The Interaction of Pax5 (BSAP) with Daxx Can Result in Transcriptional Activation in B Cells*

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Alexander V. Emelyanov, Cecilia R. Kovac‡, Manuel A. Sepulveda, and Barbara K. Birshstein§
From the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Pax5 (BSAP) is essential for B cell development and acts both as a transcriptional activator and a repressor. Using a yeast two-hybrid assay to identify potential coregulators of Pax5, we identified Daxx, a protein that is highly conserved, ubiquitously expressed, and essential for embryonic mouse development. The interaction between Pax5 and Daxx involves the partial homeodomain of Pax5 and the C-terminal fragment of Daxx. A component of promyelocytic leukemia protein nuclear bodies, Daxx has been implicated in apoptosis and characterized as a transcriptional corepressor. Upon transient transfection assay of Daxx in B cells expressing endogenous Daxx and Pax5, we observed not only transcriptional corepression but also, unexpectedly, coactivation in M12.4.1 and A20 mouse B cell lines. Pax5 domains required for coactivation were identified using 293T cells. Coactivation apparently involves recruitment of the CREB binding protein (CBP), because we precipitated complexes containing Pax5, Daxx, and CBP in B cell lines. These data suggest that Daxx can affect Pax5’s roles as an activator or repressor in B cells and describe a role for Daxx as a transcriptional coactivator.

The transcription factor Pax5 (BSAP) is essential for the development of B cells in mice (1). In Pax5-deficient mice, fetal B cells are completely absent, and B cell development in the bone marrow fails to progress past the early pro-B (pre-BI) stage (2). Pre-BI precursor cells from Pax5-deficient mice, but not from wild type mice, can give rise to a number of non-B cell hematopoietic lineages, in vitro and in vivo, including T lymphocytes and myeloid cell types, (3, 4). Accordingly, it has been shown that pre-B Pax5-deficient cells express a number of cytokine receptors (3). Pax5, therefore, appears to activate progression of early precursors to the B cell lineage and away from T cell and myeloid cell types. Introduction of wild type Pax5 into Pax5-deficient pre-BI cells rescues B cell differentiation and executes dramatically opposite effects on various genes; e.g. the myeloid M-CSF receptor and PD-1 genes are downregulated whereas CD19, N-myc, mb-1, and LEF-1 are upregulated (3, 5). Interestingly, only the N-terminal domain of Pax5 is required for activation of mb-1 and LEF-1 (5).

In addition to its role early in B cell differentiation, Pax5 is essential for later stages as well, when it influences the expression of many genes (6). We and others (7, 8) have shown that Pax5 is a transcriptional activator or repressor for the 3’-immuno-globulin heavy chain gene (Igh) enhancer and other targets (reviewed in Ref. 9) (3, 10–14). These diverse activities of Pax5 suggest that Pax5 function may be modulated by interaction with other proteins.

To identify potential coregulators of Pax5, we have used a yeast two-hybrid assay. Previously, we reported the interaction of Pax5 with importin α1 (15), one of several importin α proteins that have been implicated in the transport of proteins into the nucleus. Several other proteins have been reported to interact with Pax5 (10, 13, 16–20). Of these, only Groucho has been shown to affect Pax5’s transcriptional activity through its role as a corepressor (20). However, TBP and Rb (19) and PTIP (a novel BRCT domain-containing protein that interacts with the activation domains of several Pax proteins (21)) are also candidates for influencing the transcriptional activity of Pax5.

Here we report the interaction of Pax5 with Daxx in a yeast two-hybrid assay. The interaction was confirmed by GST pull-down assays and endogenous coimmunoprecipitation. Daxx is highly conserved and ubiquitously expressed (22) and essential for embryonic mouse development (23). Daxx is a unique protein that has been implicated in apoptosis (24–28), identified as a component of promyelocytic leukemia protein (PML) bodies (also termed ND10 or PODs (29)), and described as a transcriptional corepressor of Pax3 and Ets proteins (30–32). The experiments reported here identify Daxx as a coactivator. We have observed coactivation of Pax5 by Daxx in mature B cell lines that express Pax5, putatively through interaction with the CREB-binding protein, CBP, which has HAT activity. We also observed an inhibitory effect of Daxx on Pax5’s transcriptional activity in HS-Sultan lymphoblastoid cells and J558L plasma cells. Together, these data suggest that Daxx is a bridge protein that helps Pax5 to function as both a transcriptional activator and a repressor during B cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**

The serine/threonine-rich central domain of Pax5 (aa 144–267) (GenBank™ accession number M97013 for murine Pax5 and M96944 for human Pax5) was used as bait in a yeast two-hybrid assay, performed as described previously (15, 33). A cDNA library from Epstein-Barr virus-transformed human peripheral B cells cloned in the pACT vector (a gift from Dr. S. Eldredge, Baylor College of Medicine, Houston, TX) was screened, using an X-gal filter assay (34). Candidate clones were also assayed in an O-nitrophenyl-β-d-galactoside liquid assay (35).

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‡ Current address: Dept. of Biological Sciences, Long Island University at Brooklyn, NY 11201.
§ To whom correspondence should be addressed. Tel.: 718-430-2291; Fax: 718-430-8574; E-mail: birshtei@aeecom.yu.edu.

1 The abbreviations used are: GST, glutathione S-transferase; PML, promyelocytic leukemia protein; CBP, CREB binding protein; aa, amino acids; DBD, DNA binding domain; DTT, dithiothreitol; FMSF, phenylmethlysulfonyl fluoride; CMV, cytomegalovirus; RSV, Rous sarcoma virus; NLS, nuclear localization signal; HAT, histone acetyltransferase; FKHR, forkhead; X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactosidase; Igh, immunoglobulin heavy chain; HDAC, histone deacetylase.

11156 This paper is available on line at http://www.jbc.org
The human kidney cell line 293T and HeLa were grown in Dulbecco’s modified Eagle’s medium. The murine B cell lines M12.4.1 and A20, and the human lymphoblastoid cell lines BL/Burkitt’s lymphoma)-2 and HS-Sultan were grown in RPMI 1640 medium (Fisher). Media were supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

**Antibodies**

Goat anti-Pax5 antibody (N-19), which is directed against the N terminus of Pax5, rabbit anti-Daxx (M-112) antibody, rabbit anti-CBP antibody (A22), and normal rabbit IgG were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). We also used a custom polyclonal rabbit antibody raised against amino acids 144–391 of Pax5 (Covance).

**Cell Lines**

293T cells were transiently transfected using the LipofectAMINE reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells (2–3 × 10⁶) were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT) with protease inhibitors (1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 5 μg/ml aprotinin) and incubated for 30 min on ice. Precleared lysates were incubated for 2 h at 4 °C with goat anti-Pax5 antibody. Immunocomplexes were collected by addition of 20 μl of protein G-agarose (Sigma Chemical Co.) for 2 h at 4 °C. After extensive washing of the beads with lysis buffer, immunoprecipitated proteins were eluted, separated by SDS-PAGE, and analyzed by Western blotting.

**Transient Transfection and Reporter Assays**

The pGL3(CD19-2) luciferase reporter construct, a gift from Dr. James Hagman (National Jewish Hospital, Boulder, CO), contains four tandem copies of a low affinity Pax5 binding site from the human CD19 promoter (5′-TCGACTGGGGCCTGGGTCAGTCCGACC-3′) inserted at the XhoI site of the pGL3 promoter vector (Promega) upstream of the SV40 promoter. We carried out electrophoretic mobility shift assay analysis using nuclear extracts from B cell lines to confirm that Pax5 binding to this reporter region could be detected. (UAS)5E1bTATA Luc reporter constructs were gifts from Dr. Richard Pestell (Albert Einstein College of Medicine) and are described elsewhere (38, 39). The RSV-CBP expression construct, which contains human CBP cDNA, was a gift of Dr. Richard Pestell (Albert Einstein College of Medicine). The pcDNA3.1-p300 construct, which encodes human p300, was a gift of Drs. Nickolai Barlev and Shelley Berger from the Wistar Institute.

For reporter assays, HeLa cells and 293T cells were transfected with reporter constructs, Pax5 and Daxx expression vectors, and RSV-β-gal by the Geneporter reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s instructions. A dextran method was used for the transfection of M12.4.1 and A20 cells, as previously described (7, 40). After 48 h, cell extracts were analyzed with a luciferase assay kit (Tropix, Bedford, MA). Transfection efficiency was normalized by assay of β-galactosidase activity using a Galacto-Light plus kit (Tropix). HS-Sultan cells were transiently transfected by electroporation. 5 × 10⁶ cells in 0.8 ml of complete media were incubated for 10 min at room temperature and then electroporated at 960 microfarads/280 V. The Renilla luciferase expression vector pBL-CMV (Promega) was transfection for normalization. After 24 h, cells were harvested and firefly and Renilla luciferase activities in the cell extracts were measured using the Dual-Luciferase reporter assay system (Promega). The total amount of DNA (20–22 μg) was adjusted by addition of pEBB with no insert. All readings were taken using a Tropix LB953 luminometer (EG&G Berthold). Each experimental point was performed at least three times and within each experiment, transfections were performed in triplicate, except for M12.4.1 and A20, which were performed in duplicate. The average of the relative luciferase activity for each experiment was divided by the relative basal reporter luciferase activity, and the calculated average is shown as -fold transactivation with standard deviation.

**In Vitro Assay of Histone Acetylation and Endogenous Coimmunoprecipitation of Pax5, Daxx, and CBP**

A20 (6–8 × 10⁶), M12.4.1 (6–8 × 10⁵), 293T (1–2 × 10⁵), and 1 × 10⁶ BL-2 cells were lysed in radioimmune precipitation mild buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) with protease inhibitors (1 mM PMSF, 2 μg/ml leupeptin and pepstatin, and 5 μg/ml aprotinin) for 1 h on ice. Precleared lysates (2 mg of total protein) were incubated for 4 h at 4 °C.

**Pax5 and Daxx Constructs**

The Pax5 mammalian expression construct was generated by cloning the NotI-linked murine Pax5 cDNA from pBSK-Pax5 (a gift from Dr. Stephen Desiderio, The Johns Hopkins University, Baltimore, MD) into the pEVB vector (a modified version of pEF-BOS (36)). Pax5P5D-Pax5DH, and Pax5D5C mammalian expression constructs were prepared by cloning PCR amplified fragments from pBSK-Pax5 into ClaI-NcoI sites of pEVB. The Pax5DH construct was generated by ligating PCR-amplified fragments, aa 1–228 and aa 254–391, at an XhoI site introduced at the deletion site but without affecting the amino acid sequence. The following primers (all sequences are 5′ –3′) were used for amplification, beginning at the numbered amino acid, with restriction sites underlined and the direction (forward or reverse) (R), indicated (see Figs. 1–3): pEBB-1F, TAATATGGATCATAAGTAAAGAAATAATG; pEBB-144F, TAATATGCGGCCGCCAAGGCAGCACCGACACTT; pEBB-254F, TAATATCCTGAGGTCATCCCATCGACGAGCA; pEBB-228R, TAATATCGGATATTTCGGGTCAGTTGTGAGGA; pEBB-391R, TAATGGCGCGAGGACATGCGGCTGTCATAGCAGCCGTG; pEBB-391R, TAATGGCGCGAGGACATGCGGCTGTCATAGCAGCCGTG.

**GST-Pax5 Fusion Constructs**—These were prepared by cloning fragments of murine Pax5 cDNA amplified by PCR from pBSK-Pax5 into EcoRI-SalI sites of the pGALO vector (a gift from Dr. Leila Alland, Albert Einstein College of Medicine, Bronx, NY), which encodes the GAL4 DNA binding domain (aa 1–147) as described elsewhere (37). The following primers were used: pGALO-1F, TAATGGAAGATCATTAGTATTGAGAAATTAT; pGALO-228R, TAATATCGGATATTTCGGGTCAGTTGTGAGGA; pGALO-358R, TAATGGAAGATCATTAGTATTGAGAAATTAT; pGALO-391R, TAATGGAAGATCATTAGTATTGAGAAATTAT.

**GST-Pax5 Fusion Constructs**—These were prepared by cloning fragments of murine Daxx cDNA amplified by PCR from pBSK-Pax5 into EcoRI-SalI sites of the pGEX-5X vector (Promega). Primers used for amplification were the same as those used for creating green fluorescence protein fusion constructs, as described previously (15).

**LexA-Pax5 Constructs**—These were the same as used previously elsewhere (15), except that LexA-Pax5 (aa 144–267), containing an additional deletion of the partial homeodomain (aa 229–253), was prepared by cloning a PCR fragment amplified from pEBB-Pax5DH into the EcoRI-SalI sites of pEG202.

**Daxx mammalian expression constructs** were prepared by cloning full-length human Daxx cDNA from pEQ90-Daxx (a gift from Dr. Jerome Strauss, University of Pennsylvania Medical Center, Philadelphia, PA) into BamHI-NotI sites of pEBB. The GAL4DBD-Daxx construct was prepared by cloning full-length human Daxx cDNA into BamHI-SalI sites of pGALO. The murine Daxx mammalian expression vector (24) was a gift of Dr. Xiaolong Yang (University of Pennsylvania, Philadelphia, PA).

**GST-Daxx Fusion Constructs**—PCR-amplified fragments of human Daxx cDNA (Fig. 4A) were cloned in frame in BamHI-SalI sites of pGEX-5X vector (Promega). Primers are as follows: pGEX-1F, TAATGGATCATAATGGCCGACAGCAGCAGCAG; pGEX-240F, TAATGGAAGATCATTAGTATTGAGAAATTAT; pGEX-502F, TAATGGAAGATCATTAGTATTGAGAAATTAT; pGEX-626F, TAATGGAAGATCATTAGTATTGAGAAATTAT.

**GST Pull-down Assay**

Purified GST fusion proteins (2–5 μg) bound to glutathione-Sepharose were incubated for 1 h at room temperature with 20 μl of reaction mix, which contained [35S]methionine-labeled proteins in *vitro* synthesized by a coupled transcription/translation system (Promega) in 100 μl of basic buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1% bovine serum albumin, 1 mM DTT), supplemented with 1 mM PMSF, 2 μg/ml of leupeptin and pepstatin, and 5 μg/ml aprotinin. For *in vitro* translation, full-length murine Pax5 cDNA and human Daxx cDNA were cloned into pSP72 vector (Promega). After incubation, beads were washed five times with binding buffer containing 500 mM NaCl, and bound proteins were eluted and detected by autoradiography after separation by 10% SDS-PAGE.
with rabbit normal IgG or rabbit anti-Daxx antibody, or, also for BL-2, rabbit anti-Pax5 or rabbit anti-CBP antibody, together with 20 μl of protein A-agarose (Sigma). After extensive washing with lysis buffer, immunocomplexes were incubated in 50 μl of HAT assay buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10% glycerol, 10 mM sodium butyrate, and 0.1 mM EDTA) containing 1 mM DTT, 1 mM PMSF, 4 μg of free histones (Sigma), and 125 nCi of [3H]acetyl-CoA (Amersham Biosciences, Inc.) for 30 min at 30°C. The reaction was stopped by spotting supernatants on P81 paper filters (Whatman), which were washed 3 × in 50 mM carbonate/bicarbonate buffer (pH 9.0), and counted in a scintillation counter, LS 6800 (Beckman). Immunocomplexes, prepared as described above from A20, M12.4.1, and 293T cells, were eluted from beads, separated by SDS-PAGE, and analyzed by Western blotting.

Immunoprecipitation by antibody to CBP, Pax5, or Daxx with extracts of A20, M12.4.1, and 293T cells, were eluted from beads, separated by SDS-PAGE, and analyzed by Western blotting.

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Results

Identification of Daxx as a Pax5-interacting Protein—The central domain of Pax5 (aa 144–267) was used to screen a library of 10^7 yeast colonies containing human B cell cDNA in the yeast two-hybrid assay, and we detected one clone (isolated four times) containing human Daxx (GenBankTM accession number AFO15956) (see Fig. 1). This clone extended from bp 417 to bp 2463, coding for aa 139 through the C terminus at aa 740. Assay of truncation mutants of the Pax5 bait region (Fig. 2) showed that interaction with Daxx required the partial homeodomain but neither the octapeptide nor NLS sequences. We observed a potential weak interaction of the paired domain of Pax5 with Daxx. Human and mouse Pax5 are identical except for three amino acid residues, none of which affects the region used as bait in the two-hybrid assay (42). Human (AF15956) and mouse (AF006040) Daxx show 69% similarity (22). Although most experiments were done with human Daxx, mouse Daxx, as described below, appears to act similarly. The Potential Homeodomain of Pax5 and the C-terminal Segment of Daxx Are Essential for Interaction in Vitro—We used a GST pull-down assay to examine the physical interaction between different Pax5 truncation mutants and full-length human Daxx (Fig. 3). Consistent with the yeast two-hybrid assay, GST-Pax5 fusion proteins that contained the partial homeodomain (the shortest of which was aa 202–254) were able to bind to Daxx. However, there was no detectable interaction of the paired domain (aa 16–143) of Pax5 with Daxx, suggesting that the weak interaction observed in the yeast two-hybrid system may not be specific. Analysis of GST fusion proteins that contain different segments of human Daxx (Fig. 4) showed that only the C-terminal segment of human Daxx (aa 626–740) was able to interact with Pax5, the same region that has been shown to interact with Fas (24), Ets (30), CENP-C (43), PML (29), Pax3 (32), and sentrin and ubc-9 (44).

Daxx Modulates Pax5’s Transcriptional Activity in B Cell Lines Expressing Endogenous Pax5—It has been problematic to study the transcriptional activity of Pax5 using reporter constructs. For example, the expression of the CD19 gene is clearly linked to Pax5 expression and sequences upstream of the CD19 promoter contain two Pax5 binding sites (45). However, reporter constructs utilizing this 5′ upstream region have been ineffective in measuring Pax5-dependent activity in transient transfection assays in B cells (45, 46). In fact, measurements of Pax5-dependent transcriptional activity in non-B cells have also been difficult to achieve (12, 42). However, excised Pax5 binding sites have been successfully used for reporter assays measuring Pax5 transcriptional activity (e.g., Refs. 13, 46–48).

We tested the ability of Daxx to modulate Pax5 function in B cell lines by using the reporter, pGL3(CD19-2), which was generated by the introduction of multimerized low affinity Pax5 binding sites from the CD19 promoter to a position upstream of the SV40 promoter (see “Experimental Procedures”). In M12.4.1 and A20 murine B cells, and in HS-Sultan human cells, each of which expresses endogenous Pax5, there was more activity with pGL3(CD19-2) than with the basic reporter (pGL3), presumably reflecting the activation effect of endogenous Pax5 (see Fig. 5, A–C, compare lanes 1 and 2). Although the expression of Daxx upon transfection had no effect on reporter activity of pGL3, which lacks Pax5 binding sites (Fig. 5A–C, compare lanes 1 and 2), the expression of Daxx upon transfection resulted in an increase of ~3.5-fold in activity of pGL3(CD19-2) in three of four experiments in M12.4.1 cells and an increase of ~2.1-fold in activity in each of three experiments in A20 cells (Fig. 5, A and B, compare lanes 3 and 5). These data identify a role for Daxx in coactivation. In contrast,
an inhibitory effect of Daxx on endogenous Pax5 transcriptional activity was observed in the human cell line, HS-Sultan (Fig. 5C, compare lane 3 to lanes 4 and 5) and in the murine plasmacytoma cell line J558L (data not shown). These experiments show that Daxx can activate or repress the transcriptional activity of Pax5 in B cells.

To test whether Daxx’s coactivating function was evident only in B cells, we also used the pGL3(CD19-2) reporter in HeLa cells, which, like other non-B cells, do not express endogenous Pax5 (Fig. 5D). Expression of Pax5 resulted in a 2.6-fold increase in activity (lane 3). Coexpression of Daxx and Pax5 resulted in an additional ~2-fold increase in activity (lanes 4 and 5), whereas Daxx alone had no effect on reporter activity (lane 2). These data imply that Daxx can function as a coactivator of Pax5 in both B and non-B cell lines and suggest that factors necessary for coactivation are not restricted to B cells.

**FIG. 2.** Localization of the Pax5 interaction site for Daxx in vivo. A, schematic diagram of LexA DBD fusion deletion mutants of Pax5 and summary of a qualitative, yeast two-hybrid, X-gal filter assay, and a quantitative, β-galactosidase liquid assay.

**FIG. 3.** The Pax5 partial homeodomain is necessary for Daxx binding in vitro. A, GST fusion deletion mutants of Pax5. The numbers indicate amino acid positions. B, results of GST pull-down assay. GST alone and indicated GST fusion deletion mutants of Pax5 were used to study the interaction with in vitro translated (IVT) Daxx protein. The input lane contained 20% of the total volume of Daxx translation mix used in each assay.

**FIG. 4.** Pax5 interacts with the C-terminal region of Daxx. A, GST fusion deletion mutants of Daxx. The numbers indicate amino acid positions. B, results of GST pull-down assay. GST alone and indicated GST fusion deletion mutants of Daxx were used to study the interaction with in vitro translated (IVT) Pax5. The input lane contained 20% of the total volume of Pax5 translation mix used in each assay.
served in HeLa cells but to a lesser extent (data not shown). In addition, we detected coactivation of the E1bLuc reporter by Pax5 and murine Daxx in 293T cells (data not shown). Coactivation required GAL4DBD-Pax5, because no significant change in reporter activity was observed over that seen with GAL4DBD alone (lane 2) when Daxx was cotransfected with GAL4DBD (lane 3).

Analysis of truncation mutants of GAL4DBD-Pax5 (Fig. 1B) showed that, when the Daxx-interacting partial homeodomain was deleted from Pax5, i.e. GAL4DBD-Pax5ΔHD, the remaining Pax5 segment failed to coactivate with Daxx (Fig. 6A, lanes 8 and 9). Deletion of the entire C-terminal transactivation domain and repression domain (GAL4DBD-Pax5ΔC) resulted in a loss of transcriptional activity (compare lanes 4 and 10), as
expected, whereas deletion of the C-terminal repression domain (aa 359–391), as described in a previous study (47) (GAL4DBD-Pax5ΔRD), had no significant effect on transcriptional activity of Pax5 (compare lanes 4 and 12). Regardless, overexpression of Daxx was unable to coactivate either of these truncation mutants (Fig. 6A) (GAL4DBD-Pax5ΔC, compare lanes 10 and 11; GAL4DBD-Pax5ΔRD, compare lanes 12 and 13). We concluded that coactivation of the E1bLuc reporter by Daxx required not only the partial homeodomain of Pax5 with which Daxx interacts but also the C-terminal repression domain of Pax5.

In a reciprocal set of experiments, we found that E1bLuc is repressed by GAL4DBD-Daxx in 293T cells (Fig. 6C, lane 4). The coexpression of increasing amounts of Pax5 not only relieved Daxx-mediated repression of E1bLuc, but also resulted in a 6-fold increase in transcriptional activity (lanes 5–8). This effect was specific for GAL4DBD-Daxx: cotransfection of Pax5 with GAL4DBD alone (lane 3) showed no additional change in reporter activity over that seen with GAL4DBD (lane 2). With this reporter construct based on the E1b promoter, Daxx shows two different effects on transcriptional activity, i.e., repressive activity when assayed independently and coactivation activity when assayed together with Pax5.

The Paired Domain and the Partial Homeodomain of Pax5 Contribute to Relief of Daxx-dependent Repression of the TK Promoter—To determine whether coactivation is dependent on a particular promoter, we examined the effect of Daxx and Pax5 in 293T cell lines using the highly active TKLuc reporter. As also shown previously (32), we found that the expression of the GAL4DBD-Daxx fusion protein in 293T cells inhibited basal transcription of the TKLuc reporter (Fig. 6B, lanes 1 and 2) in a dose-dependent manner (data not shown). Coexpression of increasing amounts of Pax5 relieved Daxx-mediated repression in a dose-dependent manner (Fig. 6B, lanes 3–6), but there was no apparent coactivation. In the absence of Daxx, Pax5 had no effect on the activity of TKLuc (data not shown).

We wanted to know which segments of Pax5 were necessary for relief of repression of Daxx. Pax5ΔHD, which lacks the partial homeodomain required for interaction with Daxx, was ineffective in relieving Daxx-mediated repression (Fig. 6B, compare lanes 8 and 2). Interestingly, C-terminal segments of Pax5 were dispensable for relief of Daxx-mediated repression, because Pax5ΔC, with a deletion of the C-terminal transactivation and repressive domains (aa 269–391), was as effective as wild type Pax5 in relieving Daxx-mediated repression (compare lanes 6 and 9). This observation is in sharp contrast to the requirement for the repression domain for coactivation of Daxx and Pax5 with the E1b promoter construct. This shows that the N-terminal portion of Pax5 that extends through the paired domain and the central region, including the partial homeodomain, is sufficient to relieve repression mediated by Daxx. In accord with the idea that the paired domain contributes to relief of repression, a Pax5 mutant from which the paired domain has been deleted (Pax5ΔPD) was able to result in recovery of ~50% of basal transcription activity (lane 7). We consider the possibility that the paired domain contributes to relief of repression through interactions with proteins other than Daxx.

We performed a reciprocal experiment with GAL4DBD-Pax5 and Daxx (Fig. 6D). Like Daxx, although to a lesser extent, expression of GAL4DBD-Pax5 alone repressed this reporter (lane 3, >50% repression). Coexpression of increasing amounts of Daxx rescued basal reporter activity and activated the reporter ~2-fold (lane 6). These data indicate that GAL4DBD fusion proteins of Pax5 and Daxx, individually, can repress the TKLuc construct. If Pax5 and Daxx are expressed together, however, repression is relieved.

Pax5 and Daxx Can Interact in Vivo—To assess the formation of a Pax5-Daxx complex in vivo, we transiently expressed full-length mouse Pax5 together with human Daxx in 293T cells. Western blot analysis of immunoprecipitates showed that anti-Pax5 antibody was able to precipitate Daxx protein strongly only in the presence of Pax5 (Fig. 7A). These experiments suggest that Pax5 and Daxx are physically associated in vivo. Further experiments confirming the interaction of endogenously expressed Pax5 and Daxx in B cells are described below.

CBP Cooperates with Daxx and Pax5—That we detected a role for Daxx in coactivation in a number of B and non-B cell lines raised the possibility that Daxx is physically associated with HATs, such as CBP/p300. We found that immunocomplexes of Daxx, as isolated in the murine B cells, A20 and M12.4.1, and in 293T, contained HAT activity at levels ~20–60% of that identified in immunocomplexes formed with anti-CBP antibody, and 8- to 13-fold greater than the negative control generated with normal IgG (Fig. 7B). Furthermore, anti-Daxx antibody was able to coprecipitate CBP in these cells, indicating that CBP was associated with Daxx protein in vivo (Fig. 7D). This extends previous reports that both Daxx and CBP are present in PML bodies (50, 51), and suggests that this interaction may also occur outside of PML bodies.

Additionally, we assayed the involvement of CBP/p300 in the increase in transcriptional activity observed when human Daxx and Pax5 were cotransfected together with the E1bLuc and TKLuc reporter constructs in 293T cells (Fig. 7C). 293T cells were selected because they express SV40 T antigen and the E1A oncprotein, which both repress the activity of endogenous CBP/p300 (52, 53). Addition of CBP (lane 5), but not p300 (lane 6), resulted in a further increase in transcriptional activity over that observed for GAL4DBD-Pax5 together with Daxx (lane 3). In the presence of endogenous Daxx, CBP together with Pax5 coactivated the reporter construct but to a lesser extent (lane 4). In the absence of the partial homeodomain of Pax5 (GAL4DBD-Pax5ΔHD), neither Daxx nor CBP could activate the reporters (lane 7). These results indicate that CBP can contribute to transcriptional activation in a Daxx-dependent manner.

Building on our observations of structural and functional interactions between Pax5, Daxx, and CBP, we carried out coimmunoprecipitation studies using human BL-2 cells to identify a complex of these proteins in vivo. We considered the possibility that interactions between Pax5, Daxx, and CBP were mediated by DNA; therefore, we carried out coimmunoprecipitation in the presence of ethidium bromide, which inhibits DNA-dependent protein associations (41). Using specific antisera to Pax5, Daxx, or CBP, we detected association of all three proteins (Fig. 7E). Similar results were found in M12.4.1 cells (data not shown). These observations suggest that the interaction between Pax5, Daxx, and CBP, as detected by endogenous coimmunoprecipitation, occurs in vivo.

DISCUSSION

Two-hybrid screening has led to our identification of Daxx as a Pax5-interacting protein. The interaction involves the partial homeodomain of Pax5 and the C-terminal segment of Daxx. We have confirmed that Pax5 physically interacts with Daxx in vivo and in vitro when both proteins are overexpressed and when they are present at endogenous levels in B cell lines. Our studies are in concert with previous studies (32) that showed that Pax proteins, that contain a complete homeodomain, like Pax3 and Pax7, bind to Daxx, whereas Pax4, which has no homeodomain, does not. Pax family proteins can be divided into subfamilies, in part by their differences in the extent of their
homeodomain, ranging from complete to partial to none. Pax5 is a member of the Pax2/5/8 subfamily, which contains a partial homeodomain. We therefore predict that, in addition to binding Pax5, Daxx will also bind to Pax2 and Pax8.

Using transient transfection transcriptional assays, we detected corepression by Daxx in human HS-Sultan cells and murine J558L cells. Importantly, we found that Daxx coactivates Pax5 in M12.4.1 and A20 B cell lines, as well as in non-B cell lines, such as HeLa and 293T cells. We suggest that Daxx acts as a bridge protein to modulate Pax5’s transcriptional activity. As a corepressor, Daxx may recruit HDAC1 (31) or DNA methyltransferase 1, which plays a role in gene silencing (23). As a coactivator as described in studies reported here, Daxx may recruit CBP, which possesses HAT activity. In fact, we have detected an endogenous complex of Pax5, Daxx, and CBP.

In 293T cells, the addition of human CBP, but not p300, to GAL4DBD-Pax5 and Daxx resulted in an increase in transcriptional activity. In addition, we found that CBP could specifically acetylate the paired domain of Pax5 in vitro. Although many reports have identified functional similarity between CBP and p300, other studies have indicated some differences between these two proteins. For example, CBP, but not p300, is required for normal hematopoiesis (54 and reviewed in Ref. 55). Although our experiments implicate CBP in coactivation of Pax5, others have shown that p300 rescued repression of PU.1 by Pax5 in NIH3T3 cells (13).

A critical and as yet, unanswered question is what tips the balance between Daxx’s role in coactivation or corepression. In experiments with Pax3 and Pax3-forkhead (FKHR) fusion proteins (32), binding of Daxx did not necessarily lead to repression. Daxx repressed Pax3, but not Pax3-FKHR, although the mechanism accounting for these observations was not elucidated. Various parameters could contribute to the different outcomes of Pax5-Daxx interaction in B cells, including Ep-
stein-Barr virus transformation of HS-Sultan, as contrasted to factors leading to malignant transformation of the murine B cell lines, stochastic differences in expression of transcription factors, or differences in the stage of B cell differentiation as modeled by these different cells. Differences in transcriptional outcome of Daxx and Pax5 appear, however, to be independent of the stage of B cell differentiation, the species examined, and certain modes of malignant transformation, because we observed corepression in J558L murine plasmacytoma cells, which, like other murine plasmacytomias, contain a myc-Igh translocation and lack endogenous Pax5. Furthermore, normal human plasma cells, although not myeloma cells, have been reported to express Pax5 (56). It will be of interest to determine in future studies the nature of the additional cofactors that are required for coactivation or corepression.

In addition to the partial homeodomain of Pax5 that mediates interaction with Daxx, other segments of Pax5 contribute to the effect of Pax5 and Daxx on transcription, perhaps through the interaction with other regulatory proteins. For example, Pax5's repressor domain is necessary for the coactivation observed with Daxx using the E1bLuc reporter, which has relatively low basal activity, whereas Pax5's paired domain appears to contribute to the relief of repression of Daxx, as assayed by the TKLuc reporter, which has high basal activity. Interestingly, we did not detect involvement of the paired domain in coactivation of the E1b promoter (data not shown). Hence, coactivation and relief of repression are two different mechanisms by which Daxx and Pax5 cooperate, and differences in transcriptional coregulation may depend on the specific promoter. Interestingly, Pax5's repressor domain and paired domain have separately been implicated in the repression of PU.1 in different contexts, implying that repression occurs via different mechanisms (13). Furthermore, although Pax5 has been shown to physically interact via its octapeptide with the corepressor Groucho, its transactivation domain is also involved in repression (20).

It is challenging to attempt a synthesis of the interaction of Daxx and Pax5 in B cell biology. As analyzed by Western blots in cell lines representing different stages of B cell development, we found that Daxx is ubiquitously expressed (data not shown). However, Daxx can be up-regulated and recruited to PML bodies in splenic lymphocytes by lipopolysaccharide and ConA (27), and in a pro-B cell line by Type I (alpha and beta) interferons (57). PML bodies, with which Daxx frequently associates, have been variously considered to be transcriptional mielleus (58) or places to park excess proteins (59). Recent studies have shown that overexpression of PML could relieve Daxx-mediated transcriptional repression (31, 60, 61), presumably by recruiting Daxx to PML bodies, and hence away from interaction with HDAC and target genes. However, our studies (data not shown) showed that Pax5 was expressed throughout the nucleus, raising the likelihood that interaction between Pax5 and Daxx occurs outside of PML bodies.

Several reports support a role for Daxx in apoptosis, with potential effects on B cell selection. Apoptosis under these conditions required recruitment of Daxx to nuclear bodies by PML (26, 27). We confirmed the enhancement of Fas-mediated apoptosis by Daxx in 293T cells (24, 25), using morphological analysis (data not shown). However, we did not see any effect by Pax5 on Daxx's enhancement of apoptosis, nor have we observed any significant numbers of apoptotic cells after overexpression of either Daxx or Pax5, or both in 293T cells, HeLa, NIH3T3, or U2-OS cells. Transfection of B cells results in considerable cell death simply as a result of the procedure, and we did not observe any additional apoptosis upon transfection of Daxx.

Our studies suggest that Pax5 and Daxx can exert considerable and opposite transcriptional regulation on a variety of potential target genes in B cell lines that express Pax5, although no specific targets have been identified. Changes in the relative levels of Pax5 during B cell differentiation and Daxx after stimulation may affect not only Pax5 target genes but also indirectly regulate genes corepressed by Daxx (30). One target for Pax5 activation is the CD19 gene (3, 45), and as mentioned under “Results,” high and low affinity Pax5 binding sites have been identified in the region 5′ to the CD19 promoter (45, 46). In experiments reported here, we used the reporter, pGL3(CD19-2), which contains multimulfared low affinity Pax5 binding sites from the CD19 promoter region together with the SV40 promoter (Fig. 7), and found that activity was dependent on full-length Pax5. Under these conditions, we observed that Daxx modulated Pax5's transcriptional activity. Nonetheless, preliminary experiments using the Δ71 reporter construct, which contains a high affinity Pax5 site located upstream of the c-fos promoter (46), gave different results. In our hands, Δ71's reporter activity in NIH3T3 cells, 293T cells, and HeLa was dependent only on the paired domain of Pax5. This observation is not surprising in light of reports of Pax5 target genes that are activated dependent only on the paired domain of Pax5 (5). Daxx had no significant effect on Pax5-stimulated transcription of Δ71, suggesting that genes regulated only by the paired domain of Pax5 will not be affected by Daxx. The involvement of Daxx in the regulation of Pax5 target genes may be clarified further with the development of transgenic models.

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