower panel*). The ratio of (nondegraded) Pax-5a to Pax-5d was determined for each sample using NIH Image analysis software (Fig. 5, numbers below the samples). Together, the results suggest that, although significant protein degradation occurs in all samples, the overall levels of Pax-5a and 5d protein remain unchanged as animals age.

It has been reported that an increased number of CD5$^+$ B cells is present in aged mice (4, 8). To address the possibility that the observed posttranslational changes in Pax-5a protein reflect a change in B cell population in the spleens of aged mice, we performed Western blot analysis on four whole-cell samples of each age group, using 3×10^6 SRBs for each sample. No detectable difference in total CD5 levels between young and aged mice was observed in our aged mouse samples (mice 18–22 mo of age), using a goat polyclonal IgG to CD5 (Santa Cruz Biotechnology; results not shown). Therefore, at least in the age groups we studied, the observed changes are not the result of a change in the relative number of CD5$^+$ and CD5$^-$ B cells in aged spleens.

Effect of reduced Pax-5a DNA binding on expression of target genes

Next we wished to test whether the observed decrease in Pax-5a DNA binding activity had a measurable effect on gene expression. As target genes, we focused on the following three genes: *CD19, J chain,* and *IgH*, as all three are expressed in mature B cells and may potentially change as a result of decreased Pax-5a DNA binding activity in aging animals. Of three different technical approaches attempted (including RT-PCR, Northern blot analysis, and RNase protection assay), only RNase protection assays were sensitive enough to detect changes using the limited amount of samples available, and gave highly reproducible results. Flow cytometric analysis was performed on three 70% Percoll fractions from each age group. Results from these experiments indicated that both age groups contained similar B:T lymphocyte ratios, as determined using anti-B220 and anti-CD3 Abs (P. Zwollo and Y. Deng, unpublished observations).

Using a *CD19* riboprobe detecting exons 5–9 of the murine *CD19* gene, no significant changes in *CD19* mRNA levels were detected comparing young and aged SRB samples (results not shown). A second target gene was the *IgH* gene. An Igμ (H chain) riboprobe was designed that would enable detection of both the membrane and secreted Igμ transcripts. Resting, mature B cells (in young mice) express mostly the membrane form of Igμ, whereas activated B cells switch through a process of alternative splicing to the production of secreted Igμ, resulting in high level Ig secretion in plasma cells. IgH was a particularly important target to test as it had been reported previously that Pax-5a is likely to regulate Ig secretion in "late" mature B cells (29). Interestingly, RNase protection assays with riboprobe p(M&S) showed a significant increase in the ratio of secreted to membrane Igμ transcripts in aged mice, as shown in Fig. 6 (*left*) for four young and four aged mice. To compare for level of B cell activation, we ran RNA samples in parallel in the same gels that were derived from LPS-activated B cells from young mice (Fig. 6, *right*): Percoll-purified, LPS-activated SRBs collected 79 h after stimulation showed the expected increase in secreted μ (Fig. 6, *lane 10*), and an increase was also detectable in samples that had been activated with LPS for a short period (7 h; Fig. 6, *lane 9*) or for a long period (216 h; Fig. 6, *lane 11*). To rule out the possibility that SRB samples had been contaminated with plasma cells (which would skew the ratio toward more secreted Igμ transcripts), some of the RNA samples were also analyzed by RNase protection assay for the level of *Pax-5* transcripts (see Fig. 2). The *Pax-5* transcript levels in both age groups were identical for all samples, suggesting that similar numbers of mature B cells were present, as plasma cells do not express Pax-5 (30).

Furthermore, using NIH Image analysis software, we determined that the ratio of secreted to membrane form of the μ H chain gene increased up to 5-fold as a result of aging. It should be noted that there was considerable variation in the level of increase in the aged group, but this was observed in all aged samples (Fig. 6, S:M

FIGURE 5. Comparison of Pax-5a and 5d nuclear protein levels in young and aged mice. *A,* Western blot analysis of Pax-5a and 5d isoforms using SRB nuclear extracts from young (3 mo) and aged (20 mo) mice. Age (in months) for each sample is indicated on the *top*. Ivt Pax-5a and 5d are in the two lanes on the *far right*. The *top panel* shows Pax-5a and TFIID protein levels, using the anti-paired domain serum ED-1 incubated together with anti-TFIID Ab. The asterisk indicates a degradation product of Pax-5a (see text). The positions of isoform Pax-5a and TFIID are indicated on the *right*. The *lower panel* shows levels of Pax-5d protein in the same samples detected with mAb 6G11. Quantitative analysis: numbers underneath each lane indicate the relative density of (nondegraded) Pax-5a compared with Pax-5d for each sample (see text).

FIGURE 6. Comparison of secreted and membrane IgM transcripts in young and aged SRBs. *Left,* The p(M&S) ribo-probe detects two fragments: a 251-nt fragment covering Cµ4-S with membrane exons M1 and M2 detects the membrane form of Igµ, and a 111-nt fragment covering Cµ4-S alone detects secreted Igµ mRNA. *Lanes 1–4,* Young SRBs; *lanes 5–8,* aged SRBs. Age (in months) is indicated on the *top.* M, Membrane; S, secreted. S:M ratio is indicated at the *bottom* of the figure. *Right* (*lanes 9–11*), In the same experiment, samples from LPS-activated SRBs from young mice were used for comparison. Time of LPS activation is shown in hours. *Lane 13,* Probe alone (308 nt); *lane 12,* t, for tRNA, as negative control.
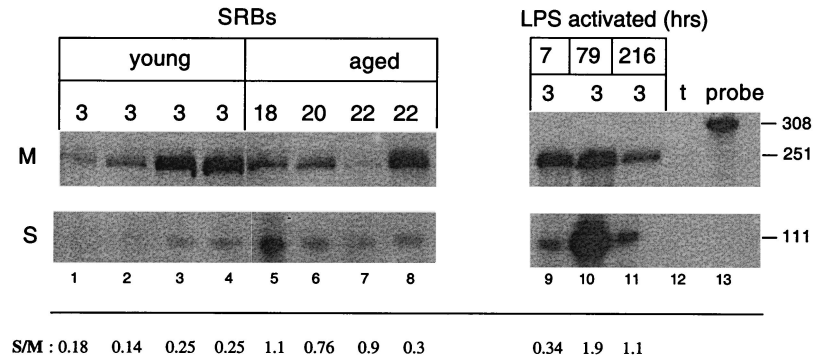
ratio per sample). In conclusion, aged mice appear to have abnormal resting B cells that produce low levels of secreted µ-chain transcripts.

Lastly, the Ig *J chain* target gene was used to determine whether decreased Pax-5a activity affects its transcription in aged mice. Previous studies had shown that Pax-5a acts as a transcriptional repressor for the *J chain* gene: increased levels of *Pax-5a* through overexpression in plasma cell lines resulted in a reduction in *J chain* RNA levels (21, 32). Here we wished to test whether the opposite situation, namely decreased levels of active Pax-5a in mature B cells, would lead to increased *J chain* expression.

J chain is expressed at basal levels until B cells become activated (21), and this may explain why our signals from RNase protection assays were too low to reliably detect changes in transcript levels between young and aged mice (not shown). As an alternative approach, we measured changes in *J chain* expression at the protein level using Western blot analysis. Whole SRBs from four 2-mo-old and four 19-mo-old mice were counted, and 3×10^6 cells were lysed and analyzed on SDS-PAGE gels.

Whole-cell lysates were used in this experiment to enable quantitative comparison of the NFs Pax-5a and TFIID as well as the cytoplasmic J chain protein. Pax-5a expression in combination with TFIID levels provided important internal controls for the relative purity of the isolated SRB fractions from young and aged mice. We had shown earlier (Fig. 5) that the protein levels of Pax-5 remain unchanged with age, thus both young and aged SRB cell fractions should have similar amounts of Pax-5 and the ratio of Pax-5 to TFIID should also remain unchanged. Given that the same total number of cells was used in each sample, this suggest that similar numbers of B cells were present in each sample, with-

out contamination of plasma cells (which do not express Pax-5) or similarly, changes in number of (Pax-5-negative) T cells (Fig. 7*A, first* and *second row*).

Using a polyclonal rabbit anti-J chain serum, very low levels of the 15-kDa J chain protein were detected in SRBs from young mice, as expected, as shown in Fig. 7*A* (*third row*). Interestingly, in aged mice the amount of J chain protein was significantly higher, while both age groups had similar Pax-5a protein levels. Similar results were obtained when PABs, isolated from 66% Percoll gradients, were analyzed, as shown in Fig. 7*B*. To confirm this result independently, cytoplasmic (S-100) fractions from SRBs were analyzed by Western blot, loading equal amounts of protein as determined by Bradford analysis (Fig. 7*C*). As a control for equal protein loading, the anti-NF-κB/p50 Ab was used, which detects a 110-kDa cytoplasmic precursor protein in the cytoplasm of SRBs (35). Using this approach, we were able to show that the levels of J chain protein are consistently higher in the cytoplasm of aged B cells as compared with young B cells. These experiments, together with the EMSAs described earlier, suggest that reduced Pax-5a activity in aged SRB cells correlates with increased expression of at least two Pax-5 target genes, the *J chain* and the *Igµ H chain* gene.

Discussion

Substantial reduction in both cellular and humoral immune responses have been associated with immune senescence observed in aging humans and rodents. Many of the observed changes in the B cell response have been correlated with changing expression patterns of B cell-specific proteins that are structurally or functionally associated with the B cell receptor. We hypothesized that some of
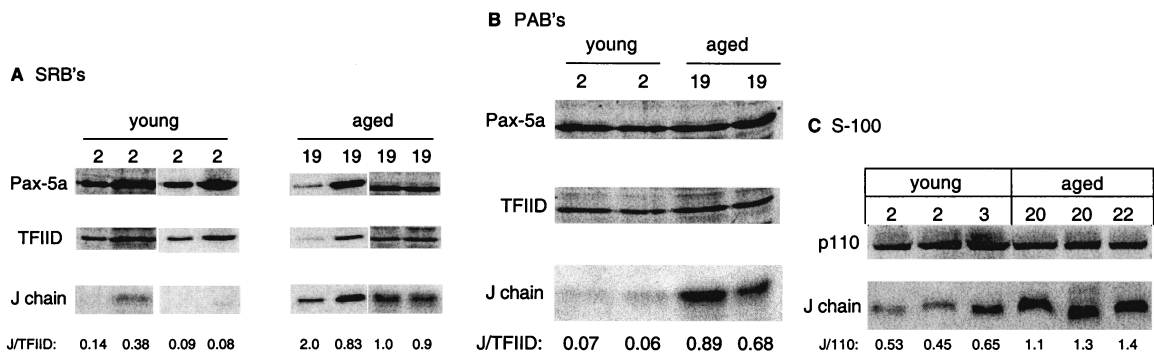
FIGURE 7. Comparison of J chain expression in young vs aged B cells. Western blot analysis. *A,* Equal numbers of SRBs were boiled and ran under denaturing conditions to allow comparison of nuclear Pax-5a and TFIID factors with the cytoplasmic J chain protein. Probings with Abs to Pax-5a, anti-TFIID, and anti-J chain (see text). The ratio of J chain to TFIID (J:T) for each sample is indicated below the figure *B,* Same experiment as in Fig. 5*A* except using PABs instead of SRBs. *C,* Western blot analysis using (25 µg) cytoplasmic fractions from SRBs purified from young and aged mice. The anti-NF-κB/p50 Ab was used as a control for equal loading. It detects the 110-kDa NF-κB/p50 precursor, which is present in the cytoplasm of resting B cells. The ratio of J chain to p110 for each sample (J:110) is indicated below the figure.

these changes have a common cause, namely the deregulation of transcription factor(s) normally expressed during B cell maturation. The molecular basis for these changes was investigated by measuring the levels of the transcription factor Pax-5, which has been shown to play essential roles during B cell development and differentiation. We focused our studies on an enriched population of mature SRBs because it comprises a well-defined and relatively uniform B cell population.

Decreased DNA binding activity of Pax-5a in aged mice

Our results show that the DNA binding activity of Pax-5a protein is strongly reduced in splenic B cells from aged mice, whereas activity or protein levels of Pax-5d do not change significantly. Decreased Pax-5a binding activity is not the result of decreased levels of Pax-5a RNA transcripts or overall protein levels, as shown by RNase protection assays and Western blot analyses. These data are in agreement with the hypothesis that a posttranslational mechanism affects Pax-5 activity in aged B cells, possibly involving proteolytic and/or redox activities.

Our results are similar to a study by Ammendola et al. who studied the transcription factor Sp1 in aged rat brain and liver tissues (36). The authors found that the DNA binding activity of Sp1 dropped (60-fold) with age, although no change either at the transcriptional or protein level was detected (36). Ammendola's study is important as it supports our finding that changes in gene expression in aged cells may occur through posttranslational modifications of transcription factors.

An interesting report by Tell et al. provides a possible explanation for the observed decreases in Pax-5a DNA binding activity in aged B cells (37). It has been well-documented that chronic exposure to reactive oxygen species (ROS) can lead to aging and age-related pathology. Tell et al. found that ROS may affect gene expression by modulating Pax-5a activity using cell lines. Importantly, the authors show that an oxidized form of Pax-5a is unable to interact with DNA, whereas the reduced form binds strongly (37). It was further shown that an intramolecular disulfide bond within the paired domain of Pax-5a causes interference with specific DNA binding (37). In a follow-up study (38), the authors show a positive correlation between exposure (of a B cell line) to the ROS-inducing substance H_2O_2 and rapid transfer of the cytoplasmic redox factor Ref-1 into the nucleus. This increase in nuclear Ref-1 is also correlated with an increase in Pax-5a binding activity in the nucleus using an in vitro assay (38).

The Tell et al. (37, 38) data are important in regard to our results, because it is likely that a changed redox state of Pax-5a is responsible for the observed reduction in its DNA binding activity in aged B cells. The notion that Ref-1 is involved in redox regulation of Pax-5a is also supported by recent reports on its role in the redox activation of other inducible transcription factors such as Pax-8, NF-κB/p50, and Jun (39–41). It is noteworthy to mention here that isoform Pax-5d, which does not appear to be sensitive to age-related processes, has two cysteine residues within its unique 42-aa C-terminal region (30). Whether these cysteine residues play a role in redox regulation and/or protein stability is currently under investigation in our laboratory. We have recently observed changes in Ref-1 levels in nuclear extracts from aged mice (P. Zwollo, unpublished observations), supporting the idea that Ref-1 function may be linked to Pax-5 activity during the aging process. Together, Tell's and our own findings provide the basis for future studies investigating the functional significance of ROS and its association with posttranslational changes in transcription factors in aged animals.

Pax-5a activity inversely correlates with both J chain and secreted Igμ expression in aging B cells

Pax-5d has no transactivation domain but has similar in vitro DNA affinity as Pax-5a (30) and is thus likely to represent a dominant-negative form of Pax-5a. The observed decrease in DNA binding activity of Pax-5a correlated with increased relative levels of DNA-bound Pax-5d in aged B cells in vitro. Resulting changes in the 5a:5d ratio in aged B cells may cause down-regulation of Pax-5 target genes that are normally expressed and, vice versa, may aid in the de-repression (or activation) of target genes that are normally suppressed.

Perhaps the most exciting results from this study suggest that decreased Pax-5a activity correlates with increased expression of two Pax-5 target genes in aged B cells, the Ig *J chain* and the secreted form of Igμ. Interestingly, Pax-5 functions as a transcriptional repressor for both genes. In the case of the *J chain* gene, earlier studies using B cell lines had already shown that *J chain* expression is largely determined by nuclear levels of Pax-5a (21, 32). Pax-5a represses J chain expression in mature B cells, whereas forced overexpression of Pax-5a in plasma cell lines leads to suppression of J chain expression (31). The observed expression of J chain in aged, resting (high-density) mature B cells clearly illustrates an "abnormality" of the aged mature B cell phenotype, because J chain is not normally expressed until the activated B cell stage.

Similarly, the observed increase in secreted Igμ suggests that in aged mature B cells, Pax-5a, which normally prevents secretion of IgM through interaction with the IgH 3′ enhancer, is no longer able to sufficiently suppress gene expression. A study by Usiu et al. (29) in which cloned late mature B cells (CH12.LX.A2) were analyzed for levels of *Pax-5a* RNA as well as both membrane and secreted Ig mRNA, observed that reduced *Pax-5* RNA was always found in high Ig-secreting clones and vice versa. This group proposed that reduction of *Pax-5a* levels play a direct role in the switch from membrane to secreted Ig transcript processing (29). Whether this alternative splicing event is modulated through the Ig 3′ enhancer remains to be determined. From our own data it appears that during the aging process, although *Pax-5* transcript levels remain the same, posttranslational changes of Pax-5a have a similar, de-repressing effect, resulting in the presence of secreted Igμ transcripts in resting B cells. This is likely to result in an increased level of secreted IgM in aged B cells. We hypothesize that decreased DNA binding activity of Pax-5a and/or increased binding of Pax-5d in aged B cells is linked to the observed "spontaneous" secretion of Abs in the absence of pathogens (8). We are currently investigating whether isoform Pax-5d actively de-represses *J chain* and/or secreted *Igμ* expression in this situation, or whether reduced Pax-5a binding by itself may be sufficient to (at least partially) release transcriptional suppression.

No effects of aging were observed for the target gene *CD19* using RNase protection assays. This may be the result of the high-affinity Pax-5 binding site on the *CD19* promoter, as compared with the lower-affinity sites on the *J chain* promoter and *Igμ H chain* 3′ enhancer, as measured by Wallin et al. (42). It has been observed that during the early stages of a B cell immune response, Pax-5a maintains its activator functions (e.g., not affecting CD19) but is relieved of its repressor functions (e.g., inducing J chain and Igμ expression) (42). Wallin's study (42) showed that activator motifs had a 20-fold higher binding affinity for Pax-5 binding sites as compared with repressor motifs. Thus when the available amount of Pax-5a decreases in aged B cells, this likely affects repressor functions first. Alternatively, it is possible that although

Pax-5a is essential for *CD19* expression during early B cell development, there may be more functional redundancy during late B cell stages.

Other effects of decreased Pax-5a activity in aged mice?

The observed reduction in class switching from IgM to IgG isotype Abs in B cells from aged mice (4) may also be caused by the decrease in Pax-5a activity. One study in which antisense Pax-5a oligonucleotides were used to down-regulate Pax-5a expression showed that this caused decreased class switching from IgM to IgG (15), although it cannot not be ruled out that the lack of isoform switching was simply the result of a lack of cell proliferation. In support for a role in cell proliferation, antisense oligonucleotides that reduce Pax-5a expression also block mature B cell proliferation in response to LPSs (14). Thus, a decrease in Pax-5a activity may play a role in the reduced B cell proliferation and/or class switching responses that have been observed in aging humans and rodents.

In summary, the reduction in Pax-5a DNA binding activity in aged mature B cells and the inappropriate expression of two of its target genes provide evidence that dysregulation of this important transcription factor may be associated with the abnormal B cell phenotype in aged mice. This is the first study to describe a change in the activity of a B cell-specific transcription factor with age, and we propose a molecular mechanism that may be central to the induction of immune senescence in aging B cells.

Acknowledgments

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