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The Epstein-Barr Virus Nuclear Protein 2 Acidic Domain Forms a Complex with a Novel Cellular Coactivator That Can Interact with TFIIE

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Epstein-Barr virus nuclear antigen 2 (EBNA 2) activates transcription of specific genes and is essential for B-lymphocyte transformation. EBNA 2 has an acidic activation domain which interacts with general transcription factors TFIIB, TFIIH, and TAF40. We now show that EBNA 2 is specifically bound to a novel nuclear protein, p100, and that p100 can coactivate gene expression mediated by the EBNA 2 acidic domain. The EBNA 2 acidic domain was used to affinity purify p100. cDNA clones encoding the p100 open reading frame were identified on the basis of peptide sequences of the purified protein. Antibody against p100 coimmunoprecipitated p100 and EBNA 2 from Epstein-Barr virus-transformed lymphocyte extracts, indicating that EBNA 2 and p100 are complexed in vivo. p100 overexpression in cells specifically augmented EBNA 2 acidic domain-mediated activation. The coactivating effect is probably mediated by p100 interaction with TFIIE. Bacterially expressed p100 specifically adsorbs TFIIE from nuclear extracts, and in vitro-translated p56 or p34 TFIIE subunit can independently bind to p100. p100 also appears to be essential for normal cell growth, since cell viability was reduced by antisense p100 RNA and restored by sense p100 RNA expression.

Epstein-Barr virus (EBV) is a human herpesvirus which establishes latent infection in B lymphocytes (for reviews, see references 31 and 32). Latently infected lymphocytes are growth transformed by virus infection, and their proliferation can be lethal in immunocompromised patients. The EBV genome encodes six nuclear proteins (EBNAs) and two integral membrane proteins (LMPs) in latently infected B lymphocytes.

EBNA 2 is one of the first two genes expressed in EBV-infected B lymphocytes (2, 47). In these cells, EBNA 2 activates transcription of the viral genes encoding LMP1 (1, 60) and LMP2 (64) and of the cellular genes CD21 (9), CD23 (56–58), and c-*fgr* (34). Molecular genetic analyses indicate that EBNA 2 is essential for EBV-mediated B-lymphocyte transformation (8, 24). Further analyses define two essential domains in EBNA 2 that are linked to its role as a transcription activator: an acidic activation domain (6, 7) and a domain that interacts with at least two cellular sequence-specific DNA-binding proteins, Jκ (CBF1) and PU.1 (22, 25, 28, 61). EBNA 2 response elements near the LMP1 (17, 55), LMP2A (63), Cp (50), and cellular CD23 (58) promoters usually have nearby Jκ and PU.1 sites. Thus, genetic and biochemical data indicate that EBNA 2 is brought to response elements by interaction with sequence-specific DNA-binding proteins and EBNA 2 then activates transcription through its C-terminal acidic domain (amino acids 426 to 483).

The experiments reported here focus on the identification of cellular proteins that interact with the EBNA 2 acidic domain. The EBNA 2 acidic domain (amino acids 426 to 483) has a core region (amino acids 449 to 462) which has about 25% of the activity of the larger domain (6). Mutation of Trp-454 to Thr (in the protein designated EBNA 2,T_{454}) is a null mutation for transcriptional activation (53). The EBNA 2 core acidic domain can be replaced by the corresponding region of the prototypic VP16 acidic domain (amino acids 439 to 451) (5). Consistent with the functional equivalence of the EBNA 2 and VP16 core acidic domains, the two acidic domains have similar affinities for TFIIB, TAF40, and the p62 subunit of TFIIH (52, 53). However, the EBNA 2 and VP16 acidic domains differ in their interactions with TATA-binding protein (TBP) and the p80/ERCC2 subunit of TFIIH (52, 53). TBP binds much better to the VP16 acidic domain, while p80/ERCC2 binds to the EBNA 2 acidic domain but not to the VP16 acidic domain. Thus, some components of the basal transcription complex interact with both the EBNA 2 and VP16 acidic domains, while others exhibit strong preference for EBNA 2 or VP16.

MATERIALS AND METHODS

Cell cultures and metabolic labeling. P3HR-1 clone 16 cells were obtained from G. Miller, Yale University (46). The P3HR-1 genome is deleted for an EBV DNA segment which encodes the last two exons of EBNA LP and the entire EBNA 2 exon. IB4 is an EBV-transformed B-lymphoblastoid cell line (33). BJAB is an EBV-negative B-lymphoma cell line (43). For metabolic labeling, 10^8 cells were grown to 10^6/ml and labeled overnight with 1 mCi of [^{35}S]methionine (New England Nuclear) in methionine-free RPMI medium (GIBCO BRL) supplemented with 10% dialyzed fetal bovine serum (GIBCO BRL). Cos and CRL1634 (human diploid fibroblast) cells were maintained as adherent cells in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Affinity binding assays. Glutathione *S*-transferase (GST)–VP16 (amino acids 413 to 490) was a kind gift from M. Green (38). GST-EBNA 2 and GST-EBNA 2,T_{454} (amino acids 427 to 483) have been described previously (53). Labeled cells (5×10^7/ml) were lysed in 150 mM NaCl–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0)–1% Nonidet P-40–2 μg of aprotinin per ml–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol–25 mM betaine (Sigma). Insoluble material was removed by centrifugation. The lysates were precleared by incubation with glutathione-Sepharose beads (Pharmacia) loaded with GST. Aliquots of precleared lysates were incubated with Sepharose beads loaded with 2 μg of GST-VP16, GST-EBNA 2, or GST-EBNA 2,T_{454} for 1 h at 4°C. The beads were washed at 4°C in lysis buffer plus 0.1% sodium dodecyl sulfate (SDS). Proteins bound to the beads were eluted with SDS sample

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buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), using 8.5% gels.

V8 peptide analysis. p120 and p100 were adsorbed by GST-EBNA 2 or GST-VP16 beads from ^{35}S-labeled cell extracts, eluted in SDS buffer, and subjected to SDS-PAGE. The proteins were visualized by Coomassie blue staining, and bands of interest were excised. The gel slices were digested in situ with V8 protease from *Staphylococcus aureus* V8 (Sigma) (4). Partially digested peptides were separated by SDS-PAGE and visualized by fluorography.

Purification and protein microsequencing of p100 and p120. A total of 4×10^9 IB4 cells were lysed. After preclearing with GST-coated beads, cell extracts were adsorbed with GST-EBNA 2 fusion protein beads for 1.5 h at 4°C. The beads were washed with lysis buffer plus 0.1% SDS; adsorbed proteins were separated by SDS-PAGE and transferred to nitrocellulose. The bands corresponding to p100 or p120 were excised. Purified p100 and p120 (~10 μg of each) were subjected to trypsin (p100) or endoproteinase Lys-C (p120) digestion, and peptides were microsequenced. Protease digestions, high-pressure liquid chromatography, mass spectroscopy, and peptide sequencing were performed at the Harvard microsequencing facility.

Cloning of p100 cDNA. An oligonucleotide was synthesized on the basis of the peptide sequence of the 3.6-kDa peptide (see Fig. 2) and mammalian codon usage (35): TACACGCCTGTGTTTGTGACAGAGATCACAGATGACCTG CACTTCTATGTGCAGGATGTGGAGACAGGC. The oligonucleotide was used as a probe to screen a cDNA library made from BL41/B95-8 cells (3). Filters were hybridized with the probe at 10^6 cpm/ml in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–100 μg of salmon sperm DNA per ml–0.05% sodium PP_i–1% SDS–20% formamide at 42°C overnight and washed three times for 20 min in 0.5× SSC–0.05% sodium PP_i–1% SDS at 42°C. Positive plaques were replated and plaque purified, and phage cDNA inserts were subcloned into pBluescript II KS (Stratagene).

p100 plasmid constructions and fusion proteins. The *Sac*II-*Msc*I fragment of p100 cDNA from pBluescript II KS was cloned into the *Xho*I site of pET-15b vector (Novagen) to generate a histidine-tagged p100 (His-p100) expression vector for *Escherichia coli* (pET-p100). To construct a glucocorticoid-inducible antisense p100 mammalian cell expression plasmid, the 1.4-kb *Eco*RI-*Hin*dIII fragment of the p100 cDNA was cloned into the *Eco*RI and *Hin*dIII sites of pGRE5-2 vector (41). To construct the inducible sense p100 expression plasmid, p100 cDNA was released from pBluescript KS by *Eco*RI digestion and cloned into the pGRE5-1 vector (41). A constitutive mammalian p100 expression vector, pSG5-p100, was generated by cloning the *Bsr*B1-*Msc*I fragment of the p100 cDNA into pSG5 (Stratagene) which had been modified by insertion of FLAG epitope-encoding DNA (IBI) 5′ to the cloning site. The p100 expressed from this plasmid is truncated for the first 20 amino acids.

GST fusion proteins were expressed in *E. coli* and purified (29). The His-p100 and histidine–β-galactosidase (His-βgal) fusion proteins were made from pET-p100 and pET-βgal (Novagen) expression plasmids and purified (Novagen).

Cloned p100 protein-protein interactions, immunoprecipitations, immunoblotting, and immunofluorescence microscopy. To study the in vitro interaction between cloned p100 and EBNA 2, p100 was ^{35}S labeled by in vitro translation (Promega) and incubated for 1 h with GST-EBNA 2 or GST-EBNA 2,T_{454} in NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) plus 0.5 mg of bovine serum albumin (BSA) per ml. Similarly, full-length EBNA 2 or EBNA 2,T_{454} was ^{35}S labeled and incubated with His-p100 or His-βgal immobilized on an Ni column. To study the interaction between p100 and TFIIE, His-p100 or His-βgal was incubated for 1 h with a TFIIE-enriched nuclear extract (26) or with in vitro-translated p56 or p34 TFIIE subunit (45) in NETN buffer plus 0.5 mg of BSA per ml. Alternatively, ^{35}S-labeled, in vitro-translated p100 was incubated for 1 h with GST-p56 or GST in NETN buffer plus 0.5 mg of BSA per ml. GST-p56 was a kind gift from M. Maxon (13). After incubation, fusion protein beads were washed four times in NETN buffer and subjected to SDS-PAGE. The bound proteins were detected by fluorography (for ^{35}S-labeled proteins) or immunoblotting (for proteins from nuclear extracts).

Rabbit antiserum against p100 was made by immunizing rabbits (Pocono Rabbit Farm & Laboratory) with His-p100 fusion protein. For p100 immunofluorescence, cells were fixed by 1:1 methanol-acetone and rabbit antisera were diluted 1:200. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.) was used at a dilution of 1:1,000. For Western blotting (immunoblotting), the rabbit antiserum was affinity purified and used at a dilution of 1:200.

For p100 immunoprecipitation, cells lysates were precleared by incubation with normal rabbit serum. Aliquots of precleared lysates were then incubated with p100 antibody for 2 h at 4°C and adsorbed to protein A-Sepharose beads for 30 min. The beads were washed in lysis buffer, and bound proteins were eluted with SDS sample buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for EBNA 2 and p100.

To quantitate the efficiency of affinity binding and immunoprecipitation, ^{35}S-labeled samples were quantitated with a PhosphorImager (Molecular Dynamics), and Western blots were scanned and processed by using Adobe Photoshop software. The quantitation was within linear range for all the samples.

Transfections and CAT assays. A total of 10^7 BJAB cells were suspended in 0.4 ml of RPMI 1640 and placed in a Bio-Rad Gene Pulser cuvette. Five micrograms of pUC-βgal, 2.5 μg of Gal-EBNA 2, 1.25 μg of a reporter plasmid with five or no Gal4 binding sites under the control of E1b promoter (6), and various amounts of pSG5-p100 as indicated were added to each cuvette. Cells were electrophorated with 220 V at 960 μF. β-Galactosidase and chloramphenicol acetyltransferase (CAT) activities were assayed after 3 days as described previously (55).

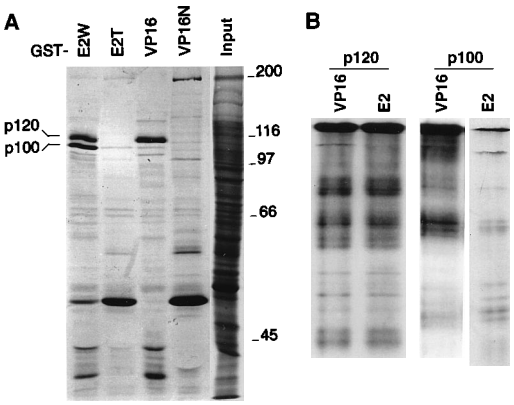

FIG. 1. The EBNA 2 acidic domain specifically binds p120, p100, and other cellular proteins. (A) Aliquots of [^{35}S]methionine-labeled lysates from EBV-transformed human B lymphocytes (IB4 cells) were incubated with beads loaded with the GST-EBNA 2 acidic domain (GST-E2W), with a null mutant GST-EBNA 2,Thr_{454} acidic domain (GST-E2T), with the GST-VP16 acidic domain, or with GST-VP16N. The bound proteins were subjected to SDS-PAGE and visualized by fluorography. The positions of p120 and p100 are indicated. Sizes on the right are indicated in kilodaltons. One percent of input labeled extracts were included (Input). (B) V8 peptide analysis of p120 and p100 adsorbed by EBNA 2 or by VP16. ^{35}S-labeled IB4 cell lysates were incubated with GST-EBNA 2 (E2) or GST-VP16 (VP16). The protein bands corresponding to p120 and p100 were excised after SDS-PAGE, subjected to V8 protease digestion, and visualized by fluorography.

Antisense inhibition of p100. BJAB cell lines stably transfected with antisense p100 were generated by cotransfecting pGRE2-p100 and a plasmid containing a hygromycin resistance gene (SV-Hyg) (59, 62) at a ratio of 10:1. Cells were selected for hygromycin resistance at 400 μg of hygromycin per ml. Resistant clones were screened for antisense p100 by PCR using primers derived from p100 cDNA and vector flanking sequence. The expression of antisense p100 was induced by incubating cells with 25 nM dexamethasone. Cell viability was determined by trypan blue staining.

Nucleotide sequence accession number. The GenBank accession number for p100 is U22055.

RESULTS

Proteins of 100 and 120 kDa associate with the EBNA 2 acidic domain. To study the comparative interactions of the EBNA 2 and VP16 acidic domains with cell proteins, the EBNA 2 acidic domain (amino acids 427 to 483), the null mutant EBNA 2 acidic domain with Trp-454 replaced by Thr (EBNA 2,T_{454}), the VP16 acidic domain (amino acids 413 to 490), or the amino-terminal part of the VP16 acidic domain (amino acids 413 to 456; VP16N) (38, 48) was expressed as a polypeptide fused to the carboxy terminus of GST (29). Equal amounts of these fusion proteins were bound to glutathione-coupled beads and incubated with [^{35}S]methionine-labeled cell extracts. After extensive washing of the beads, proteins were eluted and analyzed by SDS-PAGE and fluorography. As shown in Fig. 1A, GST-EBNA 2 specifically precipitated a number of labeled cell proteins, with the two most prominent proteins being 100 kDa (p100) and 120 kDa (p120). These proteins bound much less to GST-EBNA 2,T_{454}. Proteins of similar size also associated with GST-VP16. While p100 bound much better to GST-EBNA 2 than to GST-VP16, p120 bound similarly to GST-EBNA 2 and GST-VP16. Neither p100 nor p120 bound to GST-VP16N. Furthermore, neither p100 nor p120 was notably abundant in the labeled cell extract. The interactions of the EBNA 2 acidic domain with p100 and p120 were quantitative. Very little p100 was precipitated by GST-

EBNA 2 from cell extracts after three successive rounds of adsorption, and no p120 was precipitated by GST-EBNA 2 after one round of adsorption (data not shown).

To confirm that the p100 and p120 proteins which bind to GST-EBNA 2 and GST-VP16 are the same proteins, ^{35}S-labeled p100 and p120 were affinity purified by GST-EBNA 2 or by GST-VP16 and subjected to V8 protease digestion (Fig. 1B). The p100 proteins retrieved by GST-EBNA 2 or by GST-VP16 had the same V8 digestion patterns, and the p120 proteins retrieved by GST-EBNA 2 or by GST-VP16 also had the same V8 digestion patterns. Thus, the same p100 and the same p120 bind to the wild-type EBNA 2 or VP16 acidic domain.

p100 and p120 were purified from cell extracts by GST-EBNA 2 fusion protein affinity chromatography, the purified proteins were digested with endopeptidase, and peptide fragments were separated and microsequenced. A total of 40 sequenced amino acids from two p120 peptides matched human citrate lyase, an enzyme which catalyzes the synthesis of acetyl coenzyme A from citrate (15, 16). Although citrate lyase is a cytosolic protein which converts citrate to acetyl coenzyme A at the expense of ATP (15, 16), other metabolic enzymes or ATPases have been implicated in the regulation of gene expression. For example, the iron-responsive element-binding protein possesses aconitase activity and regulates the expression of transferrin receptor and ferritin (30); glyceraldehyde-3-phosphate dehydrogenase can bind to tRNA molecules and has been implicated in RNA export from the nucleus (49); the Sug1 ATPase interacts with the VP16 and Gal4 acidic domains and with TBP (51); and its human homolog, the Trip1 putative ATPase, interacts with the thyroid hormone receptor as well as with the VP16 and Gal4 acidic domains (36). The strong association of citrate lyase with both the EBNA 2 and VP16 acidic domains and the poor association with the null mutant EBNA 2,Thr$_{454}$ are evidence that the interaction of p120 with these acidic domains may be of some significance. However, since citric acid lyase has been extensively characterized (15, 16), we focused on p100 for further study.

Identification and cloning of p100 cDNA. A total of 104 amino acid residues of p100 were sequenced with high confidence from 3.6-, 3.5-, 2.3-, 1.9-, and 1.0-kDa trypsin-digested p100 peptides. These peptide sequences did not match any in GenBank. An oligonucleotide which can encode for the 23-amino-acid sequence derived from the 3.6-kDa peptide was synthesized on the basis of the most prevalent mammalian codon usage (35) and was used as a probe to screen a cDNA library made from BL41/B95-8 cells (3). Two cDNA clones were identified. The longer clone had a 3.5-kb insert and was sequenced. The nucleotide and the predicted amino acid sequences are shown in Fig. 2. The cDNA is likely to include the full-length open reading frame, since the mRNA is approximately 4 kb (data not shown) and the cDNA has an in-frame stop codon in the 5′ untranslated region. By the BLAST program, the predicted amino acid sequence had no significant homology to proteins from GenBank as of 8 May 1995. Searches for structural motifs by using the Prosite program revealed only potential phosphorylation sites: 12 sites for protein kinase C, 10 sites for casein kinase II, and 2 sites for tyrosine kinase. Part of the p100 cDNA sequence was found to match two human cDNA sequence tags from a heart cDNA library (37) and a cDNA sequence tag from an HL60 promyelocytic cDNA library (27, 44a).

p100 appears to be ubiquitously expressed, although levels can vary among cell lines and tissues. By Northern (RNA) blotting, a 4-kb p100 mRNA was expressed at similar levels in the pancreas, muscle, liver, lung, placenta, brain, and heart and at a significantly lower level in the kidney (data not shown). Within a two- to threefold range, p100 levels were similar in Burkitt tumor cells, EBV-transformed lymphoblastoid cell lines, and T-lymphocyte, myeloid, fibroblast, epithelial, and osteosarcoma cell lines as examined by Western blotting (data not shown). In EBV-transformed cells, we estimate the amount of p100 by Western blotting to be about 1 to 5 ng/10^6 cells relative to purified bacterially expressed p100.

p100 is a nuclear protein that localizes to specific sites in diploid fibroblasts. By immunofluorescence microscopy, p100 rabbit antibody localized p100 to the nonnucleolar nucleoplasm in Cos, HeLa, or IB4 cells (Fig. 3A and B and data not shown). Interestingly, in actively growing human diploid fibroblasts, p100 was associated with small discrete nuclear granules (Fig. 3C).

EBNA 2 interacts with p100 in vitro and in vivo. To study the interaction between EBNA 2 and cloned p100, p100 was ^{35}S labeled by in vitro translation and was incubated with GST-EBNA 2 or GST-EBNA 2,T$_{454}$. In vitro-translated p100 bound significantly better to GST-EBNA 2 (4% of input) than to GST-EBNA 2,T$_{454}$ (1% of input) (Fig. 4A and data not shown). Similarly, in vitro-translated full-length EBNA 2 bound significantly better to immobilized His-p100 (20% of input) than to His-βgal (2% of input) (Fig. 4B and data not shown). As expected, EBNA 2,T$_{454}$ bound less efficiently to His-p100 (5% of input) and did not bind to His-βgal (<1% of input) (Fig. 4B and data not shown).

To investigate the extent of stable interaction of EBNA 2 and p100 in vivo, cell extracts from EBV-transformed B lymphocytes were immunoprecipitated with an antibody against p100. The immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting for EBNA 2 or p100. As shown in Fig. 4C, antibody against p100 specifically immunoprecipitated 10% of the p100 from cells. Importantly, 10% of EBNA 2 coprecipitated along with p100 from EBNA 2-positive cells. EBNA 2 was not detected in the p100 immunoprecipitate from EBNA 2-negative B cells (P3HR-1) even though a similar amount of p100 was precipitated by the antiserum (data not shown). Further, the p100 antibody was specific for p100 and did not react with EBNA 2 on immunoblots (data not shown). To exclude the unlikely possibility that the p100 antibody selectively precipitated a small p100 fraction that is EBNA 2 associated, successive immunoprecipitations of p100 were performed. The third round of immunoprecipitation still precipitated 10% of the p100 and 10% of the EBNA 2 (Fig. 4C). The fact that in each of the three successive precipitations, p100 from cell extracts precipitates 10% of the EBNA 2 in these extracts indicates that much more than 30% of the soluble EBNA 2 in EBV-transformed lymphocytes is associated with p100.

p100 can preferentially affect transactivation mediated by the EBNA 2 acidic domain. To evaluate the potential role of p100 in EBNA 2 and VP16 acidic domain-mediated transactivation, eukaryotic expression plasmids containing the EBNA 2 or VP16 acidic domain fused to the Gal4 DNA binding domain (Gal-EBNA 2 or Gal-VP16) (6) were transfected into EBV-negative B lymphoblasts (BJAB) along with a CAT reporter plasmid containing five or no Gal4 binding sites upstream of the adenovirus E1b promoter (Gal4-E1b) (6) and various amounts of pSG5-p100 (Fig. 5). Gal-EBNA 2 increased CAT expression 97-fold, while Gal-VP16 increased CAT expression 304-fold. In five experiments, cotransfection of 5 or 10 μg of pSG5-p100 consistently increased Gal-EBNA 2-mediated activation of the Gal4-E1b promoter to 180- or 220-fold, respectively. However, 20 μg of p100 DNA had consistently less effect than 5 or 10 μg, increasing activation only to 119-fold. As expected from the lower binding of p100 to VP16, cotransfec-

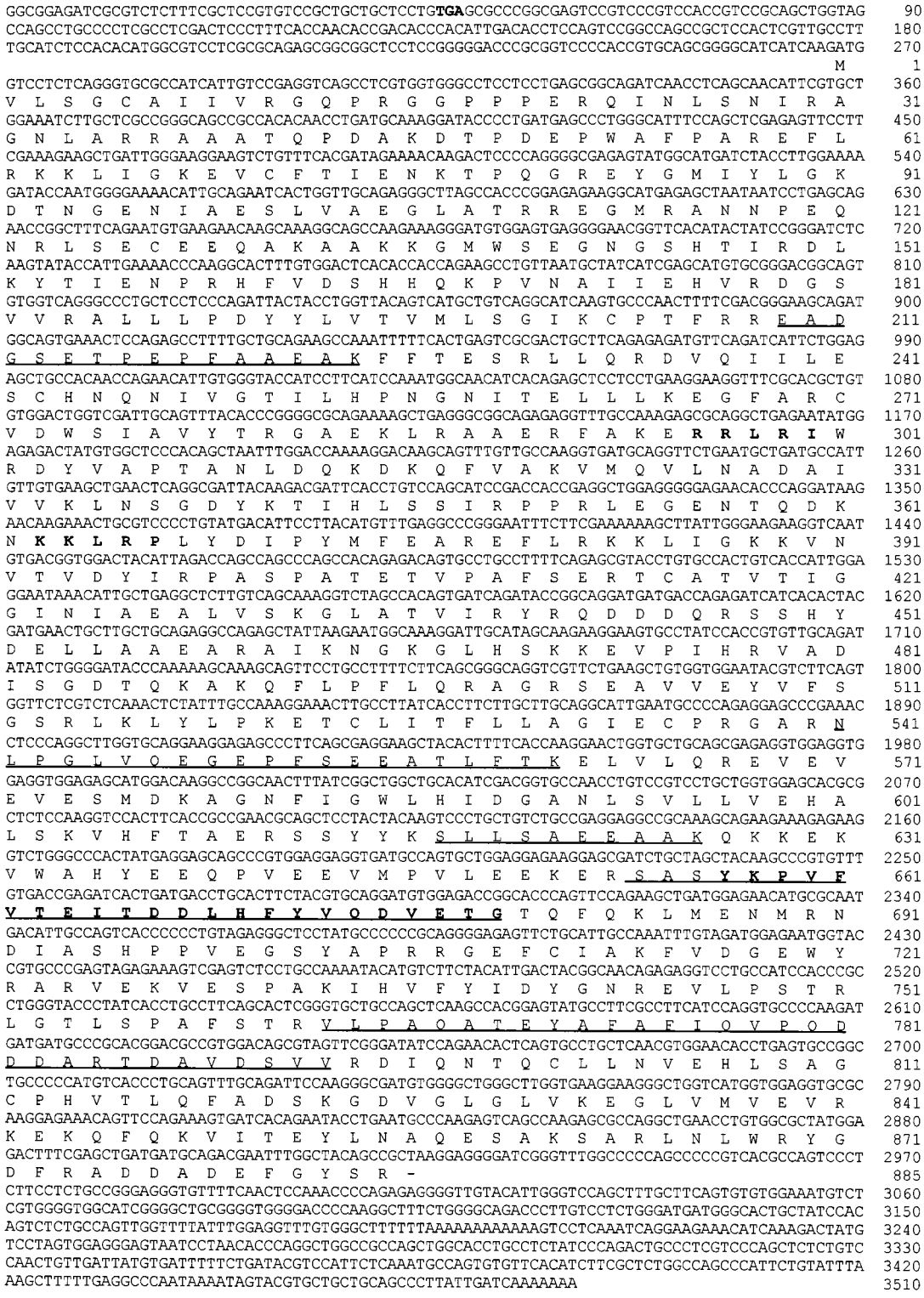

FIG. 2. Sequence of the p100 cDNA and predicted amino acid sequence. The in-frame stop codon in the 5′ untranslated region and two potential nuclear localization signals (12) are in boldface. The amino acid sequence from the 3.6-kDa peptide used to synthesize the oligonucleotide probe is underlined and in boldface. The other sequenced peptides are underlined.

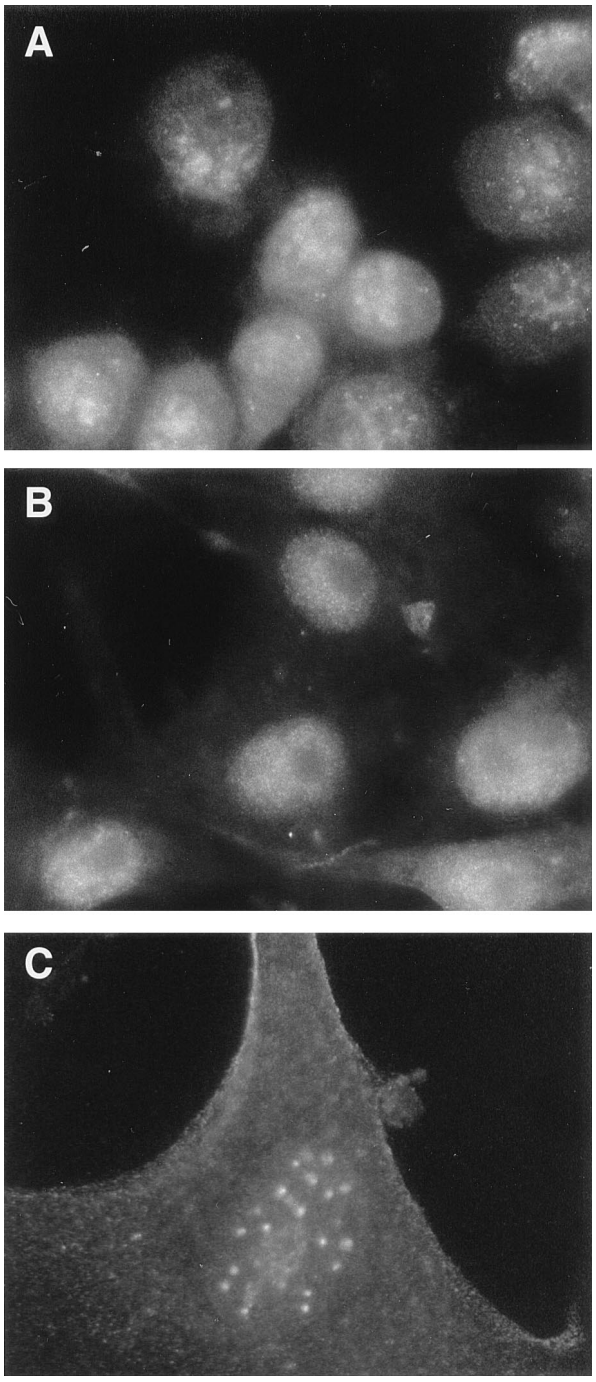

FIG. 3. Immunofluorescence microscopy of p100 in IB4 cells (A), Cos cells (B), or CRL 1634 human diploid fibroblast cells (C), using rabbit antiserum against p100 and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The cells were visualized with a Zeiss Axioskop at a magnification of ×1,000.

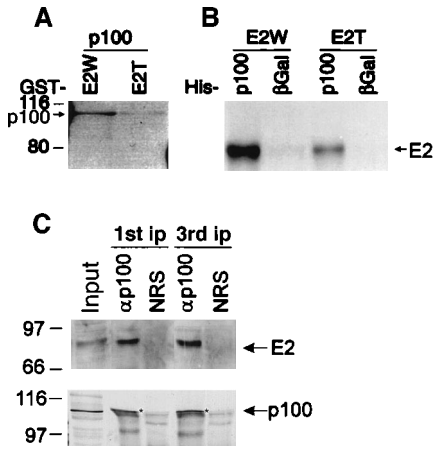

FIG. 4. Cloned p100 interacts with EBNA 2 in vitro and in vivo. (A) p100 was labeled with [^{35}S]methionine by in vitro translation and incubated with GST-EBNA 2 (GST-E2W) or GST-EBNA 2,Trp$_{454}$ (GST-E2T). (B) Full-length EBNA (E2W) or EBNA 2,Trp$_{454}$ (E2T) was labeled with [^{35}S]methionine by in vitro translation and incubated with His-p100 or His-βgal immobilized on an Ni column. p100 and EBNA 2 bound to fusion protein beads were visualized by fluorography after SDS-PAGE. (C) In vivo interaction between EBNA 2 and p100. Cell extracts from IB4 cells were immunoprecipitated by immunized rabbit serum against p100 (αp100) or normal rabbit serum (NRS). The supernatants were subjected to immunoprecipitation three times. Proteins precipitated by the antibodies were eluted with SDS sample buffer and analyzed by immunoblotting for EBNA 2 or p100. The results of the first-round (1st ip) and the third-round (3rd ip) immunoprecipitations are shown. In lanes labeled αp100, the p100 protein runs as a sharp band as indicated by asterisks. Ten percent of p100 input lysates and 3% of EBNA 2 input lysates were included (Input). The positions of EBNA 2 and p100 are indicated by arrows. Sizes on the left are indicated in kilodaltons.

tion with 5 or 10 μg of pSG5-p100 had no effect on VP16 acidic domain-mediated activation, and 20 μg of pSG5-p100 slightly increased CAT expression to 407-fold (Fig. 5). After correction for transfection efficiency, transfection of 5, 10, and 20 μg of pSG5-p100 DNA resulted in approximately 2-, 4-, and more than 10-fold increases, respectively, of p100 over endogenous p100 as measured by Western blotting (data not shown). Further, transfection of cells with up to 20 μg of p100 expression plasmid alone had no effect on basal transcription from the Gal4-E1b promoter construct (data not shown). Even though Gal-VP16 is a better transactivator than Gal-EBNA 2 in the assay, the lack of effect of p100 on Gal-VP16 activation is not due to saturation of the assay, since at high p100 concentrations we could observe a slight increase in Gal-VP16 activation. Because of the low expression level of Gal-EBNA 2 and Gal-VP16 in transfected cells, we cannot exclude the unlikely possibility that the effect of p100 cotransfection on EBNA 2-mediated activation is due to a specific effect on Gal-EBNA 2 versus Gal-VP16 protein levels. Nonetheless, these data are most compatible with a model that p100 is a specific coactivator of EBNA 2-type acidic domains.

p100 can interact with TFIIE. The association of p100 with the EBNA 2 acidic domain and its effect on transactivation could be due to p100 being a mediator between EBNA 2-like acidic domain and the basal transcription factors. To address this possibility, HeLa nuclear extracts were fractionated on a phosphocellulose column (13) and the amount of p100 in each fraction was assayed by immunoblotting. p100 was quantitatively recovered in the 0.3 M KCl fraction, which does not contain any known general transcription factor. p100 was not detected in the 0.1, 0.5, and 1.0 M KCl fractions or in more purified fractions of TFIIA/J (10), TFIIB (23), TFIIE (26), TFIIF (18), TFIIH (13, 19), or RNA polymerase (pol) II (39). Therefore, p100 is not a stable component of any general transcription factor or RNA pol II in HeLa nuclear extracts.

The potential interaction of p100 with basal transcription factors was further assayed by incubating His-p100 or His-βgal immobilized on Ni beads with nuclear extracts. Bound proteins were eluted from the beads, subjected to SDS-PAGE, and

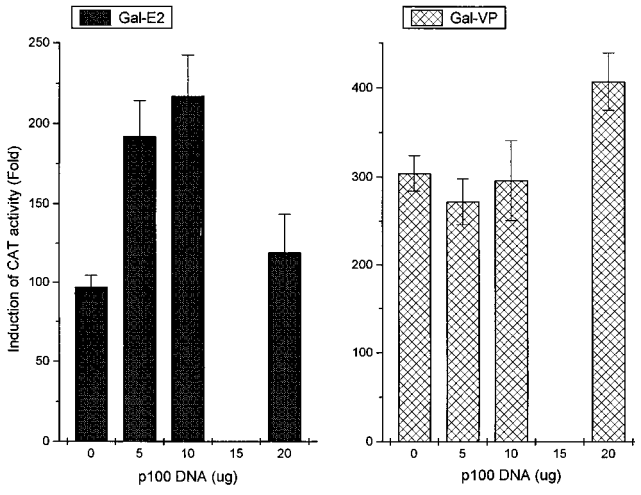

FIG. 5. Overexpression of p100 increases EBNA 2 acidic domain transactivation. Gal-EBNA 2 (Gal-E2) or Gal-VP16 (Gal-VP) was transfected into BJAB cells with reporter plasmid Gal4-E1b and various amounts of pSG5-p100 DNA as indicated. Five independent experiments were carried out. The induction of CAT activity was calculated as CAT activity with a reporter plasmid containing five Gal4 binding sites divided by CAT activity with a reporter plasmid containing no Gal4 binding site. The error bars represent standard errors. The increase of CAT activity of Gal-EBNA 2 at 5 μg ($P = 0.005$) and 10 μg ($P = 0.005$) of p100 DNA is statistically significant ($P < 0.05$); the increase of CAT activity of Gal-VP16 at 20 μg of p100 DNA is also statistically significant ($P = 0.03 < 0.05$). The variation in CAT activity at other datum points is not statistically significant.

immunoblotted for TFIIF (p74), TFIIE (p56), TBP, or TFIIB. Similarly, a TFIIH-enriched nuclear fraction (52) was incubated with His-p100 or His-βgal and immunoblotted for p89, p80, and p62 subunits of TFIIH. By comparative Western blotting 10% of the input TFIIE was detected among the His-p100 retained proteins, while TFIIF, TBP, TFIIB, and TFIIH subunits were not detected even though they were readily detected in immunoblots of the input extracts (data not shown). Further, TFIIE was not detected among the control His-βgal retained proteins (data not shown).

The basis for TFIIE interaction with p100 was further analyzed by using a TFIIE-enriched fraction (26). TFIIE is a heterodimer of 34- and 56-kDa subunits (44, 45). After incubation with His-p100 or His-βgal, 20% of input of both subunits of the TFIIE complex adsorbed to His-p100 beads, while less than 3% adsorbed to His-βgal beads (Fig. 6A). To investigate which TFIIE subunit mediates p100 binding, in vitro-translated ^{35}S-labeled p34 or p56 was incubated with His-p100. Both p34 and p56 bound to His-p100 at 10-fold-higher levels than to His-βgal, although approximately 30% of the input p34 bound to His-p100, while only 6% of input p56 bound to p100 (Fig. 6B). The p56 and p34 subunits efficiently formed a stable complex in vitro when cotranslated, as evidenced by coimmunoprecipitation of equivalent amounts of both subunits with p56 and p34 antibodies (Fig. 6C). About 10% of the input p56-p34 in vitro complex bound with fivefold-greater efficiency to His-p100 than to His-βgal (Fig. 6C). In the reciprocal experiment, ^{35}S-labeled p100 did not bind to GST, but 4% of the input p100 bound to both GST-p56 and GST-EBNA 2 (Fig. 6D). Therefore, both the p34 and p56 TFIIE subunits can specifically interact with p100.

p100 is essential for cell viability. To evaluate whether p100 is important for cell growth as might be expected for a putative coactivator, the effect of antisense p100 on cell viability was assayed. The first 1.4 kb of the p100 cDNA in an antisense orientation under the control of glucocorticoid response elements was transfected into non-EBV-infected B lymphoblasts. Three stably transfected antisense p100 cell lines and three vector control-transfected cell lines were established in parallel. The growth of the antisense p100-transfected cells under normal conditions was identical to that of vector control-transfected cells. However, when both sets of cultures were induced with dexamethasone, cells expressing antisense p100 started to die 3 days after dexamethasone addition, and the number of dead cells increased over the next 2 days so that by day 5, the cells were 30% viable; the total viable cell count remained the same as on day 1 (Fig. 7A). In contrast, the control cells remained 75% viable even at day 5, and the total viable cell count had increased three- to fivefold (Fig. 7A and data not shown). The results shown in Fig. 7A are the averages of five independent experiments. Similar effects were observed with the two other antisense and control cell lines in five independent experiments.

The decrease in cell growth and viability in response to induced antisense p100 expression correlated with a decrease in p100 protein levels. Cells transfected with antisense p100 were labeled with [^{35}S]Met for 3 h at 2 days after dexamethasone addition, and p100 was immunoprecipitated and quantitated. After correction for the slight overall decrease in total cell protein synthesis in dexamethasone-treated cultures, p100 levels were specifically reduced by 50% in the induced antisense p100-transfected cells (data not shown). In vector-trans-

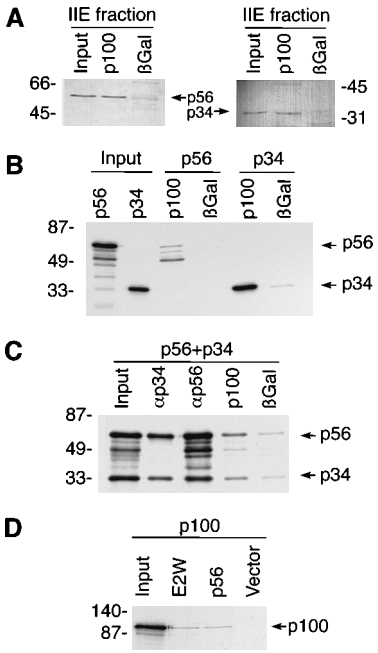

FIG. 6. p100 interacts with TFIIE. (A) p100 adsorbs TFIIE from nuclear extracts. TFIIE-enriched nuclear fraction was incubated with His-p100 and His-βgal. Bound proteins were subjected to SDS-PAGE, and the p56 and p34 subunits of TFIIE were detected by immunoblotting. (B) p100 interacts with both p56 and p34 subunits independently. p56 and p34 were individually ^{35}S labeled by in vitro translation and incubated with His-p100 and His-βgal. (C) The p34-p56 complex interacts with p100. p56 and p34 were cotranslated and immunoprecipitated with a p34 or p56 antibody (αp34 or αp56, respectively) or incubated with His-p100 and His-βgal. (D) GST-p56 binds to p100. p100 was ^{35}S labeled by in vitro translation and incubated with GST-p56, GST-EBNA 2 (E2W), and GST. In each experiment, bound proteins were visualized by fluorography. Twenty percent of input material was included for each panel except D, in which case 100% of input was included (Input). The position of each protein is indicated. Sizes on the left are indicated in kilodaltons.

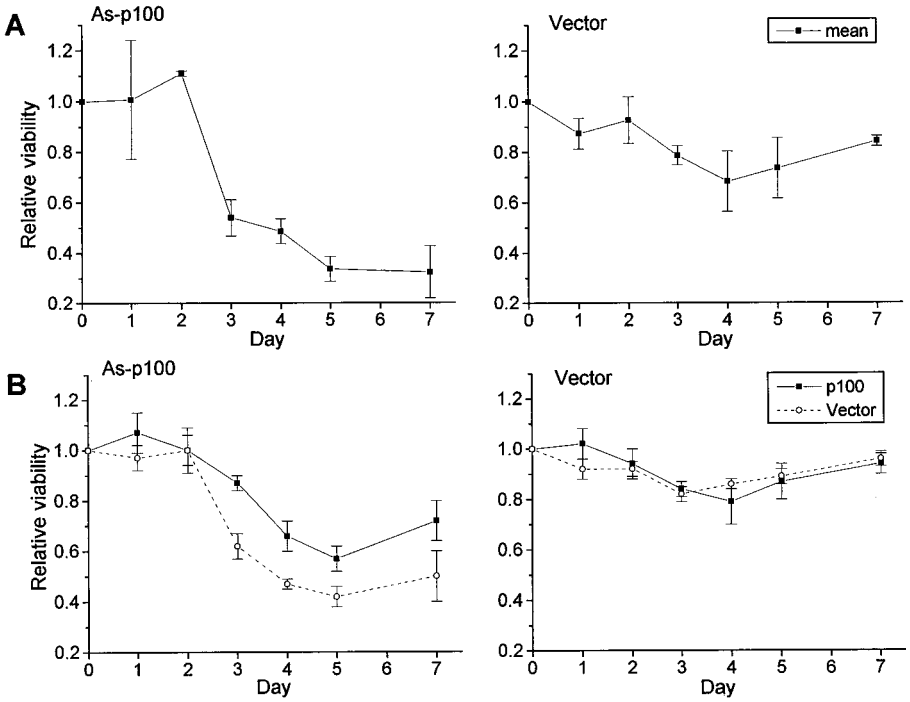

FIG. 7. Expression of antisense p100 results in cell death, and sense p100 restores cell viability. (A) Cells stably transfected with antisense p100 (As-p100) or vector control were induced by dexamethasone for antisense p100 expression, and cell viability was counted. Five independent experiments were carried out. The data presented were representative of three cell lines. (B) Cells stably transfected with antisense p100 (As-p100) or vector control were transiently transfected with sense p100 expressed from pGRE5-1,p100 (p100) or pGRE5-1 vector, respectively. Four independent experiments were carried out. The differences in cell viability between vector- and sense-p100-transfected cells were statistically significant from days 3 to 5 (day 3, $P = 0.02$; day 4, $P = 0.002$; day 5, $P = 0.03 < 0.05$). Cell viability was determined by trypan blue staining at indicated time points. Relative viability was calculated as cell viability with dexamethasone divided by cell viability without treatment. The error bars represent standard errors.

fected control cultures, the p100 level was unaffected by dexamethasone treatment. Thus, the decrease in cell viability correlated with decreased p100 levels.

To substantiate the dependence of cell viability on p100, a sense p100 expression vector with glucocorticoid response elements was introduced into cell lines stably transfected with antisense p100 before the induction of antisense expression. After induction, antisense p100 cells which had been transfected with the sense p100 expression vector exhibited 25% increased viability relative to the same cell lines transfected with the vector control (Fig. 7B). No effect was seen on the viability of cells that had been stably transfected with vector control (Fig. 7B). The 25% increase in cell viability correlated with a 25% efficiency of transfection, as determined by cotransfection with FLAG epitope-tagged p100 and immunofluorescence staining. Thus, these data confirm that p100 is important in B-lymphoblast growth.

DISCUSSION

This study demonstrates that the EBNA 2 acidic domain specifically and stably associates with p100, a novel nuclear protein. p100 can increase the ability of the EBNA 2 acidic domain to transactivate gene expression, an effect that could be mediated by the intrinsic ability of p100 to interact with both the p56 and p34 subunits of TFIIE. The significance of p100 binding to EBNA 2 is underscored by the specificity of interaction with the wild-type EBNA 2 acidic domain and not with the transcriptionally inert EBNA 2,Thr_{454} acidic domain.

As a coactivator, p100 exhibits specificity for the EBNA 2 acidic domain rather than the VP16 acidic domain. p100 binds preferentially to the EBNA 2 acidic domain over the VP16 acidic domain. Further, while low and moderate levels of p100 overexpression increased transactivation mediated by the EBNA 2 acidic domain, similar p100 levels had little or no effect on VP16-mediated transactivation. High p100 levels were required to achieve even a slight increase in VP16-mediated activation. The data are most consistent with p100 coactivation being dependent on the affinity for each specific acidic activation domain.

The interaction between p100 and TFIIE is also direct evidence that p100 can be a coactivator and a bridge between EBNA 2-type acidic domains and the basal transcription machinery (Fig. 8). TFIIE is an essential component of RNA pol II initiation complexes. In vitro, TFIIE binds to TFIID, TFIIB, TFIIF, pol II (42), and TFIIH (13, 42). In glycerol gradients of cell extracts, TFIIE is associated with pol II (20). Under basal transcription conditions in vitro, TFIIE is essential for recruitment of TFIIH (19) and regulates TFIIH enzymatic activities (40, 44). TFIIE stimulation of TFIIH kinase activity is likely to be critical because TFIIH-mediated phosphorylation of pol II is postulated to mediate the transition from initiation into elongation by pol II (for a review, see reference 11). However, binding of p100 to TFIIE probably is not sufficient to activate transcription in the absence of an appropriate activation domain, since p100 overexpression has no effect on basal transcription. Instead, p100 may serve a structural role as an adapter between the EBNA 2 acidic domain and TFIIE. Alternatively, free p100 may not be able to form a complex with TFIIE in vivo without first being anchored to promoter region by an activator. Consistent with this hypothesis, p100 did not

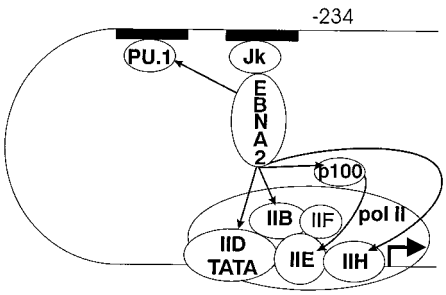

FIG. 8. EBNA 2 transactivation of the EBV LMP1 promoter. The LMP1 promoter contains two EBNA 2 response elements: one binding site (around −234 relative to the LMP1 start site) for cellular protein Jκ (CBF1) and one for PU.1 (shown as black boxes). EBNA 2 is targeted to its LMP1 response element through direct interaction with Jκ. EBNA 2 responsiveness is also mediated through interaction with PU.1. The EBNA 2 acidic activation domain stimulates transcription through interaction with TAF40, a component of TFIID; TFIIB; the p62 and p80 subunits of TFIIH; and p100, which associates with the p56 and p34 subunits of TFIIE.

copurify with TFIIE and could not be detected in p100 coimmunoprecipitation from cell extracts (data not shown).

The activity of p100 is similar to that of another recently cloned coactivator, PC4 (21). Both can bind to single-stranded DNA (data not shown), can directly bind to an activator (p100 to EBNA 2 and PC4 to VP16), and can interact with components of basal transcription machinery (p100 with TFIIE and PC4 with TFIIA). PC4 and p100 can also be developmentally regulated. PC4 is elevated in embryonic tissues and in a chemically induced rat pancreatic B-cell tumor, while p100 is elevated in differentiated promyelocytic HL60 cells (data not shown). These different coactivators may contribute to the specificity of gene regulation at different developmental stages.

Consistent with a role for p100 as a coactivator in cellular gene transcription, p100 is essential for cell viability. Antisense p100 mRNA reduced p100 levels and caused cell growth arrest and loss of viability. Sense p100 mRNA protected cells from antisense p100-induced death, further indicating that the cell death caused by antisense p100 was the specific consequence of loss of p100.

These and previous data are summarized in Fig. 8, which is a schematic model for EBNA 2-mediated transactivation of the EBV LMP1 promoter. EBNA 2 is anchored upstream of the promoter by interaction with cellular sequence-specific DNA-binding proteins, Jκ and PU.1 (22, 25, 28, 61). More than one EBNA 2 molecule appears to be necessary at a single promoter site for activation, since an EBNA 2 response element requires multiple EBNA 2 interacting sites (25, 28). Because a single Trp-454-to-Thr mutation abolishes EBNA 2 interaction with TFIIB, TAF40, TFIIH, and p100, the same binding site appears to be involved in EBNA 2 association with these factors. If so, each EBNA 2 molecule cannot simultaneously interact with p100 and TFIIB, TFIIH, or TAF40; instead, EBNA 2 molecules bound to the promoter region may interact with different factors with differential affinities and stabilities. The interaction of EBNA 2 with p100 is stable and quantitatively significant but not necessarily exclusive. The data reported here pertain only to the nucleoplasmic (isotonic salt-extractable) EBNA 2 fraction. While almost all of the p100 is nucleoplasmic (data not shown), approximately 50% of EBNA 2 is chromatin associated (54). On the basis of the absence of p100 from the chromatin fraction, chromatin-associated EBNA 2 is likely to be less complexed with p100. This possibility has not been directly assessed, since the association of EBNA 2 with p100 is unstable under the conditions for extraction of chromatin-associated proteins. EBNA 2 acidic domains which are not complexed with p100 can interact with TFIIB, TAF40, and TFIIH transiently and sequentially. EBNA 2 interactions with TFIIB and TAF40 (53) are likely to be critical to the assembly of the initiation complex with TFIID and pol II. The interactions of the EBNA 2-p100 complex with TFIIE and of free EBNA 2 with TFIIH (52) are likely to be important for the assembly of the complete transcription complex and promoter clearance. The multiplicity of intermolecular interactions can increase the potential for specific and efficient regulation.

One prediction of this model is that the equilibrium between free and p100-complexed EBNA 2 is critical in EBNA 2 acidic domain-mediated transactivation. Consistent with this model, p100 overexpression at moderate levels increased EBNA 2-mediated activation. This finding indicates that EBNA 2 association with p100 can be rate limiting. Moreover, high-level p100 expression was substantially less effective in increasing EBNA 2 transactivation, suggesting that p100 saturation of EBNA 2 acidic domains is inhibitory to their ability to transiently interact with TAF40, TFIIB, and TFIIH. Notably, in EBV-infected nucleoplasmic extracts, only 1 to 2% of EBNA 2 is stably associated with TFIIH (52), while a large fraction EBNA 2 is complexed with p100.

p100 is a large protein that appears to be fairly abundant in cells and may serve several functions. p100 is highly localized in diploid human fibroblasts. This restricted localization is consistent with the possibility that p100 is related to other localized nuclear complexes such as splicing or replication complexes. In this context, p100 could couple transcription with RNA processing or DNA replication.

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