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*J Immunol* 2001; 166:2617-2626; doi: 10.4049/jimmunol.166.4.2617
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Reduction in DNA Binding Activity of the Transcription Factor Pax-5a in B Lymphocytes of Aged Mice

Jillian Anspach,* Gail Poulsen,* Ilsa Kaattari, † Roberta Pollock, ‡ and Patty Zwollo 2 *

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.
and/or the developmental stage of the B cell, Pax-5 can act either as 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 In resting, splenic B cells, the protein level of Pax-5b is very low (M. Lowen and P. Zwollo, unpublished observations) and Pax-5e is not present at detectable levels (30).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 transactivation, 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 Igk 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% polyethyleneglycol (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 μg/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-ME containing hypoxanthine, aminopterin, and thymidine and incubated at 37°C with 5% CO2.

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 M 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, 13 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 × 10^7 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 TFIIID (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. Asctises 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 28 μg/ml. The ED-1, TFIIID, 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).

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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. 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 (TuT; Promega, Madison, WI) according to the suppliers’ instructions. From each spleen, Total cellular RNA was isolated from Percoll-purified SRBs using a RNA isolation and RNase protection assay according to the suppliers’ directions. The plasmids (pBluescript; Stratagene, La Jolla, CA) containing the iso- specific mAbs was generated using PCR amplification with sense primer cμ4F (5′-caggatgccATGTCAGACAGGAGGAATGG; lower case is the EcoRI restriction site with flanking sequence) targeting exon cμ4 and antisense primer mlgMB (5′-caggatgccACGGTGAGGGCC AACACAAGGG; lower case represents the BamHI restriction site with flanking sequence) complementary to exon M2 of the Iγ 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 EcoRI and BamHI 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. 1A. Fig. 1A shows the reactivity of 6G11 to in vitro-translated (ivt) Pax-5d, but not ivt Pax-5a (Fig. 1A, lanes 2 and 3, 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 antiserum 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 isoform.
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. 1C, 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. 2A) detects both Pax-5a/b and Pax-5d/e sequence (30), whereas a “control” tubulin probe, pm.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. 2B. 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

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**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 IgM 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/e-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 pm.100 detects a 94-nt β-tubulin transcript.
lanes 5 and the slower migrating (5a.1) position on the gel (Fig. 3) named 5a.1 (slower) and 5a.2 (faster). Ivtt Pax-5a protein runs in lanes 3–6, A, using the CD19/BSAP probe is shown. Results of this experiment showed that the complexes on the CD19 probe are Pax-5 specific, as excess wt unlabelled probe competed efficiently for binding, but mutated probe did not, as shown in Fig. 3A. Addi-
tion of the anti-paired domain antisum 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 ivtt Pax-5d forms a much weaker complex as compared with ivtt Pax-5a (Fig. 3A, lane 2), although Western blots (e.g., Fig. 1B) indicated that similar amounts of each Pax-5 isoforn protein were present in samples. This may be the result of different redox regulation or protein degradation of ivtt 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 iden-
tify 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 ivtt 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). Ivtt 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 rec-
ognized by both a N-terminal-specific anti-Pax-5 Ab N-19 and and 5a.2 complexes are shown on the left.

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 pre-
incubation 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, suggest-
ing either that 5a.1 represents a more labile protein conforma-
tion or, alternatively, that the 5a.2 complex has a protein confor-
mation 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 bind-
ing 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 dsCD19/BSAP oligonucleo-
tides. The amount of excess oligos is indicated on the left. Lane 1, Probe in the absence of protein; lane 2, ivtt Pax-5a (1 µl lysate); lane 3, ivtt 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, Ivtt 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 ivtt Pax-5a (lanes 3 and 4) or ivtt Pax-5d (lanes 5 and 6). Positions of Pax-5a.1, 5a.2, and Pax-5d complexes are shown on the left.
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 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. 4E). 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.

To 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 of a more general aging mechanism in B cells that is conserved among (mammalian) species.

**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, Ivtt Pax-5a (1 μl lysate); lane 2, Ivtt 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. 3B 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. 3B but using nuclear extracts of SRBs from *P. leucopus* mice. Lane 1, Ivtt Pax-5a (1 μl lysate); lane 2, Ivtt 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.
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 TFIIID. 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 TFIIID 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 TFIIID protein levels, using the anti-paired domain serum ED-1 incubated together with anti-TFIIID Ab. The asterisk indicates a degradation product of Pax-5a (see text). The positions of isoform Pax-5a and TFIIID 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).
Four 2-mo-old and four 19-mo-old mice were counted, and 3 at the protein level using Western blot analysis. Whole SRBs from
alternative approach, we measured changes in
script levels between young and aged mice (not shown). As an
protection assays were too low to reliably detect changes in tran-
vated (21), and this may explain why our signals from RNase
RNA levels (21, 32). Here we wished to test whether the
mature B cells, would lead to increased
expression.

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 mRNA 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 acti-
vated (21), and this may explain why our signals from RNase
protection assays were too low to reliably detect changes in transcrip-
tive 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
two 2-mo-old and four 19-mo-old mice were counted, and $3 \times 10^6$
cells were lysed and analyzed on SDS-PAGE gels.

Whole-cell lysates were used in this experiment to enable quan-
titative comparison of the NFs Pax-5a and TFIIID as well as the
cytoplasmic J chain protein. Pax-5a expression in combination with
TFIID levels provided important internal controls for the rel-
ative 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 TFIIID 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. 7A,
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. 7A (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% Per-
cell gradients, were analyzed, as shown in Fig. 7B. 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. 7C). 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 ex-
pression of at least two Pax-5 target genes, the J chain and the Ιγκ
H chain gene.

Discussion
Substantial reduction in both cellular and humoral immune re-
spones 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 pat-
terns of B cell-specific proteins that are structurally or functionally
associated with the B cell receptor. We hypothesized that some of

![FIGURE 6](http://www.jimmunol.org/)

**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 mem-
brane form of IgM, and a 111-nt fragment covering Cμ4-S
alone detects secreted IgM 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, sam-
pies 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.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Comparison of J chain expression in young vs aged B cells. Western blot analysis. A, Equal numbers of SRBs were boiled and run under
denaturing conditions to allow comparison of nuclear Pax-5a and TFIIID factors with the cytoplasmic J chain protein. Problongs with Abs to Pax-5a,
anti-TFIID, and anti-J chain (see text). The ratio of J chain to TFIIID (J:T) for each sample is indicated below the figure B. Same experiment as in Fig. 5A
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:p110) is indicated below the figure.
these changes have a common cause, namely the deregulation of transcription factor(s) normally expressed during B cell matura-
tion. 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 posttrans-
lational 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 mod-
ifications of transcription factors.

An interesting report by Tell et al. provides a possible explana-
tion for the observed decreases in Pax-5a DNA binding activity in
aged B cells (37). It has been well-documented that chronic ex-
posure 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. Impor-
tantly, 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 spe-
cific 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 H₂O₂ and rapid transfer of the cyto-
plasmic redox factor Ref-1 into the nucleus. This increase in nu-
clear 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 re-
sults, 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 regu-
lation 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-κBp50, 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 IgM 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 dominan-
tive-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. Results showing in
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DNA-bound Pax-5d in aged B cells in vitro. Results showing 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 nor-
manly 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 IgM. Interestingly, Pax-5 functions as a transcrip-
tional repressor for both genes. In the case of the J chain gene,
earlier studies using B cell lines had already shown that J chain
gene 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 sup-
pression of J chain expression (31). The observed expression of
J chain in aged, resting (high-density) mature B cells clearly illus-
trates an “abnormality” of the aged mature B cell phenotype, be-
cause J chain is not normally expressed until the activated B cell
stage.

Similarly, the observed increase in secreted IgM 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-re-
pressing effect, resulting in the presence of secreted Igα 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 se-
creted Igα 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 Igα 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
Igα 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 cell 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 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

We thank Dr. Steve Kaattari for help and advice on the generation of mAbs, Drs. Steve Kaattari, Liz Allison, and Marina Lowen for critical reading of the manuscript, and Dr. Diane Shakes for stimulating discussions. We also thank Dr. Yaping Deng for flow cytometric analysis of Percoll-purified splenic cells. We are grateful to Dr. Paul Heideman and Charles Finneran for providing young and aged P. leucopus.

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