Modulation of ppp(A2'p)_nA-Dependent RNase by a Temperature-Sensitive Mutant of Vaccinia Virus

RANDALL J. COHRS,¹ RICHARD C. CONDIT,² RADIYA F. PACHA,²
CAROL L. THOMPSON,² AND OPENDRA K. SHARMA^{1*}

Laboratory of Molecular Biology, AMC Cancer Research Center, Denver, Colorado 80214,¹ and
Department of Biochemistry, State University of New York, Buffalo, New York 14214²

Received 5 August 1988/Accepted 19 October 1988

Activation of the ppp(A2'p)_nA (2-5A)-dependent RNase was investigated during the abortive infection of BSC40 cells by a temperature-sensitive mutant of vaccinia virus, *ts22*. At the nonpermissive temperature, *ts22* has an abortive late phenotype. At the onset of late-viral-gene expression, viral mRNA is degraded and rRNA is cleaved into discrete fragments in the absence of prior interferon treatment (R. F. Pacha and R. C. Condit, *J. Virol.* 56:395-403, 1985). Concomitant with rRNA cleavage, an increase in 2-5A occurred late during infection. Discrete 18S- and 28S-rRNA degradation products from BSC40 cells infected with *ts22* at the nonpermissive temperature comigrated in denaturing agarose gels with rRNA cleaved fragments produced by the activation of 2-5A-dependent RNase in uninfected cells transfected with exogenous 2-5A. An increase in 2-5A levels and a similar discrete and characteristic degradation of rRNA were observed in BSC40 cells infected with wild-type vaccinia virus in the presence of isatin- β -thiosemicarbazone. The results show that the *ts22* lesion and the action of isatin- β -thiosemicarbazone may affect the same pathway, leading to the activation of latent 2-5A-dependent RNase and resulting in indiscriminate RNA degradation and inhibition of viral replication.

Vaccinia virus is a large DNA-containing virus distinguished by its complex morphology and cytoplasmic site of replication. Its status as the prototype of the poxvirus family, its use in vaccination in eradication of smallpox, and the recent development of recombinant vaccinia virus vaccines have emphasized the need for knowledge concerning viral gene regulation during the cell-virus interaction. The double-stranded (ds) vaccinia virus genome, approximately 186,000 base pairs in length, has the potential coding capacity for over 200 proteins (18). To determine critical gene products necessary for viral replication, conditional lethal mutations have been used. Over 30 temperature-sensitive (*ts*) complementation groups have been identified (9, 10). Fine-structure mapping by marker rescue has revealed that the majority of the *ts* lesions are restricted to the central conserved region of the genome (27). A *ts* mutant, *ts22* (9), demonstrates the abortive late phenotype at the nonpermissive temperature (40°C). Shortly after the start of late-viral-gene expression (approximately 8 h postinfection), viral and cellular mRNAs are degraded and rRNA is cleaved into discrete products (20); consequently, protein synthesis is inhibited and the viral infection is aborted. While biologically active mRNA is no longer present in the cells during the late stage of abortive *ts22* infection, transcription of viral mRNA is not affected (20). The rapid decrease in viral and cellular mRNA stability and the cleavage of rRNA into discrete products prompted our investigation of the role of ppp(A2'p)_nA (2-5A)-dependent RNase during the abortive late stages of *ts22* infection. Our results show that 2-5A-dependent RNase is activated during *ts22* infection at a nonpermissive temperature in the absence of prior interferon treatment.

The 2-5A pathway is a multicomponent system consisting of dsRNA, 2-5A synthetase, 2-5A, 2-5A-dependent RNase,

and 2'-phosphodiesterase (17). In the presence of dsRNA, 2-5A synthetase polymerizes ATP into a series of 2',5'-linked oligomers (collectively referred to as 2-5A) of the general formula p_x(A2'p)_nA, where $x = 2$ or 3 ($n \geq 2$). These molecules at nanomolar concentrations activate a latent ribonuclease, 2-5A-dependent RNase (RNase L or RNase F). The activated RNase degrades RNA and consequently inhibits protein synthesis. 2-5A is rapidly degraded by cellular 2'-phosphodiesterase; therefore, its activation of the nuclease is transient. The 2-5A pathway thus regulates stability of RNA and is a component of the antiviral activity of interferon (2, 5, 12, 19, 29, 30). 2-5A synthetase is induced after interferon treatment (17). Changes in the levels of 2-5A, 2-5A synthetase, and 2-5A-dependent RNase under altered hormonal status (7, 16, 26), cell growth (26), and differentiation (15) have led to the suggestion that the 2-5A pathway has a wider role in hormone action and cell differentiation.

BSC40 cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (GIBCO) containing penicillin and streptomycin. Cells were infected with *ts22* or wild-type virus strain WR (9) in phosphate-buffered saline (pH 7.0) containing 1 mM MgCl₂-0.01% bovine serum albumin at 15 PFU per cell and were incubated at 31 or 40°C. At various times after infection, cells were detached by treatment with 0.5% trypsin, centrifuged, washed once with phosphate-buffered saline, and stored frozen at -80°C. RNA was isolated from frozen cell pellets according to Chirgwin et al. (6) by centrifugation through cesium chloride (7). 2-5A was isolated from frozen cell pellets (approximately 2.5×10^7 cells) by homogenization in 10 volumes of 10% trichloroacetic acid. The supernatant was extracted twice with a mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (13) and was lyophilized. 2-5A was quantitated by using a functional and sensitive assay (24). Activation of 2-5A-dependent RNase, isolated by affinity binding onto 2-5A (core) cellulose, was

* Corresponding author.

TABLE 1. Intracellular levels of 2-5A in BSC40 cells infected with a *ts* mutant of vaccinia virus (*ts22*)

BSC40 cells	2-5A concn (nM) at	
	31°C	40°C
Mock infected	<1	<1
<i>ts22</i> infected (h postinfection)		
0	<1	<1
4	<1	<1
8	<1	4.9
12	<1	4.0
16	<1	16.0

measured by hydrolysis of poly(U)-[5'-³²P]Cp by exogenous 2-5A. In the presence of 0.25 to 0.5 nM exogenous standard ppp(A2'p)₃A (Pharmacia), 50% hydrolysis of poly(U) was observed. Incubation of samples with poly(U)-[5'-³²P]Cp in the absence of enzyme did not result in significant degradation of poly(U). The degradation of poly(U) by the RNase in the presence of 2-5A was specific; poly(C) was not hydrolyzed under identical conditions. The concentrations of 2-5A were based on packed-cell volumes from which extracts were made.

A significant increase in 2-5A levels occurred at 8 h postinfection, and the levels remained elevated at 12 and 16 h postinfection in *ts22*-infected cells at 40°C (Table 1). In *ts22*-infected cells at a permissive temperature (31°C) or mock-infected BSC40 cells at 31 or 40°C or BSC40 cells infected with wild-type vaccinia virus at 31 or 40°C for 12 or 16 h postinfection, ≤1 nM 2-5A was detected. The putative 2-5A isolated by trichloroacetic acid extraction from *ts22*-infected BSC40 cells 16 h postinfection (at 31 and 40°C) was further characterized. It was fractionated by high-performance liquid chromatography, and each fraction was assayed for its ability to activate 2-5A-dependent RNase (Fig. 1). The extract from *ts22*-infected cells at 40°C showed a more complex elution profile of UV-absorbing materials than did the extract from *ts22*-infected cells at 31°C. Only the fractions eluting at the positions of ppp(A2'p)₂A and ppp(A2'p)₃A' from *ts22*-infected cells at 40°C activated the RNase. None of the fractions from *ts22*-infected cells at 31°C activated the RNase. The inability to detect 2-5A from *ts22*-infected cells at 31°C was not due to the presence of inhibitory material. Trichloroacetic acid extracts from *ts22*-infected cells grown at 31°C when assayed in mixing experiments did not inhibit activation of RNase by authentic 2-5A.

Coincident with the increase in 2-5A levels during *ts22* infection at the nonpermissive temperature, degradation of RNA was also evident (Fig. 2). At 8 h postinfection, 28S rRNA was degraded, as evidenced by the appearance of a discrete degradation product (8 h lane N, band c), while more extensive rRNA degradation into discrete products (bands a, b, c, and d) occurred at 12 and 16 h postinfection. No such rRNA cleavage was observed in *ts22*-infected cells at 31°C, mock-infected cells at 31 or 40°C, or in BSC40 cells infected with wild-type vaccinia virus at 40°C. In an earlier study (20), more extensive rRNA degradation was observed at 8 h postinfection, compared with the results described here. This difference could stem from the use of different virus preparations.

Degradation of rRNA into discrete and characteristic products appears to be an indicator of 2-5A-dependent RNase activity (25). To ascertain whether the rRNA degradation products observed in *ts22*-infected cells at 40°C are produced by the activation of the 2-5A-dependent RNase,

FIG. 1. Characterization of 2-5A from *ts22*-infected BSC40 cells. 2-5A isolated by trichloroacetic acid extraction from *ts22*-infected cells (approximately 5×10^7) at 31°C (A) or 40°C (B) 16 h postinfection was resolved on a μ Bondapak C-18 column (Waters Associates, Inc.) with a gradient of methanol in 80 mM ammonium phosphate buffer (pH 7.0) (22). Fractions (1 ml) were collected, and each fraction was analyzed for its ability to activate RNase, as described in the text. The elutions of standard oligoadenylates are indicated by arrows.

authentic 2-5A, the only known activator of the RNase, was introduced into uninfected BSC40 cells by calcium phosphate coprecipitation, and the integrity of the rRNA was determined by Northern (RNA) blot hybridization (Fig. 3). rRNA was degraded into distinct products similar to those observed in *ts22*-infected cells at 40°C. The 28S-rRNA degradation products a, b, c, and d (Fig. 3A, lanes 3 and 4) as well as the 18S-rRNA-specific degradation products a', b', and c' (Fig. 3B, lanes 3 and 4) produced in uninfected BSC40 cells transfected with ppp(A2'p)₃A at 40°C migrated in denaturing agarose gels similar to those produced during

FIG. 2. Time course of rRNA degradation during abortive *ts22* infection. BSC40 cells were infected with *ts22* (15 PFU per cell) and were incubated at 31 or 40°C. RNA (10 µg) was denatured with methylmercuric hydroxide (5 mM) and was electrophoresed at 50 V on a vertical 1.8% agarose slab gel containing 5 mM methylmercuric hydroxide until the dye front migrated 13 cm (7). After electrophoresis, the gel was stained with ethidium bromide, photographed under UV light, and electroblotted onto a Zeta probe membrane (Bio-Rad Laboratories). The membrane was probed with 28S-rRNA-specific DNA I-19 (1) made radioactive with [α - 32 P]dCTP by nick translation. Lanes: M, mock-infected cells at 16 h; 0 h, 4 h, 8 h, 12 h, and 16 h, times the cells were harvested after *ts22* infection; P, 31°C; N, 40°C. Migrations of 28S rRNA and its breakdown products a, b, c, and d are indicated on the right.

the late stages of abortive *ts22* infection (Fig. 3A and B, lanes 6). rRNA was degraded into distinct products that were identical to those shown in lanes 3 and 4 when BSC40 cells were transfected with ppp(A2'p)₂A at 31°C. No such characteristic degradation of rRNA was evident when the ribosomes from BSC40 cells were treated with RNase, RNase A, RNase T₁, or RNase T₂ (data not shown). The increase in 2-5A levels and the appearance of degradation products of rRNA in *ts22*-infected cells similar to those produced in uninfected BSC40 cells transfected with exogenous 2-5A clearly show that at the nonpermissive temperature late during *ts22* infection degradation of rRNA is due to the activation of 2-5A-dependent RNase.

2-5A-dependent RNase was also activated in BSC40 cells infected with wild-type vaccinia virus in the presence of the drug isatin- β -thiosemicarbazone (IBT). The inhibition of viral protein synthesis (11) and the cleavage of rRNA into discrete products in IBT-treated wild-type vaccinia virus-infected cells are similar to those in *ts22*-infected cells at 40°C (20) or in uninfected BSC40 cells transfected with 2-5A. We have detected 10 nM 2-5A with concurrent degradation of rRNA in IBT-treated and wild-type vaccinia virus-infected cells (data not shown). No such increase in 2-5A levels or rRNA degradation was observed in mock-infected cells (with or without IBT) or cells infected with wild-type vaccinia virus in the absence of IBT.

Activation of 2-5A-dependent RNase occurs also in interferon-pretreated mouse L929 cells infected with wild-type vaccinia virus which are sensitive to virus growth inhibition by interferon (2, 12). In certain cell lines, replication of vaccinia virus is not sensitive to inhibition by interferon (21, 23). In interferon-insensitive infections, even though high levels of 2-5A are produced late in the infection, 2-5A-dependent RNase is not activated (23). There are marked differences in the kinetics of activation of 2-5A-dependent RNase in interferon-sensitive wild-type vaccinia virus-infected L929 cells and BSC40 cells infected with *ts22*. Whereas degradation of rRNA in interferon-treated virus-infected L929 cells is an early event (2, 12), occurring as

FIG. 3. Comparison of rRNA cleavage products from *ts22*-infected cells with those produced on transfection of BSC40 cells with exogenous 2-5A. ppp(A2'p)₂A (Pharmacia) was introduced into uninfected BSC40 cells by the calcium phosphate coprecipitation technique as adopted by Hovanessian and Wood (14). Growth medium from 70 to 80% confluent cells (75-cm² growth area) was aspirated, and cells were washed twice with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline (HBS). Washed cell monolayers were incubated with 5 ml of HBS containing ppp(A2'p)₂A and 125 mM CaCl₂. After incubation for 45 min at 40°C, 5 ml of serum-free growth medium was added, and incubation was continued for 90 min. The medium was aspirated, cells were washed with phosphate-buffered saline (pH 7.5) and lysed in 2 ml of guanidinium thiocyanate solution (6), and RNA was isolated as described in the legend to Fig. 2. RNA (10 µg) was denatured with methylmercuric hydroxide and resolved by electrophoresis, transferred onto a Zeta probe membrane (Bio-Rad), and hybridized with rRNA-specific DNA probes (I-19 for 28S [A] and 25-46 for 18S [B] rRNA) (1). RNA was from cells treated with HBS (lane 1), HBS-125 mM CaCl₂ (lane 2), or HBS-125 mM CaCl₂ with 1×10^{-7} M (lane 3) or 5×10^{-7} M ppp(A2'p)₂A (lane 4). For comparison, RNA from *ts22*-infected BSC40 cells at 16 h postinfection at 31°C (lane 5) and 40°C (lane 6) are included. Migrations of 28 and 18S rRNAs and their breakdown products are indicated.

early as 30 min postinfection (12), in *ts22*-infected BSC40 cells, rRNA degradation and an increase in 2-5A levels is a late event, occurring 8 h postinfection. Furthermore, in L929 cells infected with wild-type vaccinia virus, both pretreatment with exogenous interferon and virus infection are required to elicit this response, whereas during *ts22* infection or wild-type infection in the presence of IBT, exogenous interferon pretreatment is not required. The reason for this difference is not clear. It remains to be determined whether the activation of 2-5A-dependent RNase in cells late during abortive *ts22* infection or wild-type infection in the presence of IBT is mediated by endogenous interferon production.

The *ts22* lesion is mapped by marker rescue to the left end of *Hind*III fragment A (R. Pacha, R. Meis, and R. Condit, manuscript in preparation); however, the sensitivity of viral replication to IBT has not as yet been located. It is quite possible that the IBT and the *ts* gene product of *ts22* affect the same pathway involving protection from the 2-5A pathway. dsRNA is the only known activator of 2-5A synthetase (17). Formation of dsRNA from RNA isolated during vaccinia virus infection has been observed (3, 8, 28), and there is indirect evidence that dsRNA is formed in vivo during vaccinia virus infection (2, 12, 23). Inhibition of protein synthesis by cycloheximide 4 to 6 h after infection of BSC40 cells with wild-type vaccinia virus results in rRNA cleavage (20). These results indicate that soon after the onset of late-viral-gene expression the activation of the 2-5A pathway is blocked by the product of the *ts22* gene. Experiments are

in progress to characterize such a virus-coded *ts22* function(s). Inactivation of the 2-5A pathway by expression of the functional *ts22* gene product, independent of virus infection, should help elucidate the role of the 2-5A pathway in cellular RNA turnover. The gene product of the *ts22* locus could inactivate the 2-5A pathway by masking dsRNA, specifically inactivating the 2-5A synthetase, reducing intracellular ATP levels, or inactivating 2-5A and 2-5A-dependent RNase. Encephalomyocarditis virus infection is known to inactivate 2-5A-dependent RNase, and the inactivation of nuclease is prevented by interferon pretreatment of cells (4).

We thank N. Arnheim for providing molecularly cloned probes for rRNA 25-46 and I-19 and Connie Ryan for typing the manuscript.

This work was supported by Public Health Service grants HD20612 (O.K.S.) and AI18094 (R.C.C.) from the National Institutes of Health and by a gift to the AMC Cancer Research Center from Steven W. Farber.

LITERATURE CITED

1. Arnheim, N. 1979. Characterization of mouse ribosomal gene fragments purified by molecular cloning. *Gene* 7:83-96.
2. Benavente, J., E. Paez, and M. Esteban. 1984. Indiscriminate degradation of RNAs in interferon-treated, vaccinia virus-infected mouse L cells. *J. Virol.* 51:866-871.
3. Boone, R. F., R. P. Parr, and B. Moss. 1979. Intermolecular duplexes formed from polyadenylated vaccinia virus RNA. *J. Virol.* 30:365-374.
4. Cayley, P. J., M. Knight, and I. M. Kerr. 1982. Virus-mediated inhibition of the ppp(A2'p)nA system and its prevention by interferon. *Biochem. Biophys. Res. Commun.* 104:376-382.
5. Chebath, J., P. Benech, M. Revel, and M. Vigneron. 1987. Constitutive expression of (2'-5') oligo A synthetase confers resistance to picornavirus infection. *Nature (London)* 330:587-588.
6. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
7. Cohrs, R. J., B. B. Goswami, and O. K. Sharma. 1988. Occurrence of 2-5A and RNA degradation in the chick oviduct during rapid estrogen withdrawal. *Biochemistry* 27:3246-3252.
8. Colby, C., and P. M. Duesberg. 1969. Double-stranded RNA in vaccinia virus infected cells. *Nature (London)* 222:940-944.
9. Condit, R. C., and A. Motyczka. 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology* 113:224-241.
10. Condit, R. C., A. Motyczka, and G. Spizz. 1983. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. *Virology* 128:429-443.
11. Cooper, J. A., B. Moss, and E. Katz. 1979. Inhibition of vaccinia virus late protein synthesis by isatin- β -thiosemicarbazone: characterization and *in vitro* translation of viral mRNA. *Virology* 96:381-392.
12. Goswami, B. B., and O. K. Sharma. 1984. Degradation of rRNA in interferon-treated vaccinia virus-infected cells. *J. Biol. Chem.* 259:1371-1374.
13. Hersh, C. L., R. E. Brown, W. K. Roberts, E. A. Swyryd, I. M. Kerr, and G. R. Stark. 1984. Simian virus 40-infected, interferon-treated cells contain 2',5'-oligoadenylates which do not activate cleavage of RNA. *J. Biol. Chem.* 259:1731-1737.
14. Hovanessian, A. G., and J. N. Wood. 1980. Anticellular and antiviral effects of pppA(2'p5'A)n. *Virology* 101:81-90.
15. Krause, D., R. H. Silverman, H. Jacobsen, S. A. Leisy, C. W. Dieffenbach, and R. M. Friedman. 1985. Regulation of ppp(A2'p)nA-dependent RNase levels during interferon treatment and cell differentiation. *Eur. J. Biochem.* 146:611-618.
16. Krishnan, I., and C. Baglioni. 1980. Increased levels of (2'-5') oligo(A) polymerase activity in human lymphoblastoid cells treated with glucocorticoids. *Proc. Natl. Acad. Sci. USA* 77:6506-6510.
17. Lengyel, P. 1982. Biochemistry of interferons and their actions. *Annu. Rev. Biochem.* 51:251-282.
18. Moss, B. 1985. Replication of poxviruses, p. 685-704. *In* B. N. Fields, D. M. Knipe, J. L. Melnick, R. M. Chanock, B. R. Roizman, and R. E. Shope (ed.), *Virology*. Raven Press, New York.
19. Nilsen, T. W., P. A. Maroney, and C. Baglioni. 1982. Synthesis of (2'-5') oligoadenylate and activation of an endoribonuclease in interferon-treated HeLa cells infected with reovirus. *J. Virol.* 42:1039-1045.
20. Pacha, R. F., and R. C. Condit. 1985. Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. *J. Virol.* 56:395-403.
21. Paez, E., and M. Esteban. 1984. Resistance of vaccinia virus to interferon is related to an interference phenomenon between the virus and the interferon system. *Virology* 134:12-28.
22. Rice, A. P., S. M. Kerr, W. K. Roberts, R. E. Brown, and I. M. Kerr. 1985. Novel 2',5'-oligoadenylates synthesized in interferon-treated vaccinia virus-infected cells. *J. Virol.* 56:1041-1044.
23. Rice, A. P., W. K. Roberts, and I. M. Kerr. 1984. 2-5A accumulates to high levels in interferon-treated, vaccinia virus-infected cells in the absence of any inhibition of virus replication. *J. Virol.* 50:220-228.
24. Silverman, R. H. 1985. Functional analysis of 2-5A-dependent RNase and 2-5A using 2',5'-oligoadenylate-cellulose. *Anal. Biochem.* 144:450-460.
25. Silverman, R. H., J. J. Skehel, T. C. James, D. H. Wreschner, and I. M. Kerr. 1983. rRNA cleavage as an index of ppp(A2'p)nA activity in interferon-treated encephalomyocarditis virus-infected cells. *J. Virol.* 46:1051-1055.
26. Stark, G. R., W. J. Dower, R. T. Schimke, R. E. Brown, and I. M. Kerr. 1979. 2-5A synthetase: assay, distribution and variation with growth or hormone status. *Nature (London)* 278:471-473.
27. Thompson, C. L., and R. C. Condit. 1986. Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. *Virology* 150:10-20.
28. Varich, N. L., I. V. Sychova, N. V. Kaverin, T. P. Antonova, and V. I. Chernos. 1979. Transcription of both DNA strands of vaccinia virus genome *in vivo*. *Virology* 96:412-420.
29. Watling, D., H. T. Serafinowska, C. B. Reese, and I. M. Kerr. 1985. Analogue inhibitor of 2-5A action: effect on the interferon-mediated inhibition of encephalomyocarditis virus replication. *EMBO J.* 4:431-436.
30. Williams, B. R., R. R. Golgher, R. E. Brown, C. S. Gilbert, and I. M. Kerr. 1979. Natural occurrence of 2-5A in interferon-treated EMC virus-infected L cells. *Nature (London)* 282:582-586.