The Small Subunit of the Vaccinia Virus Early Transcription Factor Contacts the Transcription Promoter DNA†

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The vaccinia virus early transcription factor (VETF), in addition to the viral RNA polymerase, is required for efficient transcription of early genes in vitro. VETF is a heterodimeric protein that binds specifically to early gene promoters. In order to localize the VETF DNA binding domain, we have used photoreactive oligonucleotide probes with the sequence of the vaccinia virus growth factor promoter. The probes consisted of double-stranded oligonucleotides incorporating radiolabeled dAMP and 5-bromo-dUMP into sequences of the promoter known to contact VETF. Irradiation of a DNA probe having these nucleotides located upstream of the transcription start site in the presence of VETF resulted in the transfer of label to a polypeptide that comigrated with the small subunit of VETF. The label transfer reaction was shown to occur with the recombinant VETF small subunit in the absence of the large subunit. These results indicate that the small subunit comprises at least part of the VETF DNA binding domain and contacts the promoter in the region upstream of the transcription start site.

Vaccinia virus is a large cytoplasmic DNA virus belonging to the poxvirus family. The 191,636-bp viral genome encodes approximately 200 individual proteins (9) that are differentially expressed as a function of time after infection of a host cell (for reviews, see references 14 and 15). These genes can be operationally grouped into the early, intermediate, and late gene classes. The timing of gene expression is controlled at the level of initiation of messenger RNA synthesis elicited by the viral RNA polymerase. Biochemical studies have indicated that the specificity of transcription is dictated by class-specific factors that control promoter selection (6, 19, 22).

Transcription of early gene templates in vitro by the vaccinia virus RNA polymerase is regulated by two protein factors. Termination of transcription is mediated by the viral capping enzyme (18). The initiation of transcription requires an activator protein called the vaccinia virus early transcription factor (VETF) (6). This protein binds specifically to early promoter sites, making contact with nucleotides on both sides of the transcription start site (4). The VETF-promoter complex appears to be the basis for recruitment of the RNA polymerase to early gene promoters (10, 12). VETF also has a DNA-dependent ATPase activity (5). While the role of the ATPase activity is unclear, ATP hydrolysis has been shown to have an effect on the stability of the VETF-promoter complex (2).

Purified VETF appears to be a heterodimer of 70- and 83-kDa polypeptides (5, 6). The large and small subunits of the protein are encoded by the vaccinia virus A8 and D6 genes, respectively (3, 8). The deduced sequences of the two VETF subunits have not proven to be particularly enlightening in the identification of functional domains of the protein. There is a possible ATP binding site in the 70-kDa subunit; however, there are no readily identifiable motifs for DNA binding commonly found in other transcription activators.

In an attempt to localize the DNA binding domain of VETF, we have constructed photoreactive promoter oligonucleotides containing 5-bromouracil and radiolabeled nucleotides that potentially can be used to label DNA binding proteins. Bromouracil in the DNA provides a means to covalently couple the DNA to a bound protein (1, 16, 21). Upon irradiation with UV light, the bromine atom undergoes photolysis to yield a highly reactive uridine radical. If atoms in the bound protein are sufficiently near, a covalent bond between the uridine and the protein will result. Thus, the binding protein is tagged with a DNA adduct.

The DNA binding probes for label transfer reactions were double-stranded oligonucleotides with the sequence of nucleotides −35 to +18 (relative to the start site of transcription) of the vaccinia virus growth factor (VGF) gene promoter. This sequence has been shown previously by electrophoretic gel shift analyses (2, 4) to form a stable complex with VETF. Footprinting experiments showed that VETF contacts two regions of the VGF promoter at nucleotides −12 to −30 and +8 to +10 (4). Double-stranded oligonucleotides were constructed for labeling proteins contacting each of these sites. To label the template strand of the promoter DNA upstream of the transcription start site, an oligonucleotide with the nontemplate strand sequence from −35 to +18 was annealed to an oligonucleotide with the sequence from +1 to +18 of the template strand (Fig. 1). The remainder of the template strand was synthesized by the Klenow fragment of DNA polymerase 1 and 1 mM 5-bromo-dUTP−10 μM [α-32P]dATP (3,000 Ci/mmol)−1 mM dCTP−1 mM dGTP. This promoter probe is designated oligonucleotide A. To label the nontemplate strand of the promoter at nucleotides downstream of the transcription start site, a template strand oligonucleotide with nucleotides −35 to +18 was annealed to an oligonucleotide with nontemplate strand nucleotides −35 to −1. The oligonucleotide was made fully double stranded as described above. The latter primer probe is designated oligonucleotide B.

The ability of VETF to bind the bromouracil-labeled DNA was determined by gel shift analysis (Fig. 2). The purification of VETF and the conditions for binding and gel electrophoresis were described previously (6). The oligonucleotide

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Thus, oligonucleotides labeled in the upstream site (oligonucleotide A) readily bound VETF. The gel-shifted complex resembled that expected with unmodified DNA in terms of both the degree of the mobility shift of the DNA and the yield of the complex. Thus, replacement of thymine residues with 5-bromouracil groups in the template strand upstream region of the promoter DNA known to interact with VETF was not deleterious to VETF binding. Different results were obtained with the oligonucleotide labeled in the nontemplate strand downstream of the transcription start site (oligonucleotide B). Little or no complex was produced in the presence of VETF. This result suggested that bromine atoms on uracil groups downstream of the transcription start site interfered with VETF binding.

For the label transfer experiments, the 32P-labeled, bromouracil-substituted promoter DNA was incubated with VETF under the conditions used for gel shift assays. The complexes were then irradiated with 254-nm light (5 min at a distance of 5 cm from a Mineralight UVGL-25 lamp) in polystyrene cups. The solution was transferred to a 1.5-ml tube, and MgCl2 and DNase I were added to 5 mM and 50 μg/ml, respectively. After 30 min at 37°C, the products were resolved by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide gel (11), transferred to a nitrocellulose membrane, and visualized by autoradiography. The DNase I treatment was used to digest input DNA and to trim the DNA linked to protein in order to minimize any effect of a DNA tag on the electrophoretic mobility of the protein. DNase I has sequence preference for cleavage and does not hydrolyze every phosphodiester bond in the VETF promoter (4). The results of this experiment using oligonucleotide A revealed a labeled product with the mobility of a 70-kDa polypeptide (Fig. 2C). Another, more weakly labeled product with the mobility of a 35-kDa polypeptide was also observed. Irradiation of oligonucleotide B in the presence of VETF did not yield a detectable labeled species. Since oligonucleotide B demonstrated no propensity for interaction with VETF, all further experiments were performed with the oligonucleotide labeled in the nucleotides upstream of the promoter.

If the target of the label transfer reaction was the small subunit of VETF, then it is possible that the isolated small subunit would also undergo similar labeling. This was tested with the recombinant VETF small subunit. We have expressed VETF using the hybrid vaccinia virus-T7 expression system (13). The genes had also been engineered to encode six tandem histidine residues at the carboxy termini of the proteins to permit affinity purification on nickel–nitrilo-agarose. VETF was expressed and purified in two soluble forms. Affinity-purified protein from cells transfected with both the D6 and A8 open reading frame expression plasmids contained both subunits of VETF. The protein purified from cells transfected with the D6 open reading frame expression plasmid alone consisted of only the small subunit of VETF (13). No VETF large subunit was detected in the latter form of VETF.

Recombinant VETF heterodimer was labeled by the photo-cross-linking procedure similarly to native VETF (Fig. 3). A 70-kDa polypeptide was the acceptor of the label in both cases. The recombinant protein did not produce the 35-kDa labeled species seen with native VETF. This likely reflects the relative degree of purity of the two protein preparations. The isolated VETF small subunit was found to undergo labeling in a manner that was indistinguishable from that of the VETF holoprotein. The labeled products had the same
mobility on an SDS-polyacrylamide gel as well as similar extents of labeling. We conclude from these results that the label transfer reaction was specific for the small subunit of VETF.

The reaction requirements for the label transfer experiments suggest the labeled species to be the 70-kDa subunit of VETF. Formation of the labeled species was dependent on UV light treatment and did not occur when the DNA was irradiated in the absence of VETF (Fig. 4). The 32P-labeled product precisely comigrated with the small subunit of VETF subunits (3). These results suggest that the 70-kDa subunit of VETF was the acceptor protein in the label transfer reaction.

The DNA specificity of the label transfer reaction was tested by DNA competition. Nonradiolabeled VGF promoter DNA was used as a specific competitor for VETF labeling. A VGF promoter fragment containing a point mutation was used as a nonspecific competitor DNA. Replacement of the G residue at −21 (relative to the start site of transcription) of the 7,500-molecular-weight early promoter severely reduced promoter strength in vivo and in vitro (7). The homologous G residue at −22 of the VGF promoter has been implicated in contacting VETF by methylation interference analysis (4). We have replaced this residue in the VGF promoter with a C and have found that the affinity for VETF is reduced 10- to 20-fold relative to wild-type promoter DNA (unpublished observations). Comparison of the abilities of the wild-type and mutant VGF promoters to inhibit the photoaffinity labeling of VETF with brominated promoter DNA showed the wild-type promoter DNA to be the more effective competitor (Fig. 5). There was 50% reduction in labeling with approximately 15 pmol of wild-type promoter, whereas about 70 pmol of the mutant promoter was required for the same level of inhibition. It can be concluded from these results that the label transfer reaction reflected specific binding to the VGF promoter sequences.

The results described here indicate that the small subunit of VETF contacts the template strand of the VGF promoter upstream of the transcription start site. The reactivity of VETF with the radical generated by irradiation of 5-bromouracil residue suggests that VETF interacts with the major groove of the DNA helix in this region of the promoter. This is supported by methylation interference analyses showing that methylation of the G residue at −22 of the VGF promoter blocked VETF binding (4). Those analyses also demonstrated that VETF interacts with A residues in the minor groove of the helix. Our results also suggest indirectly that VETF interacts with the major groove of the promoter DNA downstream of the transcription start site. Replacement of thymine residues in the nontemplate strand with 5-bromomuracil resulted in an inability to complex with VETF. This suggests that VETF may form contacts with methyl groups on thymine residues downstream of the transcription start site.

It is important to emphasize that the results described here in no way exclude the possibility that the VETF large subunit also participates in DNA binding. Indeed, we were unable to identify the polypeptide contacting the VGF promoter downstream of the transcription start site, possibly because bromomuracil residues in this sequence blocked VETF binding. It is possible that the large subunit contacts this sequence. In addition, the isolated small VETF subunit readily underwent label transfer with brominated DNA; however, we have shown by gel shift analysis that the small subunit does not form a stable complex with the VGF promoter (13). These results suggest that the DNA contacts formed by the small subunit alone are relatively weak and
that some other factor (likely the large subunit) is required to stabilize the VETF-promoter complex.

The localization of a DNA binding domain to the VETF small subunit raises interesting implications as to the relationship of DNA binding and ATPase activity. Recent evidence indicates that the small VETF subunit contains the ATP binding motif of the protein. The small subunit sequence predicts an A-type ATP binding site (20) and a DEAH motif found in many nucleic acid helicases (17). Mutational analysis of these two sequences showed that they are required for ATPase activity (13). Two observations indicate that DNA binding and the ATPase activity of VETF are related. The ATPase activity is DNA dependent, being stimulated 20- to 30-fold by DNA (5, 6). In addition, ATP hydrolysis affects the stability of the VETF-promoter complex. In the presence of ATP, VETF rapidly dissociates from its binding site on the promoter (2). Thus, DNA binding renders the factor competent to bind and hydrolyze ATP, which, in turn, dissociates the factor from its binding site. The close proximity of DNA binding and ATPase motifs in VETF could account for the interdependence of the two activities. The transduction of signals between the two functional motifs is possible apparently because the two motifs reside in the same polypeptide. VETF binding to DNA could potentially induce a folding of the ATP binding pocket in the small subunit into a form favorable for ATP hydrolysis. Then, ATP hydrolysis could alter the structure of the DNA binding domain, rendering it nonfunctional. Proof of this model clearly will require a more definitive understanding of the structure of the VETF functional domains.

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REFERENCES