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Vaccinia Virus Gene D12L Encodes the Small Subunit of the Viral mRNA Capping Enzyme

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Vaccinia virus gene D12L, which lies between nucleotides 14,350 and 13,487 in the *Hin*dIII D fragment, is transcribed at early times in infection and is capable of encoding a protein 287 amino acids in length with a predicted molecular mass of 33,331. A polyclonal antiserum was raised in rabbits to a fusion protein containing 279 amino acids of the D12L protein, and this serum was used to investigate both the time of synthesis and the function of the D12L protein. A combination of Western blot analysis and immunoprecipitation from pulse-labeled and pulse-chased cell extracts demonstrated that the synthesis of a 31-kDa protein begins early in infection, that it reaches a plateau by about 4 hr, and that it is stable in the infected cell. The D12L protein was localized by Western blot analysis of detergent-solubilized virions to the sodium deoxycholate soluble fraction which suggested that it may be a virion core-associated enzyme. Due to the similarity in apparent molecular weight between the D12L protein and the small subunit of the vaccinia mRNA capping complex the anti-D12L antiserum was employed in Western blot analysis of fractions generated during the purification of the virion mRNA capping enzyme. The 31-kDa D12L protein copurified with the virus capping enzyme through chromatography on heparin–agarose and phosphocellulose and also cosedimented with the capping enzyme through a glycerol density gradient. In addition, the anti-D12L antiserum coprecipitated the large subunit of the capping enzyme, confirming that gene D12L encodes the small subunit of the viral mRNA capping enzyme. An insertion mutation which destroys the gene D12L coding sequence was constructed in a plasmid containing a portion of both genes D11L and D12L and this plasmid was used to rescue a ts mutation, in a single step, in the adjacent gene D11L. Southern blot analysis of the re-plaque-purified virus permitted the identification of the mutant virus only when the mutant was propagated in the presence of wild-type helper virus. We concluded from these data that gene D12L is essential for virus propagation in tissue culture.

INTRODUCTION

Vaccinia virus, a member of the poxvirus family, contains a double-stranded DNA genome of about 186,000 bp in length, large enough to contain about 150 simple genes. Viral genes are divided into two groups which differ in their dependence on viral DNA replication for expression. Early viral genes are transcribed soon after infection, while late genes are expressed after DNA replication begins. Poxviruses are unusual DNA viruses in that they replicate in the cytoplasm of infected cells. In order to carry on this peculiar life style, poxviruses encode many, if not all, of the enzymes employed in viral DNA replication and gene expression and they encapsidate the enzymes required to transcribe their early genes and to process early viral mRNA (reviewed in Moss, 1985).

The 5′ ends of both early and late viral mRNA possess a characteristic cap structure (Boone and Moss, 1977). A virion mRNA capping enzyme has been isolated and shown to possess two subunits, one of about 96,000 Da and another estimated to be either 31,600 or 26,000 Da (Martin *et al.*, 1975; Shuman *et al.*, 1980). The purified capping enzyme catalyzes the first three reactions in the cap formation pathway: polynucleotide 5′ triphosphatase, mRNA guanylytransferase, and mRNA (guanine-7-) methyltransferase (Martin *et al.*, 1975; Venkatesan *et al.*, 1980). In addition, this remarkable enzyme has recently been shown to be a cofactor involved in the sequence specific termination of early gene transcription (Shuman *et al.*, 1987).

Capping enzyme activity has been shown to increase in cells infected in the presence of cytosine arabinoside, an inhibitor of DNA replication (Boone *et al.*, 1977; Morgan *et al.*, 1984), suggesting that the capping enzyme subunits are virion encoded and are the products of early genes. Furthermore, Morgan *et al.* (1984) have provided evidence that vaccinia gene D1R encodes the large subunit of the capping enzyme. D1R has been sequenced (Niles *et al.*, 1986) and transcription mapping studies have shown that it is transcribed at early times in infection (Morgan *et al.*, 1984; Lee-Chen *et al.*, 1988).

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In this report we demonstrate that gene D12L encodes the small subunit of the mRNA capping enzyme complex. Furthermore, we show that the gene is expressed early in infection and that the D12 protein is stable in the infected cell. Finally, we present evidence that the capping enzyme is essential for virus replication in tissue culture.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus WR and the ts mutant C50 were propagated on BSC40 cells as described (Condit and Motyczka, 1981).

Immunological techniques. A fusion gene in which a portion of the gene D12L coding sequence was linked to the *Escherichia coli* trp E gene was constructed in the following way. A segment of the vaccinia *Hin*dIII D fragment which extends from the *Hpa*II site at 14,323, near the 5′ end of gene D12L, to the *Bam*HI site at 12,838 in the adjacent gene D11L, was ligated to the vector pATH 2 (Dieckmann and Tzagoloff, 1985). Induction of HB101 cells containing this plasmid with indole acrylic acid results in the synthesis of an insoluble fusion protein, which contains 279 of a possible 287 amino acids of the D12L protein linked to a portion of the trp E protein. The fusion protein was purified by repeated washing in 10 m*M* Tris–HCl pH 8.0, followed by preparative polyacrylamide gel electrophoresis. Antibodies were raised in rabbits by injection of 200 μg of fusion protein in Freund's complete adjuvant followed at 2-week intervals with booster injections containing 100 μg fusion protein in Freund's incomplete adjuvant. One week after the third boost, the rabbits were bled and after clot formation, the serum was separated and stored in aliquots at −20°.

Western blot analysis was carried out as described in the Bio-Rad literature using either anti-D12R antiserum or preimmune serum at a 1/500 dilution, and goat anti-rabbit antibody linked to alkaline phosphatase at a 1/3000 dilution, as the second antibody (Bio-Rad). Virus-infected cell extracts were prepared by infection of confluent monolayer cultures of BSC40 cells in 100-mm dishes, with wild-type vaccinia WR at a m.o.i. of 20. At times after initiating the infection, 1 ml of lysis buffer containing 50 m*M* Tris–HCl, 10 m*M* β-mercaptoethanol, and 0.3% SDS was added and cell lysis was effected by freezing at −20°. The frozen cultures were thawed, the dishes were scraped, and the lysate was transferred to Eppendorf microfuge tubes. Prior to electrophoresis, the extracts were boiled, cooled, and centrifuged for 5 min in a microfuge to remove any insoluble material. An aliquot of each extract was mixed with a 2 times concentrated Laemmli sample buffer solution and reboiled, and the proteins separated by electrophoresis in a 12.5% polyacrylamide gel (Laemmli, 1970). Proteins were electroblotted on to nitrocellulose and the migration positions of the cross-reacting proteins were determined by antibody staining.

The time of synthesis and the stability of the D12L protein was measured by immunoprecipitation of the D12L protein from extracts of cells labeled with ^{35}S amino acids. Cell extracts were prepared from monolayer cultures in 60-mm dishes infected with wild-type vaccinia virus WR at a m.o.i. of 20. At times after infection the medium was removed and the cells were washed with PBS (170 m*M* NaCl, 3.35 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4). Cells were pulse-labeled for 30 min at 37° with 125 μCi/ml of a mixture of [^{35}S]methionine (70%) and [^{35}S]cysteine (15%) (Translabel, ICN) in 0.8 ml of PBS. After the labeling period, the isotope was removed and the monolayers were washed twice with PBS. In pulse–chase experiments, after pulse-labeling, the monolayers were washed as described and the infected cells were overlayed with 4 ml of DME containing 10% fetal calf serum and 1 m*M* methionine. Pulse-labeled or pulsed-chased cells were lysed by the addition of 1 ml of one of three buffers: RIPA, 10 m*M* Tris–HCl, pH 8, 0.14 *M* NaCl, 1% bovine serum albumin, 0.025% sodium azide, 1 m*M* phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS); Maxi buffer: similar to RIPA with the following exceptions, 0.2% SDS, 0.2% DOC, 0.5% nonidet P40 NP40, 50 m*M* β-mercaptoethanol, 1.0% bovine hemoglobin; SDS buffer: 0.3% SDS, 12.5 m*M* Tris–HCl, pH 6.8, 50 m*M* β-mercaptoethanol. The lysed cells were frozen at −20°, and after thawing, the cell debris was transferred to an Eppendorf microfuge tube. The cell extract prepared in SDS buffer was boiled for 5 min, cooled, and cleared by centrifugation for 5 min in a microfuge. The other cell extracts were cleared by centrifugation for 15 min in a microfuge at 4°. In all cases, the supernatants were stored in aliquots at −70°. In each case, greater than 95% of the ^{35}S was found to be present in the supernatant fraction and upon gel electrophoretic analysis, there was no obvious loss of viral proteins into the insoluble fraction.

For each immunoprecipitation, 5 to 8 μl of antiserum was employed with 25 to 50 μl of extract in a total incubation of 200 μl. The extracts were precleared with 25 μl per milliliter of protein A agarose (Calbiochem). The first antiserum was incubated for 4 to 16 hr at 4° with constant rocking. The antibodies were collected by the addition of 20 μl protein A agarose and the precipitate was washed four times as described (Springer, 1987). The final pellet was resuspended in 50 μl of sample buffer and boiled, and 25 μl was applied to a polyacryl-

amide gel. The migration positions of the precipitated proteins were determined by fluorography.

Coprecipitation of the D1R protein with anti-D12R antiserum was tested as follows. Purified capping enzyme was labeled with [α-^{32}P]GTP as described by Shuman and Hurwitz (1981). After the unreacted GTP was removed by chromatography on Sephadex G-50, an aliquot of the labeled protein was mixed with 20 μl of anti-D12L antiserum in a 100-μl total volume containing 100 m*M* NaCl, 100 m*M* Tris–HCl, pH 8.0, 2 m*M* EDTA, 1% DOC, 0.5% NP40, 0.1% SDS, and incubated for 14 hr at 4°. Fifty-microliter Bio-Rad immunobeads were added and incubated for 2 hr. The antibodies were collected by centrifugation at 3000 *g* and washed three times in the incubation buffer followed by two washes in 2 *M* urea, 0.4 *M* LiCl, and 10 m*M* Tris–HCl, pH 8.0.

The vaccinia virus early transcription termination factor, mRNA capping enzyme, was purified as described by Shuman *et al.* (1987). Fractions derived from the sequential chromatography of virion extracts on heparin agarose and phosphocellulose columns followed by the sedimentation of the highly purified protein through a glycerol gradient were separated by electrophoresis in a 10% polyacrylamide gel and a Western blot analysis was carried out on each using the anti-D12L antiserum.

Wild-type vaccinia virus WR was purified and separated into subviral fractions as described (Niles and Seto, 1988). Western blot analysis was carried out on these subviral fractions using the anti-D12 antiserum at a 1/500 dilution.

Genetic analysis. In order to determine if gene D12L is essential for virus replication, the incorporation of a lethal mutation into gene D12L was attempted by the following protocol (Niles and Seto, 1988). Plasmid 810 extends from the *Bam*HI site at 12,838 to the *Xba*I site at 14,122 in the *Hin*dIII D fragment. This insert contains a region of the *Hin*dIII D fragment from the 5′ end of gene D12L to the 5′ end of gene D11L and includes the site of the base change responsible for the ts mutation C50, between 13,219 and 13,484 (Seto *et al.*, 1987). An insertion mutation was constructed in plasmid 810 by filling in the *Cla*I site at 13,850 with the Klenow fragment of DNA polymerase I, converting the *Cla*I site to a *Nru*I site, and resulting in the formation of plasmid 810 ID12, Fig. 5A. In order to confirm the presence of a frameshift mutation, the *Nru*I region of plasmid 810 ID12 was sequenced. The plasmid was cleaved at the *Eco*RI site at 13,327 and 5′ end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The base sequence of the DNA was determined according to the chemical cleavage method of Maxam and Gilbert (1980). The frameshift mutation results in the synthesis of a protein which possesses the first 164 amino acids of the D12L protein plus an additional 4 amino acids. Linearized plasmid 810 ID12 DNA was employed in the single step rescue of the mutant C50 virus. Plaques which formed at 40° were picked and propagated in 60-mm dishes. Virus DNA was isolated, and in order to determine if temperature-insensitive virus possessed an altered gene D12L, Southern transfer analysis was carried out on viral DNA cleaved either with *Cla*I or *Nru*I. The insert in plasmid 810 was isolated, labeled by nicked translation, and employed as a probe in the Southern transfer analysis.

RESULTS

Rate of synthesis and turnover of the gene D12L protein. The base sequence of gene D12L was determined and this gene was shown to be transcribed primarily at early times in infection (Niles *et al.*, 1986; Lee-Chen *et al.*, 1988). Low levels of D12L mRNA have also been reported to be present late in infection (Weinrich and Hruby, 1987; Lee-Chen *et al.*, 1988). In order to further the investigation of the expression of gene D12L, and to define the function of the gene D12L protein product, a fusion gene containing a segment of the gene D12L coding region linked to the *E. coli* trp E gene was constructed. Synthesis of a fusion protein was induced by indole acrylic acid and the isolated protein was used to produce anti-D12L antibodies in rabbits.

The antiserum was employed in Western blot analysis in order to monitor the time course of synthesis and the accumulation of the D12L protein in infected cells. Extracts of infected cells were prepared by boiling in sodium dodecyl sulfate β-mercaptoethanol and a portion of the soluble material was separated by gel electrophoresis and transferred to nitrocellulose. In Fig. 1, it can be seen that a virus-specific protein of about 31,000 Da is synthesized beginning at about 1 hr postinfection and reaches a constant level after about 4 hr. The protein is also observed in extracts prepared from cells infected in the presence of cytosine arabinoside (Fig. 1, lane 6a), an inhibitor of virus DNA replication. This protein is not observed in mock-infected cells (lane M) or in infected cells when preimmune serum is employed in the analysis (data not shown). We can conclude from these results that gene D12L is expressed early in the virus life cycle, that it encodes a product which migrates as a protein of molecular weight of about 31,000 and that the level of the D12L protein reaches a plateau after about 4 hr.

Detergent extracts were prepared from wild-type virus purified by sucrose density sedimentation and each fraction was tested for the presence of the D12L protein by Western blot analysis, Fig. 1. Cross-reacting

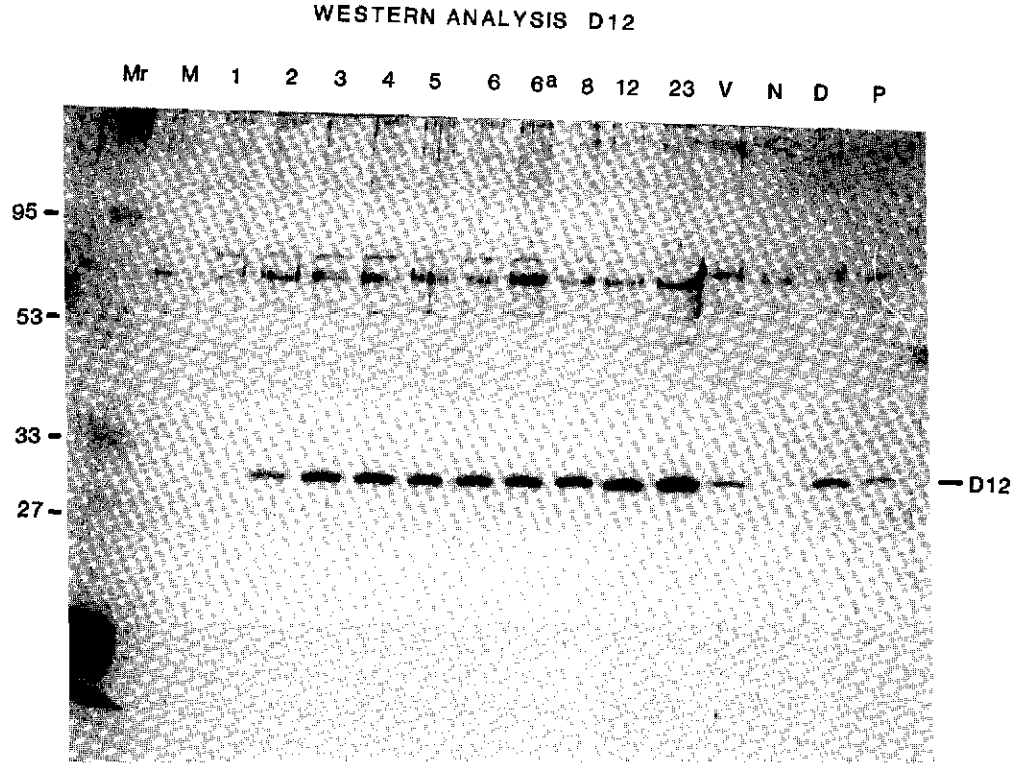


FIG. 1. Western blot analysis of the kinetics of appearance of the D12L protein. Extracts of virus-infected cell cultures were prepared by lysis in SDS and β-mercaptoethanol and the soluble proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with anti-D12L antiserum. Mr, prestained molecular weight markers; M, mock-infected cell extracts; 1 to 23, times after infection at which the cell extracts were prepared; 6a, extract prepared from cells infected for 6 hr in the presence of 100 μg/ml cytosine arabinoside; V, 11 μg of extract prepared from virus purified by sucrose density sedimentation; N, 18 μg of virus proteins soluble in 0.5% NP40 plus 80 m*M* β-mercaptoethanol; D, 17 μg of virus proteins insoluble in NP40 but soluble in 0.2% sodium deoxycholate and 10 m*M* dithiothreitol; P, 10 μg of virus proteins insoluble in either detergent solution.

material of an apparent molecular weight of 31,000 is observed in whole virus extracts (Fig. 1, lane V) and in a fraction of proteins soluble in sodium deoxycholate (Fig. 1, lane D). A minor component remains insoluble (Fig. 1, lane P). These results demonstrate that the D12L protein is a virion component that is extractable with several virion enzymes shown to be soluble in sodium deoxycholate plus dithiothreitol (Baroudy and Moss, 1980).

The rate of synthesis and stability of the D12L protein were determined by immunoprecipitation of extracts in pulse-labeling and pulse–chase analysis. In Figs. 2A and 2B, it can be seen that the D12L protein is synthesized almost exclusively at early times in infection exhibiting a maximum rate of synthesis at 2 hr postinfection. Pulse–chase studies demonstrate that the D12L protein synthesized at 2 hr of infection is stable. The combination of the Western blot analysis and pulse–chase studies are consistent with the gene D12L being expressed at early times in infection producing a stable virion protein.

The immunoprecipitation results described above are similar in the three conditions employed to prepare cell extracts, but some differences should be noted. Immunoprecipitation from the SDS extracts of pulse-labeled cells yields a single prominent product of 31,000 Da, and this protein is stable in a chase. When RIPA is employed, the kinetics of appearance are the same, but the protein appears to turn over rapidly. It is either being digested during extraction or relatively insoluble at later times in infection. In addition, there is a higher level of background proteins either brought down with preimmune serum or precipitated from mock-infected cells. Extracts prepared in maxi buffer yield a stable D12L protein but also exhibit a higher level of background proteins.

Gene D12L encodes the small subunit of the vaccinia mRNA capping enzyme. The small subunit of the mRNA capping enzyme has been reported to weigh either 26,000 (Shuman *et al.*, 1980) or 31,600 (Martin *et al.*, 1975) Da. The coding sequence of genes D4R and D12L predict the synthesis of proteins of about 25,032 and 33,331 Da, respectively (Niles *et al.*, 1986). Since the predicted molecular weights of the products of genes D4R and D12L are similar to that reported for the small subunit of the mRNA capping enzyme, we

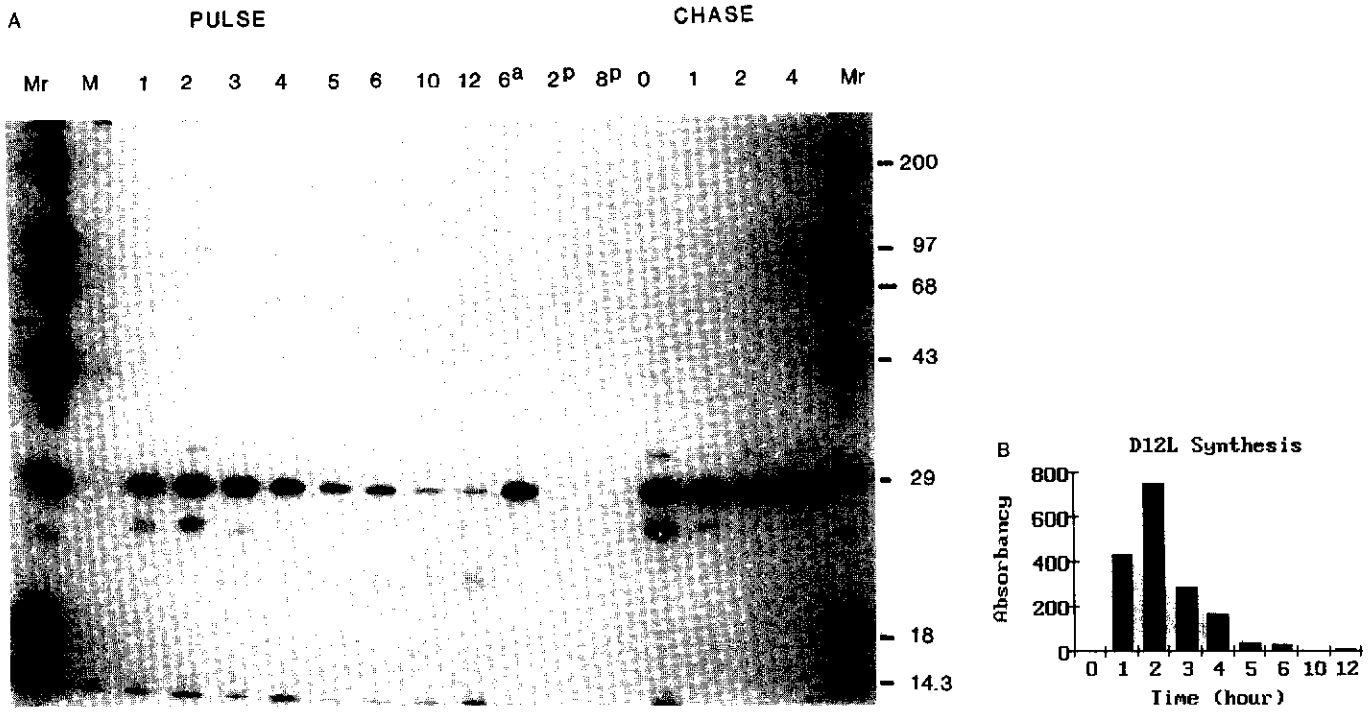


FIG. 2. (A) Synthesis and stability of the D12L protein. (A) Monolayer cultures were infected with vaccinia virus at an m.o.i. of 20, and at different times after infection cultures were labeled with 125 μCi/ml [^{35}S]methionine (70%) and cysteine (15%) (Translabel) for 30 min. In chase samples, after the 30-min labeling period, the isotope was removed and medium containing 1 m*M* methionine was returned. At different times after initiating the chase, cell extracts were prepared. Cells were lysed in 0.3% SDS and 50 m*M* β-mercaptoethanol and after boiling, insoluble material was removed by centrifugation. Each extract was precleared with protein A agarose and 50 μl was incubated with 5 μl of crude antiserum with rocking for 16 hr at 4°. The immune complexes were precipitated by the addition of 20 μl of protein A agarose and after rocking for 1 hr at 4°, the immune complexes were sedimented and the precipitates washed four times. The final pellet was resuspended in 50 μl protein gel sample buffer; after boiling, 30 μl of each sample was applied to a 12.5% gel and, after electrophoresis, the migration positions of the proteins were identified by fluorography. Pulse, samples from pulse-labeled cells precipitated with immune serum; 2^P, 8^P, pulse-labeled samples which were precipitated with 10 μl of preimmune serum; Chase, samples which were labeled for 30 min at 2 hr postinfection and chased for different lengths of time; Mr, molecular weight markers; M, mock-infected cells; 1 to 12, time after infection that the pulse was initiated; 6a, pulse-labeling for 30 min in cultures infected for 6 hr in the presence of cytosine arabinoside. (B) The amount of D12L protein in each sample was determined by densitometry of autoradiographs similar to that shown above. The area under the D12L peak was determined by triangulation and the value of each was plotted at each time after initiation of the pulse-labeling. Each point is the average of two independent determinations of the amount of D12L protein in a single set of extracts. Equivalent results have also been obtained with other extract preparations.

employed both anti-D4R and anti-D12L antiserum in Western blot analysis on fractions derived from the purification of the early transcription termination factor (mRNA capping enzyme). In Figs. 3A, 3B, and 3C, it is apparent that the anti-D12L antiserum binds to a 31,000-Da molecular protein which copurifies with the mRNA capping enzyme activity through chromatography on heparin–agarose and phosphocellulose, and sedimentation through a glycerol gradient (Shuman *et al.*, 1987). At this point of purification, the mRNA capping enzyme is estimated to be 94% pure.

If the D12L protein is the small subunit of the mRNA capping enzyme, precipitation of the D12L protein with the anti-D12L antiserum may coprecipitate the 96,000-Da large subunit. In order to determine if the anti-D12L antiserum could coprecipitate the large subunit of the mRNA capping enzyme, the purified capping enzyme was labeled with [α-^{32}P]GTP (Shuman and Hurwitz, 1981) and after removal of the unincorporated material, anti-D12L antiserum was employed to precipitate the D12L protein. The immune precipitates were washed and analyzed by gel electrophoresis and in Fig. 4, it can be seen that the addition of immune serum results in the coprecipitation of the 96,000-Da large subunit of the mRNA capping enzyme. In a correlative experiment we have found that an antiserum raised against a segment of the D1R protein will coprecipitate the D12L protein (Mah, H., Bouregois, N., and Niles, E. G., unpublished observation).

Gene D12L is essential for virus replication. In order to determine if gene D12L is essential for virus replication in tissue culture, an attempt was made to construct a virus which possessed a frameshift mutation in this gene. Following the logic previously described (Niles and Seto 1988) a plasmid which contained a portion of gene D12L, and a sequence corresponding to a

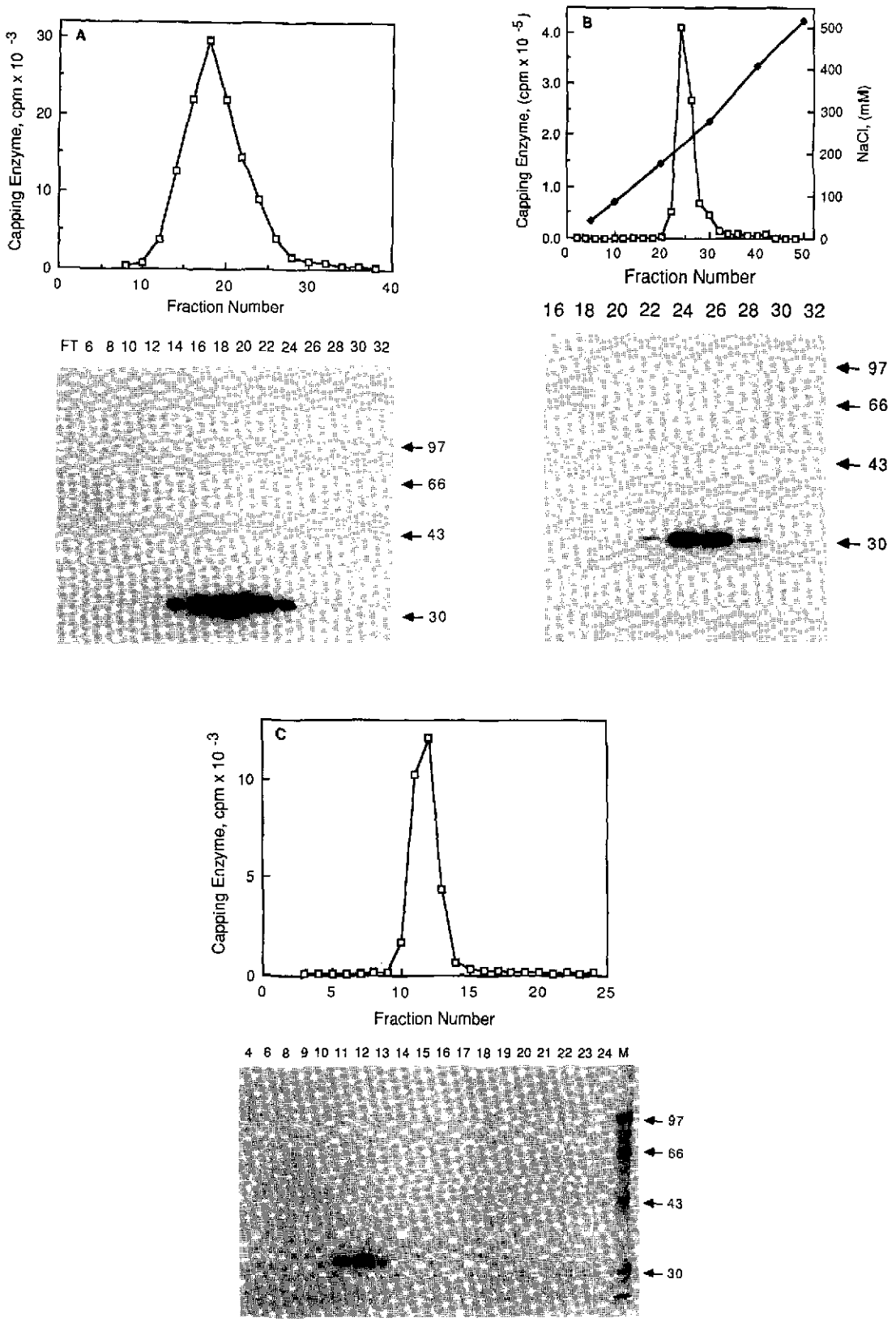


FIG. 3. Samples obtained during the purification of the vaccinia virus early transcription termination factor (mRNA capping enzyme), described by Shuman *et al.* (1987), were separated by gel electrophoresis and analyzed by Western blot analysis in which the anti-D12L antiserum was employed. Samples were derived from chromatography on (A) heparin–agarose, (B) phosphocellulose, or (C) sedimentation through a glycerol gradient. On the top of each part of the figure, the capping enzyme activity is plotted for each fraction. Below, a photograph of the Western blot is shown; M, prestained molecular weight markers; fraction numbers are indicated at the top of each lane.

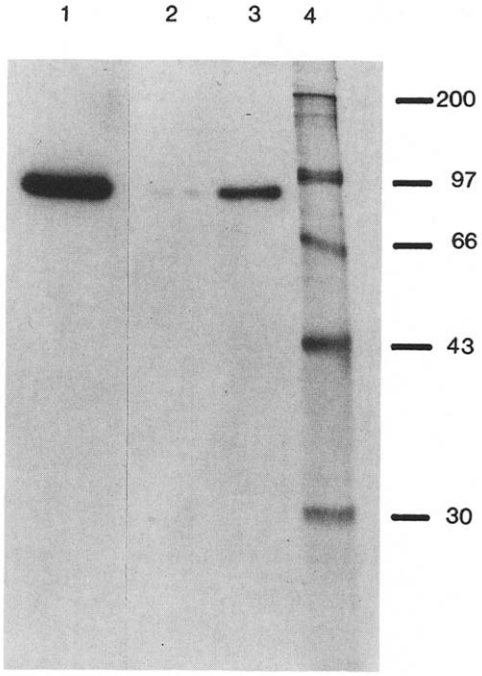


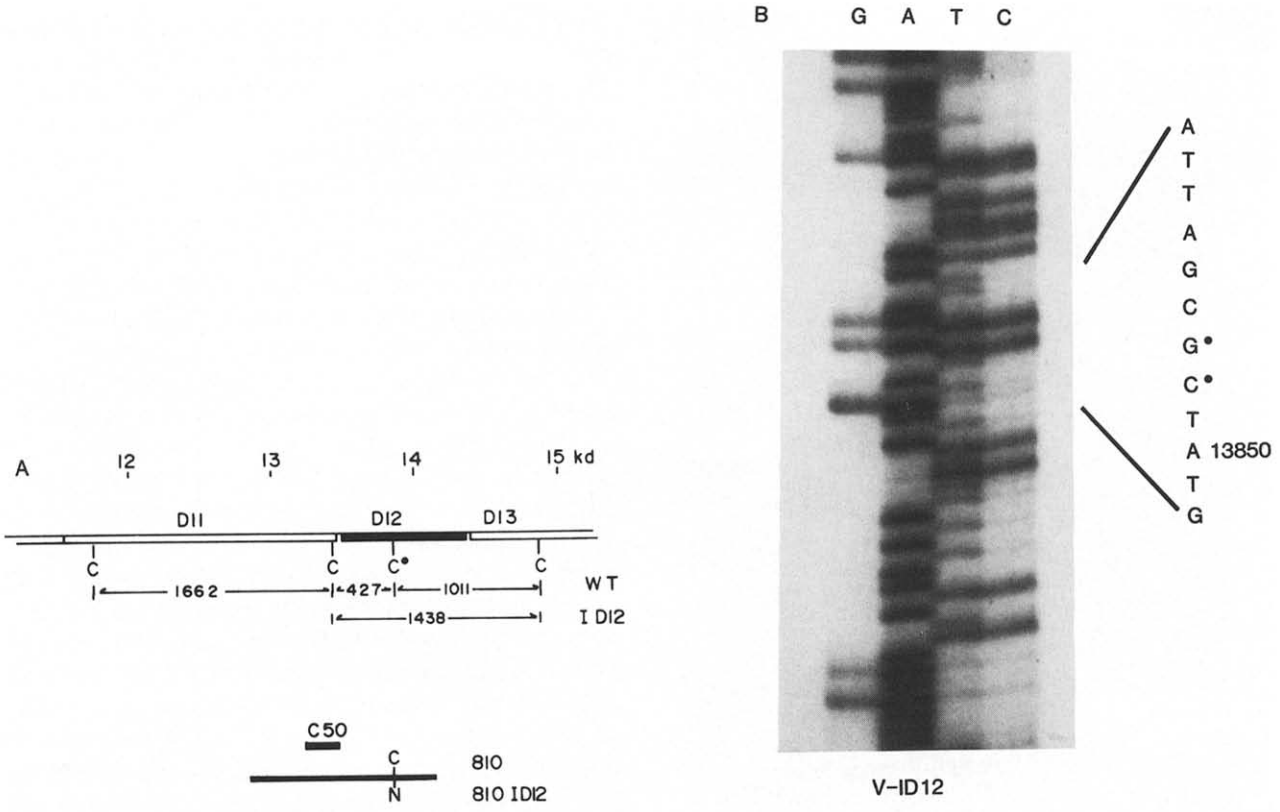
FIG. 4. Coprecipitation of the D1R protein with the D12L antibody. Capping enzyme activity was solubilized from purified virions and the 96-kDa subunit was labeled with [^{32}P]GTP as described by Shuman and Hurwitz (1981). The D12L protein was precipitated with anti-D12L antiserum, and after washing the precipitate, the final pellet was solubilized in sample buffer and analyzed by gel electrophoresis. The migration position of the [^{32}P]GTP-labeled protein was determined by autoradiography. Lane 1, labeled virus-capping enzyme; lanes 2 and 3, proteins precipitated with preimmune serum (2) and anti-D12L immune serum (3); lane 4, radioactive molecular weight markers. The sizes of the molecular weight markers are identified at the right.

wild-type version of the ts mutation C50, located in an adjacent gene D11L between 13,219 and 13,484, was altered. A *Cla*I site at 13,850, in gene D12L, was filled in with the Klenow fragment of DNA polymerase I, converting it to a *Nru*I site and generating a 2-bp insertion (Fig. 5A). The base sequence of plasmid 810 ID12 was determined and the presence of the predicted insertion mutation was confirmed (Fig. 5B). Linearized plasmid 810 ID12 was employed to rescue the ts mutation C50 in the adjacent gene D11L, in a single step (Seto *et al.*, 1987). Temperature-insensitive virus were isolated and the presence of a *Cla*I or *Nru*I site in gene D12L was determined by Southern transfer analysis. Since the ts mutation in gene D11L was converted to a wild-type sequence by recombination with plasmid DNA, a fraction of the temperature insensitive virus would be expected to also acquire the frameshift mutation in gene D12L. One would predict that if gene D12L is nonessential for virus propagation, temperature-insensitive virus would be isolated that would lack the *Cla*I site at 13,850 and acquire the *Nru*I site in gene D12L. Virus which possess a mutant gene D12L would be identifiable by the presence of a 1438-bp *Cla*I fragment and the corresponding loss of both a 1011- and 427-bp *Cla*I fragments. In addition, mutant virus would yield one new 4617-bp *Nru*I fragment which extends from the *Nru*I site at 9233 to the new site at 13,850. A second new fragment would be expected but since the *Nru*I site to the right of 13,850 is far away, cleavage at this *Nru*I site generates a very large DNA fragment which is not resolved in this gel. This new *Nru*I fragment comigrates with the wild-type *Nru*I DNA fragment, and, therefore, it cannot be differentiated from the wild-type fragment. However, if gene D12L is essential, individual DNA preparations will contain either wild-type gene D12L sequences alone, or a mixture of wild-type and mutant DNA. In Fig. 5C, the Southern transfer results for 22 independent temperature insensitive virus are presented. It can be seen that only virus No. 2 possesses both a new *Nru*I site while losing the corresponding *Cla*I site. The other virus all exhibit a wild-type gene D12L sequence. The majority of DNA sequences in the virus No. 2 preparation are wild type. In order to determine if the wild-type virus present in the virus No. 2 sample is a contaminant or a required helper virus, the virus No. 2 sample was plaque purified and the Southern transfer was repeated. The mutant DNA sequences were observed only in virus preparations in which wild-type gene sequences were also present. We conclude that since the frameshift mutation is only propagated along with a coinfecting wild-type virus that gene D12L is essential for virus propagation.

DISCUSSION

The nucleotide sequence of vaccinia virus D12L had been reported and based on this sequence, a protein product of 33,331 Da was predicted (Niles *et al.*, 1986). Transcription and translation mapping studies have demonstrated that this gene is transcribed at early times in infection (Lee-Chen and Niles, 1988; Lee-Chen *et al.*, 1988) into two mRNA which differ in their 3′ ends. Gene D12L has also been described as one member of a tandem late gene cluster (Weinrich and Hruby, 1986). Although evidence has been presented that a low level of gene D12L mRNA is present late in infection (Weinrich and Hruby, 1987; Lee-Chen *et al.*, 1988) it is not clear if this mRNA is produced by late transcription, if it is a relatively stable early mRNA which remains late in infection, or if it is present in the mRNA preparations due to asynchrony in the infection.

Through the use of a polyclonal antiserum directed against the gene D12L protein, both in Western blot analysis and in pulse-labeling studies, we have demon-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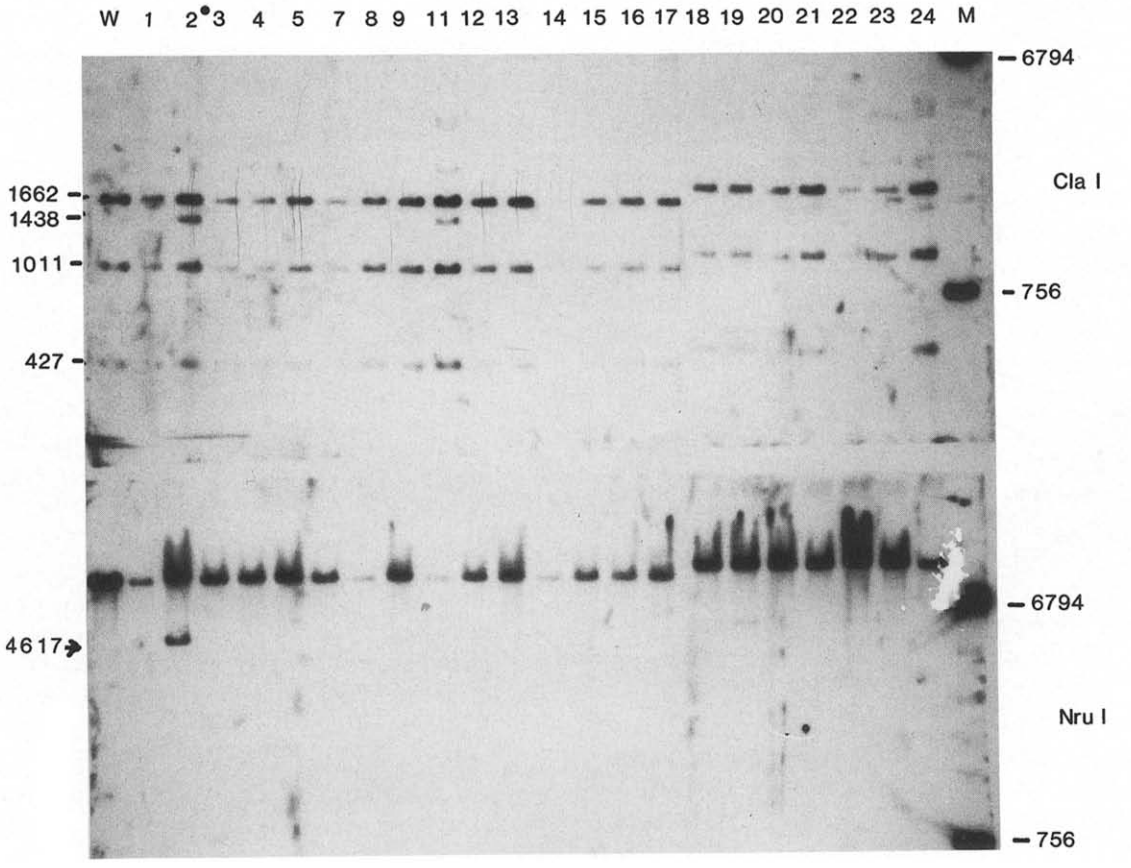
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strated that the D12L protein is synthesized primarily between 1 and 5 hr postinfection and it is stable in the infected cell. Since the D12L protein is synthesized in cells infected in the presence of a DNA replication inhibitor, cytosine arabinoside, it must be the product of an early gene. Analysis of extracts of whole virus demonstrates that gene D12L encodes a virion component. Dissociation of the virus into detergent soluble and insoluble fractions shows that the D12L protein is soluble in 0.2% DOC and 10 m*M* dithiothreitol, conditions known to solubilize many virion enzymes (Baroudy and Moss, 1980).

Since the D12L protein is similar in size to the small subunit of the viral mRNA capping enzyme, we employed Western blot analysis to determine if cross-reactivity with the anti-D12L antiserum copurifies with virion mRNA capping enzyme. Samples of capping enzyme purified by sequential chromatography on heparin–agarose, followed by phosphocellulose, and finally sedimentation through a glycerol gradient, were shown to copurify with a protein that binds the anti-D12L antiserum, suggesting that the small subunit of the enzyme is encoded by gene D12L. In order to obtain further evidence, the large subunit of the capping enzyme, the product of gene D1R, was shown to be coprecipitated along with the D12L protein by the anti-D12L antiserum. In a complementary experiment, we have been able to develop conditions which permit the coprecipitation of the D12L protein with D1R protein from infected cell extracts when anti-D1R antibody is employed (data not shown).

Transcription mapping studies demonstrate that D1R and D12L mRNA is synthesized primarily at early times in infection (Lee-Chen *et al.*, 1988; Lee-Chen and Niles, 1988). Immunoprecipitation studies of D12L protein synthesis, and D1R synthesis (H. Mah, N. Bouregois, and E. G. Niles, unpublished), show similar kinetics. These results are in agreement with the observation of Boone *et al.* (1977) who monitored capping enzyme activity during infection and concluded that it appeared early and reached a plateau by 6 hr in the infection cycle. This means that the capping enzyme represents a pair of early proteins that become packaged into progeny virions.

In order to test if gene D12L is essential for virus replication we attempted to introduce gene D12L containing a frameshift mutation into the virus. A plasmid containing the D12L sequence was mutated and used to rescue a ts mutation in the adjacent gene D11L. Temperature-insensitive virus were tested for the presence of the mutation in gene D12L. One virus plaque was identified which exhibited both a mutant and a wild-type gene D12L. In order to determine if the wild-type virus was a contaminant or a required helper virus, the mutant was re-plaque-purified. Analysis of the progeny virus genomes again showed that the mutant gene D12L could only be identified in virus preparations that also contained helper wild-type virus. Since virus which possessed the mutant D12L gene could not be found in the absence of wild-type virus, we conclude that gene D12L is essential for virus replication. We can not conclude that the mRNA capping enzyme activity is essential for virus propagation since this protein carries on other activities in the infected cells, i.e., transcription termination. In addition, since this enzyme is packaged into virus particles, its presence may be required to permit the construction of a stable virion.

Since late transcription does not terminate in response to early transcription termination signals, an alteration in either the late form of the viral RNA polymerase or the early transcription termination factor may occur at late times in infection. Since the D12L protein is a component of the early transcription termination factor (Shuman *et al.*, 1987), one might anticipate that the al-

FIG. 5. Gene D12L is essential for virus propagation. (A) The region of the *Hin*dIII D fragment containing genes D11L to D13L is presented. The scale at the top denotes the map position within the *Hin*dIII D fragment. Each gene is depicted by a box; C, *Cla*I cleavage sites; C•, *Cla*I site at 13,850 which is converted to an *Nru*I site in the mutant ID12 *Cla*I fragments are indicated. The thick black bar denotes the insert present in the plasmid 810. The black bar indicates the approximate map position of the ts mutation, C50, (Seto *et al.*, 1987). C, *Cla*I, and N, *Nru*I, denote the conversion of the *Cla*I site in 810 to a *Nru*I site in 810 ID12. (B) Nucleotide sequence in the region of the *Nru*I site in the plasmid 810 ID12. Plasmid 810 ID12 DNA was labeled at the *Eco*RI site corresponding to position 13,627 in the *Hin*dIII D fragment by T4 polynucleotide kinase and [γ-^{32}P]ATP. The plasmid was redigested with *Hin*dIII, the end-labeled DNA fragment was isolated, and the sequence was determined by the chemical cleavage method (Maxam and Gilbert, 1980). GATC, products of cleavage at G, A + G, T + C, and C, respectively; the DNA sequence in the region of the *Nru*I site is indicated along the right; the dots, •, denote the two nucleotides added by filling in the *Cla*I site at 13,850. (C) Southern transfer analysis of DNA of virus rescued by plasmid 810 ID12. ts mutant C50 was rescued to a temperature-insensitive phenotype by recombination with linearized plasmid 810 ID12 in a single step. Twenty-four plaques were picked and the virus were propagated in 60-mm dishes. DNA was isolated from a portion of each, cleaved with either *Cla*I or *Nru*I, separated by agarose gel electrophoresis, transferred to Gene Screen (NEN), and probed with the insert in plasmid 810 made radioactive by nicked translation. After removal of the unbound isotope, the migration position of the DNA fragments was determined by autoradiography. W, wild-type virus DNA; 1 to 24, temperature resistant virus DNA samples; M, molecular weight markers derived by digestion of a plasmid containing the *Hin*dIII D fragment in pBR322, with *Eco*RI. One nanogram of marker DNA was separated on the gel and transferred along with the experimental digestions. The migration of samples 18 to 24 is slower due to the fact that they were applied after the first set of samples had entered the gel. The sizes of the *Cla*I fragments and the *Nru*I fragment are indicated on the left; the lengths of the molecular weight markers are on the right.

teration in the termination factor activity might be reflected in the structure of the D12L protein. We have not observed any evidence from the Western blot analysis, or the pulse–chase studies, of a gross modification of the gene D12L protein at late times during infection.

With the identification of the genes that encode the two subunits of the capping enzyme, the availability of their base sequence, and antisera directed toward the D1R and D12L proteins, rapid progress toward elucidating the mechanism of coordinate regulation of the synthesis of the D1R and D12L proteins and the mechanism of action of the virion mRNA capping enzyme can be anticipated.

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