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E2A and E2-2 Are Subunits of B-Cell-Specific E2-Box DNA-Binding Proteins

GRETCHEN BAIN,[1] STEFAN GRUENWALD,[2]† AND CORNELIS MURRE[1]*

Department of Biology, 0116, University of California, San Diego, La Jolla, California 92130,[1] and Department of Molecular and Cellular Biology, Pharmingen, San Diego, California 92121[2]

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A class of helix-loop-helix (HLH) proteins, including E2A (E12 and E47), E2-2, and HEB, that bind in vitro to DNA sequences present in the immunoglobulin (Ig) enhancers has recently been identified. E12, E47, E2-2, and HEB are each present in B cells. The presence of many different HLH proteins raises the question of which of the HLH proteins actually binds the Ig enhancer elements in B cells. Using monoclonal antibodies specific for both E2A and E2-2, we show that both E2-2 and E2A polypeptides are present in B-cell-specific Ig enhancer-binding complexes. E2-box-binding complexes in pre-B cells contain both E2-2 and E2A HLH subunits, whereas in mature B cells only E2A gene products are present. We show that the difference in E2-box-binding complexes in pre-B and mature B cells may be caused by differential expression of E2A and E2-2.

B lymphoid cells go through many intermediate steps before becoming the plasma cells that produce and secrete immunoglobulins (Igs). The earliest stage of B-cell differentiation is represented by pro-B lymphocytes, i.e., cells that contain both the Ig heavy- and light-chain genes in germ line configuration. Both the Ig heavy- and light-chain genes are transcriptionally inactive at this stage of B-cell development. As pro-B lymphocytes differentiate into pre-B cells, the rearranged heavy chain is transcribed and translated. However, the Ig light-chain genes are not transcribed. Pre-B cells can differentiate, express the Ig light-chain genes, and become mature B lymphocytes. The mature B lymphocyte expresses both the Ig heavy and light chains, giving rise to mature surface Ig-positive B lymphocytes.

The developmental regulation of Ig gene expression is dependent on various sequences in the Ig enhancer. One class of such sequence elements is the E boxes. They share as a consensus sequence NNCANNTGNN. The E-box sites were first identified by dimethyl sulfate protection experiments (6, 12). Factors were found to protect certain sequences from methylation in the Ig heavy- and light-chain enhancer in B cells but not in non-B cells (6, 12). That the E-box elements are critical for B-cell-specific gene expression became evident from mutational analysis. Mutation of E-box sites caused a significant decrease in Ig transcription (18, 21). The most dramatic impact on Ig expression was found in mutations of elements that contain an E2 box (G/ACAGNTGT/G) (21). The E2 boxes are particularly interesting because they are also present in muscle- and pancreas-specific enhancers (3, 4, 32). Mutation of the E2-box elements present in these enhancers revealed the crucial role of these elements in regulating muscle- and pancreas-specific genes (16, 22, 26, 27, 32).

A family of proteins that bind the E2 box has recently been identified. E2-box-binding proteins share a common amino acid sequence motif that is proposed to form two amphipathic helices interrupted by a loop, designated the helix-loop-helix (HLH) motif. The HLH motif mediates homodimerization and heterodimerization with other HLH proteins (2, 23, 24, 31). While the HLH region is important for dimerization, a basic region located N terminal of the HLH region is responsible for DNA binding (10, 19, 31).

E2-box-binding HLH proteins can be divided into two classes. Class I HLH proteins include E12, E47, HEB, and the *daughterless* protein and have in common that they are ubiquitously expressed and bind as either homo- or heterodimers to the E2 box. The class I HLH proteins are structurally related and in particular are very well conserved in the region surrounding the HLH domain. They also bind the E2 box as heterodimers with class II HLH proteins. Representative members of the class II proteins are MyoD, myogenin, the *myf-5* protein, and herculin, proteins that induce myogenesis, and *Drosophila* gene products that are involved in neurogenesis, including members of the *AS-C* gene complex. Class II proteins are tissue specific and do not bind as homodimers to the E2 box.

The mammalian class I HLH proteins are closely related to the *daughterless* protein, the *Drosophila* class I protein. *daughterless* is involved in the control of at least two developmental pathways, including sex determination and neurogenesis, and is expressed ubiquitously (5, 7, 9). Like the mammalian class I HLH proteins, the *daughterless* protein binds to the E2 box either as a homodimer or as a heterodimer with members of the *achaetescute* gene family (24). Most closely related to the *daughterless* protein are the E2A gene products, E12 and E47. E12 and E47 are alternatively spliced products of the E2A gene and are identical except in the HLH region (17, 23, 30). These two proteins have been directly implicated in the regulation of B-cell-, muscle-, and pancreas-specific gene expression (8, 20, 25, 29). The E2A gene products can dimerize with tissue-specific HLH proteins to regulate cell differentiation. For example, a heterodimer of E2A and MyoD turns on muscle-specific gene expression and induces myogenesis (20) by binding E boxes in the muscle creatine kinase enhancer. Overexpression of an E47 cDNA in a pre-T-cell line activates both germ line heavy-chain gene transcription and Ig D-to-J rearrangement (29). E47 homodimers can activate transcription from a reporter construct bearing multiple E2-box sites, and both E12 and E47 can activate transcription from a construct

* Corresponding author.

containing the Ig heavy-chain enhancer (14). In addition, pancreatic, muscle, and B-cell nuclear extracts contain E2-box-binding complexes that are tissue specific (1, 3, 4, 8, 25, 32). More recently, it was shown that a double knockout of the E2A gene in mouse embryonic stem cells had no effect on differentiation of cardiac and skeletal muscle, erythrocytes, neurons, and cartilage (33). The most likely explanation is functional redundancy. In fact, E2-2 and HEB seem to have functional properties similar to those of the E2A proteins.

The E2-2 gene product is closely related to the E2A gene products but is encoded by a separate gene. In vitro studies have demonstrated binding by E2-2 to the E5 site in the heavy-chain enhancer (14). Others have shown that E2-2 can bind E2-box oligonucleotides as heterodimers with E2A, MyoD, or HEB (15, 20). E2-2 can also act as a transcriptional activator. When the coding region of E2-2 was fused to the GAL4 DNA-binding domain, the resultant fusion protein was able to activate transcription from a reporter bearing multiple copies of the Gal4 DNA-binding site (14). We have been unable to show transactivation by E2-2 alone from an E2-box reporter gene. The most likely reason is that E2-2 requires a partner for activation. In fact, cotransfection of E2-2 and MyoD expression vectors results in activation of transcription from a multimerized E2-box site (20).

Recently a new class I HLH protein, designated HEB, has been identified. HEB binds the E2 box as a homodimer or as heterodimers with MyoD, E12, or E2-2 (15). It is expressed in all tissues examined, with highest levels of expression in cell lines derived from the T-cell lineage (15). Its biological function is unknown.

Various combinations of HLH proteins obviously can impart different functional properties to different tissues or to different stages in development. Within the B-cell lineage, many HLH proteins are present, including E12, E47, HEB, and E2-2. The question of which of these class I HLH proteins is important for Ig gene expression arises. As a first approach to this question we show here that E2A and E2-2 are differentially expressed in pre-B and mature B cells. This differential expression of E2A and E2-2 results in the presence of both E2-2 and E2A gene products in complexes binding the E2 box in pre-B cells, whereas in mature B cells only the E2A gene products can be detected in the E2-box-binding complexes.

MATERIALS AND METHODS

Cell lines. Namalwa and Jurkat cells were grown in RPMI medium supplemented with 10% (vol/vol) fetal calf serum. Nalm-6 cells were grown in RPMI medium containing 10% fetal calf serum and 57 μM β-mercaptoethanol. HeLa cells were grown Dulbecco modified Eagle medium containing 10% fetal calf serum.

Northern blot analysis. RNA was isolated with guanidium isothiocyanate. Northern (RNA) blotting and hybridization were done as described previously (21a).

Plasmids. The E2-2 in vitro transcription vector was a generous gift from Tom Kadesch. The E12 and E47 in vitro transcription vectors contain the influenza virus epitope tag placed amino terminal to the full-length coding sequences.

Transcription and translation. RNA was synthesized in a 100-μl reaction mixture for 1 h at 40°C with a substrate of 2 μg of linearized DNA and with T3 or T7 RNA polymerase (Promega) in the presence of RNasin (Promega). The RNA was phenol and chloroform extracted and ethanol precipitated. The ethanol precipitate was resuspended in 20 μl of water. In vitro translation of the RNA was done with a rabbit reticulocyte lysate (Promega) in a 50-μl reaction mixture with 10% of the synthesized RNA as a substrate. Proteins were synthesized at 30°C for 1 h. Reaction conditions were as recommended by the manufacturer (Promega).

Antisera. The antibodies were made at Pharmingen according to the methods of Harlow and Lane (13).

Electropheretic mobility shift assay (EMSA). Five to seven microliters of the proteins translated in vitro in the presence of [^{35}S]methionine was incubated with a ^{32}P-labeled oligonucleotide containing the wild-type monomeric μE5 E2-box sequence. The binding reaction mixtures were incubated at room temperature and contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 0.1 to 0.5 μg of poly(dIC-dIC) as the nonspecific competitor. The complexes were resolved by electropheresis through a 5% polyacrylamide gel containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA. For supershift assays, the translated proteins were incubated for 15 min with antibody prior to incubation with labeled DNA.

Immunoprecipitations. Ten microliters of ^{35}S-labeled in vitro-translated proteins was incubated with 10 μl of antibody for 1 h on ice. One hundred microliters of the immunoprecipitation buffer (IPB) containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% deoxycholate, 2 mM EDTA, 0.02% NaN_3, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride was added along with 30 μl of a 50% slurry of protein A-Sepharose beads in IPB. This was incubated at 4°C for 1 h with rotating. Samples were washed three times with 1 ml of IPB, and beads were resuspended in 20 μl of IPB and 10 μl of 3× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 10 min, and run on an SDS–8% polyacrylamide gel. After treatment with 200 g of 2,5-diphenyloxazole [PPO] in 1 liter of acetic acid for 1 to 2 h at room temperature, gels were dried and exposed to film at −70° with an intensifying screen. For immunoprecipitations of cell extracts, 10^6 cells were resuspended in 5 ml of Dulbecco modified Eagle medium minus methionine supplemented with 10% dialyzed calf serum and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.2) and incubated at 37°C for 1 h. [^{35}S]methionine (Tran^{35}S-label; ICN) (350 μCi) was added, and incubation was continued at 37°C for 2 h. A 70% confluent 15-cm-diameter plate of HeLa cells was labeled with 700 μCi of [^{35}S]Met in 10 ml of Dulbecco modified Eagle medium minus Met. Cells were harvested, washed in PBS, and resuspended in 1 ml of IPB. After 15 min on ice, extract was spun at 45,000 rpm in a Beckman Ty65 rotor at 4°C for 30 min. The lysate was removed, and 40 μl of a 50% slurry of protein A-Sepharose beads in IPB was added and incubated at 4°C for 1 h with rotating. Beads were spun down and 10 μl of antibody was added to the lysate and incubated on ice for 1 h. Thirty microliters of a 50% slurry of protein A-Sepharose beads in IPB was added, and incubation was continued at 4°C with rotating. Beads were washed three times by resuspension in 1 ml of IPB. The immunoprecipitated material was recovered by resuspension of beads in 20 μl of IPB and 10 μl of 3× SDS-PAGE sample buffer. The samples were boiled for 10 min and loaded on an SDS–8% polyacrylamide gel. The gels were treated with PPO as above, dried, and exposed to film at −70°C with an intensifying screen.

Extract preparation and gel shift assay. Nuclear extracts were prepared as described by Dignam et al. (11). An oligonucleotide containing the wild-type monomeric μE5 E2-box sequence (same as above) was incubated with 10 μg

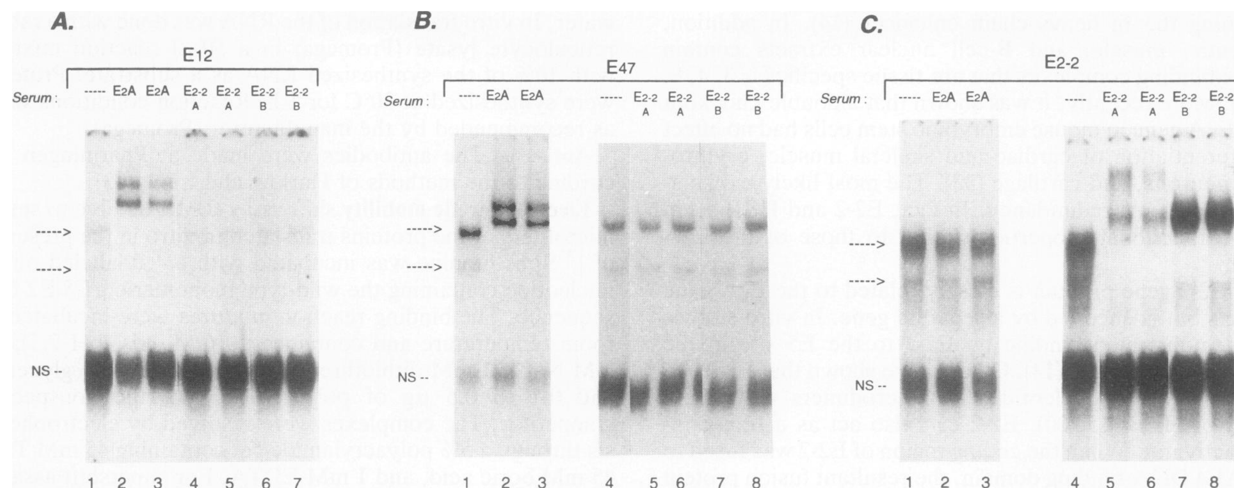

FIG. 1. EMSA of in vitro-translated proteins. (A) In vitro-translated E12 was incubated with a ^{32}P-labeled μE5 oligonucleotide under the following conditions: lane 1, no antibody; lane 2, 3 μl of E2A antibody; lane 3, 1 μl of E2A antibody; lane 4, 1 μl of E2-2 A antibody; lane 5, 1 μl of a 1:2 dilution of E2-2 A antibody; lane 6, 1 μl of E2-2 B antibody; lane 7, 1 μl of 1:2 dilution of E2-2 B antibody. (B and C) In vitro-translated E47 (B) and E2-2 protein (C) were incubated with the labeled μE5 oligonucleotide under the following conditions: lane 1, no antibody; lane 2, 3 μl of E2A antibody; lane 3, 1 μl of E2A antibody; lane 4, no antibody; lane 5, 1 μl of E2-2 A antibody; lane 6, 1 μl of a 1:2 dilution of E2-2 A antibody; lane 7, 1 μl of E2-2 B antibody; lane 8, 1 μl of a 1:2 dilution of E2-2 B antibody. Lanes 1 to 3 and 4 to 8 represent two different gels. E2-2 A and E2-2 B represent two different monoclonal antibodies used. NS indicates nonspecific complexes. Arrows indicate E2A- or E2-2-specific complexes.

of the nuclear extract. The binding reaction conditions were the same as above, except 0.5 μg of poly(dIC-dIC) was used as the nonspecific competitor. The complexes were resolved by electropheresis through a 5% polyacrylamide gel containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA. For supershift assays, nuclear extracts were preincubated with the antibody for 15 min prior to incubation with labeled DNA.

RESULTS

Antibody specificity. Previously, we and others showed that E2A-like polypeptides are present in complexes that bind the E2 box in nuclear extracts derived from B, muscle, and pancreatic cells (8, 20, 25). However, the polyclonal antibody used in these studies interacts with E12, E47, E2-2, likely HEB, and possibly other members of the class I HLH protein family. To determine precisely which HLH proteins bind to the E-box elements present in the Ig enhancers, we generated monoclonal antibodies specific for both E2A and E2-2.

To determine the specificities of the monoclonal antibodies we used both EMSA and immunoprecipitation experiments. In vitro-translated E12 homodimers bind with low affinity to the μE5 oligonucleotide (Fig. 1A, lane 1). Preincubation of in vitro-translated E12 protein with the anti-E2A antibody (Pharmingen MAb G98-271.1.3) results in a complete supershift of both complexes (Fig. 1A, lanes 2 and 3). The anti-E2-2 antibodies [Pharmingen MAb G108-302.2 (E2-2A) and G108-391.2 (E2-2B)], however, do not recognize E12 determinants (Fig. 1A, lanes 4 to 7). Similarly, in vitro-translated E47 protein incubated with the anti-E2A antibody supershifts both E47 complexes (Fig. 1B, lanes 2 and 3), whereas the anti-E2-2 monoclonal antibodies have no effect on the migration of these complexes (Fig. 1B, lanes 4 to 7). The anti-E2A antibody, however, does not distinguish between E12 and E47 (Fig. 1A and B). In both cases, preincubation of either E12 or E47 with the E2A monoclonal antibody results in more-intense supershifted complexes (Fig. 1A and B, lanes 2 and 3). This may be the result of the stabilization of DNA binding upon addition of the antibody.

E2-2 homodimers bind with a higher affinity than E12 homodimers to the μE5 oligonucleotide (compare Fig. 1A, lane 1, with Fig. 1C, lanes 1 and 4). The monoclonal antibody directed against E2A does not supershift E2-2 (Fig. 1C, lanes 2 and 3). However, in vitro-translated E2-2 is supershifted by two independent E2-2 monoclonal antibodies, designated E2-2 A and E2-2 B (Fig. 1C, lanes 5 to 8). Thus, the anti-E2A monoclonal antibody is specific for E12 and E47, and the two monoclonal antibodies directed against E2-2 specifically recognize E2-2.

Next, in vitro-translated E12, E47, and E2-2 proteins were incubated with the various monoclonal antibodies and immunoprecipitated. The data from the immunoprecipitation experiments exactly correspond to the gel shift data. In vitro-translated full-length E12 is immunoprecipitated only with the anti-E2A antibody (Fig. 2, lane 1) and not with the anti-E2-2 B monoclonal antibody (Fig. 2, lane 2). Likewise, E47 is recognized only by the anti-E2A antibody (Fig. 2, lane 4) and not by the anti-E2-2 antibody (Fig. 2, lanes 5). As expected, the E2-2 B monoclonal antibody recognizes and immunoprecipitates in vitro-translated E2-2 (Fig. 2, lane 8), whereas the anti-E2A antibody does not pull down E2-2 in immunoprecipitation experiments (Fig. 2, lane 7). The E2-2 A antibody does not recognize E2-2 under the conditions used here. This antibody will, however, recognize and immunoprecipitate the E2-2 protein under less stringent conditions (unpublished data). An influenza virus antibody used as a negative control does not immunoprecipitate the in vitro-translated proteins (Fig. 2, lanes 3, 6, and 9). Thus, in both gel shift and immunoprecipitation experiments, the anti-E2A monoclonal antibody is specific for the E2A proteins, E12, and E47, and the anti-E2-2 B antibody is specific for E2-2.

E2-2 mRNA and protein is present in cells derived from the B-cell lineage but not in T cells. Class I HLH genes, including

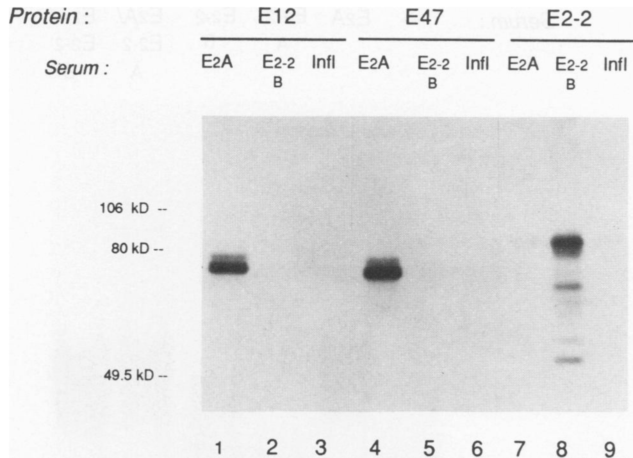

FIG. 2. Immunoprecipitation of ^{35}S-labeled in vitro-translated E2A or E2-2 protein with 10 μl of either E2A, E2-2, or influenza virus (Infl) antibody as marked.

the E2A gene, *daughterless*, and the HEB gene, are expressed in all tissues examined (15, 23). To determine whether the E2-2 protein is similarly present in all tissues, we immunoprecipitated labeled cell extracts with anti-E2A and anti-E2-2 monoclonal antibodies. An anti-influenza virus monoclonal antibody was used as a negative control. The anti-E2A antibody clearly precipitates one major band of approximately 75 kDa in all cell lines examined, consistent with its ubiquitous expression. E2A protein was immunoprecipitated in the HeLa cell, T-cell, and pre-B-cell lines examined (Fig. 3, lanes 1, 4, and 7). In contrast to E2A, E2-2 protein was expressed at high levels only at Nalm-6, a pre-B-cell line, and appeared as a band of approximately 90 kDa (Fig. 3, lane 2). Interestingly, little or no E2-2 was immunoprecipitated from HeLa or Jurkat cells (Fig. 3, lanes 5 and 8). Therefore, the pattern of E2-2 protein expression is more restricted than that of the E2A proteins and is found predominantly in B lymphoid cells. Neither the E2A nor the E2-2 protein was immunoprecipitated with the anti-influenza

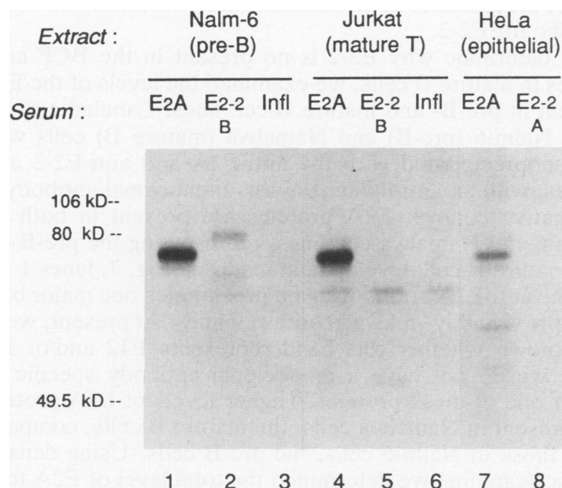

FIG. 3. Immunoprecipitation of ^{35}S-labeled cells. Nalm-6, Jurkat, or HeLa cells were labeled with [^{35}S]methionine, and extracts were immunoprecipitated as before with 10 μl of either anti-E2A, anti-E2-2, or anti-influenza (Infl) antibody as marked.

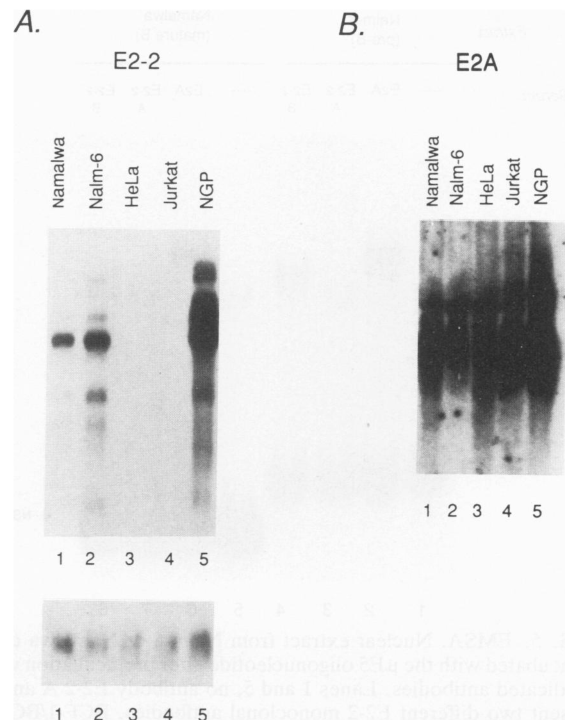

FIG. 4. (A) Northern blot analysis of E2-2 mRNA expression in various cell lines. Poly(A)$^+$ mRNA was resolved on a formaldehyde denaturing gel and transferred to nitrocellulose. The filters were hybridized to a ^{32}P-labeled probe and washed under stringent conditions before exposing to film. Lane 1, Namalwa, a human mature B-cell line; lane 2, Nalm-6, a human pre-B-cell line; lane 3, HeLa, a human epithelial cell line; lane 4, Jurkat, a human mature T-cell line; lane 5, NGP, a human neuroblastoma cell line. Below, the same RNAs were hybridized with a GAPDH probe. (B) The same RNAs as in panel A hybridized with an E2A probe.

virus antibody (Fig. 3, lanes 3 and 6). To determine whether E2-2 mRNA is present in cell lines derived from various tissues, we probed a Northern blot with a radioactively labeled E2-2 probe. E2-2 mRNA was detected in the Namalwa cell line, a mature B-cell line, and even higher levels were detected in the Nalm-6 cell line, a pre-B-cell line (Fig. 4A, lanes 1 and 2). In addition, E2-2 mRNA is expressed at very high levels in a neuroblastoma cell line (Fig. 4A, lane 5). In addition to the major transcript, several smaller E2-2 mRNAs were detected in both the B-cell lines and the neuroblastoma cell line, likely representing alternatively spliced products of the E2-2 gene. E2-2 transcripts are absent in T cells and HeLa cells, consistent with the immunoprecipitation data (Fig. 4A, lanes 3 and 4). We have examined a number of other T-cell lines for expression of E2-2 mRNA and found low to no detectable levels of E2-2 mRNA (22a). In contrast to the E2-2 mRNA, the E2A mRNA is expressed similarly in all cell lines examined (Fig. 4B). Slightly lower levels of E2A mRNA were present in HeLa cells (Fig. 4B, lane 3). Thus, E2-2 is expressed in at least two B-cell lines and in a neuroblastoma cell line but is present at very low levels in T cells, whereas E2A is expressed ubiquitously.

E2-2 is present in the BCF-1 and BCF-2 complexes in pre-B cells but not in mature B cells. E2A-like proteins are present in two B-cell-specific E2-box DNA-binding complexes termed BCF (25). The BCF complexes are present in pre-B

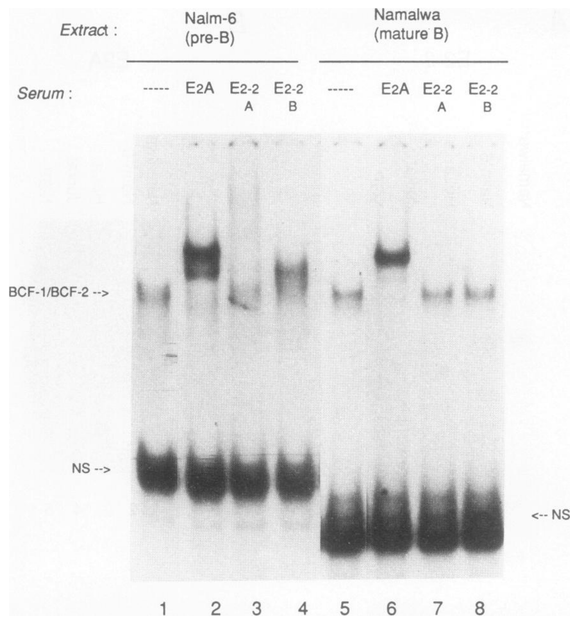

FIG. 5. EMSA. Nuclear extract from Nalm-6 or Namalwa cells was incubated with the μE5 oligonucleotide after preincubation with the indicated antibodies. Lanes 1 and 5, no antibody.E2-2 A and B represent two different E2-2 monoclonal antibodies. BCF-1/BCF-2 designates the B-cell-specific complexes identified previously. NS indicates nonspecific complexes.

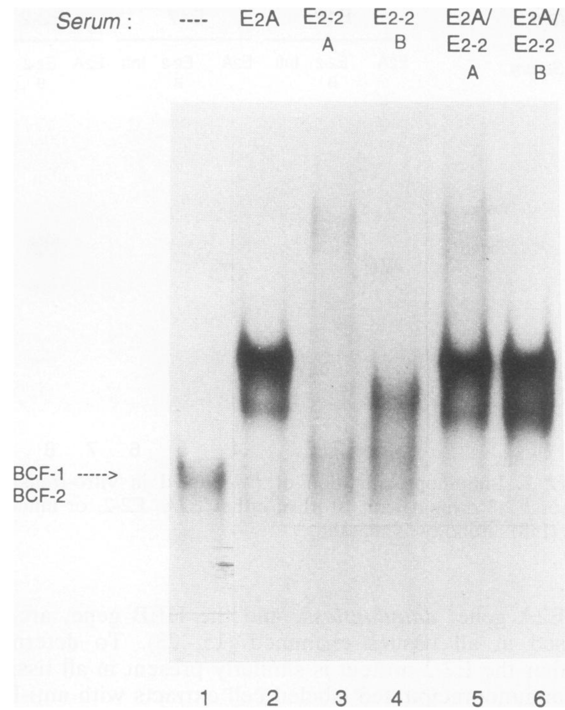

FIG. 6. EMSA. Nuclear extract from Nalm-6 cells was incubated with the μE5 oligonucleotide after preincubation with the indicated antibodies. Lane 1, no antibody. Lane 1 and lanes 2 to 6 are from different gels.

and mature B cells but not in pro-B cells (25). To determine whether both E2A and E2-2 are present in these complexes, B-cell nuclear extracts were incubated with either the anti-E2A or the anti-E2-2 antibody and assayed by EMSA. Both BCF-1 and BCF-2 supershifted in the presence of anti-E2A antibody in the pre-B (Nalm-6) and mature B (Namalwa) cells (Fig. 5, lanes 2 and 6). Thus, the BCF complexes contain E2A molecules. As with the in vitro-translated proteins, preincubation of the nuclear extract with the anti-E2A antibody results in a more intense supershifted complex, suggesting a stabilization of DNA binding. In nuclear extracts derived from a pre-B-cell line, Nalm-6, BCF is recognized by both E2-2 monoclonal antibodies (Fig. 5, lanes 3 and 4). We found similar E2-2 reactivity in another pre-B-cell line, 697 (1a). In contrast, there was very little reactivity to the anti-E2-2 monoclonal antibodies in nuclear extract from the mature B-cell line, Namalwa (Fig. 5, lanes 7 and 8). This was also observed in the EW cell line, another mature B-cell line tested (1a). Thus, BCF-1 and BCF-2 contain both E2-2 and E2A polypeptides in pre-B cells, whereas in mature B cells only E2A polypeptides are detectable.

In vitro, E2A and E2-2 proteins have the ability to form heterodimers (1a, 13a). To determine whether the BCF complexes in pre-B cells contain E2A–E2-2 heterodimers, we preincubated the B-cell nuclear extracts with a mixture of the E2A and E2-2 monoclonal antibodies and determined by EMSA whether we could further supershift the supershifted BCF complexes. The supershifts are identical when the nuclear extracts are preincubated with a mixture of E2A and E2-2 antibodies compared with when the monoclonal antibodies are added separately (Fig. 6, lanes 2 to 6). In addition, we tried, unsuccessfully, to coimmunoprecipitate E2-2 with an E2A antibody (1b). The most likely explanation is that both E2A and E2-2 bind to the E2 box in pre-B-cell nuclear extracts but do not form a heterodimer. However, our results do not exclude the possibility of an E2A–E2-2 heterodimer.

The E2A/E2-2 ratio changes in different stages of B-cell development. The finding that E2A and E2-2 polypeptides area both present in E2-box-binding complexes in pre-B cells but not in mature B cells raises the question of whether the differential binding by E2-2 in different stages of B-cell development reflects a difference in the level of E2-2 proteins present, a posttranslational modification, or, alternatively, the presence of an inhibitor molecule related to Id and specific for E2-2.

To determine why E2-2 is no present in the BCF complexes in mature B cells, we examined the levels of the E2-2 protein in pre-B- and mature B-cell lines. Labeled extracts from Nalm-6 (pre-B) and Namalwa (mature B) cells were immunoprecipitated with the anti-E2A and anti-E2-2 antibodies, with an anti-influenza virus monoclonal antibody as a negative control. E2A proteins are present in both the Nalm-6 and Namalwa cell lines, representing the pre-B-cell and mature B-cell developmental stages (Fig. 7, lanes 1 and 4). The anti-E2A antibody again precipitates one major band of approximately 75 kDa in both cell lines. At present, we do not known whether this band represents E12 and/or E47 since we do not have a monoclonal antibody specific for either one of these proteins. Higher levels of E2A proteins are present in Namalwa cells, the mature B cells, compared with those in Nalm-6 cells, the pre-B cells. Using densitometric scanning, we determined the total level of E2A to be approximately twofold higher in mature B cells than in pre-B cells (Fig. 8). A 90-kDa E2-2 polypeptide was also detected in both B-cell lines analyzed but, interestingly, was present at much higher levels in the pre-B-cell line than in the mature

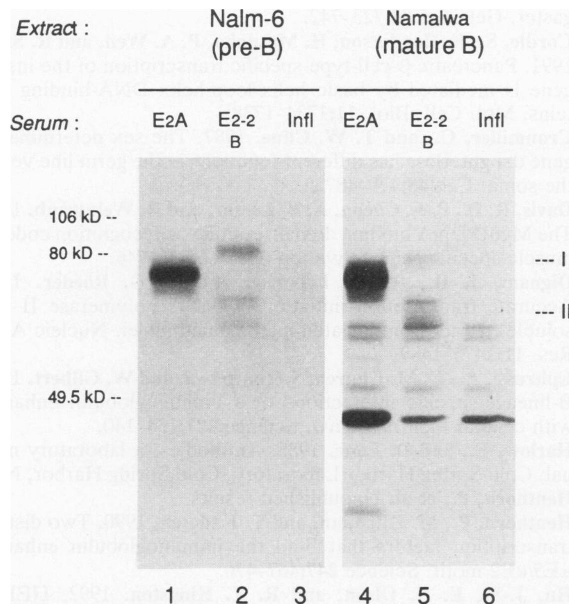

FIG. 7. Immunoprecipitation of ^{35}S-labeled cells. Nalm-6 or Namalwa cells were labeled with [^{35}S]methionine and immunoprecipitated with 10 μl of either E2A, E2-2, or influenza virus (Infl) antibody. The two polypeptides immunoprecipitated with the anti-E2-2 antibody are designated I and II.

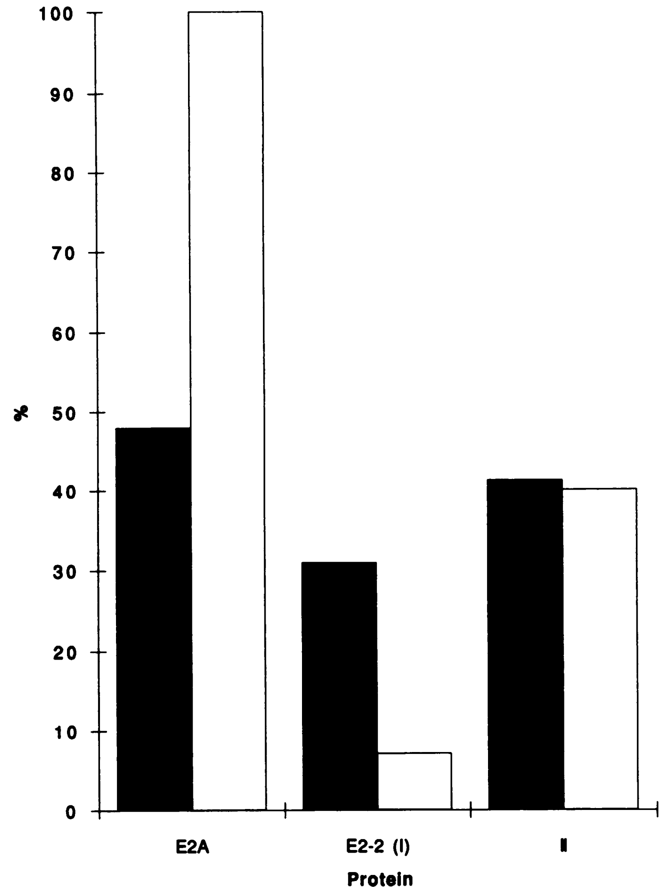

FIG. 8. Relative levels of protein immunoprecipitated from ^{35}S-labeled cells with the anti-E2A or anti-E2-2 antibodies. Protein bands on the autoradiographs were scanned with a densitometer. The resultant peaks were cut out and weighed on an analytical balance. The most intense band was set at 100%. Indicated are the relative percentages of each protein band. ■, Nalm-6 cells; □, Namalwa cells.

B-cell line (Fig. 7, lanes 2 and 5). In fact, the level of E2-2 in mature B cells is three- to fourfold lower than the level of E2-2 in pre-B cells (Fig. 8). Thus, during the transition from pre-B to mature B cells, the level of the E2A protein increases whereas the level of the E2-2 protein decreases. Also, an additional E2-2 polypeptide of lower molecular weight, designated form II, is precipitated from both the Namalwa and Nalm-6 cell lines (Fig. 7, lanes 2 and 5). The most likely possibility, given the multiple species of mRNA in the Northern blot, is that this polypeptide represents an alternative spliced product of the E2-2 gene. However, unlike the E2-2 gene product, there was no difference in the levels of form II detected in pre-B and mature B cells. There are approximately equal levels present in both stages of B-cell development.

DISCUSSION

Recently, significant progress has been made in identifying and characterizing molecules that control b-cell differentiation. One class of these molecules, the HLH proteins, plays an important role in controlling B-cell-specific gene expression. In muscle differentiation, master regulatory HLH molecules like MyoD, myogenin, and the myf-5 protein that control myogenesis in vitro have been identified. In contrast, a master regulatory molecule controlling B-cell differentiation has no yet been identified. However, recently it was shown that overexpression of an E47 cDNA in a pre-T-cell line dramatically increased the frequency of D-J rearrangement as well as germ line heavy-chain gene transcription. In addition, the levels of three lymphoid-specific gene transcripts, Oct-2, RAG-1, and RAG-2, were also increased (29). These experiments indicate the importance of HLH proteins in B-cell differentiation.

Four class I HLH proteins, E12, E47, HEB, and E2-2, have been shown to bind in vitro to the μE2, μE5, and μE4, and κE2 sequences present in the Ig heavy- and light-chain enhancers (14, 15, 23). In addition, E12 and E47 can activate the Ig enhancers when cotransfected with a reporter plasmid in non-B cells (28). Despite the well-documented in vitro studies of HLH protein interactions and DNA binding, little is known about their role in B-cell differentiation. For example, the question of which of these class I HLH proteins actually binds the E2-box elements in the B lymphocyte arises. We and others previously demonstrated that B-cell-specific complexes, designated BCF-1 and BCF-2, bind the E2 box. The BCF complexes contain E2A-like polypeptides. BCF polypeptides are present in pre-B and mature B cells but not in pro-B cells (1, 25). To determine exactly which of the class I HLH proteins are present in BCF-1 and BCF-2, we have generated monoclonal antibodies specific for both E2-2 and E2A. We demonstrate that E2A proteins bind the E2 box in pre-B (Nalm-6 and 697) and mature B (Namalwa and EW) cells. However, binding of E2-2 to the E2 box can be detected only in pre-B cells.

Interestingly, the level of E2-2 is dramatically reduced in mature B cells compared with that in pre-B cells, whereas the level of E2A proteins increases. This change in the E2A/E2-2 ratio during B-cell development may explain why there are no E2-2 polypeptides present in the BCF-1 and

BCF-2 complexes in mature B cells. It is likely that there exists a competition between E2-2 and E2A proteins for binding to the E2-box site. In pre-B cells, where the level of E2-2 is relatively high, the E2-2 protein may be able to efficiently compete with the E2A proteins for binding to the E2-box sites. The result is that both E2-2 and E2A subunits are present in the BCF complexes. However, in mature B cells, where the levels of E2-2 are much lower than the levels of the E2A proteins, E2-2 is no longer able to compete for binding. The result is little or no E2-2 present in BCF-1 and BCF-2 in mature B cells. In fact, the ratio of E2A to E2-2 is approximately 4.5 in pre-B cells, compared with 34 in mature B cells. This is a seven- to eightfold difference in the E2A/E2-2 ratio between the two different stages of B-cell development. We suggest that the difference in the E2A/E2-2 ratio in pre-B and mature B cells may explain the differences in subunits binding to the E2 box.

The growing family of HLH proteins and the formation of heterodimers obviously offers an enormous scope of complexity. As a first approach to determine the role of the various class I HLH proteins in regulating Ig gene expression, we have generated monoclonal antibodies that are specific for two class I HLH proteins, E2A and E2-2. We demonstrate here that both E2A and E2-2 bind the E2-box elements in pre-B cell lines, whereas in mature B cells only E2A subunits bind the E2 box. Recently, it has been shown that the E2A proteins are not essential for the differentiation of embryonic stem cells into cardiac and skeletal muscle, neurons, cartilage, and erythrocytes (33). However, it is possible that these proteins regulate lymphoid development in vivo. The next step should be to fully assess the roles of E2A and E2-2 in regulating Ig gene expression during B-cell differentiation by mutant mice in which the E2A and E2-2 genes are inactivated by targeted gene disruption.

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