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Molecular Genetic Analysis of a Vaccinia Virus Gene with an Essential Role in DNA Replication

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We have identified a gene encoded by vaccinia virus which is essential for DNA replication. The gene, located in the *Hin*dIII D fragment of the viral genome, is transcribed early after infection into two transcripts of 3.0 and 3.7 kilobases which share a 3′ terminus. The lesions of three temperature-sensitive DNA replication mutants with defects in this gene have been localized by marker rescue with progressively smaller DNA fragments. We have determined by hybrid selection that the gene encodes an 82-kilodalton protein. An antibody has been prepared against this polypeptide and used to quantitate expression of the protein after infection with wild-type virus or with a viral mutant whose lesion maps within this gene. The temporal pattern of expression in the mutant is unaffected, but the product encoded by the mutant is significantly more thermolabile than the wild-type protein.

Accurate DNA replication is a fundamental requirement of biological systems. Our most complete understanding of the enzymes involved in DNA replication, and of the important features of the replicating template, comes from procaryotic systems. Bacteriophages, most notably T4, have proven especially amenable to the combined approaches of genetics, biochemistry, and molecular biology. Appreciation of the complexity of the machinery required for the initiation and elongation of DNA synthesis, and for the unwinding and torsional relaxation of the DNA helix, has increased steadily as our knowledge has progressed. The analogous study of eucaryotic cells has been hampered by the inability to probe diploid cells with genetic analysis. Significant strides have been made in the analysis of simian virus 40 and adenovirus replication (for a review, see reference 6), and preliminary identification of the genetic loci required for herpes simplex virus type 1 replication has begun (7). Thorough analysis of these systems is made difficult, however, by the dependence of the virus on host functions for replication and by the physical and temporal overlap of viral and cellular processes in the nucleus.

In contrast, vaccinia virus, the prototypic member of the poxvirus family, is a DNA virus which replicates entirely within the cytoplasm of infected cells. The virus is thought to encode all of the functions required for replication and transcription of the 185-kilobase (kb) genome, and infection is accompanied by the rapid cessation of host DNA metabolism (for a review, see reference 29). The genome of vaccinia virus is a linear duplex with covalently closed termini, and by several criteria vaccinia virus represents a fruitful model system for the study of eucaryotic DNA replication. The DNA polymerase of the virus has been purified (8), the gene encoding it has been transcriptionally mapped, and genetic analysis of the polymerase is extensive (14, 22, 39, 43; Traktman et al., manuscript in preparation). The viral thymidine kinase and its gene have been similarly studied (19, 47). Viral topoisomerase (2, 17, 35a) and ribonucleotide reductase (36, 37) activities are also under study. The goal of our laboratory is to identify and analyze all of the viral genes encoding replicative functions and to dissect their regulation and the enzymes they encode. One of our approaches is the use of conditional lethal mutants to identify pertinent genes. The work described in this report concerns a complementation group of three temperature-sensitive mutants whose primary defect lies in DNA replication. We have identified and transcriptionally mapped the gene affected in these mutants, localized the lesions within the gene, and performed preliminary studies on the protein encoded by the gene in question.

MATERIALS AND METHODS

Cells and virus. Wild-type vaccinia virus, WR strain, was provided by Bryan Roberts. The temperature-sensitive (*ts*) DNA replication mutants *ts*17 and *ts*24 were provided by Richard Condit (10); *ts*69 was provided by Marcia Ensinger (15). Stocks of viral mutants were grown at the permissive temperature of 32°C on monolayers of mouse L cells (provided by Richard Condit) in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). The nonpermissive temperature used was 39.5°C. Wild-type virus was propagated at 37°C, either on monolayers of mouse L cells or in suspension cultures of L cells (provided by Joseph Kates) maintained in Joklik modified essential medium with 2.5% calf serum–2.5% horse serum (GIBCO). Virus was purified from cytoplasmic extracts by ultracentrifugation through a 36% sucrose cushion as described previously (21). The continuous line of African green monkey kidney cells, BSC-40 (provided by Richard Condit), was used for viral plaque assays and for some experiments, as described in the text. These cells were grown in monolayer culture in DMEM with 5% fetal bovine serum.

Materials. Restriction endonucleases were obtained from New England BioLabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the instructions of the manufacturer. Polynucleotide kinase, calf intestinal phosphatase, S1 nuclease, *Escherichia coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, and pancreatic RNase were from Boehringer Mannheim; DNase I was purchased from Cooper Biomedical, Inc. (West Chester, Pa.). ^{32}P-labeled nucleotide triphosphates and [^{35}S]methio-

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nine were obtained from New England Nuclear Corp. (Boston, Mass.). ^{14}C-labeled high-molecular-weight standards were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The sources of specific materials and reagents used are indicated in the text.

Preparation of plasmid DNA. The *Hin*dIII D fragment of the wild-type vaccinia virus genome cloned into pBR322 was provided by B. Moss (3). Subfragments of *Hin*dIII-D were cloned into pBR322 or pUC-8 (44) vectors by standard techniques. In some cases, DNA termini were made blunt with the Klenow fragment of DNA polymerase I and converted to novel termini carrying restriction enzyme recognition sequences by using synthetic DNA linkers (New England BioLabs). Plasmid DNA was amplified in *E. coli* HB101 and purified by the alkaline lysis procedure (20).

Marker rescue. Rescue of temperature-sensitive mutants with wild-type DNA fragments was done as described previously (11, 30, 43). Cloned DNA fragments of the wild-type genome were linearized by digestion with an appropriate restriction enzyme and purified by organic extraction and ethanol precipitation. The pellets were suspended in sterile TE (10 mM Tris, pH 7.4, 1 mM EDTA) and diluted to 20 μg/ml in sterile HBS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.0, 150 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM KCl, 6 mM dextrose). At 1 h before the transfection of infected cells, $CaCl_2$ was added to 125 mM and the DNA was allowed to precipitate at room temperature.

Dishes (60 mm) of BSC-40 cells were infected with mutant virus at a multiplicity of infection (MOI) of 0.03 PFU per cell in 0.5 ml of DMEM. After adsorption for 1 h at 32°C, the cells were fed with DMEM–5% fetal bovine serum and returned to 32°C. At 4 h after infection, 0.5 ml of the precipitated DNA solution (10 μg of plasmid) was added, and the dishes were incubated at 39.5°C for 3 days. The cells were scraped, freeze-thawed twice, and sonicated for two bursts of 15 s each (Laboratory Supplies Co. Inc., Hicksville, N.Y.; model G112SP1T). Plaque assays of serially diluted stocks were done on BSC-40 cells at 32 and 39.5°C. Cells were fixed and stained with 3.7% formaldehyde and 0.1% crystal violet, and plaques were counted to determine viral yields.

Preparation of RNA from infected cells. To prepare early viral RNA, monolayers of mouse L cells were pretreated with medium containing 100 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml for 0.5 h and then infected with wild-type virus at an MOI of 15 PFU per cell at 37°C. After 30 min of adsorption in DMEM, the cells were fed with DMEM–5% fetal bovine serum with cycloheximide (100 μg/ml). At 3 h after infection, cultures were rinsed with ice-cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and lysed in a solution of 4 M guanidinium thiocyanate, and RNA was prepared as previously described (43). Treatment of infected cells with cycloheximide has been shown to elevate the levels of early viral RNAs without disturbing the pattern of transcription (for a discussion, see reference 28). This RNA, designated cycloheximide RNA, was used for hybrid selection, S1 nuclease mapping, and RNA filter hybridization as described below. A series of viral RNAs was also prepared from cells infected in the absence of cycloheximide at 1, 2, 3.25, 4.5, and 5.75 h postinfection.

Hybrid selection and in vitro translation. (i) Hybrid selection. The indicated DNA plasmid (5 μg) was covalently bound to activated diazobenzyloxymethyl paper (Scheicher & Schuell, Inc., Keene, N.H.) as described previously (1). Cycloheximide RNA (36 μg) was incubated with each filter in hybridization buffer (0.48 μg of RNA per μl, 50% formamide [Fluka Chemical Co., Hauppauge, N.Y.], 4× SET [20× SET is 3 M NaCl, 0.4 M Tris, pH 7.8, 20 mM EDTA], 0.1% sodium dodecyl sulfate [SDS]) for 3 to 4 h at 37°C. The RNA solution was removed, and the filters were washed vigorously in 50% formamide (Fluka)–0.2× SET–0.1% SDS. Specifically bound RNAs were eluted at 65°C with 90% formamide–0.1 M Tris, pH 7.8, in the presence of tRNA (50 μg/μl) and concentrated by ethanol precipitation (25).

(ii) In vitro translation. Reticulocyte lysates (Green Hectares, Oregon, Wis.) were rendered message dependent by treatment with micrococcal nuclease (Pharmacia, Piscataway, N.J.). Hybrid-selected RNAs were translated in vitro in the presence of [^{35}S]methionine at 2 μCi/μl (32). After 1 h at 37°C and digestion with pancreatic RNase at 1.4 μg/μl, one-third of each 12.5-μl translation was suspended in sample buffer (1% SDS, 1% 2-mercaptoethanol, 50 mM Tris, 10% glycerol), heat denatured, and electrophoresed on 9 to 16% gradient polyacrylamide gels (24). Gels were examined by fluorography (5) on Eastman Kodak Co. (Rochester, N.Y.) XAR5 film.

RNA filter hybridization. Cycloheximide RNA (4 μg) or RNA prepared without cycloheximide treatment (5 μg) was electrophoresed on a vertical 1.4% denaturing agarose gel (16% formaldehyde, cast and run in MEN buffer [MEN is 20 mM morpholinepropanesulfonic acid (MOPS), pH 7.0–5 mM sodium acetate–1 mM EDTA]) at 10 V/cm. The gels were stained with ethidium bromide (1 μg/ml) and photographed under short-wave UV illumination, treated with 50 mM NaOH–100 mM NaCl, and then neutralized with 50 mM acetic acid. Gels were soaked in 20× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate), and the RNA was transferred overnight to nitrocellulose (Scheicher & Schuell) (35).

Radiolabeled probes of cloned vaccinia virus DNA fragments were prepared by nick translation (33). Baked RNA filters were prehybridized and hybridized with labeled probes at 42°C in buffer containing 6× SSC–5× Denhardt solution (50× Denhardt solution is 0.05 mg of Ficoll per ml, 0.05 mg of polyvinylpyrrolidone per ml, 0.05 mg of bovine serum albumin per ml)–0.5% SDS–50% formamide–denatured salmon sperm DNA (100 μg/μl). Filters were washed in 2× SSC–0.1% SDS at room temperature followed by 0.2× SSC–0.1% SDS at 55°C and exposed to Kodak XAR5 film at −70°C with a Cronex Lightning-Plus intensifying screen (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.).

S1 nuclease analysis. To prepare probes for S1 nuclease mapping (4, 46), plasmid DNA was linearized at the designated restriction enzyme site and labeled at the 5′ terminus with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP after dephosphorylation with calf intestinal alkaline phosphatase or at the 3′ terminus with the Klenow fragment of *E. coli* DNA polymerase I in the presence of the appropriate α-^{32}P-labeled deoxyribonucleotide triphosphate. The DNA was then subdigested with another restriction enzyme to yield fragments labeled at a single position. Labeled fragments were electrophoresed on TAE agarose gels (40 mM Tris, 20 mM acetate, 1 mM EDTA) and purified by binding to glass powder (45).

Each DNA probe was suspended in 80% formamide–1× HARTS (40 mM piperazine-*N*,*N'*-bis[ethanesulfonic acid] [PIPES], 400 mM NaCl, 1 mM EDTA) in the presence or absence of 25 μg of cycloheximide RNA as indicated (with DNA in excess). The nucleic acids were heat denatured and allowed to rehybridize for 3 h at 42°C or, in some cases, at

34°C to stabilize small duplexes. The samples were diluted with ice-cold S1 nuclease digestion buffer (0.28 M NaCl, 0.05 M sodium acetate, pH 4.6, 0.5 mM $ZnSO_4$) containing salmon sperm DNA at 20 μg/μl. S1 nuclease (200 U/μl) (Boehringer Mannheim) was added (or not) as indicated. After 30 min at 45°C, samples were extracted twice with organic solvent and precipitated with ethanol. The products were electrophoresed on 1.3% alkaline agarose gels (30 mM NaOH, 2 mM EDTA, pH 8) or, in the case of reactions carried out at 34°C, 4% acrylamide gels containing 8 M urea (cast and run in TBE [100 mM Tris, 83 mM borate, 1 mM EDTA]). Gels were examined by autoradiography.

Preparation of Trp fusion protein. A 1.6-kb intragenic restriction fragment was ligated into the pATH2 vector (T. J. Koerner, personal communication) to express a fusion polypeptide of the 82-kilodalton (kDa) gene product and *E. coli* TrpE protein. pATH is a derivative of pBR322 which contains 1 kb of the *trpE* gene under the control of an indoleacrylic acid-inducible promoter; a polylinker adjacent to the *trpE* sequence allows insertion of foreign coding sequences in the correct frame to allow their expression as a TrpE fusion protein (38). For large-scale preparation of the fusion protein, the bacteria carrying the plasmid were grown in M9 media supplemented with Casamino Acids (Difco Laboratories, Detroit, Mich.) (5 mg/ml), thiamine B1 (0.01 mg/ml), and tryptophan (20 μg/ml). The cultures were diluted into media without tryptophan and grown for 1 h at 37°C and then induced with 20 μg of indoleacrylic acid (Sigma) per ml. After 4 h at 37°C, the cultures were kept at 4°C for greater than 12 h. The cells were collected by centrifugation and suspended in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 3 mg of lysozyme per ml) on ice for 2 h. NaCl and Nonidet P-40 were added to 0.3 M and 0.6%, respectively. After 30 min on ice, lysates were sonicated with a probe sonicator (Kontes, Vineland, N.J.) to shear the DNA. Proteins were collected by centrifugation, and the pellet was suspended in 10 mM Tris, pH 7, and stored at −20°C.

To prepare the protein for injection into rabbits, the bacterial lysate was electrophoresed on a 10% SDS-polyacrylamide gel. A portion of the gel was stained with Coomassie blue to determine the position of the induced fusion protein (96 kDa). The remainder of the gel was soaked in 4 M sodium acetate; as the SDS precipitated in the salt solution, the protein bands were contrasted and the fusion protein was excised. The acrylamide slice was homogenized in phosphate-buffered saline in a tight-fitting Dounce homogenizer, emulsified in complete Freund adjuvant (Sigma), sonicated, and used to inoculate rabbits intradermally. Rabbits were boosted with additional fusion protein, and preimmune and immune sera were collected. The presence of antibodies reactive to the fusion protein was assessed by immunoblotting (41) and immunoprecipitation.

Metabolical labeling of infected cells. Confluent 60-mm dishes of L cells (4×10^6 cells per dish) were infected with wild-type or mutant virus at an MOI of 15 PFU per cell. Virus was adsorbed in DMEM at the indicated temperature for 30 min. The inoculum was removed, and the cells were rinsed and fed with DMEM–5% fetal bovine serum. At the times indicated, medium was removed and cells were washed with methionine-free DMEM and fed with methionine-free medium supplemented with [^{35}S]methionine at 100 μCi/ml. After 30 min of labeling, cells were either lysed immediately or rinsed and refed with DMEM–5% fetal bovine serum, returned to the appropriate temperature for 0.5, 1, 1.5, or 2 h, and then lysed (pulse-chase). For lysis, cells were rinsed thoroughly with phosphate-buffered saline and overlaid with ice-cold PLB (phospholysis buffer [PLB] is 1 M $NaPO_4$, pH 7.4, 1 M NaCl, 10% Triton X-100, 1% SDS, 5% sodium deoxycholate [42]). Lysis was monitored by phase-contrast microscopy; when complete, the total cell lysate was collected and centrifuged to remove the chromatin. Lysates were stored frozen at −20°C.

Immunoprecipitation. For immunoprecipitation (23, 42), a portion of the lysate described above (2×10^6 cells per ml) was preincubated with fixed *Staphylococcus aureus* (IgGSorb; The Enzyme Center, Boston, Mass.) suspended in PLB with 1 mg of bovine serum albumin per ml (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). The lysate was cleared by centrifugation, and excess immune anti-82-kDa-*trpE* antiserum was added. After 4 h on ice, IgGSorb was added to a 0.2% cell suspension and incubation was continued for a further 1.5 h. The IgGSorb was collected by centrifugation, washed twice in PLB, and boiled in sample buffer (1% SDS, 1% 2-mercaptoethanol, 50 mM Tris, 10% glycerol) to release the bound proteins. Samples were electrophoresed on 10% SDS-polyacrylamide gels, processed for fluorography, and exposed to Kodak XAR5 film at −70°C. Fluorographs were scanned with a densitometer (Quick Scan Jr.; Helena Laboratories, Beaumont, Tex.), and the relative area under each peak was assessed by weight.

RESULTS

Conditional lethal mutants have been vital tools in the identification of essential genes for a variety of biological functions. Several investigators have recently isolated a large number of temperature-sensitive mutants of vaccinia virus, grouped them into complementation groups, and mapped them to a region of the vaccinia virus genome by marker rescue (9–13, 15, 16, 40). Three complementation groups of mutants whose primary defect appears to be in DNA replication have been isolated; one group of mutants represents those with lesions in the DNA polymerase gene (39, 43). The genes affected in the other two complementation groups have not yet been identified or ascribed a function. Published data have localized the lesions in one group to a large region in the right quarter of the genome (40), and the other group has been assigned to the central *Hin*dIII D fragment of the genome (11, 31). The analysis of this *Hin*dIII-D complementation group is the focus of the work reported in this paper.

Marker rescue analysis of a complementation group of mutants mapping within *Hin*dIII-D. Temperature-sensitive mutants *ts*17, *ts*24, and *ts*69 of the WR strain were previously mapped to the same complementation group and assigned to the *Hin*dIII D fragment (10, 11, 15, 16). Our first aim was to further localize the lesion of these mutants within the *Hin*dIII D fragment and then to determine the transcript and polypeptide encoded by the affected gene. After generation of a restriction map of the *Hin*dIII D fragment, a large number of subclones were constructed and used in marker rescue experiments with *ts*17, *ts*24, and *ts*69. In all cases, cells were infected with the viral mutant, transfected with the linearized DNA fragment, and maintained at the nonpermissive temperature for 3 days. The cells were then harvested, and the virus yield was assayed at the permissive and nonpermissive temperatures. The restriction map of *Hin*dIII-D and the fragments generated is illustrated in Fig. 1; the results of marker rescue of the three mutants with these fragments are shown in Table 1. All mutants were

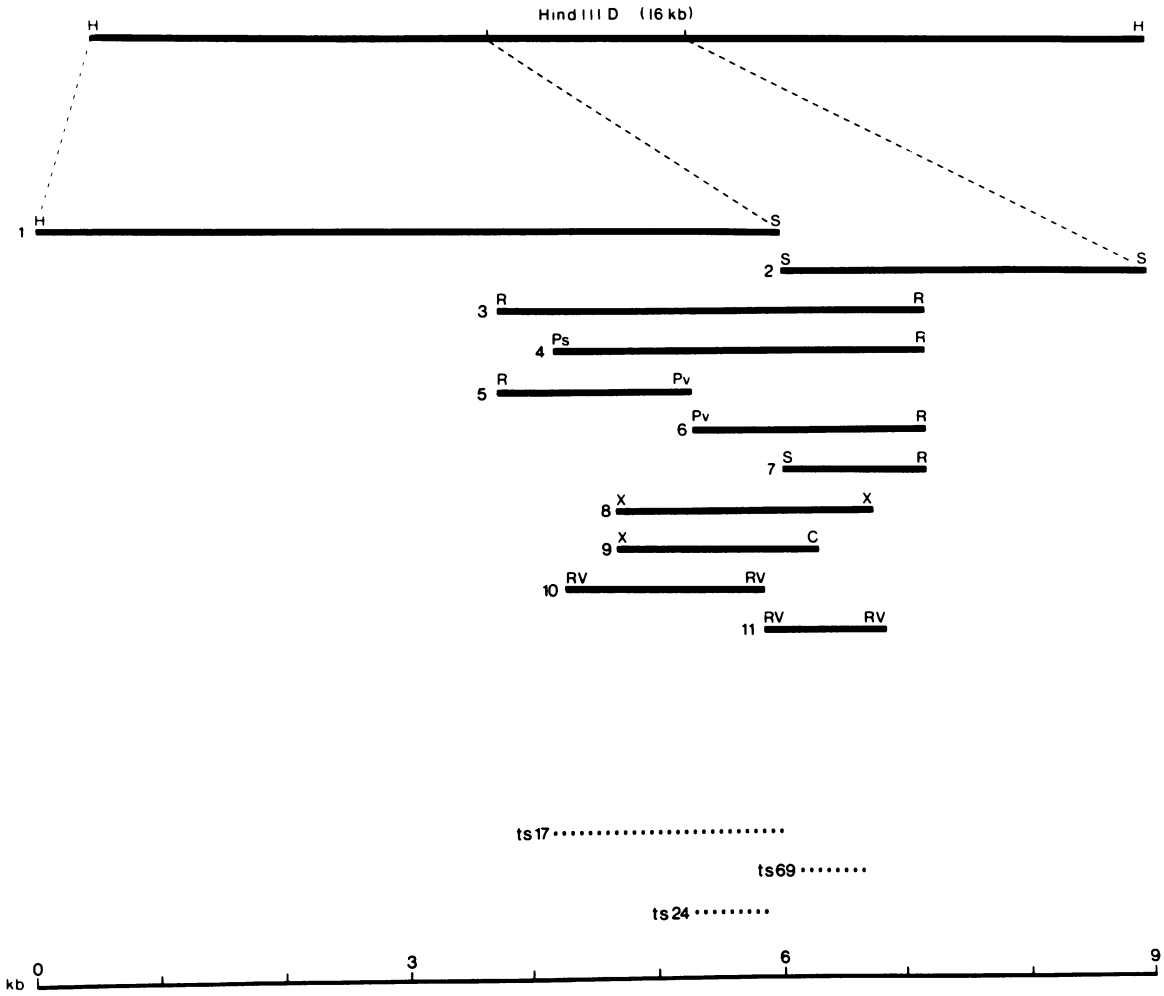

FIG. 1. Partial restriction map and constructed subclones of the vaccinia virus *Hin*dIII D fragment. Only the restriction endonuclease sites defining fragments used in marker rescue (see Table 1 and text) are shown. Sites are abbreviated as follows: H, *Hin*dIII; S, *Sal*I; R, *Eco*RI; Ps, *Pst*I; Pv, *Pvu*I; X, *Xba*I; C, *Cla*I; RV, *Eco*RV. The broken lines at the bottom of the figure summarize the localization of each lesion as determined by marker rescue (Table 1). A scale, in kilobases, is shown at the bottom.

rescued to the wild-type phenotype by a 3.4-kb *Eco*RI fragment mapping within the left half of the D fragment. The positions of the three lesions within this fragment differed; as revealed by a comparison of Table 1 and Fig. 1, the lesion in *ts*17 was localized within a 1.8-kb region (*Pst*I-*Sal*I) and those of *ts*24 and *ts*69 were localized within nonoverlapping regions of 500 (*Pvu*I-*Eco*RV) and 800 base pairs (*Sal*I-*Xba*I), respectively. The localization of the mutations within the 3.4-kb *Eco*RI fragment, as deduced from the cumulative marker rescue data, is indicated schematically at the bottom of Fig. 1.

Transcriptional map of the *Hin*dIII D fragment. Having localized the lesions of the mutants to a 3.4-kb region within the D fragment, our next aim was to determine the transcriptional map of this DNA segment. RNA harvested early after infection was analyzed by RNA filter hybridization. Replicate filters of RNA fractionated by electrophoresis in the presence of formaldehyde were probed with various DNA fragments from within the D fragment. The results are shown in Fig. 2. As previously described (26), an early RNA of 3.0 kb representing the gene encoding the large subunit of the guanylyl transferase maps at the extreme left of *Hin*dIII-D, encoded between the *Hin*dIII and *Cla*I sites (Fig. 2, lane 1) and corresponding to the first open reading frame (ORF) of the *Hin*dIII D fragment (31). In addition, two transcripts of 3.7 and 3.0 kb are seen after hybridization with the *Eco*RI 3.4-kb fragment (lane 3) and also appear after hybridization with the much smaller *Pst*I-*Pvu*I fragment (lane 4). These two transcripts of 3.7 and 3.0 kb are derived from the region of DNA which rescues the three *ts* mutants to the wild-type phenotype. The mRNA of 3.0 kb fortuitously comigrates with the guanylyl transferase mRNA, resulting in the intense signal indicated by the double arrow in lane 2.

To determine the orientation of these transcripts and to define the position of their 5′ and 3′ termini, S1 mapping analysis was done. RNA filter hybridization revealed that the two transcripts of interest detected by the *Eco*RI 3.4-kb probe appeared after hybridization with both the leftmost *Hin*dIII-*Sal*I fragment and the central *Sal*I fragment of *Hin*dIII-D (data not shown). The marker rescue data also revealed that whereas *ts*24 and *ts*17 were rescued by fragments within the leftmost *Hin*dIII-*Sal*I fragment, *ts*69, a member of the same complementation group and hence affected in the same gene, was rescued by fragments within the central *Sal*I fragment. Because these data indicated that the gene of interest spanned the leftmost *Sal*I site of *Hin*dIII-

TABLE 1. Marker rescue of *ts*17, *ts*69, and *ts*24 with subfragments of *Hin*dIII-D

DNA[a] (kb)	Titration of 39.5°C yield (PFU/ml)[b]					
	*ts*17		*ts*69		*ts*24	
	32°C	39.5°C	32°C	39.5°C	32°C	39.5°C
None	1.5×10^4	$<10^1$	2.0×10^6	$<10^3$	1.0×10^3	$<10^1$
1. H-S (6.0)	5.7×10^4	1.8×10^5	4.4×10^6	$<10^1$		
2. S (3.0)	5.1×10^3	$<10^1$	1.8×10^5	3.7×10^4		
3. R (3.4)	8.0×10^5	1.4×10^5	8.6×10^6	3.6×10^6	2.6×10^4	1.6×10^4
4. Ps-R (2.9)	1.9×10^6	2.0×10^6				
5. R-Pv (1.6)			1.4×10^5	2.0×10^3[c]	2.2×10^3	$<10^1$
6. Pv-R (1.8)			2.3×10^6	1.6×10^5[c]	8.8×10^2	4.4×10^2
7. S-R (1.1)			4.0×10^4	2.7×10^4		
8. X (2.0)			2.0×10^4	1.4×10^4	1.6×10^4	4.4×10^3
9. X-C (1.6)					2.8×10^4	1.9×10^4
10. RV (1.6)					8.0×10^4	2.0×10^3[d]
11. RV (1.0)			2.0×10^4	1.0×10^4	1.0×10^5	4.0×10^2[d]

[a] DNA fragments are named according to their delimiting restriction sites from left to right with respect to the vaccinia virus genome. Their relative positions are shown in Fig. 1.

[b] Marker rescue was done as described in Materials and Methods.

[c] In these experiments, the yield of virus from infection in the absence of transfected DNA was 3.7×10^6 PFU/ml when determined at 32°C and 9.0×10^3 PFU/ml when determined at 39.5°C.

[d] In these experiments, the yield of virus from infection in the absence of transfected DNA was 7.1×10^4 PFU/ml when determined at 32°C and 5.0×10^2 PFU/ml when determined at 39.5°C.

D, the coordinates of the RNA transcripts were determined with reference to this *Sal*I site or a nearby *Cla*I site. The data are shown in Fig. 3. RNA harvested early after viral infection was hybridized with DNA probes radiolabeled at a single 5′ or 3′ terminus. To examine the region of the transcripts mapping to the left of the *Sal*I site, probes were prepared extending from an upstream *Cla*I site to the *Sal*I site and were labeled at a single 5′ terminus (probe 2, bottom

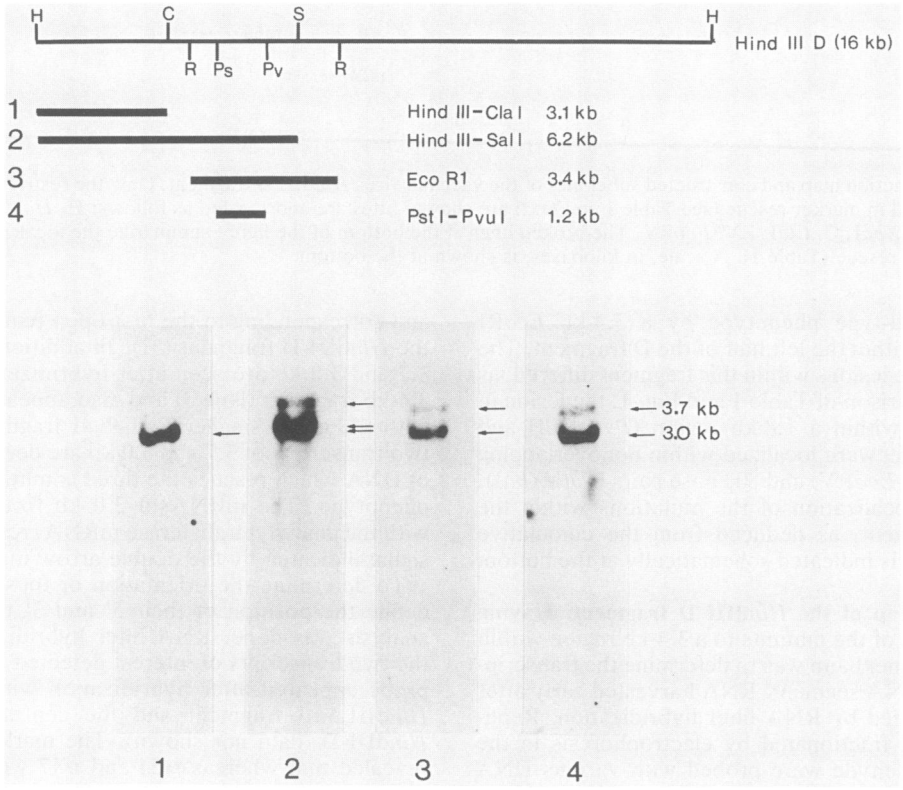

FIG. 2. Analysis of the left-hand region of *Hin*dIII-D by RNA filter hybridization. Parallel lanes of electrophoretically fractionated early RNA were transferred to nitrocellulose and probed with the nick-translated DNA subclones indicated. The sizes and positions of the probes relative to a partial restriction map of *Hin*dIII-D are diagrammed. Probes: 1, 3-kb *Hin*dIII-*Cla*I fragment; 2, 3.2-kb *Cla*I fragment; 3, 3.4-kb *Eco*RI fragment; 4, 1.2-kb *Pst*I-*Pvu*I fragment. Restriction endonuclease sites are abbreviated as in Fig. 1; only the sites which define the probes used are shown.

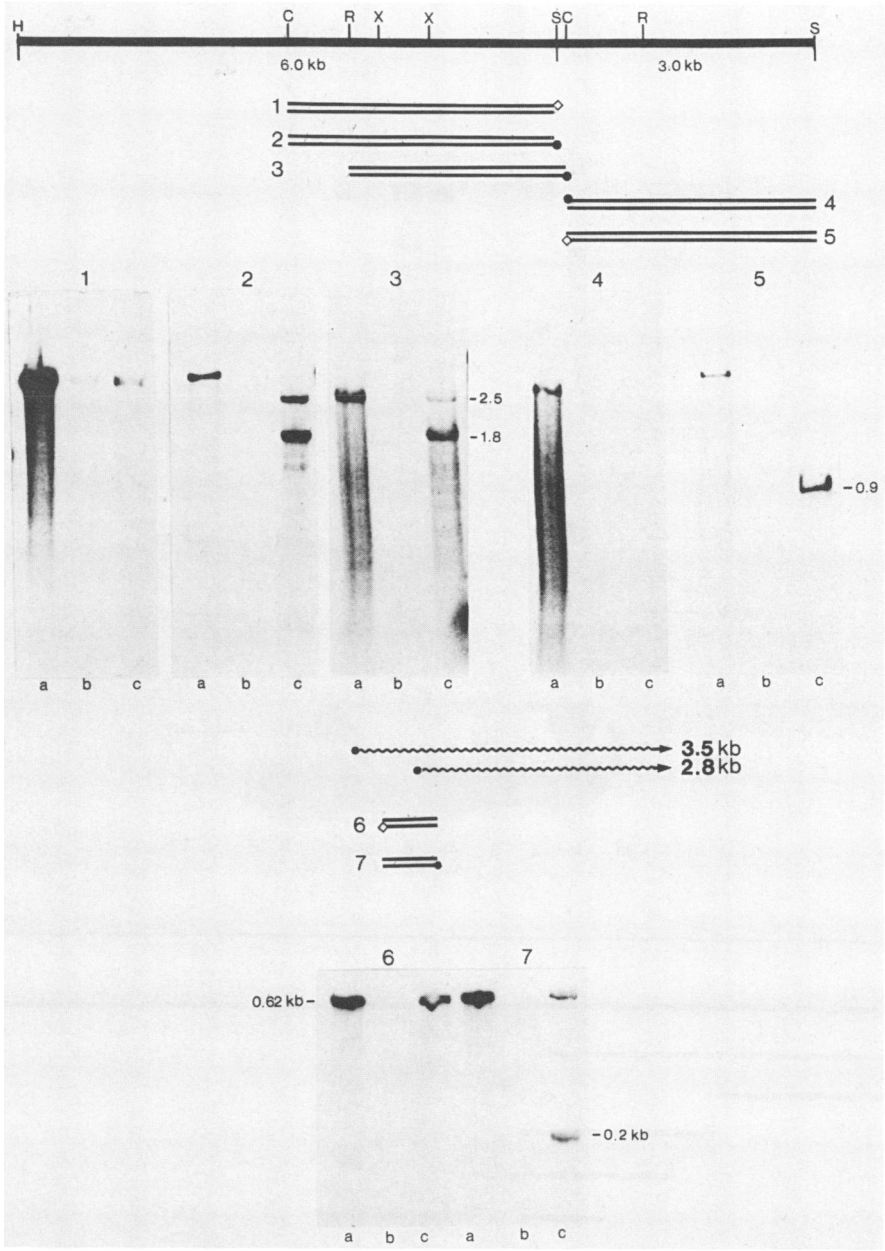

FIG. 3. S1 nuclease analysis of early transcripts mapping within the portion of *Hin*dIII-D which rescues three *ts* DNA replication mutants. 5′ (●) and 3′ (◇) radiolabeled DNA probes are shown relative to a partial restriction map of *Hin*dIII-D. Probes 1 and 2 represent a *Cla*I-*Sal*I fragment labeled at *Sal*I on the top (3′) and bottom (5′) strand, respectively. Probe 3 is an *Eco*RI-*Cla*I fragment labeled at the *Cla*I site on the bottom strand (5′). Probes 4 and 5 represent a *Cla*I-*Sal*I fragment labeled at the *Cla*I site on the top (5′) and bottom (3′) strand, respectively. Probes 6 and 7 represent an *Xba*I fragment labeled on the bottom strand at the 3′ and 5′ termini, respectively. Panels 1 to 5 show the products of the S1 nuclease reaction (hybridization at 42°C) for probes 1 to 5, fractionated by alkaline agarose gel electrophoresis and visualized by autoradiography. Panels 6 and 7 show the products of the S1 nuclease reactions for probes 6 and 7 (hybridization at 34°C) electrophoretically fractionated on urea acrylamide gels and visualized by autoradiography. For each probe, lane a represents the starting DNA fragment, lane b represents the DNA fragment incubated in the absence of RNA and treated with S1 nuclease, and lane c represents the DNA fragment hybridized in excess with early RNA and subsequently digested with S1 nuclease. Sizes of the fragments were determined relative to the migration of radiolabeled DNA markers. The map positions and polarity of the two RNAs hybridizing to this region are summarized schematically with wavy lines.

strand) or 3′ terminus (probe 1, top strand) at the *Sal*I site. Hybridization of early RNA with probe 2 yielded two protected, radiolabeled species after digestion of the hybrids with S1 nuclease (lane 2c). These data indicated the presence of two RNA transcripts whose 5′ termini map 2.4 and 1.7 kb upstream of the *Sal*I site and whose transcription occurs in a rightward direction. To confirm these data, the RNAs were also mapped with respect to a nearby *Cla*I site (probe 3). In this case, we detected a protected fragment of 1.8 kb and a species which represented the fully protected *Eco*RI-*Cla*I probe (2.5 kb). In sum, panels 2 and 3 position the approximate 5′ termini of the two mRNAs at 2.5 and 1.8 kb

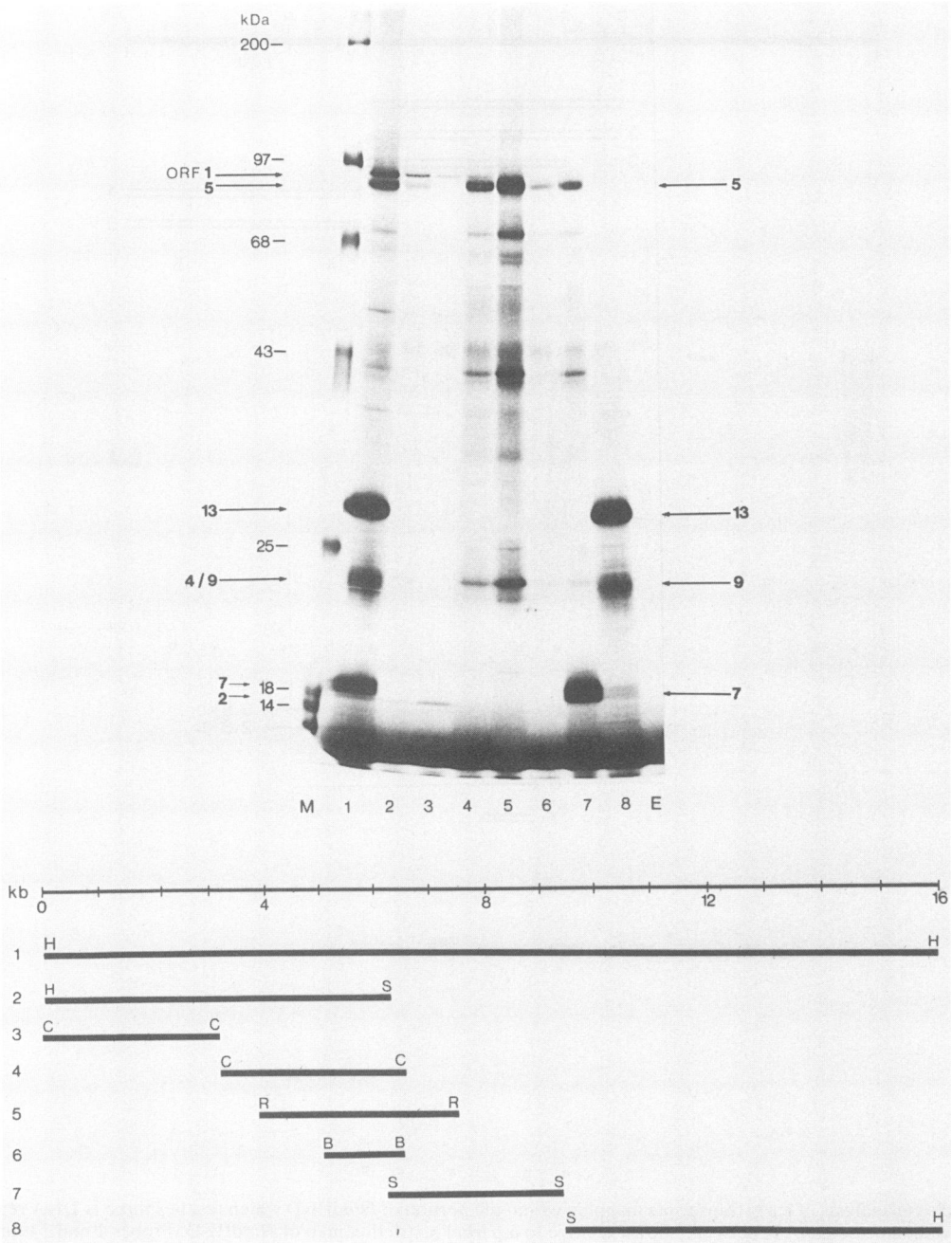

FIG. 4. Translational map of the early genes of *Hin*dIII-D by hybrid selection and in vitro translation. The products of cell-free translation of early RNA selected by hybridization to immobilized cloned fragments of *Hin*dIII-D were electrophoresed on 9 to 16% gradient polyacrylamide gels and exposed for autoradiography. The DNA subclones, depicted in order below a scale in kilobases, are: 1, the 16-kb *Hin*dIII D fragment; 2, the 6-kb *Hin*dIII-*Sal*I fragment; 3, the 3.1-kb *Cla*I fragment; 4, the adjacent 3.2-kb *Cla*I fragment; 5, the 3.4-kb *Eco*RI fragment; 6, the 1.4-kb *Bgl*II fragment; 7, the 3-kb *Sal*I fragment; 8, the 6.5-kb *Sal*I-*Hin*dIII fragment. Lane E represents the products of in vitro translation in the absence of exogenously added RNA. The molecular masses, in kilodaltons, of the ^{14}C-radiolabeled marker polypeptides in lane M are indicated by the dashes to the side. The arrows designate the translation products and identify the corresponding ORF predicted by DNA sequence analysis of *Hin*dIII-D (31). B, *Bgl*II; other restriction sites are abbreviated as described in the legend to Fig. 1.

upstream of the *Cla*I site. These data are further confirmed by measurement from an internal *Xba*I site, as shown in panel 7. No protected radiolabeled fragments were seen after hybridization of probe 1 (top strand) with early RNA, indicating that no RNAs transcribed in the leftward direction arise from this region.

To analyze the portion of the transcripts extending to the right of the *Sal*I site, hybridization was done with probes extending from the same *Cla*I site rightward to a distal *Sal*I site, and radiolabeled at the *Cla*I site at a single 3′ position (probe 5, bottom strand) or at a single 5′ position (probe 4, top strand). After hybridization of the early RNA with probe

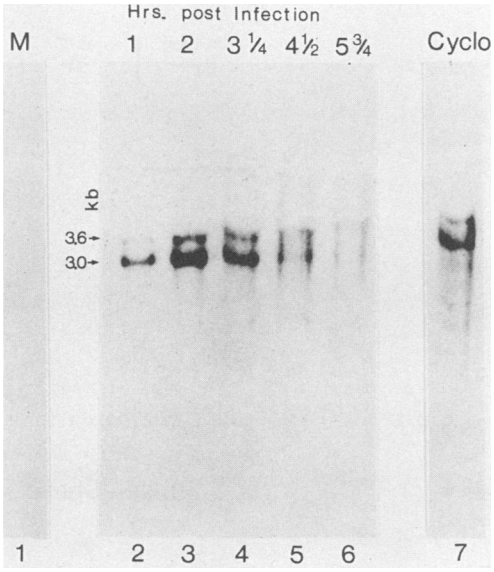

FIG. 5. Expression of the 82-kDa gene transcripts during wild-type infection: analysis by RNA filter hybridization. RNA was isolated from mock-infected mouse L cells (lane 1), from cells infected with vaccinia virus (MOI, 15 PFU per cell) after 1, 2, 3.25, 4.5, and 5.75 h of infection (lanes 2 to 6), and from cells after 3 h of infection in the presence of 100 μg of cycloheximide per ml (lane 7). The RNA (5 μg per lane) was fractionated by electrophoresis in the presence of formaldehyde, transferred to nitrocellulose, and probed with the nick-translated 3.4-kb *Eco*RI fragment depicted in Fig. 1.

5, a single protected, radiolabeled species was seen, indicating the presence of rightwardly transcribed RNA terminating 0.9 kb downstream of the *Cla*I site. No protection of probe 4 after hybridization with early RNA was seen, confirming the absence of transcripts complementary to the top strand of the genome in this region.

The position of the two overlapping transcripts deduced from these data, which have distinct 5′ termini but a common 3′ terminus, is shown schematically in Fig. 3. The predicted sizes of 3.5 and 2.8 kb are in good agreement with the 3.7- and 3.0-kb mRNAs seen by filter hybridization, with an allowance for polyadenylation. The presence of overlapping transcripts with distinct 5′ ends has been seen before in several transcriptional studies of the vaccinia genome (18, 27). A plausible model for the arrangement of the transcripts is that the longer mRNA species represents a readthrough transcript from an 0.7-kb upstream transcriptional unit into the 3.0-kb transcriptional unit. A prediction of this model would be the presence of an 0.7-kb mRNA with a 5′ terminus common to that of the 3.7-kb transcript and a 3′ terminus adjacent to the start of the 3.0-kb mRNA. RNA filter hybridization studies designed to reveal small RNAs were done but failed to detect this species (data not shown). In addition, S1 nuclease mapping designed to detect the 3′ terminus of the putative 0.7-kb mRNA species was done (Fig. 3, panel 6). No evidence of the postulated small transcript could be found (lane 6c), although control experiments indicated that protected fragments of the equivalent size could easily be detected (lane 7c, detecting 5′ terminus of 3.0-kb mRNA).

Coincident with the completion of our mapping of the *Hin*dIII-D transcripts spanning the region affected in the DNA mutant complementation group, the sequence of the *Hin*dIII D fragment was completed and kindly communicated to us by the investigators (31). The 3.0-kb transcript identified by our mapping coincides with the fifth ORF revealed by the DNA sequence analysis, and the unique upstream region of the 3.7-kb transcript coincides with the fourth ORF. Our failure to detect a small mRNA specific for ORF 4 can be at least partly explained by the absence of the consensus sequence for early transcription termination (TTTTTNT; 34, 48; L. Yuen, personal communication) in the region surrounding the 3′ end of the fourth ORF. Two copies of the termination signal are found downstream of the translational stop of the fifth ORF in a position consistent with termination of the 3.0- and 3.7-kb mRNAs at the coordinates detected by our S1 analysis. Clearly, the fine mapping of the mutants by marker rescue analysis (Fig. 1) places the lesions within ORF 5. Whether translation of ORF 5 occurs solely from the 3.0-kb mRNA or whether it can also be produced from a polycistronic 3.7-kb mRNA is not known at this time.

Translational map of *Hin*dIII-D: the gene affected in the *ts* mutants encodes an 82-kDa polypeptide. To provide a translational map of the early proteins encoded by *Hin*dIII-D and to assign a polypeptide to the gene affected in the *ts* mutants (and encoded within the 3.0-kb mRNA), hybrid selection analysis was done. Various cloned fragments of *Hin*dIII-D were covalently bound to activated filters and hybridized with RNA harvested early after infection. Complementary RNA species were eluted and translated in vitro. The [^{35}S]methionine polypeptides were fractionated on SDS-polyacrylamide gels and visualized by fluorography (Fig. 4). The data allowed us to correlate the physical map of the DNA with the encoded early polypeptides; species corresponding to the predicted ORF 1, ORF 2, ORF 4, ORF 5, ORF 7, ORF 9, and ORF 13 products were seen. Our determinations of the map positions and the molecular weights of the encoded products were consistent with those predicted by the DNA sequence (31). mRNAs were often selected with diminished intensity by downstream fragments, reflecting the transcriptional readthrough often seen in analyses of vaccinia virus gene expression (18, 27). By hybrid selection with DNA fragments completely contained within the 3.0-kb transcript and spanning the region required for marker rescue of the three *ts* mutants (Fig. 4, lane 6), the gene affected in the mutants could be unambiguously determined to encode an 82-kDa polypeptide. Consistent with its having a presumed role in DNA replication, this polypeptide, which corresponds to predicted ORF 5, was previously shown to have an affinity for DNA-cellulose (26). It is interesting that the ORF 4 gene product is detected by hybrid selection, implying that the 3.7-kb mRNA which contains both ORF 4 and ORF 5 sequences is translated into ORF 4.

Kinetic analysis of transcription of the 82-kDa polypeptide encoding mRNAs. We wished to determine the kinetics of expression of the transcript(s) encoding the 82-kDa polypeptide, the gene determined to be affected in *ts*17, *ts*24, and *ts*69. RNA was harvested at various times after infection with wild-type vaccinia virus, fractionated by denaturing electrophoresis, and analyzed by RNA filter hybridization (Fig. 5). After hybridization with a radiolabeled probe, the previously identified 3.7- and 3.0-kb mRNAs were seen. The transcripts appeared within 1 h of infection and peaked by 2 to 3 h. Transcripts were visible at late times of infection, at which time a characteristic smear of hybridization products was also visible. The 3.7- and 3.0-kb mRNAs were coordinately expressed, and, as found with the preparations of RNA harvested in the presence of cycloheximide, no 0.7-kb mRNA representing ORF 4 sequences was detected. At all

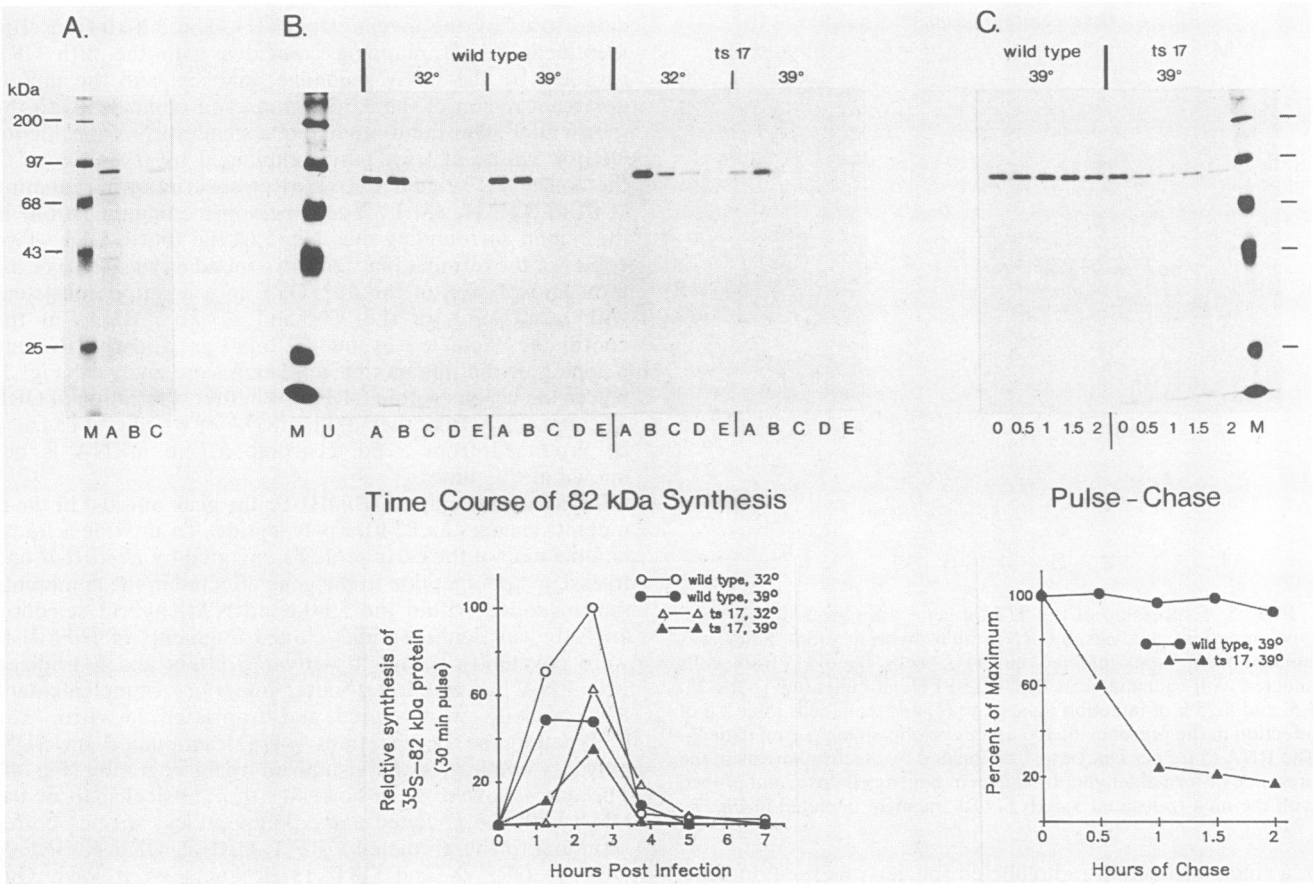

FIG. 6. Analysis of metabolically labeled 82-kDa protein by immunoprecipitation and gel electrophoresis. Fluorographs of 10% polyacrylamide gels are shown. In each case, lane M shows ^{14}C-radiolabeled protein standards whose molecular masses, in kilodaltons, are indicated to the left of panel A. (A) Comparison of the hybrid-selected 82-kDa protein and metabolically labeled proteins immunoprecipitated by the anti-82-kDa-*trpE* antiserum. Lane A represents the in vitro translation products of early RNA hybrid selected to the *Bgl*II DNA fragment (see Fig. 4). Lane B represents the products immunoprecipitated by the anti-82-kDa-*trpE* antiserum from a lysate of metabolically labeled uninfected L cells. Lane C represents the proteins immunoprecipitated by the anti-82-kDa-*trpE* antiserum from a lysate of cells infected with vaccinia virus at an MOI of 15 PFU per cell. Infected cells were labeled for 30 min with [^{35}S]methionine at 2 h postinfection and then harvested. (B) Time course of 82-kDa protein synthesis. Parallel dishes of cells infected with wild-type vaccinia virus or *ts*17 (MOI, 15 PFU per cell) at 32 and 39.5°C were metabolically labeled with [^{35}S]methionine for 30 min at successive time points and immediately lysed. In each case, lane A represents cells harvested at 1.25 h postinfection; lane B, at 2.5 h postinfection; lane C, at 3.75 h postinfection; lane D, at 5 h postinfection; lane E, at 7 h postinfection. Lane U represents a similar analysis of uninfected L cells. Each lysate was immunoprecipitated with the anti-82-kDa-*trpE* antiserum, and the products were electrophoresed on polyacrylamide gels and exposed for autoradiography. A densitometric scan of the autoradiogram is presented graphically below. (C) Stability of the 82-kDa protein in wild-type and mutant infection at the nonpermissive temperature. L cells infected with wild-type vaccinia virus or *ts*17 at 39.5°C (MOI, 15 PFU per cell) were metabolically labeled with [^{35}S]methionine for 30 min beginning at 2 h after infection and then chased with unlabeled medium. Parallel dishes were harvested immediately after labeling and at 0.5, 1, 1.5, and 2 h thereafter. Proteins precipitated from the lysates by the anti-82-kDa-*trpE* antiserum were electrophoresed and examined by autoradiography. A densitometric scan of the autoradiogram is presented graphically below.

time points (and in the absence or presence of cycloheximide), the 3.0-kb mRNA was found in greater abundance than the 3.7-kb mRNA.

Preparation of antisera to the 82-kDa polypeptide. To facilitate in vivo analysis of the 82-kDa polypeptide, we wished to prepare specific antisera. A 1.6-kb *Xba*I-*Cla*I fragment from within the 82-kDa gene was subcloned into an expression vector such that 82-kDa sequences were expressed as a *trpE*-82-kDa fusion protein after induction of the *trp* promoter. Upon addition of indoleacrylic acid, the bacterial cultures produced a 96-kDa fusion protein in high yield which was gel purified and used to produce specific antisera as described in Materials and Methods. Immunoprecipitation of metabolically labeled cultures of infected or uninfected L cells revealed that the immune serum (but not the preimmune serum, data not shown) recognized an 82-kDa polypeptide from infected cells (Fig. 6, panel A, lane C), but not from uninfected cells (lane B) which comigrated with the hybrid-selected, in vitro-translated, 82-kDa polypeptide (lane A). In addition to confirming the specificity of the antiserum, these data suggest that the 82-kDa polypeptide does not undergo extensive posttranslational processing.

Analysis of 82-kDa polypeptide expression by immunoprecipitation. The availability of the anti-82-kDa-polypeptide antiserum allowed us to investigate the time course of 82-kDa-polypeptide expression and to compare wild-type expression with that after infection with a *ts* virus containing a mutant 82-kDa allele. Cells were infected with wild-type

vaccinia virus or *ts*17 at an MOI of 15 and maintained at 32 or 39.5°C. At various times after infection, the cells were labeled with [^{35}S]methionine for 30 min, harvested, and immunoprecipitated. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 6, panel B). The wild-type and mutant polypeptides are indistinguishable with regard to electrophoretic mobility, and the overall patterns of expression are quite similar. The quantitation of 82-kDa expression is shown graphically beneath the fluorograph, and reveals that cultures maintained at 39.5°C show somewhat diminished 82-kDa polypeptide expression. Peak expression of the 82-kDa polypeptide occurs at 2.5 h after wild-type infection, a finding consistent with this protein being a classical early product and with its playing a role in DNA replication. Expression after infection with *ts*17 is diminished and delayed, as compared with wild-type infection. To examine the stability or lability of the wild-type and mutant 82-kDa proteins, pulse-chase analyses were done (Fig. 6, panel C). Infected cells (maintained at 39.5°C) were labeled with [^{35}S]methionine for 30 min at 2.0 h postinfection, refed with complete medium, and then harvested at half-hour intervals. After immunoprecipitation, the samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. As shown in Fig. 6, panel C, and quantitated in the graph below, the wild-type product was remarkably stable and showed virtually no degradation during the 2 h after labeling. The *ts*17-encoded product, however, was far more labile at 39.5°C, and only 25% of the labeled product remained intact after 1 h of chase. The decline of 82-kDa synthesis after 3 h of infection, coupled with a calculated half-life for the *ts*17 protein of 35 min, would result in the virtual absence of 82-kDa protein by 4 to 5 h after infection at 39°C. This finding offers at least a partial explanation of the temperature sensitivity of *ts*17 infection.

DISCUSSION

The genetic autonomy of vaccinia virus makes it likely that the viral genome encodes all of the enzymatic machinery required for DNA replication. Analysis of conditionally lethal mutants with primary defects in replication is a powerful means of identifying the genes encoding these enzymes. In this paper, we report the genetic, transcriptional, and biochemical analysis of one such complementation group of conditionally lethal mutants. *ts*17, *ts*24, and *ts*69 represent a complementation group of mutants previously mapped to the *Hin*dIII D fragment of the vaccinia virus genome. By performing marker rescue analyses with a series of subclones (of the *Hin*dIII D fragment) of decreasing size, we have localized the lesions of these three mutants within distinct regions of a 3.4-kb *Eco*RI fragment. RNA filter hybridization analysis and S1 nuclease mapping have led to the detection of two RNA species of 3.7 and 3.0 kb which map to this region. The mRNAs have distinct 5′ termini but share a 3′ terminus. The gene, defined by the 3.0-kb mRNA in which the three mutations map, corresponds to the fifth *Hin*dIII-D ORF recently deduced from the complete DNA sequence of the D fragment (31). Hybrid selection analyses have allowed us to map seven early polypeptide products of the *Hin*dIII D fragment and to unambiguously determine that the gene mutated in *ts*17, *ts*24, and *ts*69 encodes an 82-kDa protein. An antiserum to the 82-kDa polypeptide has been prepared by joining a 1.6-kb intragenic fragment in frame to the coding region of the *trpE* polypeptide to produce a fusion protein containing approximately 59 kDa of the 82-kDa protein. Immunoprecipitation of metabolically labeled cells has allowed the monitoring of the expression of the 82-kDa protein after infection. Expression begins within 1 h of infection and peaks by 2 to 3 h; the product encoded by *ts*17 is electrophoretically indistinguishable from the wild-type product and exhibits a similar pattern of expression. However, the *ts*17 protein appears to be significantly more thermolabile than that encoded by the wild-type virus.

By these analyses, we have identified one of the three genes now known to be essential for vaccinia virus DNA replication in tissue culture. By identification of the protein product, we have opened the way for its purification and the determination of its function in DNA replication. The rapid cessation of DNA synthesis when cultures infected with these mutants are shifted to the nonpermissive temperature is suggestive of a role for the 82-kDa protein in DNA elongation (E. Evans and P. Traktman, unpublished data). In addition, the diminished efficiency of marker rescue with these mutants, as compared with that observed in similar experiments with other DNA replication mutants (P. Traktman, unpublished data), may imply a role for the 82-kDa protein in DNA recombination. The continuation of these studies should provide insights into vaccinia virus biology and into eucaryotic DNA replication.

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