Identification and Characterization of ZIIBC, a Complex Formed by Cellular Factors and the ZII Site of the Epstein-Barr Virus BZLF1 Promoter

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The transition from latency to lytic Epstein-Barr virus replication is dependent on the Epstein-Barr virus BZLF1 gene product. Genetic and biochemical attempts to link cellular second-messenger signaling pathways that trigger this transition with the subsequent viral gene cascade have identified functional elements within the BZLF1 promoter (Zp) that appear to bind undefined cellular transcription factors. One of these previously identified sites, ZII, has homology to consensus AP-1 and CREB binding sites, implying a role for these factors in the inductive process. We have identified and characterized ZIIBC, a ZII site binding complex that is distinct from the factors previously proposed to bind this site. Active ZIIBC was found to be present in both uninduced and chemically induced cell extracts at approximately equivalent concentrations. Analysis of the DNA sequence requirements for the binding of ZIIBC to the ZII site shows that sequences homologous to AP-1 and CREB consensus sites are necessary but not sufficient for complex formation. Although the components of ZIIBC that directly contact DNA were found to be of the same molecular masses (26 and 36 kDa) in both uninduced and chemically induced cell extracts, a slight mobility difference between DNA-protein complexes formed by these two types of extracts is observable and indicates that ZIIBC is directly affected by chemical induction. The effects of ZIIBC binding to the ZII site on expression from Zp were evaluated, and they suggest that ZIIBC plays a critical role in the regulation of Zp expression.

The ability to establish latency and subsequently reactivate to a state of lytic replication is a hallmark of herpesviruses. The mechanisms involved in triggering the reactivation of latent herpesviruses are the subject of major clinical and experimental interest. In B lymphocytes infected by Epstein-Barr virus (EBV), the transition from latency to lytic replication is dependent on the EBV-encoded BZLF1 gene product Zta (22). Zta is a viral transactivator that is unique among EBV-encoded transactivators in that it alone is capable of triggering the viral lytic cycle when transfected into latently infected cells under the control of a strong heterologous promoter (9, 11, 33). Experimentally, the reactivation of latent EBV can also be triggered by treating latently infected cells with a variety of chemical agents, including tetradecanoyl phorbol acetate (TPA), calcium ionophore, butyrate, and anti-immunoglobulin (5, 12, 16, 21, 32, 34, 35). The treatment of cells with these agents triggers a variety of cellular second-messenger signaling pathways, resulting in the activation of cellular transcription factors that in turn activate transcription from the BZLF1 promoter, Zp (19).

Since the biologically relevant end point of these cellular factor cascades is transcriptional activation of the BZLF1 gene, the BZLF1 promoter is a logical target for investigations aimed at identifying cellular factors involved in this transcriptional activation via direct physical contact with Zp DNA. Several functional elements that bind cellular transcription factors have been identified in Zp; however, the protein-DNA interactions occurring at these loci have not been characterized nor have clear distinctions between the interactions observed

in extracts prepared from uninduced and chemically induced cells been noted (8, 13, 23, 31).

The regulation of BZLF1 transcription is critical for the maintenance of latency in B cells. Infected cells that are tightly latent show no detectable levels of Zta expression. However, Zta expression is rapidly induced upon chemical induction of latently infected cells (18, 28). The triggering of this switch in the regulation of Zta expression is a cellular-gene-dependent process. Thus, cellular transcription factors play a critical role in both the maintenance of latency and the triggering of reactivation. The ZII site, previously identified as a cellular factor binding site (13), is likely to be critical in this switch from latency to lytic replication. Not only is the ZII site essential in the absence of Zta and dispensable in the presence of Zta, it also contributes to the overall TPA inducibility of Zp (13). This suggests that the ZII binding factor functions in the initial cellular-factor-dependent activation of transcription from Zp and either is a distinct factor in uninduced and induced cells or is modified in some manner upon chemical induction. The presence of the ZII binding factor in uninduced cells also suggests that this factor functions as a repressor of Zp until the signals for reactivation are received.

The ZII binding site has homology with the consensus AP-1 (TGANTCA [2]) and CREB (TGANNCTCA [24]) binding sites. The region of homology between these sites is an octanucleotide core sequence (TGACATCA) that is identical to the AP-1 site found in the *c-jun* promoter, which differs from the consensus AP-1 site in that the palindromic sequences are separated by 2 bases (1). It has previously been demonstrated that Jun and Fos, the components of AP-1, isolated from baculovirus-infected insect cell extracts can bind to the ZII site (13). AP-1 binding activity is also known to be induced in extracts generated from TPA-treated cells (2, 7), lending support to the idea that the ZII binding factor is a member of the AP-1 family of transcription factors. However, it has not been

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demonstrated that Fos and Jun are the authentic factors that occupy the ZII site in B cells. Instead, several lines of evidence suggest that these are not the factors that bind the ZII site in vivo. First, cotransfection of ZpCAT constructs with a *c-jun* expression vector does not result in transactivation of the Z promoter. Second, the footprinting data of Flemington and Speck indicated equal occupancy of the ZII site in both uninduced and chemically induced cellular extracts rather than an increase in binding upon chemical induction, as has been demonstrated with other AP-1-inducible sites (2, 3, 7, 27). The CCAAT/enhancer-binding protein (C/EBP) has also been shown to be capable of protecting a region overlapping the ZII site in vitro (17); however, the functional significance of this binding has not been reported.

The data presented here identify a binding activity specific for the ZII site in fractionated extracts generated from uninfected B cells. The DNA sequence requirements for the binding of this complex to the ZII site were investigated by gel mobility shift assay and methylation interference analysis. Physical and functional characterizations of this binding activity isolated from uninduced and chemically induced extracts are also reported. The physical data demonstrate that this binding activity is distinct from those previously proposed to bind this site (AP-1 and C/EBP) and therefore represents a novel binding activity for this site. The functional data suggest that the ZII site and the identified ZII binding factor are likely to play a critical role in the regulation of transcription from Zp and, consequently, the transition from latent infection to the lytic cycle of EBV.

MATERIALS AND METHODS

Preparation of nuclear extracts. Cells were grown in 6-liter volumes and divided so that 3 liters was chemically induced (20 ng of phorbol myristate acetate per ml, 4 mM *n*-butyric acid, 7.5 μ M A23187, and 0.5 μ g of a 50:50 mix of 1,2-dioctanoyl-*rac*-glycerol and 1,2-dioctanoyl-*sn*-glycerol per ml) for 18 h while 3 liters remained uninduced. Cells were harvested, washed with hypotonic buffer (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.35], 5 mM KCl, 1.5 mM MgCl₂, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride), and then allowed to swell for 30 min in an equal volume of hypotonic buffer at 4°C. Swollen cells were broken by homogenization in a tight-fitting Dounce homogenizer until lysis was 95% complete. Nuclei were isolated by centrifugation at 1,500 $\times$ g for 10 min. Isolated nuclei were disrupted by the addition of an equal volume of high-salt extraction buffer (25 mM HEPES [pH 7.35], 1 mM DTT, 1 mM EGTA, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.5 μ g of aprotinin per ml, 0.5 μ g of leupeptin per ml, 0.5 μ g of pepstatin A per ml, 10 mM Na₂S₂O₅, 5 mM benzamide HCl, 250 mM sucrose, 0.05% Nonidet P-40, 3.6 M KCl), and the lysate was incubated for 30 min. The extract was adjusted to 0.4 M (NH₄)₂SO₄ (pH 7.9), incubated an additional 30 min, and cleared by centrifugation at 100,000 $\times$ g for 3 h. This crude extract was adjusted to 50 mM KCl by the addition of heparin agarose chromatography buffer (25 mM HEPES [pH 7.35], 1 mM DTT, 0.01% Nonidet P-40, 20% glycerol, 5 mM Na₂S₂O₅, 1 μ g of leupeptin per ml, 1 μ g of aprotinin per ml, 1 μ g of pepstatin A per ml, 1 mM phenylmethylsulfonyl fluoride). Extracts were fractionated by chromatography through heparin agarose. Bound protein was eluted with a 0.05 to 1 M linear KCl gradient in heparin agarose chromatography buffer.

EMSA. Probes for electrophoretic mobility shift assay (EMSA) analyses were generated from duplexed oligonucleotides by treatment with T4 polynucleotide kinase and [γ -³²P]ATP. All probes were purified on 10% polyacrylamide gels. Protein-DNA binding reactions were performed in a 20- μ l reaction mixture containing binding buffer (10 mM HEPES, 50 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.1% Triton X-100, 2.5% glycerol), 1 μ l of 1-mg/ml bovine serum albumin, 0.5 μ g of poly(dI-dC), 2 μ l of the protein fraction, and dH₂O (up to 19 μ l). After 10 min at 25°C, ³²P-labeled probe DNA (50,000 cpm) was added and incubation continued for an additional 20 min. Competition assays involving unlabeled competitors were done in the same manner, with unlabeled competitor being added prior to probe addition. In immunological supershift assays, 2 μ l of anti-Jun (KM-1,D), anti-Fos (K-25), and anti-ATF (25C10G) antibodies (Santa Cruz Biotechnology, Inc.) or anti-p53 antibody (Oncogene Sciences) was added to binding reactions 10 min after probe addition and incubation was allowed to continue for an additional 20 min. Reaction mixtures were separated on 5% nondenaturing polyacrylamide gels run in glycerol tolerant buffer (89 mM Tris,

28.5 mM taurine, 0.5 mM EDTA). The sequences of oligonucleotides utilized in EMSAs are indicated below.

Methylation interference assays. 3'-end-labeled probe was generated by treating the ZII oligonucleotide (5'-GATCTCCTCTGTGATGTCATGGTTTGGG3') with terminal deoxynucleotidyltransferase and [³²P]cordycepin-5'-triphosphate (DuPont NEN) and annealing the complementary-stand oligonucleotide. Duplexed probe was methylated with dimethyl sulfoxide and gel purified. The standard binding reaction was increased to a 50- μ l total volume with 5 $\times$ 10⁶ cpm of probe. EMSA gels were run as described above and transferred to DEAE-cellulose (NA-45; Schleicher and Schuell, Inc.) by semidry electrophoretic transfer (Bio-Rad). Membranes were exposed briefly to film, and both the free probe and shifted complexes were excised. Membrane slices were placed in 300 μ l of elution buffer (1.5 M sodium acetate, 1 mM EDTA) and incubated at 37°C for 30 min with occasional vortexing. Eluted DNA was extracted with chloroform and precipitated with ethanol. DNA was resuspended in cold 20 mM ammonium acetate-1 mM EDTA-1 M piperidine and incubated at 90°C for 30 min. Samples were then concentrated by extraction with butanol, dried, and analyzed on 20% sequencing gels under standard conditions (30).

UV cross-linking. An oligonucleotide containing the ZII binding site and a 17-bp 3' sequence homologous to the M13 forward sequencing primer (5'-TCTCTGTGATGGTCATGGTTTGGGACTGGCCGTCGTTTAC3') was synthesized. The M13 primer (5'-GTAAAACGACGGCCAGT3') was annealed, and duplex probe was generated by strand extension with the Klenow fragment of DNA polymerase (New England Biolabs) and Sequenase II (Amersham) in the presence of dATP, dGTP, bromo-dUTP, and [³²P]dCTP. Fully duplexed probe was purified by gel electrophoresis. Standard EMSA gels were run as described above. After electrophoresis, gels were placed directly on a shortwave UV light source (305 nm wavelength; Fotodyne) for 30 min. Protein-DNA complexes were identified by autoradiography, recovered by excision from wet gels, and eluted by overnight incubation with mixing at 37°C in 0.5 M ammonium acetate-1 mM EDTA-10 mM magnesium acetate-1% sodium dodecyl sulfate (SDS). Following elution, buffer exchange was performed by passing samples through a Bio-Spin 6 desalting column (Bio-Rad) equilibrated in nuclease digestion buffer (10 mM MgCl₂, 10 mM CaCl₂, 10 mM Tris-HCl [pH 7.5]). Samples were treated with 50 μ g of DNase I and 50 μ g of micrococcal nuclease for 20 min at room temperature prior to being run on SDS-12% polyacrylamide gels. Proteins were visualized by autoradiography of the fixed and dried gels.

Recombinant plasmids. All constructs which placed ZII sites upstream of the simian virus 40 early promoter utilized pGL2-Promoter (Promega) as a base luciferase expression vector. Duplexed ZII site oligonucleotides (5'-GATCTCCTCTGTGATGTCATGGTTTGGG3' and its complement) and mutant ZII site oligonucleotides (5'-GATCTCCTCTGTGCTATCATGGTTTGGG3' and its complement) with *Bgl*II-compatible overhangs were ligated into *Bgl*II-linearized pGL2-Promoter. The numbers and orientations of ZII and mutant ZII sites in resulting clones were determined by sequencing. The Zp fragments in ZII and mutZII, which include the start site of transcription and extend through the ZII site, were generated by PCR using ZII site primers with a unique internal *Nde*I site. The primers utilized were 5'-CAAAGGTGGCCGCAAGGTGCAAT3' and either wild-type ZII (5'-CATATGCCCAAAACCATGACATCACAGAGGA3') or mutant ZII (5'-CATATGCCCAAAACCATGATAGCATCGAGGA3'). These PCR products were cloned into the Invitrogen PCR II TA cloning vector. Additional clones were generated by *Nde*I cleavage of these TA clones and subsequent ligation with a duplexed oligonucleotide containing three copies of the ZIC sequence (5'-TATAGTTTCTAAAAGAGGAGGAGGTAGTTTCTAAAAGAGGAGGAGGTAGTTTCTAAAAGAGGAGGAGGC3' and its complement). The four resulting clones (ZII, mutZII, ZII3ZIC, and mutZII3ZIC) were subcloned into the pGL2-Basic vector (Promega) by digesting the TA clones with *Hind*III and *Eco*RV and ligating this fragment into *Hind*III- and *Sma*I-digested pGL2-Basic.

Cell culture, transfections, and luciferase assays. Cells were grown at 37°C in Opti-MEM 1 reduced-serum medium (Gibco) containing 5% fetal bovine serum (Intergen). All transfections were done by electroporation with a Bio-Rad Gene Pulser apparatus. After being washed in medium, 5 $\times$ 10⁶ cells in 0.8 ml of medium were added to 0.4-cm electroporation cuvettes containing 20 μ g of plasmid DNA. Electroporation was performed at room temperature, 960 μ F, 200 Ω , and 0.35 kV (0.25 kV for BJAB cells). For chemical induction, cells were returned to medium containing the following induction mix: 20 ng of phorbol myristate acetate (Sigma) per ml, 4 mM *n*-butyric acid (Sigma), 7.5 μ M A23187 (CalBiochem), and 0.5 μ g of a 50:50 mix of 1,2-dioctanoyl-*rac*-glycerol and 1,2-dioctanoyl-*sn*-glycerol (Sigma) per ml. Transient transfections were harvested at 18 h posttransfection and induction. Cell lysis and luciferase assays were performed by using the Promega luciferase assay system. Equivalent amounts of protein were assayed by the addition of assay reagent and immediately read in a liquid scintillation counter (LKB Wallac). All reported values are the averages of at least three separate experiments.

RESULTS

Isolation of a ZII site-specific DNA-binding activity distinct from AP-1. Although the ZII site in the BZLF1 promoter (Zp) has previously been shown by DNase I footprinting to bind a

FIG. 1. The AP-1- and ZII site-specific binding activities are separable activities. EMSA analyses of crude and heparin agarose-fractionated uninduced and chemically induced BJAB nuclear protein extracts binding to an AP-1 binding site oligonucleotide probe (5'CGCTTGATGACTCAGCCGAA3') (A) and a ZII binding site oligonucleotide probe (5'GATCTCCTCTGTGATGTCATGGTTGGG3') (B). The fraction numbers in both panels are identical and are indicated above the lanes in panel A. The mobility of the probe-specific shift is indicated by arrows to the right of each gel.

Jun/Fos complex isolated from baculovirus-infected cell extracts (13), the nature of the protein-DNA interactions occurring at this locus in B cells has not been addressed. To investigate potential protein-DNA interactions occurring within Zp, high-salt nuclear protein extracts were generated from the EBV-negative B-cell line BJAB. Extracts were prepared both from uninduced cells and from cells induced for 18 h in a chemical induction mix containing 20 ng of phorbol myristate acetate per ml, 4 mM *n*-butyric acid, 7.5 μ M A23187, and 0.5 μ g of a 50:50 mix of 1,2-dioctanoyl-*rac*-glycerol and 1,2-dioctanoyl-*sn*-glycerol per ml. These crude extracts were then fractionated by chromatography through heparin agarose, with bound protein being eluted with a 0.05 to 1 M linear KCl gradient.

As a control for the efficacy of the chemical induction protocol, protein fractions from both uninduced and chemically induced extracts were tested for the ability to bind a consensus

AP-1 binding site oligonucleotide probe in EMSAs. Figure 1A shows the DNA-binding profiles obtained with comparable protein fractions from each type of extract. The equivalence of these fractions was established by assessing the protein and salt concentrations and ubiquitous DNA-binding activities found in individual fractions. The induced extract demonstrated dramatically increased AP-1 DNA-binding activity, particularly in fractions 23 to 27, indicating the effectiveness of the induction protocol. The specificity of the binding activity in these fractions was confirmed by competition analysis and immunological supershift assay (data not shown) and indicated that these fractions displayed an AP-1-specific DNA-binding activity. Thus, the induction protocol utilizing TPA, sodium butyrate, dIC8, and A23187 was effective in inducing a binding activity known to be inducible by these chemicals and resulted in an increase in specific binding activity of greater than 10-fold.

After the AP-1 site-specific DNA-binding activity in these

FIG. 2. Cross-competition EMSA analysis of BJAB fraction 25. The peak binding activities for both AP-1 and ZII sites were found to be contained in fraction 25 (Fig. 1). This fraction was used in a cross-competition analysis to determine the specificity of the binding of this fraction to each probe. The binding of uninduced and induced fractions to the AP-1 probe (none) was tested for specificity by the addition of 25 and 125 ng of unlabeled homologous competitor (AP-1) or equivalent amounts of unlabeled ZII competitor. The results obtained with uninduced fraction 25 and the AP-1 probe were analogous to those obtained with the induced fraction upon longer exposure of the gel. Binding to the ZII site probe was analyzed in the same manner.

extracts had been characterized, the ZII site-specific binding profile of these same fractions was investigated. Surprisingly and contrary to the results obtained with the AP-1 probe, ZII-specific binding activity was found to be roughly equivalent in both uninduced and induced cell extracts (Fig. 1B). The peak fractions of ZII site-specific DNA-binding activity were fractions 23 to 27, as was the case for the AP-1 probe. However, the appearances and mobilities of the shifted complexes generated with each probe suggested that the protein-ZII and protein-AP-1 complexes are very distinct complexes. The specificities of these two binding activities were tested by cross-competition EMSA with fraction 25 from uninduced and induced extracts. As shown in Fig. 2, the formation of the AP-1 probe-specific complex is much more efficiently inhibited by unlabeled homologous competitor (AP-1) than by unlabeled ZII competitor. The affinity of this binding to the AP-1 probe is roughly 10-fold higher than that for the unlabeled ZII competitor. When the converse experiment was performed with the ZII site as the probe, it was shown that while the AP-1 competitor does compete for binding, it does so less efficiently than the unlabeled homologous competitor. Again, a 5- to 10-fold difference in binding affinity was evident.

The sequence similarity between the ZII and consensus AP-1 and CREB sites implicates a variety of Fos/Jun and ATF/CREB protein family members as possible ZII site binding factors. The finding that the peak binding activities for both the ZII and AP-1 probes were contained in the same fractions (Fig. 1), combined with the knowledge that Fos/Jun and ATF/CREB family members readily form heterologous complexes with a variety of transcriptional factors (10, 15), raised the possibility that the two observed binding activities share some components. To address this possibility, immunological supershift assays with several broadly reactive antibodies raised against various members of the *fos*, *jun*, and ATF/CREB gene

families (*c-fos*, *fosB*, *fra-1*, *fra-2*, *c-jun*, *junB*, *junD*, ATF-1, CREB-1, and CREM-1) were performed. Antibodies specific for c-Jun and p53 were also utilized. As shown in Fig. 3A, lanes 2 and 3, the AP-1 complex is specifically supershifted by the broadly reactive *fos* and *jun* antibodies. The ZII complex was unaltered in mobility and intensity by the addition of any antibody (Fig. 3B), even at antibody concentrations that completely supershifted the AP-1 complex. In addition, when both AP-1 and ZII probes were added to a single reaction mixture, two complexes of different mobilities were visualized (Fig. 3C, lane 1). These complexes were further distinguished by the ability of the anti-*fos* antibody to specifically supershift only the AP-1 complex (Fig. 3C, lane 2), lending further support to the idea that the AP-1- and ZII-specific complexes are indeed distinct and separable activities. Thus, on the basis of competition analysis and immunological supershift data, the ZII- and AP-1-specific binding activities clearly appear to be distinct cofractionating activities. The ZII site-specific activity was named ZIIBC and hereafter is referred to as such.

DNA sequence requirements for the binding of ZIIBC. With the specificity of the binding of ZIIBC established, the DNA sequence requirements for the binding of ZIIBC to the ZII site oligonucleotide were addressed. Of particular interest was the role of the 8-bp core sequence homologous to the AP-1 site in the *c-jun* promoter. A series of mutant binding sites was generated (Table 1) and utilized as probes in EMSAs and as unlabeled competitors in competition with the wild-type ZII site probe. The ZM1 mutant is based on a construct reported by Flemington and Speck (13) to result in decreased TPA inducibility of Zp. ZM3 is based on a commercially available mutant AP-1 oligonucleotide that abolishes AP-1 binding (Santa Cruz Biotechnology, Inc.). The other mutations were designed to span the length of the ZII oligonucleotide, placing

FIG. 3. The ZII-specific complex is not supershifted by antibodies to Fos/Jun and ATF/CREB complex components. The abilities of a variety of antibodies to supershift the AP-1- (A) and ZII (B)-specific complexes were tested. Lane 1, the binding of fraction 25 to the indicated probe in the absence of any antibody; lanes 2 to 6 contain antibodies raised against *fos* gene family members, *jun* gene family members, *c-jun*, ATF/CREB gene family members, and p53, respectively. (C) Fraction 25 was incubated with both ZII and AP-1 site probes in the absence of any antibody (lane 1) or in the presence of antibody raised against *fos* gene family members. The mobility of each free probe, the two distinct shifts, and the mobility of the supershifted complex observed are indicated by arrows on the right.

mutations in the 8-bp core sequence as well as in the flanking sequences.

The capacity of ZIIBC to bind directly to these oligonucleotides when utilized as probes in EMSAs was tested. The results are shown in Fig. 4A. ZM2, ZM5, and ZM6 were bound by ZIIBC as efficiently as the wild-type ZII probe was. ZIIBC bound the ZM3 mutant only minimally, while no detectable binding was evident with ZM1 and ZM4. The ability of each of these mutant binding sites to compete with the wild-type ZII site for ZIIBC binding was then tested (Fig. 4B). Mutants to which ZIIBC was shown to bind (ZM2, ZM5, and ZM6 [Fig. 4A]) were as effective as the wild-type sequence was in competing for ZIIBC binding. The ZM3 oligonucleotide, to which minimal binding was demonstrable, was able to compete approximately 10-fold less efficiently than the wild-type was. ZM1 and ZM4, as would be expected from the binding data in Fig. 4A, were not able to compete with the wild-type probe for the binding of ZIIBC. Table 1 summarizes these results and illustrates that mutations in the core sequence abolished binding, whereas mutations in the flanking sequences were tolerated.

To further define the ZII binding site and compare the

DNA-protein interactions occurring with ZIIBC isolated from uninduced and induced cells, methylation interference assays with the ZII oligonucleotide probe were performed. As shown in Fig. 5, the interference patterns from uninduced and induced complexes appear to be identical. The data indicate that interference begins at approximately 1 bp into the core (C nucleotide at 3' end) and extends to at least 2 to 3 bp 5' to the core sequence.

Physical characterization of ZIIBC. The ZIIBC-specific shift for both uninduced and induced extracts is a fairly diffuse pattern similar to those sometimes associated with multiprotein-DNA complexes. To begin the definition of the components of ZIIBC, UV cross-linking was done to identify the molecular masses of the constituents of this complex that directly contact DNA. The proteins specifically cross-linked from ZIIBC complexes isolated from uninduced and induced cells appear to be identical in molecular size (Fig. 6). A major species with an apparent molecular mass after nuclease treatment of 26 kDa and a slightly less efficiently cross-linked species with a mobility equivalent to 36 kDa were consistently detected from shifted ZIIBC-containing complexes.

TABLE 1. Summary of mutant ZII binding site oligonucleotide data^a

Oligonucleotide	Sequence (5'-3')			Mutation in core	Binding
ZII	GATCTCCT	CTG	TGATGTCA	TGGTTTGGG	
ZM1	GATCTCCT	CTG	TGATGAAT	TGGTTTGGG	No
ZM2	GATCTACC	CTG	TGATGTCA	TGGTTTGGG	Yes
ZM3	GATCTCCT	CTG	CAATGTCA	TGGTTTGGG	No
ZM4	GATCTCCT	CTG	TGCTATCA	TGGTTTGGG	No
ZM5	GATCTCCT	CTG	TGATGTCA	CGGCTTGGG	Yes
ZM6	GATCTCCT	CTG	TGATGTCA	TGGTTTAA	Yes

^a The sequences shown represent the oligonucleotides utilized in binding and competition EMSA analysis (Fig. 4). The smaller box indicates the location of the 8-bp core sequence. Each mutation is indicated by underlining and bold type. The larger box depicts the ZII site as defined by methylation interference (Fig. 5).

FIG. 4. The ZII site octanucleotide core sequence is required for the binding of the ZII binding factor. A variety of mutant ZII binding site oligonucleotides (Table 1) was generated and utilized in EMSA analyses. (A) The binding of the ZII factor to mutant sites was tested by using each oligonucleotide as a probe in the presence (+) or absence (–) of protein. (B) The ability of each mutant site to compete with the wild-type ZII probe for binding was tested. The competitor and the amount added to each reaction mixture are listed above each lane. These results are tabulated in Table 1.

In the process of characterizing ZIIBC, slight differences in the mobility of the factor isolated from uninduced and induced extracts were consistently observed by EMSAs. Figure 7 demonstrates the retardation of ZIIBC isolated from induced cells relative to ZIIBC isolated from uninduced cells. This mobility difference was also seen in additional independently prepared BJAB extracts (data not shown). The magnitude of this observed difference in mobility upon chemical induction is consistent with a minor alteration in molecular mass. As the components of each complex that directly contact DNA were shown to be of identical molecular size, protein modification may account for the observed differences.

Since phosphorylation plays a major role in many inductive pathways, the effects of dephosphorylation of ZIIBC on its DNA-binding ability were investigated. Considering that the chemical inducers utilized in the generation of the ZIIBC-containing protein extracts are known to activate a variety of

FIG. 5. Methylation interference patterns generated by uninduced and induced ZII binding factors. The DNA sequence requirements for the binding of uninduced and induced fraction 25 to the ZII site were investigated by methylation interference. Lanes B, DNA isolated from bound complexes; lanes F, flanking lanes in which the methylation pattern of the free ZII probe is shown. The sequence of the oligonucleotide probe is shown on the right.

protein kinases, including protein kinase C and the calcium- or calmodulin-dependent protein kinase, potato acid phosphatase (PAP) was used as a general protein phosphatase. PAP has previously been shown to be effective in the removal of phos-

FIG. 6. Protein components of the ZII binding factor that directly contact DNA. UV cross-linking of uninduced (lanes 2 and 5) and induced (lanes 3 and 6) ZII binding factors to the ZII site oligonucleotide indicates that both complexes consist of proteins of equivalent electrophoretic mobilities. A slight increase in mobility is seen after nuclease treatment (lanes 5 and 6). The apparent molecular masses of the isolated proteins (26 and 36 kDa) were calculated by comparison with molecular mass standards (lane 1).

FIG. 7. Uninduced and induced ZII factor DNA-protein complexes differ slightly in their relative mobilities as determined by EMSA analysis of uninduced and induced ZII factor-containing fractions. Reactions were done in triplicate to demonstrate reproducibility. The mobility of the center of each shifted complex is indicated by arrows.

phate groups from a variety of phosphoproteins (6). The treatment of ZIIBC-containing fractions with PAP resulted in decreased binding of ZIIBC isolated from both uninduced and induced cells (data not shown). Thus, it appears that ZIIBC must be phosphorylated to bind DNA. However, under these conditions we could not demonstrate an appreciable effect of dephosphorylation on the relative mobilities of the complexes formed with extracts from uninduced and induced cells.

ZIIBC functional studies. The finding that ZIIBC is abundant in nuclear protein extracts prepared from B lymphocytes, coupled with the observed alteration of its binding activity upon chemical induction, suggests that ZIIBC has the potential to play a pivotal role in the regulation of Zp. The induc-

tion-enhanced retardation of the ZIIBC-DNA complex suggests a modification that may have significant functional consequences. Conceivably, ZIIBC could act as a repressor of Zp until signals for reactivation, which then modify ZIIBC, are received. Thus, modification of this factor may relieve repression or allow ZIIBC to function as an activator of Zp. To address the possibility that ZIIBC functions as a repressor in uninduced cells, single and multiple copies of the ZII site and a mutant ZII site to which ZIIBC does not bind were cloned upstream of the simian virus 40 early promoter in the luciferase reporter construct pGL2-Promoter. These constructs were used in transient transfections in a variety of uninfected and EBV-infected cells and assayed for the expression of luciferase. Constructs consistently showed a three- to fourfold increase in expression over that of the vector alone. However, no additional statistically significant differences in the levels of expression, either positive or negative, could be demonstrated between clones containing single or multiple copies of either wild-type or mutant ZII sites (data not shown).

The role of the ZII site in the context of the authentic BZLF1 promoter was also tested. Luciferase reporter gene constructs which contained the Zp sequence from the start site of transcription up through the ZII site (–65 bp) were generated. To demonstrate a functional role for ZIIBC in the regulation of transcription from these constructs, a mutant ZII site to which ZIIBC does not bind was cloned in an analogous manner and its activity was compared with that of the wild-type sequence. These constructs were identical at all but 4 bp. The predicted levels of both basal expression and chemically induced expression from these constructs (ZII and mutZII) were low. Two additional constructs including multiple copies of the ZI repeat of Zp and therefore more closely resembling the architecture of full-length Zp were generated. These constructs were predicted to result in increased levels of expression, particularly upon TPA induction, so that any effect of a mutant ZII site on transcription would be detectable. These constructs were generated by the addition of three tandem copies of the Zp ZIC sequence to the original wild-type and mutant constructs, creating ZII3ZIC and mutZII3ZIC. All of these constructs were utilized in transient-transfection assays in a variety of cell lines, and both basal and chemically induced levels of luciferase expression were evaluated.

FIG. 8. Transcriptional activities of ZII and mutant ZII luciferase constructs. Zp luciferase constructs were tested for their level of expression in uninduced and chemically induced cells in transient-transfection assays. The ZII construct contains the Zp sequence from the start site of transcription through the ZII site cloned into pGL2-Basic. mutZII is identical at all but 4 bp. ZII3ZIC and mutZII3ZIC contain an additional three copies of the ZIC sequence. The plotted data are luciferase levels (in counts per minute) measured in a scintillation counter.

Figure 8 shows the results of these assays with BJAB cells (EBV negative) and two EBV-infected cell lines (Raji and B95-8). The overall patterns of expression in both uninfected and infected cells of all three cell lines were similar, supporting the role of cellular factors in expression from Zp. Comparison of the data obtained with uninduced and chemically induced cells likewise revealed the same overall pattern of expression; however, the differences in the magnitude of values obtained with different constructs were much greater for induced cells. In uninduced cells, the ZII construct resulted in low but consistently repeatable increased luciferase expression over vector background expression (1.5-fold). This increase in expression was abolished by the insertion of a mutant ZII site. The results obtained with induced cells support the importance of the ZII site in an even more dramatic manner. The ZII site alone results in a small but statistically significant increase in luciferase expression upon chemical induction, while the combination of ZII and 3ZIC is more highly inducible, particularly in Raji cells (sevenfold). This suggests functional cooperation between the ZII and ZIC binding factors. The three copies of ZIC contribute only slightly to the TPA inducibility of these constructs, as evidenced by the lack of significant induction of the mutZII3ZIC construct. Thus, the prevention of ZIIBC binding has significant consequences on expression from Zp, particularly in chemically induced cells.

DISCUSSION

The roles of Zta in the EBV life cycle and the inducibility of the BZLF1 promoter by external stimuli confirm the critical natures of both viral and cellular transcription factors directly affecting Zp activity in the switch from viral latency to lytic replication. Although several binding sites for putative cellular factors have been identified within Zp, the protein-DNA interactions occurring in this complex regulatory region have not been characterized. The ZII site, defined by Flemington and Speck (13, 14), was shown to be essential in the absence of Zta and dispensable in the presence of Zta and to contribute to the overall TPA inducibility of Zp. These observations defined a potentially significant functional role for the ZII binding factor in the initial cellular factor-dependent activation of transcription from Zp and established some functional criteria for assessing potential ZII binding factors.

The similarities between the ZII site sequence and the sequences of the AP-1 and C/EBP sites led to previous studies establishing the binding of AP-1 and C/EBP to the ZII site in vitro (13, 17). However, the biological significance of these protein-DNA interactions has not been evaluated for C/EBP, and the results with AP-1 have been, at best, inconclusive. In fact, it was reported that transient expression of functional *c-jun*, a component of AP-1, did not enhance the expression of Zp in Ramos cells (13).

The results presented here describe the identification and physical and functional characterizations of a cellular protein complex that specifically binds the ZII site. The accumulated data presented here demonstrate that this binding complex, ZIIBC, is distinct from AP-1 and C/EBP and therefore from the DNA-binding activities previously characterized for this site. In fact, it was reported that transient expression of functional *c-jun*, a component of AP-1, did not enhance the expression of Zp in Ramos cells (13).

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the DNA-binding activities previously characterized for this site. In contrast to previous studies that utilized footprinting techniques with crude nuclear protein extracts to reveal the location of protein-binding sites in Zp DNA, we have utilized EMSAs as the main screening assay for ZII site-specific DNA-binding activities in fractionated nuclear extracts. Previously, footprinting has permitted the identification of binding sites that have proven to be critical in the regulation of transcription. However, EMSA analyses extended by a number of other approaches, including competition and antibody supershift analyses, have enabled us to address protein composition (including those components that do not directly contact DNA), binding specificity, protein modifications, and occasionally the identities of components of the complex formed at the ZII site. For example, the specificities of related DNA-protein interaction assays, such as methylation interference and UV cross-linking, were greatly increased through the use of DNA-protein complexes isolated from EMSA gels.

ZIIBC was found to be equally abundant in extracts generated from both uninduced and chemically induced cells, demonstrating that unlike with AP-1, in which DNA binding is enhanced by TPA treatment, TPA treatment did not have a direct effect on the amount of ZIIBC DNA-binding activity. The cofractionation of the AP-1- and ZII-specific activities, as well as the propensity of Fos/Jun and ATF/CREB family members to form heterodimers with members of other transcription factor families (10, 15), raised the logical possibility that these two complexes share some components. However, this does not appear to be likely, since the two activities were readily distinguishable when both AP-1 and ZII probes were utilized in a single reaction. Furthermore, antibodies broadly reactive with the various members of the *fos* and *jun* gene families (*c-fos*, *fosB*, *fra-1*, *fra-2*, *c-jun*, *junB*, and *junD*) specifically supershifted only the AP-1-specific complex and did not alter the mobility of the ZII complex. Interestingly, the ineffectiveness of an additional *c-jun*-specific antibody in supershifting the AP-1-specific complex suggests that this complex is composed of either *junB* or *junD* rather than *c-jun*.

The sequence similarity between the ZII site and consensus CREB binding sites also suggests a role for members of the ATF/CREB family. In addition, the molecular masses of some of the members of this family are somewhat consistent with the larger cross-linked component of ZIIBC (~36 kDa). However, antibodies reactive with ATF-1, CREB-1, and CREM-1 were unable to alter the mobility and intensity of ZIIBC binding. Thus, it appears that the components of ZIIBC isolated from B cells are distinct from the purified factors previously shown to bind this site.

The presence of an octanucleotide core identical to the AP-1 site found in the *c-jun* promoter raised the possibility that AP-1 could bind this site if it were unoccupied. However, the equivalence of ZIIBC binding activities in uninduced and chemically induced extracts appears to preclude this possibility. Through mutagenesis studies and methylation interference analysis, we have defined a ZIIBC binding site that encompasses the octanucleotide core sequence and 2 to 3 bases 5' of this core. This establishes the AP-1 site as a necessary but insufficient component of the ZIIBC binding site. The inability of ZIIBC to bind to a ZII site oligonucleotide (ZM1; Table 1) containing point mutations that have previously been shown to result in decreased TPA inducibility of Zp (13) hints at the functional significance of ZIIBC and supports ZIIBC as a legitimate ZII site binding factor in B cells.

The contribution of the ZII site to overall TPA inducibility of Zp implies that the factor that binds the ZII site is either a distinct factor in uninduced and induced cells or subject to

some modification upon chemical induction. Our physical data indicate that the components of ZIIBC that directly contact DNA are of equivalent molecular masses in complexes isolated from uninduced and chemically induced cells. Thus, although it is possible, it is unlikely that the ZII site is bound by two entirely distinct complexes in uninduced and induced cells. Instead, TPA induction may result in the modification of components of the complex already present in the complex prior to chemical induction. Alternatively, the complex found in induced cells may differ from the complex isolated from uninduced cells in that TPA induction may allow the recruitment of additional, non-DNA-binding components to the complex. These options are not mutually exclusive, and both are supported by the slight but reproducible difference in the mobilities of the two complexes in EMSA analyses.

Phosphorylation is a chemical modification that is frequently used in biological systems as a mechanism for the biochemical regulation of cellular activities. The treatment of cells with chemical inducers activates a variety of cellular second-messenger signaling pathways that result in the activation of protein kinase C and the calcium- or calmodulin-dependent protein kinase (25, 26). Although these studies have precluded the participation of AP-1 in the activation of Zp via the ZII site, it is nevertheless conceivable that ZIIBC activity may be regulated in a similar manner, either directly or indirectly, by phosphorylation. Thus, the mobility difference between ZIIBC complexes isolated from uninduced and induced cells observed in EMSAs may be the result of phosphorylation. In addressing this possibility, we demonstrated that the DNA-binding activity of ZIIBC is dependent on phosphorylation events that are sensitive to treatment with PAP (data not shown). Unfortunately, these results do not distinguish between the role of phosphate residues in DNA binding by the components of ZIIBC that directly contact DNA and the role of phosphate residues in protein-protein interactions that may be necessary to form a DNA-binding-competent complex. In addition, changes in the levels or patterns of phosphorylation as a result of chemical induction, analogous to the modification of Fos and Jun in response to TPA (4, 7), are not ruled out. Resolution of these possibilities may require the cloning of ZIIBC components and analysis of the role of phosphorylation of the various components in ZIIBC function.

The physical characteristics of ZIIBC defined in this report are concordant with the functional significance of the ZII site in Zp regulation as first defined by Flemington and Speck. Although ZIIBC is present in both uninduced and chemically induced cells, it is directly affected by chemical induction. Our functional studies, in agreement with those of Flemington and Speck (13), demonstrate that the ZII site alone is not sufficient to cause the repression of a heterologous promoter (data not shown). Therefore, it does not appear that ZIIBC functions in the repression of Zp expression during latency. Instead, the ZII site stimulates expression in the context of Zp in both uninduced and induced cells. Thus, the repression of Zp in latently infected cells must be dependent on other Zp sequences that have a negative effect on Zp expression (13, 23, 31).

The ZII site appears to most significantly increase Zp expression when it is in combination with the ZIC site in chemically induced cells, suggesting that in the context of full-length Zp, the ZII site is essential in the initial triggering of transcription from Zp. The equivalence of the transfection data for various ZII site constructs in both uninfected and EBV-infected cells (Fig. 8) establishes the sufficiency of cellular factors in ZII site-mediated induction of Zp. The increased inducibility seen in Raji cells is most likely a difference in cell line

responsiveness to chemical induction, which is known to vary widely from cell line to cell line (20, 29).

The effects of the ZII site on Zp expression are directly dependent on a ZII site to which ZIIBC can bind. This establishes ZIIBC as a cellular factor complex through which chemical induction of Zp expression can be mediated. Additional studies, including the purification of ZIIBC proteins and cloning of the genes encoding individual components, will be necessary to precisely define the nature of ZIIBC and the biological consequences of ZIIBC on the maintenance of latency and the triggering of reactivation. Although ZIIBC may very well be partly composed of proteins that have previously been characterized in the context of other promoters in other systems, ZIIBC does not appear to contain the factors previously shown to bind the ZII site. Nevertheless, the interactions of multiple cellular factors are undoubtedly required for quantitative and appropriate expression of Zta; the identification and characterization of additional cellular factors that interact with Zp and possibly ZIIBC will facilitate better understanding of Zp regulation during various phases of the EBV life cycle.

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REFERENCES

- Angel, P., K. Hattori, T. Smeal, and M. Karin. 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**:875-885.
- Angel, P., M. Imagawa, R. Chui, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulator *trans*-acting factor. *Cell* **49**:729-739.
- Auwerx, J., and P. Sassone-Corsi. 1991. IP-1: a dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation. *Cell* **64**:983-993.
- Barber, J. R., and I. M. Verma. 1987. Modification of *fos* proteins: phosphorylation of *c-fos*, but not *v-fos*, is stimulated by 12-tetradecanoyl-phorbol-13-acetate and serum. *Mol. Cell. Biol.* **7**:2201-2211.
- Bauer, G., P. Hoffer, and M. Simon. 1982. Epstein-Barr virus induction by a serum factor. Characterization of the purified factor and the mechanism of its activation. *J. Biol. Chem.* **257**:11411-11415.
- Bingham, E. W., and H. M. Farrell, Jr. 1976. Removal of phosphate groups from casein kinase by potato acid phosphatase. *Biochim. Biophys. Acta* **429**:448-460.
- Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter. 1991. Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**:573-584.
- Cen, H., and J. McKnight. 1994. EBV-immortalized isogenic human B cell clones exhibit differences in DNA-protein complex formation on the BZLF1 and BRLF1 promoter regions among latent, lytic and TPA-activated cell lines. *Virus Res.* **31**:89-107.
- Chevallier-Greco, A., E. Manet, P. Chavrier, C. Monsnier, J. Dallie, and A. Sargeant. 1986. Both Epstein-Barr (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO J.* **5**:3243-3249.
- Chevray, P. M., and D. Nathans. 1992. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc. Natl. Acad. Sci. USA* **89**:5789-5793.
- Countryman, J., and G. Miller. 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterologous viral DNA. *Proc. Natl. Acad. Sci. USA* **82**:4085-4089.
- Faggioni, A., C. Zompetta, S. Grimaldi, G. Barile, L. Frati, and J. Lazzdins. 1986. Calcium modulation activates Epstein-Barr virus genome in latently infected cells. *Science* **232**:1554-1556.
- Flemington, E., and S. H. Speck. 1990. Identification of phorbol ester response elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. *J. Virol.* **64**:1217-1226.
- Flemington, E., and S. H. Speck. 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. *J. Virol.* **64**:1227-1232.
- Hai, T., and T. Curran. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* **88**:3720-3724.

16. Kallin, B., J. Luka, and G. Klein. 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. *J. Virol.* **32**:710–716.
17. Kouzarides, T., G. Packham, A. Cook, and P. J. Farrell. 1991. The BZLF1 protein of EBV has a coiled coil dimerization domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. *Oncogene* **6**:195–204.
18. Laux, G., U. K. Freese, R. Fisher, A. Polack, E. Koffler, and G. W. Bornkamm. 1988. TPA-inducible Epstein-Barr virus genes in Raji cells and their regulation. *Virology* **162**:503–507.
19. Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward. 1986. Promiscuous *trans* activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. *J. Virol.* **60**:140–148.
20. Liebowitz, D., and E. Kieff. 1993. Epstein-Barr virus, p. 107–172. *In* B. Roizman, R. J. Whitley, and C. Lopez (ed.), *The human herpesvirus*. Raven Press, New York.
21. Luka, J., B. Kallin, and G. Klein. 1979. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by *n*-butyrate. *Virology* **94**:228–231.
22. Miller, G. 1989. The switch between EBV latency and replication. *Yale J. Biol. Med.* **62**:205–213.
23. Montalvo, E. A., Y. Shi, T. E. Shenk, and A. J. Levine. 1991. Negative regulation of the BZLF1 promoter of Epstein-Barr virus. *J. Virol.* **65**:3647–3655.
24. Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682–6686.
25. Nishizuka, Y. 1986. Studies and perspectives on protein kinase C. *Science* **233**:305–312.
26. Nose, P. S., L. C. Griffith, and H. Schulman. 1985. Ca²⁺-dependent phosphorylation of tyrosine hydroxylase in PC12 cells. *J. Cell Biol.* **101**:1182–1190.
27. Papavassiliou, A. G., K. Bohmann, and D. Bohmann. 1992. Determining the effect of inducible protein phosphorylation on the DNA-binding activity of transcription factors. *Anal. Biochem.* **203**:302–309.
28. Rowe, M., A. L. Lear, D. Croom-Carter, A. H. Davies, and A. B. Rickinson. 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J. Virol.* **66**:122–131.
29. Ruf, I. K., and D. R. Rawlins. Unpublished data.
30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. Schwarzmann, F., N. Prang, B. Reichelt, S. Haist, M. Marschall, and H. Wolf. 1994. Negatively cis-acting elements in the distal part of the promoter of Epstein-Barr virus trans-activator gene BZLF1. *J. Gen. Virol.* **75**:1999–2006.
32. Takada, K., and Y. Ono. 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. *J. Virol.* **63**:445–449.
33. Takada, K., N. Shimizu, S. Sakuma, and Y. Ono. 1986. *trans* activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. *J. Virol.* **57**:1016–1022.
34. Tovey, M. G., G. Lenoir, and J. Begon-Lours. 1978. Activation of latent Epstein-Barr virus by antibody to human IgM. *Nature (London)* **276**:270–272.
35. zur Hausen, H., F. J. O'Neill, and U. Freese. 1978. Persisting oncogenic herpesvirus induced by the tumour promoter TPA. *Nature (London)* **272**:373–375.