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Early promoter-binding factor from vaccinia virions

(transcription factor/DNA–protein complex/poxvirus)

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ABSTRACT **A factor, present in transcriptionally active extracts prepared from purified vaccinia virus particles, binds to vaccinia early promoter sequences. The specificity of binding was demonstrated by electrophoretic mobility shift assays using the 5′-terminal segments of two early genes and related and unrelated competitor DNA fragments. DNase I "footprint" analysis indicated that the factor formed a complex with promoter regions of both genes and protected sequences of 10–15 nucleotides centered 21–24 nucleotides upstream of the RNA start sites. The lack of protection of a late regulatory sequence and of an early promoter with transcriptionally inactivating single-nucleotide substitutions suggested that the protein is an early transcription factor. When subjected to glycerol gradient centrifugation, the DNA-binding factor was resolved from RNA polymerase and sedimented as a 7.5S species with an estimated molecular weight of 130,000.**

Factors that bind to specific DNA sequences regulate gene expression in prokaryotes and eukaryotes. In eukaryotes, such factors may bind to promoter or enhancer regions and modulate the level of transcription or determine tissue specificity. In a few cases, the binding factors have been shown to stimulate *in vitro* transcription by RNA polymerases (1–6). The development of the gel electrophoresis mobility shift assay as well as various "footprinting" techniques (7–10) have greatly simplified and accelerated the search for specific DNA-binding proteins.

Vaccinia virus contains a linear double-stranded DNA genome of ≈185 kilobase pairs and, like other poxviruses, replicates in the cytoplasm (11). Because of the cytoplasmic habitat, vaccinia virus does not appear to depend on host nuclear enzymes—at least for early RNA synthesis. Instead, a complete transcription system is packaged within the virus core and introduced into the cell during infection (12, 13). The components include a DNA-dependent RNA polymerase with multiple virus-encoded subunits (14, 15), capping and methylating enzymes (16, 17), and poly(A) polymerase (18).

Extracts prepared from vaccinia virus particles are able to accurately and specifically initiate (19) and terminate (20) transcription of vaccinia virus early genes *in vitro*. Thus far, only one primary transcription component—RNA polymerase—has been characterized. Nevertheless, the inability of purified RNA polymerase to transcribe double-stranded DNA templates suggests that transcription factors are present in virus extracts. The recognition of specific 5′- and 3′-terminal sequences involved in initiation (21, 22) and termination (20, 32) of early RNA synthesis has made it possible to search for putative vaccinia virus DNA-binding proteins. We now report the presence of a 130-kDa factor from vaccinia virions that binds specifically to functional promoter regions of vaccinia virus early genes.

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MATERIALS AND METHODS

Vaccinia Virus Extract. A soluble deoxycholate extract of vaccinia virus was passaged through a DE 52 cellulose column to remove endogenous DNA as described (19). Glycerol gradient fractionation was performed by layering the crude extract on a 10–30% (vol/vol) glycerol gradient containing 200 mM KCl, 50 mM Tris·HCl (pH 8), 1 mM dithiothreitol, and 0.1 mM EDTA. Centrifugation was performed in a Beckman SW41 rotor at 4°C for 24 hr at 37,000 rpm. Fractions were collected and assayed for promoter-binding factor by the mobility shift assay.

DNA Fragments for Binding Studies. The plasmid pMINI was constructed by ligating a 90-base-pair (bp) vaccinia virus growth factor (VGF) promoter-containing fragment and a 175-bp terminator-containing fragment into the polylinker region of pUC19. The entire VGF minigene fragment was isolated as a 300-bp *Hin*dIII–*Eco*RI fragment from pMINI. The VGF promoter fragment was obtained from the minigene plasmid as a *Hin*dIII–*Bam*HI fragment, while the 3′ VGF terminator was excised as a *Bam*HI–*Eco*RI fragment. Plasmid pVGF was constructed by inserting the *Hin*dIII–*Bam*HI 90-bp VGF 5′ fragment between the *Hin*dIII and *Bam*HI sites of pUC19. The P7.5 promoter (derived from a gene encoding a 7.5-kDa polypeptide) was excised from plasmid pGS20 as a 160-bp *Hpa* II–*Bam*HI fragment (23).

Mobility Shift Assay. DNA fragments used in mobility shift assays were end-labeled with the appropriate deoxyribonucleoside [α-^{32}P]triphosphate (Amersham) using the Klenow fragment of DNA polymerase. A typical assay was performed in a 25-μl volume containing 600 ng of deoxycholate-solubilized vaccinia virus protein, 1 ng of ^{32}P-labeled DNA fragment, 10 mM Tris·HCl (pH 8), 1 mM dithiothreitol, 25 mM KCl, 0.02 mM EDTA, and appropriate amounts of poly(dI·dC) (Pharmacia). Binding was allowed to take place at room temperature for 20 min. Glycerol was added to a final concentration of 10% (vol/vol), and the samples were applied to a 4% polyacrylamide gel containing 6.7 mM Tris·HCl (pH 7.5), 3.3 mM sodium acetate, and 0.1 mM EDTA as electrophoresis buffer.

Construction of P7.5 Promoter Nucleotide Substitution Mutants. Plasmid pMJ4 contains the entire *Escherichia coli lacZ* protein-coding region inserted into the *Eco*RI site of the vaccinia thymidine kinase (*TK*) gene such that the *lacZ* gene and the interrupted *TK*-coding region are in opposite orientations. Plasmid pMJ21 was made by inserting a synthetic, double-stranded oligonucleotide fragment containing the wild-type early promoter of the vaccinia 7.5-kDa gene (21) into pMJ4 between the unique *Hin*dIII and *Xho* I sites immediately upstream of the *lacZ* gene. Plasmids pMJ114 and pMJ133 were constructed similarly except that the synthetic oligonucleotides contained point mutations in the P7.5 promoter. Oligonucleotides were made in an Applied Biosystems (Foster City, CA) 370B synthesizer, and sequences

Abbreviation: VGF, vaccinia virus growth factor.
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were determined after cloning in plasmids (24). Vaccinia recombinant viruses were made as described (23).

DNase I Footprint Analysis. For DNase I footprint analysis, $MgCl_2$ and DNase I (Sigma) were added at the end of the 20-min binding incubation period to achieve final concentrations of 1 mM and 2 μg/ml, respectively. Digestion was allowed to take place at room temperature for 5 min, then EDTA and glycerol were added to final concentrations of 2 mM and 10% (vol/vol), respectively, and the samples were analyzed by electrophoresis in a 4% polyacrylamide gel as described above. The bands containing factor-bound and free DNA fragments were excised, and fragments were eluted overnight at 37°C in 10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.75 M ammonium acetate. After phenol/chloroform extraction, 1:1 (vol/vol), and ethanol precipitation, the DNA samples were analyzed in a 6% sequencing gel.

RESULTS

Factor Binding to the 5′ End of the VGF Gene. We have searched for factors in transcriptionally active extracts of vaccinia virus that could bind to the regulatory regions of vaccinia virus early genes. A ^{32}P-labeled 300-bp minigene containing functional 5′-promoter and 3′-terminator segments of the VGF gene (20) was selected as a suitable substrate (Fig. 1). Excess poly(dI·dC) served as competitor to prevent nonspecific binding of proteins to the labeled DNA. After incubation of extract and DNA, polyacrylamide gel electrophoresis was performed; a decrease in mobility of the VGF minigene was used as a measure of DNA binding. Preliminary experiments indicated that the composition of the incubation buffer was critical: specific binding was not detected when the buffer was similar to that used for *in vitro* transcription. Further studies revealed that Mg^{2+}, as well as other divalent cations, destabilized the specific DNA–protein complex. Thus, when Mg^{2+} was omitted, significant DNA binding was demonstrable by a shift in the mobility of the VGF minigene (Fig. 2*A*). Appreciable binding to VGF DNA still occurred even at poly(dI·dC)/DNA base-pair ratio of 6000:1.

To locate the principal region of binding, the VGF minigene was cleaved into two segments by *Bam*HI digestion (Fig. 1). The 5′-promoter and 3′-terminator fragments were isolated and incubated separately with virus extract. Mobility shift assays showed that binding to the 3′ end of the VGF gene was completely inhibited at a 1000-fold excess of poly(dI·dC) (Fig. 2*C*), whereas binding of factor to the 5′ end of the gene was still detected at competitor/DNA ratio of 6000:1 (Fig. 2*B*). These binding experiments suggested the presence of a factor in vaccinia virus extracts capable of binding to promoter sequences of the VGF gene. A lower-affinity factor capable of binding to the 3′ region of the VGF gene was not ruled out, however.

Additional competitor DNAs were used to confirm the specificity of binding to the 5′ region of the VGF gene. Incubation of factor with the ^{32}P-labeled VGF promoter segment and poly(dI·dC) was carried out in the presence of a

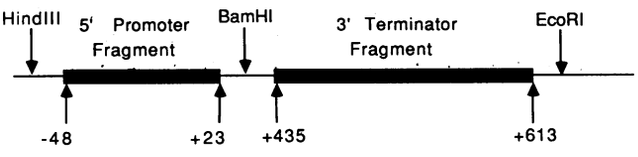

FIG. 1. VGF minigene structure. The 300-bp *Hin*dIII–*Eco*RI fragment contains the 5′ (positions −48 to +23) and 3′ (positions +435 to +613) segments of the VGF gene separated by a *Bam*HI site. The coding sequence between positions +24 and +434 has been deleted. In this numbering system, the RNA start site is at position 0 and termination occurs mainly between positions +520 and +540.

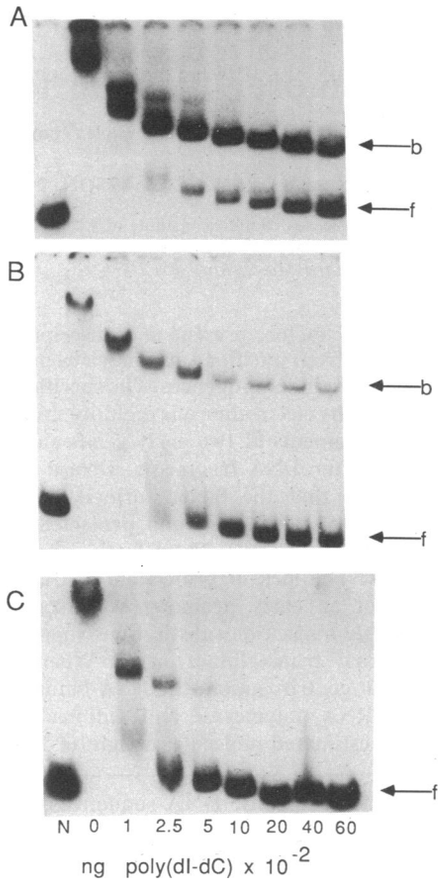

FIG. 2. Binding of factor to VGF minigene fragments. Approximately 1 ng (4000 cpm) of the end-labeled fragment below was incubated with 600 ng of a vaccinia virus extract in the presence of indicated amounts of poly(dI·dC). Factor-bound (b) and free (f) DNA species were separated by electrophoresis in a 4% polyacrylamide gel, and autoradiographs were prepared. (*A*) A 300-bp *Hin*dIII–*Eco*RI VGF minigene 3′-end-labeled with [α-^{32}P]dATP at the *Eco*RI site. (*B*) A 90-bp *Hin*dIII–*Bam*HI VGF 5′-end fragment 3′-end-labeled with [α-^{32}P]dGTP at the *Bam*HI site. (*C*) A 210-bp *Bam*HI–*Eco*RI VGF 3′-end fragment 3′-end-labeled with [α-^{32}P]dATP at the *Eco*RI site. The VGF minigene is depicted in Fig. 1.

50- or 100-fold excess of unlabeled 325-bp *Hin*dIII–*Nde* I pUC19 fragment containing the promoter segment of the VGF gene or a 250-bp *Hin*dIII–*Nde* I fragment containing the same vector sequences without VGF DNA. Whereas the vector fragment failed to inhibit complex formation, the promoter-containing fragment prevented binding at a 100-fold molar excess (Fig. 3).

DNase I Footprint Analysis. To more accurately locate the binding site within the 5′ end of the VGF gene, footprint analysis was done. After DNA binding in the presence of poly(dI·dC), the material was treated with DNase I. Because of the instability of the complex, the DNase digestion was performed in low concentrations of Mg^{2+}. Polyacrylamide gel electrophoresis was used to separate the complexed and uncomplexed DNA fragments, and both were analyzed by high-resolution polyacrylamide gel electrophoresis next to a DNA sequence ladder. Comparisons indicated that the region from ≈14 to 29 nucleotides upstream of the RNA start site of the VGF gene was protected because of factor binding (Fig. 4*A*). In addition, hypersensitive DNase sites appeared several nucleotides further downstream indicating a more extensive perturbation of DNA structure.

The 5′ end of a second vaccinia virus gene, coding for a 7.5-kDa polypeptide, was isolated to investigate the gener-

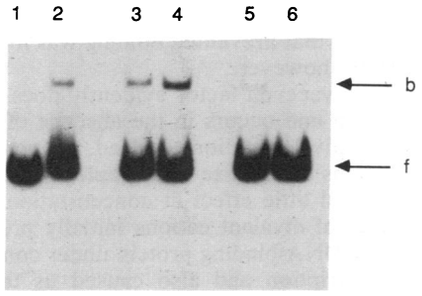

FIG. 3. Inhibition of DNA binding with competitor DNA. Plasmid pVGF was constructed by inserting the 90-bp *Hin*dIII–*Bam*HI 5′-end VGF fragment (Fig. 1) into the *Hin*dIII–*Bam*HI sites in the polylinker region of pUC19. An unlabeled 325-bp *Hin*dIII–*Nde* I fragment from pVGF containing the VGF promoter or a corresponding 250-bp *Hin*dIII–*Nde* I fragment from pUC19 was used as competitor in binding experiments. Approximately 1 ng of the 90-bp VGF 5′ promoter 3′-end-labeled with [α-^{32}P]dGTP was incubated with 600 ng of vaccinia virus extract, 1 ng of poly(dI·dC), and no competitor (lane 2), a 50 (lane 3)- or a 100 (lane 4)-fold molar excess of unlabeled 250-bp competitor, or a 50 (lane 5)- or a 100 (lane 6)-fold molar excess of unlabeled 325-bp competitor. DNA in lane 1 was not incubated with extract. Bound (b) and free (f) DNA species were electrophoretically separated in a 4% polyacrylamide gel, and an autoradiograph was prepared.

ality of the binding reaction. The P7.5 promoter has been used extensively for expression studies (22) and contains an early transcriptional regulatory sequence extending ≈30 bp upstream of the early RNA start site (21). A second RNA start site, located 50 bp upstream of the first, is regulated by an adjacent upstream late promoter (21). A 160-bp DNA fragment, containing both the early and late promoter regions, was incubated with the virus extract in the presence of excess poly(dI·dC). Specific binding was demonstrated by gel retardation of the fragment (data not shown). DNase I footprint analysis indicated that the region from 19 to 29 nucleotides upstream of the early RNA start site was protected (Fig. 4*B*). In contrast, the late promoter region located about 50 bp further upstream was not protected.

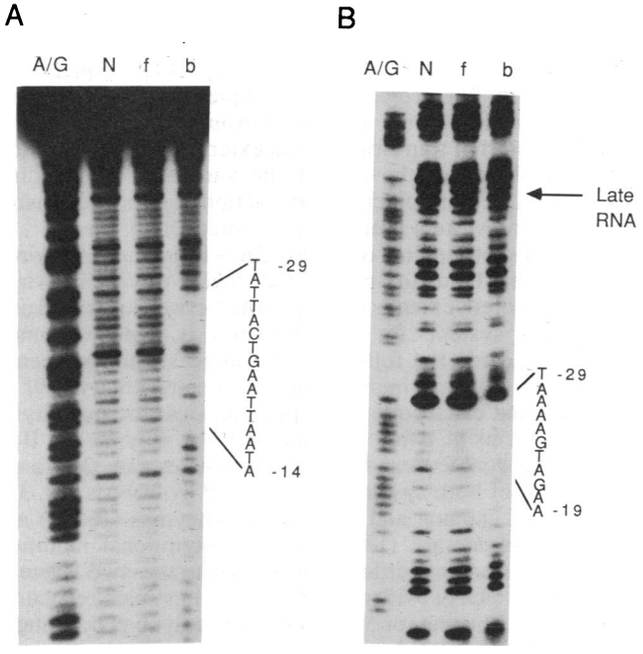

FIG. 4. DNase I footprint analysis of factor–promoter complexes. (*A*) The 90-bp VGF promoter *Hin*dIII–*Bam*HI fragment was 3′-end-labeled with [α-^{32}P]dGTP at the *Bam*HI site. (*B*) The 160-bp *Hpa* II–*Bam*HI P7.5 promoter fragment was labeled similarly. Each fragment was incubated with vaccinia virus extract and, following DNase I digestion, was electrophoresed on a 4% polyacrylamide gel. Bound and unbound DNA was purified and then analyzed by electrophoresis on a high-resolution sequencing gel. Nucleotides are numbered according to their position upstream of the early RNA start site. The location of the late RNA start site is indicated. Lanes: A/G, Maxam–Gilbert A+G reaction; N, DNA incubated without extract; f, free DNA; b, bound DNA.

Effect of Point Mutations in the Promoter Region on Factor Binding. Since factor binding occurred within the promoter regions of two vaccinia early genes, we sought evidence that this association might be involved in transcription. One approach would be to correlate the effects of promoter mutations on factor binding and gene expression. Studies (A.J.D., unpublished results) identified single-base substitutions in the P7.5 promoter that severely decreased expression of a reporter gene. To evaluate the effects of such mutations on the binding reaction, three DNA segments—the wild-type and two differing only by single-nucleotide substitutions—were incubated with the virus extract. Complex formation, as judged by gel retardation, was observed with the functional wild-type promoter from pMJ21 (Fig. 5, lane 1). In contrast, no binding was discerned with a nonfunctional promoter from pMJ114 containing a change of an adenosine to a cytidine at the −26 position (Fig. 5, lane 2). The latter mutation lies within the DNA-binding region as defined by footprint analysis. Decreased but still detectable binding occurred when the promoter had an adenosine to a cytidine mutation at the −13 position (Fig. 5, lane 3) which lies at the margin of the protected region. These results confirmed the specificity of binding and correlated binding with expression.

Glycerol Gradient Sedimentation of the DNA-Binding Factor. The inability to form a stable complex with mutated promoter segments strongly suggested that the factor is involved in transcription. The possibility that the factor is the large 500-kDa multisubunit RNA polymerase was disproved by sedimentation of virus extracts on 15–35% glycerol gradients (25). Under conditions in which the polymerase sedimented midway down the tube, most of the DNA-binding factor remained near the top (data not shown). Upon longer centrifugation in a 10–30% glycerol gradient, the DNA-

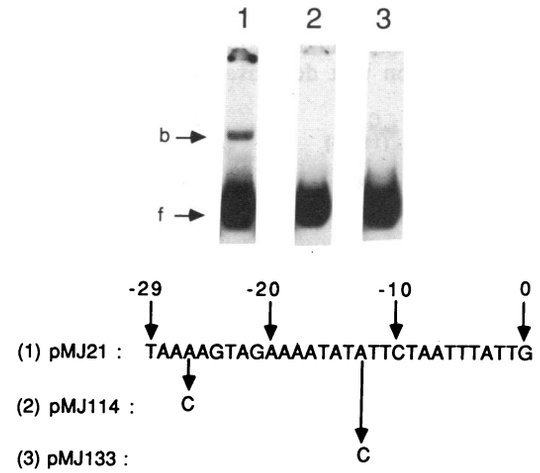

FIG. 5. Effect of point mutations in the P7.5 promoter on factor binding. The 110-bp *Bam*HI–*Pvu* II fragments containing the P7.5 promoter from either wild type (pMJ21) or point mutants (pMJ114 and pMJ133) were 3′-end-labeled with [α-^{32}P]dGTP at the *Bam*HI site. Following incubation with vaccinia virus extract, the fragments were analyzed by electrophoresis in a 4% polyacrylamide gel. Plasmids: 1, wild-type pMJ21; 2, mutant pMJ114; 3, mutant pMJ133. b, Factor-bound DNA; f, free DNA. The P7.5 promoter sequence and nucleotide substitutions of mutants are shown below the autoradiograph.

binding protein sedimented somewhat heterogeneously but with peak fractions corresponding to a 7.5S globular protein of ≈130 kDa (Fig. 6). It is possible, nevertheless, that the presence of some DNA-binding activity in the denser fractions results from gradual dissociation of the factor from RNA polymerase. The factor is probably not an RNA polymerase subunit since immunoprecipitation of the extract with a polyvalent antibody to purified RNA polymerase (14, 15) did not remove the binding factor (data not shown).

The experiment depicted in Fig. 6, as well as others, clearly shows a second DNA complex that migrates slightly faster than the major one. Thus far we have not been able to obtain sufficient material for footprinting and do not know the significance of this additional band.

DISCUSSION

In vivo (21, 22) and *in vitro* (19) studies have shown that the major transcriptional regulatory regions of vaccinia virus early genes are located within a segment that extends ≈30 bp upstream of the RNA start site. Shorter promoter segments were inactive indicating that at least part of the critical sequences is located between 24 and 30 bp from the transcription initiation site. The factor described here was isolated from transcriptionally active extracts of vaccinia virus and binds to the region that is most clearly implicated in promoter function. Moreover, single-nucleotide substitutions in this region that lead to diminution of gene expression also decreased factor binding. Glycerol gradient sedimentation clearly distinguished the 130-kDa binding activity from the much higher molecular weight vaccinia RNA polymerase. We, therefore, suggest that the binding activity is due to a transcription factor. The existence of such a factor is consistent with indications that highly purified vaccinia RNA polymerase is not able to initiate transcription on specific double-stranded DNA templates (14, 26). Since the factor did not form a stable complex with the late promoter region of one gene, it is tempting to speculate that the factor is a specific early vaccinia transcription regulatory element.

The region of factor binding was delimited by DNase I footprint analysis. Similar regions of 10–15 nucleotides, centering around 21–24 nucleotides upstream of the RNA start sites of two early genes, were protected. This footprinting procedure probably underestimates the full-contact area since one mutation that decreased binding was located 6 nucleotides downstream of the highly protected sequence. Another mutation that prevented binding was located in the protected region, however.

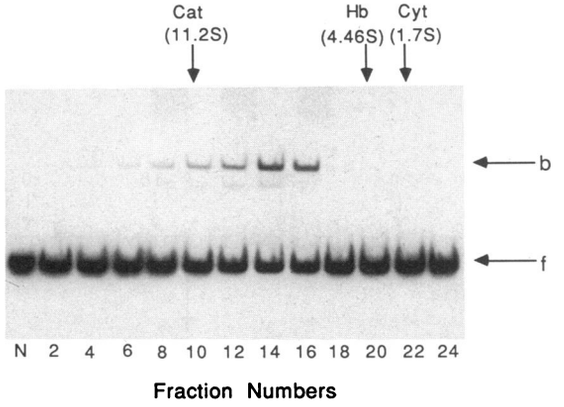

FIG. 6. Glycerol gradient sedimentation of promoter-binding factor. Vaccinia virus extract was subjected to glycerol gradient [10–30% (vol/vol)] centrifugation. Alternate fractions were incubated with [α-^{32}P]dGTP 3'-end-labeled 90-bp VGF promoter fragment and analyzed by electrophoresis on a 4% polyacrylamide gel. Peak position and sedimentation values of marker proteins are indicated. N, no extract; b, factor-bound DNA; f, free DNA; Cat, catalase; Hb, hemoglobin; Cyt, cytochrome *c*.

Binding of the vaccinia factor evidently does not require RNA polymerase and occurs in the absence of nucleoside triphosphates or divalent cations. Indeed, low concentrations of divalent cations destabilize the complex, whereas monovalent cations had little effect at concentrations up to 100 mM. The effect of divalent cations initially prevented our detection of the DNA-binding protein under conditions that mimicked transcription and also caused us to carry out DNase I footprinting under suboptimal divalent cation conditions. Such an effect of Mg^{2+} is not unique, however, since Sawadogo and Roeder (4) found that divalent cations also destabilized the specific binding of transcription factor USF. It is possible that the interaction of the vaccinia-binding protein with DNA is dynamic under transcription conditions. In this circumstance, the binding protein–promoter complex would not remain stable during multiple rounds of transcription.

Since the VGF minigene used in this study contains only 48 bp of DNA upstream of the RNA start site and the P7.5 promoter is unusual in being preceded immediately by a late promoter, we have not addressed the issue of additional factors that bind to more distant regions. However, there is as yet no evidence for enhancers or transcriptional regulatory sequences that are >30 nucleotides upstream of the cap site of early mRNA.

The factor-binding region of the promoter, 14–29 nucleotides upstream of the RNA start site, may contain the vaccinia virus equivalent of the eukaryotic "TATA box." The A+T richness of this region has been noted (27). Transcription factors that bind to the TATA region of eukaryotic genes have been isolated from mammalian cells (4) and *Drosophila* (2). The mammalian factor, known as transcription factor IID, interacts primarily with a 10-bp DNA region centered on the consensus TATA sequence at position −28 but protects DNA sequences further downstream as well (4). Binding of the *Drosophila* B factor centers about the start of transcription and extends ≈40 bp upstream (2). Elucidation of the role of the vaccinia DNA-binding protein will require biochemical fractionation of the *in vitro* transcription system prepared from vaccinia virions.

The present finding of a protein within vaccinia virus particles that binds to an A+T-rich region of vaccinia virus early promoters extends the similarity between poxviral and eukaryotic systems for mRNA synthesis. Other common features include the following: the large subunit sequence homology (28) and immunological cross-reactivity (29) of vaccinia and eukaryotic RNA polymerases; the inability of purified forms of either vaccinia or RNA polymerase II to transcribe double-stranded DNA templates (14, 26); a requirement of both systems for β,γ-ATP hydrolysis (30, 31); and use of capping and methylating enzymes (16, 17) and poly(A) polymerase (18) for posttranscriptional modifications. Outstanding differences between the two systems include the lack of evidence for vaccinia upstream regulatory sequences, splicing, or 3'-end endonucleolytic processing of vaccinia early mRNAs.

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