

A Role for the H4 Subunit of Vaccinia RNA Polymerase in Transcription Initiation at a Viral Early Promoter*

(Received for publication, February 15, 1994)

Liang Deng and Stewart Shuman

From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

The vaccinia virus H4 gene encodes an essential subunit of the DNA-dependent RNA polymerase holoenzyme encapsidated within virus particles (Ahn, B., and Moss, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 3536–3540; Kane, E. M., and Shuman, S. (1992) *J. Virol.* 66, 5752–5762). The role of this protein in transcription of viral early genes was revealed by the effects of affinity-purified anti-H4 antibody on discrete phases of the early transcription reaction *in vitro*. Anti-H4 specifically prevented the synthesis of a 21-nucleotide nascent RNA chain but had no impact on elongation of the 21-mer RNA by preassembled ternary complexes. Inhibition of initiation but not elongation was also observed with affinity-purified anti-D6 antibody directed against the 70-kDa subunit of the vaccinia early transcription initiation factor (ETF). Native gel mobility-shift assays showed that anti-H4 prevented the NTP-dependent recruitment of RNA polymerase to the preinitiation complex of ETF bound at the early promoter. Two species of ternary complexes could be resolved by native gel electrophoresis. Addition of anti-H4 to preformed complexes elicited a supershift of both ternary species but not of the preinitiation complex. Supershift by anti-D6 revealed that the more rapidly migrating species of ternary complex did not contain immunoreactive ETF. Loss of ETF from the ternary complex was time-dependent. Thus, whereas the H4 protein was a stable constituent of the elongation complex, ETF was dissociable. We suggest that H4 functions as a molecular bridge to ETF and thereby allows specific recognition of early promoters by the core RNA polymerase. H4 is unlike bacterial σ factor in that it remains bound to polymerase after the elongation complex is established.

Vaccinia virus RNA polymerase is a multisubunit enzyme devoted exclusively to the synthesis of mRNA. Viral early genes are transcribed in the cytoplasm of the host cell by RNA polymerase and accessory factors packaged within the infectious particle (1). Promoter specificity is conferred upon the viral polymerase by vaccinia early transcription factor (ETF),¹ a heterodimer of 82- and 70-kDa virus-encoded subunits that binds specifically to the early promoter and recruits RNA polymerase to the template (2–4). The subunit structure of ETF has been confirmed by reconstitution of fully active protein from the two constituent polypeptides (5). The RNA polymerase purified

* This work was supported by Grant GM42498 from the National Institutes of Health, Grant FRA-432 from the American Cancer Society, and a Scholarship from the Pew Charitable Trusts. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The abbreviations used are: ETF, early transcription initiation factor; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; OMeGMP, 3'-O-methyl GMP; OMeGTP, 3'-O-methyl GTP.

from infectious virions contains at least eight polypeptides encoded by vaccinia *rpo* genes. These are named *rpo147*, *rpo132*, *rpo35*, *rpo30*, *rpo22*, *rpo19*, *rpo18*, and *rpo7*, according to the predicted sizes (in kDa) of the gene products (6–14). Reconstitution of the polymerase from individual polypeptides has not yet been achieved.

Multiple isoforms of vaccinia RNA polymerase are obtained after extraction from virions; these may differ in chromatographic properties (15). Whereas all isoforms are capable of nonspecific RNA synthesis from single-strand DNA templates, functional and structural differences are evident. For example, polymerase preparations obtained by ion exchange chromatography or by sedimentation at low ionic strength are usually capable of initiating efficiently at an early promoter because ETF remains associated with the polymerase (16). ETF can be dissociated by sedimentation at high ionic strength (4). Polymerase depleted of ETF has been further separated into "holoenzyme," which transcribes early genes when supplemented with ETF, and "core," which synthesizes RNA nonspecifically but is largely unresponsive to ETF in promoter-dependent synthesis (17).

Ahn and Moss have detected an 85-kDa polypeptide unique to the holoenzyme (17). Peptide sequencing identified this species as the H4 gene product, a 795-amino acid polypeptide synthesized at late times during vaccinia infection and then packaged within progeny virus particles (17). The H4 protein has a predicted size of 93.6 kDa but migrates as a 85–90-kDa species during SDS-PAGE (17, 18). Specific transcription of early genes *in vitro* in the presence of ETF was confined to the H4-containing form of polymerase (17). Analysis by Kane and Shuman (18) of temperature-sensitive viruses mutated in the H4 gene demonstrated that the H4 protein is essential for vaccinia replication. We identified the H4 protein as a virion component and found, as did Ahn and Moss, that H4 is associated with the transcriptionally active holoenzyme of vaccinia RNA polymerase (18).

Free H4 was not detected in virion extracts and was not dissociable from purified polymerase under native conditions (17, 18). Moreover, our efforts to overexpress the H4 protein in bacteria or in vaccinia-infected cells have been frustrated either by a lack of significant protein accumulation or by the exclusive accumulation of H4 breakdown products. These circumstances precluded a direct biochemical approach to the function of the H4 protein during early transcription. In this communication, we address the H4 question by studying the effects of anti-H4 antibodies on discrete stages of the transcription reaction *in vitro*. We find a role for H4 during transcription initiation, specifically in the recruitment of RNA polymerase to the preinitiation complex of ETF bound at the early promoter.

EXPERIMENTAL PROCEDURES

Polyclonal rabbit antiserum was prepared against a segment of the H4 protein (amino acids 1–364) isolated from the insoluble fraction of lysates of *Escherichia coli* BL21(DE3) that had been induced to over-

express this protein encoded by a resident pET-H4(1–364) plasmid. Insoluble polypeptides were resolved by preparative SDS-PAGE. The H4(1–364) antigen was eluted from an excised gel slice. Immunization was performed at Pocono Rabbit Farm and Laboratory, Canadensis, PA. Anti-D6 serum against the 70-kDa subunit of ETF was generously provided by J. Hagler. Anti-D1(1–666) serum against the large subunit of vaccinia capping enzyme (19) was provided by P. Cong. The specificity of immune sera was confirmed by Western blotting against appropriate test antigens. Specific antibodies were affinity-purified from serum by adsorption to antigen that had been resolved by SDS-PAGE and transferred to nitrocellulose. A membrane strip containing the antigen was pretreated for 1 h with 1 ml of 3% (w/v) BSA in TN buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). The BSA/TN was removed and the membrane was incubated with 1 ml of crude serum for 12–16 h at 4 °C. Unbound material was removed, and the strip was washed sequentially with TN buffer, TN buffer containing 0.5% Nonidet P-40 plus 0.5% sodium deoxycholate, and TN buffer. Retained antibody was eluted with 0.5 ml of a solution containing 4 mM MgCl₂ and 50 µg/ml BSA. The eluate was added to 1 ml of 10 mM Tris-HCl, pH 7.4, and the sample was dialyzed extensively against 10 mM Tris-HCl, pH 7.4. Final preparations (in 3 ml of Tris-HCl, pH 7.4) were stored at -80 °C. The specificity of the affinity-purified anti-H4, anti-D6, and anti-D1 antibodies was confirmed by Western blotting against extracts of vaccinia-infected BSC40 cells and against the original antigens used for immunization. The immunoglobulin fraction from preimmune rabbit serum was adsorbed to protein A-Sepharose (Pharmacia Biotech Inc.) and eluted with 0.1 M glycine, pH 3.

RESULTS AND DISCUSSION

Although full-sized H4 could not be expressed in bacteria, we did observe robust T7-based expression from a truncated version of the H4 gene encoding the protein region from amino acids 1–364. This polypeptide, which was recovered exclusively in the insoluble fraction of bacterial lysates, was used to prepare antiserum in rabbits. The anti-H4 antibody was affinity-purified from serum. Rabbit antisera raised against the vaccinia D6 gene product (the 70-kDa subunit of ETF) and the vaccinia D1 gene product (the large subunit of capping enzyme) were also affinity-purified.

Transcription by partially purified vaccinia RNA polymerase containing ETF (3) was programmed by a linear G21 DNA template consisting of a 20-nucleotide G-less cassette driven by a synthetic early promoter (Fig. 1A). A run of 4 G residues immediately following the G-less cassette serves as an elongation block when GTP is omitted from the reaction mixtures and restricts the analysis to a single round of transcription (20, 21). As shown previously (21–23), reactions constituted in the presence of ATP, UTP, [α -³²P]CTP, and 3'-OMeGTP yielded a predominant radiolabeled 3'-OMeGMP-arrested 21-mer nascent RNA (Fig. 1B). Preincubation of the RNA polymerase with anti-H4 antibody prior to addition of template and NTPs completely inhibited synthesis of the 21-mer, as did preincubation with anti-D6 antibody. In both cases, the extent of inhibition was dependent on the amount of antibody added during preincubation (Fig. 1B). Immunoglobulin purified from preimmune serum by protein A-Sepharose chromatography had no effect on transcription (Fig. 1B, lane P). Varying the order of addition such that RNA polymerase was preincubated with template DNA prior to addition of antibody, followed 30 min later by NTPs, did not afford protection against the inhibitory effects of anti-H4 and anti-D6 (Fig. 1C). These results indicated that the H4 protein might be acting at an early stage of the transcription reaction, either during initiation *per se* or during the elongation of nascent chains to position G²¹. The effects of anti-D6 confirmed the essential function of ETF during transcription initiation.

To discriminate between anti-H4 effects on initiation *versus* elongation, transcription reactions were programmed by a novel linear template linked to paramagnetic beads. The G21-A78 transcription unit (Fig. 2A) consisted of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which was

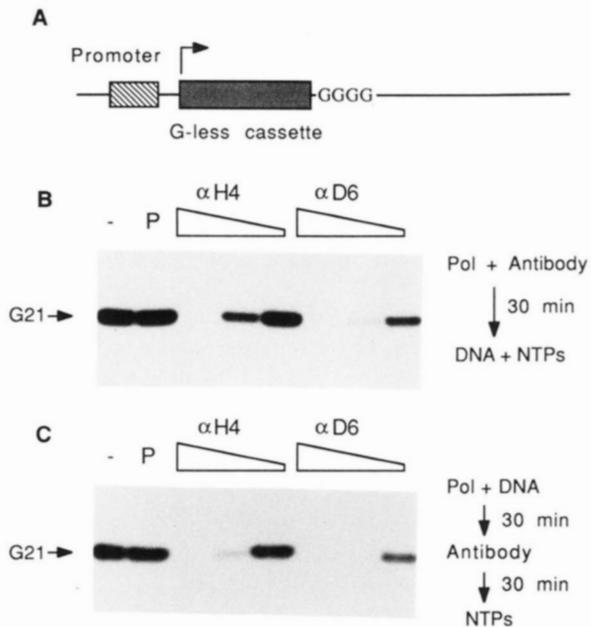


FIG. 1. Anti-H4 antibody inhibits transcription from a vaccinia early promoter. The G21 template for vaccinia transcription has been described in previous reports (21–24) and is illustrated in cartoon form in A. Standard transcription reactions contained (per 20-µl reaction volume) 20 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 2 mM DTT, 1 mM ATP, 0.1 mM UTP, 1 µM [α -³²P]CTP (1000 Ci/mmol), 0.1 mM 3'-OMeGTP, vaccinia RNA polymerase (0.07–0.1 unit of holoenzyme fraction containing ETF; Ref. 3), and G21 DNA (approximately 5 fmol of linear *Pvu*II restriction fragment). In the experiment shown in panel B, the polymerase was preincubated with affinity-purified anti-H4 or anti-D6 antibodies for 30 min on ice in transcription mixtures lacking NTPs and template. Mixtures included 170, 34, or 7 ng of anti-H4 or anti-D6 antibody, going from left to right within each series, as indicated above the lanes. Control reactions were preincubated without antibody (lane -) or with 170 ng of immunoglobulin purified from preimmune serum (lane P). Transcription reactions were started by addition of template and NTPs. The reactions were halted after a 10-min incubation at 30 °C by addition of buffer containing SDS and urea (21). Samples were extracted with phenol:chloroform, and labeled RNA was recovered by ethanol precipitation. Transcription products were resolved by electrophoresis through a denaturing 17% polyacrylamide gel (21). An autoradiogram of the gel is shown. The position of the OMeGMP-arrested G21 nascent RNA is indicated at the left. In the experiment in panel C, RNA polymerase was incubated with template DNA for 30 min on ice prior to addition of antibodies at the same concentration noted for panel B. After an additional 30 min on ice, transcription was initiated by provision of NTPs.

flanked by a run of 3 G residues at positions +21 to +23. Downstream of the G-run was inserted an A-less cassette flanked at its 3' end by a run of four A residues at positions +78 to +81. Pulse-labeling reactions containing ATP, UTP, [α -³²P]CTP, and 3'-OMeGTP yielded a 3'-OMeGMP-arrested 21-mer nascent RNA, as expected (Fig. 2B, Pulse). Template-engaged ternary complexes were recovered by centrifugation and concentration of the beads with an externally applied magnet, followed by washing the beads with buffer lacking nucleotides and magnesium (24). The integrity of the isolated ternary complexes was verified by their ability to resume elongation of the pulse-labeled RNA upon provision of unlabeled NTPs and magnesium. Omission of ATP from the elongation reaction and inclusion of 3'-dATP allowed us to "walk" the ternary complexes through the A-less cassette from G²¹ to A⁷⁸ (Fig. 2B, Walk). Note that elongation of the nascent chains beyond the induced pause site at G²¹ depended upon removal of the blocking 3'-OMeGMP moiety by a hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (25).

Synthesis of the 21-mer during the pulse phase was again blocked completely by anti-H4 and anti-D6 antibodies but was

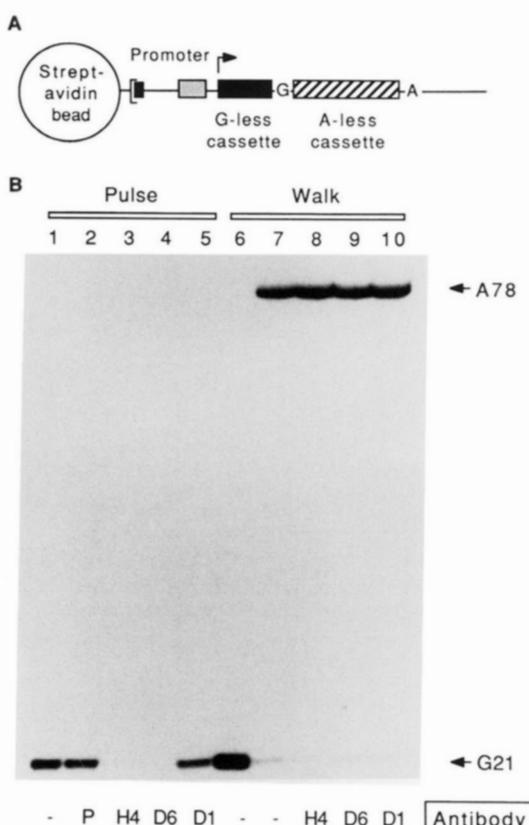


FIG. 2. Effects of anti-H4 and anti-D6 antibodies on transcription initiation and elongation. *A*, the architecture of the G21-A78 DNA template is shown in cartoon form. The DNA contains a biotinylated nucleotide incorporated uniquely at the 3' end of the template DNA strand, which serves to anchor the DNA to a streptavidin-coated magnetic bead. Biotinylation of the DNA and coupling to the beads (Dynabeads M280, Dynal) were carried out as described (24). A viral early promoter element specifies the initiation of transcription at position +1 of a 20-nucleotide G-less cassette. Three consecutive G residues immediately 3' of the G-less cassette serve to hinder polymerase elongation when transcription reactions are constituted in the absence of GTP. A 57-nucleotide A-less cassette is situated downstream of the G-less cassette. Four consecutive A residues immediately 3' of the A-less cassette arrest polymerase elongation when isolated G²¹ ternary complexes are walked down the template in the presence of GTP, CTP, and UTP. Elongation is arrested uniformly at A⁷⁸ when 3'-dATP (cordycepin triphosphate) is included in the extension reactions. *B*, “pulse” transcription reactions (*lanes 1–5*) containing 5 fmol of bead-linked G21-A78 template were performed as described in Fig. 1. Polymerase was preincubated with antibodies for 30 min on ice prior to addition of DNA and NTPs. Preimmune globulin (170 ng, *lane 2*) or affinity-purified anti-H4, anti-D6, or anti-D1 antibodies (170 ng) were included as indicated below the lanes. A control reaction was preincubated without antibody (*lane 1*). RNA synthesis was for 10 min at 30 °C. Pulse-labeled RNAs were processed for electrophoresis. In a parallel series of transcription walk reactions (*lanes 6–10*), bead-bound ternary complexes containing G21 RNA were recovered by microcentrifugation for 15 s. The beads were held in place by application of an external horseshoe magnet while the supernatant was removed and replaced with 0.1 ml of 20 mM Tris-HCl (pH 8.0), 2 mM DTT. The beads were resuspended and subjected to two further cycles of concentration and washing; after the third wash, the beads were resuspended in a small volume of the wash buffer and aliquots were distributed into individual reaction tubes to achieve approximately the same concentration of template as that used in the pulse-labeling phase. Purified complexes were incubated for 30 min on ice with affinity-purified anti-H4, anti-D6, or anti-D1 antibodies (170 ng) as indicated below the lanes. Standard elongation reactions (walk phase) were performed in 20-μl mixtures containing 20 mM Tris-HCl, 6 mM MgCl₂, 2 mM DTT, and 1 mM each of GTP, CTP, and UTP, and 0.1 mM 3'-dATP. A control reaction lacked nucleotides (*lane 6*). After incubation for 5 min at 30 °C, reactions were halted and transcription elongation products were analyzed in parallel with the pulse reaction products. An autoradiogram of the gel is shown. The positions of the G21 and A78 nascent chains are indicated at the right.

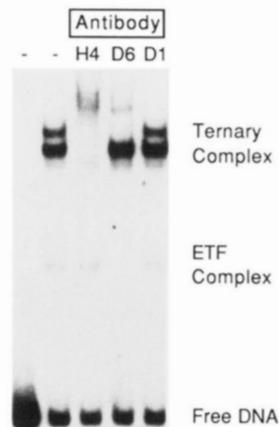


FIG. 3. Immunoreactivity of transcription complexes resolved by native gel electrophoresis. Reaction mixtures (20 μl) contained 20 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 2 mM DTT, 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.1 mM 3'-OMeGTP, polymerase, and 3' [³²P]dCMP-labeled G21-A78 template (approximately 18 fmol of a gel-purified 111-base pair HindIII/XbaI restriction fragment that was end-labeled using Klenow DNA polymerase). A control reaction lacked RNA polymerase (*lane -* at far left). Incubation was for 30 min at 30 °C. Samples were then placed on ice and supplemented with 170 ng of affinity-purified anti-H4, anti-D6, or anti-D1 antibody, or with buffer alone (−), as indicated above the lanes. Incubation with antibody was for 30 min on ice. Samples were then adjusted to 5% glycerol and electrophoresed through a nondenaturing polyacrylamide gel (4% acrylamide, 0.13% bisacrylamide) containing 0.25 × TBE as described (23). An autoradiogram of the dried gel is shown. The positions of free DNA and of protein-DNA complexes are indicated at the right.

unaffected by affinity-purified anti-D1 antibody or by preimmune globulin (Fig. 2B, *lanes 2–5*). In contrast, the elongation of the nascent 21-mer by preformed ternary complexes was impervious to incubation with either anti-H4 or anti-D6 antibodies (or with anti-D1) prior to commencement of the walk phase by provision of NTPs (Fig. 2B, *lanes 8* and *9*). These results suggested that initiation of transcription, but not elongation, was inhibited by anti-H4.

The failure of these antibodies to inhibit elongation could alternatively be explained by masking of the immunoreactive epitopes within the ternary complex or, more simply, by the absence of the antigen from the ternary complex. Native gel electrophoresis was therefore used to analyze the transcription complexes formed *in vitro* (3) and to assess their reactivity with affinity-purified antibodies. RNA polymerase associated with radiolabeled G21-A78 template in the presence of ATP, CTP, UTP, and 3'-OMeGTP to form a ternary complex; this was manifest as the appearance of novel species of retarded electrophoretic mobility (Fig. 3). At least two forms of ternary complex were resolved. A more rapidly migrating preinitiation complex of ETF bound at the early promoter was also evident (Fig. 3). That these two putative ternary complexes did indeed contain RNA was confirmed by incubating RNA polymerase with unlabeled G21 template and NTPs, including [α -³²P]CTP. The labeled nucleotide was associated with two complexes identical in mobility to the two species detected using labeled DNA as the ligand (data not shown).

Incubation of preformed G²¹ ternary complexes with anti-H4 elicited a supershift of both species of ternary complex, indicating that the immunoreactive H4 protein was present in the transcription elongation complex (Fig. 3A, *lane H4*). Anti-H4 had no effect on the ETF-promoter complex. In contrast, anti-D6 antibody supershifted only the slower ternary complex. No loss or alteration of the faster ternary complex was apparent (Fig. 3, *lane D6*). This was the case whether the experiment was performed with ternary complexes containing either labeled DNA or labeled RNA (data not shown). Anti-D6 also su-

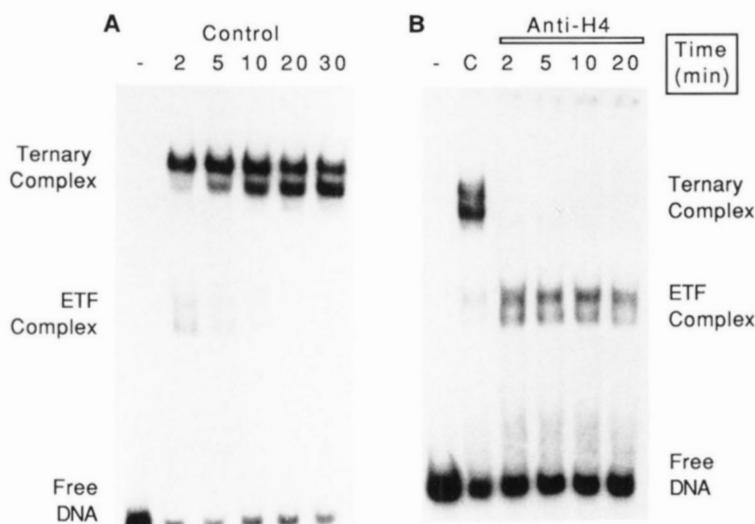


FIG. 4. Anti-H4 antibody blocks ternary complex formation. The kinetics of transcription complex formation were analyzed in the experiment shown in *panel A*. DNA binding reactions were constituted as in the legend to Fig. 3. The reactions were staggered such that samples incubated for the indicated times (min) at 30 °C could be synchronously applied to the native gel. The positions of free DNA and of protein-DNA complexes are indicated. In the experiment shown in *panel B*, the polymerase was preincubated with affinity-purified anti-H4 antibody (170 ng) for 30 min on ice in transcription mixtures lacking NTPs and labeled template. Mixtures were supplemented with NTPs and DNA and incubated for the indicated times at 30 °C before being applied to the native gel. A control reaction that had been preincubated in the absence of antibody and then supplemented with NTPs and template was analyzed in parallel. The positions of free DNA and of protein-DNA complexes are indicated.

pershifted the preinitiation complex, as expected. Thus, the two species of ternary complex differed in that the predominant, and faster, complex lacked immunoreactive ETF. Although it is conceivable that ETF might be masked within this complex, we consider it more likely that ETF was just not present, which would be consistent with faster mobility relative to the upper complex that did, in fact, contain ETF. Anti-D1 had no effect on either species of G²¹ ternary complex (Fig. 3). Note that a very minor species of protein-DNA complex, migrating just ahead of the ETF-minus ternary complex, apparently lacked both H4 and ETF, as indicated by the absence of supershift with specific antibodies (Fig. 3). The composition of this minor species is not clear.

We conclude from the results presented thus far that H4 is a stoichiometric constituent of the transcription elongation complex. The lack of effect of anti-H4 on elongation by preformed G²¹ ternary complexes argues that H4 is not required for transcription elongation. Because the pulse-walk experiments necessarily entail nascent RNA cleavage by the ternary complex, we conclude that H4 is not essential for this activity either. In light of these findings, the profound effect of anti-H4 on the synthesis of the 21-mer RNA suggests a direct role for H4 in transcription initiation. Unlike H4, ETF need not remain associated with the ternary complex once it has been assembled. Indeed, the majority of G²¹ complexes present after a 30-min incubation lack ETF, which neatly explains the finding that anti-D6 had no impact on elongation. Apparently, ETF was able to dissociate from the promoter after the ternary complex has been established.

Kinetic analysis of transcription complex formation confirmed this view. At early times, preinitiation complexes and ternary complexes were both detected by native gel electrophoresis, and the ETF-containing species was the predominant form of ternary complex (Fig. 4A). A time-dependent increase in the abundance of the faster ETF-minus species was apparent over 30 min of incubation. This occurred concomitant with a decrease in the level of ETF-promoter complex (attributable to their conversion to ternary complexes) and with a decrease in the ETF-containing ternary complex (Fig. 4A). Note that the level of free DNA template did not vary during the incubation.

Establishment of the ternary complex, rather than ETF binding to the promoter, would appear to be a rate-limiting step in early transcription. The loss of ETF from the population of elongation complexes occurred relatively slowly, more slowly, in fact, than the 2.2 min needed to synthesize and process a typical vaccinia early mRNA by the virion-encapsidated transcription system (26). Previous footprinting experiments, which demonstrated retention of the ETF footprint in the ternary complex, were performed after a 10-min incubation and at saturating levels of ETF, which insured promoter occupancy (23). The very minor species alluded to earlier became evident only after 20–30 min of incubation.

The effect of preincubating polymerase with anti-H4 prior to addition of template and NTPs was to block ternary complex formation without affecting the binding of ETF to the promoter (Fig. 4B). Indeed, preinitiation complex accumulated to higher levels after preincubation of polymerase with anti-H4 than in the mock-treated control reaction. Preincubation with anti-D6 blocked the appearance of the ETF complex and thereby precluded the formation of the ternary complex (data not shown).

A parsimonious interpretation of these results holds that H4 subunit is required for transcription initiation at a viral early promoter. An indirect effect of bound antibody on polymerase activity *per se* is unlikely in light of the pulse-walk elongation experiments showing that H4 is not essential for elongation. We imagine that the H4 subunit acts as a molecular bridge to ETF bound at the promoter, and we argue that bound anti-H4 antibody disrupts or blocks the ETF-docking site on the polymerase. Whether H4 constitutes the only point of contact between ETF and polymerase holoenzyme, or is merely one component of the protein-protein interface, remains unclear. Along these lines, we have been unable thus far to detect binary interactions between H4 and the individual subunits of ETF using the yeast two-hybrid system,² which may attest that higher order subunit contacts are involved in polymerase recruitment.

It had been suggested (17) that the recovery of both holoenzyme and core forms of RNA polymerase from virions might

² J. Hagler and L. Deng, unpublished data.

H4 (95)	SLSFNDKNTTDEMTNLYDLFFFNTLDMLRQKKISILVNDVRGDVIVSYKNSDLVSSFNA
D1 (17)	ALA-KNASELEQRS-TAYEI-NNELELFIKPPLITLVLVNISTIQESFIRFTVTN-KEGVKI V TN
H4	ELEPEIKKIPFNMKNLPPYLEKNLDQLRFSKKYLDFAYLCLRHIGIPISKKKVNVRYVFLYK :
D1	RTKIPSLSKVHLVDVKN-VQ-LVDAIDNIVWEKKSLVTENR-LHKEC-LLRLSTEERHIFYK G D
H4	IDGLSIPPIIIKDFLDVKVYVYLENTGKIYKNSFSEDHNNNSLDWGKVVIPLLKDRHLYSIEF :
D1	KYGGSSIRLELVNLIQAKTKNFTIDFKL-KYFLGSGAQSKSSLHAINHPKSRRPNTSLEIEF
H4	LSSYHLHSYYTDLIARDEPVFVKRKKLIDIEIDEPEAWKRDRVRFVAFCEHQIRLKEAMKV :
D1	TPRDNETVPYDELI-KELTTLSRHIFMASPEVILSPPINAPIK-TFMLPKQDI-V--GLDN N
H4	DANYFTKINNPFANEFIYEDGVAYCRVCGINIPFNLDAAADVICKNTVIVSTFNKTIFLSEP :
D1	ENLYVTKTODIPITIRVTNSGL-YCYFTHLGY-IIIRYPVKRIIDSEVVV--FGEAV-KDKN A
H4	YSYFVHSQRFIFNIINSFDNIMKSQTWVMKYNNIRLNIFLIDINSRQQYEKKFSSIEKR :
D1	WTVYV-I-KLI-EPVNAINDRL-EES---KYVESKLV-DICDRIVFKSKKYYEGFPETT--TS
H4	GLFFLRLSANLFESQVSSTELFYVSKMLNLNYIVALVIILNSSADFIVSYMTSKNKTVVEES :
D1	EVVMD-LSTYL-PKQPEGVILYSGPKSNIDFRIKKENTIDQTAANVVFYRMSEPIIIFGES K
H4	TLKYAISVVVIYDFLVKTRICEKGSDLTIVLTVDVYTSIMPEEELDHFQRITLELRKLVSIQ :
D1	SVEYKKFSNDKGF-PKEYGSGKIVLNGVNYLNNIYLEYINTHNEVGKSVVPIKFIAEFL Y C
H4	RSALEPNYDVESRGEELPLSALKFFDTSTIIVKTMAPVHTYIEQKIVAPTPSVEPTDASLK :
D1	VNGEILKPRID-KTMKY-INSDEYYNQHNIIVEHLDQSK1GIDFNFEDKSDVGHQYANND G L
H4	NFKELTCDEDIKILIRVHDTNATKLVIFPSHLKIEIERKKLIIPLKSLSYITNTLKYYSNS :
D1	KFR-LNPEVSYFTNKRTRGPLGI-LSNYVKTLISMCSK-TF-LDDSNKRKVLADFGNG D I
H4	YLYVFRFGDPMPFEELIDHEHVQYKINCYNIRYHLLPDSDFVVFNSNSLNREALEYAFY :
D1	ALEYKFYGEALLV-ATDPDADAIARGNERYNKLNNSGIKTYKFDYIQTIRSDTFVSSV D I RE
H4	IFLSKYVNVKQW (777) :
D1	FYFGKF-NIIDW (677)

FIG. 5. Sequence alignment of H4 protein (rpo94) with the vaccinia capping enzyme large subunit. The amino acid sequence of the H4 protein is compared to that of the capping enzyme large subunit encoded by the vaccinia D1 gene. Identical residues are indicated by a colon (:), whereas conserved residues are denoted by a period (.). The H4 sequence is displayed continuously from position 95 to 777. Single-residue discontinuities in the D1 sequence are shown as dashed lines for gaps or as extra residues below the D1 aligned sequence.

indicate a catalytic role for the H4 protein, analogous to that of bacterial σ^{70} factor. σ^{70} is the distinctive constituent of the holoenzyme of *E. coli* RNA polymerase and is required for specific promoter recognition. σ^{70} is released from the template-bound polymerase after the synthesis of a short 9–12 nucleotide nascent chain, an event coinciding with stabilization of the ternary elongation complex (27, 28). We have shown that the vaccinia ternary complex is also stabilized after the synthesis of a short nascent chain, on the order of 7–12 nucleotides (21). Elongation complexes containing 21-mer transcripts are fully stable and do not differ functionally from those containing longer RNAs (22). Thus, our finding that H4 is a constituent of the G²¹ ternary complex argues against subunit recycling (as with σ). We cannot rule out the possibility that H4 can exit the elongation complex later in the transcription cycle, perhaps even in response to extrinsic protein factors. However, in the absence of such evidence for dissociation, we are inclined to regard the H4 protein as a *bona fide* subunit of the early-specific RNA polymerase and to henceforth refer to the H4 protein as rpo94.

An analogy can be drawn between vaccinia rpo94 and the RPB4/RPB7 subunits of yeast RNA polymerase II. As with rpo94, these two yeast subunits are found in less than stoichiometric amounts in preparations of yeast polymerase II because the yeast enzyme preparations contain a mixture of isoforms (35). Polymerase II lacking these two subunits

(polymerase II Δ4/7) is competent for nonspecific RNA synthesis but is inactive in promoter-dependent transcription (35), a situation akin to the case of vaccinia holoenzyme and core polymerase isoforms. The RPB4/RPB7 subunits can be dissociated from yeast RNA polymerase in the presence of 2 M urea and subsequently added back to polymerase II Δ4/7 to reconstitute the holoenzyme (35). It has not been reported whether RPB4/RPB7 is a component of the polymerase II elongation complex.

There are no extensive similarities between the primary sequence of vaccinia rpo94 and those of other proteins involved in eukaryotic or prokaryotic transcription that would provide immediate insight into its mechanism of action. Ahn and Moss (17) did note a 27-amino acid segment of rpo94 that could be aligned with a 25-amino acid segment of RAP30, a subunit of the RNA polymerase II general transcription factor TFIIF. The two polypeptides displayed 44% identity over this short region. This is provocative, given the involvement of RAP30 in recruiting mammalian polymerase II to the preinitiation complex (29). Our own data base search using MacVector software (IBI) turned up one alignment (optimized score 143) of great potential interest; this alignment was to the large subunit of the vaccinia virus mRNA capping enzyme, which is encoded by the vaccinia D1 gene (Fig. 5). The similarity between the two proteins is moderate, with 15% sequence identity plus 19% conserved residues; however, the striking feature of this alignment is that it extends essentially continuously over a 682-amino acid segment of the H4 protein.

The vaccinia capping enzyme is a heterodimer consisting of the D1 protein and a 33-kDa subunit encoded by the viral D12 gene. The D1 subunit, an 844-amino acid polypeptide, catalyzes the γ -phosphate cleavage and transguanylylation reactions of the mRNA capping pathway (30). The small subunit, in conjunction with the large subunit, is required for cap methylation (31, 32). In addition to its role in RNA processing, the capping enzyme is a direct participant in vaccinia transcription, as a termination factor in early mRNA synthesis (16) and as an accessory factor in the transcription of the intermediate class of viral genes (33, 34). Capping enzyme is specifically required for the initiation step at intermediate promoters. Consistent with its direct involvement in transcription, the capping enzyme can bind vaccinia RNA polymerase in solution to form a binary complex (22). (It is not clear whether capping enzyme bound to the core polymerase, the holoenzyme, or both.) Association with RNA polymerase is one property that is apparently shared by rpo94 and the capping enzyme. We speculate that the sequence similarities between rpo94 and D1 define or contribute to a structural motif for interaction of promoter specificity factors with vaccinia RNA polymerase. Testing this model in the case of rpo94 will require the reconstitution of RNA polymerase holoenzyme with purified rpo94 and polymerase core.

REFERENCES

- Moss, B., Ahn, B., Amegadzie, B., Gershon, P. D., and Keck, J. G. (1991) *J. Biol. Chem.* **266**, 1355–1358
- Broyles, S. S., Li, J., and Moss, B. (1991) *J. Biol. Chem.* **266**, 15539–15544
- Hagler, J., and Shuman, S. (1992) *J. Virol.* **66**, 2982–2989
- Li, J., and Broyles, S. S. (1993) *J. Biol. Chem.* **268**, 2773–2780
- Li, J., and Broyles, S. S. (1993) *J. Biol. Chem.* **268**, 20016–20021
- Broyles, S. S., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3141–3145
- Patel, D. D., and Pickup, D. J. (1989) *J. Virol.* **63**, 1076–1086
- Ahn, B.-Y., Gershon, P. D., Jones, E. V., and Moss, B. (1990) *Mol. Cell. Biol.* **10**, 5433–5441
- Ahn, B., Jones, E. V., and Moss, B. (1990) *J. Virol.* **64**, 3019–3024
- Broyles, S. S., and Pennington, M. J. (1990) *J. Virol.* **64**, 5376–5382
- Quick, S. D., and Broyles, S. S. (1990) *Virology* **178**, 603–605
- Amegadzie, B. Y., Ahn, B., and Moss, B. (1991) *J. Biol. Chem.* **266**, 13712–13718
- Ahn, B., Rosel, J., Cole, N. B., and Moss, B. (1992) *J. Virol.* **66**, 971–982
- Amegadzie, B. Y., Ahn, B., and Moss, B. (1992) *J. Virol.* **66**, 3003–3010

15. Broyles, S. S., Yuen, L., Shuman, S., and Moss, B. (1988) *J. Biol. Chem.* **263**, 10754–10760
16. Shuman, S., Broyles, S. S., and Moss, B. (1987) *J. Biol. Chem.* **262**, 12372–12380
17. Ahn, B., and Moss, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3536–3540
18. Kane, E. M., and Shuman, S. (1992) *J. Virol.* **66**, 5752–5762
19. Cong, P., and Shuman, S. (1992) *J. Biol. Chem.* **267**, 16424–16429
20. Luo, Y., Hagler, J., and Shuman, S. (1991) *J. Biol. Chem.* **266**, 13303–13310
21. Hagler, J., and Shuman, S. (1992) *J. Biol. Chem.* **267**, 7644–7654
22. Hagler, J., and Shuman, S. (1992) *Science* **255**, 983–986
23. Hagler, J., and Shuman, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10009–10103
24. Hagler, J., Luo, Y., and Shuman, S. (1994) *J. Biol. Chem.* **269**, 10050–10060
25. Hagler, J., and Shuman, S. (1993) *J. Biol. Chem.* **268**, 2166–2173
26. Shuman, S., and Moss, B. (1989) *J. Biol. Chem.* **264**, 21356–21360
27. Straney, D. C., and Crothers, D. M. (1985) *Cell* **43**, 449–459
28. Krummel, B., and Chamberlin, M. J. (1989) *Biochemistry* **28**, 7829–7842
29. Flores, O., Lu, H., Kileen, M., Greenblatt, J., Burton, Z. F., and Reinberg, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9999–10003
30. Shuman, S., and Morham, S. G. (1990) *J. Biol. Chem.* **265**, 11967–11972
31. Cong, P., and Shuman, S. (1992) *J. Biol. Chem.* **267**, 16424–16429
32. Higman, M. A., Bourgeois, N., and Niles, E. G. (1992) *J. Biol. Chem.* **267**, 16430–16437
33. Vos, J. C., Sasker, M., and Stunnenberg, H. G. (1991) *EMBO J.* **10**, 2553–2558
34. Harris, N., Rosales, R., and Moss, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2860–2864
35. Edwards, A. M., Kane, C. M., Young, R. A., and Kornberg, R. D. (1991) *J. Biol. Chem.* **266**, 71–75