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Use of Lysolecithin-Permeabilized Infected-Cell Extracts to Investigate the *in Vitro* Biochemical

RICHARD C. CONDIT,* JACKIE I. LEWIS,* MICHELLE QUINN,† LINDA M. CHRISTEN,† and EDWARD G. NILES†,[1]

Department of Molecular Genetics and Microbiology and the Center for Mammalian Genetics, University of Florida, Gainesville, Florida 32610; and †Department of Biochemistry and Center for Molecular Biology and Immunology, State University of New York, Buffalo, New York 14214

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Lysolecithin permeabilization of vaccinia virus-infected cells was employed to prepare extracts that support faithful transcription initiation *in vitro* on plasmids possessing early, intermediate, and late viral gene promoters. Conditions which optimize transcription from each promoter were defined. The *in vitro* system was used to investigate the multifunctional viral mRNA capping enzyme, which also functions as the viral early gene transcription termination factor (VTF) and a viral intermediate gene transcription initiation factor. A low level of signal-dependent termination of early gene transcription was observed *in vitro* which could be elevated by the addition of pure mRNA capping enzyme. VTF-dependent transcription termination was found to be restricted to templates that possessed an early promoter. This restriction mimics that observed *in vivo* and demonstrates that transcription termination is limited to RNA polymerase molecules that recognize early rather than intermediate or late gene promoters. Extracts prepared from cells infected at the nonpermissive temperature with a virus containing a ts mutation in gene D12L, which encodes the small subunit of VTF, are incapable of supporting both early gene transcription termination and intermediate gene transcription initiation. Both activities are restored upon addition of the purified wild-type mRNA capping enzyme.

INTRODUCTION

Poxviruses constitute a unique family of viruses that possess a double-stranded DNA genome yet conduct their replication cycle entirely within the cytoplasm of infected cells. Poxviruses have evolved to encode the enzymes required to carry out viral DNA replication, transcription, and mRNA processing (Moss, 1990a). Vaccinia, the prototypic poxvirus, has a genome 191,686 bp in length that contains approximately 200 genes (Goebel *et al.,* 1990). Gene expression is divided into three temporal classes. Early genes are transcribed in the virus core immediately after infection; intermediate genes are transcribed subsequent to the onset of DNA replication and require prior early gene expression; late gene transcription follows the intermediate genes and requires the synthesis of three intermediate proteins (Moss, 1990b). This temporal regulation of gene transcription is mediated by the timed synthesis of class-specific transcription initiation factors. The vaccinia early gene transcription factor (VETF) is a heterodimer, derived from the D6R and A8L genes, which binds to early promoters upstream from the transcription initiation site (Broyles *et al.,* 1988; Broyles and Fesler, 1990; Gershon and Moss, 1990). VTF, the vaccinia early gene transcription terminator, a different heterodimer comprising the D1R and D12L subunits, mediates signal-dependent termination of early RNA synthesis (Shuman *et al.,* 1987). VTF is also the vaccinia virus mRNA capping enzyme. Intermediate gene transcription initiation employs three protein factors; one has been identified as the viral mRNA capping enzyme, the second, a product of gene E4L, and the third, a host protein (Vos *et al.,* 1991; Rosales *et al.,* 1994). Initiation of late gene transcription is dependent upon the synthesis of three intermediate proteins, the products of genes A1L, A2L, and G8R, and an additional unidentified early protein P (Keck *et al.,* 1990; Wright and Coroneos, 1993; Kovacs *et al.,* 1994). The vaccinia virus RNA polymerase is a large multisubunit protein (Nevins and Joklik, 1977; Baroudy and Moss, 1980). Each subunit is encoded by a viral gene and several subunits show significant homology to subunits of eukaryotic RNA Pol II (Broyles and Moss, 1986).

A genetic approach to investigating viral gene expression is available to complement the ongoing biochemical studies. Collections exist of temperature-sensitive (ts) mutations (Dales *et al.,* 1978; Condit and Motyczka, 1981; Ensinger, 1982; Condit *et al.,* 1983; Ensinger and Rovinsky, 1983) that map to genes known to encode subunits of the viral RNA polymerase (Ensinger, 1987; Thompson *et al.,* 1989; Hooda-Dhingra *et al.,* 1989, 1990), transcription initiation factors (Christen *et al.,* 1992; Carpenter and DeLange, 1992), VTF (Carpenter and DeLange, 1991), and other proteins known to play a role in viral gene expression (Kane and Shuman, 1992; Simpson and Condit, 1994; Black and Condit, 1996). In addition, reverse genetic approaches have been developed to either introduce ts mu-

[1] To whom correspondence and reprint requests should be addressed. Fax: (716) 829-2725; E-mail: CAMEGN@UBVMSA.cc.buffalo.edu.

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tations into (Hassett and Condit, 1994) or provide conditional repression of any essential viral gene (Zhang *et al.,* 1992). To take full advantage of viral genetics it is necessary to determine the biochemical consequences of each mutation. Prior reports describe class-specific transcription employing partially purified extracts of wild-type virus-infected cells (Puckett and Moss, 1983; Foglesong, 1985; Vos and Stunnenberg, 1988). To permit the facile biochemical analysis of mutations in genes that affect viral gene expression a rapid means of preparing transcriptionally active extracts of cells infected with virus at either the permissive or the nonpermissive temperature was desirable. Lysolecithin treatment of virus-infected cells has been employed in the past to generate soluble cytoplasmic extracts for analysis of RNA virus transcriptase and replicase activities (Peluso and Moyer, 1983; Carlsen *et al.,* 1985; Moyer *et al.,* 1990; Horikami and Moyer, 1991; Horikami *et al.,* 1992) and for investigating translational properties of poxvirus mRNA (Brown *et al.,* 1983).

In this report we describe an approach to combining genetic and biochemical analysis of poxvirus gene transcription. Lysolecithin treatment of wild-type or ts mutant virus-infected cells yields an extract that is active in early, intermediate, and late gene transcription *in vitro.* Furthermore, signal-dependent termination of early gene transcription occurs which can be enhanced by the addition of pure VTF. This observation permitted the demonstration that class-specific restriction of transcription termination is determined by the promoter employed in transcription initiation. Extracts prepared from cells infected with a ts mutant in the viral mRNA capping enzyme exhibited an appropriate mutant phenotype *in vitro.*

MATERIALS AND METHODS

Cells and virus

BSC40 cells, wild-type vaccinia virus strain WR, the temperature-sensitive vaccinia virus strain IHD mutant ts9383, and the conditions for virus growth, infection, and plaque titration have been described previously (Dales *et al.,* 1978; Condit and Motyczka, 1981; Condit *et al.,* 1983; Carpenter and DeLange, 1991). A549 cells, a human lung carcinoma cell line, were obtained from Dr. Sue Moyer and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersberg, MD) containing 10% defined, supplemented bovine calf serum (HyClone Laboratories, Inc., Logan, UT).

Plasmids

pC_2AT19 (Sawadogo and Roeder, 1985), containing a 375-nt G-less cassette, was obtained from Dr. Stewart Shuman (Sloan Kettering Institute, New York, NY). pSB24 (Luo *et al.,* 1991), which contains a synthetic vaccinia virus early transcription promoter placed upstream of the 375-nt G-less cassette in pC_2AT19, was also obtained from Dr. Stewart Shuman. Accurate transcription of the pSB24 G-less cassette should yield an RNA of approximately 380 nt in length. pCFW10 (Wright and Moss, 1989), which contains the vaccinia virus 11k (gene F17R) late promoter placed upstream of the 375-nt G-less cassette in pC_2AT19, was obtained from Dr. Cynthia Wright (Armed Forces Institute of Pathology, Washington, DC). Accurate transcription of the pCFW10 G-less cassette should yield a 382-nucleoside RNA which is subject to 5′ polyadenylation.

pVGFG, which contains the vaccinia virus growth factor (VGF) (gene C11R) early promoter (Broyles *et al.,* 1991) placed upstream of the 375-nt G-less cassette in pC_2AT19, was constructed as follows. Two complementary VGF promoter-containing oligonucleotides were synthesized, 5′ phosphorylated, annealed, and ligated to pC_2AT19 linearized with *Sac*I and blunt ended with T4 DNA polymerase. The inserted oligonucleotide has the sequence 5′ *AGTACT*GTTTATATTACTGAATTAATAATATAAAATTCCCAATC 3′, where the plain text represents the VGF promoter with the initiating nucleotide underlined and the italic text represents a *Sca*I site introduced for ease of identification of the desired clone. The promoter sequence in the finished clone was confirmed by DNA sequence analysis. Accurate transcription of the pVGFG G-less cassette should yield an RNA of 379 nt in length.

pG8G, containing the vaccinia virus gene G8R intermediate promoter (Baldick *et al.,* 1992), was constructed in the same fashion as pVGFG, except that the sequence of the inserted oligonucleotide was 5′ *AGTACT*CATTTAACTTTAAATAATTTACAAAAATTTAAAATA 3′. In the authentic G8R promoter, the 3′-terminal residue is a G, and the 3′-terminal ATG is the gene G8 initiation codon. In our construction, the 3′-terminal G was changed to an A for compatibility with transcription of a G-less cassette. The promoter sequence in the finished clone was confirmed by DNA sequence analysis. Accurate transcription of the pG8G G-less cassette should yield an RNA of approximately 380 nt in length.

pG8GS is a derivative of pG8G that contains the vaccinia gene G8R intermediate promoter upstream of a 3′-truncated, 188-nt G-less cassette derived from pC_2AT19. The G8R promoter and the 5′ 188 nt of the pC_2AT19 G-less cassette were PCR amplified from pG8G using the M13-40 universal sequencing primer as the upstream primer and a downstream primer which contained nucleotides 169–188 of the G-less cassette flanked with a *Sma*I site, a *Sca*I site, and a *Bam*HI site. The PCR-amplified fragment was cleaved with *Eco*RI (upstream) and *Bam*HI (downstream) and cloned into the vector portion of pC_2AT19, which had also been cleaved with *Eco*RI and *Bam*HI. The *Sma*I site at the 3′ end of the resulting truncated G-less cassette serves to efficiently arrest transcription of the G-less cassette, and the downstream *Sca*I site was used for identification of the desired clone.

Accurate transcription of the pG8GS G-less cassette should yield an RNA of approximately 195 nt in length.

p11kGX is a derivative of pCFW10 that contains the vaccinia 11k (gene F17R) late promoter upstream of a 3′-truncated, 93-nt G-less cassette derived from pC_2AT19. It was constructed in exactly the same fashion as pG8GS, except that pCFW10 was used as the template for PCR amplification, and the downstream primer contained nucleotides 73–93 of the pC_2AT19 G-less cassette. Accurate transcription of the p11kGX G-less cassette should yield an RNA of approximately 103 nt in length which is subject to 5′ polyadenylation.

To construct plasmids capable of exhibiting signal-dependent transcription termination, a 156-bp G-less cassette containing tandem termination sequences AATTTTTATAATTTTTAT in the nontemplate strand was prepared from synthesized oligonucleotides. Plasmids pSB24, pG8G, and pCFW10, containing the vaccinia virus early, intermediate, and late promoters, respectively, were linearized by digestion with *Sma*I at the 3′ end of the G-less cassette and the termination cassette was inserted. Clones containing the insert in the correct orientation were identified by DNA sequence analysis of isolated plasmid DNA and each contained the structure denoted in Fig. 5.

Infected cell extracts for transcription

Whole-cell extracts were made by a modification of the procedure described by Brown *et al.* (1983). Confluent 100-mm dishes of A549 cells were infected with vaccinia virus at a m.o.i. of 15 and incubated at 37° for 16 hr unless otherwise indicated. Infected cells were placed on a tray of ice, and all subsequent steps were done at +4°. Medium was removed from the dishes and cells were washed once with "Kwash" buffer [30 m*M* HEPES, pH 7.4 (titrated with KOH), 150 m*M* sucrose, 50 m*M* KOAc, 4.5 m*M* $Mg(OAc)_2$]. Two milliliters of Kwash buffer containing 250 μg/ml lysolethicin (L-α-lysophosphatidylcholine, palmitoyl; Sigma Biochemical Co., St. Louis, MO) was added to each dish, and the solution was distributed over the monolayers for 2 min by gently rocking the dishes. The lysolethicin was diluted by addition of 5 ml of Kwash buffer to each dish, and the buffer was immediately aspirated from each dish. The covered dishes were raised at a steep angle for 4 min to drain the remaining solution, which was then aspirated from the dishes. Two hundred-fifty microliters of MEB [25 m*M* HEPES, pH 7.4 (titrated with KOH), 50 m*M* KOAc, 1 m*M* DTT, 7.5% glycerol] was added to each dish. The infected cells were scraped into this small volume of MEB and transferred to a chilled tube. Each 100-mm dish yielded approximately 350 μl of infected cells. The lysolethicin-treated cells were triturated 5–10 times with a narrow gauge hypodermic needle or a micropipettor. $CaCl_2$ (0.1 *M* $CaCl_2$ stock solution) was added to a final concentration of 1 m*M,* and micrococcal nuclease (1 mg/ml stock solution, 15,000 units/mg; Worthington Biochemical Corp., Freehold, NJ) was added to a final concentration of 12 μg/ml. Extracts were incubated at 30° for 15 min, then placed on ice, and EGTA (0.2 *M* stock solution) was added to a final concentration of 2 m*M.* Extracts were centrifuged in a Beckman Microfuge 11 at a setting of 1.6 for 5 min to pellet nuclei and cell debris. The supernatant was removed and stored at −70° in 110-μl aliquots. Extracts are stable at −70° for at least 6 months.

In vitro transcription

Reactions (10 or 50 μl) contained 25 m*M* HEPES, pH 7.4, 4.5% glycerol, 80 m*M* KOAc, 5 m*M* $MgCl_2$, 2.6 m*M* DTT, 1 m*M* ATP, 1 m*M* UTP, 20 μ*M* CTP, 2.5 μCi [α-^{32}P]CTP (~3000 Ci/mmol stock), 100 μ*M* 3′-*O*-Me-GTP, 10 μg/ml DNA, and up to 60% by volume cell extract. Reactions were mixed on ice and incubated at 30°C for 30 min. Three hundred-fifty microliters of "PK mix" [114 m*M* Tris–HCl, pH 7.5, 14 m*M* EDTA, 150 m*M* NaCl, 1.14% SDS, 114 μg/ml tRNA, 230 μg/ml proteinase K (Life Technologies, Gaithersberg, MD)] was added and the reactions were incubated at 37° for 30 min. Reactions were extracted once with an equal volume (400 μl) of phenol/chloroform. Nucleic acids were precipitated from the aqueous phase by addition of 100 μl 10 *M* ammonium acetate and 300 μl isopropanol, incubation at room temperature for 30 min, and centrifugation for 10 min. Pellets were washed once with 70% ethanol, dried, and resuspended in 20 μl formamide loading buffer. Samples were denatured at 90°, 3 min, and 10 μl was loaded on 4 or 7% polyacrylamide urea gels in TBE. Gels were run at 250 V until the xylene cyanol reached the bottom of the gel. Gels were fixed in 10% methanol, 10% acetic acid, dried, and autoradiographed at −70° with an intensifying screen.

Immunological techniques

Western blot analyses were performed on extracts prepared from wild-type and ts mutant virus-infected cell extracts using antisera raised in rabbits against the N-terminal 26 kDa of the D1R protein or the entire D12L protein. In order to estimate the levels of the D1R and D12L proteins in the mutant virus-infected cell extracts relative to the wild-type virus-infected cell extracts, Western blots were performed in which a constant amount of mutant extract was compared to serial dilutions of the wild-type virus-infected cell extract.

Guanylyltransferase activity

The first step in the guanylyltransferase reaction, the formation of covalently bound GMP, was assayed as described (Niles *et al.,* 1994). To estimate the level of active D1R in the mutant-infected cell extract relative to the wild-type virus-infected cell extract, E-GMP formation

activity in the mutant extract was compared to dilutions of normal infected cell extract.

Preparation of capping enzyme and the methyltransferase and D1R$^{1-545}$ domains

The intact capping enzyme, the methyltransferase domain, and D1R$^{1-545}$ were purified from induced *Escherichia coli,* as described (Higman and Niles, 1992; Higman *et al.,* 1994; Myette and Niles, 1996, respectively). The concentration of each was estimated from the molar absorption calculated from the amino acid content by the GCG program Peptidesort. The $E^{1\%}_{280}$ of D1R$^{1-545}$ was calculated to be 7.4. In each case, the enzyme was purified to electrophoretic homogeneity.

RESULTS

Preparation of an infected cell extract that supports viral transcription

Several previously isolated conditionally lethal mutants of vaccinia virus display defects in gene expression which indicate that the affected gene products normally function in viral transcription. Biochemical proof of this hypothesis and further characterization of the mutants requires reproduction of the *in vivo* mutant phenotype in an *in vitro* transcription system. To this end, a method was sought for biochemical analysis of transcription defects in mutant-vaccinia-infected cells. Several factors were considered initially. In order to readily test a number of different mutants and infection conditions, a method which permitted preparation of extracts on a small scale from infected cell monolayers was desirable. In addition, since other viral and cellular components might be involved in reproduction of a particular mutant phenotype, an extraction protocol which would result in minimal disruption of the cellular architecture and yield as crude an extract as possible was preferred. An appropriate procedure was found in the published work of Brown *et al.* (1983), who used lysolethicin permeabilization of vaccinia virus-infected cells to produce cell extracts competent for *in vitro* translation. Briefly, monolayer cultures of A549 cells are infected with vaccinia virus and incubated until the infection is well into the late phase. Infected cells are then permeabilized by brief exposure to lysolethicin in an iso-osmotic sucrose solution. Permeabilized cells are harvested in a hypotonic solution and treated with micrococcal nuclease, and nuclei are removed by low-speed centrifugation to yield a transcriptionally active supernatant which can be stored frozen (Materials and Methods). The extract is active in transcription of each of three reference vaccinia virus promoters representing each gene class, early, intermediate, and late (Figs. 1–4). A single 10-cm dish of infected cells yields enough extract for 10–20 50-μl reactions.

Several variables were tested which could affect the production of extracts (not shown). First, host cells were compared. The African green monkey kidney cell line, BSC40, has been used as host for all of the genetic studies of vaccinia virus strain WR, but lysolethicin extraction of infected BSC40 cells produced extracts with variable and often low activity. The human lung carcinoma cell line, A549, which has been used successfully for production of lysolethicin extracts of Sendai-, VSV-, and measles-infected cells (Peluso and Moyer, 1983; Carlsen *et al.,* 1985; Moyer *et al.,* 1990; Horikami and Moyer, 1991; Horikami *et al.,* 1992), yielded extracts of uniformly high activity. A549 cells were therefore chosen for all subsequent experiments. Second, the effect of infection time on transcription activity in extracts was assessed. *In vitro* transcription activity was not observed at 2 hr after infection when viral early gene transcription *in vivo* is at a peak. However, both early and late *in vitro* transcription activities could be observed by 7 hr postinfection and were found to be maximal between 12 and 72 hr postinfection. Third, the effect of micrococcal nuclease treatment on extract activity was tested. Transcription in crude extracts prior to removal of nuclei demonstrated that elimination of micrococcal nuclease treatment had no effect on activity. However, without micrococcal nuclease treatment, little if any transcription activity remained in the supernatant after removal of nuclei by centrifugation. Thus, micrococcal nuclease probably solubilizes RNA polymerase or transcription factors that are otherwise removed as part of a nucleoprotein complex during centrifugation. Finally, it was observed that initial freezing results in less than 50% loss of activity; extracts are not refrozen, and extracts are stable at $-70°$ for at least 6 months.

Several variables which could affect the *in vitro* transcription itself were tested. The use of a G-less cassette in the presence of 3′-*O*-methyl GTP was found to be necessary to suppress nonspecific background transcription, which otherwise obscures the signal from promoter-specific transcription (not shown). Titration of 3′-*O*-methyl GTP revealed that a minimum of 10 μM was required to promote efficient termination of transcription at the end of the G-less cassette (not shown). Utilization of radiolabeled CTP at concentrations lower than 10 μM resulted in synthesis of shorter than normal transcripts (not shown). The effect of KOAc, NaCl, NaOAc, and NH_4OAc, in the range of 30 to 230 m*M,* was tested (Fig. 1). All salts, particularly NaCl and NaOAc, were inhibitory at concentrations in excess of 100 m*M,* consistent with previous monovalent cation titrations done on transcription assayed *in vitro* from early (Luo *et al.,* 1991; Hagler and Shuman, 1992) or late (Schwer and Stunnenberg, 1988) promoters. In the case of the early promoters, the results of Luo *et al.* (1991) and Hagler and Shuman (1992) show that inhibition by NaCl acts at the level of initiation while elongating RNA polymerase is relatively insensitive to salt. In the system described here, transcription from

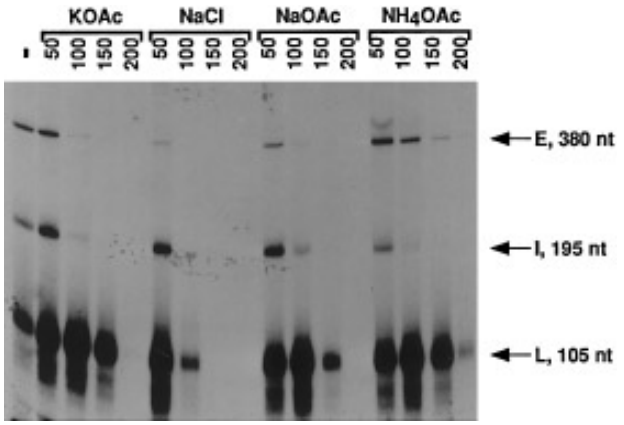

FIG. 1. Determination of salt optima in the *in vitro* transcription reaction. Reactions contained 10 μg/ml each pVGFG (E, 380-nt transcript), pG8GS (I, 195-nt transcript), and p11KGX (L, 105-nt transcript); 30 m*M* KOAc (contributed by the extract); optimum concentrations of other reagents; and added salt as indicated at the top. Reaction products were separated on a 7% polyacrylamide gel, which was dried and autoradiographed for 7 days.

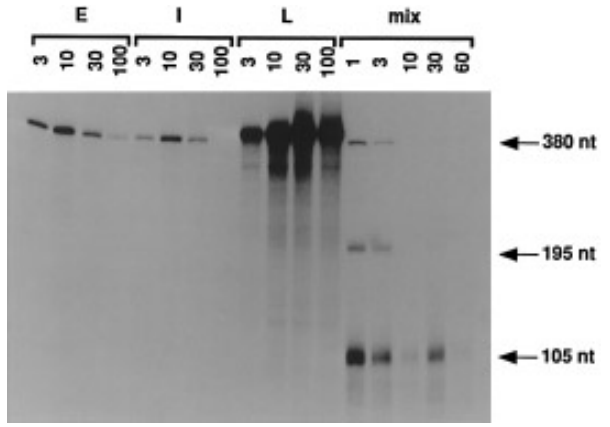

FIG. 2. Determination of optimum DNA concentration in the *in vitro* transcription reaction. Reactions contained pVGFG (E, 380-nt transcript), pG8G (I, 380-nt transcript), pCFW10 (L, 380-nt transcript), or a mixture (mix) of pVGFG (E, 380-nt transcript), pG8GS (I, 195-nt transcript), and p11KGX (L, 105-nt transcript), and optimum concentrations of other reagents. The DNA concentration, in μg/ml, is indicated at the top of each lane. In reactions containing the mixture of templates, the concentration given is the concentration of each of the three DNAs in the reaction. The signal observed with a mixture of 10 μg/ml each template in this experiment is artifactually low, probably due to poor sample recovery. Reaction products were separated on a 7% polyacrylamide gel, which was dried and autoradiographed for 4 days.

late promoters was somewhat less salt sensitive than transcription initiated at early or late promoters. KOAc gave the best stimulation; 80 m*M* KOAc was the optimum condition for coexpression of all three promoters. Identical salt titrations done with optimum concentration of each of the individual early, intermediate, and late promoters gave identical results (not shown). $MgCl_2$ and ATP optima were also determined empirically by titration (not shown). DNA titrations with templates containing each of the three classes of vaccinia virus promoters showed optimum transcription at DNA concentrations of approximately 10 μg/ml and inhibition at high concentrations of DNA (Fig. 2). The inhibition at high DNA concentrations could be mimicked by addition of an irrelevant DNA, indicating that the inhibition is a nonspecific salt or squelching effect, rather than a promoter-specific effect (not shown). Figure 2 also shows that when the system is programmed with three different promoters, each driving a different length G-less cassette, the optimum total DNA concentration for the template mixture is similar to the optimum DNA concentration for each of the individual templates. Extract titration revealed an optimum of 20–30 μl of extract per 50-μl reaction, regardless of DNA template used (Fig. 3). The extract optimum and absolute activity of each promoter is subject to slight variation when different extracts are compared.

Early gene transcription *in vitro* requires newly synthesized late proteins

Preliminary infection time course experiments described above suggested that the early transcription activity present in extracts is due to the synthesis of proteins late during infection rather than to enzymes present in infecting virions. To confirm this hypothesis we tested transcription using extracts made from cells infected in the presence of the DNA replication inhibitor hydroxyurea (HU), which permits early gene transcription but prevents intermediate and late gene transcription (Fig. 4). The results showed that extracts made in the presence of HU were incapable of initiating transcription at early and late vaccinia promoters, but were enhanced in their ability to transcribe the intermediate gene promoter. Stimulation of intermediate promoter activity probably reflects accumulation of early gene products, which include intermediate transcription factors, in the absence of intermediate and late gene transcription. The absence of late promoter activity in these extracts is consistent with the observation that three of the four known late transcription factors are the products of intermediate genes, whose transcription is inhibited in the presence of HU. The ab-

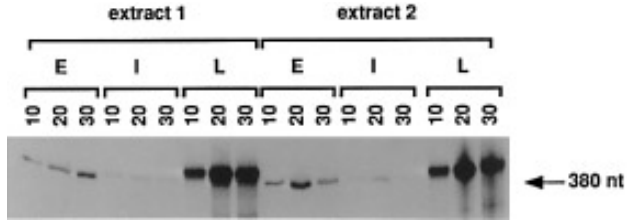

FIG. 3. Titration of extract in the *in vitro* reaction. Reactions (50 μl) contained pVGFG (E, 380-nt transcript), pG8G (I, 380-nt transcript), or pCFW10 (L, 380-nt transcript) DNA and 10, 20, or 30 μl of extract as indicated at the top, and optimum concentrations of other reagents. Two different extracts are compared. Reaction products were separated on a 7% polyacrylamide gel, which was dried and autoradiographed for 16 hr.

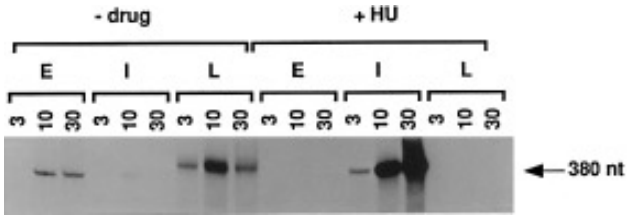

FIG. 4. The effect of hydroxyurea on infected cell extracts. Extracts were made from cells infected in the presence of 10 m*M* hydroxyurea (+HU) or in the absence of drug (− drug). Reactions contained 3, 10, or 30 μg/ml of pVGFG (E, 380-nt transcript), pG8G (I, 380-nt transcript), or pCFW10 (L, 380-nt transcript) DNA as indicated at the top, and optimum concentrations of other reagents. Reaction products were separated on a 7% polyacrylamide gel, which was dried and autoradiographed for 16 hr.

sence of early promoter activity in these extracts shows that the VETF and H4L-containing RNA polymerase are not provided by the infecting virions and suggests that each must be synthesized from late mRNA. Similar conclusions were drawn by Ahn and Moss (1992) who assayed transcription activity in extracts prepared from cytosine arabinoside-treated cells. It is noteworthy that the VETF activity observed *in vitro* is suppressed *in vivo* by unknown mechanisms, since in general early vaccinia genes are not transcribed late during infection. Our results in this regard are consistent with previously reported experiments (Zhang *et al.,* 1994).

Signal-dependent termination of transcription in infected cell extracts

To test the ability of the extracts to support early gene transcription termination, the plasmid templates were modified to contain an additional G-less region possessing a tandem early gene transcription termination signal. Synthesis of a transcript that extends from the initiation site to the end of the G-less cassette would yield a product of about 540 bases in length. Signal-dependent termination of transcription would be expected to produce a family of RNA products about 450 bases in length. Transcription of pSB24-term was carried out in the presence of various levels of exogenously added VTF and the RNA products were analyzed by gel electrophoresis (Fig. 5). In the case of the early-promoter-containing template, pSB24-term (Fig. 5A), two products are observed. The larger is the full length 540-base runthrough transcript. A minor smaller species is of the size expected for termination products. As the level of exogenous VTF is elevated there is a decrease in the yield of the 540-base RNA and a concomitant increase in the termination product. This confirms the observation reported previously that VTF is a required termination factor (Shuman *et al.,* 1987). It also demonstrates that the level of endogenous termination factor is limiting in this infected cell extract.

Transcription initiated at intermediate or late promoters fails to terminate *in vitro*

In vivo, signal-dependent transcription termination is restricted to the early class of genes (Wittek *et al.,* 1980). At intermediate or late times in infection, the TTTTTNT signal is disregarded. The mechanism of class restriction of transcription termination is unknown. However, one could speculate that VTF is inactivated or sequestered at late times or that the RNA polymerase employed at intermediate or late times is itself insensitive to VTF. In order to determine whether transcription initiated at intermediate or late gene promoters is susceptible to VTF-dependent termination *in vitro,* an extract prepared from wild-type virus-infected cells was programmed with plasmid DNA containing each promoter class, and RNA was prepared and separated by gel electrophoresis (Figs. 5B and 5C). Transcription initiated at an intermediate gene promoter is not terminated by VTF at any level tested, extending the observation of Vos *et al.* (1991). Likewise, transcription initiated at a late promoter is also insensitive to VTF-dependent termination (Fig. 5C). To test whether a pool of inactive VTF is present in the late infected cell extract, Western blot analysis was conducted employing antibodies raised against the D12L subunit of VTF (Niles *et al.,* 1989). By comparison of the intensity of the VTF in the extract to known VTF standards it was determined that the level of termination activity

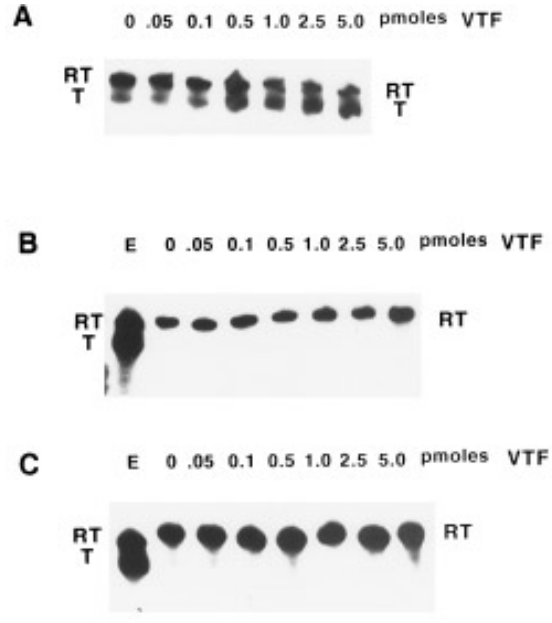

FIG. 5. Promoter-dependent termination of transcription. Transcription reactions were carried out in 10-μl volumes containing a template possessing an early, intermediate, or late gene promoter and a G-less cassette harboring a tandem termination signal. Reactions were done in the absence or presence of varying amounts of VTF. VTF, vaccinia termination factor (mRNA capping enzyme); RT, readthrough transcript; T, terminated transcript; E, products produced by transcription of pSB24-term in the presence of VTF.

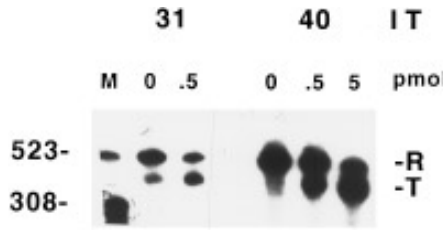

FIG. 6. Effect of a ts mutation in D12L on early gene transcription termination. An extract was prepared from cells infected at either 31 or 40° with a virus possessing a ts mutation in gene D12L. Transcription termination activity was measured in each extract using the pSB24-term template, in the absence or presence of additional VTF. IT, infection temperature, extracts employed were prepared from cells infected at 31 or 40°; R, readthrough transcript; T, termination product; M, marker lane, marker molecular weights are noted at the left.

observed in the first lane of Fig. 5A is roughly consistent with the amount of cross-reacting material in the infected cell extract (data not shown), demonstrating that the late infected cell extract does not contain a large pool of sequestered or inactivated VTF.

Analysis of transcription termination in extracts prepared from a ts mutation in the D12L subunit of VTF

Carpenter and DeLange (1991) mapped the ts9383 mutation to the D12L subunit of VTF. In order to ascertain whether the ts phenotype could be replicated *in vitro,* extracts were prepared from cells infected at 31 or 40°, the permissive and nonpermissive temperatures, respectively. When programmed with pSB24-term, the 31° extract supported synthesis of both the runthrough transcript and the termination product (Fig. 6). Exogenously added VTF enhanced the level of termination to about 70%. The extract prepared from cells infected at the nonpermissive temperature directed the synthesis of only the 540-nucleotide runthrough RNA product, demonstrating that this extract was devoid of VTF activity. Addition of pure VTF restored termination activity, providing further evidence that the small D12L subunit is essential for transcription termination, in agreement with the *in vitro* results of Luo *et al.* (1995).

The D12L subunit is required for intermediate gene transcription initiation

The mRNA capping enzyme has been shown to be an essential intermediate gene transcription factor (Vos *et al.,* 1991; Harris *et al.,* 1993). In order to assess whether the D12L subunit is required for intermediate gene transcription initiation, extracts prepared from ts mutant virus cells infected at 40° were programmed with pG8G-term, and transcription was carried out in the presence or absence of capping enzyme (VTF) (Fig. 7). In the absence of exogenously added capping enzyme, the ts mutant virus-infected cell extract does not support intermediate gene transcription. Addition of capping enzyme restores the synthesis of RNA from a template containing the intermediate promoter, providing genetic evidence for the requirement of the D12L subunit for intermediate gene transcription. The 40° ts mutant virus-infected cell extracts also lack late transcription activity (data not shown).

VTF is unstable in ts9383-infected cell extracts

In several instances, vaccinia virus ts mutant proteins synthesized at the nonpermissive temperature are rapidly degraded (Dyster and Niles, 1991; Christen *et al.,* 1992). In order to assess the stability of the D1R and D12L subunits in ts9383-infected cells, Western blot analysis was conducted and the results are presented in Fig. 8A. The level of D12L is equivalent in extracts prepared from cells infected with wild-type virus at either temperature and in an extract of cells infected at the permissive temperature with ts mutant virus. However, D12L is barely detectable, 10% or less of normal, in an extract of cells infected at 40° with the ts9383. When the D1R subunit is analyzed, it can be seen that D1R is reduced to about 40 to 50% of normal in the extract of cells infected with ts9383, at the nonpermissive temperature. Based on these observations we can conclude that while the 40° ts9383 extract is nearly devoid of the D12L subunit, a significant amount of D1R remains. To determine whether the remaining D1R subunit retains enzymatic activity, the first step of the guanylyltransferase reaction was measured. In Fig. 8B, it can be seen that the extract prepared from cells infected with ts9383 at 40° retained E-GMP formation activity. Quantitation by densitometry showed that the mutant extract retained about 36% of the wild-type extract activity, similar to the relative level of D1R protein observed in Fig. 8A. Since a substantial amount of enzymatically active D1R remains in the ts9383-infected cell extract we can conclude that in these assays, the D1R subunit alone is incapable of mediating either early gene transcription termination, consistent

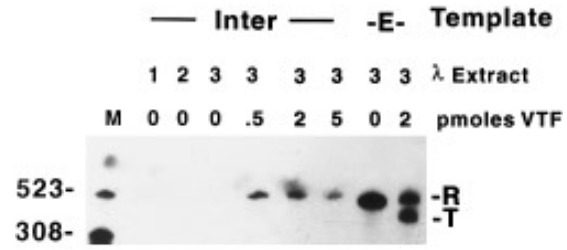

FIG. 7. Intermediate gene transcription requires mRNA capping enzyme *in vitro.* Extracts were prepared from cells infected at 40° with a virus containing a ts mutation in gene D12L. Transcription reactions were conducted in the presence of pG8G-term (intermediate) or pSB24-term (early), in the absence or presence of added VTF (mRNA capping enzyme). Template, Inter possesses an intermediate gene promoter, E possesses an early gene promoter; M, molecular weight markers, weights noted at the left; R, readthrough transcript; T, termination product.

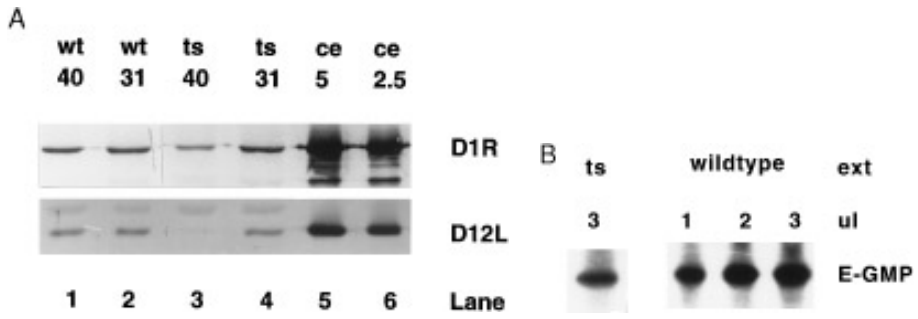

FIG. 8. Concentration of D1R and D12L subunits and guanylyltransferaes activity in extracts prepared from cells infected at the permissive and nonpermissive temperatures with wild-type and ts9383 virus. (A) Western blot analysis of D1R and D12L. Ten microliters of extracts prepared from cells infected with either wild-type virus or ts9383 at either 31 or 40°, or 2.5 or 5 pmol of capping enzyme (ce), were separated by gel electrophoresis, transferred to nitrocellulose, and incubated with antisera raised against either the N-terminal region of D1R or the entire D12L protein. (B) Guanylyltransferase activity. One to three microliters of wild-type infected-cell extract or 3 μl of ts9383 virus-infected cell extract was incubated with 1.2 μM [α-^{32}P]GTP (800 Ci/mmol) for 1 min at 0°. The samples were quenched with SDS sample buffer, separated by gel electrophoresis, and analyzed by autoradiography.

with the observations of Luo *et al.* (1995), or intermediate gene transcription initiation. Furthermore, these results suggest but do not prove that the D12L subunit plays a direct role in both processes, either through a direct interaction or by maintaining the D1R subunit in an active conformation.

Active subdomains of the mRNA capping enzyme do not support either early gene transcription termination or intermediate gene transcription initiation

The mRNA capping enzyme (VTF) consists of two functional domains. The N-terminal region of D1R harbors the mRNA triphosphatase and guanylyltransferase activities (Shuman, 1989, 1990; Shuman and Morham, 1990; Higman and Niles, 1992). A second domain containing the (guanosine-7-)methyltransferase activity consists of the small D12L subunit and the carboxyl-terminal domain of D1R (Higman *et al.,* 1994; Mao and Shuman, 1994). Each domain can be expressed in *E. coli* and isolated as two kinetically and functionally independent units (Higman *et al.,* 1994; Myette and Niles, 1996). To assess whether the subdomains are capable of supporting termination of transcription initiated at an early promoter, wild-type extracts were programmed with pSB24-term in the absence or presence of various levels of VTF or of either subdomain (Fig. 9). As seen in Fig. 5, in the absence of exogenous VTF, both a runthrough and a terminated transcript are synthesized. Addition of VTF enhances the synthesis of the termination product. The addition of neither D1R$^{1-545}$ nor the methyltransferase domain increases the production of the termination product, demonstrating that neither is capable alone of mediating early gene transcription termination. Addition of both subdomains together also fails to support termination (data not shown). These observations confirm and extend the results of Luo *et al.* (1995) who showed that neither subunit alone nor the methyltransferase domain were able to mediate early gene transcription termination *in vitro.* Furthermore, addition of either subdomain fails to diminish termination activity mediated by the endogenous VTF, demonstrating that each subdomain is incapable of acting as a dominant negative inhibitor. At very high concentrations, D1R$^{1-545}$ inhibits all transcription by an unknown mechanism.

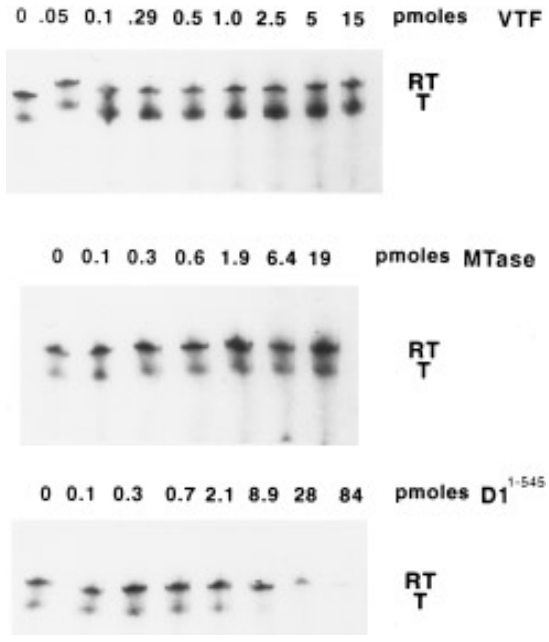

FIG. 9. Active mRNA capping enzyme subdomains lack transcription termination activity. Transcription was conducted in 10-μl reactions containing pSB24-term as the template, in the absence or presence of varying levels of capping enzyme derivatives. Top, VTF (the intact mRNA capping enzyme); middle, Mtase (the subdomain that exhibits (nucleoside-7)methyltransferase activity containing the carboxyl-terminal domain of the D1R subunit from amino acids 497 to 844 and the intact D12L subunit); bottom, D1$^{1-545}$ (the subdomain of the D1R subunit from amino acids 1 to 545, possessing the ATPase, mRNA triphosphatase, and guanylyltransferase active sites). RT, readthrough transcript; T, termination product.

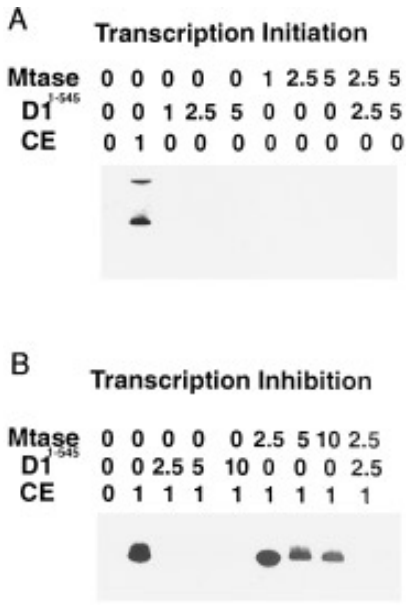

FIG. 10. Effect of mRNA capping enzyme subdomains on intermediate gene transcription. (A) Transcription analyses were carried out in 10-μl reactions containing pSB24-term DNA (lanes 1 and 2) or pG8G-term DNA (lanes 3 to 10) and varying levels of CE, mRNA capping enzyme, or the D1$^{1-545}$ or MTase subdomains. (B) Inhibition of capping enzyme-dependent intermediate gene transcription by the mRNA capping enzyme subdomains was tested in transcription reactions containing pG8G-term DNA and varying levels of mRNA capping enzyme or the capping enzyme subdomains. The amount of each protein added is noted at the top of each lane in picomoles.

To test whether either subdomain is able to support initiation of intermediate gene transcription, extracts prepared from 40° ts mutant virus infected cells were programmed with pG8-term in the presence or absence of VTF or one of the subdomains (Figs. 10A and 10B). In the absence of added VTF, there was no intermediate gene transcription. However, added VTF permitted transcription from pG8G-term. Neither subdomain alone nor both together supported intermediate gene transcription *in vitro.* To ascertain whether either subdomain was capable of acting as a dominant negative effector of intermediate gene transcription, the reaction was conducted in the presence of VTF and differing levels of D1R$^{1-545}$ or the methyltransferase domain (Fig. 10B). While neither subdomain was capable of supporting transcription initiation from an intermediate promoter, the D1R$^{1-545}$ acted as a potent inhibitor of VTF activity while the methyltransferase domain was a much weaker inhibitor.

DISCUSSION

Temperature-sensitive mutations have been isolated in a variety of vaccinia virus genes that encode proteins known to play a role in viral transcription and mRNA processing. In order to understand the biochemical basis of these mutant phenotypes, a method was developed for the rapid preparation of wild-type and mutant virus-infected-cell extracts that support faithful gene transcription *in vitro.* Specific transcription initiation of plasmids containing a G-less cassette flanked by an early intermediate or late gene promoter was observed. Conditions for optimal transcription of each plasmid were determined empirically.

In addition to RNA polymerase, transcription initiation at an early promoter requires both a specific heterodimeric protein, VETF (Broyles *et al.,* 1988), which is packaged in the virion core, and the H4L protein, a subunit of the virion RNA polymerase (Ahn and Moss, 1992). Both H4L and the two subunits of VETF are synthesized at late times in infection. Analysis of extracts prepared from cells infected with virus in the presence of a DNA replication inhibitor, hydroxyurea, demonstrates that the H4L and VETF employed in early gene transcription are newly synthesized rather than released from infecting virus cores, in agreement with the observations of Ahn and Moss (1992). The use of hydroxyurea also substantially increases the intermediate gene transcription activity found in the cell extract. It is interesting that while extracts display early promoter specific transcription activity *in vitro,* few if any early genes are transcribed *in vivo* at late times after infection when the extracts are made. Thus, the early transcription activity present late during infection *in vivo* is somehow suppressed, and this suppression is unmasked when extracts are made. The mechanism of suppression of early transcription *in vivo* is obscure; one possibility is that packaging of the early transcription apparatus into maturing virions suppresses early transcription late during infection.

Template plasmids were altered to contain an extended G-less region harboring tandem transcription termination signals. Wild-type virus-infected-cell extracts were shown to support limited termination of transcription initiated at an early promoter. The extent of termination observed was dependent on the amount of exogenously added VTF, indicating that the level of active VTF is limiting in the extract. VTF-dependent termination was found to be limited to transcription initiated at an early promoter; that is, transcription initiated at an intermediate or late promoter does not respond to VTF. This observation is consistent with *in vivo* mapping results that demonstrated that only early transcripts possess easily defined 3′ termini (Wittek *et al.,* 1980). Western blot analysis, carried out to quantify the amount of endogenous VTF in the extract, showed that a pool of inactivated VTF does not exist which might account for the inability of VTF to terminate transcription initiated at intermediate or late promoters *in vivo.* Furthermore, since addition of picomole amounts of VTF results in efficient termination *in vitro,* there is no support for a system for rapid inactivation of VTF at late times in infection. Since VTF does not have to be present at the time of transcription initiation but rather it can terminate transcription of preinitiated paused complexes (Hagler *et al.,* 1994), there is no evidence that the promoter itself or VETF plays an

essential role in generating a termination-competent RNA polymerase. Both genetic (Ensinger, 1987; Hooda-Dhingra *et al.,* 1989, 1990; Thompson *et al.,* 1989) and biochemical evidence indicate that the species of RNA polymerase employed at early times in infection is not identical to that used at later times. It was recently shown that a portion of the virion or early RNA polymerase possesses a unique additional subunit, the product of the H4L gene (Ahn and Moss, 1992; Ahn *et al.,* 1994; Kane and Shuman, 1992). RNA polymerase containing the H4L protein preferentially initiates at early promoters, while RNA polymerase lacking the H4L protein initiates transcription only at intermediate and late promoters (Ahn and Moss, 1992; Ahn *et al.,* 1994; Kane and Shuman, 1992; Zhang *et al.,* 1994; Wright and Coroneos, 1995). Immunological evidence demonstrates that the H4 subunit remains associated with the elongating RNA polymerase molecule (Deng and Shuman, 1994). One appealing model proposes that the H4L subunit of the early RNA polymerase is an essential cofactor employed in early gene transcription termination.

The mRNA capping enzyme (VTF) can be separated into two active subdomains, one possessing the ATPase, mRNA triphosphatase, and guanylyltransferase activities and the other possessing the (nucleoside-7-)methyltransferase active site (Shuman, 1989, 1990; Shuman and Morham, 1990; Higman and Niles, 1992; Higman *et al.,* 1994; Myette and Niles, 1996). Our results show that neither subdomain alone is capable of supporting termination of early gene transcription *in vitro,* consistent with previously reported observations (Luo *et al.,* 1995). Furthermore, neither subunit exhibits the ability to inhibit termination activity of the endogenous VTF. However, at high concentrations, $D1R^{1-545}$ is a general inhibitor of transcription by an unknown mechanism. Likewise, neither subdomain, alone nor in combination, is able to support initiation of intermediate gene transcription. Furthermore, the D1R remaining in ts9383-infected-cell extracts is inactive in intermediate gene transcription, demonstrating that the D12L subunit plays a role beyond stabilizing the D1R subunit. Unlike the early gene transcription termination activity of VTF, the subdomains do act as inhibitors of capping enzyme's ability to support intermediate gene transcription. $D1R^{1-545}$ is a particularly potent inhibitor, suggesting that this domain of the capping enzyme may be a site of interaction with the other intermediate gene transcription initiation factors. This interaction appears to be essential for transcription initiation (Rosales *et al.,* 1994).

A ts mutation was mapped to gene D12L which encodes the small subunit of the mRNA capping enzyme (Carpenter and DeLange, 1991). Extracts prepared from cells infected at the permissive temperature exhibit normal VTF activity. Extracts derived from cells infected at 40°, however, are unable either to terminate early gene transcription or to initiate intermediate gene mRNA synthesis. Both activities can be restored by the addition of pure mRNA capping enzyme. These results provide genetic evidence in confirmation of the known role for the capping enzyme in both activities. More importantly, this approach provides a convenient null extract to analyze the activity of capping enzyme mutants in both early gene transcription termination and intermediate gene transcription initiation.

Interestingly, *in vivo,* the D12L ts mutant displayed no apparent defects in either early transcription termination or intermediate transcription initiation, but rather was defective in telomere resolution and morphogenesis (Dales *et al.,* 1978; Carpenter and DeLange, 1991). Thus the *in vitro* phenotype of this mutant differs significantly from the *in vivo* phenotype, and the *in vitro* phenotype is more consistent with the known activities of the D12L protein. This observation suggests that the *in vivo* phenotype of a ts mutant may sometimes be misleading, and that analysis of mutant extracts *in vitro* may be a useful supplement to understanding the true biochemical consequences of mutation of a given gene. It must be kept in mind that the *in vivo* observation that normal virus protein synthesis was maintained at the nonpermissive temperature in ts9383-infected cells, reported by Carpenter and DeLange (1991), was obtained during the initial 12 hr of infection. Our results were obtained employing extracts prepared from cells infected for 20 hr at the nonpermissive temperature.

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