RNA Synthesis of Vesicular Stomatitis Virus

V. INTERACTIONS BETWEEN TRANSCRIPTION AND REPLICATION

STANLEY M. PERLMAN AND ALICE S. HUANG

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 3 August 1973

The temperature-sensitive mutant *ts114* of vesicular stomatitis virus is temperature-sensitive in both primary and secondary transcription, but not in the replication of 40S RNA. The synthesis of 40S RNA is specifically inhibited when protein synthesis is shut off. The addition of cycloheximide to cells infected by *ts114*, rapidly inhibits RNA replication at the permissive and nonpermissive temperatures. However, the addition of cycloheximide at the nonpermissive temperature also results in almost complete recovery of transcription to the level found at the permissive temperature. Other inhibitors of protein synthesis applied at the nonpermissive temperature result in the same recoverability of the apparent temperature-sensitive lesion. Ribonucleic acid products synthesized by *ts114* at the nonpermissive temperature in the presence of cycloheximide are identical to virus-specific messenger RNA by the criteria of size and complementarity to virion RNA. When mutant-infected cells are shifted to the nonpermissive temperature, incubated for a period of time, and then treated with cycloheximide, the ratio of transcriptive to replicative activity varies depending on the time at which radioactive precursor is added to cells. This suggests an interdependence between replication and transcription during virus-specific RNA synthesis.

RNA synthesized in cells infected with B particles of vesicular stomatitis virus (VSV) consists of virion RNA, 40S RNA complementary to virion RNA (M. Stampfer, Ph.D. thesis, Mass. Institute of Technology, 1972), messenger RNA with sedimentation coefficients of 13 to 15S and 28S (7) and partially double-stranded 25 to 35S RNA intermediates (14). Whereas the process of transcription has been partially elucidated, that of replication involving the synthesis of virion RNA and its 40S complementary counterpart is not well understood.

Transcription includes two phases. Primary transcription is carried out by input virions and their associated polymerase. It takes place independent of de novo protein synthesis (7, 9). Secondary transcription by definition is messenger RNA copied from newly synthesized virion RNA and is thus dependent upon prior protein synthesis. Replication also only begins after viral protein synthesis has commenced. The synthesis of 40S RNA and that of 40S RNA complementary to virion RNA is tightly coupled. When 40S RNA synthesis is detected under a variety of conditions intracellular 40S RNA always contains the same proportion of virion RNA and complementary 40S RNA.

The study of temperature-sensitive mutants defective in RNA synthesis should aid in understanding replication and transcription. Five complementation groups of the Indiana serotype of VSV have been identified (3, 4, 11). Group I and group IV mutants, as defined by Pringle, are defective in RNA synthesis (12). Group II mutants, of which only two at present have been identified, may also be defective in some aspect of RNA synthesis (12).

In a previous communication, we characterized several group I and group IV mutants. The results suggest that a member of either group may be deficient in replication, transcription, or both. One of the group I mutants, *ts114*, is greatly inhibited at the nonpermissive temperature in both primary and secondary transcription. These results agree with those of Szilagy and Pringle, who showed that the *in vitro* polymerase activity of *ts114* is inactive at the nonpermissive temperature (15).

This investigation shows that the lesion in *ts114* is more complex than previously recognized, and suggests that the replicative and transcriptive functions strongly interact with one another.

Part of this investigation was presented at the

ICN Virus Meeting at Squaw Valley on 19 to 22 March 1973.

MATERIALS AND METHODS

Cells and viruses. Chinese hamster ovary (CHO) cells were grown as previously described (M. Stampfer, Ph.D. thesis, Mass. Institute of Technology, 1972). *ts114*, a temperature-sensitive mutant of the Indiana serotype of VSV was obtained from Craig Pringle of the Institute of Virology in Glasgow. *ts114* belongs to complementation group I, as defined by Pringle (12). During one-step growth in CHO cells the ratio of the production of plaque-forming *ts114* at the nonpermissive temperature of 37 C and at the permissive temperature of 31 C was less than 0.00006.

ts114 was grown in CHO cells and purified. Only standard B particles of *ts114* were used in this study.

For biochemical experiments with suspension cultures of CHO cells, the permissive temperature was 31 C and the nonpermissive temperatures were 37 to 37.5 C. The temperature of the cultures was controlled by submersion in water baths which varied from the set temperature by no more than ± 0.1 C.

Infection of cells, labeling with radioactive precursors, and preparation and analysis of RNA. CHO cells were infected as described previously (7). At the end of the indicated incubation periods, the infected cells were harvested and VSV RNA prepared as described previously (7). RNA was analyzed by centrifugation in a Beckman SW41 rotor in 15 to 30% sucrose gradients containing SDS buffer (100 mM NaCl, 1 mM ethylenediaminetetraacetate, 0.5% SDS, 10 mM Tris, pH 7.4). The conditions of centrifugation are described in the figure legends. After centrifugation, the gradients were analyzed (7).

Materials. Actinomycin was a kind gift from Merck Sharp & Dohme, Rahway, N.J. Other reagents were obtained as previously described (7).

RESULTS

Effect of cycloheximide on the RNA species synthesized at the permissive temperature (31 C). In the absence of any viral protein synthesis, infection of cells by VSV results in only primary transcription and replication does not begin (7, 9). The effect of inhibition of protein synthesis at later times during the infectious cycle on RNA synthesis has been studied in cells infected with paramyxoviruses (2, 8, 10, 13). In all cases, cessation of protein synthesis causes an inhibition in the synthesis of RNA of the size of virion RNA without affecting the synthesis of messenger RNA.

To determine whether inhibition of protein synthesis has a similar effect on VSV RNA synthesis, a sample of *ts114*-infected cells was treated at 31 C with cycloheximide after 3 h of growth at this temperature. As shown in Fig. 1, 40S RNA synthesis was completely inhibited, whereas transcription was only slightly decreased. The effect of cycloheximide on replication was very rapid, resulting in inhibition of

FIG. 1. Sucrose-gradient patterns of RNA synthesized by *ts114* at the permissive temperature in the presence and absence of protein synthesis. A 2-ml sample of 8×10^6 CHO cells was infected with *ts114* at a multiplicity of 16. After adsorption for 30 min at 31 C, the cells were diluted twofold. Actinomycin was added to a final concentration of 5 μ g/ml. After 3 h of incubation at 31 C, cycloheximide was added to one-half of the sample at a final concentration of 100 μ g/ml. A 50- μ Ci amount of ³H-uridine was added 5 min later to each half and the incubation continued for 60 min. Cytoplasmic extracts were prepared and analyzed on 15 to 30% sucrose-sodium dodecyl sulfate gradients by centrifugation for 14 h at 23,000 rpm as described in Materials and Methods. Symbols: ○, control; ●, cycloheximide-treated.

40S RNA synthesis within a few minutes of its addition.

Cycloheximide had the same effect on wild-type VSV RNA synthesis. Transcription was unaffected, whereas replication was rapidly and completely inhibited (manuscript in preparation). Protein synthesis is inhibited by 95% at the concentrations of cycloheximide used in all

the experiments reported in this communication (7).

Effect of cycloheximide on the RNA species produced by *ts114* at the nonpermissive temperature (37°C). We have shown previously that when *ts114*-infected cells are shifted to 37°C after a period of incubation at 31°C, replication of both strands of 40S RNA is unaffected and transcription is greatly inhibited (see Fig. 2). If addition of cycloheximide under nonpermissive conditions rapidly inhibits replication, it would be possible to prelabel 40S RNA at the nonpermissive temperature, add cycloheximide, and then follow the processing of newly synthesized virion RNA in the absence of labeled messenger RNA.

As shown in Fig. 2, cycloheximide added at the same time as the infected cells were transferred from 31°C to 37°C did rapidly inhibit the synthesis of 40S RNA. In addition, surprisingly, this treatment also greatly accelerated the rate of synthesis of 13 to 15S and 28S messenger RNA in an apparent partial reversal of the temperature-sensitive transcriptive lesion in *ts114*. The amount of 13 to 15S messenger RNA recovered at 37°C after treatment with cycloheximide was nearly as great as the amount found at the permissive temperature in the absence of the inhibitor, whereas 28S messenger RNA was only slightly increased. Pactamycin and puromycin, two other inhibitors of protein synthesis and structurally unrelated to cycloheximide, had the same effect on VSV RNA synthesis at the nonpermissive temperature. With both drugs, replication was rapidly inhibited and some transcriptive activity recovered, although puromycin was less effective than pactamycin or cycloheximide in recovering this activity.

Complementarity of RNA products synthesized at 37°C in the presence of cycloheximide to virion RNA. The RNA synthesized at the nonpermissive temperature in the presence of cycloheximide had sedimentation coefficients similar to that of previously identified VSV messenger RNA. VSV messenger RNA has been shown to be complementary to virion RNA (6). To insure that the products synthesized at 37°C in the presence of cycloheximide have the same polarity as messenger RNA and were not cleavage products of 40S RNA, the products were annealed to virion RNA. Almost all of the RNA synthesized at the nonpermissive temperature in the presence of cycloheximide (89%) and that synthesized at the permissive temperature (92%) was complementary to virion RNA (Table 1). This suggests that the RNA made at 37°C in the absence of protein synthesis was identical to messenger RNA.

Effect of longer incubations at 37°C on RNA

FIG. 2. Sucrose-gradient patterns of RNA synthesized by *ts114* at the nonpermissive temperature in the presence and absence of protein synthesis. A 1.5-ml amount of 5×10^6 CHO cells was infected with *ts114* at a multiplicity of 16. After adsorption for 30 min at 31°C, the cells were diluted twofold. Actinomycin was added to a final concentration of 5 μ g/ml. After 5 h of incubation at 31°C, the cells were divided into three samples. One sample was incubated at 31°C, one at 37°C, and one at 37°C in the presence of 100 μ g of cycloheximide per ml. Five minutes later, each sample was labeled with 50 μ Ci of 3 H-uridine for 60 min. Cytoplasmic extracts were prepared and analyzed by centrifugation for 17 h at 20,000 rpm as described in Materials and Methods. Symbols: $\circ$, 31°C; $\times$, 37°C; $\bullet$, 37°C with cycloheximide.

species synthesized in the presence of cycloheximide. The results of the previous experiments suggest that a protein factor must be continuously synthesized for replication to proceed normally. The experiment presented in Fig. 2 may be interpreted to indicate that an equilibrium between replication and transcription exists, and that the lesion in *ts114* makes replication more favorable relative to transcription. When cycloheximide was added replication was inhibited and the equilibrium was shifted towards transcription. In the next set of experiments, cells infected with *ts114* were incubated for longer periods at 37°C before the addition of cycloheximide to see if longer incubations at the nonpermissive temperature

TABLE 1. Annealing of RNA synthesized at 31 C and at 37 C in the presence of cycloheximide^a

Labeled RNA species ^b	Temperature of synthesis (C)	Cycloheximide	Counts/min		Ribonuclease resistance (%)
			Total	Ribonuclease resistance	
13 to 34S	31	—	11,725	10,706	92
13 to 34S	37	+	1,530	1,347	89

^a Each sample of labeled RNA was mixed with 4 μ g of unlabeled RNA which had been extracted from purified virions with phenol. Detailed procedures for the preparation and annealings have been described (7).

^b A 1-ml sample of 4×10^6 CHO cells was infected with ts114 at a multiplicity of 12 under conditions identical to that shown for Fig. 1. After 3 h of incubation at 31 C, one-half of the cells was shifted to 37 C and treated with cycloheximide (100 μ g/ml). Five minutes later, both samples were labeled with 50 μ Ci of ³H-uridine for 110 min. Cytoplasm was prepared and analyzed by centrifugation for 17 h at 21,000 rpm. The RNA from the 13 to 34S region of each gradient was pooled and precipitated with ethanol prior to annealing.

would alter the equilibrium more strongly towards replication even in the presence of the drug.

In the next experiment, shown in Fig. 3, a sample of ts114-infected cells was incubated at 31 C for 3 h before being transferred to 37 C. Two hours later, at 5 h after the initiation of infection, cycloheximide and radioactive precursor were added to the cells for another hour. Under these conditions, 40S RNA continued to be synthesized to the same degree in the absence or presence of protein synthesis. At the same time, the addition of cycloheximide resulted in only a small recovery of transcriptive activity.

If replication is only temporarily made independent of protein synthesis, then longer pretreatments with cycloheximide at the nonpermissive temperature before addition of ³H-uridine should result in an inhibition of the synthesis of 40S RNA with a concomitant increase in messenger RNA synthesis. Cells were incubated for 3 h at 31 C and then another hour at 37 C before cycloheximide was added. ³H-uridine was added 1 h after the drug, at 5 h after the initiation of infection. Under these conditions, the pattern of RNA synthesized was very similar to that observed when cycloheximide was added to cells at the time of shift to 37 C (Fig. 3). Although the synthesis of 40S RNA was greatly inhibited a small amount was still produced.

The synthesis of messenger RNA was greatly increased compared to that seen at 37 C in the absence of the inhibitor.

When ts114-infected cells were transferred to 37 C after incubation for 3 h at 31 C, there was a slow decay in the ability of the infected cells to synthesize total RNA. Several experiments with cycloheximide at the nonpermissive temperature showed a variability in the relative distribution of products made by transcriptive or

FIG. 3. Sucrose-gradient patterns of RNA synthesized by ts114 in the absence of protein synthesis after longer preincubations at the nonpermissive temperature. A 1.5-ml sample of 6×10^6 CHO cells was infected with ts114 at a multiplicity of 20. After adsorption for 30 min at 31 C, the cells were diluted twofold. Actinomycin was added to a final concentration of 5 μ g/ml. After 3 h of incubation, the sample was shifted to 37 C and divided in three. One hour later (4 h postinfection), 200 μ g of cycloheximide was added to one sample. At 5 h postinfection, 200 μ g of cycloheximide was added to a second sample. All three samples were then labeled for 70 min with 50 μ Ci of ³H-uridine. Cytoplasmic extracts were prepared and analyzed by centrifugation for 4 h at 40,000 rpm as described in Materials and Methods. Symbols: O, untreated, labeled 70 min, 5 h postinfection; ●, treated with cycloheximide and labeled 70 min, 5 h postinfection; ×, treated with cycloheximide at 4 h postinfection and labeled 70 min, 5 h postinfection.

replicative processes, but at any given time the total amount of RNA synthesizing activity remained constant from experiment to experiment.

DISCUSSION

Replication and transcription appear to be tightly coupled in VSV-infected cells. The interaction between the two functions is most apparent when *ts114*-infected cells are incubated at the permissive temperature for a few hours prior to incubation at the nonpermissive temperature. Under these conditions *ts114* contains a temperature-sensitive lesion in secondary transcription and not in replication. However, if protein synthesis were inhibited at the time that the infected cells were transferred to the nonpermissive temperature, secondary transcription would be almost completely recoverable. When addition of an inhibitor of protein synthesis was delayed at the nonpermissive temperature, transcriptive activity was only partially recoverable, and replicative activity only partially shut off. Under these conditions longer periods of inhibition of protein synthesis at the nonpermissive temperature eventually led to a greater shift from replicative to transcriptive functions. Upon prolonged incubation under nonpermissive conditions, some thermal inactivation of transcriptase activity did occur as evidenced by a gradual decrease in its recoverability.

This dissociation of replication from transcription by inhibiting protein synthesis occurs for wild-type VSV as well (manuscript in preparation) and has been reported to occur for paramyxoviruses (2, 8, 10, 13). The finding that the replicative function is rapidly labile when protein synthesis is inhibited suggests that attempts to detect cell-free synthesis of virion 40S RNA must be done under conditions when the replicative function is stabilized. One such condition may be when *ts114* is incubated at the nonpermissive temperature, thus permitting an accumulation of the factor(s) necessary for 40S RNA synthesis.

Also, from these observations on secondary transcription, primary transcription by *ts114* in the presence of cycloheximide should be detected. However, primary transcription was not detected in *ts114*-infected cells (unpublished observations), and *in vitro* the polymerase activity of *ts114* is temperature sensitive (15). Our inability to detect primary transcription may be due to the rapid thermal inactivation of the enzyme molecules initially associated with input *ts114* virions. The recovery of secondary transcription with *ts114* at the nonpermissive tem-

perature may be a reflection of the amplification of the number of transcriptase molecules in the cell, thus allowing detection of their activity during gradual inactivation. An alternative explanation for the lack of detectable primary transcription by *ts114* in the presence of cycloheximide is suggested by the fact that *de novo* protein synthesis is not required for primary transcription to occur. Secondary transcription, by definition, occurs on newly synthesized 40S RNA template and, therefore, requires the initiation of protein synthesis. The presence of *de novo* virus-specific protein(s) may partially stabilize transcriptase molecules to thermal inactivation. Preliminary analysis of the decay of primary and secondary transcription by *ts114* at the nonpermissive temperature favors the second interpretation.

Experiments with three other group I mutants (*ts11*, *ts12*, *ts13*) and a group IV mutant (*ts41*) show that inhibition of protein synthesis also caused a partial to complete recovery of transcriptive activity at the nonpermissive temperature (S. M. Perlman, *in press*). Thus, every mutant preliminarily characterized as RNA⁻ which we have tested has had some recoverable secondary transcriptase activity at 37°C in the presence of cycloheximide.

Before considering the complex interactions between transcription and replication, it is important to note that any analysis of VSV RNA synthesis is complicated by the finding that the active template for transcription and possibly replication is not just single-stranded 40S virion RNA, but rather this RNA enclosed in a nucleocapsid structure (1; Huang, unpublished observations). Thus, a model of VSV RNA synthesis may include interactions between the transcriptase, the replicase, and the nucleocapsid proteins, as well as interactions of these proteins with the RNA template. Moreover, host factors may also participate in VSV RNA synthesis. This is evidenced by the differences in size of the complementary RNA pieces transcribed *in vitro* and *in vivo* (7) as well as by host effects on virus-specific RNA replication during interference by defective particles (5). With so many possible interactions, complex models for VSV RNA synthesis can be designed. One simple model is that one enzyme molecule, the virion-associated transcriptase, has multiple functions. This enzyme is hypothesized to interact with other polypeptides which determine its transcriptive and replicative functions. Such a model would involve only a change in the recognition of initiation and termination sites during VSV RNA synthesis. Obviously, *in vitro* reconstruction of VSV RNA synthesis and

further *in vivo* biochemical experiments will test the validity of this model.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-10100 from the National Institute of Allergy and Infectious Diseases and by American Cancer Society research grant VC-63. S. M. P. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research and A. S. H. is a Career Development Awardee of the Public Health Service.

We thank Craig Pringle for kindly sending us his mutants and Louise Chu for excellent technical assistance.

LITERATURE CITED

1. Bishop, D. H. L., and P. Roy. 1972. Dissociation of vesicular stomatitis virus and relation of the viral proteins to the viral transcriptase. *J. Virol.* 10:234-243.
2. East, J., and D. Kingsbury. 1971. Mumps virus replication in chick embryo lung cells: properties of RNA species in virions and infected cells. *J. Virol.* 8:161-173.
3. Flamand, A. 1969. Etude des mutants thermosensibles du virus de la stomatite vésiculaire. Mise au point d'un test de complementation. *C. R. Acad. Sci. Paris* 268D:2305-2308.
4. Holloway, A. F., P. K. Y. Wong, and D. V. Cormack. 1970. Isolation and characterization of temperature-sensitive mutants of vesicular stomatitis virus. *Virology* 42:917-926.
5. Huang, A. S., and D. Baltimore. 1970. Defective viral particles and viral disease processes. *Nature (London)* 226:325-327.
6. Huang, A. S., D. Baltimore, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. *Virology* 42:946-957.
7. Huang, A. S., and E. K. Manders. 1972. Ribonucleic acid synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. *J. Virol.* 9:909-916.
8. Kaverin, N., and N. Varich. 1971. Virus-specific RNA formed in Newcastle disease virus-infected cells after suppression of protein synthesis by cycloheximide. *Arch. Gesamte Virus Forsch.* 35:378-384.
9. Perrault, J., and J. J. Holland. 1972. Absence of transcriptase activity or transcription-inhibiting ability in defective interfering particles of vesicular stomatitis virus. *Virology* 50:159-170.
10. Portner, A., and D. Kingsbury. 1972. Identification of transcriptive and replicative intermediates in Sendai virus-infected cells. *Virology* 47:711-725.
11. Pringle, C. R. 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine and ethyl methane sulfonate. *J. Virol.* 5:559-567.
12. Pringle, C. R., and I. B. Duncan. 1971. Preliminary physiological characterization of temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* 8:56-61.
13. Robinson W. S. 1971. Sendai virus RNA synthesis and nucleocapsid formation in the presence of cycloheximide. *Virology* 44:494-502.
14. Stampfer, M., D. Baltimore, and A. S. Huang. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. *J. Virol.* 4:154-161.
15. Szilagyi, J. F., and C. R. Pringle. 1972. Effect of temperature-sensitive mutations on the virion-associated RNA transcriptase of vesicular stomatitis virus. *J. Mol. Biol.* 72:281-292.