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The ZEBRA Activation Domain: Modular Organization and Mechanism of Action

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An RNA polymerase II activator often contains several regions that contribute to its potency, an organization ostensibly analogous to the modular architecture of promoters and enhancers. The regulatory significance of this parallel organization has not been systematically explored. We considered this problem by examining the activation domain of the Epstein-Barr virus transactivator ZEBRA. We performed our experiments in vitro so that the activator concentrations, stabilities, and affinities for DNA could be monitored. ZEBRA and various amino-terminal deletion derivatives, expressed in and purified from *Escherichia coli*, were assayed in a HeLa cell nuclear extract for the ability to activate model reporter templates bearing one, three, five, and seven upstream ZEBRA binding sites. Our data show that ZEBRA contains four modules that contribute to its potency in vitro. The modules operate interchangeably with promoter sites to determine the transcriptional response such that the loss of modules can be compensated for by increasing promoter sites. Potassium permanganate footprinting was used to show that transcriptional stimulation is a consequence of the activator's ability to promote preinitiation complex assembly. Kinetic measurements of transcription complex assembly in a reconstituted system indicate that ZEBRA promotes formation of a subcomplex requiring the TFIIA and TFIID fractions, where TFIIA acts as an antirepressor. We propose a model in which the concentration of DNA-bound activation modules in the vicinity of the gene initiates synergistic transcription complex assembly.

Activation of transcription is a common regulatory strategy for controlling development, differentiation, and response to extracellular signaling in eukaryotic cells. To provide a framework for the problem, consider the organization of a typical RNA polymerase II (Pol II) promoter. A typical promoter contains a TATA box 25 bp upstream from the start site (43, 57). Further upstream are the binding sites for two or more activators that act synergistically to stimulate transcription; synergy provides a mechanism to control both the level and diversity of gene expression (2, 41, 42). The current hypothesis is that activators stimulate transcription by interacting, via DNA looping, with components of the general transcription machinery to facilitate assembly of a transcription complex over the start site.

Biochemical analysis of the general transcription machinery has led to the identification of seven factors, in addition to RNA Pol II, required for transcription from promoters bearing a TATA box (57). In the absence of activators, these factors assemble, in an ordered pathway, into a stable preinitiation complex (1, 12, 16, 43, 57). This assembly is nucleated by binding of the TATA-binding protein (TBP) to the TATA motif. TBP is a component of TFIID, a multisubunit complex containing at least six TBP-associated factors (TAFs) (8, 50). One or more TAFs present in TFIID are required to allow the general transcription machinery to respond to activators. TFIIA and TFIIB associate with the TBP-TATA complex to form an intermediate that is recognized by TFIIF and RNA polymerase. This event is followed by sequential binding of TFIIE, TFIIH, and TFIIJ to complete formation of the preinitiation complex (57). After the assembly is complete, or nearly so, ATP is hydrolyzed to melt the DNA encompassing the start site. This forms what has historically been referred to as the open complex (54). The ATP requirement may be for TFIIH, which acts as both a kinase and an ATP-dependent helicase (31).

Activators are often classified according to the amino acid composition of their activation domains or regions. It has been proposed that different classes of activation domains may function by different mechanisms (37). Acidic activation regions are rich in acidic amino acids and have a net negative charge. Recent mechanistic studies have shown that GAL4-derived acidic activators (5, 28), containing the GAL4 DNA binding domain fused to various acidic activation regions (41), act by promoting transcription complex assembly (29, 54). Furthermore, the synergistic effect of the GAL4 derivatives on transcription (a single activator is either impotent or marginally active, whereas two or more activators elicit a greater than additive response [5]) correlates with a synergistic increase in the number of open complexes (55). Recruitment and kinetic experiments suggest that acidic activators target early steps in preinitiation complex formation (8, 18, 29, 47, 53, 55, 56), including assembly of subcomplexes requiring TFIIA and TFIID (55) or TFIIB and TFIID (29, 47). Although a mechanism for how acidic activators function is emerging, little is known about how other classes of activation domains work. It is noteworthy, however, that affinity chromatography and far-Western blotting experiments have implicated factors involved in early steps in complex assembly as potential targets of both acidic and nonacidic activators (23, 25, 27, 30, 46).

An activator often contains two or more activation regions, or subdomains, distinguished by their locations within the activator and by their chemical compositions (37). Depending upon the particular study, these activation regions act either synergistically, additively, or redundantly to influence the activator's potency (13, 17, 21, 32, 38, 48, 49, 52). It has been noted (13) that the organization and function of the multiple activation regions are superficially analogous to the modular structure of promoters and enhancers (7, 39,

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45). The regulatory significance of this observation has not been systematically examined. Analyses of several studies suggest that the effect of the subdomains often depends on the promoters used in the study. For example, the two glutamine-rich activation regions of Sp1 were redundant when assayed on a reporter template bearing six Sp1 binding sites (6) but were synergistic when assayed on a template containing only two sites (40). Although the mechanism underlying promoter-dependent variability is not known, studies on Sp1 suggest that the number of promoter sites may be a key determinant.

We have been interested in this problem as it relates to an Epstein-Barr virus (EBV) transactivator called ZEBRA (also called Zta). ZEBRA's role is to mediate a genetic switch in the viral life cycle. In B lymphocytes, EBV exists in a latent state that can be disrupted by select extracellular signals that cause the virus to switch to a lytic pathway (36). The signaling event initially activates expression of ZEBRA, which, in turn, binds upstream of and stimulates a series of responsive genes; the net effect is synthesis of infectious virus. The promoters of responsive genes contain between one and eight recognizable ZEBRA binding sites. An understanding of how the design of ZEBRA and promoter organization control transcription of this family of genes could provide a paradigm for how cellular activators differentially regulate transcription.

ZEBRA contains a carboxy-terminal basic region, responsible for DNA binding specificity, and a coiled-coil dimerization domain (10, 26). Full-level transcriptional activation is conferred by a nonacidic, amino-terminal region rich in alanine, proline, and glutamine but displaying little overall homology to previously characterized motifs. Deletion analysis of the activation region suggests that it is composed of several critical regions (11, 15), but it is not known how these contribute to ZEBRA's potency or whether they can act independently.

In this report, we dissect the activation region of ZEBRA biochemically and address its mechanism of action. We use deletion mutants and domain swap experiments to show that ZEBRA contains four regions or modules that mediate activation. By comparing the activities of these mutants on promoters bearing different numbers of upstream ZEBRA sites, we show that the four modules function interchangeably with promoter sites to mediate the transcriptional response. We then show that ZEBRA turns on a gene by promoting formation of an active preinitiation complex. The limiting step in this process is ZEBRA-mediated assembly of a subcomplex requiring the TFIIA and TFIID fractions. We discuss the ramifications of our study on the design and function of eukaryotic activation regions.

MATERIALS AND METHODS

Cloning, overexpression, and purification of ZEBRA and its deletion mutants. Expression vectors encoding ZEBRA and the deletion mutants were constructed as follows. Oligonucleotide primers flanking selected regions of the ZEBRA cDNA sequence were synthesized with an *Nco*I site upstream and a *Bam*HI site downstream of the desired coding sequence. To introduce the *Nco*I site, it was necessary that the first codon following the initiator ATG begin with a G. This affected our selection of deletion mutants, which we tried to space 25 to 30 amino acids apart. The polymerase chain reaction (PCR) was used to synthesize fragments of the intervening DNA. These PCR fragments were cloned into the *Nco*I-*Bam*HI-digested T7 RNA polymerase expression vector pET11d (Novagen). Representative constructs encoding Z(2–245), Z(51–245), Z(99–245), and Z(131–245) (for an explanation of nomenclature, see Results) were cloned in duplicate from two independent PCRs. The expression vectors were subsequently transformed into *Escherichia coli* BL21(DE3) (Novagen) or, for Z(99–245), *E. coli* BL21(DE3)pLysS.

For each protein, six liters of *E. coli* bearing the expression vectors was grown at 37°C to an A_{600} of 0.5. Isopropylthiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. After 3 h [or 6 h for Z(99–245)], the cells were harvested by centrifugation and washed in a buffer mixture containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0) and 200 mM NaCl. The cell pellets were resuspended in 150 ml of buffer A (20 mM HEPES [pH 7.0], 1 mM EDTA, 25 mM 2-mecaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin and pepstatin per ml) containing 0.6 M NaCl. The cells were lysed by sonication, and insoluble debris was removed by centrifugation at 10,000 × *g* for 10 min. For all proteins except Z(25–245), polyethyleneimine was added to the sonication supernatant to a final concentration of 0.1%. After 10 min of gentle stirring at 0°C, the precipitate was removed by centrifugation for 10 min at 10,000 × *g*. The ZEBRA derivatives were precipitated by addition of solid ammonium sulfate to 0.2 g/ml of supernatant. After 20 min of gentle stirring at 0°C, the precipitates were collected by centrifugation at 10,000 × *g* for 10 min. The pellets were resuspended in buffer A to a conductivity equal to that of buffer A–0.2 M NaCl and loaded onto a 5-ml heparin-Sepharose (Pharmacia) column preequilibrated with the same buffer. For Z(25–245), an equal volume of buffer A was added to the sonication supernatant, which was then loaded onto a 5-ml DEAE-cellulose column preequilibrated with buffer A–0.3 M NaCl. The flowthrough was loaded onto a 5-ml heparin-Sepharose column preequilibrated in buffer A–0.3 M NaCl. The columns were washed with 10 ml of buffer A–0.3 M NaCl and developed with a linear gradient of buffer A containing 0.4 to 1.2 M NaCl. The proteins eluted with a broad peak centered at 0.6 M NaCl. The purest fractions, as determined by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels, were pooled and used in our experiments. Z(2–245), Z(77–245), Z(99–245), and Z(131–245) were 90% pure, while Z(25–245) and Z(51–245) were 50% pure.

To generate the GAL4-ZEBRA fusions, PCR products bearing the desired portions of ZEBRA were cloned in frame to the C terminus of GAL4(1–94) in a pGem3 vector. Synthetic mRNAs were synthesized by using SP6 RNA polymerase and subsequently translated in a wheat germ extract as instructed by the manufacturer (Promega).

In vitro transcription and open complex formation in HeLa nuclear extracts. The 40-μl reaction mixtures contained 90 or 150 μg of HeLa nuclear extract, 200 ng of DNA (200 ng of reporter template or the indicated amount of reporter with carrier pGem3), 12.5 mM HEPES (pH 7.9), 60 mM KCl, 8 mM $MgCl_2$, 12.5% glycerol, 0.6 mM dithiothreitol, and 60 μg of bovine serum albumin per ml. The transcriptional activities of ZEBRA and the deletion derivatives were measured by adding nucleoside triphosphates to a final concentration of 0.5 mM. After 1 h at 30°C, the reactions were terminated and mRNA was isolated and quantitated as described previously (5). DNase I footprinting or gel mobility shift assays were used to normalize the binding of the activators to the template sites. Transcription titrations were performed to determine concentrations of activator sufficient to produce

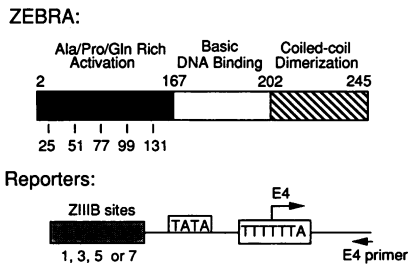

FIG. 1. Schematic representation of ZEBRA, its derivatives, and reporter templates used in this study. ZEBRA consists of a carboxy-terminal coiled-coil dimerization domain (positions 202 to 245), a basic DNA recognition domain (167 to 202), and a region bearing the transcriptional activation function (2 to 167). The ZEBRA used in our study begins at position 2 because wild-type ZEBRA contains two methionines at the amino terminus and only one was cloned into our expression vector. The vertical lines indicate the amino-terminal endpoints of the deletion mutants. The reporter templates contain the indicated number of ZIIIB sites (TTAGCAA), centered 14 bp apart, upstream of the adenovirus E4 TATA box and initiation site (3). The sequence of the T-rich E4 initiation region is shown.

optimal levels of transcription. These concentrations were used for our experiments. To measure open complex formation, α-amanitin and dATP were added to the mixture to final concentrations of 2 μg/ml and 0.5 mM, respectively. The permanganate probing was performed as described previously (54), with the following modifications. After phenol and chloroform extraction of the reaction mixture, the permanganate-modified templates were precipitated with ethanol in the presence of 2 M ammonium acetate. The pellets were washed with 80% cold ethanol and air dried. The DNA was subjected to primer extension as described elsewhere (5).

Kinetic analysis of preinitiation complex assembly using partially purified transcription factors. The fractionation of general transcription factors from HeLa nuclear extracts and the purification of recombinant TFIIB have been described previously (55). The 40-μl reaction mix contained 10 ng of Z_7E4T, 200 ng of pGem3 carrier DNA, 8 mM $MgCl_2$, 12.5% glycerol, 0.6 mM DTT, 10 μl of TFIIA, 10 to 20 μl of TFIID fraction, 30 ng of recombinant TFIIB, 3 μl of TFIIE/F/Pol fraction, 2 μg of α-amanitin per ml, and 0.5 mM dATP. All reactions were performed at 30°C. Open complexes were probed as previously described (54). To determine the rate of open complex formation, the template was coincubated with all of the factors in the presence of ZEBRA for 0, 5, 10, and 30 min before probing with permanganate. To dissect the assembly pathway, the template was first preincubated with the indicated subsets of factors for 30 min. The remaining components, including α-amanitin and dATP, were then added back, and incubation continued for 5, 10, and 30 min before probing.

RESULTS

Experimental design. ZEBRA and a series of amino-terminal deletion derivatives, each retaining the DNA binding domain but lacking portions of the activation region (Fig. 1), were overexpressed in and purified from *E. coli*. The designations used in the text refer to the remaining amino acid sequence; Z(25–245), for example, refers to a derivative containing amino acids 25 through 245 of ZEBRA, hence a deletion of the amino-terminal 24 amino acids. The templates used in our assay contained one, three, five, or seven synthetic ZEBRA binding sites positioned immediately upstream from the TATA box of the adenovirus E4 gene; we designate these promoters Z_nE4T, where *n* represents the number of binding sites (3). We chose these simple model templates to avoid the complications introduced by natural promoters bearing sites for other regulatory proteins that might confound our analysis. The transcriptional potencies of ZEBRA and the mutant proteins were compared by incubating site-saturating amounts of each, as determined by DNase I footprinting and transcription assays, with a particular DNA template in a HeLa cell nuclear extract. The in vitro system was used both to circumvent the difficulty of accurately controlling for the protein and DNA concentrations in vivo and to address the mechanism of ZEBRA-mediated transactivation biochemically. We have previously shown that ZEBRA's ability to stimulate transcription in vitro correlates well with its activity in cell culture transfection assays (3). The mRNA synthesized in vitro was assayed by primer extension, and the results were measured by laser densitometry. We used a potassium permanganate modification assay to assess the activator's ability to assemble an active preinitiation complex. Permanganate modifies thymine residues in single-stranded DNA. DNA melting by Pol II, likely the last or penultimate step in transcription complex formation, exposes six thymine residues in the start site of the E4 reporter templates (Fig. 1). Permanganate-modified thymines in this region were detected and quantified by primer extension using an E4 primer (54). Thus, the sensitivity to permanganate served as a direct measure of initiation complex formation.

ZEBRA contains four activation modules. Figure 2A is an autoradiograph of a typical in vitro transcription result. Neither ZEBRA nor its amino-terminal deletion derivatives stimulate transcription on a template bearing a single site (3). On three sites, ZEBRA is moderately active, Z(25–245) and Z(51–245) are less active, and the remaining mutants are relatively inactive. In contrast, when measured on five sites, Z(77–245) and Z(99–245) activate at least as well as ZEBRA does on the three-site template. Although the activity of Z(131–245) on five sites is low, it is measurably higher than on three sites. The data indicate that the inactive mutants, as assayed on a template bearing three sites, contain potential transcription activity that is manifested on a template bearing five sites.

Autoradiographs from representative experiments were quantitated and plotted as a bar graph (Fig. 2B). The vertical axis represents the percentage of transcriptional activity of the mutants relative to ZEBRA; ZEBRA is assigned a value of 100% on both three and five sites, although the absolute level of stimulation is higher on five sites (Fig. 2A). The data show that progressive deletions of ZEBRA lead to roughly three significant drops in transcriptional activity. The first drop occurred with mutants lacking the first 24 [Z(25–245)] or 50 [Z(51–245)] amino acids. In this case, the activity decreased to 57% on Z_3E4T or to 71% on Z_5E4T. The second drop occurred with further deletions removing either 76 [Z(77–245)] or 98 [Z(99–245)] amino acids; the activity decreased to 10% on Z_3E4T but to only 39% on Z_5E4T, a point that we address below. The third drop occurred when amino acids up to 131 were deleted; activity fell to 3% on Z_3E4T or to 11% on Z_5E4T. Deletions into the DNA binding domain produced proteins that were unstable in *E. coli*. We conclude that ZEBRA contains at least four modules involved in transactivation. These are summarized schematically at the bottom of Fig. 2B. Module I is from amino acids 2 to 50,

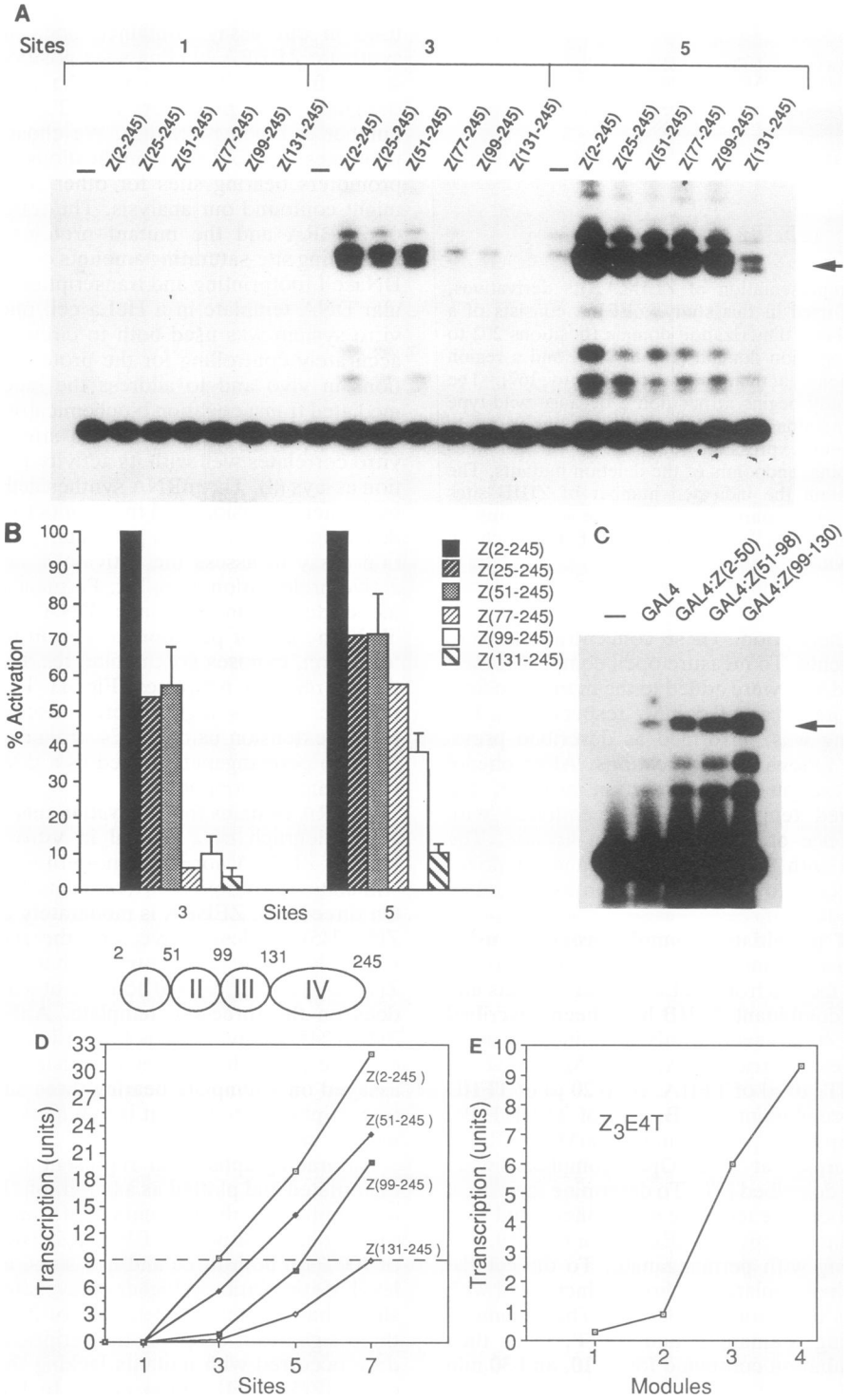

FIG. 2. Transcriptional potency of ZEBRA and its derivatives in a HeLa cell extract. (A) Templates bearing the indicated number of ZIIIB sites were incubated in a HeLa cell extract either alone (−) or with the indicated ZEBRA derivatives. After 1 h, the products were isolated and quantitated by primer extension, and the primer extension products were fractionated on a 10% polyacrylamide–urea gel. An autoradiograph of the gel is shown. The arrow indicates the position of the E4 primer extension products. Note that the basal level increases as the number of sites is raised from one to five. This was a reproducible effect but was not observed with GAL4-responsive templates. The elevated level is likely due to small amounts of cellular activators, present in the HeLa extracts, that recognize the ZEBRA sites. (B) ZEBRA contains four activation modules. The bar graph compares the relative activities of deletion mutants with ZEBRA. The activities of ZEBRA were assigned a value of 100% on both Z_3E4T and Z_5E4T, although the absolute amount of stimulation differs. The data for representative mutants in each class, including Z(2–245), Z(51–245), Z(99–245), and Z(131–245), were pooled from five separate experiments and averaged. The error bars indicate standard deviations. The data for Z(25–245) and Z(77–245) are averages of two separate experiments. Below is a schematic representation of the four modules mapped in our study. (C) The ZEBRA modules activate transcription when fused to the GAL4 DNA binding domain. GAL4-ZEBRA fusion proteins were synthesized by in vitro transcription and translation in a wheat germ extract.

module II is between 51 and 98, module III is between 99 and 130, and module IV is between 131 and 245, within the DNA binding domain of ZEBRA. We note that we were unable to find a particular sequence motif characterizing the modules, although modules I through III are generally rich in alanine, proline, and glutamine.

Figure 2C shows that each of the first three modules stimulates transcription independently when fused to the GAL4 DNA binding domain, GAL4(1–94), and assayed on a GAL4-responsive template. Although GAL4(1–94) (GAL4 in Fig. 2C) is marginally active on its own, the ZEBRA modules significantly increase that stimulatory potential. The fourth module clearly functions independently of the other three when assayed in the context of Z(131–245) on a ZEBRA-responsive template (Fig. 2A).

ZEBRA binding sites and activation modules function interchangeably to determine the transcriptional response. Figure 2A shows that mutants that were inactive when assayed on three-site templates became active when tested on a template bearing five sites. Thus, increasing the number of ZEBRA sites compensated for the loss of modules. To further analyze this relationship, we compared the transcriptional activities of four representative amino-terminal deletion derivatives on the one-, three-, five-, and seven-site reporter templates and plotted the results as a line graph (Fig. 2D).

The graph illustrates two points. First, the amount of stimulation is a function of the numbers of both promoter sites and activator modules. A given level of transcription could be obtained by an activator bearing more modules on fewer sites as well as by an activator bearing fewer modules on more sites. By interpolation on the graph shown in Fig. 2D (dashed line), we found that Z(2–245), bearing all four activation modules, required three sites to stimulate transcription to 9 U. However, for a similar level of activation Z(131–245), bearing one module, needed seven sites. A similar trend was observed for other levels of stimulation.

The second point is that for each activator, the transcriptional response to increasing promoter sites is initially synergistic. Thus, ZEBRA was inactive (0 U) on a template bearing 1 site, but transcription was activated to 9 U with three sites. This effect is synergistic because the greater than ninefold increase in transcription is larger than the threefold increase in sites. The quotient of the increase in transcription divided by the increase in sites provides a relative measure of the synergy. In the case described above, this quotient is greater than threefold. However, when sites were increased from three to five and from five to seven, the corresponding transcription levels were enhanced almost additively from 9 to 19 U (1.3-fold synergy) and from 19 to 32 U (1.2-fold synergy), respectively. The same trend was apparent for Z(51–245) or Z(99–245), though more sites were needed to support the response. Figure 2E is a graph showing that the modules themselves act synergistically to increase the potency of ZEBRA when measured on a template bearing three sites. Thus, an activator bearing one [Z(131–245)] or two [Z(99–245)] modules is only marginally active, while increasing the number of modules to three [Z(51–245)] generates a synergistic increase in transcription from 0.9 to 6.0 U. The 6-fold increase in transcription is achieved by a 1.5-fold increase in modules (4.4-fold synergy). However, the effect was less pronounced when the number of modules increased from three to four [Z(2–245)]; in this case, transcription was enhanced from 6.0 to 9.3 U (1.2-fold synergy).

Taken together, our data demonstrate that promoter sites and activation modules function interchangeably to stimulate transcription. The initial effect is a synergistic increase between barely detectable and activated transcription, after which additional modules or sites stimulate transcription additively. It is not known whether transcription might still be increased synergistically if a different class of activation module (i.e., acidic) were delivered. The four modules do not appear to be equally potent in our assays (Fig. 2B and C). This does not substantially affect our conclusions, however, since the four modules are qualitatively similar in function.

Transcription complex formation stimulated by ZEBRA and its deletion mutants. Figure 3 demonstrates that ZEBRA and its derivatives stimulate transcription by assembling active preinitiation complexes over the start site. We have previously shown that GAL4-derived acidic activators facilitate assembly of a preinitiation complex that, in the presence of ATP or dATP, is converted to an open complex measurable by potassium permanganate sensitivity (54). We wished to determine whether ZEBRA, bearing a nonacidic activation region, would also promote formation of such a complex and, if so, whether open complex assembly correlated with the transcription levels elicited by ZEBRA and its deletion mutants on the test promoters. Recall that the permanganate sensitivity of six thymine residues within the E4 start site is a measure of the amount of open complex formed (54, 55).

In our experiment, a DNA template bearing seven upstream ZEBRA sites was incubated in a HeLa cell nuclear extract in the presence or absence of ZEBRA. After a 28-min preincubation, dATP and α-amanitin were added for 2 min, after which the mixture was treated with permanganate to detect start site melting. Note that 28 min was sufficient for full-level open complex formation as determined in a time

Equivalent amounts of the synthetic GAL4-ZEBRA fusion proteins, as determined by gel mobility shift analysis, were incubated with 2 ng of a GAL4-responsive template, G_9E4T, in a HeLa nuclear extract. The resulting RNA products were measured by primer extension. GAL4, the DNA binding domain, amino acids 1 to 94; GAL4:Z fusions, the GAL4 DNA binding domain fused to the indicated amino acids from the ZEBRA activation domain. Each ZEBRA fusion represents a separate module identified by the deletion analysis in Fig. 2B. We resorted to in vitro-synthesized protein because GAL4:Z(2–50) and GAL4:Z(51–98) were not produced efficiently in *E. coli*. Lane − indicates the level of transcription generated by a mock-translated extract. (D) The promoter sites and activator modules are functionally interchangeable. Four representative activators, Z(131–245), Z(99–245), Z(51–245), and Z(2–245), bearing one, two, three, and four activation modules, respectively, were assayed on templates bearing one, three, five, and seven ZEBRA sites. Autoradiographs from three typical experiments were scanned on a laser densitometer. The transcription signal was normalized as described below, averaged, and plotted against the template sites; the amounts of stimulation are in arbitrary units representing the integrated areas under the curves. Background transcription was subtracted from these values. As the background transcription on each reporter template was very reproducible from experiment to experiment, we used the average to normalize the data sets. (E) Modules act synergistically to activate transcription. This graph plots the values obtained by each of the representative deletions, bearing one, two, three, and four modules, on a template containing three sites. Note that for the dimers on three sites, the total numbers of modules are 6, 12, 18, and 24.

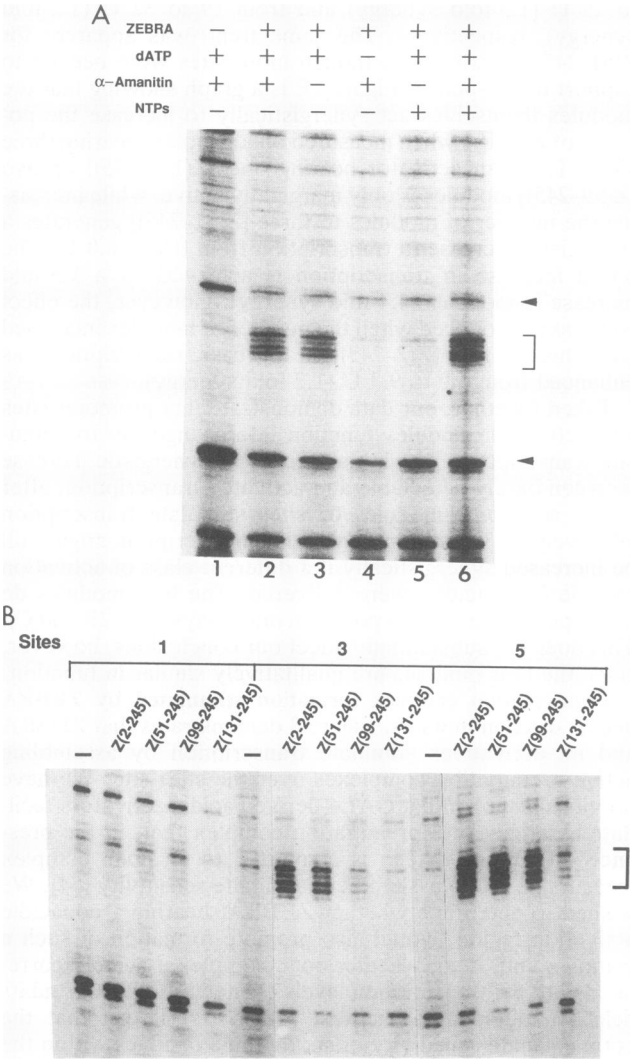

FIG. 3. Open complex formation by ZEBRA and its derivatives in a HeLa extract. (A) ZEBRA stimulates functional open complex formation. Z_7E4T (20 ng) was incubated in a HeLa cell nuclear extract with the indicated combinations of ZEBRA, dATP (0.5 mM), α-amanitin (2 μg/ml), and nucleoside triphosphates (NTPs; 0.5 mM). After 28 min, the reaction mixtures were probed with permanganate. The purified DNA was then subjected to primer extension, and the products were fractionated on a 6% polyacrylamide–urea gel. The positions of the six permanganate-sensitive thymines in the E4 start site are marked by a bracket. The arrowheads denote the hypersensitive residues at −9 and +17 which appear to result from TFIID binding (54). (B) Open complex formation by ZEBRA and the derivatives parallels their corresponding transcriptional activities. The indicated DNA templates (20 ng) were incubated in HeLa cell nuclear extract with the indicated ZEBRA derivatives in the presence of α-amanitin and dATP. Open complex formation was measured after 1 h. Incubation was for 1 h to parallel the incubation time in our standard in vitro transcription reaction. The thymines in the start site are indicated with a bracket. Ideally, the conditions used in this experiment would be identical to our standard in vitro transcription assay, which employs 200 ng of template DNA. However, the signal-to-noise ratio is lower with higher template concentrations. This is likely a result of a decreased proportion of active templates. To reduce the background resulting from nonspecific pausing of *Taq* polymerase, 20 instead of 200 ng of template was used. These changes do not affect the relative transcriptional activities (data not shown).

course experiment (data not shown). In the absence of ZEBRA, thymines in the start site region of the test promoter reacted poorly to permanganate attack (Fig. 3A, lane 1). Inclusion of ZEBRA in the preincubation mixtures greatly increased the sensitivity (lane 2). The effect was dependent on a high-energy cofactor because omission of dATP resulted in loss of the hypersensitivity (lane 4). A nonhydrolyzable β-γ imido analog of ATP (AppNHp) failed to substitute (data not shown), suggesting that the requirement was either energetic or for a phosphorylation event. Note that we often use dATP rather than ATP, as the latter reagent can be used as a substrate for transcription. We conclude that ZEBRA stimulates assembly of a preinitiation complex that likely uses ATP hydrolysis to form the open complex.

The open complexes are functional and contain RNA Pol II as measured by two criteria. First, addition of nucleoside triphosphates to reactions containing ZEBRA and dATP resulted in disappearance of the hypersensitive signal, consistent with elongation of Pol II away from the start site and reannealing of the DNA behind it (Fig. 3A, lane 5). Second, the addition of low concentrations of the Pol II elongation inhibitor α-amanitin to this mixture antagonized the effect of nucleoside triphosphates and prevented disappearance of the open complex signal (lane 6). α-Amanitin neither enhanced nor inhibited appearance of the open complex signal in the absence of nucleoside triphosphates (compare lanes 2 and 3).

The ability of the activation modules to stimulate transcription correlates with their ability to promote transcription complex assembly. Figure 3B shows the results obtained by assaying four representative deletion derivatives on templates bearing one, three, or five sites. By comparing the open complex levels in Fig. 3B with the transcriptional results in Fig. 2A, we found that the effects of the deletions, relative to ZEBRA, were comparable in both assays. This suggests that the effect of the activator is primarily on initiation. If the activator affected both initiation and elongation, there would likely be a discrepancy in the transcription versus open complex levels.

Kinetic dissection of the preinitiation complex assembly pathway. The following experiments suggest that ZEBRA stimulates transcription complex assembly by promoting formation of a subassembly requiring TFIIA and TFIID. First, the factor requirements for open complex formation were investigated in a reconstituted system. The system was composed of recombinant TFIIB, HeLa fractions TFIIA and TFIID, and a fraction containing TFIIE, TFIIF, and RNA Pol II (TFIIE/F/Pol [55]). All of the fractions and ZEBRA were required for open complex formation on Z_7E4T (Fig. 4A); omitting either TFIIA, TFIID, TFIIB, or TFIIE/F/Pol prevented appearance of the signal. Similarly, all of these factors and ZEBRA are necessary and sufficient for transcription from Z_7E4T (data not shown).

We used a kinetic assay to define the step that ZEBRA affects to promote assembly of the open complex. This assay was previously used to study the mechanism of GAL4-AH (55). We first determined the time course of open complex formation in our reconstituted system. Figure 4B shows that the open complex, assembled from a complete set of components, is detectable at 5 min and reaches a peak at 30 min. Longer incubations for up to 60 min did not further increase open complex levels (data not shown). We then preincubated various subsets of the factors with DNA template. After 30 min, the remaining components were added back to the mixture, and the incubations were allowed to continue

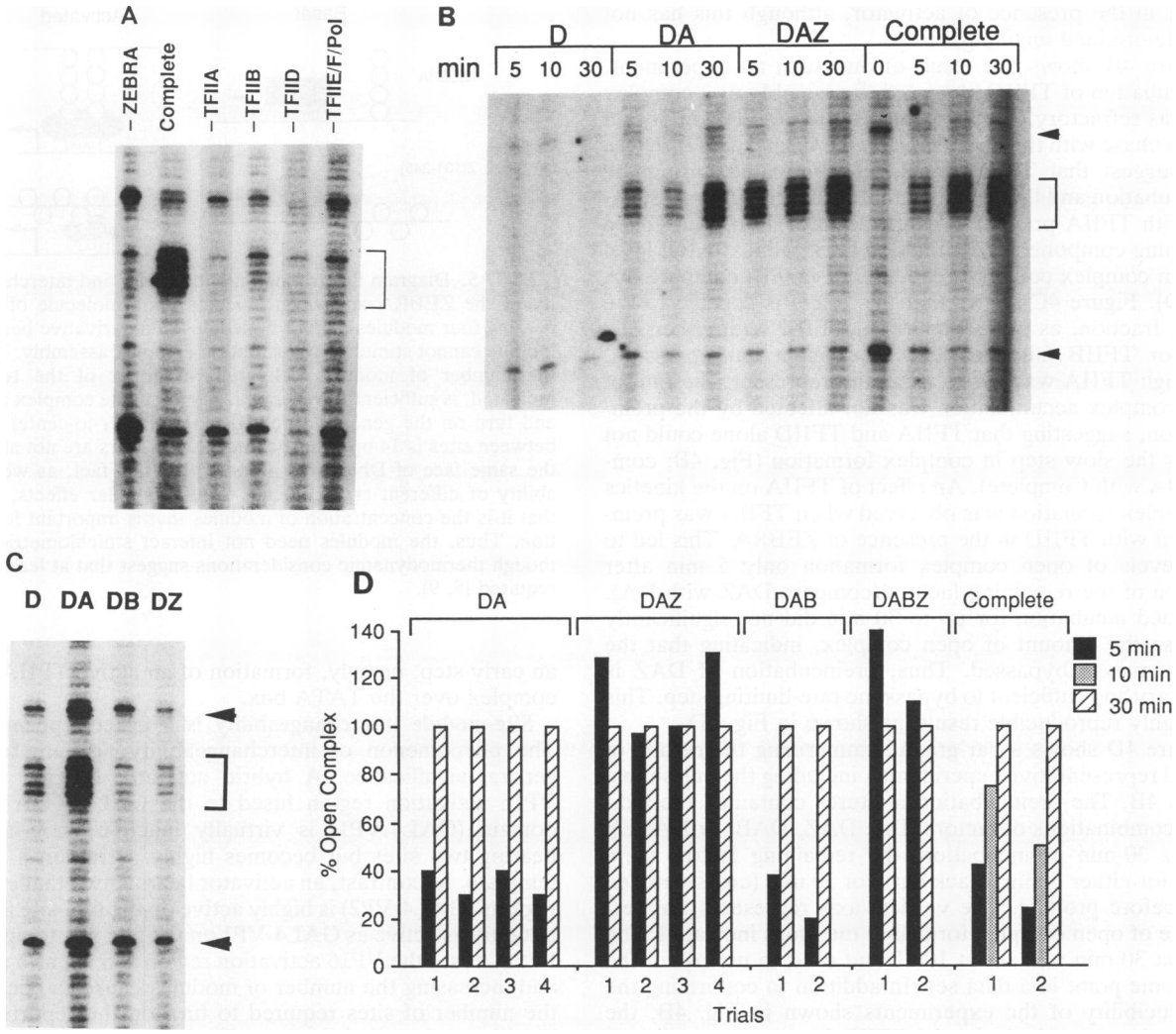

FIG. 4. Kinetic analysis of preinitiation complex assembly. (A) Factor requirements for open complex formation. The complete (Complete) reaction mixtures contained 10 ng of Z_7E4T, ZEBRA, and a full complement of general transcription factors required for transcription in vitro. Alternately, the indicated fraction was omitted from the reaction. After 30 min, open complex formation was measured. The multiple thymines in the start site are bracketed. (B) Effect of preincubation on open complex formation. Either TFIID (D), TFIID and TFIIA (DA), or TFIID, TFIIA, and ZEBRA (DAZ) were preincubated with Z_7E4T. After 30 min, the missing factors were added back with dATP and α-amanitin, and the incubation was allowed to continue for the indicated times before probing with permanganate. In the control experiment, which lacked a preincubation, a complete (Complete) set of components was mixed simultaneously and the open complex was probed various times thereafter. (C) The TFIIA fraction antirepresses TFIID inactivation. TFIID was incubated for 30 min alone (D) or with TFIIA (DA), TFIIB (DB), or ZEBRA (DZ). The missing factors were then added back and incubated for 30 min before the open complex was measured. The bracket indicates the thymines at the start site, and arrows denote hypersensitive sites described previously (52). (D) Bar graph comparing the kinetic effect of 30-min preincubations of DA, DAZ, DAB, and DABZ. During the course of the study, these different preincubation mixtures were tested for their effects on eliminating the lag in open complex formation. To compare many different experiments, the level of open complex achieved after a 30-min chase with the remaining components was set as 100%; it was our experience that this level represented the peak. We then compared the levels achieved by different preincubations at 5 min postchase, the earliest reproducible time point that we measured. Each combination of 5- and 30-min preincubations is considered a separate trial. "Complete" represents the time course of open complex formation starting from free factors. The results were reproducible in two trials. Autoradiographs from representative experiments, including the one shown in panel B, were scanned with a laser densitometer. The intensity of the open complex signal was normalized to the DNA concentration in the samples, using the background bands located from +10 to +15. These bands were due to nonspecific stalling by *Taq* polymerase. In DNA titrations, they served as a linear measure of the amount of template (data not shown). The quantitation was performed to normalize for DNA recovery, which varies in some experiments performed in the reconstituted system. Note that with some batches of TFIID, a full level of open complex could not be obtained even when the preincubations contained TFIIA, a result observed previously when the effects of acidic activators were assayed (55).

for various times before addition of potassium permanganate. If a full level of open complexes formed rapidly upon addition of the missing components, this would be taken as evidence that preincubation had bypassed a slow step in the pathway leading to complex assembly. The assembly pathway deduced in the absence of activators is known to be TFIID and then TFIIA followed by TFIIB and the remaining factors (1, 12). We assumed that the pathway would be

similar in the presence of activator, although this has not been determined unambiguously.

Figure 4B shows the result of one such an experiment. Preincubation of TFIID alone led to assembly of a complex that was refractory to open complex formation even after a 30-min chase with the missing components and dATP. These data suggest that TFIID has been inactivated during the preincubation and this effect cannot be reversed. Preincubation with TFIIA prevented inactivation of TFIID; after the remaining components were added back, almost a full level of open complex could be recovered (Fig. 4B; compare DA with D). Figure 4C shows that this effect is specific to the TFIIA fraction, as preincubation of TFIID with either ZEBRA or TFIIB failed to lead to efficient antirepression. Although TFIIA was acting as an antirepressor, the rate of open complex accumulation was not affected by the preincubation, suggesting that TFIIA and TFIID alone could not bypass the slow step in complex formation (Fig. 4B; compare DA with Complete). An effect of TFIIA on the kinetics of complex formation was observed when TFIIA was preincubated with TFIID in the presence of ZEBRA. This led to high levels of open complex formation only 5 min after addition of the remaining factors (compare DAZ with DA). Extended incubation for up to 30 min did not significantly increase the amount of open complex, indicating that the slow step was bypassed. Thus, preincubation of DAZ is necessary and sufficient to bypass the rate-limiting step. This is a highly reproducible result, as shown in Fig. 4D.

Figure 4D shows a bar graph summarizing the results of several representative experiments, including the one shown in Fig. 4B. The preincubation mixtures contained the indicated combinations of factors (DA, DAZ, DAB, or DABZ). After a 30-min preincubation, the remaining factors were added for either 5 min (black bars) or 30 min (cross-hatched bars) before probing. The vertical axis represents the percentage of open complex formed at the times indicated. The value at 30 min was set at 100% and used to normalize the other time point in a data set. In addition to confirming the reproducibility of the experiments shown in Fig. 4B, the graphs show that inclusion of TFIIB in the preincubations had no effect on the kinetics of complex assembly in two separate trials. Thus, once a complex involving TFIID, TFIIA, and ZEBRA forms on the template, the remaining components, namely, TFIIB, TFIIE/F/Pol, and other factors, can rapidly assemble into a functional transcription complex. The slow step is not due to slow binding of ZEBRA to the promoter, since binding was complete within 5 min when measured by DNase I footprinting (data not shown). We conclude that TFIIA can prevent inactivation of TFIID by forming a ZEBRA-responsive TFIIA-TFIID complex. This assembly is converted by ZEBRA, in a rate-limiting step, into an active form onto which the remaining factors can be rapidly added.

DISCUSSION

The transcriptional activation region of ZEBRA in vitro is composed of four modules. A critical number of modules is required to turn on a reporter gene in an effect that is synergistic. Increasing the number of modules delivered either by activators containing more modules or by a promoter bearing more sites elicits a similar transcriptional response. This interchangeability reflects a common mechanistic theme underlying the organization of a promoter and an individual activator. The role of the modules in ZEBRA is to promote assembly of a transcription complex by acting at an early step, namely, formation of an active TFIIA-TFIID complex over the TATA box.

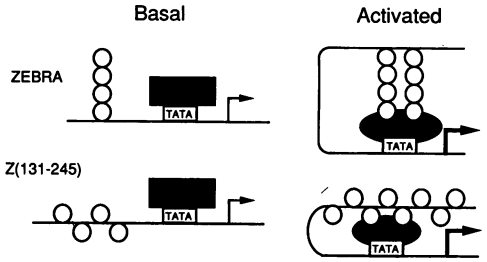

FIG. 5. Diagram illustrating the modularity and interchangeability of the ZEBRA activation modules. One molecule of ZEBRA bearing four modules or four molecules of a derivative bearing one module cannot stimulate transcription complex assembly. Doubling the number of modules delivered, in either of the two ways indicated, is sufficient to synergistically stimulate complex assembly and turn on the gene. We note that the center-to-center distance between sites is 14 bp. Thus, adjacent activators are not aligned on the same face of DNA. We propose that this fact, as well as the ability of different arrangements to elicit similar effects, suggests that it is the concentration of modules that is important for activation. Thus, the modules need not interact stoichiometrically, although thermodynamic considerations suggest that at least two are required (5, 9).

Site-module interchangeability is a general phenomenon. The phenomenon of interchangeability appears to be of general significance. A hybrid activator bearing a single VP16 activation region fused to the GAL4 DNA binding domain (GAL4-VP1) is virtually inactive on a template bearing two sites but becomes highly active on a five-site template. In contrast, an activator bearing two tandem VP16 regions (GAL4-VP2) is highly active on the two-site template but only as active as GAL4-VP1 on the five-site template (9). In this case, the VP16 activation region can act as a module, and increasing the number of modules causes a decrease in the number of sites required to turn on the reporter gene. However, four modules on one site could not turn on the gene, whereas two modules on two sites could, suggesting that in addition to the number of modules, at least two sites are required. Such a finding is not surprising, since the ability of a single activator to turn on a gene might compromise the cell's ability to use combinatorial control as a mechanism for gene regulation. The finding that a natural activator uses modularity in a manner similar to that of our GAL4-VP16 hybrids suggests that our finding has a physiological basis.

Implications of site-module interchangeability on assembly of a transcription complex. Figures 2 and 3 show that a threshold number of modules, variously arranged, act synergistically to assemble a transcription complex. Thermodynamic considerations suggest that synergy results from simultaneous contacts between the complex, or a subassembly thereof, and two or more modules. One view is that a threshold free energy is required to overcome a barrier in complex assembly. As each module binds, a quantum of free energy would be added (released) until the threshold is reached. This would lead to an exponential increase ($\Delta G = -RT \ln K$) in the affinity of the target for multiple modules versus a single module (5, 28), and the exponential increase in affinity would be manifested as a synergistic increase in transcription.

The ability of various arrangements of the modules, distributed within the protein or along the DNA, to elicit similar

transcriptional effects argues for a nonstoichiometric, dynamic model in which a limited number of modules contact the transcription complex but a certain threshold concentration is required. This model is illustrated schematically in Fig. 5. The targets can constantly dissociate but are recaptured by neighboring modules; continual sampling would promote stable association of the targets with the activation surface. This model contrasts with a simple stoichiometric model in which the cooperativity would result from all of the modules simultaneously contacting the transcription complex. A stoichiometric model, however, is not unreasonable given that the surface of the >2 million-Da transcription complex would be very large and likely able to accommodate multiple interactions.

Although we have considered synergy as an equilibrium problem, it is equally plausible that it is the result of kinetic effects (19). In this type of model, the modules would contact two components and accelerate two rate-determining steps in the transcription pathway (28). Cooperative binding of the activators to naked DNA or to chromatin (51) might also contribute to synergy, although the former has not been observed with ZEBRA and the latter has not yet been tested. We have also not tested whether superactivation, a phenomenon involved in synergistic activation by Sp1 (6, 40), contributes to the synergy. We note, however, that superactivation involves direct protein-protein interactions among Sp1 protomers, and we have not yet been able to detect this for ZEBRA.

Tuning activator potency. ZEBRA, like many other eukaryotic activators, controls a family of responsive genes. Those gene products probably need to be expressed at different levels and, in some cases, at different times during the lytic switch. Thus, mechanisms that program both the level and timing of expression must be in place. The main variables to consider are the number, affinity, and arrangement of promoter sites and the concentration and potency of ZEBRA. It is likely that these parameters coevolved to fit the biological constraints of the viral expression programs. The modular nature of eukaryotic promoters and activators is likely to facilitate such coevolution by providing it a mechanism to adjust promoter or activator strength in small, quantum steps.

Previous studies from our laboratory suggested one mechanism by which ZEBRA programs the extent and timing of EBV gene expression. EBV promoters contain between one and eight known ZEBRA binding sites. Using the model system described in this report, we found that templates bearing more ZEBRA binding sites are turned on to higher levels and by lower concentrations of ZEBRA than are templates bearing fewer sites (3). Because ZEBRA concentration rises gradually during induction, then promoters bearing more sites may be turned on more rapidly and to higher levels than are promoters bearing fewer sites.

The potency of the activator must be tuned to fit this scheme. If ZEBRA was a weak activator, as with Z(131–245), then a differential response would be possible but would require many sites to initially turn on the gene and even more to achieve high levels of activation. There will be a limit to the number of sites, as activator potency is known to decrease with distance (4). In contrast, if ZEBRA was a strong activator like VP16, which is approximately three times stronger in transient transfection assays (unpublished), then maximal level transcription would be elicited from only a few sites (three) and the differential response to sites would be lost. This situation might be suitable for a limited family of genes (VP16 is thought to control only five herpes simplex virus genes through three or fewer upstream binding sites [33]) but not for a larger family in which variability in expression levels might be crucial.

Evolution has apparently employed several strategies to tune the strength of the activator. One is to modify the amino acid composition of the activation region. This is best exemplified by the wide range of potencies displayed by acidic activation regions (5, 14, 22). The others are to change the number and arrangement of modules within a region (9), the extreme case being GCN4, whose activation domain consists of repetitive seven-amino-acid units (22). The strategies apparently coexist, resulting in activators bearing multiple regions with, in some cases, highly divergent amino acid compositions. Both strategies possibly change the potency by adjusting the free energy of interaction between an individual activator and its target.

We note that all of our experiments have been performed in vitro, and the ability to extrapolate these conclusions to in vivo situations must be regarded with caution. The in vitro system was essential, however, for controlling parameters such as activator concentration, site occupancy, stability, and other variables which are difficult to manipulate in cell culture transfection studies.

The mechanism of ZEBRA action. ZEBRA stimulates transcription by promoting preinitiation complex formation. There appears to be a checkpoint where the complex can enter either inactive or productive pathways. In our experiments, this is regulated by components present in the TFIID fraction that favor an inactive pathway. The repression is not reversible during the time course of our assay because addition of the missing components cannot rescue open complex formation after preincubation of TFIID with template. Inclusion of the TFIIA fraction in the preincubation suffices to prevent TFIID from becoming refractory to open complex formation. Repressors of transcription that work by competing with TFIIA and TFIIB for binding TFIID, including NC1, NC2 (34, 35), and Dr1 (24), have been described. However, it is also possible the inhibition is due to a novel repressor.

The activation of TFIIA-TFIID complexes by ZEBRA was apparently rate limiting because preincubation of TFIID, TFIIA, and ZEBRA shortened the time period required for full-level open complex formation compared with preincubations containing TFIIA and TFIID alone. Preincubations lacking ZEBRA took as long as 30 min to form a full level of open complex after ZEBRA was added back, similar to incubations beginning from free components. Preincubations containing ZEBRA, however, took only 5 min. One interpretation of these data is that TFIIA enters into the assembly pathway early and acts as an antirepressor by switching TFIID to a productive pathway. ZEBRA then enters the pathway and slowly converts the TFIIA-TFIID complex into an active form. ZEBRA might accomplish this by direct contact with TBP (27), TAFs, or even TFIIA. Our results do not preclude the involvement of ZEBRA in subsequent assembly steps. Our model in Fig. 5 suggests, in fact, that the ZEBRA modules may affect two different components or steps in the process.

Of particular interest is our finding that distinct types of activation regions, the alanine-proline-glutamine-rich activation modules of ZEBRA and the acidic regions of the GAL4 derivatives, appear to stimulate transcription by acting on the TFIIA-TFIID complex. The ability of different classes of activators to target identical steps in complex assembly may indicate a general mechanism of transcriptional stimulation. The requisite role of TAFs in complex assembly (TBP itself

does not mediate any of the steps described above) suggests a provocative area of future research. TAFs were hypothesized to be, in some cases, the direct targets of activators (8), a prediction confirmed with Sp1 (20). It is possible that ZEBRA mediates assembly of the TFIIA-TFIID complex through direct interactions with TAFs.

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REFERENCES

1. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56**:549–561.
2. **Carey, M.** 1991. Mechanistic advances in eukaryotic gene activation. Curr. Opin. Cell. Biol. **3**:452–460.
3. **Carey, M., J. Kolman, D. Katz, L. Gradoville, L. Barberis, and G. Miller.** 1992. Transcriptional synergy by the Epstein-Barr virus transactivator ZEBRA. J. Virol. **66**:4803–4813.
4. **Carey, M., J. Leatherwood, and M. Ptashne.** 1990. A potent GAL4 derivative activates transcription at a distance in vitro. Science **247**:710–712.
5. **Carey, M., Y.-S. Lin, M. R. Green, and M. Ptashne.** 1990. A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. Nature (London) **345**:361–364.
6. **Courey, A., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55**:887–898.
7. **Dynan, W. S.** 1989. Modularity in promoters and enhancers. Cell **58**:1–4.
8. **Dynlacht, B. D., T. Hoey, and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66**:563–576.
9. **Emami, K. H., and M. Carey.** 1992. A synergistic increase in potency of a multimerized VP16 transcriptional activation domain. EMBO J. **11**:5005–5012.
10. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8**:127–132.
11. **Flemington, E., A. Borras, J. Lytle, and S. Speck.** 1992. Characterization of the Epstein-Barr virus BZLF1 protein transactivation domain. J. Virol. **66**:922–929.
12. **Flores, O., H. Lu, M. Killeen, J. Greenblatt, Z. F. Burton, and D. Reinberg.** 1991. The small subunit of transcription factor TFIIF recruits RNA polymerase II into the preinitiation complex. Proc. Natl. Acad. Sci. USA **88**:9999–10003.
13. **Gerster, T., C.-G. Balmaceda, and R. G. Roeder.** 1990. The cell type-specific octamer transcription factor OTF-2 has two domains required for the activation of transcription. EMBO J. **9**:1635–1643.
14. **Gill, G., I. Sadowski, and M. Ptashne.** 1990. Mutations that increase the activity of a transcriptional activator in yeast and mammalian cells. Proc. Natl. Acad. Sci. USA **87**:2127–2131.
15. **Giot, J.-F., I. Mikaelian, M. Buisson, E. Manet, I. Joab, J.-C. Nicolas, and A. Sergeant.** 1991. Transcriptional interference between the EBV transcription factors EB1 and R: both DNA-binding and activation domains of EB1 are required. Nucleic Acids Res. **19**:1251–1258.
16. **Greenblatt, J.** 1991. RNA polymerase-associated transcription factors. Trends Biochem. Sci. **16**:408–411.
17. **Hardwick, J. M., L. Tse, N. Applegren, J. Nicholas, and M. A. Veliuona.** 1992. The Epstein-Barr virus R transactivator (Rta) contains a complex, potent activation domain with properties different from those of VP16. J. Virol. **66**:5500–5508.
18. **Hawley, D.** 1991. Transcriptional activation: enter TFIIB. Trends Biochem. Sci. **16**:317–318.
19. **Hershlag, D., and F. B. Johnson.** 1993. Synergism in transcriptional activation: a kinetic view. Genes Dev. **7**:173–179.
20. **Hoey, T., R. O. J. Weinzierl, G. Gill, J.-L. Chen, B. D. Dynlacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of drosophila TAF110 reveal properties expected of coactivators. Cell **72**:247–260.
21. **Hollenberg, S. M., and R. M. Evans.** 1988. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. Cell **55**:899–906.
22. **Hope, I., S. Mahadevan, and K. Struhl.** 1988. Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein. Nature (London) **333**:635–640.
23. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenlatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature (London) **351**:588–590.
24. **Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg.** 1992. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. Cell **70**:477–489.
25. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67**:365–376.
26. **Lieberman, P. M., and A. J. Berk.** 1990. In vitro transcriptional activation, dimerization and DNA binding specificity of the Epstein-Barr virus Zta protein. J. Virol. **63**:3040–3050.
27. **Lieberman, P. M., and A. J. Berk.** 1991. The Zta trans-activator protein stabilizes TFIID association with promoter DNA by direct protein-protein interaction. Genes Dev. **5**:2441–2454.
28. **Lin, Y.-S., M. F. Carey, M. Ptashne, and M. R. Green.** 1988. GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell **54**:659–664.
29. **Lin, Y.-S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator in vitro. Cell **64**:971–981.
30. **Lin, Y.-S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature (London) **353**:569–571.
31. **Lu, H., L. Zawel, L. Fisher, J. M. Egly, and D. Reinberg.** 1992. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. Nature (London) **358**:641–645.
32. **Ma, J., and M. Ptashne.** 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48**:847–853.
33. **Mackem, S., and B. Roizman.** 1982. Structural features of the α gene 4, O, and 27 promoter regulatory sequences which confer a regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
34. **Meisterernst, M., and R. G. Roeder.** 1991. Family of proteins that interact with TFIID and regulate promoter activity. Cell **67**:557–567.
35. **Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder.** 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. Cell **66**:981–933.
36. **Miller, G.** 1990. The switch between latency and replication of Epstein-Barr virus. J. Infect. Dis. **161**:833–844.
37. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence specific DNA binding proteins. Science **245**:371–378.
38. **Muller-Immergluck, M. M., W. Schaffner, and P. Matthias.** 1990. Transcription factor Oct-2A contains functionally redundant activating domains and works selectively from a promoter but not from a remote enhancer position in non-lymphoid (HeLa) cells. EMBO J. **9**:1625–1634.
39. **Ondek, B., L. Gloss, and W. Herr.** 1988. The SV40 enhancer contains two distinct levels of organization. Nature (London) **333**:40–45.
40. **Pascal, E., and R. Tjian.** 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. **5**:1646–1656.
41. **Ptashne, M.** 1988. How transcriptional activators work. Nature (London) **335**:683–689.
42. **Ptashne, M.** 1992. A genetic switch, 2nd ed. Blackwell Press, New York.

43. **Roeder, R. G.** 1991. The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. Trends Biochem. Sci. **16**:402–408.
44. **Schaeffer, L., R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J. H. J. Hoeijmakers, P. Chambon, and J. M. Egly.** 1993. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science **260**:58–63.
45. **Schaffner, G., S. Schirm, B. Muller-Baden, F. Weber, and W. Schaffner.** 1988. Redundancy of information in enhancers as a principle of mammalian transcription control. J. Mol. Biol. **201**:81–90.
46. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345**:783–786.
47. **Sundseth, R., and U. Hansen.** 1992. Activation of RNA polymerase II transcription by the specific DNA-binding protein LSF. J. Biol. Chem. **267**:7845–7855.
48. **Sutherland, J. A., A. Cook, A. J. Bannister, and T. Kouzarides.** 1992. Conserved motifs in Fos and Jun define a new class of activation domain. Genes Dev. **6**:1810–1819.
49. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2, interdependent activation domains induce Oct-2 phosphorylation. Cell **60**:375–386.
50. **Tanese, N., B. F. Pugh, and R. Tjian.** 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev. **5**:2212–2224.
51. **Taylor, I. C. A., J. Workman, T. J. Schuetz, and R. E. Kingston.** 1991. Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential functions of DNA binding domains. Genes Dev. **5**:1285–1298.
52. **Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon.** 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell **59**:477–487.
53. **Wampler, S. L., and J. T. Kadonaga.** 1992. Functional analysis of Drosophila transcription factor IIB. Genes Dev. **6**:1542–1552.
54. **Wang, W., M. Carey, and J. D. Gralla.** 1992. Polymerase II promoter activation: closed complex formation and ATP-driven start-site opening. Science **255**:450–453.
55. **Wang, W., J. Gralla, and M. Carey.** 1992. The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. Genes Dev. **6**:1716–1727.
56. **White, J., C. Brou, J. Wu, Y. Lutz, V. Moncollin, and P. Chambon.** 1992. The acidic activator GAL4-VP16 acts on preformed template-committed complexes. EMBO J. **11**:2229–2240.
57. **Zawel, L., and D. Reinberg.** 1992. Initiation of transcription by RNA polymerase II: a multistep process. Prog. Nucleic Acid Res. Mol. Biol. **44**:67–108.