Characterization of a Temperature-Sensitive Mutant of Vaccinia Virus Reveals a Novel Function That Prevents Virus-Induced Breakdown of RNA

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Received 1 April 1985/Accepted 7 August 1985

We have attempted to characterize the molecular defect in a temperature-sensitive mutant of vaccinia virus, *ts22*, which has an abortive late phenotype. At the nonpermissive temperature, *ts22* displays normal viral protein synthesis until 8 h postinfection. Between 8 and 10 h after infection all viral protein synthesis ceases abruptly. Characterization of *ts22* revealed that (i) primary transcription of late viral genes was not grossly impaired, (ii) late viral mRNA was biologically inactive since it could not stimulate *in vitro* protein synthesis, and (iii) extensive cleavage of rRNA and late viral mRNA occurred at the time that viral protein synthesis aborted *in vivo*. These data suggest that *ts22* is defective in a function which prevents host rRNA and viral mRNA from being degraded. Inhibitor studies with cytosine arabinoside and cycloheximide showed that induction of and protection from rRNA breakdown occurred at approximately the same time during infection and required late viral gene expression. The viral protein synthesis pattern observed in vaccinia virus-infected cells treated with the drug isatin- β -thiosemicarbazone was strikingly similar to that observed in *ts22*-infected cells at the nonpermissive temperature (J. Cooper, B. Moss, and E. Katz, *Virology* 96:381-392, 1979). Analysis of rRNA integrity in isatin- β -thiosemicarbazone-treated, vaccinia virus-infected cells revealed extensive cleavage of rRNA, suggesting that the *ts22* and drug inhibitor may function in the same pathway.

Vaccinia virus, a prototypical member of the poxvirus family, contains a linear double-stranded DNA genome of approximately 180,000 base pairs. It therefore contains sufficient information to encode 100 to 200 polypeptides. Unlike other eucaryotic DNA viruses, which have an obligatory nuclear phase, the poxviruses replicate exclusively in the cytoplasm of susceptible host cells. Vaccinia virus therefore encodes an array of enzymes essential for DNA and RNA metabolism (reviewed in references 12 and 22). Vaccinia virus provides a unique system not only for studying the regulation of eucaryotic gene expression independently of the host but also for studying virus-host interactions.

Several laboratories have recognized the need for a thorough genetic analysis of the vaccinia virus. This has resulted in the isolation and characterization of a large number of temperature-sensitive mutants of vaccinia virus (4-6, 11, 15, 16). The isolation and preliminary characterization of 65 temperature-sensitive mutants of vaccinia virus representing 32 complementation groups have been described (5, 6). These studies revealed four biochemical phenotypes based on the ability of the mutants to synthesize viral DNA and viral proteins at the permissive and nonpermissive temperatures: (i) a normal phenotype (mutants are indistinguishable from the wild type [WT]), (ii) a DNA-negative phenotype (mutants do not synthesize viral DNA or late proteins at the nonpermissive temperature), (iii) a defective late phenotype (normal DNA synthesis and weak or delayed late viral protein synthesis at the nonpermissive temperature), and (iv) an abortive late phenotype (normal DNA synthesis, but late viral protein synthesis which starts normally at the nonpermissive temperature and then ceases abruptly).

The abortive late phenotype is particularly interesting because it suggests either a defect in the synthesis, function,

or stability of late viral mRNA or a perturbation in some other host-virus interaction necessary for continued late viral protein synthesis. These hypotheses prompted us to characterize further the defect in one abortive late mutant, *ts22*.

In this report, we show that late during a *ts22* infection at the nonpermissive temperature, at the time when viral protein synthesis aborts, rRNA and viral mRNA are degraded. This indicates that vaccinia virus encodes a function which prevents a virus-induced breakdown of host rRNA and viral mRNA.

The abortive late protein synthesis pattern observed during infection by *ts22* under nonpermissive conditions (5) is virtually identical to the pattern observed when a vaccinia virus infection is carried out in the presence of the antipoxvirus drug isatin- β -thiosemicarbazone (IBT) (24, 28, 34). We report here that rRNA is degraded at the time that viral protein synthesis aborts in a vaccinia virus infection in the presence of IBT. This suggests that the *ts22* and IBT inhibitors may function in the same pathway.

The pattern of rRNA degradation in a *ts22* infection at the nonpermissive temperature is strikingly similar to that observed in interferon-treated, vaccinia virus-infected mouse L cells (17, 18). Therefore, we must consider the possibility that the phenomenon we are observing is related to the defense of the virus against an antiviral state. This possibility will be discussed.

MATERIALS AND METHODS

Cells and virus. BSC40 cells, WT vaccinia virus strain WR, *ts22*, and all conditions for virus growth, infection, and plaque titration have been described in detail previously (5, 6). IBT (Pfaltz and Bauer, Inc.) was dissolved in acetone at a concentration of 5 mg/ml and then diluted with 4 volumes of 0.25 M NaOH immediately before use. A further 290-fold dilution was made by adding the IBT-containing solution to

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the medium before use. IBT was added at the end of the 30-min virus absorption period. The final concentration of IBT was 15 μ M (9).

Protein synthesis. Pulse-labeling infected cells with [35 S]methionine, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and autoradiography procedures have been described previously (5). Temperature shifts were accomplished by moving dishes of infected cells from 31 to 40°C or from 40 to 31°C without changing the medium.

RNA synthesis. Dishes (35 mm) of BSC40 cells were infected with 0.25 ml of the WT or *ts22* in phosphate-buffered saline (PBS), pH 7, at a multiplicity of infection of 20. After a 30-min absorption period at 31 or 40°C, 2 ml of warm medium was added to the infected dishes, and incubation was continued. At various times after infection, the medium was removed and the infected cells were pulse-labeled with 0.5 ml of [3 H]uridine (20 μ Ci/ml in PBS, specific activity 14 Ci/mmol; ICN Chemicals and Radioisotopes Division) for 12 min. The label was then removed, and 1 ml of lysis buffer containing 10 mM Tris hydrochloride, pH 8.0, 1 mM MgCl₂, and 1% Triton X-100 was added. The cells were scraped from the dishes and transferred to chilled tubes. The samples were incubated on ice for 10 min and vortexed occasionally. The cells were then spun at 1,000 $\times$ g for 10 min at 4°C to remove the nuclei. Cold 10% trichloroacetic acid (TCA) (1 ml) was added to 1 ml of the supernatant. A 1-ml amount of this solution was filtered on a Whatman GF/C filter, washed twice with cold 5% TCA and once with ethanol, air dried, and counted in a liquid scintillation counter.

Isolation of total RNA from infected cells. Dishes (100 mm) of BSC40 cells were infected with 1.5 ml of WT or *ts22* virus in PBSAM (PBS containing 1 mM MgCl₂ and 0.01% bovine serum albumin) at 15 PFU/cell and incubated at 31 or 40°C. At various times after infection, infected cells were rinsed once with 1 ml of cold PBSAM and then lysed in 1 ml of cold guanidinium solution. (The guanidinium solution contained 4 M guanidinium thiocyanate [Fluka Chemicals], 25 mM sodium citrate, pH 7.0, and 0.5% sodium lauryl sarcosine; β -mercaptoethanol was added to a final concentration of 0.1 M after the solution was filter sterilized. The guanidinium solution can be stored in the dark at 4°C.) The extract was passed through a 23-gauge needle four to five times to reduce viscosity. A 2-ml amount of the homogenate was layered on top of a 3-ml cushion of 5.7 M CsCl (special biochemical grade 99.9%; Gallard and Schliesenger)-0.1 M EDTA, pH 7.0, in Beckmann SW50.1 cellulose nitrate tubes. The tubes were centrifuged in a Beckmann SW50.1 rotor at 35,000 rpm at 15°C for at least 12 h. The supernatant (3 ml) was removed, and the walls were gently rinsed three times with cold guanidinium solution to remove attached DNA, each time leaving 2 ml of the CsCl. The tube was then cut just above the meniscus of CsCl. The CsCl was decanted, and the pellet was rinsed twice with cold 70% ethanol. The RNA pellet was dissolved in 10 mM Tris hydrochloride-1 mM EDTA, pH 7.0, adjusted to 150 mM sodium acetate, and precipitated with 2.5 volumes of 95% ethanol at -70°C. The RNA pellet was harvested by centrifugation at 12,000 $\times$ g for 15 min and dissolved in distilled water at a concentration of 5 mg/ml. The RNA was routinely stored at -70°C. RNA concentration was determined by assuming that 1 A₂₆₀ unit was equivalent to 45 μ g of RNA per ml. Generally, 100 to 175 μ g of RNA was obtained from 10⁷ cells. The RNA was essentially free of protein and viral DNA, as judged from the A₂₆₀/A₂₈₀ ratio.

RNA dot-blot hybridization. Total RNA was immobilized

on a nitrocellulose filter and hybridized as described previously (32). An equimolar mixture of purified cloned vaccinia virus *Hind*III DNA fragments D through O (provided by B. Moss) was labeled by nick translation with 32 P-labeled deoxynucleotides (700 Ci/mmol; ICN) as described previously (27) and used as a hybridization probe. Autoradiograms were made with Kodak XAR-5 film and an intensifying screen (Cronex Lightning-Plus, du Pont) at -70°C. After autoradiography, individual dots were excised, and radioactivity was determined in a liquid scintillation counter.

In vitro translation. Total RNA was translated in an mRNA-dependent reticulocyte system (lysate was obtained from Green Hectares, Oregon, Wis.) essentially as described by Cooper and Moss (8) and modified by Hruby and Ball (19). To monitor protein synthesis, 0.4 to 0.6 mCi of [35 S]methionine per ml (final concentration) (1,000 Ci/mmol; New England Nuclear Corp.) was added to reaction mixtures containing 19 nonradioactive amino acids, each at a concentration of 100 μ M. Incubation was at 30°C for 90 min for experiments involving RNA extracted at 14 h after infection and 60 min for in vitro translation of RNA extracted at 8 h after infection. Samples (2 μ l) were spotted on Whatman 3MM filters and processed to determine the hot TCA-precipitable radioactivity as described previously (7).

Electrophoretic analysis of RNA. Glyoxylated samples of RNA were electrophoresed on a 1.8% agarose gel in 5 mM sodium phosphate buffer (pH 7.0) and stained with ethidium bromide (29). Glyoxal (Matheson, Coleman and Bell, Norwood, Ohio) was deionized and stored as described previously (32). For Northern analysis, RNA gels were transferred to hybridization transfer membranes (Gene Screen, New England Nuclear Corp.) essentially as described by Southern (30). Before transfer, the membrane was soaked in 12.5 mM sodium phosphate buffer (pH 6.5) for 15 to 20 min. The transfer buffer used was 12.5 mM sodium phosphate buffer (pH 6.5). After transfer, the membrane was rinsed in 12.5 mM sodium phosphate buffer (pH 6.5) to remove residual agarose. The membrane was dried, baked at 80°C for 3 h, and prehybridized at 42°C for 12 h. The prehybridization buffer contained 50% formamide (deionized), 0.2% polyvinylpyrrolidone (molecular weight [MW] 40,000), 0.2% bovine serum albumin, 0.2% Ficoll (MW 400,000), 0.05 M Tris hydrochloride (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate (MW 500,000), and denatured salmon sperm DNA (>100 μ g/ml). A cloned 2.0-kilobase (kb) *Cla*I-*Bgl*III subfragment of the *Hind*III-E region of the vaccinia virus genome (obtained from P. Traktman), which contains half of the vaccinia virus DNA polymerase gene (33), was labeled by nick translation as described above and used as a hybridization probe. The probe was denatured by boiling at 100°C for 15 min, followed by quick cooling. Hybridization was carried out at 42°C for 18 h with the prehybridization buffer containing the denatured radioactive probe. After hybridization, the membrane was washed twice for 20 min in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, then once in 2 $\times$ SSC containing 1.0% SDS at 65°C for 1 to 2 h, and finally in 0.1 $\times$ SSC at room temperature for 1 to 2 h. The membrane was dried and autoradiographed as described above.

RESULTS

In vivo protein synthesis in *ts22*-infected cells. Synthesis of viral proteins during a normal vaccinia virus infection is regulated in at least two temporal classes. Early genes are expressed prior to DNA replication or under conditions in

FIG. 1. In vivo protein synthesis in *ts22*-infected cells. BSC40 cells were infected with *ts22* (20 PFU/cell) and pulse-labeled for 15 min with [35 S]methionine (20 μ Ci/ml) at various times after infection. Labeled proteins were analyzed by electrophoresis on 10% SDS-polyacrylamide slab gels, followed by autoradiography. Approximate MWs (in thousands) are shown at the left. The first nine lanes in each autoradiogram represent infections maintained continuously at 31 (A) or 40°C (B). The last three lanes represent temperature shifts performed at 12 h postinfection from 40 to 31°C (A) or 31 to 40°C (B).

which DNA replication is prevented. Expression of late genes is coupled to viral DNA replication. Early viral protein synthesis is initiated between 0 and 1 h after infection, peaks at 2 to 3 h after infection, and is shut off by 4 to 5 h after infection. Coincident with the onset of early protein synthesis is the rapid inhibition of host protein synthesis. The initiation of late viral protein synthesis occurs 3 to 4 h after infection. During WT infection, late viral protein synthesis can continue for as long as 16 to 24 h after infection. It has been shown previously that during infection by the abortive late mutant *ts22*, viral protein synthesis at 40°C is normal until 8 h postinfection; however, between 8 and 10 h postinfection, all viral protein synthesis ceases abruptly (5). The data shown in Fig. 1 confirm and extend this observation. At 31°C, the pattern of viral protein synthesis in a *ts22* infection was identical to that observed in WT-infected cells. At 40°C, *ts22* showed a pattern of viral protein synthesis indistinguishable from that observed in WT-infected cells until 8 h postinfection, including host shutoff, synthesis and shutoff of early viral proteins, and initiation of late viral protein synthesis. However, between 8 and 10 h postinfection, all protein synthesis ceased abruptly. Two other mutants (*ts4* and *ts23*) in the same complementation group as *ts22* had patterns of viral protein synthesis virtually identical to that of *ts22*-infected cells at 40°C (data not shown). Of the 32 complementation groups previously described, only one has this peculiar phenotype. No other genetic analysis of vaccinia virus has revealed a similar phenotype.

To determine whether the abortive late phenotype is reversible, we analyzed the effects of a temperature shift on the time course of virus protein synthesis. When *ts22*-infected cells were shifted from 40 to 31°C (Fig. 1A) at any time after in vivo protein synthesis had aborted, protein synthesis did not resume. This result indicates that the cessation of late protein synthesis is irreversible. When *ts22*-infected cells were shifted from 31 to 40°C (Fig. 1B) late during infection, protein synthesis stopped within 2 h. This suggests that the *ts22* gene product is required continuously

late during infection for maintenance of protein synthesis. When the temperature was shifted from 31 to 40°C or 40 to 31°C at early times (before 8 h), *ts22*-infected cells assumed the phenotype characteristic of the new temperature (data not shown).

Viral RNA synthesis in infected cells. The results described above suggest that the abortive late phenotype could be due to a defect in the synthesis, translation, or stability of viral mRNA. We therefore compared the synthesis, structure, and function of viral RNA from *ts22*- and WT-infected cells at 31 and 40°C.

We assayed viral RNA synthesis by pulse-labeling with tritiated uridine and by RNA dot-blot hybridization. Since vaccinia virus replicates in the cytoplasm of host cells, virus-specific RNA can be identified by its site of synthesis. When infected cells are pulse-labeled with tritiated uridine for less than 12 min, virtually all of the labeled material in the cytoplasm is viral RNA (2). Therefore, viral RNA synthesis in WT- and *ts22*-infected cells at 31 and 40°C was compared by measuring incorporation of tritiated uridine into TCA-precipitable material in the cytoplasm of infected cells (Fig. 2). Since the vaccinia virus infectious cycle proceeds faster at 40 than at 31°C, the rate of incorporation of [3 H]uridine into vaccinia virus mRNA was greater at 40 than at 31°C. The pattern of uridine labeling during *ts22* and *wt* infection at 40°C was virtually identical (Fig. 2).

The steady-state concentration of viral RNA sequences in *ts22*- and WT-infected cells at 31 and 40°C was quantitated by RNA dot-blot hybridization (Fig. 3A). Individual dots were excised, and radioactivity was determined (Fig. 3B). The steady-state concentration of vaccinia virus-specific RNA present late during a *ts22* infection at 40 and 31°C was comparable. The data from the pulse-labeling and hybridization experiments together imply that the viral transcriptional apparatus is intact and that viral mRNA sequences are present late during a *ts22* infection at 40°C.

In vitro translation of viral RNA. The experiments described above show that late protein synthesis aborts in

FIG. 2. RNA synthesis as measured by [^3H]uridine incorporation in cells infected with WT (Δ , $\blacktriangle$) and *ts22* ($\circ$, $\bullet$). BSC40 cells were infected (20 PFU/ml) and incubated at 31 ($\circ$, Δ) or 40°C ($\bullet$, $\blacktriangle$). At various times after infection, infected monolayers were pulse-labeled with [^3H]uridine (20 $\mu\text{Ci/ml}$) for 12 min, cytoplasmic extracts were prepared, and incorporation into acid-insoluble material was measured.

ts22-infected cells at 40°C despite the presence of appreciable quantities of virus-specific late mRNA sequences. To investigate the functional integrity of viral RNA present late during a *ts22* infection at 40°C, we tested its ability to support protein synthesis in vitro. Template activity of viral RNA was measured in vitro with a micrococcal nuclease-treated rabbit reticulocyte lysate. It has been shown previously that this in vitro system is capable of accurately translating both early and late vaccinia virus mRNAs (8). The products of the in vitro translation reactions were analyzed by SDS-polyacrylamide gel electrophoresis (data not shown) and by incorporation of [^{35}S]methionine into TCA-precipitable material (Fig. 4). Total RNA obtained at 8 h postinfection from WT- and *ts22*-infected cells at 31 and 40°C stimulated amino acid incorporation to a similar extent (Fig. 4A). In contrast, total RNA isolated from *ts22*-infected cells at 40°C at 14 h postinfection did not stimulate the incorporation of [^{35}S]methionine into proteins (Fig. 4B). In vitro translation reactions were also done with total RNA extracted at 2, 4, 6, 10, 12, and 16 h postinfection from both WT- and *ts22*-infected cells incubated at 31 and 40°C. RNA isolated up to 8 h postinfection from *ts22*-infected cells at 40°C stimulated protein synthesis in vitro, whereas late viral RNA (10 h and thereafter) was defective. Results of mixing experiments show that late RNA extracted from *ts22*-infected cells at 40°C did not inhibit the translation of early RNA (data not shown). These results suggest that *ts22* is

FIG. 3. Dot-blot hybridization of RNA extracted from cells infected with WT ($\circ$, Δ) or *ts22* ($\bullet$, $\blacktriangle$). BSC40 cells were infected (15 PFU/ml) and incubated at 31 ($\circ$, $\bullet$) or 40°C (Δ , $\blacktriangle$). Total RNA was extracted at 12 h postinfection as described in Materials and Methods. Various concentrations of total RNA (in micrograms, indicated below dots) were blotted on a nitrocellulose filter and hybridized to an equimolar mixture of ^{32}P -labeled cloned vaccinia virus *Hind*III DNA fragments D through O. (A) Autoradiograph. (B) Individual dots from the filter were excised, and radioactivity was measured as described in Materials and Methods.

defective in the maintenance of mRNA function late during infection.

A close comparison of in vivo and in vitro protein synthesis shows that the decay in template activity of mRNA as assayed in vitro lagged behind the decay of [^{35}S]methionine

FIG. 4. Cell-free protein synthesis directed by RNA extracted from cells infected with WT (Δ , $\blacktriangle$) or *ts22* ($\circ$, $\bullet$). Total RNA was extracted from infected cells (15 PFU/ml) incubated at 31 ($\circ$, Δ) or 40°C ($\bullet$, $\blacktriangle$) as described in Materials and Methods at 8 h (A) and 14 h (B) postinfection. Various concentrations of each RNA preparation were tested for stimulation of [35 S]methionine incorporation in vitro with a micrococcal nuclease-treated rabbit reticulocyte system. Incorporation was measured by TCA precipitation and is shown as 10^4 counts per minute.

labeling seen in vivo by 2 to 4 h. Possible explanations for this lag will be discussed (see Discussion).

Structural analysis of RNA from infected cells. A plausible explanation for all of the above observations is that late during a *ts22* infection at 40°C viral mRNA is cleaved to a functionally inactive form. We therefore decided to measure the size of viral RNA by Northern analysis (32). Total RNA extracted from WT- and *ts22*-infected cells at 31 and 40°C at 2, 4, 6, 8, and 14 h postinfection was denatured and electrophoresed on agarose gels. Before Northern analysis, the RNA was visualized by staining with ethidium bromide (Fig. 5). We discovered that rRNA was degraded into discrete products at 8 and 14 h postinfection in *ts22*-infected cells at 40°C (Fig. 5). This was not observed in a WT infection at 31 or 40°C, in a *ts22* infection at 31°C, or early during a *ts22* infection at 40°C (Fig. 5). Specifically, degradation of 28S rRNA was virtually complete. It is not clear whether the band comigrating with 18S rRNA represents intact 18S rRNA or partially degraded 28S rRNA. The time during infection that degradation of rRNA was observed coincided with the abrupt shutoff of viral protein synthesis.

To measure the size of the late viral transcripts by Northern analysis, it would be necessary to use a specific vaccinia virus late gene as a probe. However, late vaccinia virus transcripts are extremely heterogeneous in length at their 3' ends, presumably due to inefficient termination of transcription (10, 21). Thus, a discrete late gene may encode a family of transcripts, many of which are much larger than necessary to synthesize the polypeptide encoded by the gene. Precise information about the structural integrity of the late viral mRNA could be complicated by this size heterogeneity in the normal transcripts. We found, however, that almost any DNA probe in the Northern analysis yielded evidence of mRNA cleavage. An example is shown (Fig. 6) for a DNA fragment from the vaccinia virus DNA polymerase gene. This gene is transcribed early during infection into two mRNAs, 3.9 and 3.4 kb long (33). Early during WT or *ts22* infection at 31 or 40°C (2 h postinfection), the probe hybridized to the 3.9- and 3.4-kb transcripts (33; data not shown). At 6 and 8 h postinfection, in addition to the DNA polymerase transcripts, RNA in the size range of >5 kb was

also detected. This may represent transcription from a late promoter through the DNA polymerase gene. In *ts22*-infected cells at 40°C at 8 h postinfection, the 3.4-kb DNA polymerase transcript disappeared and a 2.6-kb transcript, a 1.8-kb transcript, and RNAs of 800 base pairs and smaller were detected instead. The simplest interpretation of these observations that is consistent with the data presented in Fig. 4 and 5 is that the new, smaller transcripts result from cleavage of larger RNAs. The results strongly suggest that *ts22* is defective in a function that in a normal vaccinia virus infection protects rRNA and viral mRNA from degradation.

Effect of inhibitors on RNA stability in infected cells. The results suggest a model in which at least two discrete events, which we will term induction and protection, are triggered during a vaccinia virus infection. The induction phenome-

FIG. 5. Analysis of infected-cell RNA by agarose gel electrophoresis. Total RNA was extracted from WT- and *ts22*-infected cells (15 PFU/ml) incubated at 31 or 40°C at 2, 4, 6, 8, and 14 h postinfection as described in Materials and Methods. A 15-μg amount of each RNA preparation was denatured with glyoxal and analyzed by electrophoresis on a 1.8% agarose gel. A photograph of the ethidium bromide-stained gel is shown. 28S, 18S, and 5.8S rRNA species are indicated. Lanes: M, mock-infected cells; a, WT, 31°C; b, WT, 40°C; c, *ts22*, 31°C; d, *ts22*, 40°C.

FIG. 6. Northern analysis of infected-cell RNA. BSC40 cells were infected with WT or *ts22* (15 PFU/ml) and incubated at 31 or 40°C. Total RNA was extracted at 6 h (first four lanes) and 8 h (last four lanes) postinfection as described in Materials and Methods. RNA was denatured and electrophoresed on a 1.8% agarose gel. RNA was transferred to membranes and hybridized to a ³²P-labeled vaccinia virus DNA clone that contained half of the vaccinia virus DNA polymerase gene. The sizes of the prominent RNA bands are shown at the left.

non, which ultimately results in rRNA breakdown, can be blocked by a protection function synthesized by the virus. In this fashion, the balance between the two opposing processes is maintained for a productive viral infection. In the abortive late mutant this balance is completely tilted in favor of the induction process, which continues unabated. Therefore, *ts22* provides a system for assaying the induction process in the absence of interference from the protection phenomenon. Deductions can be made about the protection phenomenon by comparing *ts22* infection with WT infection, in which both induction and protection processes occur.

To determine whether induction requires early or late viral gene expression, we assayed for rRNA breakdown late during a *ts22* infection carried out at 40°C in the presence of cytosine arabinoside (ara-C), which prevents late vaccinia virus gene expression. If induction requires early viral protein synthesis, rRNA cleavage should occur in the presence of ara-C; but if induction requires only late viral protein synthesis, then rRNA breakdown should be prevented by ara-C. Cells were infected with *ts22* and incubated at 40°C for 10 h in the presence or absence of ara-C. Total RNA extracted from these cells was denatured, electrophoresed on an agarose gel, and stained with ethidium bromide (Fig. 7, lane e). rRNA was intact in *ts22*-infected cells treated with ara-C, demonstrating that induction requires late viral gene expression.

We attempted to determine whether protection requires early or late viral gene expression by examining the effect of adding cycloheximide (Cx) at different times on rRNA integrity in WT- and *ts22*-infected cells. In this experiment, Cx was added at 0, 2, 4, 6, and 8 h after a WT infection or at 0, 4, and 6 h after a *ts22* infection. Infected cells were incubated at 40°C for a total period of 10 h. At the end of the incubation period, total RNA was extracted, denatured, electrophoresed on an agarose gel, and stained with ethidium bromide (Fig. 7). When viral protein synthesis was inhibited at 0 or 4 h after infection with *ts22*, rRNA breakdown was not observed. When Cx was not added until 6 h postinfection, rRNA breakdown occurred. This suggests that induction requires protein synthesis between 4 and 6 h postinfection. This result is consistent with the conclusion drawn from the experiment with ara-C, that induction is a late viral phenomenon. rRNA breakdown was less pronounced when Cx was added at 6 h after infection (Fig. 7, lane c) than in an untreated control (Fig. 7, lane d). This suggests that complete induction requires continued protein synthesis between 6 and 10 h postinfection. During a WT infection, rRNA breakdown was observed when Cx was added at 4 or 6 h after infection but not when it was added at 0, 2, or 8 h after infection. The rRNA breakdown was much less pronounced than in a *ts22* infection. The results obtained for a WT infection represent the combined effects of the induction and protection processes. Although significant protection was apparent by 6 h postinfection, it was still not sufficient to completely block the induction process. However, by 8 h in a WT infection, the induction process had been completely inhibited. rRNA breakdown was observed earlier in the WT infection than in the *ts22* infection, probably because of

FIG. 7. Effect of inhibitors on the structural integrity of RNA. BSC40 cells were infected with *ts22* (lanes a through e) or WT (lanes f through k) at 15 PFU/ml. Cx (300 µg/ml) was added to the monolayers 20 min before infection (lanes a and f) or 90 min (lane g), 4 h (lanes b and h), 6 h (lanes c and i) or 8 h (lane j) after infection. Ara-C (40 µg/ml) was added to *ts22*-infected cells 20 min before infection (lane e). Lanes d and k, RNA extracted from *ts22* and WT-infected cells, respectively, in the absence of any inhibitor. Incubation was continued for a total of 10 h at 40°C. Total RNA was extracted as described in Materials and Methods. RNA (15 µg) was fractionated on a 1.8% agarose gel after denaturation with glyoxal. A photograph of the ethidium bromide-stained gel is shown. 28S, 18S, and 5.8S rRNA species are indicated.

slight variations in the overall time course of gene expression between the two viruses.

In conclusion, the results with the WT and *ts22* together show that induction and protection occur simultaneously and require protein synthesis from 4 through 8 h after infection.

Effect of IBT on RNA stability in infected cells. IBT, a potent inhibitor of poxvirus replication (28), severely restricts the synthesis of late viral proteins (24, 34). Studies with IBT have shown that inhibition of protein synthesis cannot be entirely accounted for by an inhibition in mRNA synthesis, degradation of mRNA, or the functional integrity of the mRNA (9). The abortive late protein synthesis pattern in IBT-treated, vaccinia virus-infected cells is virtually identical to the pattern of protein synthesis observed in *ts22*-infected cells at 40°C (5). This similarity prompted us to examine the structural integrity of RNA in IBT-treated, vaccinia virus-infected cells. rRNA was degraded to discrete products late during vaccinia virus infection in the presence of IBT (Fig. 8). IBT did not affect the integrity of rRNA before 8 h postinfection (data not shown). The pattern of rRNA breakdown and the time during infection that rRNA cleavage occurred were indistinguishable from those observed in *ts22*-infected cells at 40°C. In the absence of a vaccinia virus infection, IBT does not precipitate rRNA breakdown. rRNA breakdown in the presence of IBT was not observed in cells infected with a vaccinia virus mutant resistant to IBT. These results indicate that IBT inhibits a viral function that is required to protect rRNA from being degraded. It seems likely that the *ts22* gene product and IBT may function in the same pathway. It is even conceivable that IBT inhibits the same function for which *ts22* is temperature sensitive.

DISCUSSION

We attempted to identify the molecular defect in an abortive late temperature-sensitive mutant of vaccinia virus, *ts22*. At the nonpermissive temperature, the mutant displayed normal viral protein synthesis up to 8 h after infection, whereas between 8 and 10 h postinfection all viral protein synthesis ceased abruptly. Characterization of the mutant revealed a novel aspect of viral gene expression: late during infection at the nonpermissive temperature, extensive cleavage of rRNA and mRNA occurred. Our data show that under these conditions (i) primary transcription of late viral genes is not grossly impaired (Fig. 2 and 3), (ii) late viral mRNA is biologically inactive since it cannot stimulate *in vitro* protein synthesis (Fig. 4), and (iii) a late viral component serves as an inducer for rRNA breakdown (Fig. 7). rRNA was similarly degraded in vaccinia virus-infected, IBT-treated cells (Fig. 8), which suggests that the *ts22* and IBT inhibitors may function in the same pathway.

Cleavage of both rRNA and mRNA resulted in a characteristic electrophoretic pattern of breakdown products. The reasons for this specific cleavage pattern are unclear. One possibility is that the enzyme responsible for RNA cleavage is sequence specific. Alternatively, the pattern may be dictated by the complex secondary structure of the 28S and 18S rRNA molecules or by protection by ribosomal proteins. The specific cleavage products observed in vaccinia virus mRNAs could be explained in a similar fashion.

We must consider the possibility that the rRNA and mRNA degradation observed in *ts22*-infected cells is not the primary effect of a mutation in the *ts22* gene but rather a generalized, indirect effect of inhibition of all protein synthesis. However, two different situations in which viral

FIG. 8. Effect of IBT on the structural integrity of RNA. BSC40 cells were infected with WT or an IBT-resistant vaccinia virus mutant (IBT^R) (15 PFU/ml). IBT (15 μ M) was added to monolayers after the virus had adsorbed to cells for 30 min. Incubation was continued at 40°C for 12 h (WT) or 10 h (IBT-resistant mutant). Total RNA was extracted as described in Materials and Methods. RNA (15 μ g) was denatured and electrophoresed on a 1.8% agarose gel. A photograph of the ethidium bromide-stained gel is shown. 28S, 18S, and 5.8S rRNA species are indicated. Lanes M, Mock-infected cells.

protein synthesis was inhibited did not result in the breakdown of RNA: (i) when vaccinia virus-infected cells were treated with Cx (Fig. 7) and (ii) during infection with NG26, a temperature-sensitive, phosphonoacetic acid-resistant vaccinia virus mutant in which viral protein synthesis ceased at 40°C at 8 h postinfection (data not shown). These results indicate that breakdown is directly related to a specific lesion in *ts22*. We therefore favor the interpretation that breakdown is actually a cause of the abortive infection rather than an artifact of a nonspecific inhibition of protein synthesis.

We have noted that the decay of mRNA template activity as assayed *in vitro* lags behind the decay measured *in vivo* by 2 to 4 h (compare Fig. 1B, 8 h, with Fig. 4A). There are two possible explanations for this lag. First, it is possible that the *in vitro* protein synthesis system is not as sensitive to mRNA degradation as the *in vivo* system is. A more interesting explanation is that the decay of protein synthesis observed *in vivo* represents the combined effects of rRNA and mRNA breakdown. Thus, when partially degraded mRNA from infected cells is added to an *in vitro* system containing intact ribosomes, more protein synthesis is observed than was observed *in vivo*.

The simplest explanation of the rRNA degradation phenomenon is that viral infection triggers two processes, induction and protection. We define the induction phenomenon as a process whose activation ultimately results in the cleavage of rRNA and late viral mRNA. The induction pathway was inhibited by ara-C (Fig. 7, lane e). This implies that the first component in the induction pathway is either a late viral gene product, late viral RNA, or replicated DNA itself. It is possible that induction is a multistep process involving other viral and host factors. It is reasonable to speculate that the last step in the induction pathway is the activation or synthesis of an RNase activity. The RNase could be a cellular or a viral gene product. The protection phenomenon involves a viral function which intervenes at some stage in the induction pathway and suppresses RNA breakdown. *ts22* is defective in the protection function, so that at the nonpermissive temperature the induction pathway dominates the infection. The experiments with Cx (Fig. 7) demonstrate that induction and protection occur at approx-

imately the same time during infection and require late viral protein synthesis.

It is interesting to speculate on the reasons for the existence of the induction and protection pathways. The induction pathway may have evolved as part of a host defense mechanism in response to viral infection. According to this hypothesis, infected cells may use some product unique to a viral infection as an inducer of the pathway that ultimately results in rRNA and mRNA breakdown. Vaccinia virus may have evolved a protection function to combat this host defense mechanism and create a productive infection.

We noted a striking resemblance between the rRNA breakdown in *ts22*-infected cells at the nonpermissive temperature and that in interferon-treated cells infected with vaccinia virus (17, 18), encephalomyocarditis virus (29), or reovirus (23). This similarity raises the possibility that the phenomenon observed in *ts22*-infected cells is in some way related to that seen in interferon-treated, virus-infected cells.

It has been proposed that the rRNA breakdown observed in interferon-treated, virus-infected cells results from the activation of a 2-5A-dependent RNase, also called RNase L. The activation of RNase L is apparently the last step in a cascade of events which together constitute one pathway of the interferon-induced antiviral state. Specifically, interferons induce a double-stranded RNA (dsRNA)-activated 2-5A synthase, which in the presence of dsRNA (which may be synthesized during a viral infection [3]) and ATP converts ATP to a series of 2'-5'-linked adenylate oligomers, called 2-5A. 2-5A in turn activates RNase L, resulting in the cleavage of rRNA and mRNA (reviewed in reference 20). This 2-5A pathway is only one of at least two mechanisms proposed for the antiviral effects of interferon. In the second pathway, viral dsRNA activates a protein kinase which phosphorylates the small subunit of a eucaryotic initiation factor, eIF-2, thereby inactivating it and leading to inhibition of protein synthesis (reviewed in reference 1). The only feature common to both pathways (besides blocking viral growth) is that the inducer is viral dsRNA.

Given the similarity in rRNA degradation patterns in *ts22*-infected cells and interferon-treated, vaccinia virus-infected cells, it is tempting to hypothesize that the phenotype of a *ts22* infection represents the uncontrolled induction of the RNase L pathway. A logical extension of this hypothesis is that the protector function that we believe is defective in *ts22* normally serves to counteract the antiviral state. There is biological evidence to support this speculation. First, vaccinia virus can rescue vesicular stomatitis virus from the inhibitory effects of interferon in doubly infected interferon-treated cells (13, 31). Second, vaccinia virus is refractory to inhibition by interferon in certain cell lines, such as HeLa, CV1, and L929, despite an active 2-5A-dependent RNase (25). Attempts to precisely characterize the vaccinia virus-mediated inhibition of the antiviral state have revealed the existence of a virus-encoded inhibitor of the dsRNA-dependent protein kinase (14, 26). It has also been proposed that vaccinia virus may encode an inhibitor of RNase L (25).

Despite the similarity of the rRNA degradation pattern, several observations are inconsistent with the hypothesis that the phenomenon seen in *ts22*-infected cells is directly correlated to the interferon-induced RNase L. First, in all of the studies cited above, pretreating the infected cells with interferon is obligatory, whereas in a *ts22* infection interferon is not added. Second, in several attempts (e.g., rescue of vesicular stomatitis virus and the protein kinase inhibitory factor) to determine whether the defense function encoded

by vaccinia virus against the antiviral state is induced early or late, it has been shown to be an early viral function (13, 31). In contrast, preliminary evidence from our studies with Cx (Fig. 7) indicate that protection is a late viral phenomenon. Third, rRNA degradation in interferon-treated, vaccinia virus-infected cells occurs as early as 30 min postinfection, and by 2 h after infection much of the 28S and 18S species have been degraded (17, 18). It is therefore apparent that the induction of interferon-induced RNase L is an early viral event. In contrast, ara-C experiments in *ts22*-infected cells (Fig. 7) showed that induction of rRNA breakdown is a late viral phenomenon. In light of these inconsistencies, it is important to reemphasize that the only similarity is in the pattern of rRNA degradation. It seems possible that the phenomenon we observed in the abortive late mutant may involve interferon-induced RNase L, but in ways we do not fully comprehend.

ACKNOWLEDGMENTS

We thank E. Niles and M. Garrick for critical reading of the manuscript. We also thank E. Niles for helpful discussions and for bringing to our attention the rRNA degradation phenomenon in vaccinia virus-infected, interferon-treated cells. We thank E. Paoletti for the generous gift of the IBT-resistant mutant of vaccinia virus. We thank P. Traktman for providing the vaccinia virus DNA polymerase clone and the procedure for isolating RNA from vaccinia virus-infected cells. We thank B. Moss for providing the cloned *HindIII* DNA fragments of vaccinia virus.

This work was supported by Public Health Service grant 2R01-AI1809404 (to R.C.C.) from the National Institutes of Health.

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