MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 6185–6191
0270-7306/91/126185-07$02.00/0

Vol. 11, No. 12

Repression of Immunoglobulin Enhancers by the Helix-Loop-Helix Protein Id: Implications for B-Lymphoid-Cell Development

ROBERT B. WILSON,[1] MEGERDITCH KILEDJIAN,[1] CHUN-PYN SHEN,[1] ROBERT BENEZRA,[2] PATTY ZWOLLO,[3] SUSAN M. DYMECKI,[3] STEPHEN V. DESIDERIO,[3] AND TOM KADESCH[1]*

Howard Hughes Medical Institute and the Department of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6072[1]; Department of Cell Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021[2]; and Howard Hughes Medical Institute and the Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205[3]

Received 31 July 1991/Accepted 25 September 1991

It has been proposed that the helix-loop-helix (HLH) protein Id serves as a general antagonist of cell differentiation by inhibiting bHLH (HLH with an adjacent stretch of basic amino acids) proteins specifically required for developmental programs (such as MyoD). We show here that ectopic expression of Id represses in vivo activity of the bHLH protein E2-5 (encoded by the E2A gene) and of both the immunoglobulin heavy-chain (IgH) and kappa-light-chain gene enhancers to which E2-5 binds. Id does not affect the activity of the bHLH-zip protein, TFE3, which also binds these enhancers. We examined a large panel of B-cell lines that represent different stages of lymphoid development and found only two that express Id mRNA. The cell lines Ba/F3 and LyD9 have been categorized previously as early B-lymphoid-cell progenitors. Unlike their more mature B-lymphoid-cell counterparts, Ba/F3 and LyD9 cells do not express Iμ sterile transcripts, which are indicative of IgH enhancer activity. Moreover, Ba/F3-derived nuclear extracts lack E2-box-binding activity, indicating the absence of free bHLH proteins, and transfected Ba/F3 cells fail to support the activity of the IgH enhancer. Hence, expression of Id correlates inversely with bHLH protein activity and enhancer function in vivo. These results suggest that Id may play a role early in B-lymphoid-cell development to regulate transcription of the IgH locus.

The helix-loop-helix (HLH) motif is found in a variety of proteins involved in transcriptional and/or developmental regulation (4, 5, 9, 11, 29, 42, 43, 46, 52, 60, 62). It mediates heteromeric interactions between many of these proteins and, in conjunction with an adjacent stretch of basic amino acids (bHLH), is essential for DNA binding (18, 43, 44, 61). Recently, three developmentally regulated HLH proteins that lack these basic amino acids have been described. These proteins, *Drosophila* EMC (encoded by the *extramacrochaetae* gene) and mammalian Id and HLH462, repress activity of other bHLH proteins (6, 13, 21, 23). Experiments with Id indicate that it inhibits DNA binding of MyoD and of the immunoglobulin enhancer-binding proteins E12 and E47 by forming HLH-mediated heteromeric complexes. Ectopic expression of Id has also been used as an indicator of bHLH protein involvement in insulin (16) and kappa 3′ (51) enhancer activity.

The immunoglobulin heavy-chain (IgH) and kappa-light-chain (Igκ) gene enhancers both bind at least two structurally distinct HLH transcription factors. One of these is of the bHLH class and is represented collectively by proteins encoded by the E2A gene and produced through alternative splicing: E2-5 (previously ITF-1), E47, and E12 (29, 33, 43, 47, 59). All three of these related proteins bind the μE5 and κE2 motifs. The HLH regions of E2-5 and E47 are the same; the proteins differ only in what may be a reflection of alternate first-exon usage (30, 33). Another HLH transcription factor, TFE3, is of the bHLH-zip class and binds the μE3 motif (4). Although E2A and TFE3 transcripts are ubiquitously expressed, overexpression of both E2-5 and TFE3 is required to activate the IgH enhancer in nonlymphoid cells (53).

Little is known concerning how or when the IgH enhancer is activated during B-lymphocyte development; this contrasts sharply with what is known about activation of the Igκ enhancer. Igκ enhancer activity appears to be regulated primarily through the activation of NF-κB at a point that defines the boundary between pre-B cells and B cells (38, 56). IgH enhancer activity can be detected in pre-B, B, and plasma cells (all of these cell types express rearranged IgH genes) and in some T-cell lines (27). In addition to activating transcription of rearranged genes, the IgH enhancer serves as the promoter of Iμ sterile transcripts (39, 58). Hence, sterile transcription can also be used as an indicator of IgH enhancer activity. Sterile transcripts have been observed in a variety of pro-B cells, including some that actively carry out heavy-chain gene rearrangement (pro-B cells are those that have not yet undergone heavy-chain rearrangement and, therefore, do not express a cytoplasmic μ heavy-chain protein), and in some T-cell lines (including those that support activity of a transfected enhancer-dependent transcription unit) (27). The rare B-cell lines that have been postulated to be more developmentally immature than Iμ-positive pro-B cells have not been characterized with respect to enhancer activity and/or Iμ transcription.

In this report, we examine the relationship of Id expression to IgH enhancer activity and to B-lymphoid-cell development. Our results show that Id can inhibit the in vivo activity of the E2A-encoded enhancer-binding protein E2-5 but not of TFE3, a bHLH-zip protein. When Id is transfected into B cells, it can also inhibit activity of both IgH and Igκ gene enhancers. Id expression appears to be limited to cells representing very early stages of lymphoid or myeloid

* Corresponding author.

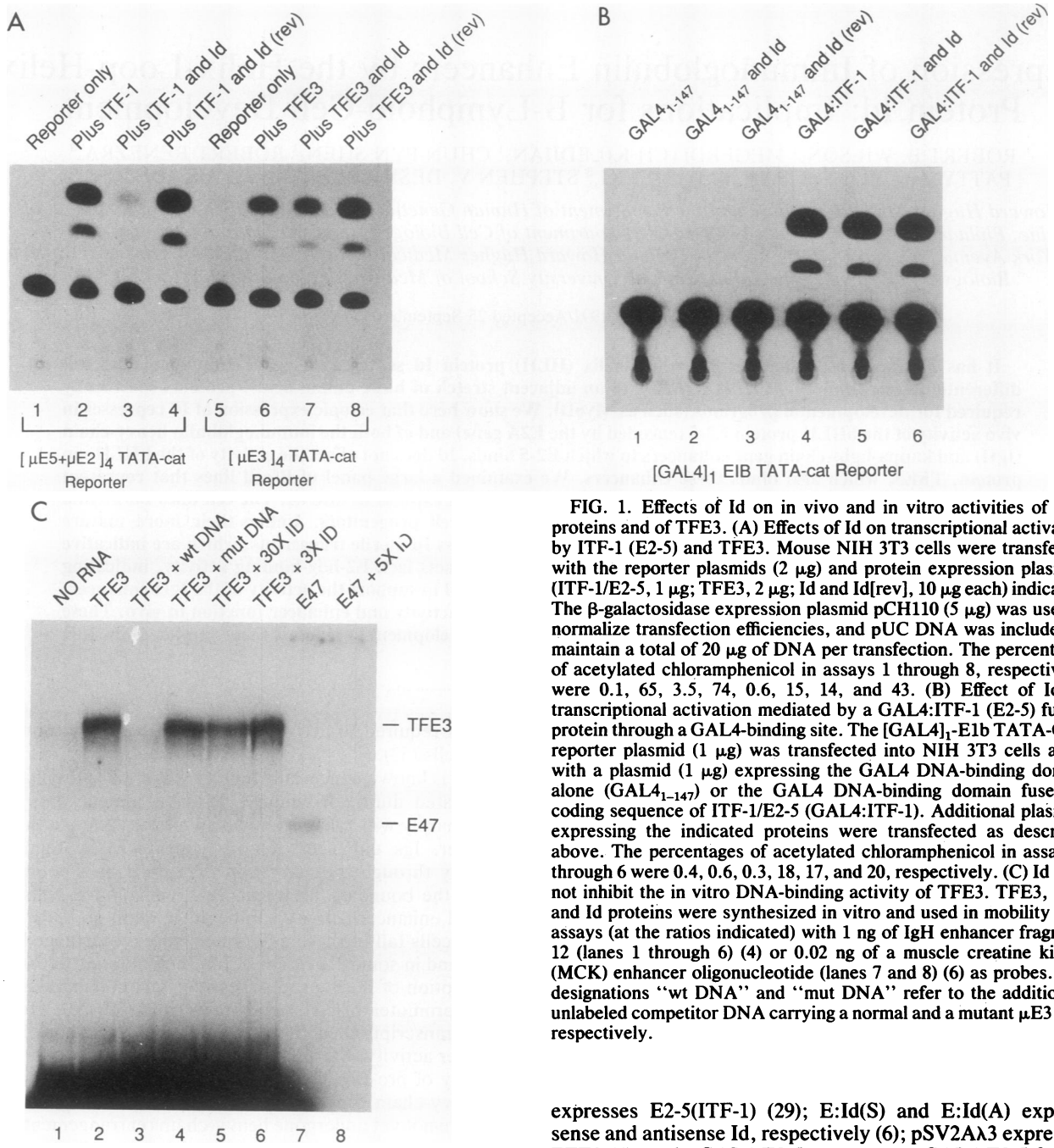

FIG. 1. Effects of Id on in vivo and in vitro activities of E2A proteins and of TFE3. (A) Effects of Id on transcriptional activation by ITF-1 (E2-5) and TFE3. Mouse NIH 3T3 cells were transfected with the reporter plasmids (2 μg) and protein expression plasmids (ITF-1/E2-5, 1 μg; TFE3, 2 μg; Id and Id[rev], 10 μg each) indicated. The β-galactosidase expression plasmid pCH110 (5 μg) was used to normalize transfection efficiencies, and pUC DNA was included to maintain a total of 20 μg of DNA per transfection. The percentages of acetylated chloramphenicol in assays 1 through 8, respectively, were 0.1, 65, 3.5, 74, 0.6, 15, 14, and 43. (B) Effect of Id on transcriptional activation mediated by a GAL4:ITF-1 (E2-5) fusion protein through a GAL4-binding site. The $[GAL4]_1$-E1b TATA-CAT reporter plasmid (1 μg) was transfected into NIH 3T3 cells along with a plasmid (1 μg) expressing the GAL4 DNA-binding domain alone ($GAL4_{1-147}$) or the GAL4 DNA-binding domain fused to coding sequence of ITF-1/E2-5 (GAL4:ITF-1). Additional plasmids expressing the indicated proteins were transfected as described above. The percentages of acetylated chloramphenicol in assays 1 through 6 were 0.4, 0.6, 0.3, 18, 17, and 20, respectively. (C) Id does not inhibit the in vitro DNA-binding activity of TFE3. TFE3, E47, and Id proteins were synthesized in vitro and used in mobility shift assays (at the ratios indicated) with 1 ng of IgH enhancer fragment 12 (lanes 1 through 6) (4) or 0.02 ng of a muscle creatine kinase (MCK) enhancer oligonucleotide (lanes 7 and 8) (6) as probes. The designations "wt DNA" and "mut DNA" refer to the addition of unlabeled competitor DNA carrying a normal and a mutant μE3 site, respectively.

development. We have shown that early progenitor cells neither contain free bHLH proteins nor express the IgH enhancer; these results are consistent with the fact that Id is functional in these cells. Hence, the down-regulation of Id may be necessary at an early stage for cells to activate the IgH locus and to enter into a B-lymphoid-cell developmental program.

MATERIALS AND METHODS

Plasmids and transfections. All in vivo expression plasmids have been described previously as follows. pSV2βE2-5 expresses E2-5(ITF-1) (29); E:Id(S) and E:Id(A) express sense and antisense Id, respectively (6); pSV2Aλ3 expresses TFE3 (4); and pGAL4:E2-5 expresses a GAL4:E2-5 fusion protein (29). The following minimal promoter-chloramphenicol transferase (CAT) reporter plasmids have also been described previously as follows: $p[E5+E2]_4$TATA:CAT (four E2-5-binding sites linked to the liver bone kidney alkaline phosphatase TATA box [29]), $p[E3]_4$TATA:CAT (four TFE3-binding sites linked to the liver bone kidney alkaline phosphatase TATA box [4]), and [GAL4]E1b TATA:CAT (one GAL4-binding site linked to the adenovirus E1b TATA box [40]). Reporter plasmids containing the IgH enhancer (34) and Igκ enhancer (3) have also been described.

NIH 3T3 cells and P3-X63Ag8 cells were transfected by the calcium phosphate coprecipitation technique of Graham and van der Eb (25) as outlined in the works of Beckmann et

al. (4) and Kiledjian et al. (34), respectively. Ba/F3 cells (the gift of R. Palacios, Basel Institute of Immunology) and LyD9 cells (the gift of T. Kinashi, Harvard Medical School) were both grown in RPMI 1640 supplemented with 10% fetal bovine serum and 10% WEHI-3-conditioned medium (as a source of interleukin-3) (49). Ba/F3 cells were transfected by electroporation (500 V/cm, 1,080 μF) (14). Cells were harvested and assayed for CAT expression (24) 2 days after transfection. Transfection efficiencies were normalized to relative expression levels of either β-galactosidase or placental alkaline phosphatase by including plasmids pCH110 (28) or pSV2Apap (31), respectively.

RNA analyses. Northern (RNA) analyses were carried out with 20 μg of total cellular RNA by standard procedures (2).

Nuclear extracts and mobility shift assays. Nuclear extracts of Ba/F3, HAC6, BFO3, and 3T3 cells were prepared by using the procedure of Schreiber et al. (55), modified to include the following protease inhibitors in the nuclear resuspension buffer: leupeptin (10 μg/ml), pepstatin A (1 μg/ml), aprotinin (1 mg/ml), and PMSF (phenylmethylsulfonyl fluoride) (1 mM). BJA-B nuclear extracts were prepared by the method of Dignam et al. (19).

Mobility shift assays were carried out with oligonucleotide probes, as previously described (53), carrying either a μE5 site (μE5 + μE2mut oligo) or a μE3 site (μE3 oligo). Oligonucleotides were radiolabeled by filling 3′ overhangs with DNA polymerase I (large fragment) plus dCTP, dTTP, [α-^{32}P] dATP, and [α-^{32}P]dGTP. Binding reactions (15 μl) contained 1 to 2 μl of nuclear extract, 0.5 ng of labeled oligonucleotide (plus 25 ng of wild-type or mutant competitor, if used), and 2 μg of poly(dI · dC) in 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH (pH 7.9)–4 mM Tris-HCl (pH 7.9)–60 mM KCl–1 mM EDTA–1 mM dithiothreitol–12% glycerol. Samples were resolved on 5% acrylamide gels (29:1, acrylamide to bisacrylamide) at room temperature with 0.5× TBE (45 mM Tris-borate, 1 mM EDTA [pH 8]).

Mobility shift assays utilizing in vitro-translated TFE3 (expressed from pT7β-λ3) (4), E47, and Id were carried out essentially as described previously (6).

RESULTS

Id inhibits activity of E2A proteins in vivo. As shown in Fig. 1A, transfection of NIH 3T3 cells with a plasmid that expresses E2-5 resulted in transcriptional activation of a minimal promoter carrying μE5-binding sites (lanes 1 and 2). However, in the presence of a plasmid that expresses Id, this transcriptional stimulation was reduced 20-fold (lane 3). Repression was not observed with a plasmid that carries the Id cDNA in reverse orientation [Id(rev)] (lane 4). Transcriptional activation of a minimal promoter that carries a GAL4-binding site by a fusion protein consisting of the DNA-binding domain of yeast GAL4 (amino acids 1 through 147) and intact E2-5 was not repressed by Id (Fig. 1B). We conclude that Id inhibits in vivo activity of E2-5 by forming heteromeric complexes that are unable to bind DNA. When these heteromeric complexes are allowed to bind through an alternate DNA-binding motif (GAL4), transcriptional activation is not affected. Id had no effect on the ability of TFE3 to stimulate transcription of a reporter gene through its cognate μE3 motif (Fig. 1A, lanes 5 through 8), nor was Id able to inhibit DNA binding of in vitro-translated TFE3 (Fig. 1C). Hence, Id does not inhibit the activity of all HLH proteins.

It is known that E2-5 (and TFE3) can stimulate activity of the IgH enhancer in transfection assays (53). However, it is not known whether bHLH proteins, in general, or E2A proteins (E2-5, E12, and E47), in particular, contribute to the enhancer activity normally observed in B cells. To address this issue, we examined the effect of Id on IgH enhancer activity in B cells. As shown in the histogram depicted in Fig. 2, transfection of the plasma cell line P3-X63Ag8 with a plasmid that expresses Id resulted in a roughly twofold reduction in the activity of a transcription unit whose expression is entirely dependent on the IgH enhancer (columns 2 and 3); increasing the amount of Id in the transfections did not give a further reduction in activity (data not shown). A plasmid that expresses antisense Id had no inhibitory effect [Id(rev)] (column 4). As expected from the binding specificity of E2-5 (it binds to μE5 and to a lesser extent to μE2) (53), an enhancer carrying a deletion that removes the μE1, μE2, and μE5 motifs was not inhibited by Id (data not shown). We conclude that bHLH proteins, possibly those encoded by the E2A gene, contribute to IgH enhancer activity in mature B cells.

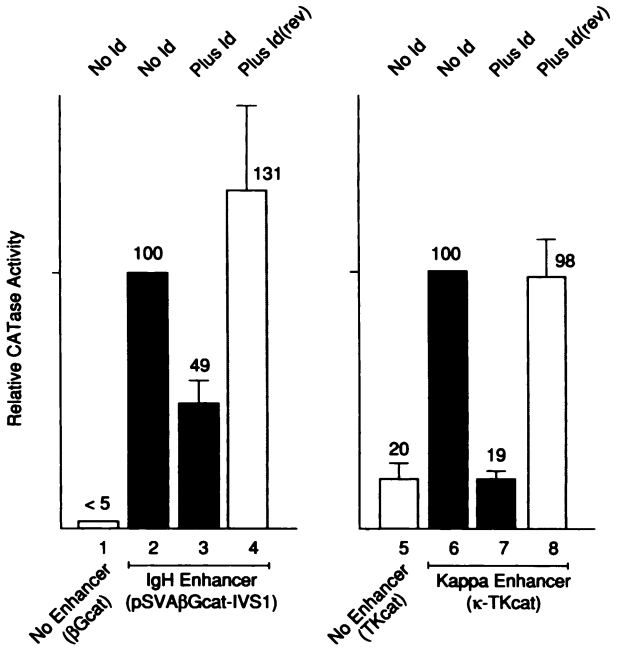

FIG. 2. Id inhibits activity of the IgH and Igκ enhancer in B cells. Plasmids (10 μg) carrying the IgH or Igκ enhancer linked 3′ to a β-globin-CAT transcription unit or a thymidine kinase-CAT transcription unit, respectively, were transfected into P3-X63Ag8 cells. In addition, cells were transfected with plasmids (80 μg) expressing Id or antisense Id [Id(rev)] as indicated. The placental alkaline phosphatase expression plasmid pSV2Apap (10 μg) was included to normalize transfection efficiencies, and pUC DNA was added to maintain a total of 100 μg of DNA per transfection. CAT values for each reporter in the absence of Id or Id(rev) were set equal to 100, and the effects of Id and Id(rev) for each reporter are indicated as the numerical mean relative to that standardized value. Bars represent standard errors.

E2A proteins also bind the κE2 motif within the Igκ enhancer, and, as expected, this enhancer was also repressed by Id (Fig. 2, columns 6 and 7). Id had a much more deleterious effect on the Igκ enhancer than it did on the IgH enhancer and reduced Igκ enhancer activity to undetectable levels in our assays. Mutations of the κE2 motif have been shown to depress Igκ activity 10-fold (38). Hence, the pronounced effect of Id could be explained simply by its

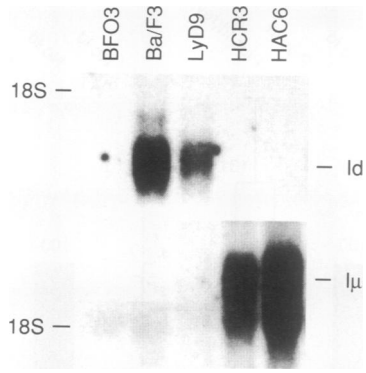

FIG. 3. Expression of Id in B-lymphoid-cell lines correlates inversely with endogenous IgH enhancer activity. Total RNA from mature B cells (BFO3) and pro-B cells (Ba/F3, LyD9, HCR3, and HAC6) were subjected to Northern analysis by using probes corresponding to the Id cDNA (top panel) and the Cμ constant region (lower panel). BFO3 cells express IgA and have deleted sequences corresponding to Cμ. Quantitation of imput total RNA was determined spectrophotometrically and confirmed by ethidium bromide staining of 18S and 28S RNAs. In addition to the cell types indicated, Id mRNA was not detected in the following cell types: for pro-B-cell lines, HAFTL-1-14.5, HAFTL-1-14.6, HS1C5, H58C1, JP2, JP4, JP7, 2.1A3753, FE1NC3, FE2NC1, KFFTL-1, BAFTL-1, BASC2/C6, 22D6.5, and 38B9.7; for pre-B-cell lines, PD31 and 70Z/3; for mature B-cell lines, 1-7, M12.14.5, A20/2J, and LK; for plasma cell lines, SP2/0 and S194; and for T-lymphoid-cell lines, CTLL, HT-2, EL4, R1.1, and 2B4.11. All of these latter cell types are described in the work of Dymecki et al. (20).

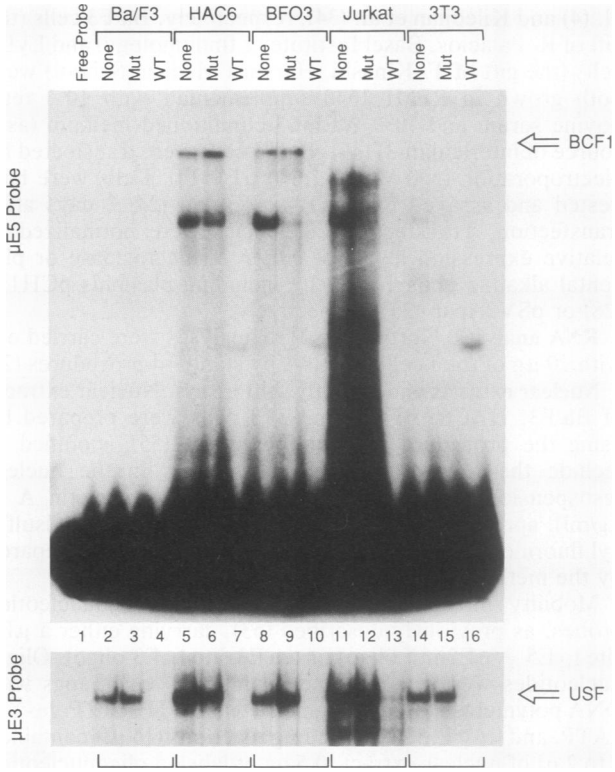

FIG. 4. Ba/F3 cells do not express BCF1 bHLH protein activity. Mobility shift assays were carried out with the nuclear extracts indicated and either a μE5 probe (top panel) or a μE3 probe (detecting upstream stimulatory factor [USF]; bottom panel). Competitions were carried out with a 50-fold molar excess of unlabeled wild-type (WT) or mutant (Mut) oligonucleotide.

abrogating the binding of bHLH proteins to the κE2 motif. However, since the NF-κB-binding site is also known to be crucial for Igκ enhancer activity (38), we tested directly whether Id can inhibit activity of a minimal promoter linked to two NF-κB-binding motifs (58). Although this promoter showed a high level of κB-dependent activity in B cells, it was not inhibited by Id (data not shown).

Id expression is limited to early B lymphoid progenitor cells. We next sought to determine whether Id is down-regulated during B-lymphoid-cell development. It has been reported previously that Id is expressed in a variety of cell types, including pre-B (B18-8) and plasma (B16) cell lines (6). However, levels of Id RNA vary widely from cell type to cell type, and those reported for B-lineage cells were found to be exceedingly low. On the basis of our present findings that Id inhibits both the IgH and Igκ enhancers, it is unlikely that such low-level expression is functionally significant. We therefore extended the analysis by looking at Id expression in a large number of cell lines representing different stages of lymphoid-cell development. In our study, Id mRNA was not detected in a variety of plasma, mature B-, pre-B-, and T-cell lines (data not shown; for a complete list of cell lines tested, see Fig. 3 legend). It was, however, detected in 2 of the 19 pro-B-cell lines, designated Ba/F3 and LyD9, that we examined (Fig. 3). The level of Id mRNA in the Ba/F3 cell line, for example, is equivalent to that in murine erythroleukemia (MEL) cells (data not shown) and is, therefore, at least 20 to 50 times that reported previously for the B18-8- and B16-lymphoid-cell lines (6).

The Id-positive Ba/F3 and LyD9 cell lines have been reported to represent early lymphoid and/or early myeloid progenitor cells. Ba/F3 was initially grouped with the Ba/C1 cell line (49) as being interleukin-3 dependent and B-220 positive. However, unlike a variety of cell lines that were isolated in the same screen, Ba/F3 cells, like Ba/C1 cells, were unable to undergo B-cell maturation when they were introduced into irradiated mice (47a). We have noted that the Ba/F3 cells in our hands no longer express the B-220 marker (generally associated with B cells) (15), do express Mac-1 (generally associated with myeloid cells and certain B-cell lymphomas) (17, 57), are still interleukin-3 dependent, and are blastlike in appearance (data not shown). LyD9 cells have been shown to undergo B-cell maturation when they are introduced into irradiated mice (49) and when they are cocultured in vitro with stromal cells (35).

Ba/F3 cells do not contain E2-box-binding (bHLH) activity or support IgH enhancer activity. If Ba/F3 cells express functional Id protein, then one would not expect free bHLH dimers to exist in the cells. Specifically, one would not expect to find μE5-binding activity. Accordingly, nuclear extracts were made from Ba/F3 cells and from a variety of other cell types representing more mature B-lineage cells (HAC6, HCR3, and BFO3), T cells (Jurkat), and fibroblast cells (3T3), and mobility shift assays were carried out with a labeled μE5 oligonucleotide (Fig. 4, top panel). Extracts of HAC6 cells (pro-B; lanes 5 through 7), HCR3 (pro-B; data not shown), and BFO3 cells (mature B; lanes 8 through 10) gave rise to a shifted complex, denoted here as BCF1. Two closely-migrating complexes, designated previously as BCF1 and BCF2, have been shown to be B-cell specific and to contain E2A protein epitopes (45). As expected for BCF1, the complex we detected was not observed in the presence of excess by a homologous cold oligonucleotide (WT; lanes

TABLE 1. IgH enhancer activity in reporter gene transfections of P3-X63Ag8 and Ba/F3 cells[a]

Reporter	Activity (relative units) in:	
	P3-X63Ag8	Ba/F3
pSV2ACAT (simian virus 40 enhancer)	100	100
pSVAβGCAT-IVS1 (IgH enhancer)	470 ± 150[b]	9 ± 2
pSVAβGCAT (no enhancer)	8 ± 1	4 ± 2

[a] Transfections of P3-X63Ag8 and Ba/F3 cells were carried out by using 20 μg of pSV2ACAT and 80 μg each of pSVAβGCAT and pSVAβGCAT-IVS1. Transfections were normalized to alkaline phosphatase levels obtained by using 10 μg of pSV2Apap, and total DNA per transfection was brought to 100 μg with pUC19.

[b] Values represent the means and standard errors of the mean derived from four separate transfections.

7 and 10) and not by a mutant oligonucleotide (Mut; lanes 6 and 9). Furthermore, the complex was not detected in Ba/F3, Jurkat, or 3T3 cells (lanes 1 through 3, 1 through 13, and 14 through 16, respectively). All extracts gave rise to roughly equivalent levels of upstream stimulatory factor activity (binding to a μE3 probe; lower panel). Previous studies by Murre et al. (45) have also shown that LyD9 cells, as well as Ba/F3 cells, lack BCF1- (and BCF2-) binding activity.

Ba/F3 cells were assayed for IgH enhancer activity by using reporter gene transfections and by measuring Iμ sterile transcription. When an IgH enhancer-dependent reporter (pSVAβGCAT-IVS1) (34) was transfected into Ba/F3 cells, no activity could be detected although these cells expressed a simian virus 40 enhancer-responsive reporter (pSV2A CAT) perfectly well (Table 1). These results contrast with those obtained by using a plasma cell line (P3-X63Ag8) in which the IgH enhancer-driven activity was approximately four times that of the simian virus 40 control. Transfections of LyD9 cells resulted in high levels of enhancer-independent transcription from these reporters, and, hence, we were unable to confirm these results in LyD9 cells.

Although Iμ sterile transcripts could be detected in two of the B-cell lines not expressing Id mRNA, none were found in Ba/F3 and LyD9 cells (Fig. 3) (note that BFO3 cells express IgA and would not be expected to give rise to an RNA that hybridizes with the μ constant region probe used in this experiment). Given that Iμ transcription is a direct result of IgH enhancer activity (58), we conclude that the endogenous IgH enhancers in Ba/F3 cells and LyD9 cells are inactive.

DISCUSSION

On the basis of their physical properties, HLH proteins can be subdivided into three groups, designated bHLH, bHLH-zip, and dnHLH (dn stands for dominant negative). The bHLH proteins bind DNA and are characterized by their ability to form heterodimers promiscuously. Members of this class include the E2A proteins, MyoD, myogenin, daughterless proteins, and proteins encoded by the *achaete-scute* complex of *Drosophila* (43, 44). Given that bHLH heterodimers often bind DNA more avidly than their respective homodimers, it has been proposed that cell-type-specific bHLH proteins (e.g. MyoD, myogenin, *achaete-scute*) must form heterodimers with the more widely expressed bHLH proteins (e.g., E2A and daughterless proteins) to bind DNA and activate transcription in vivo. The bHLH-zip proteins are characterized by the presence of a leucine zipper positioned adjacent to HLH helix 2. Members of this group include TFE3, TFEB, USF, AP4, members of the Myc family of proteins, and Max (4, 8, 10, 26, 32). These proteins bind DNA but do not heterodimerize promiscuously. Although Max forms heterodimers with c-Myc, L-Myc and N-Myc, other members of this group—TFE3, USF, and AP4—are thought to function only as homodimers. The HLH-proximal leucine zipper has been shown to dictate the restricted dimerization specificity in these proteins (4, 32). The dnHLH proteins heterodimerize with bHLH proteins, are unable to bind DNA and, as heterodimers, render bHLH proteins unable to bind DNA. The results presented here concerning Id and TFE3 confirm that they do not interact with bHLH-zip proteins. The dnHLH proteins are represented thus far by Id, HLH462, and EMC (6, 13, 21, 23). On the basis of the initial studies with Id, the genetics of *emc*, and the profile of HLH462 expression during embryonic development, it is possible that dnHLH proteins serve as general antagonists of the differentiated state by inhibiting development-specific bHLH proteins.

The results presented in this report demonstrate an inverse correlation between Id expression and IgH enhancer activity. This was measured in several ways. First, transfection assays demonstrated that Id can inhibit in vivo activity of E2-5, an E2A-encoded protein capable of stimulating the IgH enhancer. This result was expected, given that Id has already been shown to inhibit DNA binding of the related E47 protein in vitro (6). Second, Id overexpression in mature B cells was shown to inhibit activity of both IgH enhancer- and Igκ enhancer-dependent transcription. It is likely that this was the direct consequence of Id inhibiting the activity of endogenous E2A-containing homodimers and/or heterodimers which are required for enhancer activity in B cells. Third, we only detected Id expression in the very early hematopoietic progenitor cell lines, Ba/F3 and LyD9. A variety of other cell lines, representing more mature B cells and including 17 other pro-B-cell lines, were found not to express the normal Id message. Several of the pro-B cells were shown to express Iμ sterile transcripts and, thus, indicated that their endogenous IgH enhancers were active. Ba/F3 cells expressed neither exogenous nor endogenous IgH enhancers. Furthermore, unlike the pro-B cells tested, Ba/F3 cells did not contain detectable levels of BCF1, a B-cell-specific E2-box-binding activity known to contain E2A proteins (45).

In cotransfection experiments, we found that Id reduced IgH enhancer activity by 50%; it is, therefore, unlikely that the almost complete absence of enhancer activity in Ba/F3 cells is due to Id expression alone. We have previously reported that in 3T3 cells, the μE5 site inhibits the ability of TFE3 to activate transcription through the μE3 site, which is situated 30 bp away (53). Presumably, 3T3 cells contain a repressor that binds the μE5 site and is able to inhibit activity mediated through other sites. Ba/F3 cells apparently carry the same (or a related) repressor, because we could not obtain transcriptional activation with TFE3 in the presence of an intact μE5 site (61a). Hence, enhancer activity in Ba/F3 cells may be inhibited both by Id and by a pleiotropic repressor that binds to the μE5 site in place of the Id-sequestered bHLH protein. Furthermore, levels of E2A RNA in Ba/F3 and LYD9 cells are lower than those found in the more mature cell lines (data not shown). This suggests that activation of the enhancer may require concomitant Id down-regulation and E2A up-regulation, a situation that parallels that observed with Id and MyoD during myoblast differentiation (6). Given that overexpression of E2-5 is able

to activate an IgH enhancer-dependent reporter in Ba/F3 transfections, it is likely that Id does not exist in vast excess (data not shown).

We propose that full activation of the IgH enhancer requires Id down-regulation and that this represents a very early event in B-lymphoid-cell differentiation. Ba/F3 and LyD9 cells represent a developmental stage prior to Id shutoff. The fact that our clone of Ba/F3 cells has lost B-220 expression (generally indicative of B cells) (15) and has increased Mac-1 expression (generally associated with myeloid cells) (57) does not invalidate this conclusion. It is known that LyD9 cells, which are B-220 positive and Mac-1 positive, can generate both mature B lymphoid (IgM-secreting) and myeloid subclones (35, 36, 48). LyD9 cells have also been reported to lack BCF1 and BCF2 activities (45), possibly because they also express Id. The early distinctions that separate myeloid precursors from lymphoid precursors are likely to be quite fluid and not to be discriminated by Id expression per se. In fact, we have recently shown that differentiation of a myeloid cell line, 32D, is accompanied by a transient decrease in Id expression and can be blocked by the ectopic expression of Id (37). Hence, myeloid and B-lymphoid cell developmental pathways may share a number of common features, including an obligatory shutdown of Id expression.

Although our data demonstrate an appropriate correlation, they do not address whether E2A-containing complexes (e.g., BCF1 and BCF2) are responsible for explicitly directing the early phases of lymphoid cell development. It is almost certain that such proteins stimulate immunoglobulin gene transcription in pre-B, B, and plasma cells. Given the possible role that sterile transcription plays in immunoglobulin gene rearrangement (1, 7, 22, 54), it is likely that these proteins indirectly affect DNA recombination as well. Immunoglobulin gene rearrangement is central to the ultimate function of a B cell, and, therefore, the E2A proteins in B cells may indeed be loosely considered master regulatory proteins. However, it is not known whether expression of these proteins initiates a cascade of transcriptional regulatory events, similar to what occurs with the bHLH proteins involved in myogenesis. Furthermore, it is not clear whether the BCF complexes consist of E2A homodimers or of heterodimers between E2A and B-cell-specific MyoD-like proteins. Present information on the BCF complexes indicates that they consist of E2A homo-oligomers (45). Support for this stems from two observations. First, BCF1 and BCF2 together comprise a mobility shift pattern that is very similar to that obtained with transfected or in vitro-translated E47 homodimers, and second, E2A antiserum is highly reactive with the BCF complexes and with E47 homodimers but is much less reactive toward MyoD:E47 heterodimers. Additional experiments are needed to confirm the composition of the BCF complexes and to address their role in directing B-cell development.

ACKNOWLEDGMENTS

R.B.W. and M.K. contributed equally to this work.

We thank Harold Weintraub for critical reading of the manuscript.

This work was supported by funds (to T.K. and S.V.D.) from the Howard Hughes Medical Institute.

REFERENCES

1. **Alessandrini, A., and S. V. Desiderio.** 1991. Coordination of immunoglobulin DJ_H transcription and D-to-J_H rearrangement by promoter-enhancer approximation. Mol. Cell. Biol. **11**:2096–2107.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
3. **Atchison, M. L., and R. P. Perry.** 1988. Complementation between two cell lines lacking κ enhancer activity: implications for the developmental control of immunoglobulin transcription. EMBO J. **7**:4213–4220.
4. **Beckmann, H., L.-K. Su, and T. Kadesch.** 1990. TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. Genes Dev. **4**:167–179.
5. **Begley, C. G., P. D. Aplan, S. M. Denning, B. F. Haynes, T. A. Waldmann, and I. R. Kirsch.** 1989. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc. Natl. Acad. Sci. USA **86**:10128–10132.
6. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
7. **Blackwell, T. K., M. W. Moore, G. D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F. W. Alt.** 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature (London) **324**:585–589.
8. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science **251**:1211–1217.
9. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–832.
10. **Carr, C. S., and P. A. Sharp.** 1990. A helix-loop-helix protein related to the immunoglobulin E box-binding proteins. Mol. Cell. Biol. **10**:4384–4388.
11. **Chen, Q., J.-T. Cheng, L.-H. Tsai, N. Schneider, G. Buchanan, A. Carroll, W. Crist, B. Ozanne, M. J. Siciliano, and R. Baer.** 1990. The tal gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J. **9**:415–424.
12. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
13. **Christie, B. A., L. K. Sanders, L. F. Lau, N. G. Copeland, N. A. Jenkins, and D. Nathans.** 1991. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc. Natl. Acad. Sci. USA **88**:1815–1819.
14. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
15. **Coffman, R. L., and I. L. Weissman.** 1981. B-220: a B cell-specific member of the T200 glycoprotein family. Nature (London) **289**:681–683.
16. **Cordle, S. R., E. Henderson, H. Masuoka, P. A. Weil, and R. Stein.** 1991. Pancreatic β-cell-type-specific transcription of the insulin gene is mediated by basic helix-loop-helix DNA-binding proteins. Mol. Cell. Biol. **11**:1734–1738.
17. **Davidson, W. F., T. N. Fredrickson, E. K. Rudikoff, et al.** 1984. A unique series of lymphomas related to the Lyt1+ lineage of B lymphocyte differentiation. J. Immunol. **133**:744–753.
18. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60**:733–746.
19. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
20. **Dymecki, S. M., J. E. Niederhuber, and S. V. Desiderio.** 1990. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. Science **247**:332–336.
21. **Ellis, H. M., D. R. Spann, and J. W. Posakony.** 1990. Extramacrochaetae, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. Cell **61**:27–38.

22. **Ferrier, P., B. Krippl, T. K. Blackwell, A. J. W. Furley, H. Suh, A. Winoto, W. D. Cook, L. Hood, F. Costantini, and F. W. Alt.** 1990. Separate elements control DJ an dVDJ rearrangement in a transgenic recombination substrate. EMBO J. **9**:117–125.
23. **Garrell, J., and J. Modolell.** 1990. The Drosophila extramacrochaetae locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. Cell **61**:39–48.
24. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
25. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
26. **Gregor, P., M. Sawadogo, and R. G. Roeder.** 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. **4**:1730–1740.
27. **Groschedl, R., and D. Baltimore.** 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell **41**:885–897.
28. **Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of Escherichia coli lac Z fusions in mammalian cells. J. Mol. Appl. Genet. **2**:101–110.
29. **Henthorn, P., M. Kiledjian, and T. Kadesch.** 1990. Two distinct transcription factors that bind the immunoglobulin enhancer mE5/kE2 motif. Science **247**:467–470.
30. **Henthorn, P., R. McCarrick-Walmsley, and T. Kadesch.** 1990. Sequence of the cDNA encoding ITF-1, a positive-acting transcription factor. Nucleic Acids Res. **18**:677.
31. **Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch.** 1988. Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. Proc. Natl. Acad. Sci. USA **85**:6342–6346.
32. **Hu, Y.-F., B. Luescher, A. Admon, N. Mermod, and R. Tijan.** 1990. Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. Genes Dev. **4**:1741–1752.
33. **Kamps, M. P., C. Murre, X. Sun, and D. Baltimore.** 1990. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell **60**:547–555.
34. **Kiledjian, M., L.-K. Su, and T. Kadesch.** 1988. Identification and characterization of two functional domains within the murine heavy-chain enhancer. Mol. Cell. Biol. **8**:145–152.
35. **Kinashi, T., K. Inaba, T. Tsubata, K. Tashiro, R. Palacios, and T. Honjo.** 1988. Differentiation of an interleukin 3-dependent precursor B-cell clone into immunoglobulin-producing cells in vitro. Proc. Natl. Acad. Sci. USA **85**:4473–4477.
36. **Kinashi, T., K. Tashiro, K. Inaba, T. Takeda, R. Palacios, and T. Honjo.** 1989. An interleukin-4-dependent precursor clone is an intermediate of the differentiation pathway from an interleukin-3-dependent precursor clone into myeloid cells as well as B lymphocytes. Int. Immunol. **1**:11–19.
37. **Kreider, B., R. Benezra, T. Kadesch, and G. Rovera.** Submitted for publication.
38. **Lenardo, M., J. W. Pierce, and D. Baltimore.** 1987. Protein-binding sites in Ig-gene enhancers determine transcriptional activity and inducibility. Science **236**:1573–1577.
39. **Lennon, G. G., and R. P. Perry.** 1985. Cμ-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5′-nontranslatable exon. Nature (London) **256**: 495–497.
40. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) **338**:39–44.
41. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
42. **Mellentin, J. D., S. D. Smith, and M. L. Cleary.** 1989. lyl-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. Cell **58**:77–83.
43. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, myoD, and myc proteins. Cell **56**:777–783.
44. **Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lasser, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.
45. **Murre, C., A. Voronova, and D. Baltimore.** 1991. B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. Mol. Cell. Biol. **11**:1156–1160.
46. **Nelson, C., L.-P. Shen, A. Meister, E. Fodor, and W. J. Rutter.** 1990. Pan: a transcriptional regulator that binds chymotrypsin, insulin, and AP-4 enhancer motifs. Genes Dev. **4**:1035–1043.
47. **Nourse, J., J. D. Mellentin, N. Galili, J. Wilkinson, E. Stanbridge, S. D. Smith, and M. L. Cleary.** 1990. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell **60**:535–545.
47a. **Palacios, R.** Personal communication.
48. **Palacios, R., H. Karasuyama, and A. Rolink.** 1987. Lyl+ Pro-B lymphocyte clones. Phenotype, growth requirements and differentiation in vitro and in vivo. EMBO J. **6**:3687–3693.
49. **Palacios, R., and M. Steinmetz.** 1985. IL3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell **41**:727–734.
50. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**:1482–1486.
51. **Pongubala, J. M. R., and M. L. Atchison.** 1991. Functional characterization of the developmentally controlled immunoglobulin kappa 3′ enhancer: regulation by Id, a repressor of helix-loop-helix transcription factors. Mol. Cell. Biol. **11**:1040–1047.
52. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. **3**:2050–2061.
53. **Ruezinsky, D., H. Beckmann, and T. Kadesch.** 1991. Modulation of the IgH enhancer's cell type specificity through a genetic switch. Genes Dev. **5**:29–37.
54. **Schlissel, M. S., and D. Baltimore.** 1989. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell **58**:1001–1007.
55. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. Nucleic Acids Res. **17**:6419.
56. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
57. **Springer, T., G. Galfre, D. S. Secher, and C. Milstein.** 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. **9**:301–306.
58. **Su, L.-K., and T. Kadesch.** 1990. The immunoglobulin heavy-chain enhancer functions as the promoter for Iμ sterile transcription. Mol. Cell. Biol. **10**:2619–2624.
59. **Sun, X.-H., and D. Baltimore.** 1991. An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. Cell **64**:459–470.
60. **Tapscott, S. J., R. L. Davis, M. J. Thayer, P. F. Cheng, H. Weintraub, and A. B. Lassar.** 1988. MyoD: a nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. Science **242**:405–411.
61. **Voronova, A., and D. Baltimore.** 1990. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc. Natl. Acad. Sci. USA **87**:4722–4762.
61a. **Wilson, R., and T. Kadesch.** Unpublished observations.
62. **Wright, W. E., D. A. Sasson, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**:607–617.