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Expression and function of ZEBRA: the Epstein-Barr viral replication activator

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EXPRESSION AND FUNCTION OF ZEBRA:

THE EPSTEIN-BARR VIRAL REPLICATION ACTIVATOR

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ABSTRACT

EXPRESSION AND FUNCTION OF ZEBRA:

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Epstein-Barr virus (EBV) encodes a protein, ZEBRA, which enables the virus to switch from a latent to a lytic life cycle. ZEBRA expression was studied in lymphoid cells harboring either standard virus or a mixture of standard and defective viruses. ZEBRA protein could not be detected in cells latently infected with standard EBV but was constitutively expressed in cells containing both defective and standard EBV genomes. Experiments indicated that regulation of transcription of the ZEBRA gene (BZLF1) is a pivotal event in the control of EBV replication. ZEBRA specific BZLF1 transcripts were spontaneously expressed in cells harboring defective virus but were only synthesized by the latent virus after chemical induction. BZLF1 transcription was sensitive to inhibition of protein synthesis but not to inhibition of lytic viral DNA synthesis. This suggests that ZEBRA is an early replicative protein whose expression is dependent on the synthesis of a viral or cellular factor.

The basic domain of the ZEBRA protein is homologous to the Fos/Jun oncogene family and shares their ability to bind

the canonical AP-1 site (TGAGTCA). However, ZEBRA does not contain a leucine zipper domain; a motif necessary for DNA binding of Fos/Jun proteins. Additionally, ZEBRA binds to sites which deviate from the AP-1 consensus sequence. Thus, it was of interest to study the DNA binding properties of the ZEBRA protein.

Deletional mutagenesis of the BZLF1 cDNA indicated that amino acids 172-227, representing the basic and putative dimerization domains, were required for specific binding to AP-1 and divergent sites. Mutagenesis of three basic amino acids, which are conserved in Fos, abrogated ZEBRA binding to all target sequences. Additionally, ZEBRA was determined to bind DNA as a homodimer.

DNA binding studies of ZEBRA and a Fos-GCN4 chimera indicated that although these proteins have homology in their basic DNA binding domains, they have different cognate binding specificities. The autoregulated BZLF1 promoter contains three divergent AP-1 sequences; Fos-GCN4 uniquely recognized one of the sites while ZEBRA bound only the other two sequences. Additionally, ZEBRA, but not Fos, was found to be phosphorylated by casein kinase II in-vitro. This phosphorylation abrogated ZEBRA binding to all of its target DNA sequences. Regulation of ZEBRA's DNA binding activity may be required for the progression of the EBV replicative cycle.

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CHAPTER I

INTRODUCTION

Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus which was first identified in cultured Burkitt's lymphoma cells (BL) (Epstein et al., 1964). The virus has also been linked to nasopharyngeal carcinoma (NPC), a tumor commonly found in southern China (deSchryver et al., 1969). Although EBV has not been proven to be the etiologic agent in either of these malignancies, it has been shown to produce a benign lymphoproliferative disease, infectious mononucleosis (Henle et al., 1968, Niederman et al., 1968). This lymphoproliferation can become fatal in patients who have inherited or acquired immunodeficiencies (Hanto et al., 1981, Purtillo, 1981). Additionally, infection with EBV in-vitro was found to result in the immortalization of fresh neonatal lymphocytes (Henle et al., 1968, Pope et al, 1968, Miller et al., 1969).

Lymphoid cells immortalized with the Epstein-Barr virus generally maintain the virus in the latent state. These cells can harbor up to 800 EBV genomes as unintegrated extra-chromosomal plasmids or episomes (Sugden et al., 1979). The virus is maintained in a tightly latent state in both Burkitt's lymphoma and nasopharyngeal carcinoma (Raab-Traub et al., 1983, Rowe et al., 1986, Tugwood et al., 1987). Conversely, EBV was found to be highly replicated in

oral hairy leukoplakia (OHL), an AIDS associated lesion (Greenspan et al., 1985). OHL is associated with chronic productive EBV infection in the epithelial cells of the tongue. In contrast with BL and NPC cells, replicating virus and mature EBV virions are found in OHL lesions (Greenspan et al., 1985). Thus, Epstein-Barr virus is associated with the pathogenesis of disease in both its latent and lytic states. This study focuses on the role of the EB viral protein, ZEBRA, which regulates the switch between the latent and lytic viral life cycles.

The Epstein-Barr viral genome is a double stranded DNA molecule of approximately 172,000 base pairs (bp) (Pritchett et al., 1975, Hayward and Kieff, 1977, Baer et al., 1984). Whereas the EBV genome is linear in virions, it exists as a covalently closed circular plasmid in latently infected cells (Lindahl et al., 1976, Pritchett et al., 1975). Circularization occurs at the terminal repeats; 4-12 direct reiterations of a 500 bp sequence (Given et al., 1979, Kintner and Sugden, 1979). Only rarely have integrated copies of the EBV genome been identified (Henderson et al., 1983, Matsuo et al., 1984).

EBV infection is restricted to cells expressing the 140 Kd C3d complement receptor which has been designated CR2 or CD21 (Fingeroth et al., 1984, Frade et al., 1985). Resting B lymphocytes as well as epithelial cells of the orophaynx and cervix and some T cell lines express this receptor on

the cell surface (Robinson et al., 1979, Sixbey et al., 1986, 1987, Stocco et al., 1988). Binding of the virus is mediated through the interaction of CR2 and the EBV glycoprotein, gp 350 (Hoffman et al., 1980).

EBV infection results in a continuous proliferation of lymphocytes in-vitro, a process termed immortalization. Cells immortalized by EBV maintain the virus in the latent state and express only a few virally encoded genes. Ten viral gene products have been identified in these cells including six EB nuclear antigens (EBNAs, labeled EBNA-1 to EBNA-6), the latent membrane protein (LMP), two small RNAs (EBER-1 and EBER-2), and the terminal protein (TP) (reviewed in Miller, 1990). Although the process of immortalization is not well understood, the EBNA-2 protein appears to play a critical role. EB viral strains which contain deletions in this region (such as P3-HR-1) are immortalization incompetent (Bornkamm et al., 1982, Rabson et al., 1982).

Replication of the latent EBV genome in synchrony with host cell DNA synthesis results in a stable partitioning of the virus to daughter cells (Adams, 1987, Hampar et al., 1974). This process requires the interaction of the EBNA-1 protein with a 1.8 Kb subfragment of the EBV genome termed oriP, origin of plasmid replication (Yates et al., 1984). EBNA-1 binds a 30 bp repeated sequence located within oriP (Rawlins et al., 1985).

Latent EB virus can be activated, in-vitro, by a

variety of external stimuli such as butyrate, phorbol esters and serum factors (Bauer et al., 1985, Luka et al., 1979, zur Hausen et al., 1979). At least 55 distinct viral genes are expressed during the replicative cycle (Hummel and Kieff, 1982, Weigel et al., 1983). Early antigens (EA) as well as late gene products such as the viral capsid antigen (VCA) (a protein sensitive to inhibition of viral DNA synthesis) (Summers and Klein, 1976) are expressed following induction. Raji cells, which do not replicate viral DNA from the lytic origin, can produce early but not late antigens (Goodman et al., 1978).

Replicative antigens are spontaneously expressed at a low level in some "lytic producer" strains of EBV. The non-immortalizing HR-1 strain is a lytic virus as defined by its ability to induce EA in superinfected latent Raji cells (Henle et al., 1970). The HR-1 cell line was derived as a subclone of the Burkitt lymphoma cell line, Jijoye (Hinuma and Grace, 1967). Restriction analysis of the HR-1 virus revealed submolar restriction fragments in addition to the standard fragments, suggesting the presence of at least two different classes of molecules in these cells (Delius and Bornkamm, 1978, Bornkamm et al., 1980). The EBV sequences contained within the submolar restriction fragments have been designted 'het', for heterogeneous DNA (Heston et al., 1982).

Miller and colleagues subcloned the HR-1 cell line and

recovered two types of clones. The majority of clones contained only the standard EBV genome. However, one subclone, HR-1 clone 5, contained hypermolar levels of 'het' DNA (Rabson et al., 1983). Jenson et al. (1986, 1987) demonstrated that het DNA comprises an extensively rearranged and deleted HR-1 genome (Fig. 1). The defective genome is maintained by cell-to-cell spread and can be lost from cell culture (Miller et al., 1985, Kolman, J.L. personal communication). HR-1 clone 5 cells spontaneously enter viral replication and release a mixture of standard and het viruses (Rabson et al., 1983). Additionally, only clones harboring het DNA maintain the ability to induce early antigen expression in latent EBV (Miller et al., 1984).

Countryman and Miller (1985) then showed that the capacity to activate replication can be mapped to one het DNA fragment. The het fragment responsible for disrupting latency is a 2700 bp fragment termed WZhet. It represents a recombination and inversion of two regions of EBV DNA (BamHI W and BamHI Z) which are usually more than 50 Kb apart on the standard EBV genome (Fig. 2) (Countryman et al., 1985, Jenson and Miller, 1988). WZhet contains only one complete open reading frame, BamHI Z leftward reading frame 1 (BZLF1), which is inverted relative to its position in the standard genome (Fig. 2) (Jenson et al., 1986, Jenson et al., 1987). It was not immediately clear whether het DNA

disrupted latency by encoding a novel protein or alternatively, that rearrangements in het DNA activated expression of a wild type EB viral protein. It has since been determined that both the standard and het BZLF1 open reading frames maintain the ability to disrupt the EBV latent cycle (Chevallier-Greco et al., 1986, Countryman et al., 1987, Takada et al., 1986). We have termed the protein encoded by BZLF1, ZEBRA (Z EB Replication Activator).

Expression and regulation of the ZEBRA protein in the standard and HR-1 het EB viruses was analyzed. ZEBRA is differentially synthesized in these viruses suggesting that the lytic nature of HR-1 is due to an abnormal regulation of ZEBRA expression. Additionally, the DNA binding activity of ZEBRA was studied. ZEBRA has a broad DNA binding specificity which is inhibited by casein kinase II phosphorylation. The potential importance of these properties in regulating the EBV lytic cycle will be discussed.

Figure 1. Physical map of Epstein-Barr viral DNA in one cellular subclone of the HR-1 cell line. The boxes represent defective (or het) DNA. Boxes with similar shading symbolize het DNA sequences which are adjacent to each other (from Miller et al., 1984).

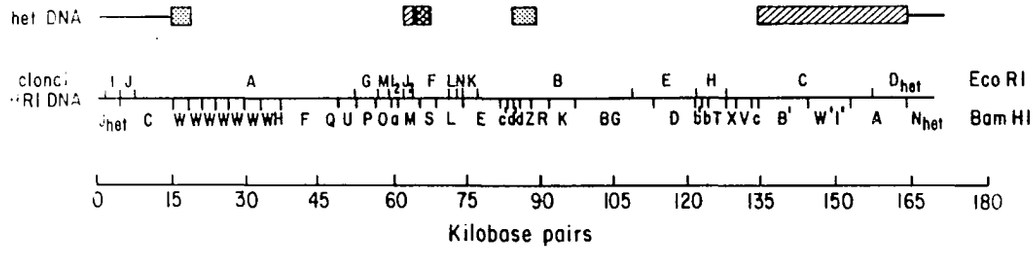
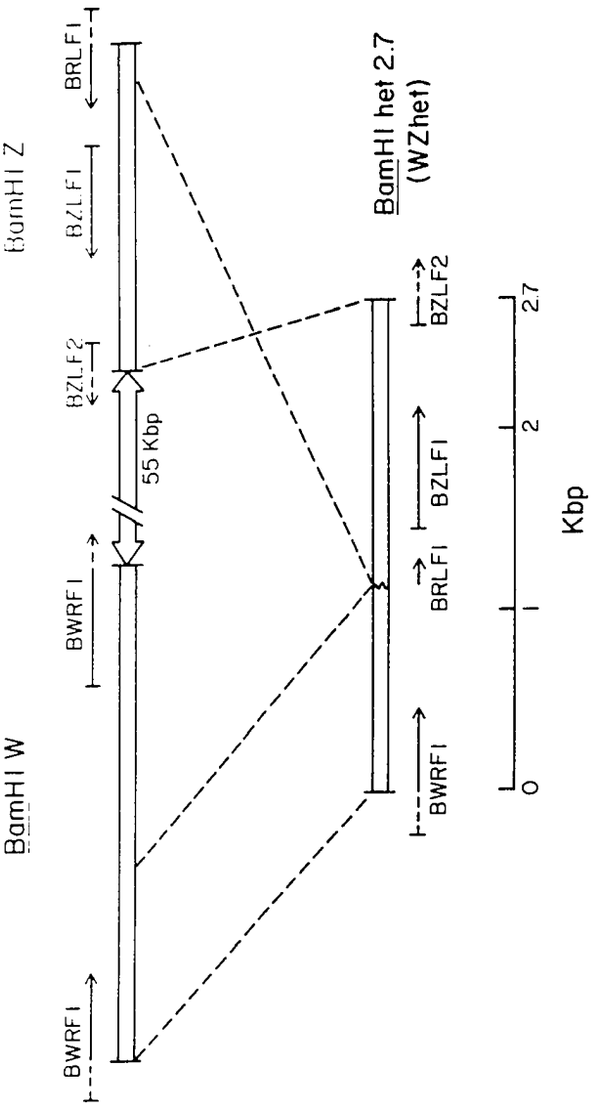


Figure 2. Map of WZhet (BamHI het 2.7). The 2.7 Kb WZhet fragment is capable of disrupting latency. The fragment represents intramolecular recombination and inversion of two regions of EBV DNA (BamHI W and BamHI Z) which are more than 55 Kb apart on the standard EBV genome. All open reading frames (horizontal arrows) except BZLF1 are truncated (from Jenson and Miller, 1988).



CHAPTER II

EXPRESSION OF THE BZLF1 LATENCY-DISRUPTING GENE DIFFERS IN STANDARD AND DEFECTIVE EPSTEIN-BARR VIRUS

INTRODUCTION

Epstein-Barr virus is maintained in immortal B lymphocytes predominantly in the latent state. Expression of lytic cycle products occurs spontaneously in a small proportion of EBV-infected lymphocytes; however viral replication can be induced in many more cells by such agents as phorbol esters, butyrate and serum factors (Bauer et al., 1985, Luka et al., 1979, zur Hausen et al., 1979). Virus from the P3HR-1 subline of the Jijoye Burkitt's lymphoma also has the capacity to induce EB viral replication (Henle et al., 1970, Miller et al., 1984).

The P3HR-1 cell line contains two forms of EBV DNA, a standard genome and a defective (heterogeneous or het) genome which is extensively deleted and rearranged (Cho et al., 1984, Heston et al., 1982). Experiments with cellular subclones of the P3HR-1 cell line implicated the het DNA in activating replication of the standard EBV genome (Heston et al., 1982). HR-1 clone 16 (HH514-16), which is representative of the majority of HR-1 subclones, contains the standard genome but has lost het DNA and with it the capacity to synthesize viral replicative products

spontaneously. HR-1 clone 5 (HH543-5), a rare clone which contains hypermolar levels of het DNA as well as standard EBV DNA, spontaneously enters viral replication (Rabson et al., 1983). Clone 5 cells release a mixture of standard and het viruses, the latter of which activate viral replication when added to cells bearing a latent genome.

The capacity to activate replication has been mapped to the BZLF1 open reading frame, which encodes a protein termed ZEBRA (Z EB Replication Activator) or EB1 (Chevallier-Greco et al., 1986, Countryman and Miller, 1985). ZEBRA is responsible for activation of early gene expression (Countryman et al., 1987). Whereas the BZLF1 open reading frame is present in both standard and het genomes, in the latter the reading frame is inverted relative to its position in the standard genome. In the het genome, BZLF1 has new upstream and downstream regions as the result of intramolecular recombinations (refer to chapter I, Fig. I-11) (Jenson et al., 1986, Jenson et al., 1987). This altered genome configuration suggests that the BZLF1 gene might be aberrantly regulated in het DNA.

Previous experiments which characteerized the ability of the BZLF1 gene product to disrupt viral latency relied exclusively on gene transfers of plasmids in which expression of the BZLF1 gene was driven by strong heterologous promoters (Chevallier-Greco et al., 1986, Countryman et al., 1987, Hardwick et al., 1988, Takada et

al., 1986). The purpose of the present experiments was to study the pattern of ZEBRA expression during the life cycle of standard and defective EB viruses in Burkitt lymphoma cells, a natural target.

RESULTS

Properties of the ZEBRA Fusion Protein. A ZEBRA fusion protein was obtained by cloning the 679 bp NaeI-PvuII subfragment of WZhet in frame with the trpE coding sequences contained in the pATH 11 vector (Dieckmann and Tzagaloff, 1985). Since a genoomic fragment was used in the expression construct, the ZEBRA fusion protein made in bacteria would not be identical to that made in eukaryotic cells if eukaryotic ZEBRA is expressed exclusively from spliced mRNAs (Biggin et al., 1987). The EBV BZLF1 open reading frame (ORF) should encode 218 amino acids: 9 amino acids upstream of the two methionines at the 5' end of the ORF; 167 amino acids from exon 1 of BZLF1 and 42 amino acids downstream from the first intron. If the BZLF1 gene is not spliced in eukaryotic cells, the 218 amino acid insert included in the bacterial fusion protein would encompass the entire 209 amino acid BZLF1 ORF.

The calculated molecular weight of the protein encoded by the EBV insert is 23.3 KDa (Jenson et al., 1988). The pATH 11 vector contains sequences coding for a 36 KDa trpE polypeptide. The observed electrophoretic mobility of the trpE-BZLF1 fusion protein, 72 KDa, was about 13 KDa larger than calculated. The ZEBRA polypeptide from eukaryotic cells also migrates about 13 KDa slower than expected (Jenson et al., 1988). The synthesis of fusion protein was highly inducible by addition of indoleacrylic acid to HB101

cells transformed with the pATH 11/BZLF1 construct. After treatment of the bacterial cell lysate with DNAse I, the fusion protein accounted for about 50% of the total protein in the insoluble pellet (Fig. 1).

Detection of Polymorphic ZEBRA Polypeptides by Rabbit Antisera Raised to the TrpE-BZLF1 Fusion Protein. Initial experiments evaluated the specificity of the antisera obtained from rabbits immunized with the trpE-BZLF1 fusion protein. Post-immune rabbit sera recognized a nuclear antigen in COS-1 cells transfected with pSV2neo-WZhet and in HR-1 clone 16 cells treated with TPA (Fig. 2). Preimmune rabbit sera did not react with COS-1 cells or HR-1 cells by immunofluorescence or by immunoblotting (not shown). The post-immunization rabbit sera did not detect antigens in untransfected COS-1 cells nor in uninduced clone 16 cells in an immunofluorescence assay (not shown).

It was known from immunoblot analysis using polyvalent human sera, as well as a weakly reactive rabbit antiserum raised to a lac Z-BZLF1 fusion protein, that ZEBRA polypeptides from various EBV strains have different electrophoretic mobilities (Countryman et al., 1987). By means of chimeric mutants and DNA sequence analysis it was previously determined that 3 point mutations at amino acid positions 25, 51 and 73 in BZLF1 account for the 3 KDa difference in electrophoretic mobility of HR-1 het ZEBRA (39KDa) and standard HR-1 ZEBRA (36KDa) (Countryman et al.,

1987, Jenson et al., 1988). There is also a reproducible difference in electrophoretic mobility of about 1-2 KDa between ZEBRA from standard HR-1 and EBV strain FF41. This mobility difference disappeared when the proteins were further denatured in 8 M urea; however, even after denaturation in urea, HR-1 het ZEBRA migrated about 3 KDa slower than standard HR-1 ZEBRA (D. A. Katz, unpublished data).

The rabbit antiserum to TrpE-BZLF1 recognized the polymorphic ZEBRA proteins made after transfection of COS-1 cells with the BZLF1 gene from various EBV strains (Fig. 3). Furthermore, using this serum we showed that the ZEBRA polypeptide made in lymphoid cells from intact virus comigrated with the polypeptide made when the encoding region from that virus was transfected into COS-1 cells. For example, the ZEBRA polypeptide made in butyrate induced clone 16 cells co-migrated with ZEBRA expressed in COS-1 cells transfected with pSV2neo- BamHI Z (HR-1) (Fig. 3). Three higher molecular weight bands of 70-90 KDa were also detected with antibody to BZLF1 on immunoblots of extracts prepared from induced clone 16 cells. These products may represent ZEBRA oligomers which were not completely denatured prior to electrophoresis.

Kinetics of ZEBRA expression in cells containing only a standard EBV genome. The antiserum was used in a time course study to determine whether ZEBRA was an early or late

viral protein. ZEBRA could be detected within 6 hrs after addition of butyrate to clone 16 cells. Expression reached near maximal levels within 2-3 days after addition of butyrate and remained elevated throughout a two week period. However, cells cultured for 2 weeks in the absence of inducing agent failed to express detectable levels of ZEBRA. The higher molecular weight immunoreactive proteins were first detected about 3 days after butyrate treatment (Fig. 4).

Additional kinetic experiments were performed in which clone 16 cells were induced with butyrate in the presence or absence of inhibitors of EBV viral DNA synthesis, phosphonoacetic acid (PAA) or acyclovir (ACV). Treatment with these inhibitors did not alter ZEBRA expression. The higher molecular weight proteins reactive with the antibody to BZLF1 were also made in the presence of inhibitors of viral DNA synthesis (Fig. 5 and data not shown). The efficacy of the inhibitors was monitored by assessing the synthesis of a late viral protein, p21. This protein, which was induced in clone 16 cells and recognized by the RM antiserum 3 and 5 days after induction, was blocked by PAA (Fig. 5). When ACV was used, it was found that the drug had blocked viral DNA replication. Linear EBV, representing replicated genomes, was present following butyrate induction only when ACV was absent (Katz et al., 1989). As expected, neither ACV nor PAA affected expression of latent products,

EBNAs, recognized by the RM antiserum (Fig. 5). These experiments demonstrated that in clone 16 cells ZEBRA was not expressed during latency and behaved as an early replicative protein following chemical induction.

Constitutive Expression of ZEBRA in Cells Bearing Both Standard and Defective EBV Genomes. It was of interest to compare ZEBRA expression in clone 16 cells with its expression in spontaneously replicating clone 5 cells. The polymorphisms of ZEBRA encoded by WZhet and standard BamHI Z (Fig. 3) were used to monitor expression of the two ZEBRA variants in clone 5 cells which harbor both standard and defective genomes. Clone 5 cells expressed ZEBRA spontaneously, without addition of inducing agents (Fig. 6). There was generally a lag in ZEBRA expression after subculture into fresh medium, but by 2-4 days after subculture ZEBRA was detected and persisted thereafter. The het variant was predominant and was detected before standard ZEBRA.

The effects of two inducing agents, TPA and butyrate, were tested in clone 5 cells (Fig. 7). At 3 and 4 days after subculture het ZEBRA was made spontaneously, in the absence of inducer. In the presence of butyrate, standard ZEBRA as well as het ZEBRA, were activated. In many experiments (not shown), butyrate preferentially activated expression of standard ZEBRA. TPA seemed to induce het ZEBRA to a greater extent than standard ZEBRA. These

experiments suggested that there are separate controls on expression of ZEBRA from the two genomes.

Since ZEBRA persisted in viral cultures at maximal levels for two weeks after induction (Fig. 4), it was of interest to determine the stability of the ZEBRA protein. Following induction with butyrate, clone 5 cells were treated with either cycloheximide or actinomycin D to abolish translation or transcription, respectively. As expected, the level of ZEBRA protein increased in butyrate treated cells during the next 48 hours (Fig. 8). However, no decrease in ZEBRA protein was observed in cycloheximide or actinomycin D treated cells during the same time period (Fig. 8). These results suggest that ZEBRA is a stable protein whose half life is greater than 48 hours.

Exogenous Infection with Virus Stocks Containing Both Standard and Defective Genomes. Virus stocks prepared from clone 5 cells, containing a mixture of standard and defective EBV genomes, were inoculated into EBV genome negative Burkitt Lymphoma (BL) cells which had been stably converted by HR-1 (CL16) and B95-8 virus strains (Calender et al., 1987, Seibl et al., 1986). The converted lines were used as targets in order to compare expression of the endogenous standard virus with the superinfecting het and standard virus mixture.

All 4 EBV converted BL cell lines maintained the virus in a tightly latent state. In these lines, neither TPA nor

butyrate stimulated expression of the ZEBRA from endogenous EBV genome (Fig. 9 and data not shown). The same viruses (HR-1 clone 16 and B95-8), however, could be induced in other cell backgrounds. When the EBV converted cells were superinfected by clone 5 virus stocks, het ZEBRA was markedly induced by TPA treatment (Fig. 9). Het ZEBRA expression in these 4 Burkitt Lymphoma lines was not accompanied by synthesis of standard ZEBRA, as it was in clone 5 cells. Nonetheless, expression of the het ZEBRA protein was associated with the production of a large number of early antigens (data not shown).

Transcription of BZLF1 in HR-1 Cells Bearing Standard and Defective EBV Genomes. Northern blot analysis was performed to determine whether the observed differences in standard and het ZEBRA expression were due to regulation at the transcriptional level. RNA from clone 16 and clone 5 cells was probed with a 523 bp fragment representing 264 bp upstream of BZLF and 259 bp of the first coding exon. Two prominent mRNAs of 3.5 Kb and 0.9 Kb were detected one and two days after butyrate treatment (Fig. 10). In clone 5 cells the same two mRNAs were expressed spontaneously without addition of an inducing agent. In uninduced clone 16 cells there were no transcripts identified with this probe. The experiments indicated that differences in ZEBRA expression by clone 16 and clone 5 cells were likely to be the result of differences in transcriptional control.

Northern blots were then probed with fragments specific for the BZLF1 and BRLF1 open reading frames in an attempt to distinguish between the 3.5 Kb and 0.9 Kb mRNAs. In induced clone 16 cells, both mRNAs were recognized by the BZLF1 specific probe while only the 3.5 Kb mRNA was recognized by the BRLF1 specific probe (Fig. 11). These results corroborate the finding that the BRLF1 specific mRNA is colinear with BZLF1 at the 3' end (Biggin et al., 1987, Seibl et al., 1987). B95-8 cells, a marmosset EBV line, expressed a 15 Kb BZLF1 specific transcript in addition to the 3.5 Kb and 0.9 Kb transcripts (Fig. 12).

The synthesis of both BZLF1 mRNAs was sensitive to treatment with cycloheximide, a protein synthesis inhibitor (Fig. 13). Additionally, neither mRNA was detected if cycloheximide was added 8 hours following induction of clone 16 cells with butyrate (Fig. 13).

Kinetics and Stability of BZLF1 specific mRNAs. RNA was isolated at various time points following TPA induction of B95-8 cells in an attempt to determine the kinetics of transcription of the BZLF1 specific 3.5 Kb and 0.9 Kb mRNAs. Both mRNAs were detected 2 hours after induction and both reached maximal levels at 13 hours post induction (Fig. 14). The half life of these mRNAs was determined by treating butyrate induced clone 5 cells with actinomycin D and assessing RNA levels by Northern blot analysis. Again, both the 3.5 Kb and 0.9 Kb mRNAs had approximately the same

kinetics of expression with a half life of 3.5 hours (Fig. 15). The half life of these mRNAs is also 3.5 hours in clone 16 cells and B95-8 cells (data not shown).

DISCUSSION

Since the BZLF1 gene product can activate the expression of many EBV genes in transfection experiments, it has been assumed that this gene plays a pivotal role in the switch between latency and replication in the context of the intact virus (Chevallier-Greco et al., 1986, Countryman and Miller, 1985, Hardwick et al., 1988, Seibl et al., 1986). However, little is known about the regulation of this gene in natural infection. There have been no reports describing the effects of different variables on expression of ZEBRA protein in lymphoid cells. Regulation of expression of the BZLF1 gene differs in cells with or without het DNA and these results are summarized in Figure 15. These experiments define at least three sets of variables which affect ZEBRA expression: presence of defective virus, cell background, and addition of chemical inducers such as sodium butyrate and TPA.

Defective Virus. The presence of defective virus was associated with constitutive ZEBRA expression. The defective genomes are easily lost from clone 5 cells (Miller et al., 1985). Those clone 5 cells which contain the defective virus expressed ZEBRA, while those which have lost het DNA no longer synthesize the ZEBRA polypeptide (Kolman, J.L., unpublished data). Parental HR-1 cells, which contain several different families of defective EBV DNA molecules,

express distinct polypeptides which are reactive with the anti-BZLF1 serum. Many defective populations activate ZEBRA synthesis while the loss of defective virus is usually associated with an absence of spontaneous ZEBRA expression (J. Kolman, unpublished data).

Seibl et. al. observed a 40 KDa BZLF1 specific protein in hybrid-selected translations with RNAs from induced HR-1 cells. They also immunoprecipitated a 35 KDa BZLF1 protein from metabolically labelled cells. Although they concluded that the 35 KDa protein was processed from a 40 KDa precursor, their studies could not distinguish beween expression of products from the standard and defective HR-1 EBV genomes (Seibl et al., 1986). However, this system enabled me to distinguish between the role of standard and defective viruses in ZEBRA expression.

The polymorphisms of the ZEBRA polypeptide provided a powerful tool with which to assess ZEBRA expression from the defective and standard genomes. In the absence of inducers, most of the ZEBRA in clone 5 cells was derived from the defective genome (Fig. 6,7). When virus stocks containing both types of genomes were used to infect EBV converted BL cells, het ZEBRA was exclusively expressed (Fig.9). These results suggest that aberrant controls on ZEBRA expression in the defective genome are responsible for its high level of expression. In related experiments using gene transfer into BL cells ZEBRA was expressed at high levels from

plasmids containing the rearranged EcoRI het 16 palindromic fragment; ZEBRA was not made when it was introduced on a standard BamHI Z fragment (Rooney et al., 1988). Thus, the acquisition of new positive regulatory signals as the result of the genome rearrangements are likely to explain the high level of spontaneous het ZEBRA expression in clone 5 cells.

A fraction of the ZEBRA protein expressed in clone 5 cells bearing het DNA is derived from the BZLF1 open reading frame in the standard genome. This finding suggests that an autoregulatory loop may exist; het ZEBRA may activate the expression of standard ZEBRA, either directly or by the action of other EBV products whose expression is stimulated by ZEBRA. The long half life of the ZEBRA may be important in its role in regulating the EBV lytic cycle and its possible function during the late phase of viral replication (Fig. 8).

Cell Background. Cell background provides an important influence on ZEBRA expression. Upon de novo infection of primary B cells with virus stocks, with or without defectives, ZEBRA synthesis is not detected (Rooney et al., 1989). In EBV converted Burkitt's lymphoma lines there is "tight" regulation of ZEBRA expression; inducing agents such as TPA or butyrate, are incapable of activating standard ZEBRA expression. This may be explained by the recent finding that EBV infection of Burkitt's lymphoma lines results in the integration of the virus rather than its

maintenance as an extrachromosomal plasmid (Hurley et al., 1991). Expression may be differentially regulated in integrated EBV as compared with an EBV episome.

Preferential expression of het ZEBRA in these cells may be due to a gene dosage effect or may be the result of the juxtaposition of a TPA responsive element in the het virus. Nonetheless, high levels of het ZEBRA expression in a BL cell background does not activate expression of standard ZEBRA as it does in other cells (Figs. 6 ,7, and 9).

In cell lines in which EBV is less tightly latent, such as clone 16 (Fig. 3), Raji, or <u>in vitro</u> EBV immortalized cord blood B lymphocytes such as X50-7, ZEBRA is not synthesized spontaneously but can be activated with chemical inducers and het DNA. The B95-8 and FF41 marmoset cell lines, which spontaneously replicate EBV without requirement for defective DNA or an inducing chemical, spontaneously synthesize ZEBRA. Thus, the tendency of an EBV containing cell line to exhibit a tightly latent or a productive phenotype correlates with stringent versus loose control on ZEBRA expression. This correlation has been strengthened by the observation that transfection of a ZEBRA-VP16 fusion protein can activate high levels of standard ZEBRA expression in clone 16 cells but not in an EBV converted BL cell line, BL30/clone 16.(Baumann, Grogan, Gradoville, and Miller, manuscript in preparation).

The nature of these host cell-specific controls can

only be conjectured. They may represent positive or negative regulatory elements.

Chemical Induction. It seems likely that the pathways by which chemicals such as TPA and butyrate activate EBV early gene expression intersect with those which control ZEBRA gene expression. This hypothesis can be tested since inducing agents vary in their effects on cell lines carrying different EBV genomes. For example, TPA is a much more potent inducing stimulus of early antigen synthesis in Raji cells than butyrate and it was found that ZEBRA expression is activated to a greater extent in Raji cells by TPA than by butyrate (data not shown). However, these experiments cannot distinguish which replicative genes are TPA inducible.

The inducing drugs differentially affect standard and defective EBV. The standard EBV genome in clone 16, is more efficiently induced to express ZEBRA by butyrate than by TPA (Fig. 7, and data not shown). The defective genome in clone 5, however, is more strongly affected by TPA. Additionally, Rooney et. al. have shown through gene transfers that in the genetic environment of het DNA, het ZEBRA expression is markedly induced by TPA (Rooney et al., 1988). A probable site for the TPA responsive element in the defective DNA is the promoter element for the MS-EA gene which is positioned downstream of BZLF1 in het DNA. This promoter contains a binding site for the AP-1 transcription factor which is TPA

inducible (Angel et al., 1987, Lee et al., 1987). Butyrate may activate expression of ZEBRA by altering the nucleosome configuration in the cell. Butyrate has been shown to inhibit histone deacetylation resulting in histone hyperacetylation (Candido et al., 1978, Sealy and Chalkley, 1978).

However, the differential effects of chemical inducers on het versus standard ZEBRA are transient (Fig. 7). A likely explanation is that both het and standard ZEBRA proteins are each capable of stimulating the other. This hypothesis is supported by the finding that spontaneous expression of het ZEBRA protein is eventually accompanied by expression of the standard ZEBRA protein.

Nature of the control. These experiments indicate that the major controls on ZEBRA expression are at the transcriptional level. Chemical induction of cells containing only standard virus such as clone 16, Raji and B95-8 is invariably associated with marked increases in BZLF1 mRNA (Figs. 9, 11, 13, and data not shown). However, in clone 5 cells which contain a defective virus, BZLF1 specific transcripts are made constitutively and are not always increased by butyrate treatment. The level of ZEBRA mRNAs in these cells varies with culture conditions such as temperature, cell density, and interval since feeding (not shown). These variables probably reflect cellular controls on the het virus.

Since this work was completed, Manet et al. reported the isolation of four BZLF1 specific cDNAs clones: a spliced BZLF1 with three exons; two bicistronic BRLF1-BZLF1 clones, encoding an unspliced BRLF1 ORF and the spliced BZLF1 gene; and a spliced BZLF1 clone with a deleted 5' terminus (Manet et al. 1989). In these experiments, only two BZLF1 specific mRNAs were detected in a number of EBV positive cell lines; corresponding to the spliced BZLF1 mRNA and one bicistronic BRLF1-BZLF1 message (Figs. 10, 11, 12). However, the 0.9 Kb message is generally of higher abundance than the 3.5 Kb transcript and may represent more than one BZLF1 transcript. Additionally, a 15 Kb BZLF1 specific message was detected in induced B95-8 cells (Fig. 12). This message may correspond to the leftward transcript hybridizing to BamHI-W previously described by Rogers and Speck (1990). Thus, the message encoding the entire ZEBRA protein is contained within at least three transcripts. However, it has still not been determined whether ZEBRA is translated from all these mRNAs in-vivo.

Studies of the kinetics of expression of the 3.5 and 0.9 Kb messages suggest that they may be regulated by similar cellular controls. Both messages were detected within two hours following induction (Fig. 14) and both have a half life of approximately 3.5 hours (Fig. 15).

These results help clarify previously published results on BamHI Z transcripts. Biggin et. al. detected two

transcripts of 2.8 Kb and 1.0 Kb from BRLF1 and BZLF1, respectively, in HR-1 superinfected Raji cells treated with protein synthesis inhibitors and suggested that these "immediate early" transcripts are derived from the defective genome (Biggin et al., 1987). However, under the conditions of their experiment, they could not ascertain whether the transcripts were expressed from the Raji genome, the HR-1 standard genome, or the HR-1 defective genome.

Laux et al. showed that these two transcripts could be detected in the defective Raji cell line after induction with TPA (Laux et al., 1988). However, in contrast to transcripts made after superinfection, those induced by TPA were not detected in the presence of a protein synthesis inhibitor, cycloheximide. They suggested that the mechanism of activation of replication by superinfection and by TPA are different (Laux et al., 1988).

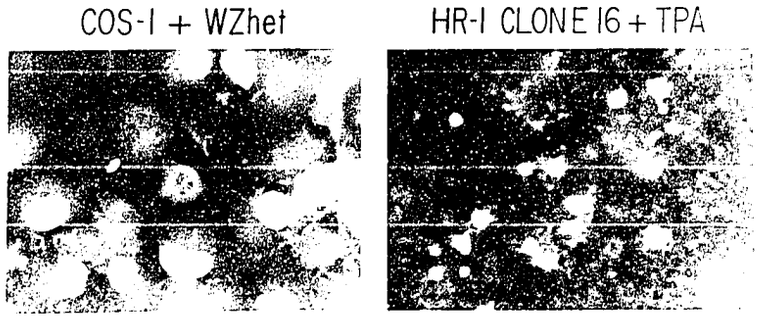
These experiments support the findings of Laux et al. since synthesis of both the 3.5 Kb and 0.9 Kb BZLF1 specific mRNAs in clone 16 cells were sensitive to inhibition of protein synthesis with cycloheximide (Fig. 13). Therefore, a viral, or possibly cellular protein must be made before ZEBRA mRNA can be synthesized in cells with a standard unrearranged genome. Additionally, de novo protein synthesis may be continually required for BZLF1 transcription from standard virus since no ZEBRA specific transcripts were detected in cells which were treated with

cycloheximide 8 hours following addition of butyrate (Fig. 13). Although BZLF1 transcripts are not detected in any of the cycloheximide + butyrate treated cells, RNA extracts from these cells, but not from uninduced cells, consistently demonstrate a high level of background hybridization with BZLF1 specific probes. This suggets that de-novo protein synthesis may be required for the stabilization of BZLF1 transcripts. However, in cells containing a defective virus, transcripts are made constitutively and do not appear to require new protein synthesis.

Future work will entail a search for differences in the control mechanisms on BZLF1 in the standard and defective viruses. Control may involve repressors encoded by the virus or the cell as well as transcription factors which are activated by inducing agents.

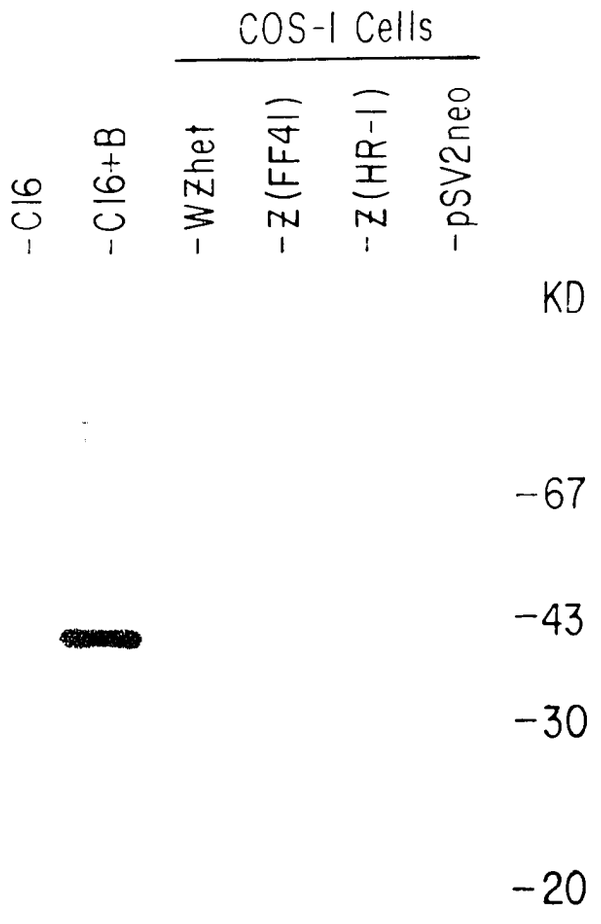
Figure 1. Appearance of the TrpE BZLF1 fusion protein on a polyacrylamide gel stained with Coomassie brilliant blue.

Figure 2. Reactivity of rabbit antiserum to the TrpE-BZLF1 fusion protein by immunofluorescence. COS-1 cells were transfected 3 days previously with pSV2neo/WZhet. HR-1 clone 16 cells were treated with TPA for 3 days. The rabbit antiserum was used at a 1:30 dilution; rhodamine conjugated anti-rabbit Ig at 1:50.



ANTISERUM: ANTI BZLF1

Figure 3. Reactivity of rabbit antiserum to the TrpE-BZLF1 fusion protein by immunoblotting. Extracts were prepared from HR-1 clone 16 cells which were untreated (C16) or treated with butyrate for 3 days (C16+B). COS-1 cells were transfected with pSV2neo plasmids containing WZhet, the BamHI Z fragment from EBV strains FF41, HR-1, or no insert. The immunoblot was probed with a 1:100 dilution of rabbit antiserum to BZLF1.



ANTISERUM: ANTI-BZLF1

Figure 4. Kinetics of ZEBRA expression after butyrate induction of HR-1 clone 16 cells. Each time point represents an extract of 2 X 10^6 cells which was electrophoresed through a 10% SDS-polyacrylamide gel. The immunoblot was probed with rabbit antiserum to the TrpE-BZLF1 fusion and I^{125} - protein A.

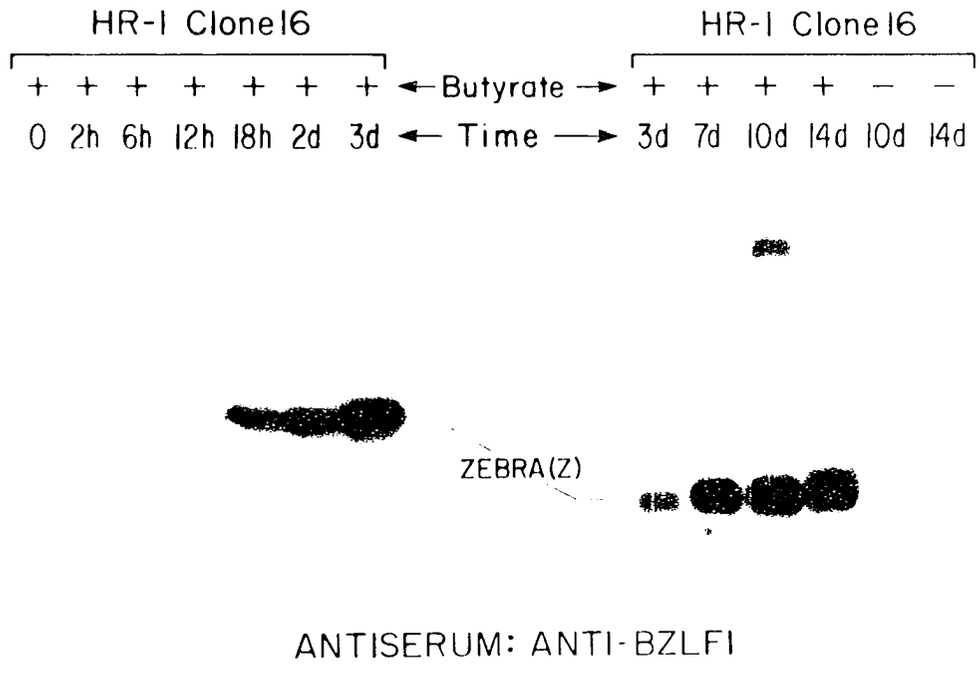
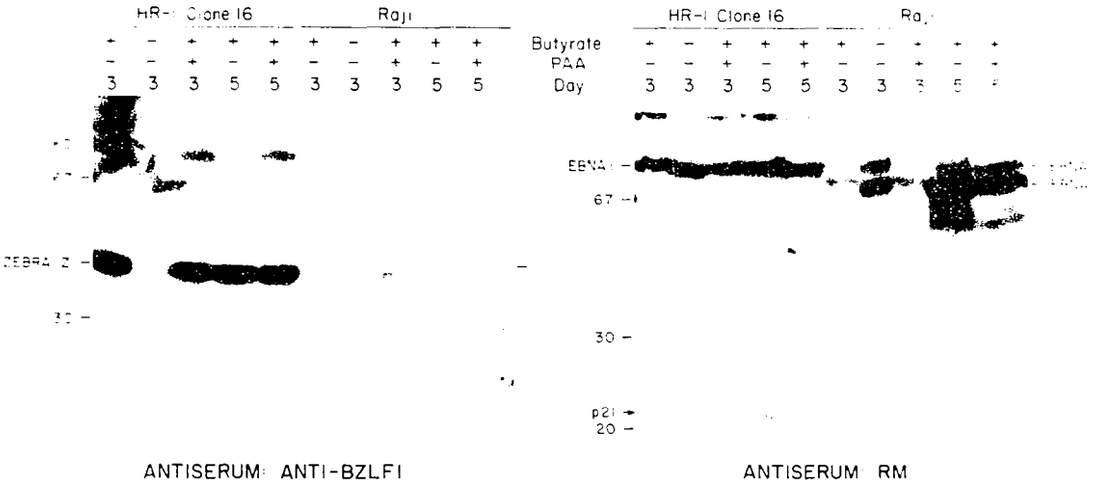


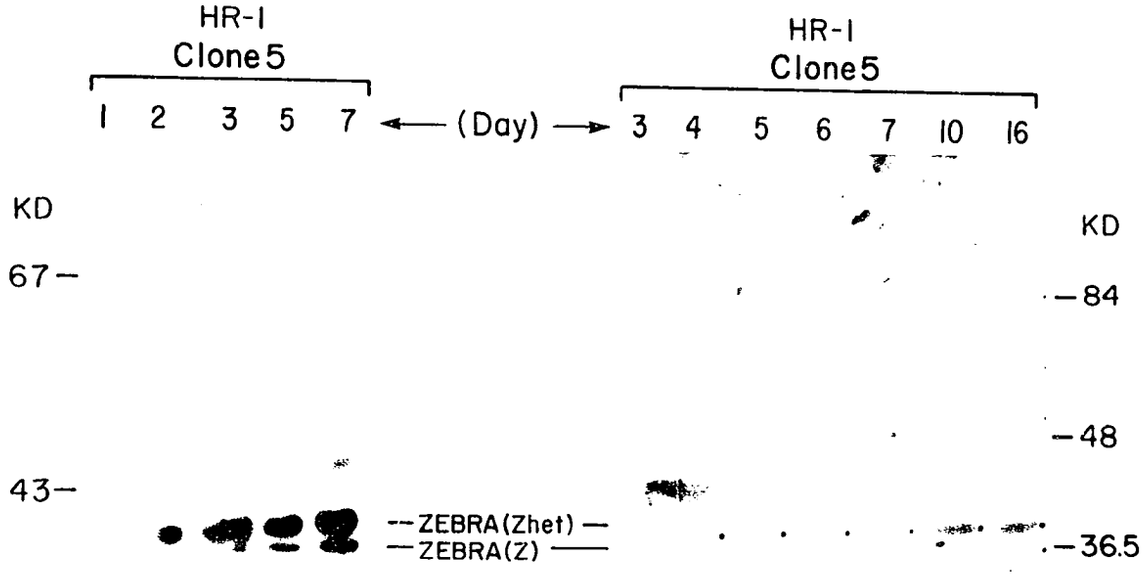
Figure 5. Lack of effect of phosphonoacetic acid (PAA) on ZEBRA expression in HR-1 clone 16 cells. Cells were uninduced or induced with butyrate. An aliquot of induced cells was also treated with PAA. Cell extracts were analyzed after 3 or 5 days on duplicate immunoblots with rabbit anti-BZLF1 serum or polyvalent human antiserum, RM, which recognizes a p21 late protein. ZEBRA expression is unaffected by PAA but p21 synthesis is inhibited.



ANTISERUM: ANTI-BZLF1

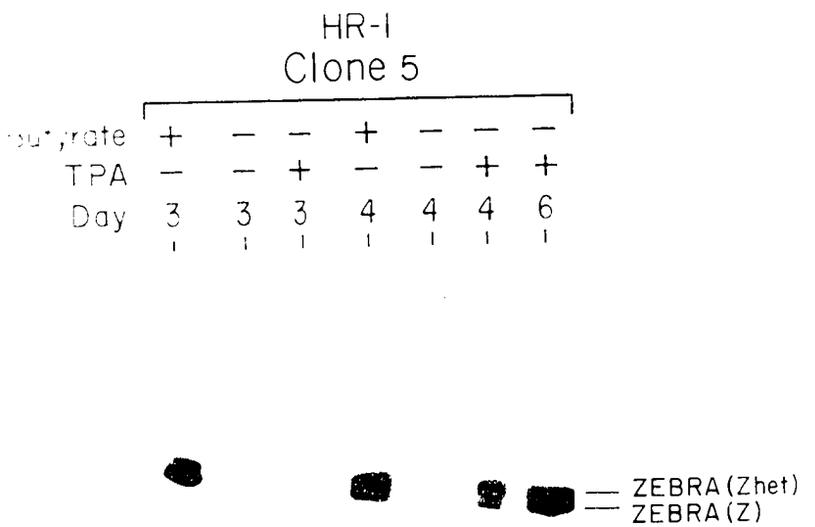
ANTISERUM: RM

Figure 6. Constitutive Expression of ZEBRA in HR-1 clone 5 cells. Duplicate experiments are illustrated. Extracts were prepared at intervals after splitting the cells at a ratio of 1:4 (left) or 1:5 (right). There is a delay in ZEBRA expression after the cells are split. Beginning at 2-4 days after subculture ZEBRA is expressed spontaneously, without addition of an inducing agent. The het variant is more abundant than standard ZEBRA.



ANTISERUM: ANTI-BZLF1

Figure 7. Effects of inducing agents on expression of the two ZEBRA variants in HR-1 clone 5 cells. Uninduced cells express mainly the het variant; after addition of TPA or butyrate both the het and standard ZEBRAs are expressed. TPA has a greater effect on het ZEBRA whereas butyrate preferentially stimulates the standard variant.



ANTISERUM: ANTI-BZLF1

Figure 8. Stability of the ZEBRA protein. Clone 5 cells were induced for three days with butyrate at which time transcription or protein synthesis were inhibited with actinomycin D or cycloheximide, respectively. Cells were then harvested at various time points following inhibition. Each time point represents an extract of 1 X 10^6 cells which were electrophoresed through a 10% SDS-polyacrylamide gel. The immunoblot was probed with rabbit anti-BZLF1 serum.

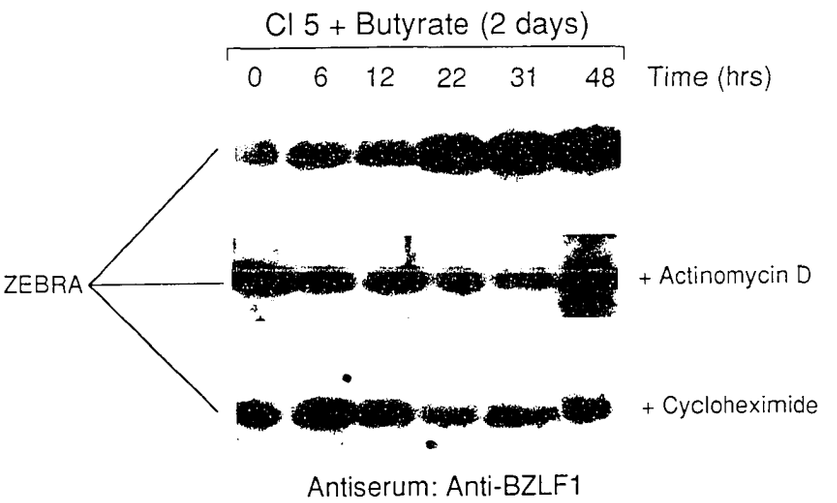
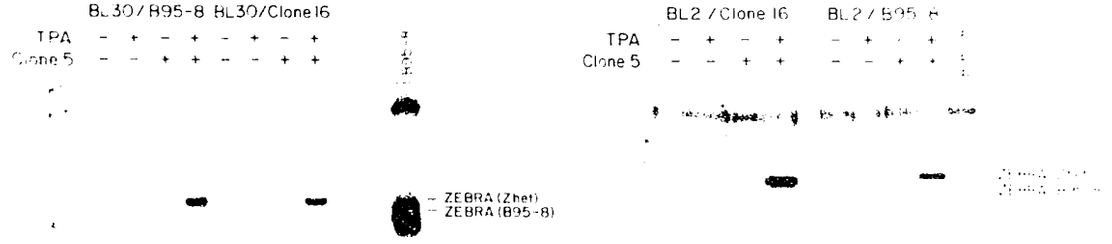
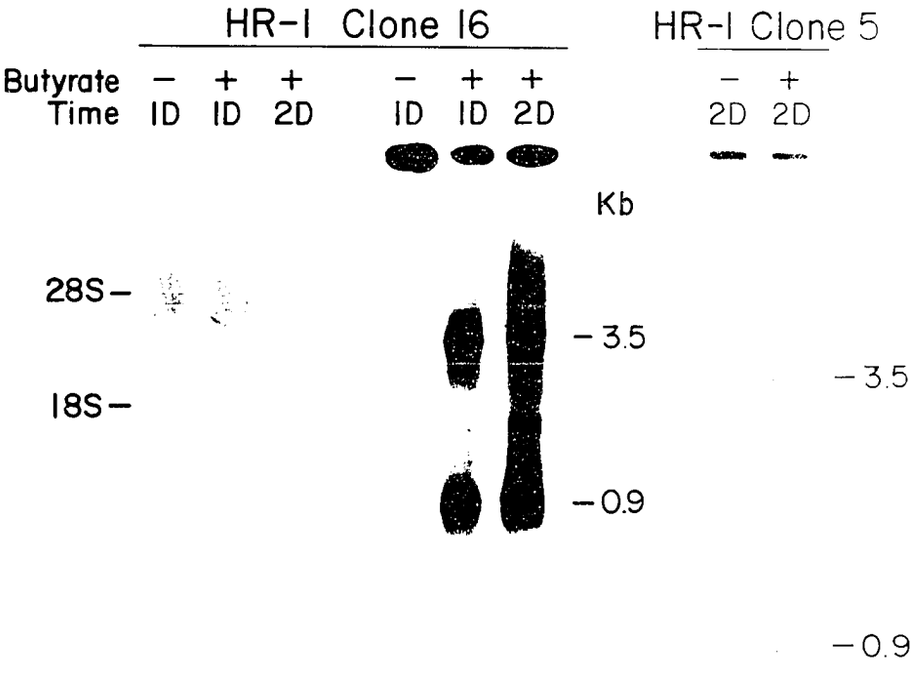


Figure 9. Expression of ZEBRA after infection of Burkitt lymphoma cell lines BL30 and BL2 stably converted with the B95-8 and HR-1 clone 16 strains of EBV. The cells were treated (+) or untreated (-) with TPA and infected (+) or uninfected (-) with clone 5 virus stocks. Western blots prepared 48 hrs after infection were reacted with anti-BZLF1 serum. Following infection of converted BL30 or BL2 cells the het ZEBRA is expressed in the presence of TPA. However, in these cells, the standard ZEBRA from clone 16 or B95-8 virus is not expressed after TPA induction or superinfection with clone 5.



ANTISERUM ANTI-BZLF1

Figure 10. Northern blot analysis of BZLF1 transcription in HR-1 clone 16 and clone 5 cells. RNA was prepared from uninduced clone 16 cells 1 day after subculture and from butyrate treated clone 16 cells at one and two days after subculture. RNA was prepared from clone 5 cells two days after subculture in the absence or presence of butyrate. Both blots were probed with a 523 bp subfragment of mutant 80 of WZhet (Countryman et al., 1987). The filter containing clone 16 RNAs was stained with methylene blue to demonstrate that the level of ribosomal RNAs from the uninduced and induced cells was similar.



ribosomal RNAs Probe: BZLF 1

Figure 11. Localization of BZLF1 specific transcripts. RNA was prepared from uninduced and butyrate treated clone 16 cells one day after induction. RNAs were electrophoresed in duplicate and Northern blots were probed with a 395 bp BZLF1 specific fragment or a 538 bp BRLF1 specific fragment. Both the 3.5 Kb and 0.9 Kb mRNAs are detected with the BZLF1 probe whereas only the 3.5 Kb mRNA is detected with the BRLF1 specific probe.

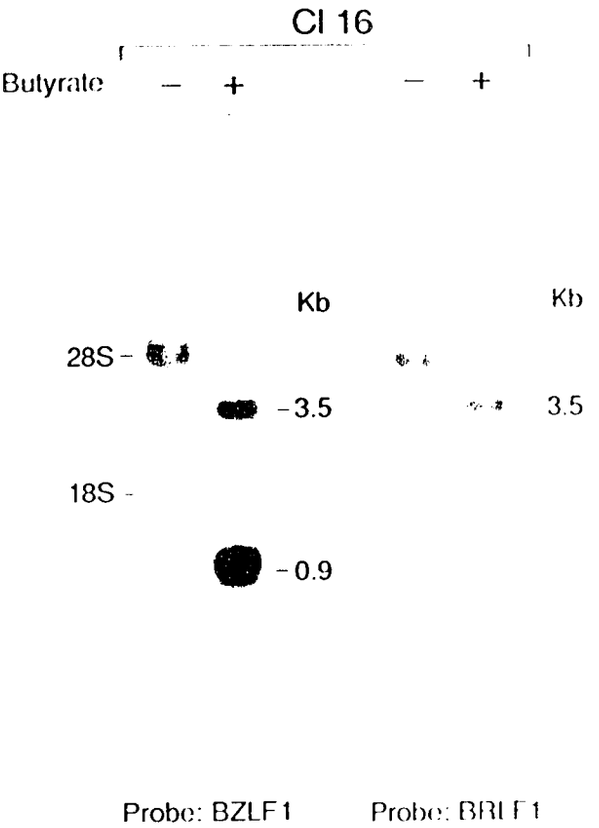
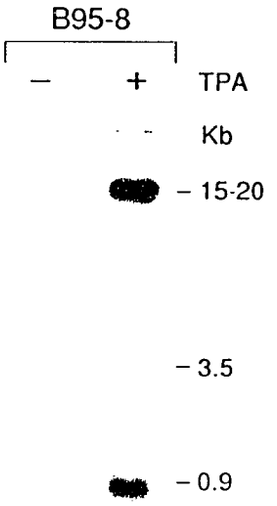
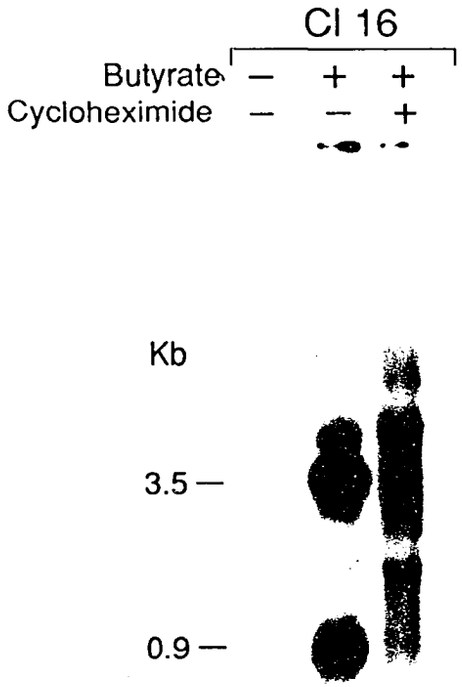


Figure 12. Northern blot analysis of BZLF1 transcription in B95-8 cells. RNA was prepared from uninduced and TPA treated B95-8 cells. The blot was probed with a 627 bp subfragment of BZLF1. A 15 Kb mRNA is detected in addition to the 3.5 Kb and 0.9 Kb BZLF1 specific mRNAs.

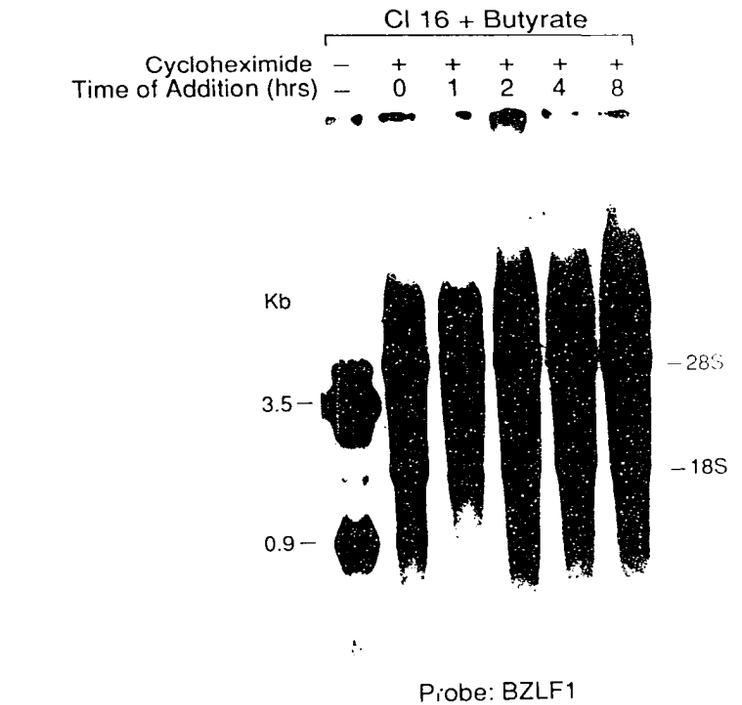


Probe: BZLF1

Figure 13. Protein synthesis is required for BZLF1 transcription following butyrate induction. Clone 16 cells, which contain only standard EBV DNA, were untreated or induced with butyrate. A) An aliquot of the butyrate treated cells was treated with the protein synthesis inhibitor, cycloheximide. After 24 hours RNA was harvested from the three groups of cells and probed on a Northern blot with a 627 bp subfragment of BZLF1. The two BZLF1 specific mRNAs, 3.5 Kb and 0.9 Kb, are detected in butyrate treated cells but are not expressed in the presence of cycloheximide. B) Cycloheximide was also added to different cell aliquots at 1, 2, 4, and 8 hours following butyrate induction. RNA was harvested 48 hours after induction and similarly probed on a Northern blot with the BZLF1 subfragment. The BZLF1 specific mRNAs were not detected in any of the cyloheximide treated cells.

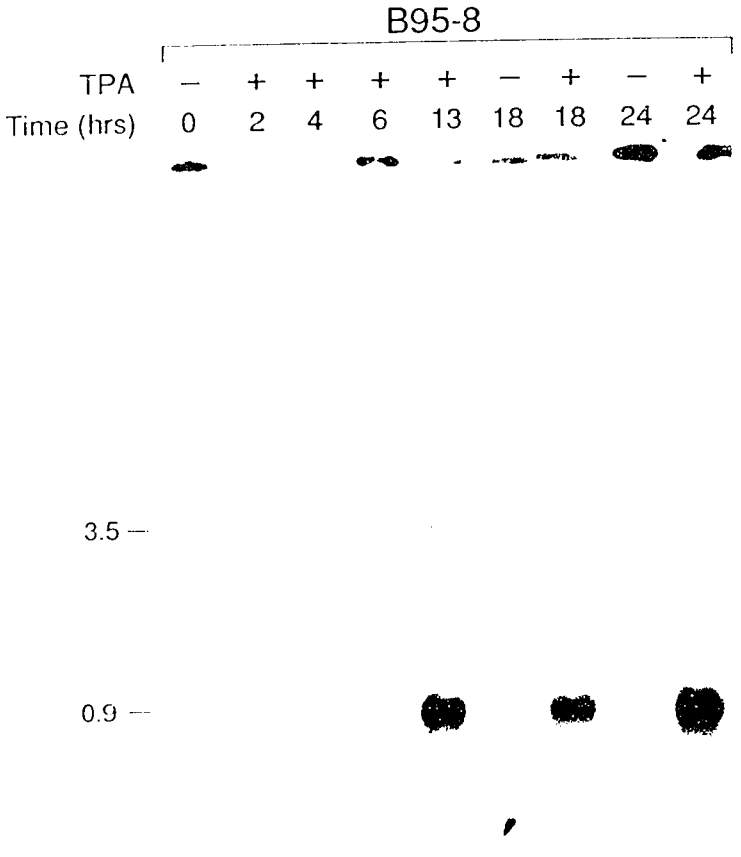


Probe: BZLF 1



Probe: BZLF1

Figure 14. Kinetics of BZLF1 transcription in TPA induced B95-8 cells. Each time point represents RNA harvested from 1 X 10^6 cells. The Northern blot was probed with the 627 bp BZLF1 subfragment. The 3.5 Kb and 0.9 Kb BZLF1 specific mRNAs are detected simultaneously.



Probe: BZLF1

Figure 15. Stability of BZLF1 specific mRNAs in butyrate induced clone 5 cells. Clone 5 cells were induced with butyrate for 2 days prior to inhibition of transcription with actinomycin D. RNA was harvested at various time points. Each time point represents RNA from 1 X 10^6 cells and the blot was probed with the 627 bp BZLF1 subfragment. The 3.5 Kb and 0.9 Kb BZLF1 specific mRNAs have similar stabilities.

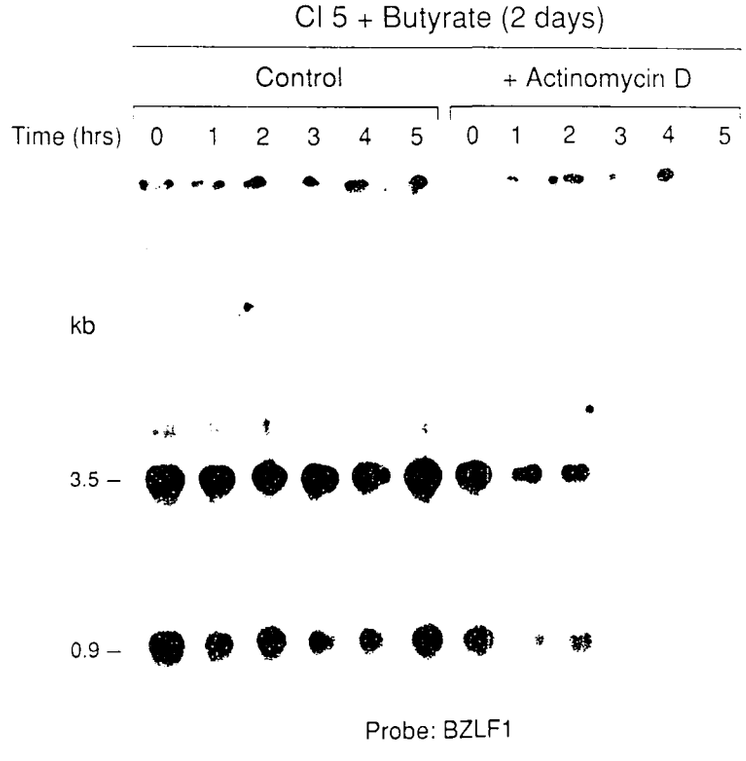
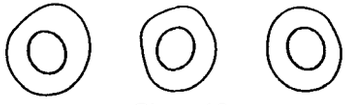


Figure 16. Differences in regulation of BZLF1 expression in HR-1 cells with or without het DNA. Clone 16 cells, which lack het DNA, express latent products (shaded nuclei) and spontaneously enter the replicative cycle at a low rate (0.1% of cells). Clone 5 cells, which harbor het DNA, spontaneously produce virus. BZLF1 expression was studied in untreated cells or after induction with TPA or butyrate. BZLF1 specific transcripts were detected by Northern blotting and ZEBRA protein expression was assayed by Western blotting with a monospecific antiserum (from Miller, 1990).

DIFFERENCES IN REGULATION OF BZLF1 IN HR-1 CELLS WITH OR WITHOUT HET DNA

HR-1 Cell Subclone	Het DNA	BZFL1 EXPRESSION			
		spontaneous		induced	
		mRNA	protein	mRNA	protein
 Clone 16	–	–	–	+	+
 Clone 5	+	+	+	+	+

CHAPTER III

DNA BINDING ACTIVITY OF ZEBRA: THE EPSTEIN-BARR VIRAL REPLICATION ACTIVATOR

INTRODUCTION

Epstein-Barr virus infection of lymhoid cells, either in-vitro or in-vivo, results in lymphocyte immortalization and viral latency (reviewed Miller, 1990a). Several groups have now shown that expression of the EB viral gene, BZLF1 results in a switch to the lytic viral life cycle (Countryman and Miller, 1985, Chevallier-Greco et al, 1986, Takada et al., 1986). The mechanism by which the BZLF1 gene product, ZEBRA, activates the replicative cycle has not yet been determined.

Recently, Farrell and co-workers determined that exon II of ZEBRA shares homology with the basic domain of the Fos/Jun oncogene family (Fig. 1) (Farrell et al., 1989). Fos/Jun related proteins have previously been shown to bind the canonical AP-1 site (TGAGTCA), and Farrell et al. (1989) showed that ZEBRA bound an AP-1 consensus sequence located in the promoter of an EBV early gene, MS-EA (Angel et al., 1987, Lee et al., 1987, Hope and Struhl, 1987, Farrell et al., 1989). All of the Fos/Jun related proteins require dimerization for DNA binding and have been shown to dimerize through a leucine zipper motif, a region containing leucines at heptad intervals (Abel and Maniatis, 1989, Agre et al.,

1989, Gentz et al., 1989, Kouzarides and Ziff, 1988, 1989, Landschulz et al., 1988, Neuberg et al., 1989a, Sassone-Corsi et al., 1988, Schuerman et al., 1989, Sellers and Struhl, 1989, Vinson et al., 1989). ZEBRA, however, does not contain a leucine zipper dimerization domain downstream of its basic domain. Additionally, ZEBRA has been shown to bind to sites which deviate from the AP-1 consensus sequence (Chang et al., 1990, Flemington and Speck, 1990b, Lieberman and Berk, 1990, Packham et al., 1990, Taylor et al., 1991a, Urier et al., 1989). Thus, it was of interest to define the domains of the ZEBRA protein required for DNA binding and determine whether ZEBRA binds DNA as a dimer.

RESULTS

Determination of the ZEBRA DNA Binding Domain by Deletional Mutagenesis. A BZLF1 cDNA obtained from Raji cells was cloned in frame with the trpE coding sequences contained in the pATH 11 expression vector (Fig. 2B). The 75 kilodalton (Kd) fusion protein bound a 20 base pair (bp) oligonucleotide which contains an AP-1 consensus sequence (MS-AP-1) derived from the promoter of the EBV early gene, MS-EA (Figs. 2C, 2D, Z_{1-245}). Several deletion mutants defined the domain of the protein which bound the AP-1 site (Figs. 2C, 2D). All of the mutants exhibited some temperature sensitive DNA binding activity. Mutant $Z_{141-245}$ encoded the smallest protein which behaved like the wild type ZEBRA (Z_{1-245}) in binding DNA both at room temperature and at 4C (Fig. 2A). However, even this mutant did not bind DNA as well at 4C as the wild type Z_{1-245}. The region of ZEBRA encoded by exon II, which shares homology with the Fos basic domain (Farrell et al., 1989), was insufficient to elicit DNA binding activity (Fig. 2C, 2D, $Z_{168-202}$). Although $Z_{111-159}$ bound DNA unstably (see smear, Fig. 2C) at room temperature (22°C) and $Z_{172-245}$ did not bind DNA under these conditions (Fig. 2C), both mutant proteins bound DNA at 4°C (Fig. 2D). Deletion of the 18 carboxyl amino acids of ZEBRA resulted in a mutant (Z_{1-227}) which bound the AP-1 site at room temperature, but not at 4°C (Figs. 2C, 2D). Furthermore, all the ZEBRA deletion mutants showed the same

pattern of binding to the divergent AP-1 sites, ZIIIA and ZIIIB, present in the BZLF1 promoter (data not shown, refer to chapter IV). Thus, ZEBRA amino acids 172-227 are required for DNA binding although neighboring sequences may influence protein folding or stability under some temperature conditions.

General DNA Binding Activity of ZEBRA mutants. DNA binding of ZEBRA mutants was also analyzed by the ability of ZEBRA proteins to bind an array of DNA sequences. Z_{1-245} as well as the unspliced ZEBRA protein from rearranged het DNA, $WZ_{1-167(+41)}$ (which contains 167 amino acids from the first exon of Zhet plus 41 amino acids from the first intron), were electrophoresed in an SDS-polyacrylamide gel, blotted onto nitrocellulose and incubated with radiolabeled pSV2neoWZhet DNA. Suprisingly, both proteins bound DNA, and the unspliced Zhet protein appeared to have a greater intrinsic DNA binding potential than the wild type ZEBRA protein, Z_{1-245} (Fig. 3A). Whereas pSV2neoWZhet contains an AP-1 binding site, identical results were obtained when the proteins were incubated with radiolabeled pATH vector DNA (data not shown). In an attempt to determine whether a bacterial protein, or TrpE, which is present at the 5′ terminus of the ZEBRA constructs is affecting DNA binding, the DNA binding of bacterial extracts as well as TrpE was analyzed. Bacterial extracts, cells transformed with the trpE vector as well as TrpE- $WZ_{1-167(+41)}$ were electrophoresed and stained

with Coomassie brilliant blue (Fig. 3B). An identical gel was transferred to nitrocellulose and probed with pSV2neo WZhet. While several low molecular weight bacterial bands were observed in all lanes, TrpE did not bind DNA and TrpE-$WZ_{1-167(+41)}$ again bound DNA strongly (Fig. 3C).

The location of this general DNA binding domain was mapped by deletional mutagenesis of TrpE- $WZ_{1-167(+41)}$. Construction of the deletions is described in the Experimental Procedures and the extent of the deletions is shown in Fig. 4A. All deletion proteins were stably expressed as assayed by coomassie blue staining of an SDS-polyacrylamide gel (data not shown). The results of the general DNA binding assay indicated that the smallest clone capable of binding DNA was $WZ_{133-167(+41)}$ (Fig. 4B). Interestingly, $WZ_{133-167(+4)}$ did not bind DNA suggesting that intron sequences from WZhet were important for the general DNA binding ability of this protein (Fig. 4B). A clone containing only the basic domain of the ZEBRA protein encloded by exon II, $Z_{168-202(+4)}$, was not able to bind DNA in this assay (data not shown).

Site Directed Mutagenesis of the ZEBRA Basic Domain. The region of ZEBRA encoded by the second exon of BZLF1 (aa 168-202) is partially homologous to the basic region of Fos (Fig. 1, 5A). It has recently been shown that amino acids 144-146 and 154-156 are required for the DNA binding ability of Fos (Neuberg et al.,1989b). These basic amino acids are

conserved at positions 178-180 and 188-190 of ZEBRA exon II. Whereas ZEBRA exon II sequences were not necessary for the general DNA binding of the protein, they were necessary for binding to the AP-1 heptamer (Figs. 2 and 4). Therefore, we were interested in determining whether the same basic amino acids contribute to the specific DNA binding activity of ZEBRA. The ZEBRA residues were mutated from lys-arg-tyr to glu-glu-leu and arg-lys-cys to glu-glu-ser, respectively (Fig. 5A, courtesy E. Flemington). These mutations abrogated DNA binding to the consensus (MS-AP-1) as well as divergent AP-1 sites (ZIIIA and ZIIIB) (Fig. 5B, courtesy E. Flemington). Equivalent amounts of the in vitro translated wild type and mutant proteins were used in each reaction (data not shown). These results demonstrate that the same basic residues in ZEBRA and Fos are required for binding to AP-1 and related sequences. However, deletional mutagenesis showed that these basic residues are necessary but not sufficient for sequence specific DNA binding (Fig. 2).

ZEBRA Binds DNA as a Stable Homodimer. Proteins which bind palindromic DNA binding sites have generally been shown to bind DNA as a dimer, where each protein binds the dyad half site (reviewed in Johnson and McKnight, 1989). Since ZEBRA binds a consensus sequence with two dyad half sites (TGAGTCA) and contains homology to the Fos/Jun dimeric proteins (Abate et al., 1990, Farrell et al., 1989, Hope and Struhl, 1987, Kouzardies and Ziff, 1988, Landschulz et al.,

1988, Sassone-Corsi et al., 1988), the hypothesis that ZEBRA also binds DNA as a homodimer was tested.

The method used by Hope and Struhl (1987) to show that the GCN4 protein bound DNA as a dimeric protein was utilized. When proteins are complexed to DNA on an acrylamide gel, the mobility of the complex is proportional to the molecular weight of the protein. When Z_{1-245} was analyzed by gel retardation analysis its mobility was less than that of the truncated ZEBRA binding protein, $Z_{141-245}$ (Figs. 2 and 6). In order to determine whether ZEBRA bound DNA as a homodimer, a protein lysate containing Z_{1-245} and $Z_{141-245}$ were denatured together in 6M urea and then renatured prior to analysis by gel mobility. A shifted complex of intermediate mobility, in addition to the two complexes observed when either of these proteins bound DNA separately, was observed (Fig. 6, lane $(Z_{1-245} + Z_{141-245})u$). The detection of an intermediate sized band suggests that this complex is composed of a heterodimer of the individual proteins.

This method has been successfully used to show that the bovine papilloma virus E2 protein and yeast transcriptinal activator bind DNA as a dimer and trimer, respectively (Dostatni et al., 1988, Giri and Yaniv, 1988, McBride et al., 1989, Sorger and Nelson, 1989). Furthermore, no heterodimer complex was formed if the ZEBRA proteins were mixed following renaturation; indicating that complex association occurs immediately after dissociation and is

stable in the absence of DNA (Fig. 6, lane $((Z_{1-245})u + (Z_{141-245})u)$.

DISCUSSION

A Comparison of ZEBRA and Fos DNA Binding Domains. The construction and expresssion of ZEBRA deletion mutants in E. coli enabled a determination of the domains within the ZEBRA protein which are required for DNA binding. These studies indicate that the region 3′ to the ZEBRA basic domain (aa 199-227) is required for DNA binding. The carboxy-terminal 18 amino acids (228-245), while not strictly required for DNA binding, may also contribute to dimer formation and/or protein stability since mutants lacking this region bind DNA in a temperature sensitive fashion (Figs. 2C and 2D). These results are consistent with those of Packham et al. who found that mutants which deleted the same regions of exon 1 or 3 bound DNA in a salt dependent manner, whereas the wild type protein bound DNA under all conditions (Packham et al., 1990).

Site directed mutagenesis of c-fos previously identified the amino acids necessary for DNA recognition of the Fos/Jun complex (Neuberg et al., 1989b). Mutagenesis of Fos amino acids 144-146 or 154-156 completely abrogated DNA binding (Neuberg et al., 1989b). The corresponding amino acids are conserved in the basic domain of ZEBRA exon II and proteins mutated in these positions were unable to bind any of the DNA binding sites (MA-AP-1, ZIIIA, or ZIIIB) (Fig. 5). These results suggest that while the DNA binding specificities of Fos and ZEBRA differ, the same two clusters

of basic amino acids are necessary for the specific DNA binding ability of both proteins. Further work is needed to define the amino acids in the basic region which confer the unique binding specificities of Fos and ZEBRA (refer to chapter IV).

General DNA Binding of the ZEBRA Protein. The region responsible for the ability of the unspliced het ZEBRA protein to bind DNA in a nitrocellulose filter binding assay has been localized to the 35 carboxy-terminus amino acids in exon I (aa 133-167) and the 41 amino acids present in the first intron (Fig 4). The residues within the carboxy terminus as well as the intron are highly basic, a characteristic of many DNA binding domains. It is not clear whether het ZEBRA is ever expressed in-vivo in this unspliced form, but a cDNA corresponding to the unspliced standard ZEBRA has never been detected (Biggin et al., 1987, Manet et al., 1989).

This filter binding method has previously been used to map the intrinsic DNA binding domains of the v-myb and c-myc proteins (Klempnauer and Sippel, 1987, Dang et al., 1989). The intrinsic DNA binding domain of v-myb has also been shown to confer sequence specific DNA binding potential (Klempnauer and Sipper, 1987, Biedenkapp et al., 1988, reviewed in Luscher and Eisenman, 1990b). However, the region of c-myc necessary for its nonspecific DNA binding does not include the basic, helix-loop-helix and leucine

zipper domains of the protein which were required for its sequence-specific DNA binding potential (Blackwood and Eisenman, 1991, Blackwell et al., 1990, Dang et al., 1989, Prendergast and Ziff, 1989, reviewed in Luscher et al., 1990a). Thus, the importance of nonspecific DNA binding domains in both c-myc and ZEBRA has yet to be determined.

Function of ZEBRA Exon III. Unlike all other identified transcription factors which bind the AP-1 consensus sequence, ZEBRA does not contain a leucine zipper directly adjacent to the basic region (Landschulz et al., 1988, Vinson et al., 1989). While ZEBRA exon III (aa 199-245) is not homologous to any known dimerization domain (Vinson et al., 1989), ZEBRA binds DNA as a homodimer (Fig. 6) and this region is required for specific binding (Figs. 2C and D). It has been hypothesized that this domain forms an alpha helix and that dimerization involves a coiled coil interaction (Flemington and Speck, 1990c, Kolman et al., 1991, Lieberman and Berk, 1990, Packham et al., 1990, Taylor et al., 1991a). Moreover, O'Shea and colleagues (1989) have hypothesized that the leucine zipper motif forms a coiled coil structure and have recently shown that the x-ray structure of the leucine zipper is a coiled coil (Rasmussen et al., 1991). Both ZEBRA exon III and the leucine zipper domain contain the 4-3 hydrophobic repeat necessary for generation of a coiled coil structure (O'Shea et al., 1989, Flemington and Speck, 1990c).

The existence of different leucine zipper and coiled coil motifs is important in regulating DNA binding of various proteins. ZEBRA, as well as the Fos and Jun protooncogenes and the yeast regulatory protein, GCN4 all bind the TPA response element (AP-1, TGAGTCA). However, the dimerization potentials of these proteins are very specific. A GCN4 leucine zipper homodimerizes whereas a Fos zipper dimerizes specifically with Jun and Jun dimerizes with itself and Fos (Kouzarides and Ziff, 1989, Neuberg et al., 1989a, Sellers and Struhl, 1989, Nakabeppu and Nathans, 1989). While ZEBRA has the potential to bind DNA as a homodimer, heterodimerization with a leucine zipper protein or a yet unidentified protein may be important for the induction of cellular genes and/or the negative regulation of ZEBRA responsive genes.

Figure 1. Homology among basic DNA binding domains of BZLF1 and the AP-1 family of proteins. Data are derived from Farrell et al. (1989). Positions of the various amino acids are as follows: BZLF1, amino acids 175-196; cFos,140-161; cJun, 264-285; and GCN4, 228-249. Most of the homology consists of positively charged residues (arginines, R and lysines, K) (from Miller, 1990b).

Homology among DNA Binding Domains

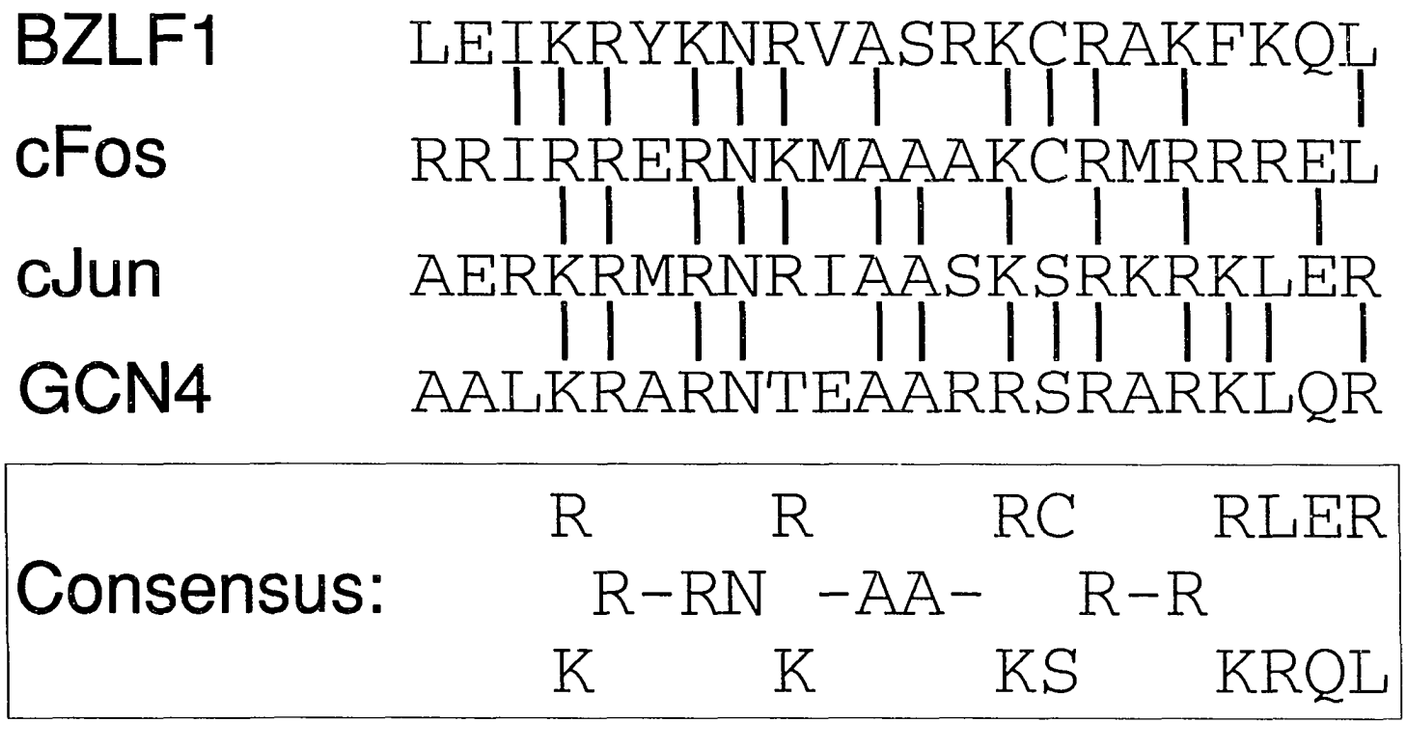


Figure 2. DNA binding activity of ZEBRA mutants. (A) Structure of ZEBRA deletion mutants. The ZEBRA cDNA is shown at the top (Manet et al., 1989) and clones are designated according to the ZEBRA amino acids present in the construct. $Z_{\Delta 111-159}$ is named according to the amino acids which have been deleted. Z_{1-167} was derived from genomic WZhet DNA (Taylor et al., 1989). Translated sequences which are derived from ZEBRA intron sequences are indicated by a hatched bar and sequences from the expression plasmid polylinker are indicated by a stippled bar. (B) Expression of TrpE-ZEBRA fusion proteins in bacteria. Total protein from bacteria which carried various TrpE-ZEBRA deletion proteins was resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The fusion protein is indicated with dots in each lane. (C) Gel retardation assay showing binding of TrpE-ZEBRA deletion mutants to a 20 bp ^{32}P-labeled oligonucleotide containing the AP-1 consensus sequence (Farrell et al., 1989). Extract from bacteria transformed with the trpE plasmid, pATH 11, was used as a negative control (vector lane). Binding reactions and electrophoresis were performed at room temperature (RT,22C). Complexes were electrophoresed on a 4% acrylamide gel in 0.5X TBE. (D) Gel retardation assay of the same TrpE-ZEBRA deletion mutants performed as in (C) except that binding reactions and electrophoresis were carried out at 4C.

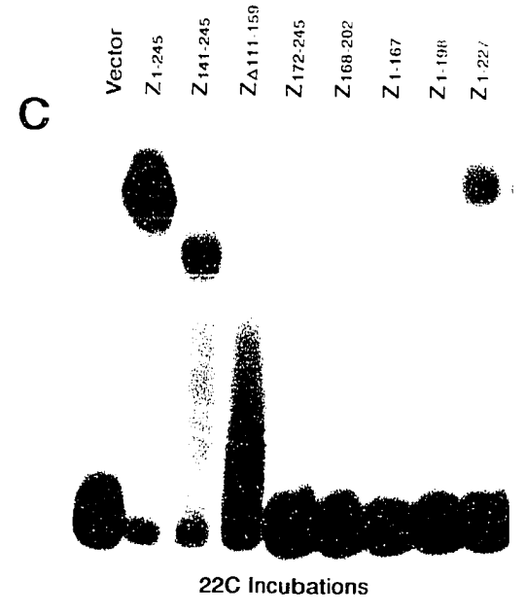


Figure 3. Non-specific DNA binding of ZEBRA proteins. (A) Z_{1-245} and $WZ_{1-167(+41)}$ were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose. The blot was then incubated with ^{32}P-labeled PSV2neoWZhet and analyzed by autoradiography. (B) Protein extracts from E. coli AG1 cells, AG1 cells transformed with the trpE pATH expression vector, and cells transformed with trpE-$WZ_{1-167(+41)}$ were analyzed by Coomassie brilliant blue staining of an SDS-polyacrylamide gel. (C) An identical gel was transferrred to nitrocellulose and analyzed by autoradiography following incubation with ^{32}P-labeled PSV2neoWZhet. Identical results were obtained following incubation with ^{32}P-labeled pATH vector DNA (data not shown).

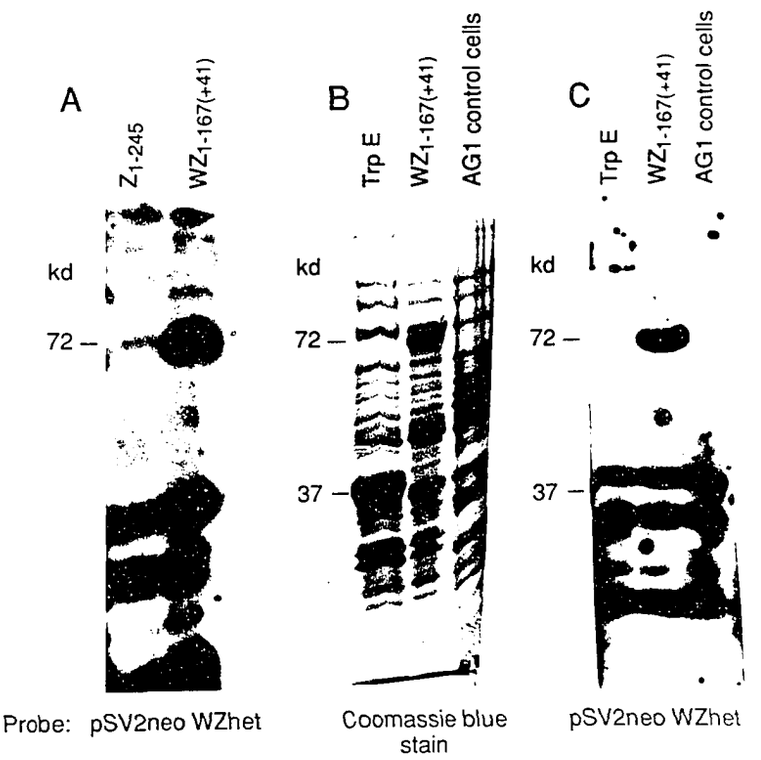
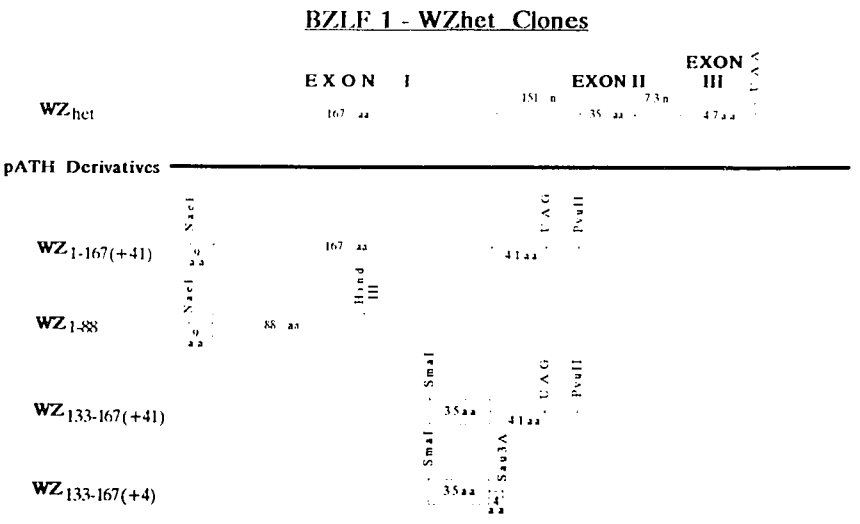
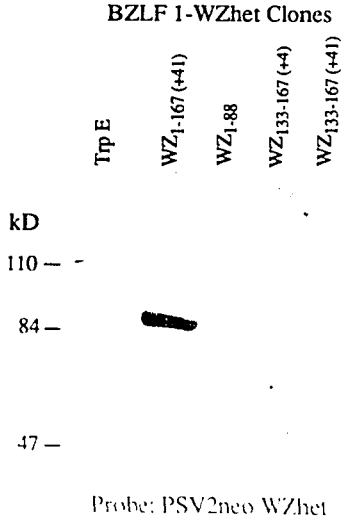


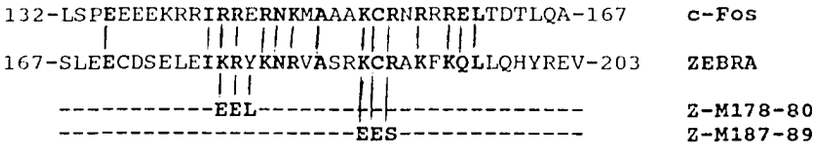
Figure 4. Mapping the non-specific DNA binding domain of $WZ_{1-167(+41)}$. (A) Structure of WZ deletion mutants. The structure of WZhet is shown at the top; a bar indicates an exon and a line denotes an intron (Jenson et al., 1988). Clones are designated according to the WZhet amino acids present in the construct. Amino acids denoted in parentheses refer to those amino acids which are translated from intron sequences. (B) Proteins extracts were prepared from induced AG-1 cells harboring trpE expression plasmids containing $WZ_{1-167(+41)}$, WZ_{1-88}, $WZ_{133-167(+41)}$, and $WZ_{133-167(+4)}$. Extracts were electrophoresed on an SDS-polyacrylamide gel and then transferred to nitrocellulose. The blot was probed with ^{32}P-nick translated PSV2neoWZhet and analyzed by autoradiography.





Probe: PSV2neo WZhet

Figure 5. Effect of point mutations in the basic domain of ZEBRA. (A) Amino acid sequences of human c-fos exon III (aa 132-167) (van Straaten et al., 1983) and BZLF1 exon II (aa 168-202) (Biggin et al., 1987). The conserved residues in this highly basic domain are indicated by lines (Farrell et al., 1989). ZEBRA mutants were obtained by site directed mutagenesis of the BZLF1 cDNA cloned in SP64. Amino acids 178-180 and 187-189 were changed as shown for Z-M178-80 and Z-M187-89, respectively. (B) Gel retardation assay demonstrating the inability of ZEBRA constructs mutated at aa 178-180 (Z-M178-80) or 187-189 (Z-M187-89) to bind MS-AP-1, ZIIIA, or ZIIIB. The wild type (Z-WT) and mutated ZEBRA constructs (Z-M178-180 and Z-M187-189) were transcribed in-vitro with SP6 polymerase and translated in a wheat germ extract. Equivalent amounts of the resulting translated proteins were each incubated with the following ^{32}P-labeled oligonucleotide probes: MS-AP-1, ZIIIA, and ZIIIB (see Experimental procedures for a listing of these sequences). These complexes were then resolved by gel electrophoresis on a 4% acrylamide gel in 0.5X TBE.



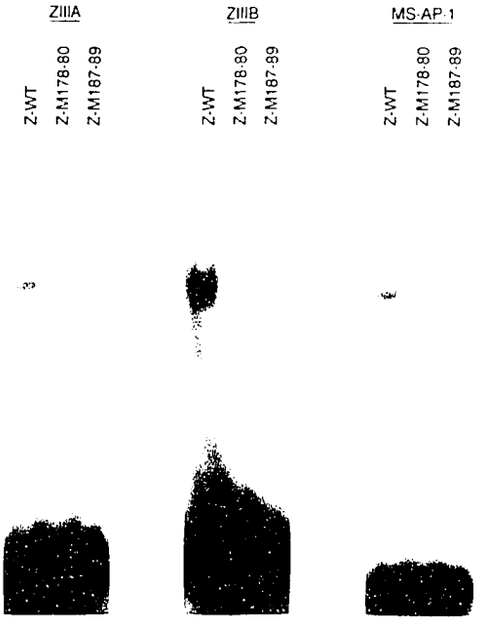
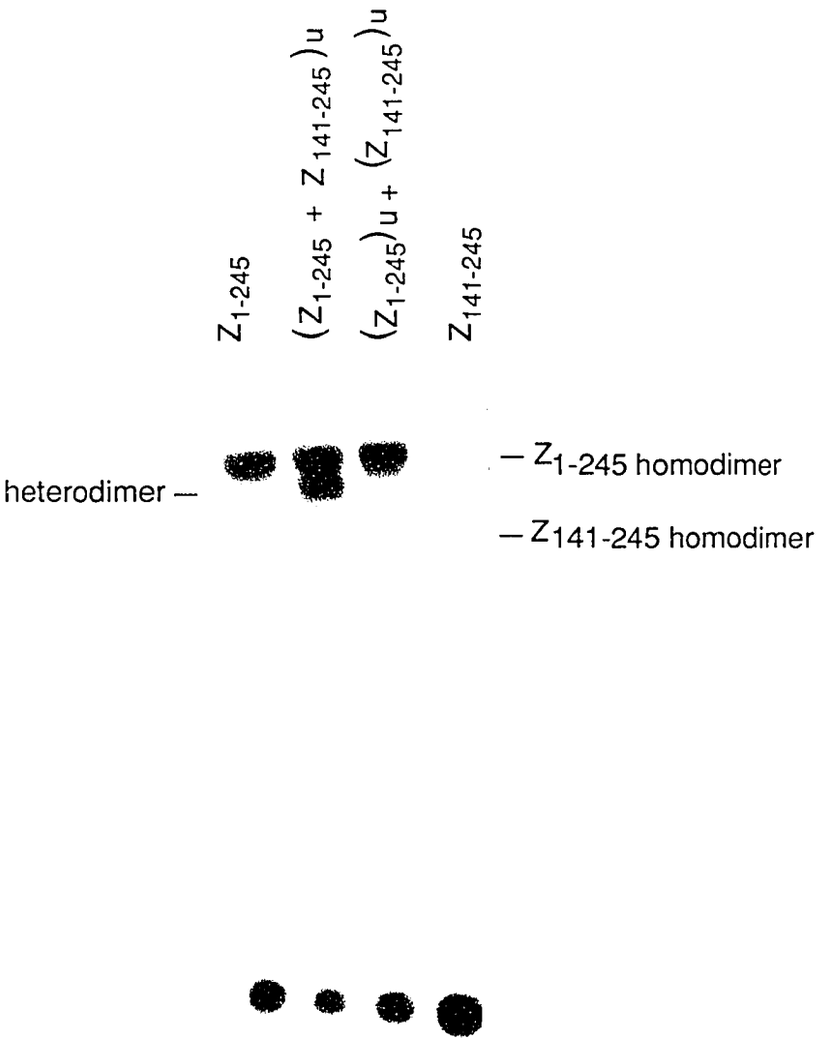


Figure 6. ZEBRA binds DNA as a stable homodimer. Z_{1-245} and $Z_{141-245}$ were dissociated with urea and then reassociated either separately ($Z_{1-245}u$ + $Z_{141-245}u$) or together ((Z_{1-245} + $Z_{141-245}$)u) prior to gel retardation analysis. Proteins were used in a gel binding assay with the 20 bp ^{32}P-labeled oligonucleotide containing the AP-1 consensus sequence. The various protein extracts were incubated with the oligonucleotide probe and 1 ug poly dIdC in binding buffer for thirty minutes prior to electrophoresis. Binding reactions and electrophoresis were performed at room temperature (RT,22C) and complexes were electrophoresed on a 4% acrylamide gel in 0.5X TBE.



MS-AP-1

CHAPTER IV

ZEBRA AND FOS DIFFER IN THEIR DNA BINDING SPECIFICITIES FOR SITES IN THE EPSTEIN-BARR VIRAL BZLF1 PROMOTER

INTRODUCTION

Epstein Barr Virus (EBV) persists in lymphoid cells in a latent state but can be activated *in vitro* by phorbol esters, butyrate or expression of a virally encoded gene, ZEBRA (Chevallier-Greco et al., 1986, Countryman et al., 1985, Gradoville et al., 1990, Luka et al., 1979, Taylor et al., 1989, van Straaten et al., 1983). The mechanism by which ZEBRA initiates the cascade of viral replication has been the focus of much study. Numerous groups have found that ZEBRA directly transactivates several promoters of lytic cycle genes, including a region integral to the EBV lytic origin of replication (Chavrier et al., 1989, Chevallier-Greco et al., 1986, Flemington et al., 1990, Gradoville et al., 1990, Hammerschmidt et al., 1988, Hardwick et al., 1988, Kenney et al., 1989, Lieberman et al., 1990, Urier et al., 1989).

Exon II of ZEBRA is homologous to the basic domain of the Fos/Jun proto-oncogene family (Farrell et al., 1989). In Fos/Jun complexes, this domain has been shown to interact directly with a DNA heptamer known as the TRE or AP-1 site

(TGAGTCA) (Angel et al., 1987, Lee et al., 1987, Neuberg et al., 1989b). It was determined that ZEBRA shares the ability of the Fos/Jun complex to bind an AP-1 consensus site (Farrell et al., 1989). An AP-1 consensus sequence (MS-AP-1) is located in the promoter of an EB early gene, BSLF2-BMLF1 (MS-EA) (Farrell et al., 1989). ZEBRA can transactivate transcription through this site, although it is not yet clear whether AP-1 sequences are required for transactivation of EB viral genes in infected lymphocytes (Holley-Guthrie et al., 1990, Urier et al., 1989).

ZEBRA also binds sites on the EBV genome which deviate from the AP-1 consensus sequence (Flemington and Speck, 1990b, Lieberman and Berk, 1990, Lieberman et al., 1990, Packham et al., 1990, Urier et al., 1989). The autoregulated BZLF1 (ZEBRA) promoter contains three divergent AP-1 elements (Flemington and Speck, 1990b). Two sites, TGAGCCA (ZIIIA) and TTAGCAA (ZIIIB), 105-129 bp upstream of the transcriptional start site are arranged in a tail to tail fashion and are separated by 6 nucleotides. An additional AP-1 sequence, TGACATCA (Z-AP-1 octamer), is located further downstream at -67. This sequence, which was first identified in the jun promoter by Angel et al., mediates activation by TPA and Jun/AP-1 (Angel et al., 1988). Recent work has shown that although this site is TPA inducible in the ZEBRA promoter, it does not appear to be transactivated by ZEBRA (Flemington and Speck, 1990a,

1990b).

Since both ZEBRA and Fos/Jun are involved in transcriptional regulation of gene expression it was important to compare the DNA binding activities of these proteins. Therefore, we studied the relative DNA binding affinities of ZEBRA and a Fos-GCN4 chimera for the divergent AP-1 sites found in the autoregulated BZLF1 promoter.

RESULTS

Binding of ZEBRA and Fos-GCN4 to Oligonucleotides Bearing Single AP-1 and "AP-1 Like" Sequences. It was previously shown that ZEBRA footprinted sites ZIIIA at -129 (TGAGCCA) and ZIIIB at -116 (TTAGCAA), but not the Z-AP-1 octamer sequence (TGACATCA) 67 bp upstream of the BZLF1 transcriptional start site (Flemington and Speck, 1990b, Fig. 1A). A gel retardation assay was used to compare the relative affinities of ZEBRA and Fos-GCN4 for these sites in the BZLF1 promoter. The Fos basic domain has been shown to bind the AP-1 consensus site as a homodimer when fused to the GCN4 leucine zipper (Hope and Struhl, 1987, Kouzarides and Ziff, 1989, Sellers and Struhl, 1989). A Fos-GCN4 chimera, expressed as a trpE fusion protein, bound the MS-AP-1 consensus site but not ZIIIB or ZIIIA. ZIIIB differs from the consensus AP-1 site at 3bp and ZIIIA contains a single base pair change from the consensus heptanucleotide AP-1 sequence (Fig. 3B). Fos-GCN4 bound the Z-AP-1 octamer with high affinity whereas ZEBRA interacted only weakly with this sequence. Thus, Fos-GCN4 binds the AP-1 octamer and heptanucleotide sequences while ZEBRA binds the MS-AP-1, ZIIIA and ZIIIB sites with relatively high affinity (Fig. 1B). The trpE-ZEBRA and trpE-Fos-GCN4 fusion proteins were derived from the urea solubilized fraction of total E. coli protein extracts. ZEBRA and Fos-GCN4, expressed in this system, bind DNA as homodimers (refer to chapter III, Kolman

et al., manuscript in preparation, Taylor et al., 1991b). A control extract of bacterial protein did not exhibit specific binding to any of these sites (data not shown).

Binding of ZEBRA and Fos-GCN4 to an Oligonucleotide Containing Two ZEBRA Response Elements, ZIIIA and ZIIIB. Since the ZIIIA and ZIIIB sites are only separated by 6 nucleotides in the ZEBRA promoter, we studied the ability of ZEBRA and Fos-GCN4 to bind both sites simultaneously. An oligonucleotide termed "Double" which includes both sites in the orientation found in the BZLF1 promoter was used in gel band shift assays. ZEBRA was able to bind two sites on this oligonucleotide simultaneously as demonstrated by the appearance of two band shift products in a mobility shift assay (Fig. 1B). Suprisingly, the Fos-GCN4 chimera, which did not bind a single ZIIIA or ZIIIB site, was able to bind the Double oligonucleotide (ZIIIA + ZIIIB) generating a slower migrating complex (Fig. 1B).

The order of binding to sites on the Double oligonucleotide was then studied. Serial dilutions of ZEBRA and Fos-GCN4 were incubated with 4 X 10^{-13}M of ^{32}P labelled Double probe. At low protein concentrations (0.25 ul), ZEBRA only bound a single site on the oligonucleotide (one shifted complex) while at high concentrations (5 ul) it began to interact with both sequences on a single oligonucleotide molecule (two shifted complexes) (Fig. 2A). However, Fos-GCN4 generated a single complex of slower

mobility with the Double probe, regardless of protein concentration (Fig. 2B).

Nucleotide Specificity of ZEBRA and Fos-GCN4 Binding Sites. Point mutations were introduced into the MS-AP-1, ZIIIA, and ZIIIB oligonucleotides in an attempt to establish which base pairs mediate specific DNA binding by ZEBRA and Fos-GCN4. The oligonucleotides created were MS-AP-1* (TTAGTCA), ZIIIA* (TGAGCAA) and ZIIIB* (TGAGCAA). ZIIIA* and ZIIIB* contain an identical 7 bp core recognition site (TGAGCAA), but differ in their surrounding sequences (see Experimental procedures). Additionally, the ZIIIA and ZIIIB oligonucleotides were mutated at 4 positions to generate the ZIIIAm and ZIIIBm oligonucleotides.

ZEBRA bound with relatively high affinity to the MS-AP-1, ZIIIA and ZIIIB sequences containing a single point mutation (MS-AP-1*, ZIIIA*, ZIIIB*) (Fig. 3A). However, it was unable to bind either the ZIIIAm or ZIIIBm sites which contain 4 base pair changes (Fig. 3A). The lack of binding to the ZIIIAm or ZIIIBm sites confirms the importance of the identified core element. Fos-GCN4 was more fastidious in its binding site requirements; it recognized the ZIIIB* (TGAGCAA) sequence, but did not interact with any of the other mutated oligonucleotides (Fig. 3B). ZIIIA* and ZIIIB* differ from the AP-1 consensus at 2 nucleotides, while ZIIIB differs at 3 positions and ZIIIA differs at only one. ZIIIA* and ZIIIB* contain the same 7 bp recognition sequence

but their surrounding sequences vary. These results suggest that ZEBRA is considerably more tolerant of changes within the 7 bp recognition sequence than Fos-GCN4.

Competition Analysis to Determine the Affinities of ZEBRA and Fos-GCN4 for AP-1 and ZEBRA Promoter Sequences. The relative affinities of ZEBRA and Fos-GCN4 for AP-1 related sequences were assayed by competition analysis (Fig. 4). Complex formation between ZEBRA or Fos-GCN4 and the MS-AP-1 probe were assayed in the presence of three concentrations of cold competitor oligonucleotides. The relative reduction of AP-1 complex formation is proportional to the affinity of ZEBRA or Fos-GCN4 for the oligonucleotide competitor. Disruption was quantified by comparing the integrated intensity of each shifted complex as a function of the concentration of oligonucleotide competitor (Figs. 4C and D). The ZEBRA complex formed with the MS-AP-1 site was most efficiently competed with MS-AP-1, ZIIIA*, and ZIIIB* (Figs. 4A and 4C). A 5 fold molar excess of these oligonucleotides eliminated ZEBRA binding to ^{32}P labelled MS-AP-1, whereas a 50 fold molar excess of ZIIIA, ZIIIB, or Double oligonucleotide was required to achieve a similar level of inhibition (Figs. 4A and C). The ZEBRA/MS-AP-1 complex was minimally reduced by the addition of a 50 fold molar excess of Z-AP-1 octamer and was not affected by the addition of unlabeled ZIIIAm, ZIIIBm, or a control oligonucleotide with no AP-1 like sequences (Figs. 4A, 4C and data not shown).

Thus, ZEBRA binds with highest affinity to TGAGTCA (MS-AP-1) and TGAGCAA (ZIIIA* and ZIIIB*) sites and with a lower affinity to TTAGCAA (ZIIIB), TGAGCCA (ZIIIA), TTAGTCA (MS-AP-1*), and the Double oligonucleotide (Figs. 4C and 5).

The Fos-GCN4/MS-AP-1 complex was efficiently competed with a 5 fold molar excess of Z-AP-1 octamer, 20 fold excess of MS-AP-1 or a 50 fold molar excess of ZIIIB* (Figs. 4B and 4D). Suprisingly, the Double oligonucleotide which was bound by Fos-GCN4 in a gel retardation assay was unable to disrupt the formation of a complex at the MS-AP-1 site (Figs. 2, 4B and 4D). None of the remaining divergent AP-1 sites competed with the formation of a Fos-GCN4/MS-AP-1 at a 50 fold molar excess (data not shown). A second competition experiment was also quantified; the relative affinities of ZEBRA and Fos for the various oligonucleotides was found to be the same in both experiments (data not shown).

DISCUSSION

Binding Site Specificities of ZEBRA and Fos-GCN4. ZEBRA and Fos bind similar DNA sequences, and both ZEBRA expression and TPA treatment result in activation of EBV lytic viral DNA replication (Chevallier-Greco et al., 1986, Countryman and Miller, 1985, Gradoville et al., 1990, Taylor et al., 1989, zur Hausen et al., 1978). An underlying biological question is whether Fos, or other cellular AP-1 proteins, mediate TPA induction of the EBV lytic cycle. While this question has not been addressed directly, I have begun by assessing the binding affinities of ZEBRA and Fos-GCN4 for several sites in the BZLF1 and MS-EA promoters. I chose to compare ZEBRA binding to that of Fos-GCN4 since the first indication that ZEBRA was a DNA binding transactivator came from a recognition of the homology between the basic domains of ZEBRA and c-fos (Farrell et al., 1989). Although Fos generally binds in-vivo as a Fos/Jun heterodimer, an artificial Fos-GCN4 construct, containing the Fos basic domain fused to the GCN4 leucine zipper, was used in order to allow Fos to bind DNA as a homodimer. Many groups have previously shown that that the target site specificity of a protein is not affected by the nature of its leucine zipper (Kouzarides and Ziff, 1989, Nakabeppu and Nathans, 1989, Neuberg et al., 1989a and Sellers and Struhl, 1989).

ZEBRA bound a much broader range of DNA sequences than did Fos-GCN4 (Fig. 2). ZEBRA bound the palindromic AP-

1 site, but also bound the ZIIIA and ZIIIB sites which do not contain perfect dyads. Unexpectedly, oligonucleotides with base pair substitutions which disrupt the partial dyad nature of ZIIIA or ZIIIB (TGAGCCA to TGAGCAA or TTAGCAA to TGAGCAA) were bound by ZEBRA with increased affinity (Fig. 3, Fig. 5). This departs from the proposed "scissors-grip" model of DNA binding by dimeric proteins, in which each protein monomer is hypothesized to bind an identical DNA "half-site" (Vinson et al., 1989). Although it is known that ZEBRA (expressed in vitro or as a trpE fusion protein) binds DNA as a homodimer (Chang et al., 1990, Flemington and Speck, 1990c, Kolman et al., 1991, Lieberman and Berk, 1990, Taylor et al., 1991b), it is unclear whether both monomers contact or recognize symmetrical units on DNA. There are two possibilities which could explain ZEBRA binding to assymetric sites: first, only one subunit of the ZEBRA homodimer contacts DNA; or second, each subunit recognizes a wide variety of half-site sequences, thereby recognizing asymmetric sequences. Our preliminary data suggests that the second hypothesis accounts for ZEBRA's asymmetrical recognition of DNA (Kolman et al., 1991, Taylor et al., 1991b). It has also been found that the homodimeric glucocorticoid receptor binds DNA asymmetrically and is more sensitive to mutations in the right half of its binding site (reviewed in Beato, 1989).

Fos-GCN4 binds as a homodimer to the palindromic AP-1

site (Hope and Struhl, 1987, Kouzarides and Siff, 1989, Sellers and Struhl, 1989, Fig. 1). Both Fos/Jun heterodimers and artificial Fos homodimers have been shown to recognize the AP-1 consensus sequence asymmetrically (Risse et al., 1989). This suggests that the Fos basic domains in a homodimer interact with heptamer target sequences in a manner equivalent to a Fos/Jun heterodimer. A Fos-GCN4 homodimer did not bind any of the divergent AP-1 sites with high affinity. It was unable to recognize ZIIIA, ZIIIB, or ZIIIA*, all of which were recognized by ZEBRA homodimers (Figs. 2 and 5). Fos-GCN4 bound ZIIIB* (TGAGCAA) but not ZIIIA* even though both oligonucleotides contain the same heptamer core sequence. This result suggests that flanking nucleotides influence binding ability. ZIIIB*, but not ZIIIA*, contains the bases CA and TG adjacent to the recognition sequence increasing the dyad symmetry of the site, **CA**TGAGCAA**TG**. The importance of flanking sequences in the binding affinity of Fos was noted by Nakabeppu and Nathans (1989). They found that substitutions of cytosine (C) and guanosine (G) for adenine (A) and thymine (T) bases at flanking positions 1 and 10 of the cyclic AMP response element resulted in greater than a 90% decrease of homodimeric Fos or Fos/Jun binding activity (Nakabeppu and Nathans, 1989). This novel result indicates that the binding site specificity of Fos is broader than originally proposed; Fos-GCN4 can bind an asymmetric heptamer sequence

with two mismatches from the AP-1 consensus in an appropriate flanking oligonucleotide environment.

While ZEBRA has a broader specificity than Fos-GCN4 in recognizing various heptamer sequences related to the AP-1 consensus, only Fos-GCN4 binds the Z-AP-1 octamer sequence (TGACATCA) located in the BZLF1 promoter with high affinity. This is interesting since this site was originally defined as the Jun/AP-1 response element in the jun promoter and is TPA responsive (Angel et al., 1988b). Thus, ZEBRA is unable to bind a subset of the known AP-1/TPA responsive elements.

Fos/Jun as well as artificial Fos homodimers are known to bind the octamer cAMP response element (CRE) (TGACGTCA) which differs from Z-AP-1 octamer at the 5th position (G to A) (Chang et al., 1990, Flemington and Speck, 199a, Nakabeppu and Nathans, 1989, Nakabeppu et al., 1988, Risse et al., 1989, Sellers and Struhl, 1989). While the sites are similar, Deutsch et al. have shown that they have different signal-responsive properties, that is, CREs are not responsive to TPA (Deutsch et al., 1988). Chang et al. have shown that ZEBRA does not bind a CRE element (Chang et al., 1990). Although ZEBRA does not bind the TPA responsive Z-AP-1 octamer site, binding to this site may be important in the induction of the EBV lytic cascade. For example, Fos or another cellular AP-1 protein may bind this site in vivo (Angel et al., 1988b, Angel et al., 1987, Deutsch et al., 1988, Lee et al., 1987) leading to low levels of transcrip-

tion from the BZLF1 promoter. ZEBRA could then bind to the ZIIIA and ZIIIB sites in the BZLF1 promoter resulting in autoactivaton of this gene (Flemington and Speck, 1990b). **Binding of ZEBRA and Fos-GCN4 to an Oligonucleotide with Two Binding Sites.** We studied ZEBRA and Fos-GCN4 binding to the Double oligonucleotide in which the ZIIIA and ZIIIB heptamers occur as they are arranged in the BZLF1 promoter: oriented head to tail and separated by six base pairs. Two ZEBRA response elements, ZRE1 (TGTGTAA) and ZRE2 (TGAGCAA) (identical to the heptamer core sequence in the ZIIIA* and ZIIIB*), are arranged in a similar manner, separated by 10 bp in ori lyt (Lieberman et al., 1990). This arrangement may be a common mode of targetting ZEBRA transcriptional control in the EBV genome.

These results show that two ZEBRA homodimers can bind ZIIIA and ZIIIB simultaneously on the Double oligonucleotide (Fig. 1). We have not determined which site is filled initially although footprinting data has demonstrated that ZEBRA binds ZIIIB with higher affinity than ZIIIA (Flemington et al., 1990b, Lieberman and Berk, 1990). Gel shift experiments with limiting dilutions of ZEBRA protein show that both sites are bound only after a significant portion of single sites are filled. Furthermore, competition experiments indicate that ZEBRA does not have a greater affinity for the Double oligonucleotide than for either single site (Figs. 4A and 4C). Thus, ZEBRA binds

non-cooperatively to these sites. However, binding of ZEBRA to these two adjacent sites may allow for synergistic transactivation.

Fos-GCN4, which did not bind oligonucleotides bearing single ZIIIA or ZIIIB sites, bound the Double oligonucleotide (containing ZIIIA and ZIIIB) as a high molecular weight complex (Figs. 1 & 2). The association of this slow mobility complex can be disrupted by addition of ZEBRA protein suggesting that the two proteins compete for a similar sequence on the Double probe (unpublished data). However, the Double oligonucleotide did not interfere with Fos-GCN4/MS-AP-1 complex formation (Figs. 4B and D). One explanation for this phenomenon is that these associations are non-competitive because the two probes bind different Fos-GCN4 complexes. The MS-AP-1 probe is bound by a Fos-GCN4 homodimer whereas the Double probe may only bind a Fos-GCN4 complex with more than two monomers and perhaps with other proteins. Alternatively, Fos-GCN4 might bind the Double oligonucleotide indirectly through a second protein which is not required for binding to the MS-AP-1 site.

Several groups have recently examined ZEBRA binding activity in EBV promoters and the origin of EBV lytic replication (ori lyt) (Farrell et al., 1989, Flemington and Speck, 1990b, Hardwick et al., 1988, Kenney et al., 1988, Lieberman and Beerk, 1990, Lieberman et al., 1990, Packham et al., 1990, Urier et al., 1989) but there had been no

studies directly comparing the ability of Fos and ZEBRA to bind the same sites. Since our work was completed Chang et al. (1990) reported on the binding specificities of ZEBRA and Fos/Jun in ori lyt. Our work on the binding specificities of ZEBRA and Fos for sites in the BZLF1 and MS-EA promoters provides both complementary and novel observations. Chang et al. demonstrated that ZEBRA binds an AP-1 consensus site with higher affinity than divergent heptamer sites in ori lyt, whereas we have found that it binds equivalently to an AP-1 site as well as a divergent sequence (ZIIIA* or ZIIIB*) (Chang et al., 1990). ZIIIA* and ZIIIB* contain the same core heptamer sequence found in ZRE2 in ori lyt (Chang et al., 190). The two studies show that Fos/Jun and Fos-GCN4 are restrictive in binding divergent AP-1 heptamer sites. However, we have found novel binding of a Fos-GCN4 homodimer to a divergent heptamer sequence flanked by dyad symmetry (ZIIIB*).

The interaction of ZEBRA with cellular AP-1 sequences may result in the displacement of Fos or other cellular AP-1 factors, thereby precluding their activity. Alternatively, by binding AP-1 sites, ZEBRA may transactivate (or possibly transrepress) cellular genes in a manner analogous with cellular AP-1 transcription factors. AP-1 proteins are probably not able to activate transcription of EBV genes through ZEBRA responsive elements because at least one AP-1 binding protein, Fos-GCN4, is unable to bind those sites.

The divergent nature of the ZEBRA responsive sites makes it unlikely that cellular AP-1 proteins can have an ongoing role promoting viral transcription and activating the EBV lytic cycle. Additionally, the ability of Fos to bind the TPA responsive Z-AP-1 octamer and CRE sites, which are not bound by ZEBRA, indicates that ZEBRA can not simply substitute for Fos. The ability of these related proteins to bind similar as well as distinct sites adds yet another level of complexity to the relationship of these transactivators in regulating cellular and viral gene expression.

Figure 1. Binding specificity of ZEBRA and Fos-GCN4 for AP-1 and BZLF1 promoter sequences (Z_p). (A) Sequences of the ZEBRA (BZLF1) and MS (BSLF2-BMLF1) promoters. AP-1 like sequences are underlined (Farrell et al., 1989, Flemington and Speck,1990b) and the transcriptional start site of the BZLF1 gene is marked. (B) Gel retardation assay showing the specificities of trpE (vector), trpE-ZEBRA (Z_{1-245}) and trpE-Fos-GCN4 (FOS-GCN4) proteins for oligonucleotides containing the AP-1 and Z_p sequences; Z-AP-1 octamer, ZIIIA, and ZIIIB (Flemington and Speck, 1990b). The Double oligonucleotide contains sites ZIIIA and ZIIIB in the orientation found in the BZLF1 promoter. Equivalent amounts of bacterially expressed fusion proteins were incubated with each oligonucleotide (4 X 10^{-13}M) for thirty minutes prior to analysis of the retardation complexes by gel electrophoresis.

A

ZEBRA (BZLF1) Promoter

```
          .          . ZIIIA    .   ZIIIB .          .
-140  GAAACTATGCATGAGCCACAGGCATTGCTAATGTACCTCATAGACACACC
      CTTTGATACGTACTCGGTGTCCGTAACGATTACATGGAGTATCTGTGTGG

          .          .          . Z-AP-1 OCTAMER     .
-90   TAAATTTAGCACGTCCCAAACCATGACATCACAGAGGAGGCTGCTGCTT
      ATTTAAATCGTGCAGGGTTTGGTACTGTAGTGTCTCCTCCGACGACGAA

          .          .          .          .          .
-40   GGCTTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGCACCTT
      CCGAAATTTCCCCTCTACAATCTGTCCATTGAGTGATTTGTAACGTGGAA
```

MS-EA (BSLF2-BMLF1) Promoter

```
          .          . MS-AP-1.          .          .
-120  ACGGTCACCTTCATGAGTCAGTGCTTCGCCGGTCGCTGTGGGGCCAATCA
      TGCCAGTGGAAGTACTCAGTCACGAAGCGGCCAGCGACACCCCGGTTAGT
```

B

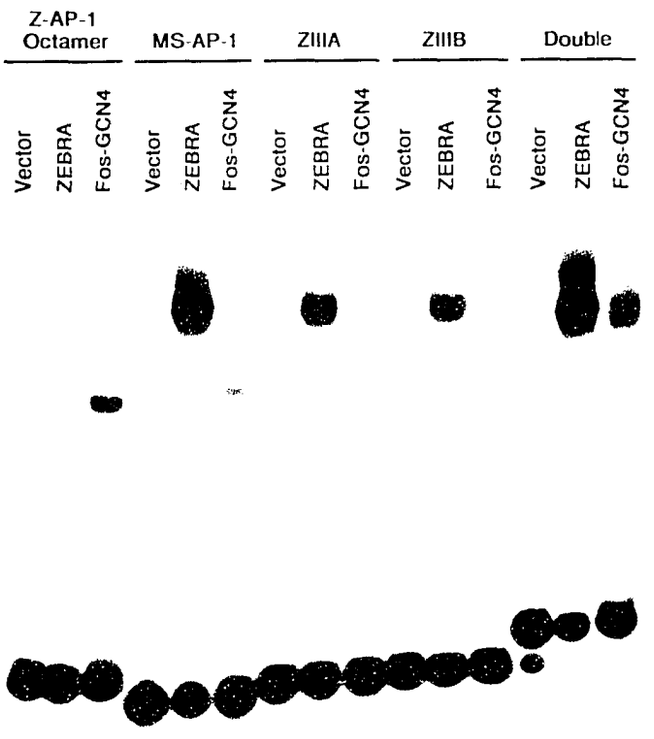


Figure 2. Binding of ZEBRA and Fos-GCN4 to the Double oligonucleotide. ZEBRA and Fos-GCN4 proteins were titrated in a gel retardation assay with 4 X 10^{-13}M ^{32}P-labeled MS-AP-1 or Double (ZIIIA + ZIIIB) probe. Various volumes of protein extract (shown as ul in the figure) were incubated with the MS-AP-1 or Double probe in the presence of poly dIdC. ZEBRA and Fos-GCN4 both bind to the MS-AP-1 site as a single complex indicated as **S**. Mobility shift complexes with a slower mobility are indicated as **D**.

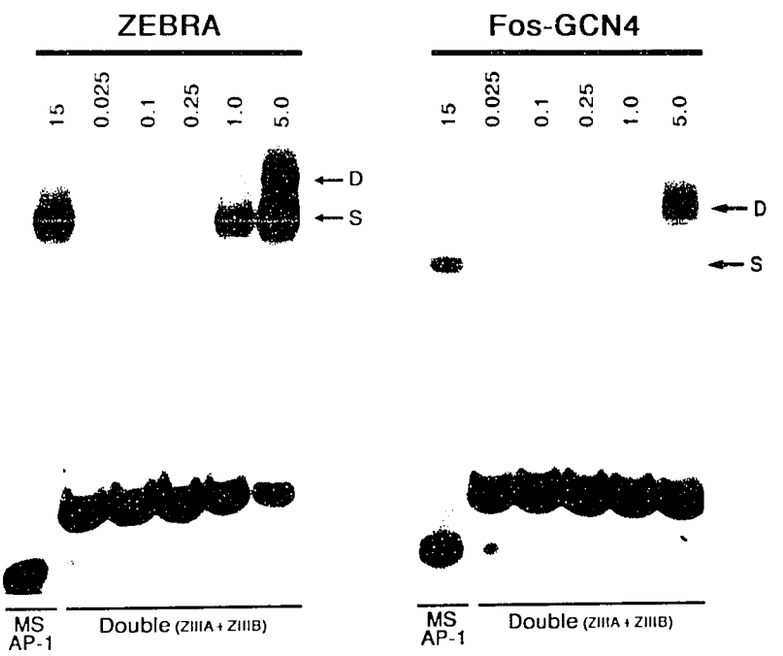
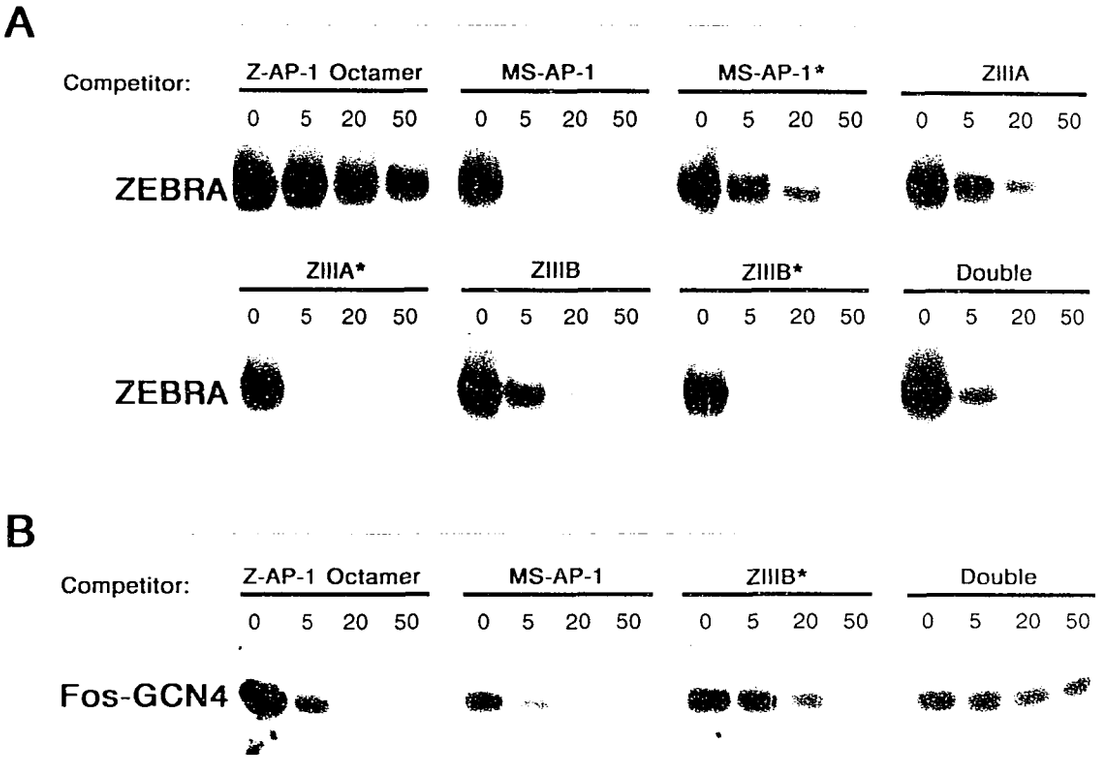


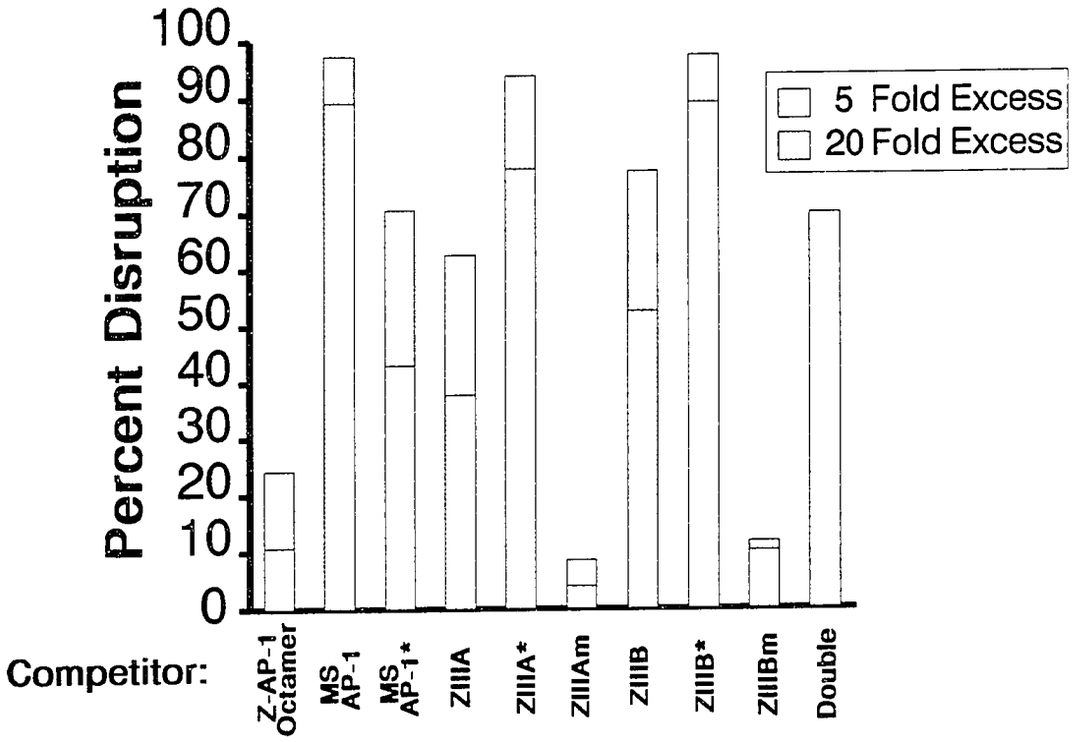
Figure 3. Gel retardation assay illustrating the binding specificities of ZEBRA and Fos-GCN4 for mutant recognition sequences. MS-AP-1, ZIIIA, and ZIIIB oligonucleotides were mutated at a single position (noted by a *); the base substitution is underlined. Two oligonucleotides mutated at multiple positions are designated ZIIIAm and ZIIIBm. The control oligonucleotide contains no AP-1 related sequences. Bacterially expressed trpE-ZEBRA (A) or trpE-Fos-GCN4 (B) was incubated with these ^{32}P-labeled oligonucleotides and binding was analyzed by a mobility shift assay.

Figure 4. Competition experiment to evaluate the affinity of ZEBRA and Fos-GCN4 for AP-1 and ZEBRA promoter sites. 4 X 10^{-13}M ^{32}P- labeled MS-AP-1 oligonucleotide was incubated with bacterially expressed trpE-ZEBRA (A) or trpE-Fos-GCN4 (B) in the presence of three concentrations of cold competitor DNA. Complex formation was resolved by gel electrophoresis. Only the shifted complexes are shown and the nature of the competitor and its molar excess over the MS-AP-1 probe is indicated. (C) These results were quantified by scanning the relative intensity of each shifted complex. The percent disruption of the ZEBRA/MS-AP-1 complex is plotted for each oligonucleotide competitor. The stippled portion of each bar represents the percent disruption with a 5 fold molar excess of oligonucleotide competitor, while the shaded area represents the additional disruption with a 20 fold molar excess of competitor. (D) Results were quantified as in (C) for disruption of the Fos-GCN4/MS-AP-1 complex.



C

Oligonucleotide competition of ZEBRA/MS-AP-1 Complexes



D

Oligonucleotide Competition of Fos-GCN4/MS-AP-1 Complex

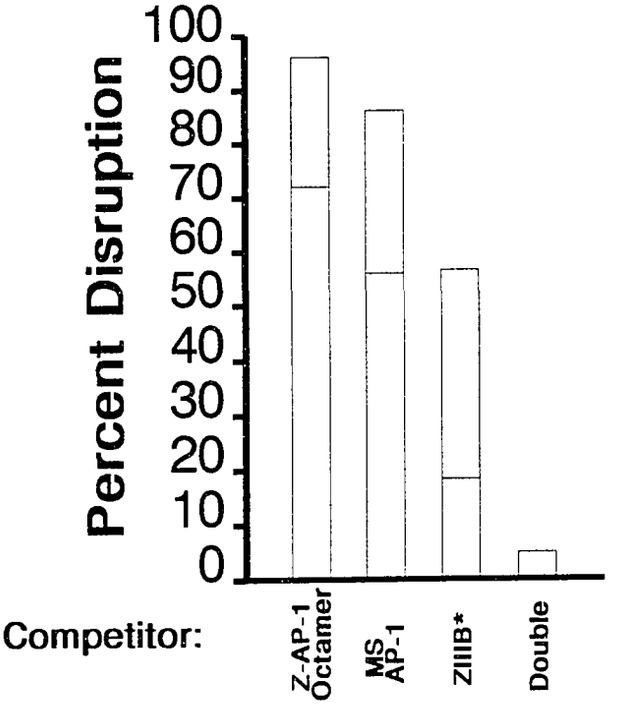


Figure 5. Evaluation of ZEBRA and Fos-GCN4 binding site affinities. Affinities were determined from the competition analysis in Fig. 4. Nucleotide substitutions in the recognition sequences are noted by bold type. The relative affinities are marked with 1 representing the highest affinity while a dash (-) indicates that the protein did not bind the sequence specifically.

Affinities of ZEBRA and Fos/GCN4 for AP-1 and Related Sequences

Name	*Promoter*	*Oligonucleotide*	ZEBRA	Fos/GCN4
MS-AP-1	BSLF2	TCTTCATGAGTCAGTGCTTC	1	2
MS-AP-1*	—	TCTTCATTAGTCAGTGCTTC	2	–
Z-AP-1 (Octamer)	BZLF1	CTAGAAACCATGACATCACAGAGGATC	3	1
ZIIIA	BZLF1	CTAGCTATGCATGAGCCACAGATC	2	–
ZIIIA*	—	CTAGCTATGCATGAGCAACAGATC	1	–
ZIIIB	BZLF1	CTAGCAGGCATTGCTAATGTACCGATC	2	–
ZIIIB*	—	CTAGCAGGCATTGCTCATGTACCGATC	1	3

CHAPTER V

THE UNIQUE DNA BINDING ACTIVITY OF ZEBRA IS INHIBITED BY CASEIN KINASE II PHOSHPORYLATION

INTRODUCTION

I have recently studied the DNA binding activity of the Epstein-Barr viral replication activator, ZEBRA (Taylor et al., 1991a). This protein has sequence homology to the Fos/Jun family in its basic domain (exon II) and shares the ability of these proteins to bind an AP-1 consensus site (Farrell et al., 1989). However, ZEBRA is also able to bind divergent AP-1 sites not bound by Fos or Jun (Chang et al., 1990, Flemington and Speck, 1990b, Lieberman et al., 1990, Packham et al., 1990, Taylor et al., 1991, Urier et al., 1989).

Both ZEBRA and Fos contain potential casein kinase II (CKII) phosphorylation sites directly upstream of their basic DNA binding domains (Fig. 1). Casein kinase II is a ubiquitous protein kinase present in both the cytoplasm and nucleus of eukaryotic cells (Hathaway and Traugh, 1982, Edelman et al., 1987). Studies using synthetic peptides have shown that CKII preferentially phosphorylates serine or threonine residues situated 5′ to clusters of acidic amino acids (Marin et al., 1986, Kuenzel et al., 1987). CKII also appears to selectively phosphorylate residues adjacent to

predicted B-turns (Hathaway and Tragh, 1982).

CKII activity is stimulated by mitogens such as serum, TPA, insulin, insulin-like growth factor and epidermal growth factor (Ackerman and Osheroff, 1989, Carroll and Marshak, 1989, Carroll et al., 1988, Karlund and Czech, 1988, Sommercorn et al., 1987). This suggests that CKII may play a role in the regulation of cell growth. Additionally, it has been determined that a large number of nuclear oncoproteins satisfy the substrate requirements for casein kinase II (CKII) phosporylation (Carroll et al., 1988, Krebs et al., 1988). The SV40 large T antigen, P53 tumor suppressor protein, HPV E7 oncoprotein, c-Myc, c-Myb and serum response factor (SRF) have all been shown to be specifically phosphorylated by CKII (Barbosa et al., 1990, Carroll et al., 1988, Grasser et al., 1988, Krebs et al., 1988, Luscher et al., 1990, 1989, Manak et al., 1990, Meek et al., 1990).

While the physiological role of kinases has not been definitively determined for any system, they have been implicated in the regulation of various protein functions. CKII has recently been shown to uniquely affect DNA binding of two unrelated proteins. CKII phosphorylation inhibits DNA binding by the c-myb oncogene but enhances the binding activity of the serum response factor, a factor which mediates Fos transcription (Luscher et al., 1990, Manak et al., 1990). Phosphorylation of a protein with an acidic

activator domain, such as that present in GAL4 or GCN4, has been hypothesized to enhance transcriptional activity by increasing net negative charge (Carroll et al., 1988, Hope and Struhl, 1988, Ma and Ptashne, 1987, and Ptashne, 1988). Both GAL4 and GCN4 contain potential CKII sites within the acidic domain (Carroll et al., 1988). Although this hypothesis has not been proven, protein phosphorylation has been shown to upregulate transcriptional activation of CREB and the yeast heat shock factor as well as DNA replication by SV40 (Sorger and Pelham, 1988, Virshup et al., 1989, Yamamoto et al., 1988, Dwarki et al., 1990, reviewed, Edelman et al., 1987).

Thus, it was of interest to determine whether the potential CKII acceptor sites found within the acidic domains of ZEBRA and the related Fos protein are phosphorylated. I have determined that only ZEBRA is phosphorylated by CKII in-vitro. Additionally, I have found that CKII specific phosphorylation of ZEBRA results in a reversible inhibition of DNA binding.

RESULTS

CKII Differentially Affects ZEBRA and Fos Phosphorylation. A TrpE-ZEBRA fusion protein as well as purified ZEBRA protein (expressed in E.coli) were used to study the ability of CKII to specifically phosphorylate the ZEBRA protein (Carey et al., 1991, Taylor et al., 1991a). These proteins were incubated with purified CKII and [γ-^{32}P]ATP and analyzed by autoradiography following SDS-polyacrylamide gel electrophoresis (Carroll et al., 1988, Carroll and Marshak, 1988). Labelled phosphate was transferred to a casein substrate as well as to the TrpE-ZEBRA and purified ZEBRA proteins (Fig. 2). However, a control E. coli extract with trpE protein was not phosphorylated by CKII (Fig. 2). Additionally, labeled phosphate was not incorporated in the ZEBRA protein when [γ-^{32}P]ATP was added without CKII (data not shown).

Since ZEBRA has been hypothesized to be related to the Fos oncogene protein and the two proteins have related DNA binding specificities, we were interested in determining whether the two proteins may be similarly modified by kinase activity. Both proteins contain potential CKII sites in similar positions, directly 5′ to the basic DNA binding domain (Fig. 1) (Carroll et al., 1988, Farrell et al., 1989, Taylor et al., 1991a). CKII phosphorylation of a trpE-Fos-GCN4 fusion protein was studied. This fusion protein contains the basic DNA binding domain of Fos (aa 126-162,

which includes the potential CKII phosphorylation site) fused in frame to the GCN4 leucine zipper. It has previously been shown that the GCN4 leucine zipper does not affect the binding specificity of the Fos protein but allows it to bind DNA as a homodimer (Abate et al., 1990, Kouzarides and Ziff, 1989, Nakabeppu and Nathans, 1989, Sellers and Struhl, 1989). Unlike ZEBRA, this protein was not phosphorylated by CKII (Fig. 3). Additionally, a TrpE-Fos fusion protein containing only Fos amino acids 126-162 was not phosphorylated (data not shown).

Mapping the CKII Specific Phosphorylation Sites in ZEBRA. A panel of ZEBRA deletion mutants were utilized to map the CKII specific phosphorylation sites (Taylor et al., 1991a). These proteins, which were all expressed as TrpE fusion proteins in E. coli, were incubated with purified CKII plus [γ-^{32}P]ATP and analyzed by autoradiography following polyacrylamide electrophoresis (Fig. 3, Taylor et al., 1991). The smallest ZEBRA deletion proteins which could be phosphorylated by CKII were $Z_{141-245}$, $Z_{\Delta 111-159}$, Z_{1-198}, and $Z_{168-202}$ localizing the CKII phosphorylation region to amino acids 160-198 (Fig. 3 and data not shown). The two serines which are potentially phosphorylated in this region are S-167 and S-173 (refer to Fig. 1). Although the ZEBRA deletion protein, $Z_{172-245}$, was not phosphorylated in this assay, $Z_{168-202}$ was a substrate, suggesting that S-173 is an acceptor site for CKII phosphorylation but requires a few of the upstream

acidic residues for its phosphorylation. S-167, if it were an acceptor site, would not be expected to be phosphorylated in the ZEBRA deletion mutant WZ_{1-167}. CKII phosphorylation requires the presence of acidic residues downstream of the acceptor site (i.e. aa 168-176) not present in this construct (Fig. 3) (Marin et al., 1986, Kuenzel et al., 1987).

CKII Phosphorylation Inhibits ZEBRA Specific DNA Binding. ZEBRA has previously been shown to bind a consensus AP-1 sequence as well as divergent heptamer sites (Chang et al., 1990, Farrell et al., 1989, Flemington and Speck, 1990b, Liberman et al., 1990, Packham et al., 1990, Taylor et al., 1991a, Urier et al., 1989). We analyzed the effects of CKII phosphorylation on ZEBRA's ability to bind AP-1 as well as divergent sites. Purified ZEBRA protein was preincubated with CKII and ATP and then exposed to the ^{32}P-labeled AP-1 oligonucleotide probe. DNA binding was assayed by determining the level of retarded complex in a mobility shift gel. Untreated ZEBRA protein bound the AP-1 oligonucleotide with high affinity as did ZEBRA extracts preincubated with only CKII or ATP (Fig. 4). However, binding to the AP-1 oligonucleotide was almost completely inhibited when ZEBRA protein was preincubated with both ATP and CKII (Fig. 4). CKII phosphorylation also inhibited ZEBRA binding to divergent AP-1 sites located in the ZEBRA promoter (ZIIIA, ZIIIB, and AP-1-octamer) (data not shown,

refer to Experimental Procedures or Taylor et al., 1991, for sequences of oligonucleotides).

It has previously been shown that protein phosphatase 2A (PP2A) can reverse CKII phosphorylation (Luscher et al., 1990, reviewed Cohen and Cohen, 1989). Therefore, following a 15 minute preincubation of ZEBRA with ATP and CKII, extracts were treated with PP2A for an additional 15 minutes. When ZEBRA from these extracts was analyzed for DNA binding activity, a partial recovery of DNA binding activity was obtained (Fig.4). This result suggests that phosphorylation of the ZEBRA protein does not irreversibly inhibit its DNA binding activity.

DISCUSSION

CKII Phosphorylation of ZEBRA. We have demonstrated that ZEBRA serves as a substrate for CKII phosphorylation in-vitro. ZEBRA contains two potential CKII acceptor sites at serine-167 and serine-173. ZEBRA deletion mutants have been used to show that these serines are contained within the region phosphorylated by CKII (aa160-198). The data presented here suggest that serine-173, and possibly S-167, are phosphorylated by CKII (Fig. 3). Further biochemical characterization including sequence analysis of two dimensional phosphopeptide maps will be conducted to confirm this result.

Although there is no data concerning the phosphorylation state of ZEBRA in-vivo, ZEBRA was specifically phosphorylated by CKII in-vitro. Another serine-threonine protein kinase, the cAMP dependent protein kinase (Slice and Taylor, 1989), was unable to phosphorylate the ZEBRA protein in-vitro (Taylor, unpublished data).

Interestingly, the expression and activity of CKII in-vivo is influenced by factors which also affect ZEBRA expression. ZEBRA, which triggers activation of the EBV lytic cycle, is activated by phorbol esters (zurHausen et al., 1978, Biggin et al., 1987, Laux et al., 1988). Similarly, CKII activity is elevated in homogenates of cells treated with various mitogens including the phorbol ester, TPA (Carroll et al., 1988). Alternatively, ZEBRA may be

phosphorylated in-vivo by the EB viral gene product, BGLF4, which has sequence homology with other serine/threonine kinases (Kolman, J.L. , personal communication, Smith and Smith, 1989).

CKII Phosphorylation of Fos. Fos, which has a potential CKII acceptor site (Carroll et al., 1988) in a region similar to ZEBRA, was not phosphorylated by CKII in-vitro (Fig. 3, Curran and Marshak, personal communication). Several potential CKII sites in c-myc were also not phosphorylated by CKII in-vitro (Luscher et al., 1989). Thus, CKII phosphorylation may not be determined solely by a protein's primary structure.

However, the c-Fos serine-133, which corresponds to S-167 in ZEBRA, is also conserved in Jun (S-249) and GCN4 leucine zipper proteins. Since this work was completed, it has been determined that c-Jun is phosphorylated in-vitro at S-249 as well as T-239 and S-243 by glycogen synthase kinase 3 (Boyle et al., 1991). Protein kinase C dephosphorylation of c-Jun at one or more of these sites resulted in increased DNA binding (Boyle et al., 1991). Therefore, phosphorylation, albeit by different kinases, may similarly affect ZEBRA and some of the related basic-leucine zipper proteins.

Effects of CKII Phosphorylation on ZEBRA DNA Binding. The effects of CKII phosphorylation on DNA binding have previously been studied for c-myb and the serum response

factor, SRF (Luscher et al., 1990, Manak et al., 1990). *In-vitro* DNA binding activity of SRF was enhanced by CKII phosphorylation, while binding of c-myb was inhibited by phosphorylation (Luscher et al., 1990, Manak et al., 1990). We have found that ZEBRA, like c-myb, is negatively regulated by CKII phosphorylation. CKII phosphorylation abrogated ZEBRA's ability to bind all of its target DNA sequences (Fig. 4 and data not shown).

While ZEBRA's ability to bind DNA is crucial for its role in transactivating early EB viral genes, the potential function of the ZEBRA protein in the late phase of lytic replication has not yet been studied (Flemington and Speck, 1990b, Kenney et al., 1989, Packham et al, 1990, Rooney et al., 1989, Urier et al., 1989). Our data suggests that ZEBRA is a very stable protein with a half life of greater than 48 hours (Taylor and Hagmeier, unpublished results, refer to chapter II). Additionally, it has recently been shown that ZEBRA associates with a late EB viral protein (Katz et al., manuscript in preparation). Therefore, regulation of ZEBRA's ability to bind DNA may be required during the late phase of EB viral replication if the protein serves additional functions in the lytic cascade.

Effects of CKII Phosphorylation on ZEBRA Transactivation. CKII phosphorylation has been hypothesized to increase the level of transactivation by acidic activators by increasing their net negative charge (Carroll et al., 1988, reviewed

Ptashne, 1988). Phosphorylation by protein kinase A and other serine/threonine kinases has been shown to increase the transcriptional activity of CREB and yeast heat shock factor (Yamamoto et al., 1988, Sorger and Pelham, 1988).

However, it is difficult to explain how CKII phosphorylation could increase transcriptional activation in the case of ZEBRA or c-myb. Since phosphorylation of these two proteins inhibits their DNA binding activity, one would have to invoke a model in which ZEBRA or c-myb transactivates transcription as a DNA binding protein and/or as a soluble factor. It is of interest to note that v-myb has been reported to transactivate certain promoters (e.g., human hsp70) in the absence of its DNA binding domain (Klempnauer et al., 1989). Alternatively, phosphorylated ZEBRA may interact with a second DNA binding factor, thereby activating transcription at novel sequence elements. The extent of phosphorylation of the herpes simmplex virus gene product, ICP4, has been shown to modulate its ability to form complexes on specific DNA sequences (Papavassiliou et al., 1991).

The activation domains of the ZEBRA and myb proteins have not been definitively determined. It is possible that they do not contain acidic activators and that CKII phosphorylation affects DNA binding but not transactivation. This hypothesis is presently being studied by using ZEBRA proteins mutated at serine-167 in transactivation assays

(Kolman,J.L. personal communication).

Effects of CKII Phosphorylation on ZEBRA Nuclear Localization. Recent evidence suggests that CKII phosphorylation may also affect the relocalization of proteins between the cytoplasm and nucleus (Rihs et al., 1991). Rihs and coworkers have shown that the rate of nuclear transport of SV40 large T antigen was affected by mutagenesis of two serines which are phosphorylated by CKII in-vitro (Grasser et al., 1988, Rih et al., 1991). Although the SV40 T antigen nuclear localization signal (NLS) was necessary and sufficient for nuclear localization, wild type large T-antigen accumulated in the nucleus after 30 minutes while the mutant protein required 12 hours to achieve the same degree of partition (Rih et al., 1991).

The CKII acceptor site in SV40 large T antigen is located 13 aa 5' to the basic NLS (Rih et al., 1991). Potential CKII sites are located approximately 15 amino acids upstream of many proteins harboring nuclear localization sequences, including polyoma T, adenovirus E1a, c-myc, p53 and c-rel (Rih et al., 1991). Most of these proteins have not yet been tested for CKII phosphorylation. However, ZEBRA fits the CKII site/NLS motif well. The ZEBRA NLS has recently been localized to a region within the basic domain of the protein (Giot et al., 1991), located immediately downstream of the CKII phosphorylation site. The effects of CKII on the rate of nuclear transport of ZEBRA

may be another mechanism by which phosphorylation regulates ZEBRA's role in the EB lytic cycle.

Figure 1. Schematic representation of ZEBRA and Fos. (A) Several structural domains in both proteins are indicated: potential casein kinase II phosphorylation sites in both ZEBRA and Fos (denoted CKII); the basic domains of both proteins; the coiled-coil domain in ZEBRA and the leucine zipper motif in Fos. (B) Amino acid sequences of the potential CKII phosphorylation sites and basic domain of ZEBRA and Fos (Biggin et al., 1987, van Straaten et al., 1983). The serines which are possible acceptor sites for CKII phosphorylation are indicated in bold type (**S**). Conserved residues in the ZEBRA and Fos basic domains are marked by lines (Farrell et al., 1989).

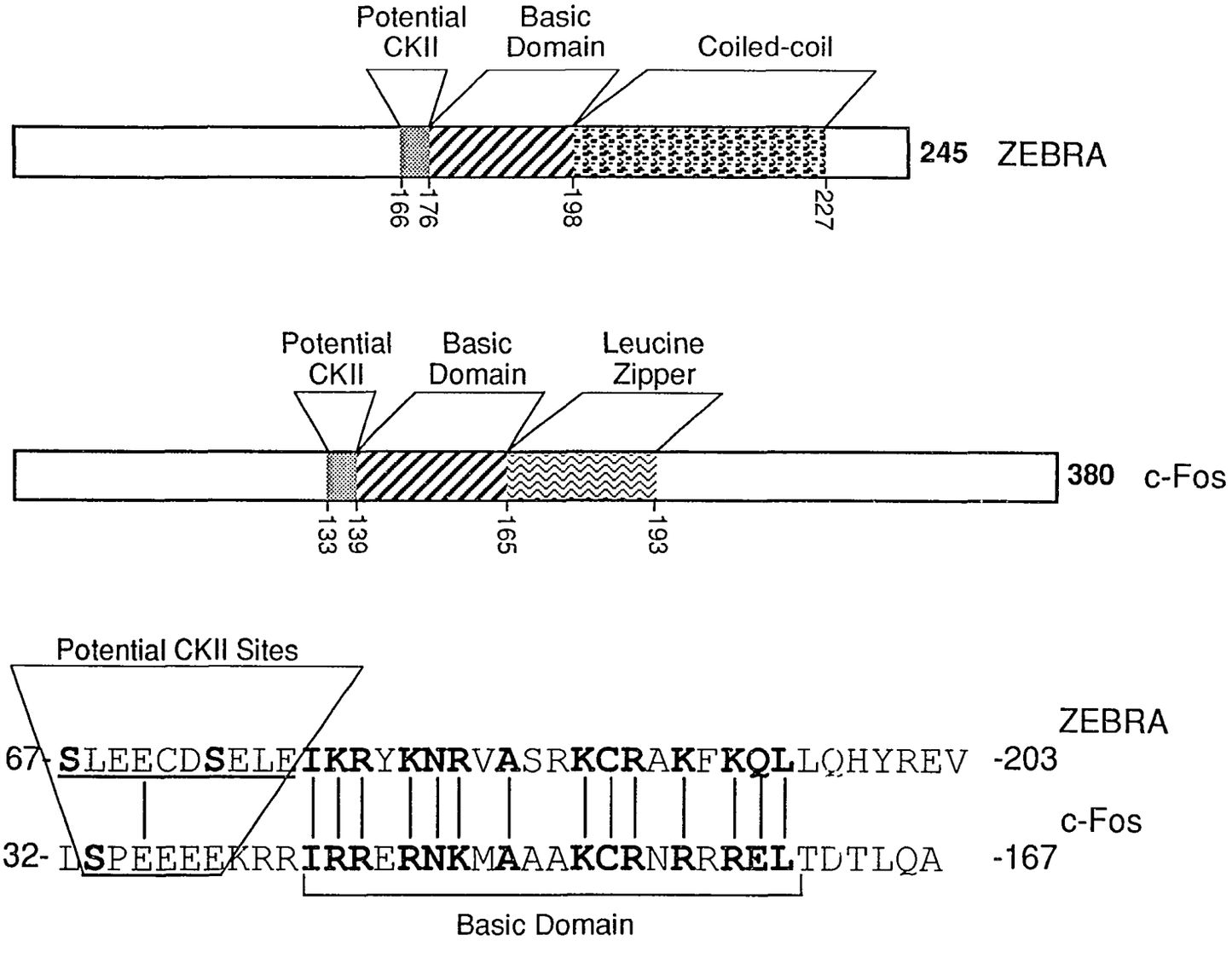
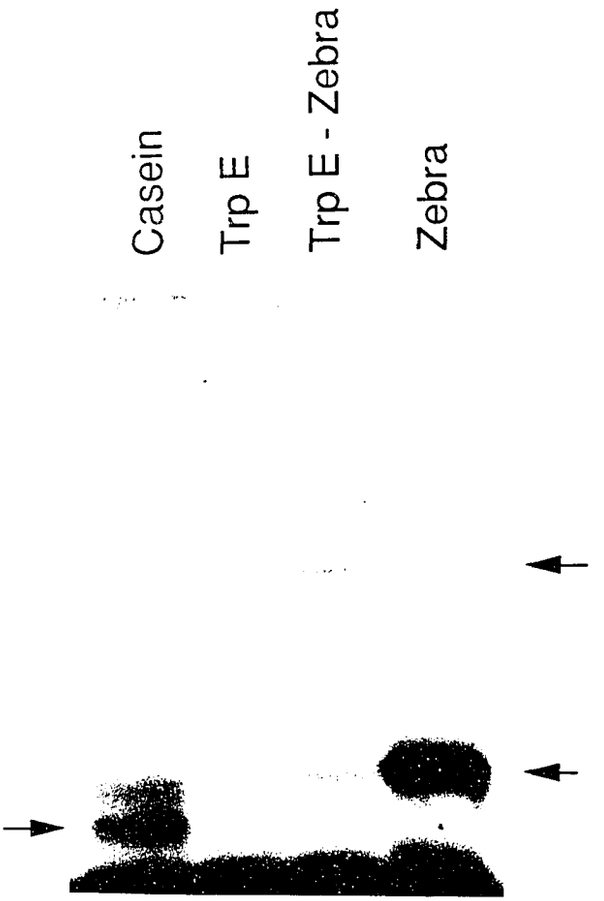


Figure 2. Phosphorylation of ZEBRA by casein kinase II. ZEBRA protein purified from E. coli (Carey et al., 1991) or expressed as a TrpE fusion protein was incubated with CKII in the presence of [γ-^{32}P]ATP at 37C for 15 minutes. Proteins were then electrophoresed on an 8% SDS-polyacrylamide gel and analyzed by autoradiography. Phosphorylation of casein was used as a positive control and trpE protein was used as a negative control.



CK II Phosphorylation

Figure 3. Mapping the casein kinase II phosphorylation site in ZEBRA and Fos. (A) Various ZEBRA deletion mutants were used to map the sites phosphorylated by CKII in ZEBRA. Total protein from bacteria which carried various TrpE-ZEBRA deletion proteins was resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. (B) Protein extracts from each of the ZEBRA deletion mutants as well as a Fos-GCN4 construct was incubated with CKII in the presence of [γ-^{32}P]ATP at 37C for 15 minutes. Proteins were then electrophoresed on an 8% SDS-polyacrylamide gel and analyzed by autoradiography. These mutants localize the CKII phosphorylation site(s) in ZEBRA can to amino acids 160-198. A Fos-GCN4 construct containing the potential Fos CKII site was not phosphorylated in vitro.

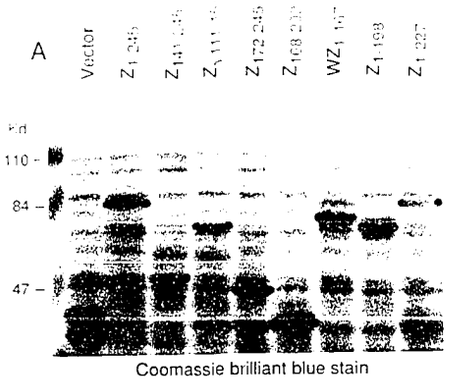
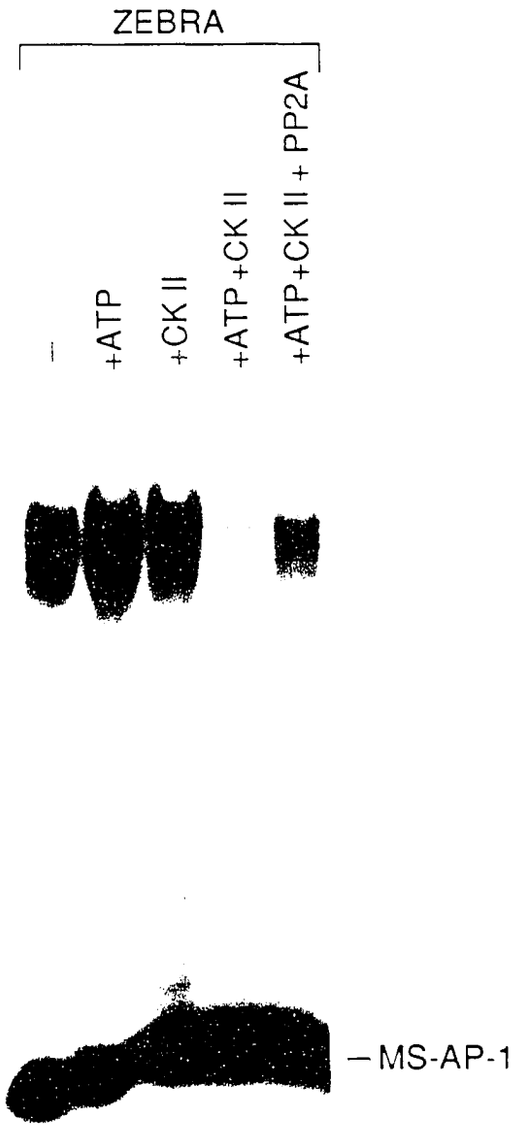


Figure 4. Phosphorylation of ZEBRA by CKII inhibits DNA binding. Purified ZEBRA protein was preincubated with CKII, ATP, or CKII + ATP for 15 minutes at 37C. Protein phosphatase 2A (PP2A) was added for 15 minutes following pretreatment with CKII + ATP. All extracts were then incubated with a ^{32}P-labeled oligonucleotide containing the AP-1 consensus sequence for 20 minutes at room temperature (22C). Complexes were electrophoresed on a 4% acrylamide gel in 0.5X TBE.



CHAPTER VI

CONCLUSIONS

Activation of the Latent EBV Genome. The discovery that ZEBRA induces lytic EB viral DNA replication suggested that it may function as a transactivator of lytic genes (Countryman et al., 1985, 1987, Gradoville et al., 1990, Rooney et al., 1988, Takada et al., 1986). Several groups have shown that promoters in the EB origin of lytic replication (orilyt) and several early genes: BZLF1, BRLF1 (R), BMLF1 (MS-EA) and BMRF1 (EA-D) are responsive to ZEBRA activation (Chavrier et al., 1989, Chevallier-Greco et al., 1986, Flemington et al., 1990b, Gradoville et al., 1990, Kenney et al., 1989a, Lieberman et al., 1989, Sinclair et al., 1991, Takada et al., 1986, Urier et al., 1989). However, ZEBRA expression is not sufficient to induce release of infectious EB virions in Raji cells or a tightly latenty X50-7.17 subclone (Gradoville et al., 1990, reviewed in Miller, 1990b). This suggests that although ZEBRA can directly activate a group of EBV early genes, additional factors are required for late gene expression and virion production.

A second EBV transactivator encoded by the BRLF1 open reading frame (termed R) has been identified (reviewed in Hayward and Hardwick, 1991). This protein is also expressed early in the lytic cycle, but is incapable of inducing viral

replication in the absence of ZEBRA (Cox et al., 1990). The R transactivator is encoded as a bicistronic mRNA; the complete BRLF1 open reading frame is encoded together with the downstream BZLF1 sequences (Biggin et al., 1987, Manet et al., 1989). R transactivates expression from the EBV origin of lytic replication (orilyt), the BMLF1 early gene promoter, the HIV-1 long terminal repeat (LTR) and positively regulates its own expression (BRLF1 promoter) (Chevalier-Greco et al., 1989, Cox et al., 1990, Hammerschmidt and Sugden, 1988, Hardwick et al., 1988, Kenney et al., 1989b, Quinlivan et al., 1990, Sinclair et al., 1991). Recent evidence suggests that R and Z may act synergistically to activate transcription of a subset of EBV early genes (Cox et al., 1990, Giot et al., 1991, Kenney et al., 1989b). Furthermore, R has been shown to be one of the factors necessary for ZEBRA activation in EBV negative cells (Kenney et al., 1989b).

Transcription of ZEBRA. The work presented in chapter II indicates that ZEBRA expression is differentially regulated in standard and defective EB viruses. Novel upstream and downstream sequences present in het DNA are responsible for activation of ZEBRA expression (Fig I-2, Rooney et al., 1988). Two BZLF1 specific mRNAs, 3.5 Kb and 0.9 Kb, are spontaneously expressed in cells harboring a het virus but are only synthesized by the standard latent virus after chemical induction (Fig II-10, Taylor et al., 1989).

Initiation of the two transcripts has been mapped to the BRLF1 and BZLF1 promoters, respectively (Manet et al., 1989, Biggin et al., 1987). The 3.5 Kb mRNA represents the bicistronic message encoding both the R and ZEBRA transactivators (Fig. II-11, Biggin et al., 1987, Manet et al., 1989).

It is interesting to note that no novel BZLF1 mRNAs were detected in cells (clone 5) harboring the het virus (Fig. II-10, II-15). The 0.9 Kb BZLF1 specific mRNA can be transcribed by either the het or standard EB genome, whereas the 3.5 Kb message can only be transcribed by the standard virus. The rearrangements in HR-1 clone 5 het DNA result in a deletion of most of the BRLF1 open reading frame (refer to Fig. I-II). The kinetics of transcription and decay of both mRNAs were identical in clone 5 cells (Fig. II-15, Taylor and Hagmeyer, unpublished data). Thus, even when ZEBRA expression is aberrantly regulated in the defective genome, transcription of BRLF1 and BZLF1 are closely linked. Coordinate expression of these two mRNAs may be mediated by different mechanisms since Sinclair et al. (1991) recently showed that the BZLF1 promoter is responsive to TPA while the BRLF1 promoter is not. Alternatively, stimulation of transcription at both the BZLF1 and BRLF1 promoters may be mediated by low levels of the ZEBRA transactivator (Flemington and Speck, 1991b). WZhet may spontaneously produce enough het ZEBRA to activate the standard promoters

in clone 5 cells. TPA, on the other hand, activates endogenous AP-1 transcription factors (Angel et al., 1987, Lee et al., 1987) which may induce transcription of the standard BZLF1 promoter. ZEBRA produced in this way could then auto-stimulate the BZLF1 promoter (0.9 Kb transcript) and initiate transcription from the BRLF1 promoter (3.5 Kb transcript).

In contrast to the results of Giot et al. (1991), I did not observe a decreased level of transcription of the BRLF1 and BZLF1 bicistronic mRNA when ZEBRA was expressed spontaneously from the het genome (Fig. II-10). The importance of the R transactivator in regulating induction of the EBV lytic cycle awaits the development of a system for recovering mutant viral genomes.

ZEBRA DNA Binding. DNA binding is often required for transcriptional activation by transactivators. The demonstration that ZEBRA shares homology with members of the cFos family (Farrell et al., 1989) suggested that it may bind a consensus AP-1 site and activate promoters which carry a copy of this sequence. Indeed, ZEBRA has been shown to transactivate the c-fos promoter in a chloramphenicol acetyl transferase (CAT) assay although it was unable to activate the c-jun promoter (Flemington and Speck, 1990d). Furthermore, AP-1 transactivators may regulate ZEBRA expression via an AP-1 octamer sequence located in the BZLF1 promoter (refer to chapter III, Flemington and Speck, 1990b,

Taylor et al., 199a). This finding suggests that ZEBRA and some AP-1 proteins may function together to regulate induction of the lytic cycle.

ZEBRA directly binds an array of related DNA motifs including a consensus heptamer AP-1 sequence (refer to chapter III, Chang et al., 1990, Farrell et al., 1989, Flemington and Speck, 1990b, Packham et al., 1990, Lieberman and Berk, 1990, Lieberman et al., 1990, Taylor et al., 1991a, Urier et al., 1989). The various EBV genes which were found to be transactivated by ZEBRA, including BZLF1, BRLF1, orilyt, BMRF1 and BMLF1, all contain ZEBRA binding sites in their promoters (Chang et al., 1990, Farrell et al., 1989, Flemington and Speck, 1990b, Kenney et al., 1989b, Packham et al., 1990, Lieberman and Berk, 1990, Lieberman et al., 1990, Sinclair et al., 1991, Taylor et al., 1991a, Urier et al., 1989). Although many of the ZEBRA binding sites do not contain a dyad symmetry, ZEBRA binds DNA as a dimer and requires two intact basic domains for DNA binding activity (refer to chapter III, Chang et al., 1990, Kolman et al., 1991, Taylor et al., 1991a, 1991b).

Regulating ZEBRA DNA binding activity. Regulation of ZEBRA DNA binding activity may be important in the EBV lytic cycle. It has recently been shown that DNA binding activity can be regulated by phosphorylation, redox potential and dimerization (refer to chapter V, Abate et al., 1990b, Boyle et al., 1991, Luscher et al., 1990, Manak et al., 1990, Sun

and Baltimore, 1991). Additionally, phosphorylation of an inhibitory protein, termed IP-1, inhibits DNA binding of Fos-Jun heterodimers (Auwerx and Sassone-Corsi, 1991). ZEBRA, but not the related Fos protein, can be phosphorylated in-vitro by casein kinase II (CKII). ZEBRA phosphorylation results in an abrogation of its DNA binding activity to all target sequences (refer to chapter V).

Abate and co-workers have found that DNA binding of Fos-Jun heterodimers is modulated by a reduction-oxidation of a single conserved cysteine in the basic DNA-binding domains of the two proteins (Abate et al., 199ob). This cysteine is conserved in the basic domain of ZEBRA, and as is the case for Fos-Jun heterodimers, ZEBRA is unable to bind DNA in the absence of a reducing agent (DTT) (Taylor, N., unpublished data). Thus, regulation of the redox or phosphorylation state of ZEBRA (or a related regulatory protein) may influence ZEBRA DNA binding activity in-vivo.

Regulation of the dimerization potential of leucine zipper and helix-loop-helix (HLH) proteins is important for DNA binding activity and specificity. As discussed in chapter IV, DNA binding of basic-leucine zipper (bzip) proteins is regulated by dimerization potential. A GCN4 leucine zipper homodimerizes whereas a fos zipper dimerizes specifically with jun and jun dimerizes with itself or fos (Kouzarides and Ziff, 1989, Neuberg et al., 1989, Sellers and Struhl, 1989, Nakabeppu and Nathans, 1989). The binding

specificities of these proteins is also modulated by dimerization (Benbrook and Jones, 1990, Gounari et al., 1990, Kara et al., 1990, Ivashkiv et al., 1990). Additionally, the E12 bHLH protein can form homo-or heterodimers, but only binds DNA as a heterodimer and the myc bHLH-zip protein only binds DNA as a heterodimer (Blackwood and Eisenman, 1991, Sun and Baltimore, 1991).

ZEBRA, which dimerizes via a coiled-coil interaction (Flemington and Speck, 1990c), homodimerizes and fails to interact with the leucine zipper domains of GCN4 or jun (refer to chapter III, Chang et al., 1990, Kolman et al., 1991, Taylor et al., 1991b). Target proteins which heterodimerize with ZEBRA, thereby regulating DNA binding activity and transactivation, may be identified by screening cDNA expression libraries. One candidate for heterodimerization is the human heat shock protein, hsp70, which shares homology with ZEBRA at amino acid positions important for the formation of a coiled-coil (Kolman, J.L., personal communication). Additionally, ZEBRA associates with a late EB viral protein which may modify its activity (Katz et al., manuscript in preparation).

Regulation of ZEBRA activity. ZEBRA transactivation is regulated by EBV and cellular factors: ZEBRA activation of orilyt, BRLF1, BMRF1 and BMLF1 is enhanced by an interaction with R; ZEBRA acts synergistically with the HIV-1 tat transactivator to activate the HIV-1 LTR; and ZEBRA acts

synergistically with c-myb to activate the BMRF1 and SV40 early promoters (Chevallier-Greco et al., 1989, Holley-Guthrie et al., 1990, Kenney et al., 1989a, manuscript submitted, Lieberman et al., 1988, Mallon et al., 1990, Sinclair et al., 1991). Although the importance of these various factors in regulating ZEBRA activity in-vivo has not yet been determined, these results suggest that interactions with cellular and viral transcription factors are required for disruption of EBV latency.

The properties of monomer proteins determines the transactivation activities of Fos/Jun heterodimers (Chiu et al., 1989, Nakabeppu and Nathans, 1991, Schutte et al., 1989). Heterodimers of c-Fos or c-Jun with Jun-B or a naturally truncated form of FosB, respectively, are transactivation deficient (Schutte et al., 1989, Nakabeppu and Nathans, 1991). A truncated BZLF1 cDNA containing BRLF1 sequences fused to 3' exon I sequences of BZLF1 has been identified (Manet et al., 1989). While the function of this protein has not been determined, it may heterodimerize with ZEBRA and thus, alter its target recognition sequence or transactivation potential.

Several groups have recently attempted to map the ZEBRA activation domain (Baumann et al., manuscript in preparation, Carey et al., manuscript in preparation, Giot et al., 1991, Rooney et al., 1990). It is generally agreed that the activation domain is encoded by exon I of ZEBRA but

there is some disagreement as to the precise amino acids responsible for activation. Additionally, Baumann and colleagues have found that the ZEBRA transactivation domain may be distinct from a second domain required for disruption of EBV latency (Baumann et al., manuscript in preparation). Thus, the process by which ZEBRA and associated proteins induce the EBV lytic cycle promises to be complex.

The role of EBV replication in disease. Activation of EBV has been observed in oral hairy leukoplakia, an AIDs associated lesion (Greenspan et al., 1985). Defective viral DNA containing a rearrangement of BamHI W to BamHI Z sequences, similar to the recombination found in P3HR-1, has been identified in 2 of 10 oral hairy leukoplakia lesions (Patton et al., 1990). Furthermore, antibodies to ZEBRA as well as spontaneous lytic EB viral DNA replication are found in AIDS patients, but not in control individuals. These data suggest that activation of ZEBRA (by recombination or other processes) is important in the pathogenesis of OHL (Alsip et al., 1988, Patton et al., 1990, Joab et al., 1991). The mechanisms by which ZEBRA expression is suppressed and activated in-vivo may remain an enigma for a few more years.

EXPERIMENTAL PROCEDURES

Cell Lines

HH514-16 (clone 16) is a prototypic HR-1 subclone which contains standard Epstein-Barr virus but lacks het virus. Clone HH543-5 (clone 5) harbors hypermolar levels of het DNA in addition to standard EB virus (Rabson et al., 1982). The B95-8 cell line is a marmoset cell line derived by cocultivation of marmoset cells with a human lymphoblastoid cell line (Miller et al., 1982). These cell lines were grown in RPMI 1640 with 8% fetal calf serum, penicillin, amphotericin B (Fungizone) and streptomycin.

COS-1 cells were grown in Dulbecco modified Eagle medium with 8% fetal bovine serum.

Chemical Induction and Inhibition

HR-1 cells were induced with sodium butyrate (3 mM) or TPA (20 ng/ml). In some experiments, induction was accompanied by inhibition of viral DNA replication with 100 ug of phosphonoacetic acid (PAA) per ml (Summers and Klein, 1976).

Transcription and translation were inhibited with 5 ug/ml actinomycin or 10 ug/ml cyclohexamide, respectively.

Transfections

COS-1 cells were transfected by the DEAE-dextran method, followed by chloroquine treatment (Luthman and Magnusson, 1983). COS-1 cells were seeded at a 1:5 split

ratio in 100-mm dishes 72 hours prior to transfection. The medium was removed and the cells were incubated with 10 ug of plasmid DNA and 300 ug DEAE-dextran suspended in TS (0.14M NaCl, 0.005M KCl, 0.7M Na_2HPO_4, 0.025M Tris HCl, 0.002M $MgCl_2$, 0.002M $CaCl_2$, pH 7.4) for 60 minutes at 37C in 5% CO_2. The cells were washed once with TS and were then resuspended in DMEM with 5% FCS containing 100 um chloroquine (Sigma). Following a 4 hour incubation, the medium was replaced with DMEM plus 5% FCS and incubation continued for 72 hours.

Cells used for immunoflourescence were seeded in 60-mm dishes containing coverslips. Transfections were carried out as described above.

Plasmids

PSV2neo plasmids containing either WZhet (the het BamHI fragment from defective EBV containing the BZLF1 open reeading frame) or the standard BamHI Z fragment from EBV FF41 or HR-1 have been described by Countryman et al. (1987). A 679 bp NaeI-PvuII fragment encompassing the unspliced BZLF1 open reading frame, WZ_{1-167}, (including exon I and 41 amino acids from the first intron) was isolated from WZhet. The overhanging end produced by PvuII digestion was filled in with the Klenow fragment of DNA polymerase and deoxynucleotide triphosphates. The fragment was inserted into the unique SmaI site of the trpE bacterial expression bector pATH 11, which was the gift of T.J. Koerner via M.

Carlson (Taylor et al., 1989, Dieckmann and Tzagaloff, 1985). Deletion mutants of $WZ_{1-167(+41)}$ were constructed as follows: WZ_{1-88} was cloned by excision of the HindIII fragment of $WZ_{1-167(+41)}$; $WZ_{133-167(+41)}$ was cloned as a SmaI-HindIII fragment into pATH 10; and $WZ_{133-167(+4)}$ was cloned as a SmaI-Sau3A fragment into pATH 10.

The full length BZLF1 cDNA (aa1-aa245) (Manet et al., 1989) was cloned as a NaeI-BamHI fragment into the SmaI-BamHI sites of pATH 11. The BZLF1 cDNA was the gift of A. Sergeant. BZLF1 deletion mutants were constructed as follows: $Z_{141-245}$ was cloned as a NheI-BamHI fragment, $Z_{172-245}$ was cloned by deletion of sequences upstream of an internal BsmI site in Z_{1-245}, Z_{1-198} was cloned as a NaeI-PstI fragment, and Z_{1-227} was cloned from a B95-8 ZEBRA cDNA (Farrell et al., 1989) as a BamHI-HincII fragment. All fragments were cloned into the appropriate sites of pATH vectors. $Z_{\Delta 110-159}$ was constructed by R. Baumann following BAL31 digestion of Z_{1-245} at the unique NheI site (aa 141). The 2nd exon of BZLF1, $Z_{167-202}$, was cloned from genomic BamHI Z as a PvuII-HincII fragment.

Mutant ZEBRA proteins were constructed by E. Flemington using site directed mutagenesis. The BZLF1 open reading frame cloned in SP64 was the gift of P. Farrell (Farrell et al., 1989). A HindIII -EcoRI fragment containing aa88-245 was cloned into the XhoI-EcoRI sites of pBluescript KS (Stratagene). Amino acids 178-180 and 187-189 were then

mutated from KRY to EEL and RKC to EES, respectively, in two additional constructs. Site directed mutagenesis was performed as described by the manufacturer (BioRad).

A trpE Fos-GCN4 chimera was constructed by John Kolman. The fos basic domain (aa 126-162) was fused to the GCN4 leucine zipper (aa 251-281) at an introduced XhoI site. This construct was the kind gift of T. Kouzarides and E. Ziff (Kouzarides & Ziff, 1989). This clone was inserted into the BamHI and XbaI sites of the pATH 1 trpE expression vector and is termed Fos-GCN4 or FG, fos basic domain (F) fused to a GCN4 leucine zipper (G). The inserts were sequenced by John Kolman using the Sanger dideoxynucleotide protocol modified for use with double stranded DNA clones.

Expression of TrpE-Fusion Proteins

TrpE fusion proteins were expressed as described previously (Kleid et al., 1981). Escherichia coli AG1 containing the various TrpE-fusion proteins were grown overnight in LB. The cells were then diluted 1:10 in M9 medium containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 100 ug/ml of ampicillin. Cells were induced for four hours with 20 ug/ml of indoleacrylic acid after having reached an optical density of 0.2 at 600 nm. Bacteria were pelleted and stored at -20C.

Fusion protein was enriched by preparation of an insoluble protein fraction (Kleid et al., 1981, Klempnauer and Sippel, 1987). One hundred ml of bacteria were pelled

and resuspended in 10 ml TEN (10mM Tris, pH 7.8; 1mM EDTA; 50mM NaCl) with 1 mg/ml lysozyme. 220 ul Nonidet P-40 (NP40) was added after 15 min and incubation continued for 10 min on ice. 15 ml of a Nacl-Mg solution (1.5M NaCl; 12 mM $MgCl_2$) containing 100 ul of 2 mg/ml DNAse was added and the sample was nutated at 4C for one hour. Insoluble material was washed twice with TEN.

Fusion protein was either resuspended in SDS-sample buffer for gel electrophoresis or dissolved in 6m urea for DNA binding reactions. Proteins used for DNA binding reactions were first visualized by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue in order to approximate the level of induced fusion protein in each preparation. The volume of cells containing equivalent amount of fusion protein (3.5 to 5 mls) were pelleted and resuspended in 1 ml of 6m urea (Angel et al., 1988a). Cells were sonicated twice for 20 s each. The urea solubilized protein fraction was dialyzed three times for 45 min at 4C against 15mM Hepes, pH 7.9; 75mM KCl; 0.2mM EDTA; 1mM DTT and 7.5% glycerol. In some heterodimer experiments, equivalent amounts of two fusion proteins were solubilized together in urea prior to dialysis.

Immunizations

TrpE-$WZ_{1\text{-}167(+41)}$ (ZEBRA exon I) fusion protein expressed from 50 ml of cells was recovered in the insoluble protein fraction and electrophoresed on an 8% preparative SDS-

polyacrylamide gel. The gel was stained with Coomassie brilliant blue and strips containing the fusion protein were ground and emulsified in complete Freund adjuvant (0.5 ml per rabbit). Rabbits were immunized by subcutaneous injection and boosted every 2 weeks with an equivalent amount of protein (approximately 25 mg) emulsified in incomplete Freund adjuvant (0.5 ml per rabbit). Serum was collected 1-2 weeks following each boost (Taylor et al., 1989).

Immunofluorescence

Following transfections on coverslips for 72 hours, coverslips were air-dried, fixed in a 2:1 acetone:methanol solution and stored at -20C prior to use. The coverslips were incubated for 1 hour at 37C with a 1:50 dilution of ZEBRA specific antibody in PBS^{++} (PBS plus 1mM $CaCl_2$ and 0.5mM $MgCl_2$). Coverslips were washed with PBS^{++} for 10 minutes and then incubated with a 1:50 dilutin of a rhodamine-conjugated anti-rabbit immunoglobulin for an additional 1 hour at 37C. Final washes were in PBS^{++} for 2 minutes (X5) followed by an H_2O rinse. Coverslips were inverted and mounted in buffered glycerol (90% glycerol buffered with 0.4M Na-bicarbonate, pH9.5) on standard microscope slides.

Protein Electrophoresis and Western Blotting

Proteins were electrophoresed by the method of Laemmli (Laemmli, 1970). Protein samples were suspended in sample

buffer (200 mM Tris, pH 6.8; 15% 2-mercaptoethanol; 1% SDS; 30% glycerol; 0.002% bromophenol blue). Mammalian cell extracts were sonicated for 20s and all samples were heated at 100C for 5 minutes. Samples were electrophoresed in a discontinuous buffer system using vertical slab electrophoresis at 200 volts. The stacking gels were 4% acrylamide (30:0.8 polyacrylamide:bis-acrylamide) in 0.125M Tris pH 6.8 and the resolving gels were typically 8% polyacrylamide in 0.375M Tris pH 8.8.

Proteins were either detected directly by staining or were transferred to nitrocellulose for immunoblotting. Proteins were directly stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue in 30% methanol and 10% glacial acetic acid) and destained with 30% methanol, 10% acetic acid.

Alternatively, proteins were electrotransferred to nitrocellulose filters in 0.25M Tris, 0.15M glycine, 0.1% SDS, and 25% methanol using a Bio-Rad transblot apparatus for 2 hours at 200 mAmps at 4C (Towbin et al., 1971, Burnette et al., 1981). Nitrocellulose filters were blocked in Blotto (5% non-fat dry milk, 10^{-4}% anti-foam A (Sigma), 0.5 uM thimerisol) at room temperature for 1-12 hours with gentle agitation (Johnson et al., 1984). The filters were nutated for 1 hour in Blotto with 1:100 to 1:1000 dilutions of a ZEBRA-specific rabbit antibody or a polyvalent human serum, RM, which detects latent EBV proteins as well as a

p21 late viral protein (Rowe et al., 1987). Filters were washed twice for 20 minutes in TS (10 mM Tris, pH 7.4; 0.9% NaCl; 0.05% Tween-20) and incubated with a 1:2,000 dilution of ^{125}I-labelled staphylococcal protein A (0.5-1.0 uCi) (Amersham) in Blotto for 60-90 minutes. Filters were then further washed in TS, dried and exposed to Kodak XAR-5 or BB-5 films with intensifying screens.

Protein Phosphorylations

Purified ZEBRA protein as well as fusion proteins were phosphorylated in casein kinase buffer (75mM KCl; 5 mM $MgCl_2$; 10 mM Hepes, pH 7.9; 1 mM EGTA and 0.5mM DTT). Fusion proteins (100 ng, 2 ul) were made as described above while ZEBRA protein (250 ng) was purified by ammonium sulfate precipitation following overexpression in E. coli using the tac expression vector pRW76. The protein was 90% pure as judged by coomasie blue staining of SDS-polyacrylamide gels (Carey et al., manuscript submitted). Proteins were phosphorylated in the presence of 10uCi of [γ-^{32}P] ATP and 5 ul of CKII. Following incubation at 37C for 15 minutes samples were denatured by boiling and electrophoresed on an 8% SDS-polyacrylamide gel as described above.

Radiolabeled DNA

Purified DNA fragment probes for northern blot analysis were electroeluted from TBE-polyacrylamide gels and radiolabelled by the random primer method (Feinberg and Vogelstein, 1983).

DNA fragments which were used as probes are as follows: the 5' BamHI-HindIII fragment of WZhet mutant 80 which contains 264 bp upstream of BZLF1 and 259 bp of the first BZLF1 coding exon; a 395 bp fragment from the HindIII site in BZLF1 encoding the 3' region of exon I; a 627 bp BamHI-PstI fragment from the BZLF1 cDNA which encodes exons I and II; and a 538 bp SacI-BamHI fragment which is specific for the BRLF1 open reading frame. Typically, 100 ng of double stranded DNA was denatured by incubation at 100C for 5 minutes in the presence of random hexamers (Pharmacia) and then cooled on ice for 5 minutes. The DNA was then labelled with 33 uCi of ^{32}P-dCTP (Amersham) and 5 units of the Klenow fragment of E. coli DNA polymerase in Klenow buffer (50 mM Tris-HCl, pH 7.2; 10 mM $MgSO_4$, 0.1 mM DTT, 50 ug /ml bovine serum albumin) containing 3 uM unlabelled dATP, dGTP, and dTTP. Reactions were incubated at room temperature for several hours. DNA probes for south-western analysis were prepared by nick translation (Rigby et al., 1977). DNA (100 ng) was incubated for 90 minutes at 15C in Klenow buffer with 1.25 ng of DNAse (Worthington) and 10 units of E. coli DNA polymerase 1 (New England Biolabs) containing 3 uM unlabelled dATP, dGTP, and dTTP and 33 uCi of ^{32}P-dCTP. Oligonucleotides were end-labelled in Klenow buffer in the presence of 5 units of Klenow and ^{32}P-labelled deoxynucleotides for 30 minutes at room temperature. DNA fragment probes were purified from unincorporated label

using G-50 sephadex columns while oligonucleotide probes were purified over G-4 columns.

Northern Blots

Total cellular RNA or cytoplasmic RNA was harvested from uninduced cells and at various times following induction. Total RNA was isolated by the guanidium isothiocyanate method essentially as described by Chirgwin et al. (1979). Approximately 5 X 10^7 cells were resuspended in ice cold GIT (4M guanidine isothiocyanate; 0.025M NaOAC, pH 6.0; 1.5M B-mercaptoethanol) and layered on top of 4 ml cesium chloride buffer (5.7M CsCl; 0.0125M NaOAC, pH 6.0) in an SW41 polyallomer tube. Tubes were filled completely with GIT and centrifuged in an SW41 rotor at 32,000 RPM for 21 hours at 20C. The RNA pellet was precipitated in ethanol and stored at -70C prior to use.

Cytoplasmic RNA was isolated by lysis of cells in an NP40 buffer. 5 X 10^7 cells were resuspended in 187 ul of 150 mM NaCl and 10 mM Tris, pH 8.0 containing 5 units of RNAsin on ice. Lysis was achieved by the addition of 14 ul of 10% NP-40 and cytoplasmic RNA was isolated in the supernatant following centrifugation in a microfuge for 1 minute at 4C. The supernatant was removed into an equal volume of a urea solution (7M urea; 350mM NaCl; 10 mM Tris, pH 7.5; 20 mM EDTA; 1% SDS) and extracted twice with phenol: chloroform (1:1) prior to precipitation with ethanol.

RNA was electrophoresed on 1% agarose gels containing

1X MOPS (20mM MOPS; 5mM NaOAC; 1mM EDTA) and 6% formaldehyde at 200V in a running buffer which contained 1X MOPS and 6% formaldehyde. RNA was resuspended in 1X MOPS, 6% formaldehyde and 50% formamide and heated at 70C for 10 minutes prior to the addition of gel loading buffer (xylene cyanol and bromophenol blue in 25% glycerol). Gels were transferred to nytran overnight in 20X SSC following washes in ddH_2O; 50mM NaOH and 10 mM NaCl; 0.1M Tris, pH 7.5; and ddH_2O.

rRNA bands were visualized by soaking the filter in 5% acetic acid and then staining with 0.004% methylene blue in 0.5M NaOAC, pH 5.2 (Davis et al., 1986).

Blots were hybridized according to the method of Church and Gilbert (1984). Blots were prehybridized for 3 hours - overnight at 65C in a sodium phosphate mix (0.5M NaP_i, pH 6.8; 7% SDS; 1% BSA; 0.1 mM EDTA) and denatured probe was then added directly to the mix for an additional 16-24 hours at 65C. Radiolabelling of probes is described in a separate section. Blots were washed 3X for 15 minutes at 65C in 40 mM NaP_i, 0.5% BSA, 5% SDS, and 1mM EDTA. Two final washes were carried out in 40 mM NaP_i, 1% SDS and 1mM EDTA. Filters were exposed to Kodak XAR-5 or BB-5 film with intensifying screens.

Gel Retardation Assays

DNA binding reactions using gel shift mobility assays were first described by Fried and Crothers (1981). The gel

retardation assays performed in this study were modified from the procedures of Farrell et al. (1989). Reactions containing 4 X 10^{-13}M of the appropriate ^{32}P end-labelled double stranded oligonucleotide and 2 ul of individual trpE fusion proteins (approximately 100 ng) or 4 ul of a mixture of two proteins were incubated in 25 microliters of binding solution (15 mM Hepes, pH 7.9; 75mM KCl; 0.2mM EDTA; 1mM DTT; 7.5% glycerol; 1 ug poly dIdC) for 30 minutes at room temperature or 4C. Competition assays were conducted with 5, 20 and 50 fold molar excesses of unlabelled competitor over the ^{32}P labelled MS-AP-1 oligonucleotide. (The Double oligonucleotide was used in 2.5, 10 and 25 fold molar excesses since it contains two potential binding sites (ZIIIA and ZIIIB)). The reactions were electrophoresed under non-dissociating and non-denaturing conditions in 4% polyacrylamide-0.5X TBE (44 mM Tris-borate, 1 mM borate, 1 mM EDTA) at room temperature or 4C. Gels were then dried on a Bio-Rad slab gel drying apparatus and autoradiographed.

Gel retardation assays involving casein kinase II were performed in a 30 ul volume of casein kinase buffer (described above). 250 ng of purified ZEBRA protein was incubated at 37C for 15 minutes in the presence of 5 ul of casein kinase II (purified from bovine testes,Carroll et al., 1988, 1989) and 1 ul of 10 mM ATP (Pharmacia). Some extracts were then subject to treatment with 1 ul protein phosphatase 2A (PP2A) (10^{-5} units) for an additional 15

minutes at 37C. PP2A was stored in 50 mM Tris, pH 7.5 and 0.1% BSA and was the kind gift of Jonathan Chernoff. ^{32}P labelled oligonucleotide probe and 1 ug of poly dIdC were added to each sample and the reactions were incubated for an additional 30 minutes at 22C. Samples were then electrophoresed as described above.

Films from competition experiments were quantified by densitometer scanning on a Kodak BioImage densitometer with Visage 2000 software. Disruption of complex formation was determined by comparing the ratio of shifted complex in lanes with and without competitor.

Synthetic Oligonucleotides

The synthetic double stranded oligonucleotides used in binding studies were as follows (sequence reflects top strand in a 5' to 3' direction): Core sequences are underlined and mutated bases are noted in bold type.

MS-AP-1:	TCTTCATGAGTCAGTGCTTC
MS-AP-1*:	TCTTCATTAGTCAGTGCTTC
ZIIIA:	CTAGCTATGCATGAGCCACAGATC
ZIIIA*:	CTAGCTATGCATGAGC**AA**CAGATC
ZIIIAm:	CTAGCTATGCA**GAATTC**ACAGATC
ZIIIB:	CTAGCAGGCATTGCTAATGTACCGATC
ZIIIB*:	CTAGCAGGCATTGCT**CA**TGTACCGATC
ZIIIBm:	CTAGCAGG**ATCC**GCTAATGTACCGATC
Double:	GACTATGCATGAGCCACAGGCATTGCTAATGTACCGA
	(contains ZIIIA + ZIIIB)

Z-AP-1 octamer: CTAGAAACCATGACATCACAGAGGATC

Control oligo: TGGCATGCTGCTGACATCTGGC

All oligonucleotides had overhanging XbaI sites at the 5' end and overhanging BamHI sites at the 3' end, with the exception of the AP-1 and Double oligonucleotides which had CT and AG overhangs at the 5' and 3' ends, respectively.

Nitrocellulose DNA-binding Assays

Immunoblotting was performed as described above. Incubation with radiolabeled DNA (south-westerns) followed the procedure described by Bowen et al. (1980) and Klempnauer and Sipper (1987). Following blocking of immunoblots for 1 hour in Blotto, the blots were washed three times (20 minutes each) with 6M urea; 0.2% NP-40 and four times (30 minutes each) with DNA-binding buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA; 50 mM NaCl) at room temperature. The blots were incubated with approximately 5 X 10^6 c.p.m. of nick-translated ^{32}P-labelled DNA in 10 ml of DNA-binding buffer for 30 minutes. Blots were then washed three times (10 minutes each) with DNA-binding buffer prior to analysis by autoradiography.

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