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Interaction of the 82-kDa subunit of the vaccinia virus early transcription factor heterodimer with the promoter core sequence directs downstream DNA binding of the 70-kDa subunit

(protein–DNA crosslinking/DNA bending/DNA-binding specificity/aryl azide)

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ABSTRACT **The vaccinia virus early transcription factor (VETF), a heterodimeric protein composed of 82- and 70-kDa subunits, interacts with viral early promoters at both a sequence-specific core region upstream and a sequence-independent region downstream of the RNA start site. To determine the VETF subunit-promoter interactions, ^{32}P-labeled DNA targets were chemically synthesized with uniquely positioned phosphorothioates to which azidophenacyl bromide moieties were coupled. After incubating the derivatized promoter with VETF and exposing the complex to 302-nm light, the protein was denatured and the individual subunits with or without covalently bound DNA were isolated with specific antiserum and analyzed by SDS/polyacrylamide gel electrophoresis. Using a set of 26 duplex probes, with uniquely positioned aryl azide moieties on the coding or template strands, we found that the 82-kDa subunit interacted primarily with the core region of the promoter, whereas the 70-kDa subunit interacted with the downstream region. Nucleotide substitutions in the core region that downregulate transcription affected the binding of both subunits: the 82-kDa subunit no longer exhibited specificity for upstream regions of the promoter but also bound to downstream regions, whereas the binding of the 70-kDa subunit was abolished even though the mutations were far upstream of its binding site. These results suggested mechanisms by which the interaction of the 82-kDa subunit with the core sequence directs binding of the 70-kDa subunit to DNA downstream.**

Vaccinia virus, a large double-stranded DNA virus that replicates in the cytoplasm of infected cells, has nearly 200 genes that can be divided into three temporal classes—early, intermediate, and late—which are regulated at the transcriptional level by a cascade mechanism (1). The macromolecular components necessary for transcription of viral early genes are contained within the virus particle and brought with it into the host cell during infection (2–4). Early transcription has been reconstituted *in vitro* using a viral early promoter template, purified vaccinia virus early transcription factor (VETF), and purified viral RNA polymerase containing RAP94, an associated transcription specificity factor (5–8). The nucleotide sequence of a cis-acting core promoter region, located from nucleotides −13 to −28 relative to the RNA start site, determines the level of transcription (9) and stability of binding to VETF (10).

VETF is a multifunctional protein: it recruits RNA polymerase to the template (11, 12), induces promoter bending (13), and has intrinsic DNA-dependent ATPase activity (14, 15). VETF is composed of 70- and 82-kDa subunits, which are encoded by the viral D6R and A8L open reading frames, respectively (16, 17). The 70-kDa subunit contains the ATPase catalytic site (18); no activity has been associated with the 82-kDa subunit. DNA footprinting and methylation interference analyses showed that VETF interacts with viral promoters at sites upstream (−29 to −12) and downstream (+8 to +10) of the RNA start site, even though the latter region provides no sequence specificity for binding (13). Little is known regarding the specific subunit interactions with the promoter. Broyles and Li (19) incorporated 5-bromo-dUMP residues into the promoter template strand upstream of the RNA start site and reported crosslinking by 254-nm light to the 70-kDa polypeptide alone or as a subunit of VETF. Crosslinking products were not detected when the nontemplate strand contained bromouracil moities downstream of the RNA start site; nor was crosslinking of the 82-kDa subunit of VETF to DNA detected in that study.

To further analyze the interactions between VETF and early promoters, we chose a crosslinking technique that has recently been employed in the study of other DNA-binding proteins (20, 21). An important advantage of this new method is that a phosphorothioate, to which the crosslinking agent azidophenacyl bromide (AZPB) is coupled, can be positioned at any site within either DNA strand. Upon long wave length (302 nm) ultraviolet irradiation, the reactive azido group covalently links contact points on the DNA-binding protein. We modified the analysis to determine the crosslinking of individual subunits of VETF to the promoter. Here, we report that the 82- and 70- kDa subunits of VETF interact primarily with upstream and downstream regions of the promoter, respectively.

MATERIALS AND METHODS

Purification of VETF. VETF was isolated from vaccinia virus virions by a method similar to that of Broyles *et al.* (7). Soluble proteins were extracted from purified virions and chromatographed successively on two DEAE-cellulose columns and a single-stranded DNA-agarose column.

Preparation of Azidophenacyl Modified Oligonucleotides. The nucleotide sequence of the vaccinia virus growth factor early promoter is shown in Fig. 1*A*. A series of oligonucleotides with two adjacent phosphorothioate substitutions in one strand were chemically synthesized (Cruachem, Herndon, VA) and designated by the position of the nucleotide flanked by phosphorothioates, relative to the RNA start site. The oligonucleotides were gel-purified, 5′ end-labeled (to a specific activity between 0.32 and 0.98 μci/pmol) with T4 polynucleotide kinase, and coupled to AZPB as described (21). Approximately 20 pmol of each radiolabeled oligonucleotide was incubated at room temperature for 1 hr in 100 μl of 20 mM sodium bicarbonate (pH 9.0)/45% (vol/vol) dimethyl sulfox-

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Abbreviations: AZPB, azidophenacyl bromide; VETF, vaccinia virus early transcription factor.

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ide/5.0 mM AZPB, extracted four times with isobutyl alcohol, and ethanol precipitated. These modified oligonucleotides were subsequently annealed with 1.5 × the molar amount of the complementary oligonucleotide for 5 min at 55°C followed by incubation at room temperature for 2 hr.

DNA–Protein Crosslinking. In individual wells of a 96-well plate, 1 pmol of the labeled duplex DNA was incubated with 4 pmol of VETF in a reaction mixture containing 50 pmol of poly(dI-dC)/50 mM KCl/10 mM Tris·HCl, pH 8.0/0.2 mM EDTA/0.05% Nonidet P-40/5% glycerol for 20 min in the dark at room temperature. The plates were placed on ice and irradiated with 302-nm light at 5 cm for 2 min. Short wavelength light of less than 300 nm was screened out by the polystyrene cover of the plate (21).

Immunoprecipitation of Crosslinked Products. Samples containing 0.2 pmol of irradiated DNA-protein complexes were treated with 1% SDS at 85°C for 3 min. The reaction mixtures were diluted with 10 vol of 50 mM Tris·HCl, pH 7.4/150 mm NaCl/1% Nonidet P-40, and then incubated for 16 hr at 4°C with 20 μl of polyclonal antiserum raised to the 82- or 70-KDa subunit of VETF (17). Alternatively, the SDS treatment step was omitted and the samples containing irradiated DNA-protein complexes were incubated directly with antibody. In either case, antigen–antibody complexes were then incubated with 100 μl of protein A-Sepharose (Pharmacia) suspension (20% vol/vol) for 1 hr at 4°C, collected by centrifugation, and washed twice with 50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% Triton X-100. After the addition of SDS/PAGE loading buffer and incubation at 85°C for 3 min, the supernatant was applied to a 10% polyacrylamide/SDS gel. The gels were dried and exposed to Kodak BioMax MR x-ray film with two DuPont Cronex Lightening Plus KE screens for 4 days.

RESULTS

Crosslinking of VETF to a Viral Early Promoter. The promoter (Fig. 1*A*) selected for our crosslinking study was previously used to determine VETF–DNA interactions (13, 19). Based on DNA footprinting analysis, we designed the first probe with a phosphorothioate on each side of nucleotide −25, relative to the RNA start site of the coding strand. The oligonucleotide was 5′ end-labeled with ^{32}P, derivatized with AZPB, and hybridized to its complementary unmodified strand. Control probes were also synthesized without derivatization. The double-stranded DNAs were incubated with VETF and irradiated with 302 nm light. Analysis of the products by a gel mobility-shift assay indicated that the chemical modifications had not interfered with complex formation (Fig. 1*B*, lanes 3 and 4). Samples from identical reaction mixtures were treated with SDS to dissociate noncovalently linked complexes and analyzed by SDS/PAGE. Covalent linking of VETF to DNA only occurred when the DNA was coupled to AZPB and UV-irradiated (Fig. 1*C*, lane 2). In this experiment, approximately 5% of the labeled probe was covalently linked to VETF. Nonspecific coupling of DNA to bovine serum albumin was not detected (Fig. 1*C*, lane 4).

Immunoprecipitation of Crosslinked Products. The crosslinking experiment described above was consistent with footprinting data, indicating that VETF contacted the core region of the viral promoter. However, because of the similar sizes of the two subunits and the added mass of the DNA probe, we could not determine which VETF subunit was crosslinked. To resolve this question, a sensitive immunoprecipitation protocol was devised. SDS was added to the reaction mixtures to dissociate noncovalently linked components; then the individual subunits, with or without attached ^{32}P-labeled DNA, were captured with subunit-specific antibody and analyzed by SDS/PAGE and autoradiography.

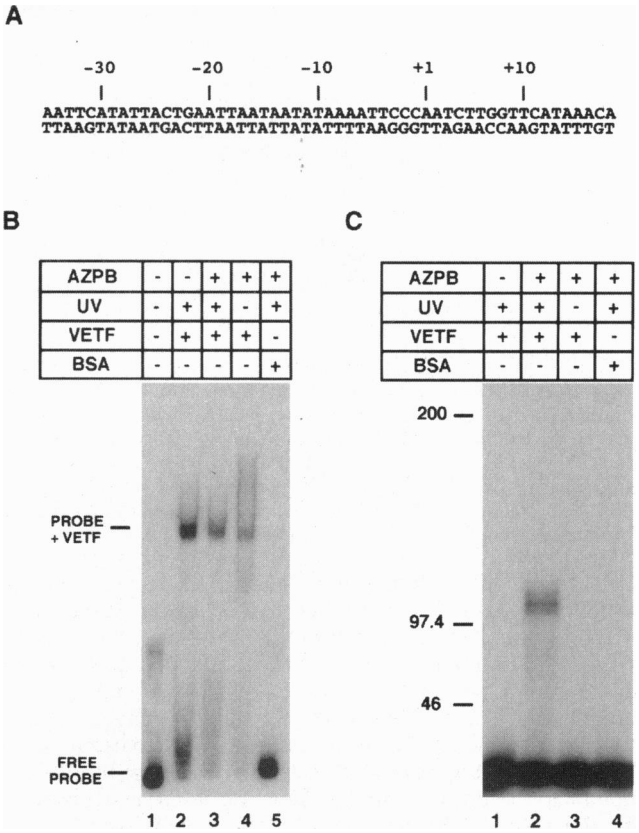

FIG. 1. Photoaffinity-labeling of VETF. (*A*) Sequence of coding (top) and template (bottom) strands of an early promoter. The RNA start site is designated +1. (*B*) Gel mobility-shift assay of VETF incubated with an unmodified (lanes 1 and 2) or an AZPB derivatized (lanes 3–5) ^{32}P-labeled promoter. The derivatized probe contains a pair of aryl azides flanking nucleotide −25 of the coding strand. The components of each reaction are indicated above the lane. The reaction mixtures were analyzed by electrophoresis in a 4% nondenaturing gel and an autoradiograph was made. (*C*) Crosslinking of VETF to a derivatized ^{32}P-labeled promoter. The components of the reaction mixtures are indicated as above. After incubation, the reaction mixtures were treated with SDS, resolved by SDS/PAGE, and autoradiographed.

Two radioactive probes were used to test the procedure: one contained aryl azides flanking the −25 position, and the other the +12 position, of the coding (top) strand (Fig. 1*A*). In each case, the complementary strand was not derivatized. SDS/PAGE analysis of the immunoprecipitated complexes indicated that both probes were crosslinked to VETF (Fig. 2, lanes 2 and 3). After SDS dissociation, only the antiserum against the 82-kDa subunit immunoprecipitated protein linked to the aryl azides at the −25 position (Fig. 2, lane 4). Conversely, the protein linked to the aryl azides at the +12 position was immunoprecipitated only with antiserum to the 70-kDa subunit (Fig. 2, lane 5). The antisera were raised against recombinant proteins eluted from SDS gels, accounting for the more efficient immunoprecipitation of denatured proteins compared with native ones (17). Preimmune sera did not immunoprecipitate the crosslinked complexes (data not shown).

Systematic Analysis of Subunit Crosslinking. We surveyed the entire promoter using a set of 26 duplex probes, each containing a pair of aryl azides flanking a single nucleotide on either the coding or template strand. Analysis was carried out as described above by immunoprecipitation of the crosslinked material before or after SDS dissociation (Fig. 3). Aryl azides between bases −29 and +4 in the coding strand and −29 and −1 in the template strand were principally crosslinked to the 82-kDa subunit. By contrast, aryl azides between bases +8 and

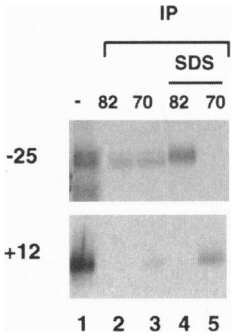

FIG. 2. Identification of photoaffinity-labeled VETF subunits. Two probes containing aryl azides flanking nucleotide −25 or nucleotide +12 of the coding strand were incubated with VETF and irradiated. Reaction products were immunopurified with antiserum against the 82-kDa (lanes 2 and 4) or the 70-kDa (lanes 3 and 5) subunit before (lanes 2 and 3) or after (lanes 4 and 5) SDS denaturation. The total (lane 1) or immunopurified (lanes 2–5) complexes were resolved by SDS/PAGE and autoradiographed.

+16 in the coding strand and +4 and +12 in the template strand were crosslinked principally to the 70-kDa subunit. This preference was not absolute, however, since some crosslinking of the 70-kDa subunit to aryl azides at −29 and at −9 to +4 of the coding strand and some crosslinking of the 82-kDa subunit to aryl azides between +8 and +16 of the template strand were detected. Nevertheless, the overall pattern of the 82-kDa subunit binding to the upstream region and the 70-kDa subunit to the downstream region was evident as depicted in the summary of the data in Fig. 4*B*. Taken together, the binding of the two subunits may account for the previous DNA footprinting and methylation interference data (Fig. 4*A*).

DNA-Binding Specificity of VETF. To determine if the binding pattern of the VETF subunits was dependent on the promoter core sequence, we introduced point mutations at positions −28, −23, and −14 because similar nucleotide substitutions had been shown to reduce transcription drastically (9). Two probes were produced for both the unmutated and mutated promoters: one contained aryl azides flanking nucleotide −25 and the other flanking position +12 of the coding strand. As also shown in the preceding section, the unmutated probes with aryl azides at the −25 and +12 positions crosslinked to the 82- and 70-kDa subunits, respectively (Fig. 5*A*, lanes 4 and 5). Surprisingly, the mutated probes with aryl azides at the −25 and +12 positions both crosslinked to the 82-kDa subunit (Fig. 5*B*, lane 4), but neither probe crosslinked efficiently to the 70-kDa subunit (Fig. 5*B*, lane 5). Thus, the crosslinking pattern with the mutated +12 derivatized probe (Fig. 5*B*, lanes 4 and 5) was opposite to that exibited by the unmutated +12 derivatized probe (Fig. 5*A*, lanes 4 and 5). Apparently, the core promoter mutations resulted in promiscuous 82-kDa binding and abrogation of 72-kDa binding.

To further explore the binding specificities, aryl azides were attached to phosphorothioates flanking position −25 of promoter probes that had 8, 18, or 28 nucleotides deleted from the downstream end of the promoter. As illustrated in Fig. 6*A*, VETF crosslinked to each of the templates. Immunoprecipitation with each subunit-specific antibody revealed that the 82-kDa, but not the 70-kDa subunit, bound to each of the truncated promoters (Fig. 6*B*).

DISCUSSION

In the photo-crosslinking procedures of Yang and Nash (21) and Mayer and Barany (20), aryl azides are chemically linked to phosphorothioate residues at specific positions within the polynucleotide chain. A distance of 11 Å separates a phosphorous atom

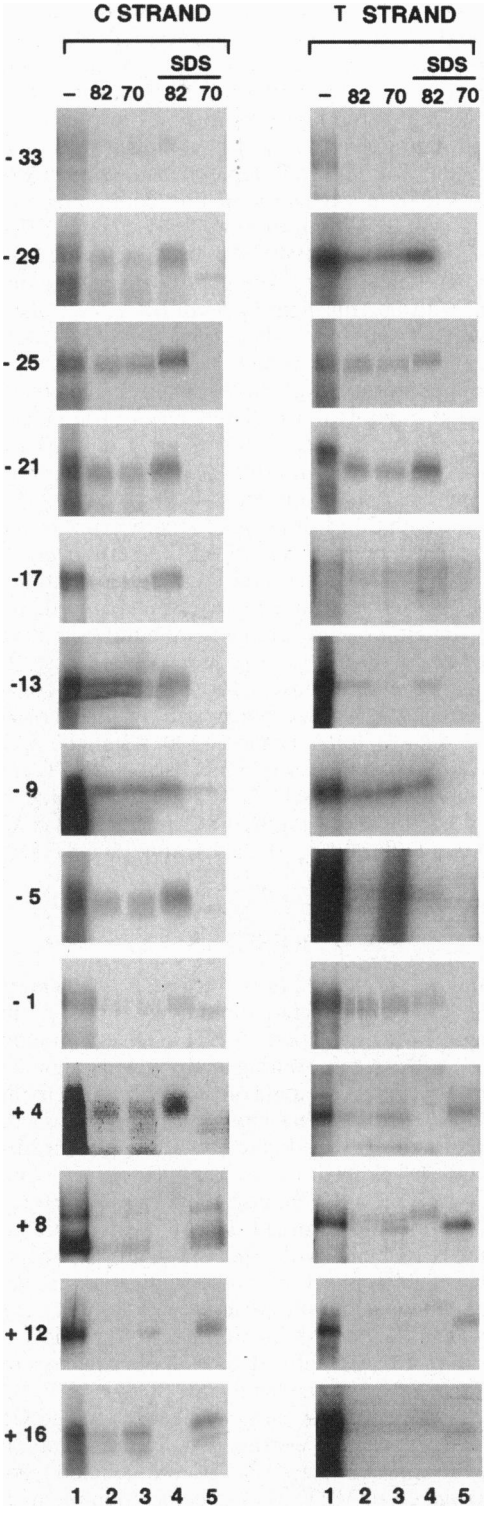

FIG. 3. Systematic screening of promoter–VETF subunit interactions. Each probe contained a pair of aryl azides flanking the nucleotide indicated to the left of the coding (C) or template (T) strand. The probes were incubated with VETF and irradiated. The entire reaction mixture (lane 1), or protein–DNA complexes immunoprecipitated with antiserum to the 82-kDa subunit (lanes 2 and 4) or 70-kDa subunit (lanes 3 and 5) before (lanes 2 and 3) or after (lanes 4 and 5) SDS denaturation, were resolved by SDS/PAGE and autoradiographed.

and the reactive nitrogen of a coupled aryl azide (21). Protein that is bound to such DNA can be covalently crosslinked by long wavelength UV irradiation, which will not crosslink protein to

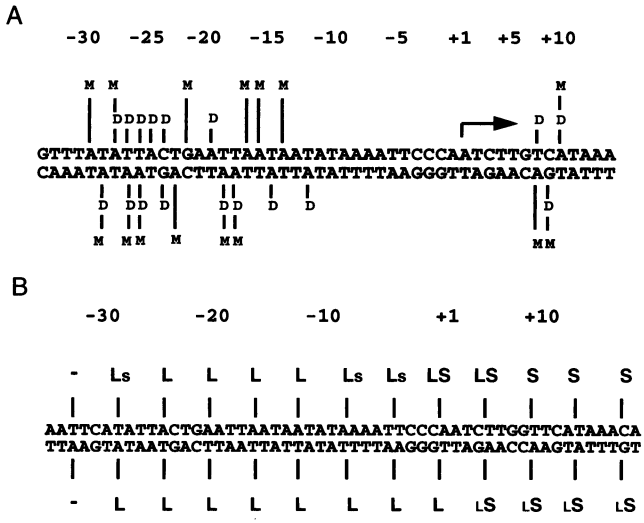

FIG. 4. Comparison of DNA footprinting and subunit crosslinking. (*A*) The interaction of VETF with the early promoter was previously determined by footprinting and methylation interference assays (13). The nucleotides that were protected by DNaseI digestion are designated D, whereas those nucleotides whose methylation interferes with VETF binding are designated M. (*B*) Interaction of VETF subunits with the promoter as determined by crosslinking experiments. Crosslinking of the 82-kDa subunit to the promoter is indicated by L (for large subunit) and the crosslinking of the 70-kDa subunit is indicated by S (for small subunit). In positions where crosslinking of both subunits occurred, the fainter band is denoted by a small letter. The symbol − indicates that no crosslinking was detected.

unmodified DNA. Moreover, the rigid nature and relatively short length of the crosslinking substituent restricts its access to a small segment of the protein. We have adapted this procedure to analyze the interactions of multisubunit proteins with ^{32}P-labeled DNA. The irradiated complexes were treated with SDS and the individual subunits, with or without covalently linked DNA, were captured by immunoprecipitation and separately analyzed by PAGE and autoradiography.

Previous transcription studies had mapped the sequence-specific functional elements of a vaccinia virus early promoter to the core region from −13 to −28, relative to the RNA start

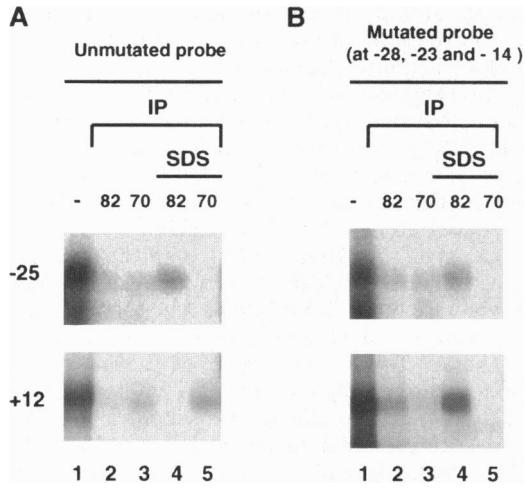

FIG. 5. Effect of promoter core mutations on crosslinking of VETF. The probes contained aryl azides flanking nucleotides −25 or +12 of the coding strand. In the mutated template, the nucleotides at positions −28, −23, and −14 were substituted with C. Each template was incubated with VETF and irradiated. Aliquots were analyzed directly (lane 1) or after immunoprecipitation with antiserum against the 82-kDa (lanes 2 and 4) or the 70-kDa (lanes 3 and 5) subunit before (lanes 2 and 3) or after (lanes 4 and 5) SDS denaturation.

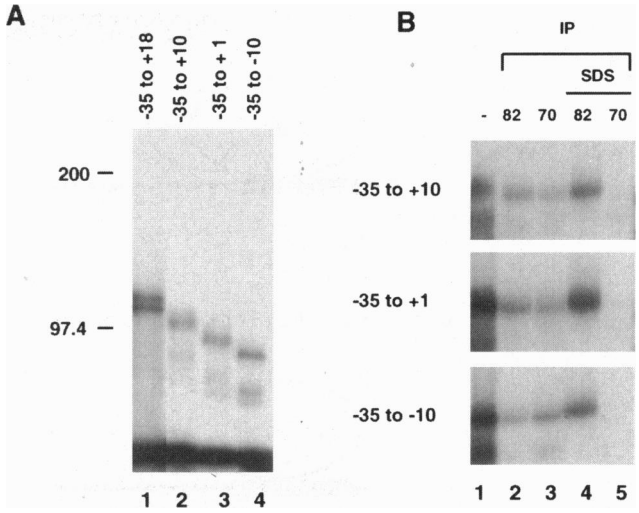

FIG. 6. VETF crosslinking to truncated promoters. (*A*) The crosslinked products, formed by irradiation of reaction mixtures containing VETF and probes consisting of the early promoter sequence from −35 to +18 (lane 1), −35 to +10 (lane 2), −35 to +1 (lane 3), or −35 to −10 (lane 4), were analyzed by SDS/PAGE and autoradiography. (*B*) SDS/PAGE analysis of the immunoprecipitated crosslinked subunits. Aliquots of the reaction mixtures were analyzed directly (lane 1) or after immunoprecipitation with antiserum to the 82-kDa subunit (lanes 2 and 4) or the 70-kDa subunit (lanes 3 and 5) either before (lanes 2 and 3) or after (lanes 4 and 5) SDS dissociation.

site (9). By contrast, footprinting and methylation interference experiments had revealed that VETF interacted with a non-sequence-specific region downstream of the RNA start site in addition to the sequence-specific core region (13). To resolve this apparent paradox, we analyzed the interactions of VETF to DNA upstream and downstream of the RNA start site by the crosslinking procedure described above. We prepared 1 set of 13 oligonucleotides, each containing a single pair of aryl azides flanking a nucleotide in the coding strand of a vaccinia virus early promoter and a second set similarly modified in the template strand. In this manner, the reactivity of azido groups next to every fourth base of each strand in the 53-bp DNA segment was analyzed. We found that the upstream core region interacted principally with the 82-kDa subunit, whereas the downstream region interacted principally with the 70-kDa subunit. These results suggested that the 82-kDa subunit is responsible for the sequence specificity of the heterodimeric VETF and that both subunits contribute to the footprinting data. It would be interesting to examine the interactions of the free 82-kDa subunit with DNA, but the latter has not been obtained in soluble form (18).

Broyles and Li (19) analyzed the crosslinking of VETF to promoters of similar length and sequence as those used in the present study, except that one probe had 5-bromouracil moieties substituted for all 16 thymidines from −6 to −35 of the template strand and the other contained six 5-bromouracil moieties substituted for all thymidines of the coding strand between +3 and +13. With the upstream probe they found crosslinking of the 70-kDa subunit but not the 82-kDa subunit; with the downstream probe no crosslinking was detected. Differences between those results and ours may be due to the extensive bromouracil modification of the DNA.

Surprisingly, base substitutions in the core region designed to destroy the function of the promoter did not prevent crosslinking of the 82-kDa subunit to DNA, but instead prevented crosslinking of the 70-kDa subunit at its normal downstream position. Furthermore, the 82-kDa subunit no longer exhibited positional specificity: it was crosslinked to downstream as well as upstream regions of the mutated promoter. The 82-kDa subunit was also crosslinked to probes

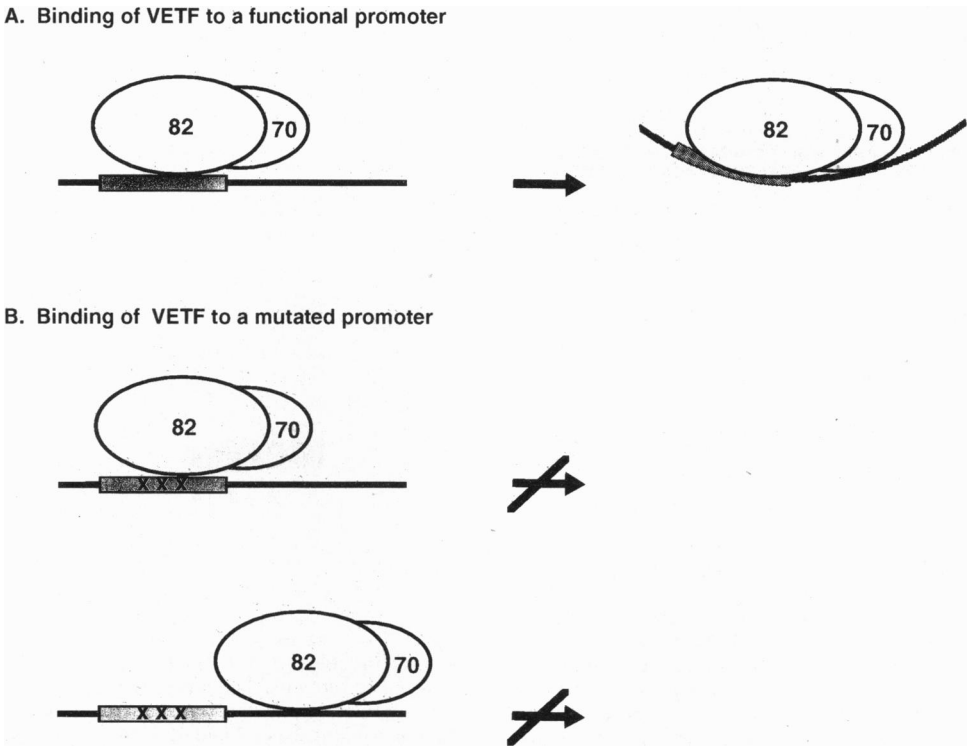

FIG. 7. Model depicting the binding of VETF subunits to an early promoter. (*A*) The 82-kDa subunit of VETF is shown binding to the core region of an early promoter (hatched box) resulting in the bending of the DNA and binding of the 70-kDa subunit. (*B*) The 82-kDa subunit of VETF is shown binding to the core region or to the downstream region of a mutated promoter (hatched box with XXX). Bending of DNA and binding of the 70-kDa subunit do not occur.

in which the downstream region was deleted and that are too short to demonstrate gel mobility shifts (13). Thus, the 82-kDa subunit can be crosslinked to DNA lacking either the sequence specificity or length of a functional promoter. Crosslinking of the 70-kDa subunit appears to be very stringent, occurring only with a functional promoter.

The present results, together with data obtained from studies of VETF footprinting and DNA bending (13), can be accommodated by the model depicted in Fig. 7*A*. We propose that binding of the 82-kDa subunit to the specific core sequence induces DNA bending and close interaction of the 70-kDa subunit with the downstream sequence. For some DNA-binding proteins, and perhaps for VETF as well, DNA bending results from intercalation of an amino acid side chain into the DNA helix (22). We suggest that amino acid intercalation occurs only when the 82-kDa subunit interacts with the early promoter consensus sequence. Stringent sequence specificity is not required, however, for binding of the 82-kDa subunit to DNA, as shown by crosslinking to mutated promoters. However, under the latter conditions, the 70-kDa subunit does not interact with DNA (Fig. 7*B*). The additional interactions of the 82- and 70-kDa subunits, which occur when VETF binds to a functional promoter sequence, may stabilize the complex and account for the predominant crosslinking of the 82-kDa subunit to the core sequence. A contribution of the 70-kDa subunit to the stability of the VETF–DNA complex is consistent with the destabilizing effect of ATP hydrolysis observed by Broyles (15) and the evidence discussed below that the 70-kDa subunit contains the ATPase catalytic site. When a truncated promoter is used for binding of VETF, the 82-kDa subunit might still interact specifically with the core sequence, but the length of the DNA would be insufficient to contact the 70-kDa subunit. We also considered an alternative model in which VETF undergoes a conformational change, upon binding to the core sequence, which brings the 70-kDa subunit in proximity to DNA. In fact, both DNA bending and conformational changes in VETF could occur simultaneously.

We have considered how our findings relate to the DNA-dependent ATPase activity of VETF (14). Although this ATPase activity is stimulated by DNA, there is no evident DNA sequence specificity requirement (14). The presence of both an A-type ATP-binding motif and a DEAH sequence suggested that the catalytic site resides in the 70-kDa subunit of VETF (16, 17). Consistent with this, Li and Broyles (18) showed that VETF molecules containing point mutations in these motifs still exhibited promoter binding but lacked ATPase and transcripitional activities. However, the 70-kDa subunit alone had little ATPase activity even in the presence of DNA (18) suggesting that the 82-kDa subunit contributes to catalysis or DNA binding. Since our data showed crosslinking only of the 82-kDa subunit when VETF binds to nonconsensus promoter sequences, stimulation of ATPase activity may be mediated through association of the 82-kDa subunit with DNA and subsequent protein–protein interactions.

Promoter-specific DNA-binding and DNA-dependent ATPase activity are also features of transcription initiation by RNA polymerase II (23, 24) and some functional analogies can be drawn between VETF and components of the eukaryotic system. The A+T-rich core region of vaccinia virus early promoters has similarities to the TATA box. The TATA box-binding protein, like the 82-kDa subunit of VETF, binds to a short core promoter region and is capable of bending DNA. The 70-kDa subunit of VETF may be compared with one or more of the TATA box-binding protein accessory factors that bind DNA downstream of the TATA box. The DNA-dependent ATPase activity of VETF suggests that the 70-kDa subunit may also provide some functions of the basal transcription factor TFIIH.

In summary, we have demonstrated an association of the 82- and 70-kDa subunits of VETF to upstream and downstream regions, respectively, of a vaccinia virus early promoter. It

should now be possible to identify the amino acids of VETF that interact with DNA and a similar approach might be used to study RNA polymerase subunit interactions with DNA.

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