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E2A Expression, Nuclear Localization, and In Vivo Formation of DNA- and Non-DNA-Binding Species during B-Cell Development

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A monoclonal antibody (Yae) was characterized and shown to specifically recognize E2A proteins in vivo, including the E2A-Pbx1 fusion gene products, $p77^{E2A\text{-}Pbx1}$ and $p85^{E2A\text{-}Pbx1}$. E2A proteins of a predominant molecular mass of 72 kDa, which comigrated with in vitro-produced rat E12 and rat E47, were detected in human pro-B, pre-B, mature B, and plasma cell lines. The Yae antibody detected an E2A-containing μE2 enhancer element-binding complex (BCF-1) in pre-B- and mature B-cell lines in electrophoretic mobility shift assays which displayed a migration rate similar to that of in vitro-produced rat E12 and rat E47. A new E2A-containing μE2-binding species (P-E2A) was identified in plasma cells by using electrophoretic mobility shift assays. E2A proteins were detected in pro-B cells but were unable to bind the μE2 site. These observations suggest that the μE2 site is the target of stage-specific E2A regulatory complexes during B-cell development. Immunostaining analyses demonstrated the predominant nuclear localization of E2A proteins. Finally, we have identified an E2A form, designated I-E2A, which is unable to bind DNA. Our observations demonstrate novel in vivo mechanisms for the regulation of transcription by E2A proteins during B-cell development.

B lymphocytes play an important role in the humoral immune response. Mammalian B cells begin to form as early as the embryonic stage of development in both the liver and the spleen (14, 41). In adult mammals, B cells are derived from the multipotential stem cells of bone marrow (40, 47). Differentiation of B cells involves a complex set of events that leads to the generation of mature B cells. Immunoglobulin genes inherited by precursor B cells are in germ line configuration and must recombine in order to give rise to the diversity observed among immunoglobulins produced by different B cells. The recombination of immunoglobulin genes is a well-timed series of steps that begins with the rearrangement and then expression of the heavy-chain gene to give rise to pre-B cells, followed by light-chain gene rearrangement and subsequent expression of the light-chain gene to result in mature B cells (1, 7, 27, 53). The immunoglobulin heavy-chain (IgH) gene is transcribed at all stages of B-cell development, in contrast to the kappa light-chain (Ig κ) gene, which is efficiently transcribed only after rearrangement. The transcription rate of immunoglobulin genes also increases severalfold when mature B cells terminally differentiate to immunoglobulin-secreting plasma cells.

B-cell-specific enhancers which regulate the expression of the IgH and Ig κ genes have been identified (4, 6, 10, 13, 16, 30, 31, 38, 43, 45, 46). These enhancers are composed of multiple functional elements, including elements of the E-box (CANNTG) class (10, 13). There are five E boxes in the IgH enhancer (μE1 to μE5) and three in the Ig κ enhancer (κE1 to κE3). Mutations of any single E box significantly lower IgH and Ig κ gene expression (23, 25, 28, 42, 58). Multiple, unique cDNAs that encode proteins which bind the μE2, μE5, and κE2 sites have been isolated by expression cloning (15, 19, 20, 34, 37, 63). These E-box-binding proteins, which include E12, E47, ITF-1/E2-5, ITF-2/E2-2, and HEB, preferentially recognize the consensus sequence CA G/C/T C TG T/C, a subset of the E-box motif (20, 34, 37, 63). These proteins are all of the basic helix-loop-helix (bHLH) family and display extensive regions of conserved amino acids in their bHLH and DNA-binding domains (19, 20, 37). E12, E47, ITF-1/E2-5, ITF-2/E2-2, and HEB transcripts are all expressed in B cells (19, 20, 34). Three of these proteins, E12, E47, and ITF-1/E2-5, are the products of a single gene (E2A) and are derived by alternative RNA splicing events (19, 37, 55).

E12 and E47, which are encoded by partial cDNAs coding for peptides of 440 and 131 amino acids, respectively, result from an alternative splicing event which chooses between exons containing related bHLH motifs (34, 55). A cDNA designated E2A.E12, encoding a full-length E12 protein of 654 amino acids, termed E2Aα, has been isolated (21). The sequence of full-length E47 has not been reported but is presumed to encode a protein identical to E2Aα, with the exception of a 72-amino-acid region containing the bHLH motif in the C terminus. ITF-1/E2-5 is identical to full-length E47 starting at amino acid 101 of full-length E47, the result of a proposed additional alternative splicing event of E2A RNA which results in a different N terminus (19). cDNA sequences which encode full-length ITF-1 have not been reported; therefore, the extent of the N-terminal differences with E47 are not known. E2Aα, full-length E47, and ITF-1 are collectively known as E2A proteins. Rat cDNAs encoding full-length rat E12 (rE12; known as Pan-2) and all but the N-terminal 9 amino acids of rat E47 (rE47; also known as Pan-1) which encode predicted full-length proteins of 649 and 647 amino acids, respectively, have been isolated (37). The predicted rE12 and rE47 proteins are identical with the exception of a 72-amino-acid divergent region in the C terminus and a single amino acid insertion at amino acid 168 of rE47, which may be due to a splice stutter (37).

The bHLH motif is a protein dimerization motif. In addition to the bHLH dimerization region, E2A proteins contain a putative leucine zipper dimerization motif (34, 37). bHLH proteins are proposed to dimerize with other mem-

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bers of the bHLH family to form cell-specific transcriptional regulatory complexes. In support of this model, in vitro-produced E12, E47, HEB, and ITF-2 form heterodimers with in vitro-produced MyoD and myogenin, transcription factors that play a role in muscle cell differentiation (12, 20, 24, 35, 62). In addition, studies using polyclonal antisera generated against E12 fusion proteins and MyoD for immunoprecipitation assays have reported that MyoD forms heterodimers with E12/E47-like proteins in vivo (24). The sizes of proteins immunoprecipitated in these studies by the E12 antisera are different from the predicted molecular masses of full-length E12 and E47, and the immunoprecipitated proteins may be other bHLH proteins. In support of this explanation, the anti-E12 antiserum also recognizes ITF-2, the product of a different gene (24). However, the molecular masses of E2Aα, full-length E47, and ITF-1 present in vivo are not known, and the antiserum may have immunoprecipitated alternative forms of E2A proteins.

Although E2A transcripts have been shown to be expressed in different cell lineages (22, 34, 37, 63), the distribution and form of E2A proteins have not been defined. Northern (RNA) blot analyses performed on rat and mouse tissues detected ubiquitously expressed 3-kb E2A transcripts (37, 63). However, other studies conducted with human cell lines have indicated a heterogeneously sized population of E2A mRNAs (22, 34).

In this report, we have characterized a new monoclonal antibody, Yae, generated against rE12, to study the role of E2A proteins in B-cell development, by examining their expression and ability to bind the IgH enhancer. We demonstrate the presence of E2A proteins in stage-specific complexes which correlate with the expression of the IgH gene during B-cell development. We present evidence that E2A proteins are present in both DNA and non-DNA-binding forms in vivo, forming a novel mechanism for the regulation of transcription by E2A proteins.

MATERIALS AND METHODS

Whole-cell, nuclear, and cytoplasmic lysates (Western blots [immunoblots]). Log-phase cultured cells were centrifuged for 10 min at 600 × *g* to pellet cells, and the supernatant was aspirated. The cell pellet was then resuspended in fresh RPMI 1640 medium, and cells were counted with a hemocytometer. Approximately 2×10^6 cells were then centrifuged again as described above, and the medium was aspirated. The pelleted cells were used to generate either whole-cell, nuclear, or cytoplasmic lysates. The whole-cell lysate was obtained by immediately lysing the cell pellet at 95°C for 5 min in 1 ml of sodium dodecyl sulfate (SDS) lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. The nuclear and cytoplasmic fraction lysates were generated as follows. Approximately 10^6 pelleted cells were resuspended and incubated in 250 μl of hypotonic lysis buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg of pepstatin A per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) for 5 min on ice. Then Nonidet P-40 was added to a final concentration of 0.1 to 0.2% and mixed gently by inversion to lyse the cells. Complete cell lysis was monitored by phase-contrast light microscopy. The nuclei were pelleted by centrifugation at 5,000 × *g* for 4 min at 4°C. The supernatant was removed and centrifuged at 10,000 × *g* for 10 min at 4°C to remove any nuclei remaining in the supernatant. The supernatant was then mixed with 250 μl of SDS lysis buffer and heated at 95°C for 5 min. This crude lysate was stored as the cytoplasmic fraction. The pelleted nuclei were resuspended in 500 μl of lysis buffer and heated at 95°C for 5 min. This crude lysate was stored as the nuclear fraction. After heating, all lysates were sonicated for 10 s in a Sonifier cell disruptor (Branson Sonic Power Co.) with settings of 50% duty cycle and continuous pulse to shear chromosomal DNA. For rapid and conventional fractionations, the method was similar to the foregoing procedure except that 100 μl of the buffer A containing 20% glycerol, 100 mM KCl, and 0.1 mM EDTA was used to lyse the cells. The nuclei were centrifuged at 1,000 × *g* for 30 s in the rapid method and 5,000 × *g* for 4 min for the conventional method. Both nuclear and cytoplasmic fractions were resuspended in equal volumes (500 μl) of SDS lysis buffer, boiled, and sonicated as described above. All lysates were stored at −80°C until use.

Nuclear and cytoplasmic extracts (electrophoretic mobility shift assay [EMSA]). Cultured cells in log phase were centrifuged, resuspended in fresh medium, and counted as described above. Approximately 10^6 cells were centrifuged as described above, and the medium was aspirated. Cells were then washed once with 1 ml of phosphate-buffered saline (PBS; pH 7.5) lacking magnesium and calcium. The cells were resuspended and incubated in 250 μl of hypotonic lysis buffer A and lysed with Nonidet P-40, and nuclei were pelleted as for whole-cell, nuclear, and cytoplasmic lysates (see above). The supernatant was centrifuged at 10,000 × *g* to remove remaining nuclei. The supernatant was removed and adjusted to 75 mM (final concentration) NaCl–20% (vol/vol) (final concentration) glycerol and stored as the cytoplasmic fraction. The nuclei were resuspended and incubated with gentle mixing in 250 μl of extraction buffer B (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg of pepstatin A per ml, 1 μg of leupetin per ml, 1 μg of aprotinin per ml) for 20 min at 4°C. The salt-extracted mixture was then centrifuged at 10,000 × *g* for 15 min at 4°C to remove the nuclei and particulate matter. Glycerol was added to the supernatant to a final concentration of 20% (vol/vol). All extracts were stored at −80°C until use.

In vitro transcription and translation. RNAs coding for full-length rE12 and full-length rE47 were generated by in vitro transcription of linearized plasmids PAR5 (rE12) and PAR5/2 (rE47). In vitro transcription was performed by using T7 RNA polymerase to synthesize rE12 and rE47 RNAs as instructed by the manufacturer (Promega). Radiolabeled full-length rE12 and rE47 were synthesized by translation of their RNA templates in rabbit reticulocyte lysate in the presence of [^{35}S]methionine as instructed by the manufacturer (Promega). Truncations of rE47 were generated by digestion of PAR5/2 with either *Hae*II or *Pst*I prior to in vitro transcription. *Hae*II and *Pst*I restriction enzyme digestions generated cDNA templates which give rise to predicted N-terminal truncation products of 208 and 163 amino acids, respectively (37).

Antibody purification. Yae monoclonal antibody secreted by the 3C6 hybridoma cell line into tissue culture medium was purified by protein A-Sepharose affinity chromatography. Approximately 50 ml of 3C6 tissue culture medium was centrifuged at 600 × *g* for 10 min at 4°C to pellet the cells, and the supernatant was removed and centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was then applied to a 2-ml protein A-Sepharose (Pharmacia) column, and the flowthrough was reapplied to the column. Then 20 ml of 10 mM Tris-HCl (pH 7.5) was added to the column to wash off

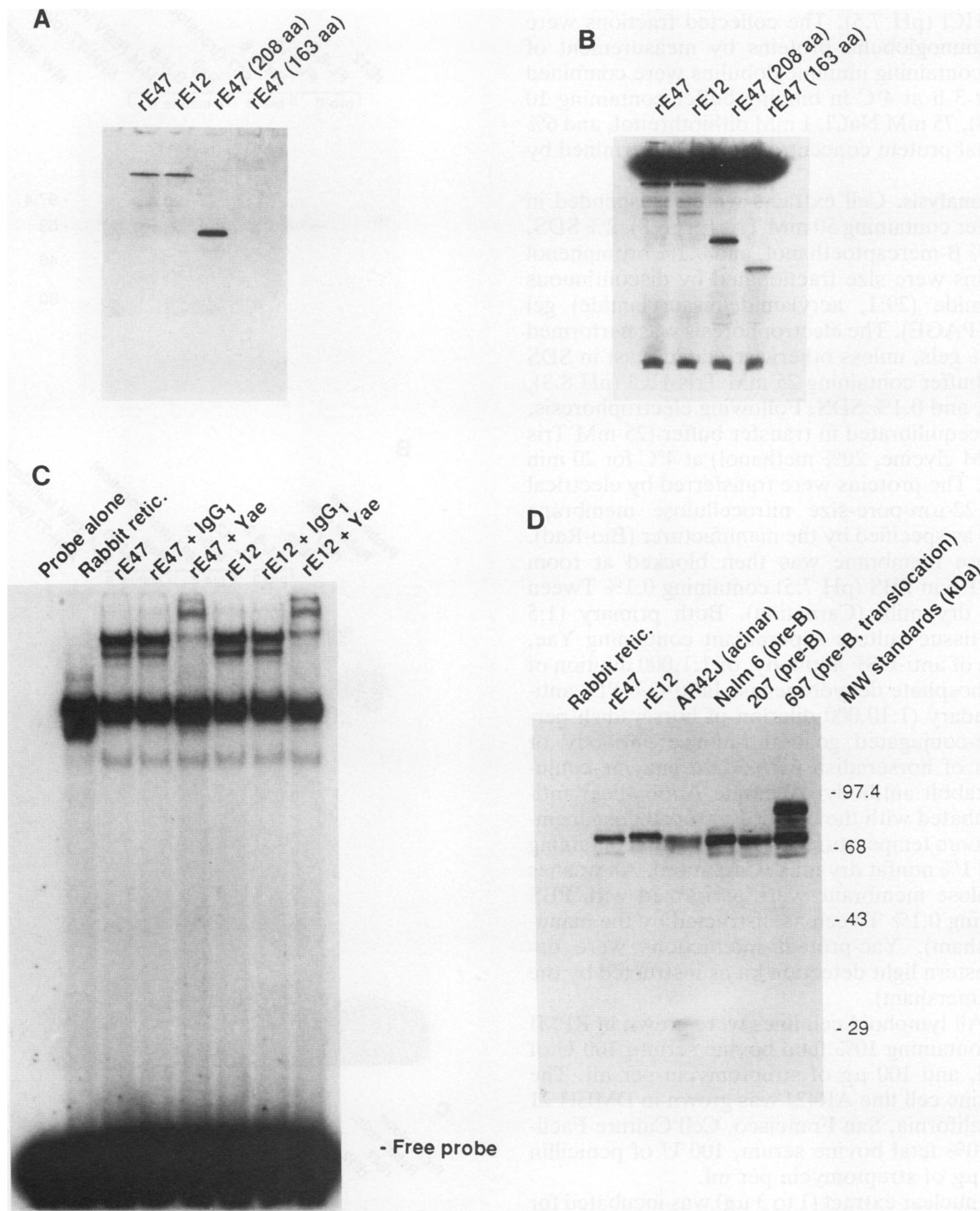


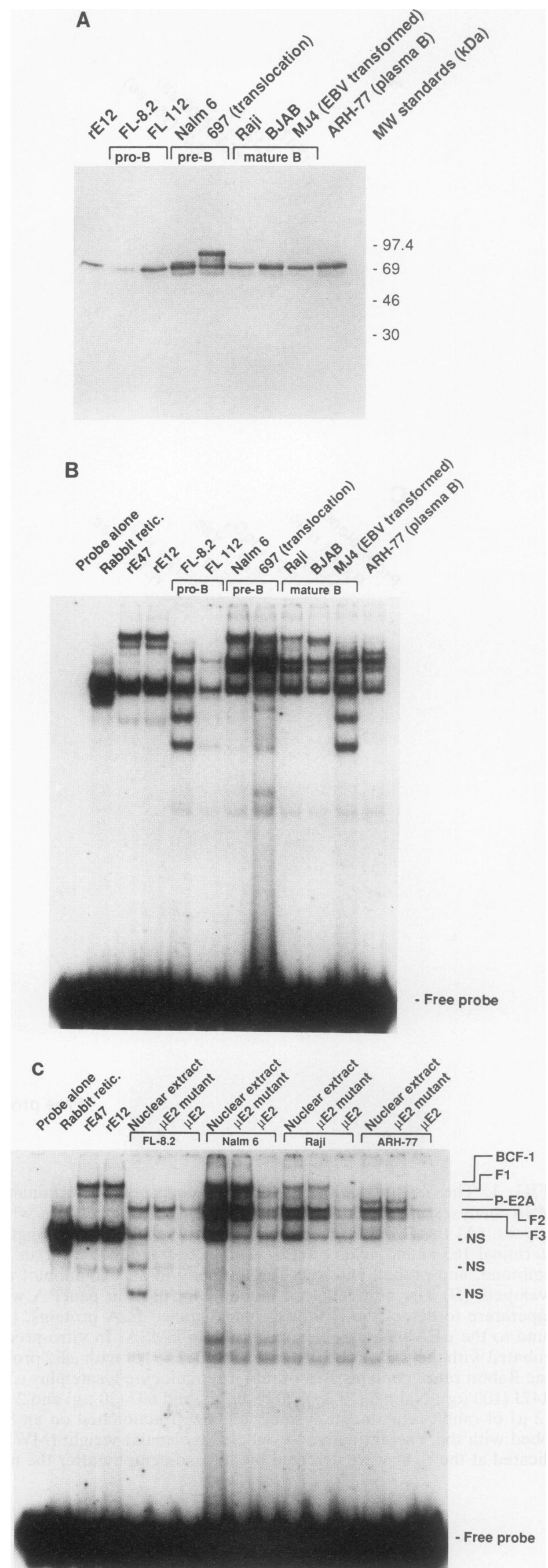
FIG. 1. The Yae monoclonal antibody recognizes a determinant present in both in vitro-produced rE12 and rE47 in Western blot and EMSA analyses and detects a predominant 72-kDa species in whole-cell lysates prepared from rat and human cell lines in immunoblot analyses. (A) In vitro-produced, [^{35}S]methionine-labeled full-length rE12, full-length rE47, N-terminal 208 amino acids (aa) of rE47, and N-terminal 163 amino acids of rE47 were size fractionated on an SDS–14% denaturing polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the Yae antibody in an immunoblot analysis. The blot was exposed to X-ray film for approximately 2 s and developed. (B) The nitrocellulose immunoblot used in panel A was dried overnight at room temperature and exposed for 24 h at room temperature to detect the [^{35}S]methionine-labeled E2A proteins. (C) The Yae antibody recognizes both in vitro-produced rE12 and rE47 bound to the μE2 enhancer element probe in EMSA. In vitro-produced full-length rE12 and rE47 in rabbit reticulocyte lysate (1 μl) were incubated with the μE2 probe (lanes rE12 and rE47) or with μE2 probe plus 1 μg of IgG1 control monoclonal antibody or 1 μg of Yae antibody. Lane Rabbit retic. contains 1 μl of rabbit reticulocyte lysate plus μE2 probe as a control. (D) Whole-cell lysates prepared from the cell lines AR42J (100 μg), Nalm 6 (20 μg), 207 (40 μg), and 697 (30 μg) and 2 μl of rabbit reticulocyte lysate containing in vitro-produced rE12 or rE47 or 2 μl of rabbit reticulocyte lysate were size fractionated on an SDS–10% denaturing gel, transferred to a nitrocellulose membrane, and probed with the Yae antibody. ^{14}C-labeled molecular weight (MW) markers of 97.4, 68, 43, and 29 kDa (Bethesda Research Laboratories) indicated at the right were detected by autoradiography after the nitrocellulose membrane was dried overnight at room temperature.

unbound proteins. The antibodies were eluted with sequential 1-ml volumes of 100 mM glycine (pH 3.0). Approximately 1-ml fractions were collected in tubes containing 100 μl of 1 M Tris-HCl (pH 7.5). The collected fractions were assayed for immunoglobulin proteins by measurement of A_{280}. Fractions containing immunoglobulins were combined and dialyzed for 3 h at 4°C in binding buffer containing 10 mM Tris (pH 7.5), 75 mM NaCl, 1 mM dithiothreitol, and 6% glycerol. The final protein concentration was determined by A_{280}.

Western blot analysis. Cell extracts were resuspended in SDS sample buffer containing 50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromphenol blue. The proteins were size fractionated by discontinuous SDS-polyacrylamide (29:1, acrylamide/bisacrylamide) gel electrophoresis (PAGE). The electrophoresis was performed at 3 V/cm in 10% gels, unless otherwise stated, cast in SDS electrophoresis buffer containing 25 mM Tris-HCl (pH 8.3), 250 mM glycine, and 0.1% SDS. Following electrophoresis, the gels were preequilibrated in transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 20% methanol) at 4°C for 20 min prior to transfer. The proteins were transferred by electrical field onto a 0.22-μm-pore-size nitrocellulose membrane (Trans-Blot cell) as specified by the manufacturer (Bio-Rad). The nitrocellulose membrane was then blocked at room temperature for 1 h in PBS (pH 7.5) containing 0.1% Tween and 5% nonfat dry milk (Carnation). Both primary (1:5 dilution of 3C6 tissue culture supernatant containing Yae, 1:25,000 dilution of anti-USF antibody, or 1:1,000 dilution of anti-glucose-6-phosphate dehydrogenase [anti-G-6-PD] antibody) and secondary (1:10,000 dilution of horseradish peroxidase enzyme-conjugated goat anti-mouse antibody or 1:10,000 dilution of horseradish peroxidase enzyme-conjugated goat anti-rabbit antibody; Accurate Antibodies) antibodies were incubated with the blocked nitrocellulose membrane for 1 h at room temperature in PBS (pH 7.5) containing 0.1% Tween and 1% nonfat dry milk (Carnation). All washes of the nitrocellulose membrane were performed with PBS (pH 7.5) containing 0.1% Tween as instructed by the manufacturer (Amersham). Yae-protein interactions were detected with a Western light detection kit as instructed by the manufacturer (Amersham).

Cell cultures. All lymphoid cell lines were grown in RPMI 1640 (GIBCO) containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The pancreatic exocrine cell line AR42J was grown in DMEH 21 (University of California, San Francisco, Cell Culture Facility) containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

EMSA. Crude nuclear extract (1 to 3 μg) was incubated for 15 min on ice with 15 fmol (10^5 cpm) of ^{32}P-5′-end-labeled DNA probe in a 15-μl final reaction volume containing 10 mM Tris-HCl (pH 7.5), 5 to 15 μg of poly(dI-dC), 75 mM NaCl, 1 mM dithiothreitol, 6% glycerol, and 20 μg (final concentration) of protein adjusted with bovine serum albumin (BSA) fraction V. In vitro-synthesized rE12 and rE47 were incubated with the probe as described above except that 20 to 100 ng of poly(dI-dC) was used. Competition assays with a 30-fold molar excess of either wild-type or mutant unlabeled DNA oligonucleotide were carried out by incubation for 10 min on ice with the crude nuclear extract prior to addition of the labeled probe. Antibodies were incubated with the crude nuclear extract or rabbit reticulocyte lysate for 20 min on ice, and the final protein concentration was adjusted to 20 μg with BSA prior to addition of the labeled probe. The reaction mixture was loaded onto a



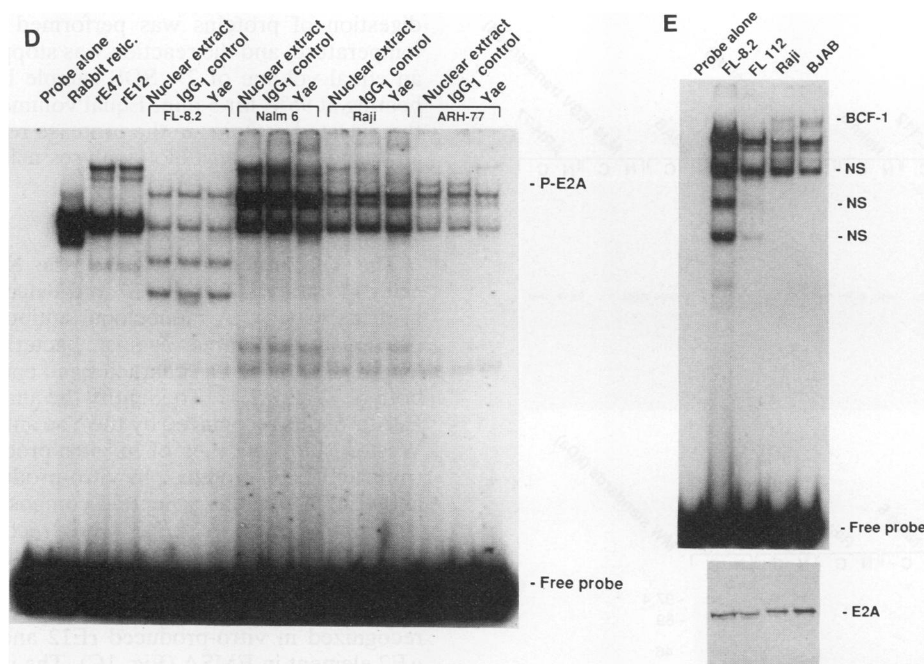


FIG. 2. E2A proteins are present in all stages of B-cell development investigated, and E2A-containing species present in human pre-B, mature B, and plasma cell lines specifically bind the μE2 enhancer element in EMSA. In all panels, lanes Rabbit retic. contain 1 μl of rabbit reticulocyte lysate and lanes rE12 and rE47 hold 1 μl of rabbit reticulocyte lysate containing in vitro-produced full-length rE12 and rE47, respectively. (A) Immunoblot analysis, using the Yae antibody, of whole-cell lysates containing the stated amount of protein prepared from human cell lines were loaded in lanes FL-8.2 (150 μg), FL112 (150 μg), Nalm 6 (30 μg), 697 (30 μg), Raji (60 μg), BJAB (60 μg), MJ4 (60 μg), and ARH-77 (60 μg). Molecular weight (MW) standards are indicated on the right. (B) Two micrograms of FL-8.2, FL112, or ARH-77 nuclear extract or 1 μg of all other nuclear extracts was incubated with the μE2 probe and analyzed by EMSA. The cell line source of nuclear extract is designated above each lane. (C) Binding competition experiments were performed in EMSA with a 30-fold molar excess of wild-type or mutant μE2 site, using binding conditions identical to those for panel A for the cell lines FL-8.2, Nalm 6, Raji, and ARH-77. The content of each lane is designated at the top. NS, nonspecific complex. (D) The Yae antibody detects E2A-containing μE2-bound species present in nuclear extracts prepared from pre-B, mature, and plasma cell lines. One microgram of the Yae antibody or 1 μg of a control IgG1 subclass monoclonal antibody was incubated in the binding reaction conditions used for panel A, using FL-8.2, Nalm 6, Raji, and ARH-77 nuclear extracts. Lanes Nuclear extract contain nuclear lysate from the designated cell line and μE2 probe but no antibody. (E) Similar amounts of E2A proteins in nuclear extracts of FL-8.2 (100 μg), FL112 (100 μg), Raji (10 μg), and BJAB (10 μg) cells detected by the Yae antibody in Western blot analysis (bottom) were used in EMSA (top) to detect μE2-binding species. The content of each lane is indicated at the top.

6% nondenaturing polyacrylamide (29:1, acrylamide/bisacrylamide) gel cast in 0.25× Tris-borate-EDTA (TBE) electrophoresis buffer containing 225 mM Tris-borate and 0.5 mM EDTA at pH 8.3. Electrophoresis was carried out in 0.25× TBE electrophoresis buffer at 13 V/cm for 75 min at room temperature. The μE2 probe used in all EMSA assays consists of the oligonucleotide sequence 5′CCCACAGCAG GTGGCAGGAA3′; the mutant μE2 consists of the sequence 5′CCCACAGTCTCCAACAGGAA3′. All probes used were blunt-ended double-stranded oligonucleotides.

Immunofluorescence. Cells attached to poly-L-lysine-coated microscope slide coverslips were fixed with 3% paraformaldehyde at room temperature for 15 min, washed, and blocked with PBS containing 1% BSA for 10 min at room temperature. The fixed cells were then permeabilized with 100% methanol for 5 min at −20°C. Permeabilized cells were incubated with primary antibody (1:40 dilution of mouse ascites fluid containing Yae) for 20 min at room temperature. Excess primary antibody was washed, and cells were incubated with secondary antibodies (1:80 dilution of fluorescein isothiocyanate-labeled goat anti-mouse antibody; Southern Biotechnology) for 20 min at room temperature. Cells were washed for 10 min in PBS containing 1% BSA for 10 min at room temperature, and coverslips were mounted on microscope slides, using gel mount (Biomeda) containing 1 mg of antifade reagent (*p*-phenylenediamine) per ml. Photographs were taken with a 100× oil objective.

Liquid chromatography. Nuclear and cytoplasmic extracts prepared from Raji cells were resuspended in buffer C (20 mM HEPES [pH 7.9], 100 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40). Nuclear and cytoplasmic extracts containing equal amounts of E2A proteins as determined by Western blot analysis using the Yae antibody were applied to a salmon sperm DNA-Sepharose column as follows. Two milliliters of nuclear extract (containing 2 mg of protein) and 2 ml of cytoplasmic extract (containing 10 mg of protein) were loaded onto 1-ml salmon sperm DNA-Sepharose columns twice and allowed to bind. The loaded columns were washed with 2 ml of buffer C. Proteins were eluted off the columns by using a 2-ml step gradient of increasing NaCl salt concentrations of 0.2, 0.3, 0.4, 0.5, and 1.0 M in buffer C. All procedures were performed at 4°C and at a flow rate of 0.1 ml/min. The collected fractions were stored at −80°C until use.

Protease digestion. Similar amounts of E2A proteins in the nuclear and cytoplasmic extracts derived from Raji cells were resuspended in equal volumes of buffer C and incu-

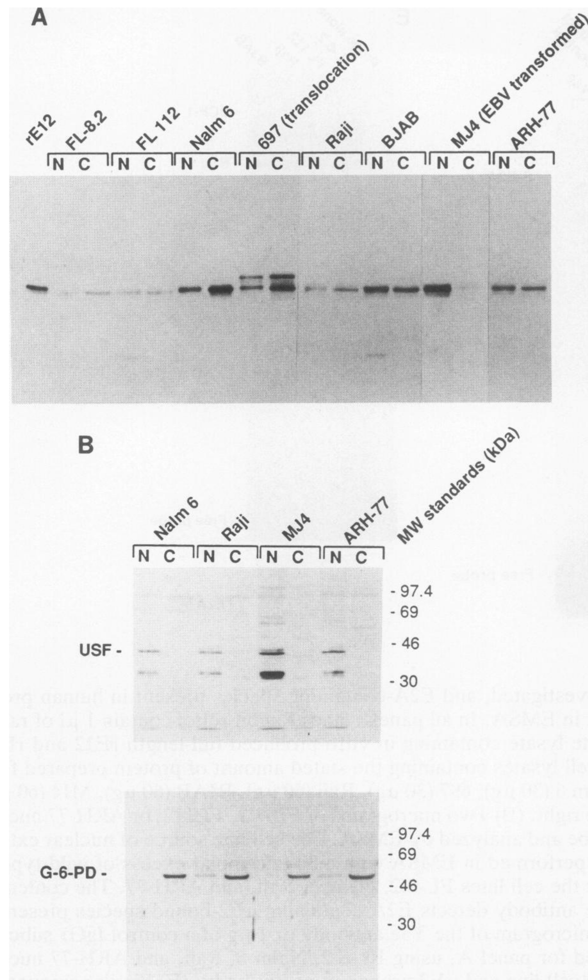


FIG. 3. E2A proteins of a predominant 72-kDa molecular mass are detected in nuclear and cytoplasmic lysates prepared from human pro-B, pre-B, mature B, and plasma cell lines in Western blot analyses. Proteins were size fractionated on SDS–10% denaturing polyacrylamide gels, transferred to nitrocellulose membranes, and probed for the presence of E2A proteins with the Yae monoclonal antibody. (A) Equal volumes (10 μl) of boiled nuclear (N) or cytoplasmic (C) fractions prepared from human cell lines and containing the indicated amount of protein were analyzed in lanes N FL-8.2 (10 μl; 39 μg), C FL-8.2 (10 μl; 56 μg), N FL112 (10 μl; 43 μg), C FL112 (10 μl; 64 μg), N Nalm 6 (10 μl; 12 μg), C Nalm 6 (10 μl; 19 μg), N 697 (10 μl; 14 μg), C 697 (10 μl; 26 μg), N Raji (10 μl; 23 μg), C Raji (10 μl; 30 μg), N BJAB (10 μl; 27 μg), C BJAB (10 μl; 33 μg), N MJ4 (10 μl; 37 μg), C MJ4 (10 μl; 60 μg), N ARH-77 (10 μl; 15 μg), and C ARH-77 (10 μl; 26 μg); 2 μl of rabbit reticulocyte lysate containing in vitro-produced rE12 was loaded in lane rE12. (B) Immunoblot analyses performed with antisera raised against a nuclear (USF transcription factor) and a cytoplasmic (G-6-PD) protein demonstrate the efficient separation of nuclear and cytoplasmic cellular components. The same amounts of nuclear and cytoplasmic fractions from Nalm 6, Raji, MJ4, and ARH-77 cells as used in panel A were analyzed in a Western blot assay using either an anti-USF (top) or an anti-G-6-PD (bottom) antiserum. Molecular weight (MW) standards (Amersham) are indicated at the right.

bated with *Staphylococcus aureus* V8 protease (Promega) at a protease/protein concentration of 1:100. The controls received equal volume of buffer C to make up for the added protease volume in the digestion reactions. The partial digestion of proteins was performed for 10 min at room temperature, and the reaction was stopped by the addition of an equal volume of 2× SDS sample buffer and immediate heating at 95°C for 5 min. Equal volumes of the controls and the digested proteins in the protease reaction mixtures were analyzed by Western blot analyses using the Yae antibody.

RESULTS

The Yae antibody recognizes the N-terminal 208 amino acids of both rE12 and rE47 and detects E2A and E2A-Pbx proteins in vivo. A monoclonal antibody (designated Yae) previously generated against bacterially expressed full-length rE12 (36a) was characterized and shown to recognize both rE12 and rE47. To identify the antigenic determinant of E2A proteins recognized by the Yae antibody, we performed Western blot analyses of in vitro-produced full-length and truncated E2A proteins. In vitro-produced full-length rE12 and rE47 as well as a peptide composed of the N-terminal 208 amino acids of rE47 were recognized by the Yae antibody (Fig. 1A and B). However, the Yae antibody was unable to detect a peptide composed of the N-terminal 163 amino acids of rE47 (Fig. 1A and B). The Yae antibody also recognized in vitro-produced rE12 and rE47 bound to the μE2 element in EMSA (Fig. 1C). The interaction of the Yae monoclonal antibody with μE2-bound rE12 or rE47 resulted in a slower-migrating or supershifted species (Fig. 1C).

The ability of the Yae antibody to specifically recognize E2A proteins in vivo was demonstrated by Western blot analyses of whole-cell lysates derived from rat and human cell lines. A predominant species of 72 kDa which comigrated with in vitro-produced rE12 and rE47 was detected by the Yae antibody in lysates prepared from the rat AR42J cell line, from which the cDNAs encoding rE12 and rE47 were isolated, and two pre-B-cell lines, Nalm 6 and 207 (Fig. 1D). Two minor species of 63 and 77 kDa were also observed. The 72-kDa species corresponds closely to the predicted molecular masses of rE12 (67.7 kDa) and rE47 (67.4 kDa), based on cDNA analysis (37). Moreover, no proteins were detected in control rabbit reticulocyte lysate.

To further characterize and confirm the specificity of the Yae antibody for E2A proteins, we performed Western blot analysis of whole-cell lysate prepared from the pre-B acute lymphoblastic leukemia (ALL) cell line 697 (Fig. 1D). The 697 cell line bears a t(1;19)(q23;p13.3) chromosomal translocation which joins *E2A* sequences with those of a second gene, *pbx1* (22, 29, 39). This translocation results in two major E2A-Pbx1 fusion proteins of 77 kDa ($p77^{E2A\text{-}Pbx1}$) and 85 kDa ($p85^{E2A\text{-}Pbx1}$) (21). These fusion proteins contain the N-terminal 483 amino acids of E2A proteins and would be expected to carry the determinant recognized by the Yae monoclonal antibody if the epitope is conserved between rat and human proteins. Because one native chromosomal *E2A* gene is retained in the diploid cell type, native E2A proteins as well as the E2A-Pbx1 fusion proteins should be present in 697 cells. As predicted, the Yae antibody detects the 72-kDa species as well as 80- and 87-kDa species in 697 whole-cell lysate (Fig. 1D). In addition, the 80- and 87-kDa species were not detected in whole-cell lysates prepared from Nalm 6 and 207 pre-B ALL cell lines, which do not carry the t(1;19)(q23; p13.3) chromosomal translocation (Fig. 1D). These results demonstrate the high specificity of the Yae recognition of E2A proteins.

The Yae antibody detects E2A-containing μE2 element-binding complexes in all stages of B-cell differentiation with the exception of pro-B cells. To study the expression and

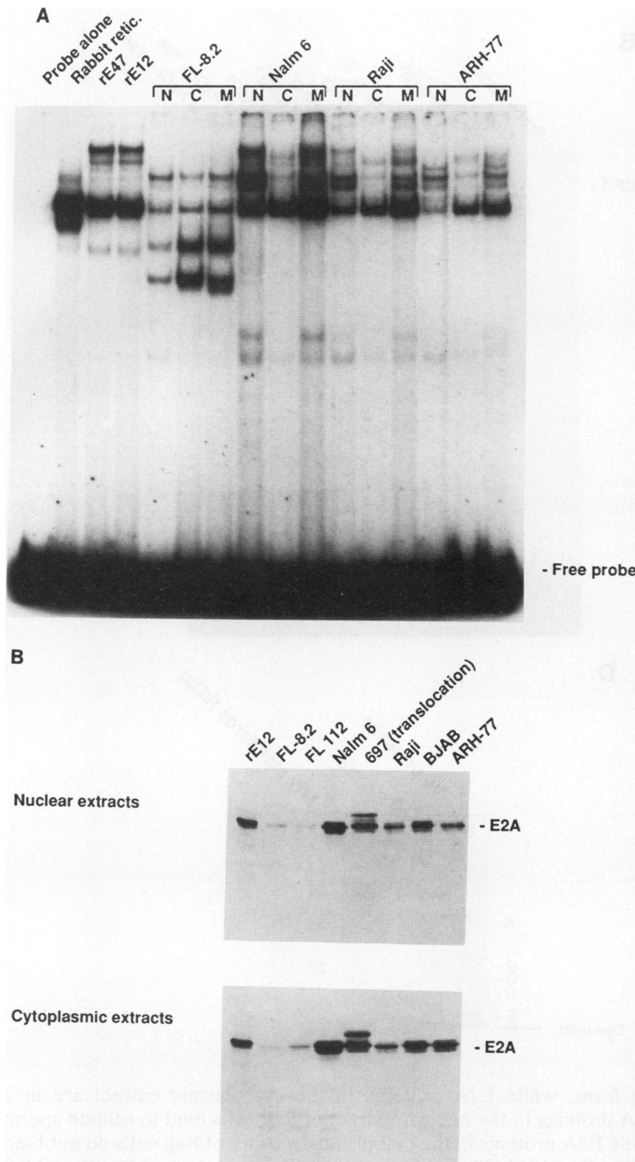


FIG. 4. Cytoplasmic E2A species do not bind the μE2 site in EMSA. (A) Nuclear and cytoplasmic extracts prepared from the FL-8.2, Nalm 6, Raji, and ARH-77 cell lines were analyzed by EMSA for μE2-binding species. Two micrograms of nuclear (lanes N) or 10 μg of cytoplasmic extract (lanes C) of FL-8.2 and ARH-77 cells was analyzed; 1 μg of nuclear (lanes N) or 5 μg of cytoplasmic extract (lanes C) of Nalm 6 and Raji cells was used. Lanes M contain the same amount of nuclear and cytoplasmic extracts present in both lanes N and C mixed together. Lane Rabbit retic. contains 1 μl of rabbit reticulocyte lysate. Lanes rE12 and rE47 have 1 μl of rabbit reticulocyte lysate containing in vitro-produced rE12 and rE47, respectively. Similar amounts of E2A proteins are present in the amounts of nuclear and cytoplasmic extracts used in binding reactions, as determined by Western blot assay (B). Nuclear extracts used in EMSA binding reactions were prepared by 0.4 M NaCl extraction of isolated nuclei as detailed in Materials and Methods. This results in the enrichment of E2A proteins on a total protein basis, requiring higher amounts of cytoplasmic extract to be added to binding reactions to achieve similar amounts of E2A proteins. (B) Nuclear (top) and cytoplasmic (bottom) extracts used in EMSA contain intact E2A proteins. Twenty micrograms of nuclear or 100 μg of cytoplasmic lysates was examined in immunoblots using the Yae antibody to test for E2A protein degradation and establish the relative abundance of E2A proteins used in EMSA. Western blot analyses demonstrate no significant proteolytic degradation of E2A proteins in nuclear extracts (top) or in pre-B, mature B, and plasma cytoplasmic lysates (bottom) used in EMSA. Some apparent E2A degradation was observed in pro-B cytoplasmic extracts, although a substantial portion of pro-B cytoplasmic E2A proteins remained intact.

forms of E2A proteins during B-cell differentiation, we examined lysates prepared from pro-B, pre-B, mature B, and plasma cell lines for the presence of E2A proteins. In all cases, at least two independently derived cell lines representing each stage of B-cell development were analyzed for the presence and abundance of E2A proteins by Western blot analysis using the Yae antibody. E2A proteins which comigrated with in vitro-produced rE12 were detected in whole-cell lysates prepared from all stages of B-cell differentiation examined (Fig. 2A).

Nuclear extracts prepared from different stages of B-cell development were probed with the Yae antibody in EMSA to identify E2A species which bind the μE2 site. Multiple μE2-bound species were observed during all stages of B-cell differentiation (Fig. 2B). Binding competition experiments performed with a 30-fold molar excess of unlabeled wild-type or mutant μE2 site demonstrated the presence of μE2-specific and nonspecific binding species (Fig. 2C). μE2-binding species containing E2A proteins were identified by the interaction of specific species with the Yae antibody in EMSA. E2A-containing μE2-binding species were observed in pre-B, mature B, and plasma cells but not in pro-B cells (Fig. 2D). The E2A-containing species identified in both pre-B and mature B cells is likely to be the previously reported BCF-1 factor (36). BCF-1 was stated to contain E12/E47-like proteins, based on immunoreactivity of the BCF-1 species with a polyclonal antisera generated against E12 fusion protein. Here, we demonstrate that the BCF-1 species contains E2A proteins. A newly identified E2A-containing μE2-binding species (designated P-E2A, for plasma E2A) which migrates faster than the BCF-1 species was detected in the plasma cell line ARH-77 (Fig. 2C and D). The BCF-1 species greatly decreased in abundance in the plasma cell line coincident with the appearance of P-E2A, suggesting a developmental switch of E2A-containing complexes. A species which comigrates with P-E2A is present in the Epstein-Barr virus (EBV)-transformed mature B-cell line MJ4 (Fig. 2B) and the CESS cell line (data not shown) but not in two other mature B-cell lines, Raji and BJAB. BCF-1 is also greatly reduced in MJ4 and CESS cells. Three μE2-binding species of similar mobility, designated factor 1 (F1), factor 2 (F2), and factor 3 (F3) (Fig. 2C), that were not recognized by the Yae antibody in EMSA were present during all stages of B-cell development (Fig. 2D).

No Yae-reactive μE2-binding species were detected in nuclear extracts of pro-B cells in EMSA (Fig. 2D), although E2A proteins were present in both FL-8.2 and FL112 pro-B-cell nuclear extracts (see Fig. 4B, top). Because E2A proteins are of a relatively low abundance in pro-B cells compared with later stages of B-cell development, the failure to detect E2A-containing binding species may have been due to a low abundance of E2A proteins. To test this premise, the amounts of FL-8.2 and FL112 pro-B nuclear extracts used for EMSA binding studies were increased and adjusted to contain E2A proteins similar to the amounts of E2A proteins present in Raji and BJAB nuclear extracts, which produced easily observable BCF-1 species in EMSA (Fig.

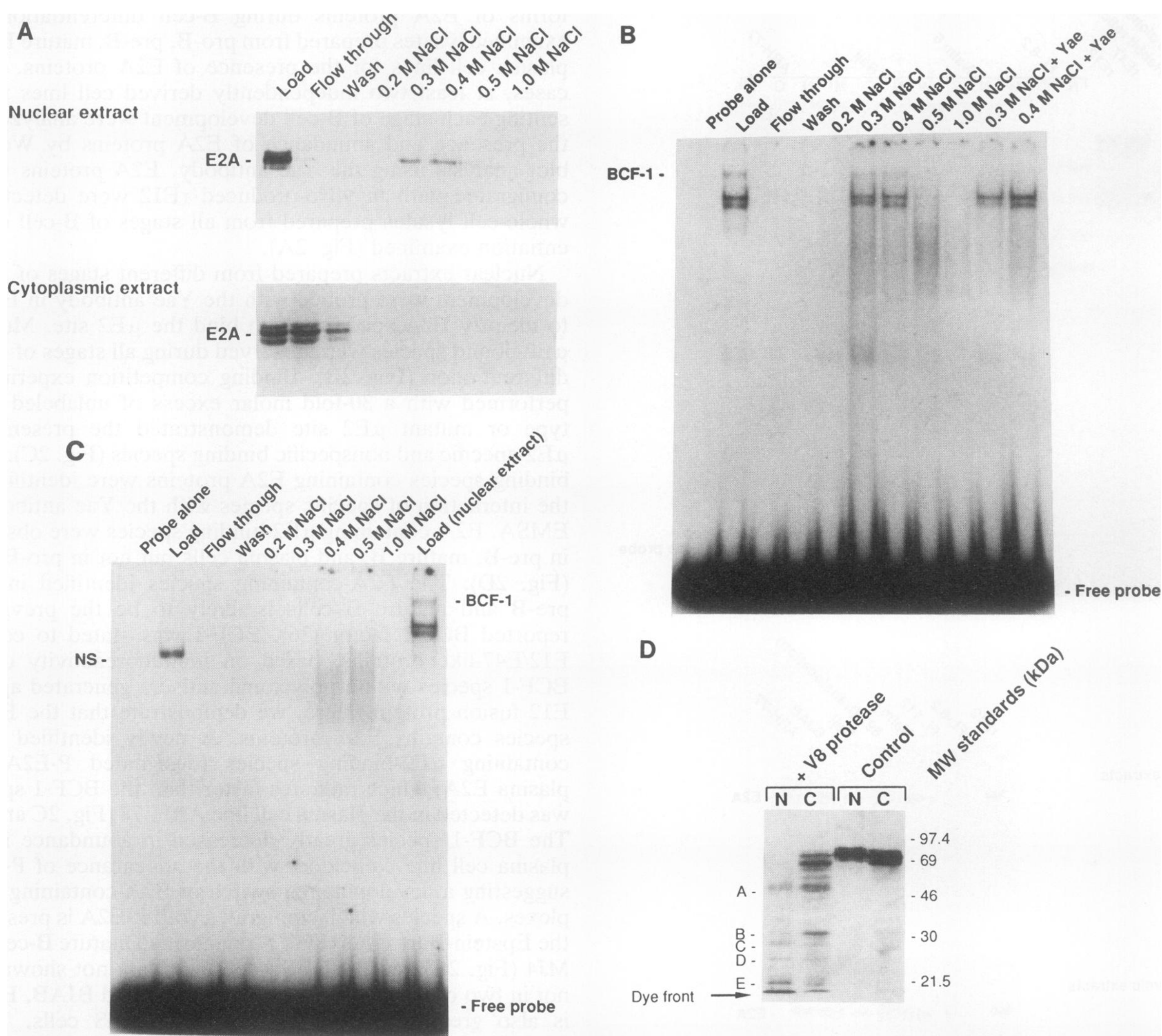


FIG. 5. E2A proteins in the nuclear extract are in a DNA-binding form, while E2A proteins in the cytoplasmic extract are in a non-DNA-binding form. (A) Western blot analysis demonstrating that E2A proteins in the nuclear extract of Raji cells bind to salmon sperm DNA-Sepharose column and elute at 0.3, 0.4, and 0.5 M salt fractions, while E2A proteins in the cytoplasmic extract of Raji cells do not bind and are detected in the flowthrough and wash fractions. Equal volumes (20 μl) of load (20 μg), flowthrough (2.8 μg), wash (2.3 μg), and 0.2 (2.6 μg), 0.3 (2.4 μg), 0.4 (2.3 μg), 0.5 (2.8 μg), and 1.0 (2.4 μg) M salt eluates from nuclear extract (top) and equal volumes (20 μl) of load (100 μg), flowthrough (71.2 μg), wash (12.2 μg), and 0.2 (3.2 μg), 0.3 (2.4 μg), 0.4 (2.2 μg), 0.5 (2.3 μg), 1.0 (2.0 μg) M salt eluates from cytoplasmic extract (bottom) collected from the salmon sperm DNA-Sepharose column were tested for the presence of E2A proteins in Western blot analyses using the Yae antibody. The lanes containing various fractions are indicated at the top. (B) Equal volumes (1 μl) of the same fractions used in panel A (top) were tested for μE2-binding activity in EMSA. One-microgram amounts of Yae interacted with BCF-1 species present in the 0.3 and 0.4 M salt fractions are shown in the far right lanes. (C) EMSA analysis using the μE2 element in volumes (1 μl) equal to those of the same fractions used in panel A (bottom). The far right lane contains 1 μl of load nuclear extract used in panel B. NS, nonspecific complex. (D) Partial *S. aureus* V8 protease digestion of Raji nuclear and cytoplasmic protein extracts resulted in peptides (labeled A to E) similar in size, as detected by the Yae antibody in Western blot analysis. Twenty micrograms of V8 protease-digested or control nuclear extract and 100 μg of V8 protease-digested or control cytoplasmic extract was used. The content of each lane is indicated at the top. Molecular weight (MW) standards are indicated at the right.

2E). However, no additional μE2-binding species were observed in pro-B nuclear extracts with use of these increased amounts of nuclear extracts (Fig. 2E).

E2A proteins are detected in the nuclear and cytoplasmic fractions of cell lysates prepared from all investigated stages of B-cell development. To examine the subcellular distribution of E2A proteins in B cells, we performed immunoblot analyses of nuclear and cytoplasmic fractions of B-cell lysates (Fig. 3A). Similar amounts of E2A proteins were detected in equal volumes (also containing similar amounts of protein) of the prepared nuclear and cytoplasmic fractions of two pro-B-cell lines (FL-8.2 and FL112) which were derived from fetal liver (59, 60), two mature B-cell lines (Raji and BJAB), the plasma cell line (ARH-77) (Fig. 3A), and the CESS cell line (data not shown). However, E2A proteins were more abundant in the cytoplasmic fraction than in the nuclear fraction of the two pre-B-cell lines (Nalm 6 and 697) (Fig. 3A). In contrast, E2A proteins were predominantly

present in the nuclear fraction prepared from MJ4 (Fig. 3A), which is a lymphoblastoid cell line derived from mature B cells by EBV transformation (61). An analysis of the distribution of E2A and E2A fusion proteins, $p77^{E2A\text{-}Pbx1}$ and $p85^{E2A\text{-}Pbx1}$, in the pre-B ALL cell line 697 revealed similar proportions of both E2A and E2A fusion proteins in the cytoplasmic and nuclear fractions (Fig. 3A).

Antisera specific to the nuclear protein marker, USF transcription factor, and the cytoplasmic protein marker, G-6-PD, were used in Western blot analyses to confirm efficient separation of nuclear and cytoplasmic fractions (44, 52, 54, 65). The USF transcription factor is also a member of the bHLH family, possesses a leucine zipper motif, and binds E-box sequences (8, 17, 32, 51). The majority of G-6-PD was detected in the cytoplasmic fraction (Fig. 3B, bottom), and the majority of USF was detected in the nuclear fraction (Fig. 3B, top).

E2A proteins present in the cytoplasmic fraction do not bind the μE2 element or nonspecific DNA. To investigate the ability of the E2A species present in the cytoplasmic fraction to bind the μE2 site, nuclear and cytoplasmic fractions were prepared from pro-B, pre-B, mature B, and plasma cell lines and tested in EMSA experiments for μE2-binding activity. Although similar amounts E2A proteins from nuclear and cytoplasmic extracts were used in EMSA, no Yae-reactive μE2-bound species were detected in cytoplasmic extracts (Fig. 4A). No significant proteolytic degradation of E2A proteins was detected in any of the nuclear lysates used in EMSA (Fig. 4B, top). No significant E2A degradation was observed in the pre-B, mature B, and plasma cytoplasmic extracts used in EMSA (Fig. 4B, bottom). Apparent E2A degradation was detected in pro-B cytoplasmic extracts, but there remained a significant portion of intact E2A proteins (Fig. 4B, bottom). To test for the presence of an inhibiting activity in the cytoplasmic fraction, nuclear and cytoplasmic extracts were mixed and used for binding. No inhibition of binding was observed in EMSA (Fig. 4A).

The ability of E2A proteins present in the cytoplasmic fraction to bind DNA was tested by using liquid chromatography. Similar amounts of E2A proteins present in the nuclear and cytoplasmic extracts prepared from Raji cells were applied to a salmon sperm DNA-Sepharose column, and bound proteins were eluted with increasing salt concentrations. The collected fractions were analyzed for the presence of E2A proteins by Western blot analyses and EMSA. E2A proteins in the nuclear extract bound to the DNA and eluted in the 0.3, 0.4, and 0.5 M salt fractions, as shown by both Western blot (Fig. 5A, top) and EMSA (Fig. 5B) analyses. In contrast, E2A proteins in the cytoplasmic extract did not bind to DNA and were detected in the flowthrough in the Western blot analysis (Fig. 5A, bottom). Moreover, E2A proteins in the flowthrough did not recognize the μE2 site in EMSA (Fig. 5C). Partial *S. aureus* V8 protease degradation of detected E2A species in the nuclear and cytoplasmic extracts of Raji cells used in these chromatographic experiments gave rise to similar-size peptides detected by the Yae antibody in Western blot analysis (Fig. 5D). This finding confirms that the detected non-DNA-binding Yae-reactive species in the cytoplasmic extract are E2A proteins. These observations demonstrate that the E2A proteins are present in DNA-binding and non-DNA-binding forms in vivo. We have designated the non-DNA-binding species I-E2A, for inhibited E2A.

Immunostaining analyses detect E2A proteins predominantly in the nucleus of B cells. Immunostaining of Raji mature B cells using the Yae antibody was performed to accurately determine the subcellular localization of E2A proteins in vivo. In contrast to the immunoblotting results, which detected significant amounts of E2A proteins in both nuclear and cytoplasmic fractions of B-cell lysates, E2A proteins were predominantly localized to the nucleus in Raji cells (Fig. 6A) as well as cells representing other stages of B-cell development (data not shown). Because the E2A proteins present in cytoplasmic fractions are in a non-DNA-binding form and hence would not be expected to be associated with the chromatin, this finding raised the possibility that the E2A proteins detected in the cytoplasmic fractions leaked out of the nucleus during subcellular fractionations in a time-dependent manner. This premise was examined by performing very rapid cell lysis and fractionations and examining nuclear and cytoplasmic fractions by immunoblot analysis. Consistent with the immunostaining results, E2A proteins were predominantly detected in the nuclear fraction of cell lysates prepared by very rapid fractionation (Fig. 6C). To confirm the presence of non-DNA-binding E2A proteins in the rapidly prepared nuclear extract, the nuclear fraction was applied to the salmon sperm DNA column to detect non-DNA-binding and DNA-binding forms of E2A proteins. A significant amount of E2A proteins present in the rapidly prepared nuclear fraction were unable to bind the nonspecific salmon sperm DNA affinity matrix (data not shown). The ratio of DNA-binding and non-DNA-binding forms of E2A proteins present in the rapidly prepared nuclear lysate was similar to the ratio of E2A proteins observed in the conventionally prepared nuclear and cytoplasmic fractions of B cells, respectively, consistent with the explanation that nuclear non-DNA-binding E2A proteins partition from the nucleus during conventional subcellular fractionation.

DISCUSSION

We have characterized a new monoclonal antibody (Yae) generated against rE12. The Yae antibody recognized E2A proteins of approximately 72 kDa in both rat and human cell lines in Western blot assays (Fig. 1D), consistent with the predicted molecular masses of E2A gene products. Full-length E12 and full-length E47 are presumed to be identical in the region which contains the Yae cognate determinant. Therefore, because the Yae antibody recognizes rE12 and rE47 and human proteins which comigrate with in vitro-produced rE12 and rE47 in SDS-PAGE, the Yae antibody likely recognizes both full-length E12 and full-length E47 human proteins. ITF-1 diverges from full-length E47 in the N terminus and regains identity with full-length E47 from amino acids 101 to 654, inclusive. Yae recognition of rE12 and rE47 is lost at amino acid 163. Further characterization of the Yae recognition site has localized the determinant to between amino acids 195 and 208 of rE12 (66). Therefore, the Yae antibody would also recognize ITF-1. Thus, it is likely that the Yae antibody recognizes all reported E2A splice variants. The Yae antibody detected the fusion proteins, $p77^{E2A\text{-}Pbx1}$ and $p85^{E2A\text{-}Pbx1}$, resulting from the t(1;19)(q23;p13.3) E2A gene translocation present in the pre-B ALL cell line 697. The Yae antibody also interacts with the majority of the E2A-containing BCF-1 and P-E2A species in EMSA experiments. These collective results suggest that the Yae monoclonal antibody is highly specific for the E2A proteins. This reagent should prove to be valuable in studies of E2A regulation of gene expression because no reagents which recognize E2A proteins in vivo with such a high specificity have been reported.

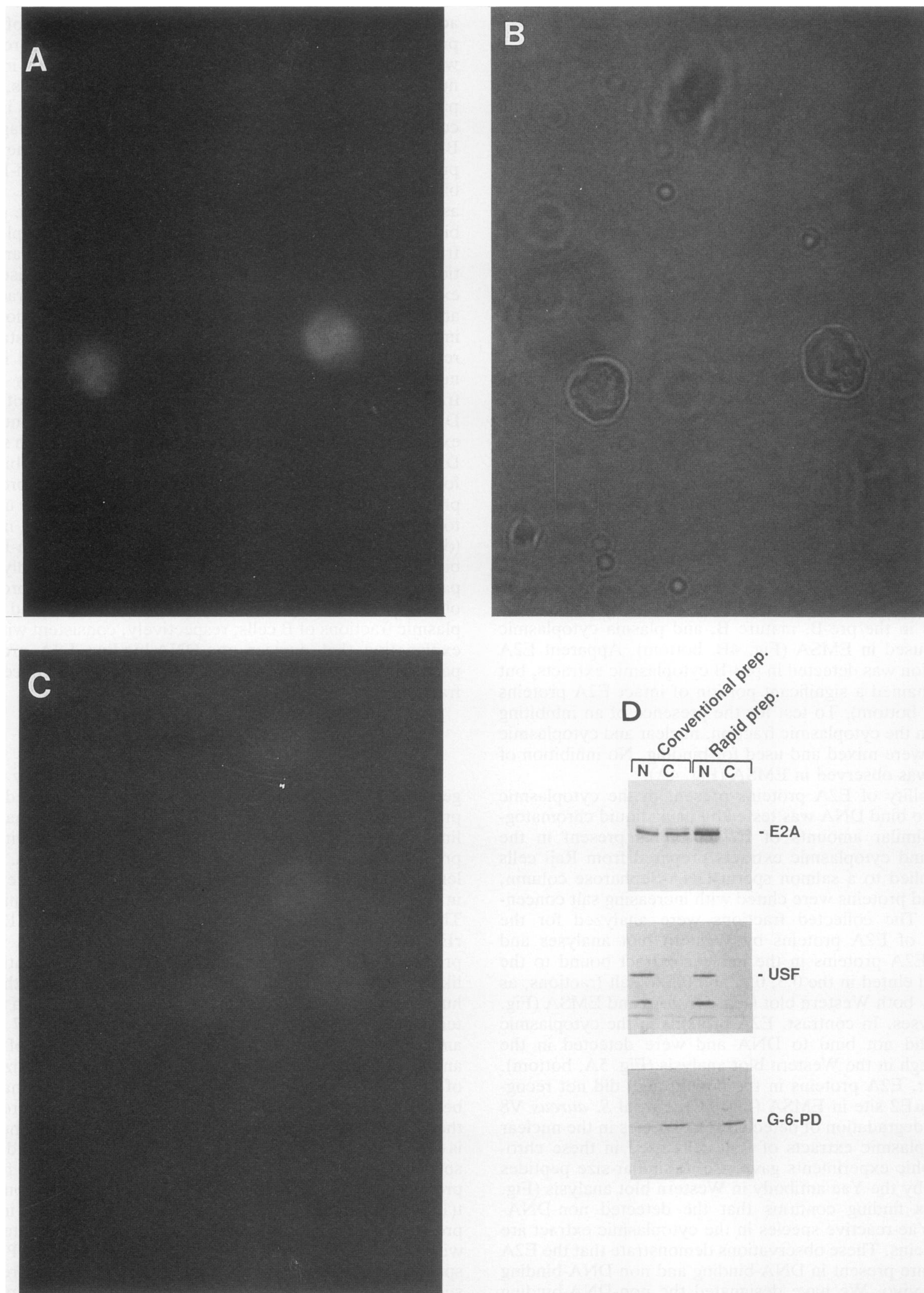


FIG. 6. E2A proteins are predominantly nuclear. (A) Immunofluorescence staining of Raji cells demonstrating nuclear localization of Yae-detected E2A proteins. (B) Phase-contrast photograph of the cells in panel A. (C) Control primary antibody (control mouse ascites fluid; Sigma) plus fluorescein isothiocyanate-labeled goat anti-mouse antibodies. (D) Western blot analyses of nuclear and cytoplasmic fractions of Raji cells derived by conventional preparation (Conventional prep.) and rapid preparation (Rapid prep.) of subcellular fractions demonstrate

The Yae antibody was used to study the role of E2A proteins in B-cell development. We have shown that E2A proteins are expressed during all stages of B-cell development (Fig. 2A). The synthesis of E2A proteins at the pro-B stage may have great implications in the etiology of the class of pre-B ALLs containing the t(1;19)(q23;p13.3) translocations of the E2A gene, which is associated with poor prognosis of patients carrying this chromosomal translocation (11). In these translocations, fusion proteins with transforming properties that are composed of the N-terminal 483 amino acids of E2A proteins under *E2A* promoter control fused to C-terminal *Pbx1* sequences (22, 39) are produced (21). Because E2A proteins are synthesized at the pro-B stage, fusion proteins arising from E2A translocations at this stage would be expressed as early as the pro-B stage of development.

Although E2A proteins of identical molecular mass were detected in nuclear lysates prepared from all B-cell stages, no E2A-containing μE2-binding species were detected in the pro-B-cell stage. An E2A-containing μE2-binding species (BCF-1) was detected in pre-B and mature B stages, and a different E2A-containing μE2-binding species (P-E2A) was observed in the plasma cell stage (Fig. 2D). P-E2A migrates at a significantly faster rate than in vitro-produced rE12 and rE47 bound to the μE2 site. This observation strongly suggests that E2A proteins are complexed with heterologous proteins in plasma cells. The BCF-1 species comigrates with the slowest-migrating species of the doublet comprised of in vitro-produced rE12 or rE47 bound to the μE2 site (Fig. 2C), but an equivalent B-cell species which comigrates with the faster-migrating in vitro-synthesized species is not observed. Thus, the BCF-1 species may consist of E2A heteromers, which might explain the absence of the doublet which is observed with use of in vitro-synthesized E2A proteins. The failure of E2A species in pro-B nuclear lysates to bind the μE2 site may be due to an interaction with Id proteins or a different sequence specificity of pro-B E2A species. The inability of the Yae antibody to interact with the F1, F2, and F3 species indicates that these species do not contain the Yae determinant and therefore do not contain E2A proteins (Fig. 2C and D). The highly sequence related λE2 and κE2 sites of the λ and κ light-chain enhancers also bind different species present in mouse J558L B-cell nuclear extract (49). The detection of multiple μE2-binding species in all stages of B-cell development is reminiscent of beta-cell insulin Nir element (E box)-binding species which demonstrate μE1, μE2, or μE3 sequence binding preferences (33). Conversion of the Nir sequence to these related sequences results in different functional effects (33); only the μE2 sequence which selectively binds a E2A-containing species is active as a transcriptional element (15, 33). Differential regulation of transcription at the Nir site may therefore involve specific complex binding, and a similar mechanism may exist for transcriptional regulation mediated by the μE2 site in B cells. The μE2 site is apparently the target of developmental regulation. In pro-B cells, no E2A-containing species are bound. At the pre-B and mature B stages, BCF-1 is bound, and at the plasma stage, BCF-1 is replaced by P-E2A. This stage-specific distribution of E2A enhancer element-binding complexes correlates with the transcriptional state of the IgH and Ig κ genes during B-cell development. In pro-B cells, the IgH gene is transcribed very weakly, and then the transcription rate increases significantly upon entering the pre-B and mature B-cell stages, reaching a maximum upon terminal plasma cell stage differentiation.

Although immunostaining demonstrated the localization of E2A proteins to the nucleus, significant amounts of E2A proteins were shown to be present in the cytoplasmic and nuclear fractions of cells at all stages of B-cell differentiation examined (Fig. 3A) by using conventional cell lysis and fractionation methods. This observation is in marked contrast to the results obtained for E2A proteins in pancreatic acinar and beta-cell lines, in which E2A species were present predominantly in the nuclear fraction of cell lysates (61a). In addition, USF, an HLH protein which also recognizes the μE2 site, was detected predominantly in the nuclear fraction of B-cell lysates.

The HLH E2A proteins in the cytoplasmic extract neither recognized the μE2 element in EMSA (Fig. 4A) nor bound to nonspecific DNA in chromatography studies (Fig. 5A and C), providing a ready explanation for the partitioning of non-chromatin-bound E2A proteins to the cytoplasmic fraction during cell lysis. Chromatography experiments using heparin-Sepharose demonstrated that E2A proteins present in both nuclear and cytoplasmic extracts bound to the matrix. However, E2A proteins present in the cytoplasmic extract eluted below 0.5 M NaCl salt concentration, while E2A proteins in the nuclear extract eluted at higher salt concentrations (data not shown). This result further defines the differences between E2A proteins present in the nuclear and cytoplasmic extracts. Experiments using the detergent deoxycholate, which has been shown to induce the NF-κB DNA-binding activity (2, 3), were unable to activate E2A-binding activity (data not shown). In addition, we have been unable to detect metabolically [^{35}S]methionine radiolabeled E2A proteins by immunoprecipitation using the Yae antibody, to examine the possibility that an associated protein may be inhibiting DNA binding. Partial *S. aureus* V8 protease digestion of nuclear and cytoplasmic protein extracts gave rise to similar-size peptides detected by the Yae antibody in Western blot analysis (Fig. 5D). However, microheterogeneity in E2A forms, which could account for the failure of some species to bind DNA, would not have been detected by this experiment. Therefore, the mechanism of the restriction of DNA binding awaits further purification and examination of these forms. These results suggest a selective in vivo transcriptional regulation mechanism of E2A bHLH proteins, by the inhibition of DNA binding.

bHLH E2A proteins are able to dimerize with Id class HLH proteins, which lack the basic region and therefore form heterodimers which are unable to bind DNA (5, 56). It is possible that E2A-Id complex formation is the cause of the non-μE2-binding E2A species detected in pro-B cells because this is consistent with previous reports which demonstrated that Id mRNAs are present at high levels in pro-B cells (56, 64). However, Id mRNAs are very low or unde-

the nuclear localization of E2A proteins. Ten microliters (60 μg) of nuclear (N) and 10 μl (75 μg) of cytoplasmic (C) extracts prepared by the conventional method or 10 μl (95 μg) of nuclear (N) and 10 μl (35 μg) of cytoplasmic (C) extracts prepared by a rapid method (see Materials and Methods) were probed for the presence of E2A proteins (top) by using the Yae antibody. The same amounts of extracts were probed for the presence of USF (middle) and G-6-PD (bottom) by using anti-USF and anti-G-6-PD antibodies, respectively, to show efficient separation of nuclear and cytoplasmic fractions of cell lysates.

tectable in pre-B and mature B cells (56, 64), and thus it is less likely that an E2A-Id complex gives rise to the non-DNA-binding E2A species detected in pre-B and mature B cells. In support of this hypothesis, the E2A-Pbx1 fusion proteins p77$^{E2A-Pbx1}$ and p85$^{E2A-Pbx1}$, which lack the E2A bHLH region, were also detected in the nuclear and cytoplasmic fractions of the 697 cell line in a ratio that paralleled that of the wild-type E2A proteins present in 697 cells and the pre-B-cell line Nalm 6 (Fig. 2A). At this time, it is unclear why a significant proportion of E2A proteins are in a non-DNA-binding form.

In contrast to all other B-lineage cell lines studied, E2A proteins were observed predominantly in the nuclear fraction of an EBV-transformed mature B-cell line, MJ4 (Fig. 2A). In addition, the MJ4 line lacked the BCF-1 species and contained the P-E2A species characteristic of the plasma cell line studied (Fig. 2B). EBV is known to infect resting B cells and induce the expression of B-cell activation antigens and cell adhesion molecules (18, 48, 57) which are normally expressed during physiological B-cell activation. This result suggests that the non-DNA-binding E2A form may be activated to a DNA-binding form upon B-cell activation. Further understanding of the role that E2A proteins play in the regulation of B-cell function and the potential for EBV interaction with E2A-regulated pathways awaits additional biochemical studies.

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