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The 62- and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein–Barr virus nuclear protein 2

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ABSTRACT **EBNA 2 (Epstein–Barr virus nuclear antigen 2) is an acidic transactivator essential for EBV transformation of B lymphocytes. We show that EBNA 2 directly interacts with general transcription factor IIH. Glutathione *S*-transferase (GST)–EBNA 2 acidic domain fusion protein depleted transcription factor IIH activity from a TFIIH nuclear fraction. The p89 (ERCC3), p80 (ERCC2), and p62 subunits of TFIIH were among the proteins retained by GST–EBNA 2. Eluates from the GST–EBNA 2 beads reconstituted activity in a TFIIH-dependent *in vitro* transcription assay. The p62 and p80 subunits of TFIIH independently bound to GST–EBNA 2, whereas the p34 subunit of TFIIH only bound in the presence of p62. A Trp → Thr mutation in the EBNA 2 acidic domain abolishes EBNA 2 transactivation *in vivo* and greatly compromised EBNA 2 association with TFIIH activity and with the p62 and p80 subunits, providing a link between EBNA 2 transactivation and these interactions. Antibodies directed against the p62 subunit of TFIIH coimmunoprecipitated EBNA 2 from EBV-transformed B lymphocytes, indicating that EBNA 2 associates with TFIIH *in vivo*.**

Epstein–Barr virus (EBV) nuclear antigen 2 (EBNA 2) is essential for primary B lymphocyte transformation by EBV (1, 2). The transforming properties of EBNA 2 appear linked to its ability to activate expression of viral and cellular genes. These genes include the major EBV-transforming gene *LMP1* (3, 4), and the cellular *CD21* (5), *CD23* (6–8), and c-*fgr* genes (9). Characterization of the EBNA 2 activation domain revealed that it is enriched in acidic residues. Interestingly, EBNA 2 does not exhibit sequence-specific DNA-binding activity, yet EBNA 2-responsive elements have been defined in the latent membrane protein 1 (10, 11), latent membrane protein 2 (12), *Cp* EBNA (13) promoters, as well as the cellular *CD23* promoter (8). Recent studies have demonstrated that EBNA 2 is tethered to these responsive elements by the recombination signal-binding protein Jk (also called CBF1 and RBP 2N) (14–17) and transcription factor PU.1 (18). It is presumed that once EBNA 2 is loaded onto the promoter via Jk or PU.1, specific protein–protein contacts occur between the activation domain of EBNA 2 and components of the RNA polymerase II (RNAPII) transcription machinery. Indeed, recent studies have demonstrated a direct and specific interaction between the activation domain of EBNA 2 and transcription factor IIB (TFIIB) and the TATA-binding protein-associated factor 40 (TAF40) (19). The integrity of the EBNA 2 activation domain is critical for transactivation. Mutation of Trp-454 to either threonine or alanine abolishes transactivation and the ability of EBNA 2 to interact with the basal machinery (19).

Activator proteins, like EBNA 2, can potentially interact with various components of the basal transcription machinery. It is known that RNAPII requires seven general transcription factors to accurately initiate RNA synthesis from protein coding genes (for review, see ref. 20). Interactions of an activator with one or more of these general transcription factors could influence the rate of complex formation, promoter clearance, or elongation.

In an attempt to further characterize the mechanism by which EBNA 2 facilitates transcription rates, we isolated a protein complex from nuclear extracts that specifically interacts with the EBNA 2 activation domain. This complex contains helicase, kinase, and ATPase activities. Here we report that some of these activities can be attributed to general transcription factor IIH (TFIIH). TFIIH is a multisubunit complex consisting of approximately eight polypeptides ranging in size from 89 to 34 kDa. TFIIH is the only general transcription factor known to possess enzymatic activities. It contains a DNA-dependent ATPase, an ATP-dependent helicase and a kinase specific for the carboxyl-terminal domain (CTD) of the large subunit of RNAPII (for review, see ref. 21). Biochemical studies have implicated TFIIH in catalyzing promoter clearance (22). Interestingly, analysis of the TFIIH polypeptides revealed that most subunits are DNA excision repair proteins (23–27) and that TFIIH is required for DNA nucleotide excision repair (26, 28, 29). Hence, it appears that interaction of an activator with the multifunctional TFIIH complex could have enormous impact on the enzymatic rate of transcription initiation and perhaps even DNA repair.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. P3HR-1 clone 16 cells were obtained from G. Miller, Yale University (30). The P3HR-1 EBV genome is deleted for an EBV DNA segment that encodes the last two exons of EBNA leader protein and the entire EBNA 2 exon. IB4 is an EBV-transformed B-lymphoblastoid cell line (31). All cells were maintained in RPMI 1640 medium/10% fetal bovine serum.

Construction of Plasmid. Glutathione *S*-transferase (GST)–VP16 (aa 413–490) is from M. Green, University of Massachusetts, Worcester, MA (32), GST–EBNA 2 (aa 427–483) and GST–EBNA 2 [aa 427–483(T_{454})] are described (19) and are referred to as GST–EBNA 2W and GST–EBNA 2T.

Purification of Fusion Proteins. GST fusion proteins were expressed in *Escherichia coli* after induction with 0.1 mM isopropyl β-D-thiogalactopyranoside (33). Cells were harvested 3 hr after induction. After sonication and centrifugation, the extracts were incubated with glutathione–agarose beads (Pharmacia) for 1 hr at 4°C. The beads were precipitated and washed with NETN buffer [20 mM Tris (pH 8.0)/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40].

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Abbreviations: EBV, Epstein–Barr virus; EBNA 2, EBV nuclear antigen 2; TFIIH, transcription factor IIH; GST, glutathione *S*-transferase; CTD, carboxyl-terminal domain; RNAPII, RNA polymerase II.

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Depletions and Affinity Binding Assays. An SP-Sepharose TFIIH fraction (26) was diluted in NETN buffer to a final 120 mM NaCl and incubated for 1 hr at 4°C with 2 μg of GST–VP16, GST–EBNA 2W, GST–EBNA 2T, or GST that had been adsorbed to glutathione beads. The supernatants were further adsorbed with fresh fusion protein beads for a total of three depletions. Proteins were eluted from the beads by incubation with 20 mM glutathione/NETN buffer/100 mM Tris, pH 8.0 for 30 min at room temperature, and the eluate was used in transcription assays. Alternatively, the proteins were eluted with SDS sample buffer, run on SDS/PAGE, transferred to nitrocellulose, and analyzed by immunoblot for the p89, p80, and p62 subunits of TFIIH (for review, see ref. 21). To study direct interactions between EBNA 2 and the TFIIH subunits, p89, p80, p62, p44, and p34 were radiolabeled by *in vitro* translation with [^{35}S]methionine (Promega). In addition, p62 was tagged at the N terminus with six histidine residues and purified from *E. coli* by Ni-affinity chromatography (provided by Tae-Kyung Kim, Howard Hughes Medical Institute, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School). Recombinant histidine-tagged p62 and the ^{35}S-labeled proteins were incubated with 2 μg of GST–VP16, GST–EBNA 2W, GST–EBNA 2T, or GST in NETN buffer containing bovine serum albumin at 0.5 mg/ml and 150 mM NaCl. Bound proteins were analyzed by SDS/PAGE and immunoblot (for histidine-tagged p62) or fluorography (for ^{35}S-labeled proteins). For the study of TFIIE interaction, HeLa cell nuclear extracts were incubated with 2 μg of GST–VP16, GST–EBNA 2W, GST–EBNA 2T, or GST, and the adsorbed proteins were analyzed by SDS/PAGE and immunoblot using antibody to the p56 subunit of TFIIE.

Immunoprecipitation of p62 and EBNA 2. IB4 or P3HR-1 cells were lysed in buffer (5×10^7 cells per ml of lysis buffer) containing 150 mM NaCl, 10 mM Hepes (pH 8.0), 1% Nonidet P-40, aprotinin at 2 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol. Insoluble material was removed by centrifugation. The lysates were precleared by incubation with normal rabbit serum. Aliquots of precleared lysates were incubated for 2 hr at 4°C with rabbit serum immunized against p62. Protein A-Sepharose beads were added for 30 min. The beads were washed in lysis buffer, and adsorbed proteins were eluted with SDS sample buffer, run on SDS/PAGE, transferred to nitrocellulose, and analyzed by immunoblot for p62 and EBNA 2.

***In Vitro* Transcription Assay.** TFIIH-dependent transcription was reconstituted *in vitro* by using recombinant TATA-binding protein, transcription factor IIB, transcription factor IIE, and transcription factor IIF, purified transcription factor IIA/J, RNAPII, and a plasmid containing the adenovirus major late promoter driving transcription of a 392-nt G-less cassette (34). Purified TFIIH or eluates from the GST "pull-downs" were added to the transcription reaction. Reaction mixtures (40 μl) were incubated for 1 hr at 30°C and processed as described (34).

RESULTS

The EBNA 2 Acidic Activation Domain Depletes TFIIH Activity from a Crude Nuclear Fraction. In our initial attempts to identify cellular proteins that interact with EBNA 2, we fractionated nuclear extracts over an EBNA 2 acidic activation domain column. The proteins eluted contained kinase and helicase activity and a polypeptide composition similar to general transcription factor TFIIH (data not shown). To determine whether TFIIH could indeed be a cellular target for EBNA 2, a wild type (EBNA 2W) and a transcriptional null mutant (Trp-454 → Thr; EBNA 2T) version of the EBNA 2 activation domain were expressed as GST fusion proteins (19, 33). As a positive control, we used the herpes simplex virus activator VP16 because it was recently shown to interact with TFIIH (35). The fusion proteins were tested for their ability to deplete TFIIH activity from a crude nuclear TFIIH fraction. Residual TFIIH activity in the depleted extracts was measured in a TFIIH-dependent *in vitro* transcription assay. GST–VP16 and GST–EBNA 2W depleted nearly all of the TFIIH activity; whereas GST–EBNA 2T and GST had little adverse effect (data not shown). Importantly, eluates from VP16 and EBNA 2W fusion protein beads restored TFIIH activity; whereas eluates from EBNA 2T and GST fusion protein beads did not (Fig. 1*A*). The ability of the VP16 and EBNA 2W acidic domains to adsorb essential factor(s) from the crude TFIIH fraction and the inability of the null mutant EBNA 2T to adsorb these factors provides a biochemical link between EBNA 2 transactivation *in vivo* and TFIIH depletion *in vitro*.

To confirm that EBNA 2 and VP16 were specifically depleting TFIIH, purified TFIIH, TFIIB, or TFIIE was added to the GST–VP16- or GST–EBNA 2-depleted supernatants. As shown in Fig. 1*B*, only TFIIH reconstituted basal transcription activity to the supernatants.

TFIIH Subunits Adsorb to the EBNA 2 Acidic Domain, Whereas TFIIE Subunits Do Not. TFIIH is a multisubunit complex composed of approximately eight polypeptides. The largest subunit, p89, is identical to the DNA excision repair protein XPB/ERCC3 (23), and the p80 subunit is identical to the XPD/ERCC2 excision repair protein (24, 26, 27). Both proteins possess ATP-dependent helicase activity (36–38). Three other TFIIH subunits have been cloned: p62 (39), p44, and p34 (25). The 44-kDa protein contains a DNA-binding zinc finger and is the homologue of the yeast SSL1 gene product involved in DNA repair. The 34-kDa subunit also contains a zinc finger and is hydrophobic. Until recently, identification of the CTD kinase subunit(s) had not been achieved. Now evidence exists that the CTD kinase is the cyclin-dependent kinase (CdK)-activating kinase (for review, see ref. 40), which is composed of cdk-7 and cyclin H (D. O. Morgan and D.R., unpublished work).

To determine which TFIIH subunits had specifically adsorbed to the GST–VP16 or GST–EBNA 2W beads in the above experiments, the bound proteins were eluted by SDS

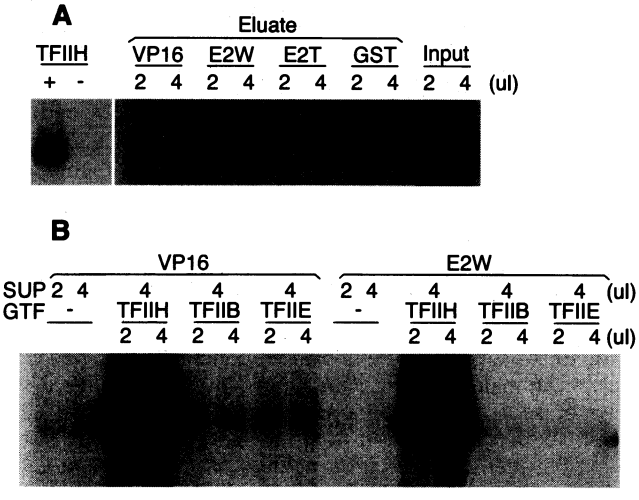

FIG. 1. EBNA 2 depletes TFIIH activity from crude TFIIH fraction. A crude SP-Sepharose TFIIH nuclear fraction (26) was incubated with either GST–EBNA 2W, GST–EBNA 2T, GST–VP16, or GST beads. Bound proteins were eluted with glutathione and assayed. (*A*) TFIIH-dependent transcription assays (indicated by + and − TFIIH) were supplemented with 2 and 4 μl of eluted proteins from the indicated beads or with 2 and 4 μl of untreated crude TFIIH fraction (Input). (*B*) Supernatants (SUP) (2 or 4 μl) after depletion by GST–VP16 (VP) or GST–EBNA 2W (E2W) were added directly to transcription reactions alone or in combination with purified general transcription factors TFIIH, TFIIB, or TFIIE (2 or 4 μl). Two microliters of TFIIH, TFIIB, and TFIIE are saturating in the reconstituted assay.

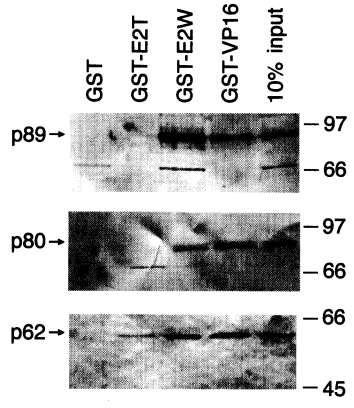

FIG. 2. EBNA 2 acidic domain interacts with TFIIH subunits *in vitro*. A crude TFIIH fraction was incubated with GST–VP16 (VP16), GST–EBNA 2 (E2W), GST–EBNA 2T (E2T), or GST. Bound proteins were eluted by SDS sample buffer, and analyzed by immunoblot for p89 and p80 using affinity-purified rabbit antibodies and for p62 by monoclonal antibodies. Ten percent of total input extracts were also included. The position of each subunit is indicated by an arrow.

sample buffer and analyzed by immunoblot with antisera to the p89, p80, or p62 subunits. As shown in Fig. 2, p89, p80, and p62 had adsorbed to GST–VP16 or to GST–EBNA 2W but had not adsorbed to GST–EBNA 2T or GST. Thus, the EBNA 2 and VP16 activation domains adsorb at least three of the TFIIH subunits. Based on our observation that the eluates from these beads contain TFIIH transcription activity, it is likely that the entire complex was retained by the EBNA 2 and VP16 activation domains.

The enzymatic activities of TFIIH have been shown to be subject to regulation by another general transcription factor, TFIIE (26, 41–43). TFIIE is a heterodimer of 34- and 56-kDa subunits (44–47). Studies in the human system have demonstrated that recombinant TFIIE can stimulate the CTD kinase and ATPase activities of TFIIH and, depending on conditions, either inhibit (26) or stimulate (43) its helicase function. Because TFIIE can directly interact with TFIIH (26, 48), we wanted to determine whether the interaction between EBNA 2 and TFIIH could be mediated through TFIIE. Fig. 3 shows that TFIIE p56 was readily detectable in nuclear extracts but did not adsorb to VP16 or EBNA 2 fusion proteins. Thus, although TFIIE and TFIIH are closely associated, only TFIIH interacts with EBNA 2 and VP16 acidic domains.

EBNA 2 Interacts Directly with the TFIIH p80 and p62 Subunits But Interacts Indirectly with p34. To determine which of the five cloned TFIIHs can directly bind to EBNA 2, ^{35}S-labeled, *in vitro*-translated p89, p80, p62, p44, and p34 were tested for their ability to bind to the VP16 or EBNA 2 activation domains. In addition, recombinant histidine-tagged p62 was analyzed. *In vitro* translated or bacterially expressed

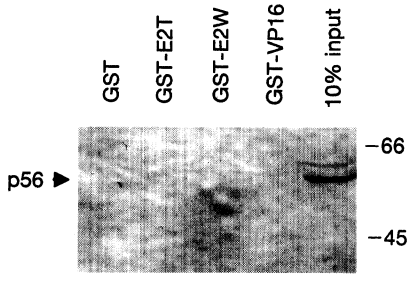

FIG. 3. EBNA 2 acidic domain does not interact with TFIIE. HeLa cell nuclear extracts were incubated with GST–VP16 (VP16), GST–EBNA 2 (E2W), GST–EBNA 2T (E2T), or GST. Bound proteins were eluted by SDS sample buffer and analyzed by immunoblot for the p56 subunit of TFIIE. An aliquot of nuclear extracts was also included (input).

histidine-tagged p62 preferentially bound to GST–VP16 and GST–EBNA 2W as compared with GST–EBNA 2T (Fig. 4 *A* and *B*), indicating that p62 can directly interact with the VP16 and EBNA 2 acidic activation domains.

Interestingly, the p34 subunit did not bind to GST–EBNA 2W by itself but did bind to GST–EBNA 2W when cotranslated with the p62 subunit (Fig. 4*B*). Similar results were obtained when p62 and p34 were translated in separate reactions and then mixed (data not shown). These data thus indicate that p34 directly contacts p62 in the TFIIH complex and also demonstrate that the p34/p62 complex stably interacts with the EBNA 2 acidic domain.

Like p62, the TFIIH p80 subunit can also directly bind to EBNA 2. As shown in Fig. 4*C*, *in vitro*-translated p80 bound to GST–EBNA 2W with higher affinity than to GST–EBNA 2T or to GST. Surprisingly, p80 had very low affinity for GST–VP16. The binding of p80 to GST–VP16 was not significantly above background in several experiments (Fig. 4*C* and data not shown).

In contrast to p62, p80, and p34, the p89 or p44 TFIIH subunits did not bind specifically to GST–VP16 or GST–EBNA 2 when translated independently or in the presence of either p62 or p80 (data not shown).

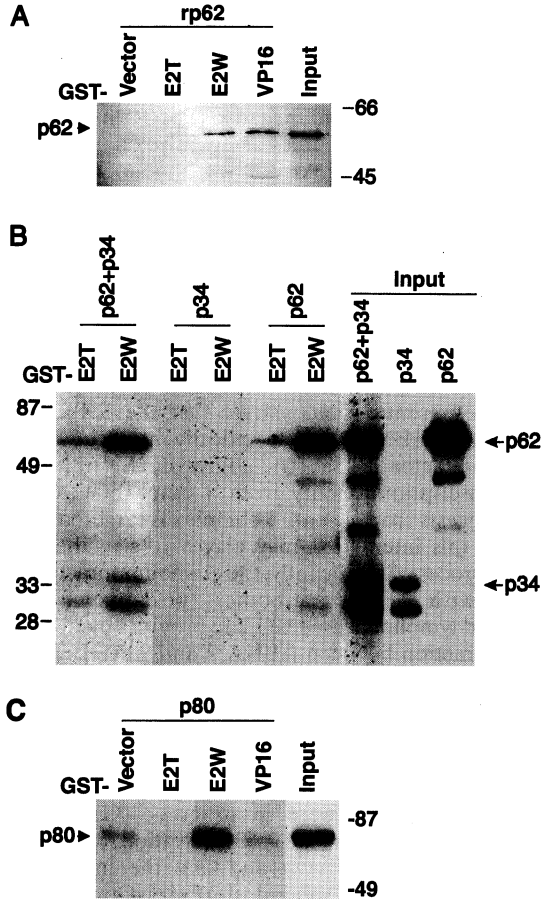

FIG. 4. EBNA 2 directly binds to the p80 and p62 subunits of TFIIH and pulls down the p34 subunit through p62. (*A*) Bacterially expressed recombinant p62 (rp62) was incubated with GST–VP16 (VP16), GST–EBNA 2 (E2W), GST–EBNA 2T (E2T), or GST. Proteins bound to GST fusion protein beads were analyzed by SDS/PAGE and immunoblot for p62 by using monoclonal antibodies. (*B*) p62 and p34 were *in vitro* translated either in separate reactions (p62 or p34) or cotranslation (p62 + p34) in the presence of [^{35}S]methionine. (*C*) p80 was ^{35}S labeled by *in vitro* translation. The translation lysates were incubated with the various GST fusion protein beads. Bound proteins were analyzed by SDS/PAGE and visualized by fluorography. An aliquot of *in vitro* translation mixture was included (Input).

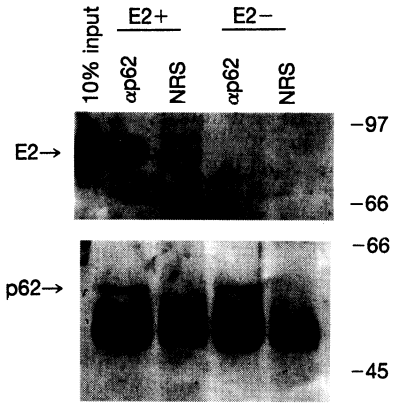

FIG. 5. EBNA 2 interacts with TFIIH *in vivo*. Extracts from EBNA 2-positive IB4 cells (E2+) or EBNA 2-negative P3HR-1 cells (E2−) were immunoprecipitated by immunized rabbit serum against p62 (αp62) or normal rabbit serum (NRS). Proteins precipitated by the antibodies were eluted with SDS sample buffer and analyzed by immunoblot for p62 and EBNA 2. Ten percent of extracts from EBNA 2-positive cell line were also included. The positions of EBNA 2 and p62 are indicated by arrows.

EBNA 2 Interacts with TFIIH *in Vivo*. To evaluate whether the interaction observed *in vitro* between the EBNA 2 activation domain and TFIIH is relevant *in vivo*, the TFIIH complex was specifically immunoprecipitated from nuclear extracts of EBNA 2-positive (IB4) and -negative (P3HR-1) EBV-infected human B lymphocyte cell lines using polyclonal antibodies to the p62 subunit (Fig. 5). An EBNA 2 monoclonal antibody specifically detected 1–2% of the extractable EBNA 2 in the p62 immune complex from EBNA 2-positive IB4 cells. Comparable amounts of p62 were precipitated from both cell lines (Fig. 5). Thus 1–2% of EBNA 2 appears to be stably complexed with TFIIH *in vivo*.

DISCUSSION

The results presented demonstrate that the EBV transcriptional activator EBNA 2 can specifically interact with general transcription factor TFIIH. The ability of EBNA 2 to deplete TFIIH transcription activity from a crude nuclear fraction strongly suggests that the entire complex is targeted by EBNA 2 and that this interaction may play a role in the EBNA 2 activation process. Significantly, the coimmunoprecipitation of EBNA 2 with TFIIH antibodies (p62) from an EBV-transformed lymphoblastoid cell line provides evidence for an *in vivo* interaction between EBNA 2 and TFIIH. The observation that a Trp → Thr mutation in the activation domain of EBNA 2 abolishes transactivation as well as the interaction with TFIIH is compatible with an important role for TFIIH in EBNA 2 transactivation of cellular and viral genes in EBV induction of cell growth transformation.

While these studies were in progress, Xiao *et al.* (35) reported that the VP16 and p53 activation domains could interact with TFIIH *in vitro* and that the interaction was mediated by the 62-kDa subunit of TFIIH. Our data confirm that VP16 interacts directly with TFIIH via p62. However, our analysis using the five cloned human TFIIH subunits revealed some differences in the fashion by which EBNA 2 and VP16 interact with TFIIH. Specifically, although both EBNA 2 and VP16 could interact with the 62-kDa subunit, only EBNA 2 exhibited an affinity for the 80-kDa subunit that is the DNA excision repair protein ERCC2. Moreover, we recently showed that VP16 can interact strongly with TATA-binding protein, but that EBNA 2 displayed a much weaker affinity for TATA-binding protein (19). Therefore, although both VP16 and EBNA 2 are acidic activators that can interact with TFIIH, the functional significance of the interactions may differ and may reflect nuances in the way the acidic activators interact with and influence the basal machinery.

The observation that p34 can associate with GST–EBNA 2W only in the presence of p62 is evidence that p34 can interact with p62 in the absence of the other subunits. Surprisingly, p89 or p44 independently or together did not interact with the EBNA 2 acidic domain in the presence of p62 and/or p80. This result may indicate that p89, p80, and p44 cannot associate with p62/p34 in the absence of the cyclin-dependent kinase-activating kinase (D. O. Morgan and D.R., unpublished work) and the other uncloned subunits of TFIIH. It may also suggest that the association may require posttranslational modifications of certain protein interfaces.

The activity of a number of transcriptional regulators can be modulated by posttranslational modifications (49). Interestingly, the original complex isolated from extracts by the EBNA 2 activation domain contained a kinase activity that could phosphorylate the EBNA 2 acidic activation domain itself (X.T., unpublished results). Because TFIIH contains a kinase activity, we investigated whether TFIIH could phosphorylate EBNA 2 *in vitro*. An EBNA 2 kinase activity was indeed associated with the TFIIH fraction during its purification through the standard phosphocellulose, DEAE-Sephacel, SP-Sepharose, phenyl Superose, and Mono S chromatographic steps (34). However, when the TFIIH preparation was further purified on a Superdex 200 gel filtration column, the TFIIH activity eluted at ≈250 kDa, whereas the EBNA 2 kinase activity eluted between 40 and 80 kDa (R.D., unpublished results). Therefore the GST–EBNA 2 kinase is not TFIIH. The identity of the kinase was not further pursued because the kinase associated with and phosphorylated GST–EBNA 2W and GST–EBNA 2T equally.

TFIIH is postulated to be involved in promoter clearance, a stage between formation of an open complex and transcription elongation (22). Protein–protein and protein–DNA contacts established during initiation must be disrupted so that RNAPII can escape from the promoter and processively transcribe the template DNA strand. The TFIIH helicase activity probably catalyzes DNA unwinding during promoter clearance, and the intrinsic TFIIH kinase probably contributes to polymerase clearance by phosphorylating the CTD of the largest RNAPII subunit. Recent studies have revealed that the interaction between acidic activators and TFIIH has dramatic functional consequences. These activators can directly enhance promoter clearance rates of RNAPII in a manner dependent on TFIIH (P. Kumar and D.R., unpublished work).

The EBNA 2 acidic domain does not promiscuously bind to proteins such as TATA-binding protein (19) or TFIIE but selectively interacts with TFIIB, TAF40, and TFIIH. The interactions appear to depend on the same EBNA 2 acidic domain configuration because the EBNA 2 Trp-454 → Thr mutation abolishes all three interactions. The various EBNA 2 interactions support the notion of multiple targets for an activator and may reflect alternate or sequential interactions (50). It is becoming clear that activator proteins can target at least two components of the RNAPII transcription machinery: TFIIB and TFIIH. The consequence of these interactions is revealed by the observation that TFIIB loads RNAPII onto the promoter before initiation and TFIIB is released after initiation of transcription (L. Zawel and D.R., unpublished work). Recruitment of TFIIB back to the promoter by activators could facilitate polymerase loading onto the promoter (32). However, recruitment of TFIIB alone could not accelerate transcription rates significantly unless the rate at which the polymerase escapes the promoter is concomitantly enhanced. This is the function attributed to TFIIH and hence, recruitment by activators of both TFIIB and TFIIH facilitates activation in a concerted fashion.

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