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RNA Synthesis by Newcastle Disease Virus Temperature-Sensitive Mutants in Two RNA-Negative Complementation Groups

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The temperature-sensitive RNA-negative mutants of Newcastle disease virus comprise two complementation groups, group A (seven members) and group E (one member). The RNA-synthesizing activities of four representative members of group A and the single member of group E were compared with the activity of the wild type. These mutants were defective to varying extents in primary transcription at the nonpermissive temperature, ranging from mutant A1, which had no activity, to mutant E1, which lost only 50% of its activity. All of the mutants were also defective in a postprimary transcriptive process since after preincubation at the permissive temperature in the presence of cycloheximide, there was no subsequent RNA synthesis at the nonpermissive temperature upon removal of the cycloheximide. Similarly, in experiments in which cycloheximide was not used, shifts from the permissive temperature to the nonpermissive temperature before 3 h postinfection did not result in RNA synthesis. However, later shifts to the nonpermissive temperature did allow RNA synthesis. With the exception of mutant A1, all of the mutants maintained this RNA-synthetic ability for at least 3 h, suggesting that RNA synthesis from progeny genomes was not the major postprimary transcriptive defect in these mutants. In contrast, the RNA-synthetic ability of mutant A1 rapidly decayed at the nonpermissive temperature, suggesting that the A gene product is involved in RNA synthesis from progeny genomes. The postprimary transcriptive defect(s) of the other mutants may be in the processing or stability of a protein, in the processing of mRNA, or in replication. Plaque-forming revertants (ts^+) of all of the mutants coreverted for RNA synthesis. This finding strengthens the relationship between temperature sensitivity for plaquing and both the primary and postprimary RNA-negative phenotypes.

Temperature-sensitive (*ts*) mutants have been isolated from several paramyxoviruses, including measles virus (2, 16), respiratory syncytial virus (14, 15), Sendai virus (1, 30), and Newcastle disease virus (NDV) (31, 36, 42). When these mutants are tested for RNA-synthesizing ability, a relatively large fraction of them are RNA negative (RNA^-; i.e., they show reduced synthesis of virus-specific RNA at nonpermissive temperatures) (2, 16, 30, 32, 42). For NDV, one group of Sendai virus, and measles virus, these RNA^- mutants fall into two, two, and three complementation groups, respectively (1, 5, 42), which is consistent with the involvement of two or three proteins in RNA synthesis. In contrast, seven complementation groups have been identified for another group of Sendai virus mutants (30). For most of these viruses, as well as the rhabdovirus vesicular stomatitis virus (VSV) (43), the majority of the RNA^- mutants fall into one large complementation group.

The *ts* mutants of NDV isolated by Tsipis and Bratt (42) fall into five complementation groups (designated A, B, C, D, and E) and an additional group, group BC, which complements neither group B nor group C. All members of groups A (seven members) and E (one member) were identified as RNA^- simply by measuring trichloroacetic acid (TCA)-precipitable radioactivity in secondary chicken embryo cells labeled with [^{3}H]uridine for 5 h in the presence of actinomycin D (42). We have tentatively identified group A as representing the gene for the L protein and group E as representing the gene for the P protein (27a). Both protein L and protein P are probably involved in RNA synthesis. In this paper we describe further experiments designed to localize the defective process(es) for each

group A and E mutant. For these detailed experiments, a system more easily manipulatable than chicken embryo cells in monolayer cultures and with more precise temperature control was required. Suspension cultures of Chinese hamster ovary (CHO) cells maintained in constant temperature water baths met these criteria, for in most respects, including RNA synthesis patterns and virus production, NDV infections of CHO cells are similar to infections of chicken embryo cells (Bratt, unpublished data). The single group E mutant and four of the group A mutants were analyzed for primary and postprimary transcriptive activities at permissive and nonpermissive temperatures and after shifts to nonpermissive temperatures. Each of these mutants was defective in primary transcription, as well as in at least one postprimary transcriptive process.

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MATERIALS AND METHODS

Virus purification and cell culture procedures. NDV wild-type strain AV (Australia-Victoria, 1932; designated AV-WT) has been described previously by Bratt and Gallaher (4). This virus was grown in embryonated hen eggs at 37.5°C and was concentrated by centrifugation from the allantoic fluid, as previously described (44). Isolation and preliminary characterization of mutants were performed as described by Tsipis and Bratt (41, 42). For each of the *ts*RNA$^-$ mutants, spontaneous revertants were isolated from plaques formed in secondary chicken embryo cells at the nonpermissive temperature (41.8°C) and were subsequently plaque purified three times. CHO cells were grown in spinner cultures as previously described (25).

Chemicals and medium. The growth medium used for CHO cells has been described previously (7). For the experiments described below, 40 mM HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was included, giving a constant pH of 7.0. Actinomycin D was generously provided by Merck Sharp & Dohme. [5,6-^{3}H]uridine (34 Ci/mmol) was obtained from New England Nuclear Corp., and cycloheximide was obtained from Sigma Chemical Co.

Infection and labeling. CHO cells were suspended at a concentration of 2.7×10^6 cells per ml and, while spinning, were infected at the appropriate multiplicity of infection (MOI). The MOI was 10 for all experiments except the primary transcription experiments, in which the MOI was 50. The temperature during this period (37.5, 41.2, or 41.8°C) had no effect on the results (data not shown). After 30 min the cells were diluted to a concentration of 4×10^5 cells per ml and then incubated at the appropriate temperature. With the exception of the experiments shown in Table 1 and Fig. 4, the RNA labeling data reported below represent the averages of duplicate 0.5-ml samples that were removed from spinner cultures, diluted with 0.5-ml portions of medium containing actinomycin D (final concentration, 5 μg/ml) and [^{3}H]uridine (final concentration, 10 μCi/ml), and maintained without spinning for 1 h. The levels of virus-specific radioactivity were calculated by subtracting the radioactivity incorporated into uninfected cultures under similar conditions, as described in the legend to Fig. 1. (The results obtained in this way were identical to the results obtained in more cumbersome experiments, in which [^{3}H]uridine was added to cultures previously treated for 30 min with actinomycin D [Bratt, unpublished data].) In all of the figures illustrating kinetic experiments, the data points are plotted at the beginning of the labeling period.

Primary transcription. Cells preincubated with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml for 30 min were infected at an MOI of 50 and then maintained in the presence of actinomycin D and cycloheximide. This concentration of cycloheximide inhibited [^{35}S]methionine incorporation by 99% in both uninfected and infected cells (data not shown). Cycloheximide allows primary transcription to continue but blocks replication. Cells were labeled in spinner cultures with [^{3}H]uridine (150 μCi/ml) beginning 30 min after infection and were subjected to TCA precipitation 2 h later.

Extraction and velocity sedimentation of RNA. RNA was extracted with proteinase K, phenol, and chloroform-isoamyl alcohol as previously described (7). The extracted RNA was layered onto linear 12-ml 15 to 30% sucrose gradients and centrifuged at 22°C and 39,000 rpm in a Spinco SW41 rotor for 4 h, as described previously (44). Gradients were collected from the bottom through a Beckman model 25 spectrophotometer, which measured absorbance at 260 nm, and then fractionated into 0.3-ml samples with a Gilson Microfractionator. Fractions were TCA precipitated with yeast RNA as a carrier, and radioactivity was determined with a scintillation counter.

TCA-precipitable and soluble radioactivity. After each labeling period, the cells were pelleted from the labeling medium and suspended in 1 ml of solubilizing buffer (0.1 N NaCl, 0.01 M Tris-hydrochloride, pH 8.5, 0.002 M EDTA, 1% sodium dodecyl sulfate, 1% mercaptoethanol). After an equal volume of 25% TCA was added at 4°C, samples were blended in a Vortex mixer and then kept at 4°C for at least 10 min before they were filtered through membrane filters (type HA; pore size, 0.45 μm; Millipore Corp.). The filters were rinsed with cold 5% TCA and ethanol, dried under a heat lamp, and counted in a toluene-Omnifluor (New England Nuclear Corp.) cocktail in a scintillation counter. For determinations of TCA-soluble radioactivity, 20 μl of the sample liquid passing through the filter was counted in Aquasol (New England Nuclear Corp.).

RESULTS

RNA synthesis at 37.5 and 41.8°C. Figure 1 shows the rates of actinomycin D-resistant [^{3}H]uridine incorporation into uninfected and AV-WT-infected cells. Acid-precipitable incorporation into uninfected cells was slower at 41.8°C (Fig. 1B) than at 37.5°C (Fig. 1A), as

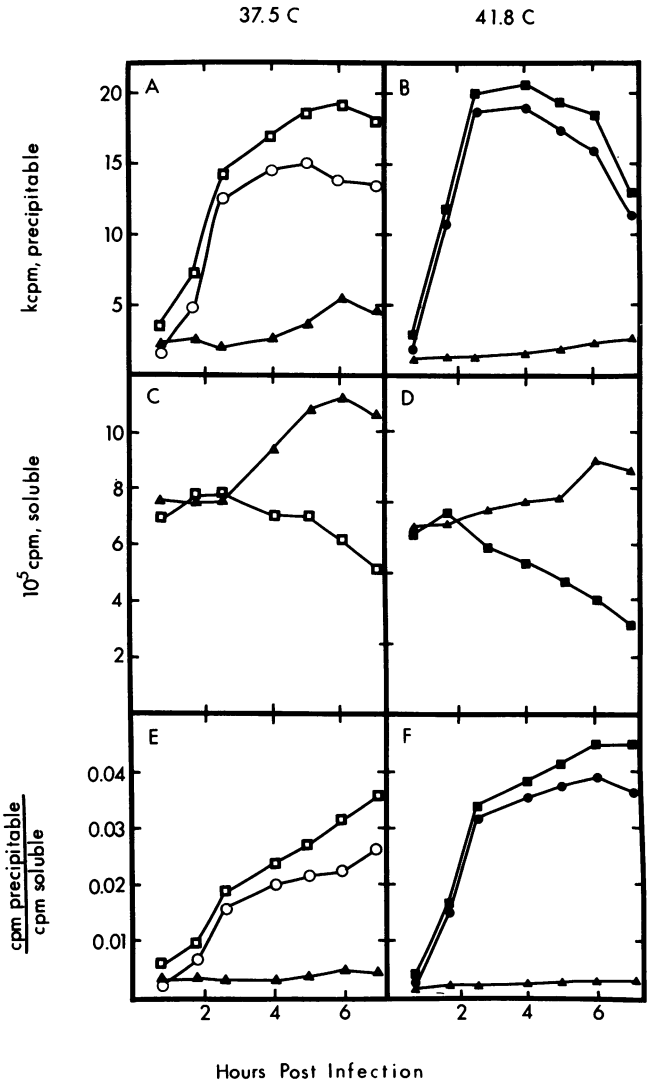

FIG. 1. Acid-precipitable and -soluble [^{3}H]uridine incorporation into actinomycin D-treated AV-WT-infected and uninfected cells. Cells were infected with AV-WT (MOI, 10) or mock infected at 41.8°C. After 30 min, the cultures were diluted and maintained under spinning conditions at 37.5°C (A, C, and E) or 41.8°C (B, D, and F). Cells were labeled in 1-h pulses. Uninfected cell background radioactivity (△ and ▲) was subtracted from AV-WT-infected cell radioactivity (□ and ■), resulting in virus-specific incorporation (○ and ●). (A and B) Acid-precipitable radioactivity. (C and D) Acid-soluble radioactivity. (E and F) Ratio of precipitable radioactivity to soluble radioactivity.

might be expected since 41.8°C is supraoptimal for mammalian cells. In contrast, the rate of total incorporation into infected cells was greater at all times at the higher temperature. Greater incorporation at the higher temperature was not due to a general increase in [^{3}H]uridine uptake since less [^{3}H]uridine was actually taken up by cells at 41.8 than 37.5°C (Fig. 1C and D). Greater incorporation of [^{3}H]uridine at 42.5 than 34°C has also been reported in cells infected with the Beaudette C strain of NDV (36). Therefore, virus-specific incorporation, which was calculated as the difference between total actinomycin D incorporation in infected and uninfected cells, was greater at 41.8°C, as expected for an avian virus. (Similar results for AV-WT were found previously in chicken embryo cells, although in that case incorporation into the uninfected cells was also greater at the higher temperature, consistent with the higher in vivo temperature of avian cells [42].) Little virus-specific incorporation occurred before 2 h at either 37.5 or

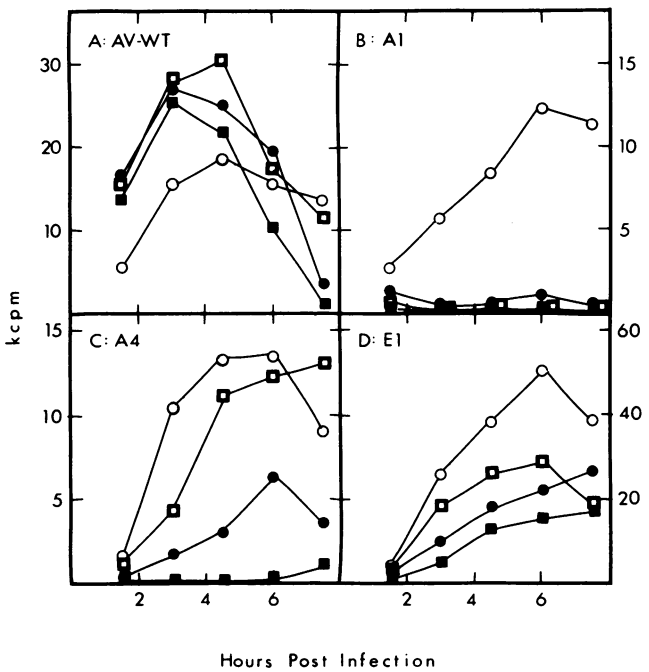

FIG. 2. Temperature dependence of [^{3}H]uridine incorporation in mutant infections. Cells infected for 30 min at 41.8°C with AV-WT (A), mutant A1 (B), mutant A4 (C), or mutant E1 (D) at a MOI of 10 were diluted and divided into four equal portions, which were incubated at four different temperatures. At the times indicated, duplicate samples from each spinner tube were divided into portions and pulsed with actinomycin D and [^{3}H]uridine for 1 h at 37.5°C (○), 40.8°C (□), 41.2°C (●), and 41.8°C (■). An uninfected cell background value of 1,000 to 2,000 cpm was determined at each temperature and subtracted at each point.

41.8°C. Subsequently, the rate increased more rapidly, reached a higher plateau, and decreased more rapidly at the higher temperature.

Temperature dependence of [^{3}H]uridine incorporation in mutant infections. The temperatures at which RNA synthesis could be studied effectively for each *ts* mutant were determined by measuring the rates of virus-specific [^{3}H]uridine incorporation at temperatures between 37.5 and 41.8°C (Fig. 2). The rate of virus-specific incorporation in AV-WT-infected cultures (Fig. 2A) was again lowest at 37.5°C and appeared to be close to optimal at 40.8°C, with slightly lower rates at 41.2 and 41.8°C and more rapid decreases at the higher temperatures. In contrast, the rate of incorporation into mutant-infected cells was maximal at 37.5°C; this rate was similar to the rate observed in AV-WT-infected cultures at that temperature. Mutant A1 (Fig. 2B) demonstrated the greatest temperature sensitivity, with virtually no incorporation at any temperature above 37.5°C. Mutants A4 (Fig. 2C) and E1 (Fig. 2D) showed gradual losses of activity at temperatures above 37.5°C; mutant A4 activity was completely shut off only at 41.8°C, but mutant E1 activity was never completely shut off, even at 41.8°C. Results similar to those obtained with mutants A4 and E1 were obtained with mutants A3 and A5 (data not shown). In the experiments described below, 37.5°C was used as the permissive temperature, and either 41.2 or 41.8°C was used as the nonpermissive temperature.

At late times after infection, the rate of RNA synthesis tended to level off or decrease, especially for AV-WT. This could be attributed to decreasing levels of [^{3}H]uridine incorporation during infection, since correcting the precipitable radioactivity (Fig. 1A and B) for soluble radioactivity (Fig. 1C and D) resulted in apparently steadily increasing rates of RNA synthesis beginning 2 to 3 h postinfection (Fig. 1E and F). (Although correcting for this artifact of [^{3}H]uridine uptake might have been done for each experiment, this procedure was not adopted because it was too cumbersome and the overall conclusions would not have been affected.)

Primary transcription. The first virus-specific RNA-synthetic event in a paramyxovirus infection is the synthesis of mRNA from the input genome RNA by the accompanying transcriptase. This process of primary transcription can

TABLE 1. Primary transcription[a]

Virus	Transcription ratios		
	Mutant (37.5°C)/ AV-WT (37.5°C)	Mutant (41.2/37.5°C)/ AV-WT (41.2/37.5°C)	Mutant (41.8/37.5°C)/ AV-WT (41.8/37.5°C)
AV-WT	1.0	1.0	1.0
A1	0.89 ± 0.09	0.30 ± 0.025	−0.067
A3	0.59 ± 0.14	0.36 ± 0.041	0.08
A4	0.38 ± 0.13	0.36 ± 0.026	0.32
A5	0.50 ± 0.14	0.32 ± 0.070	0.10
E1	0.46 ± 0.14	0.89 ± 0.085	0.55 ± 0.05

[a] In each primary transcription experiment, cultures were labeled with [^{3}H]uridine beginning 30 min after infection. The cumulative virus-specific acid-precipitable [^{3}H]uridine incorporation was determined at 1, 2, and 3 h. Only the 2-h results are presented, although the 1- and 3-h results were similar. Results from mutants tested between two and six times are presented as means ± standard deviations. The group A mutants which were obviously *ts* for primary transcription at 41.2°C were tested only once at 41.8°C.

be isolated and quantified by infecting cells at a high MOI and labeling with [^{3}H]uridine in the presence of both actinomycin D and cycloheximide (6, 12, 18, 23, 29, 35).

The primary transcriptive activity of AV-WT was about 50% greater at 41.2 than 37.5°C and 90% greater at 41.8 than 37.5°C (data not shown), similar to the total RNA synthesis measured late in infection (Fig. 1). Compared with AV-WT, the group A and E mutants were all less active (38 to 89%) at 37.5°C (Table 1). At 41.2°C the group A mutants were clearly *ts* for primary transcription. At 41.8°C all of the mutants, including mutant E1, showed a twofold or greater temperature sensitivity. In fact, mutant A1 was completely inactive.

Postprimary transcriptive processes. If primary transcription were the only defect in each of these mutants, allowing only primary transcription to occur at the permissive temperature (by including cycloheximide in the medium) and then shifting to the nonpermissive temperature (and removing the cycloheximide) should have permitted subsequent virus-specific RNA synthesis at that temperature. Figure 3 shows the results of such an experiment. The virus-specific RNA-synthetic activities in cells infected by mutants A1, A4, and E1 (as well as mutants A3 and A5 [data not shown]) were no better after the cultures were shifted to 41.8°C after initial incubation at 37.5°C in the presence of cycloheximide than they were in similarly treated cells continuously maintained at 41.8°C. Thus, each mutant appeared to be defective in at least one process after primary transcription.

To study the block(s) in postprimary transcriptive RNA synthesis, cultures were incubated at the permissive temperature for different periods of time and then tested for virus-specific [^{3}H]uridine incorporation after being shifted to 41.2°C (Fig. 4). Mutants incubated throughout infection at 41.2°C (and at 41.8°C [data not shown]) were clearly *ts*, as observed previously. For mutant-infected cultures shifted from 37.5 to 41.2°C as early as 2 to 3 h postinfection, no subsequent virus-specific [^{3}H]uridine incorporation could be detected. When the cultures were shifted between 3 and 6 h postinfection, virus-specific [^{3}H]uridine incorporation could be detected in all cases. For cells infected by mutants A4 and E1 (as well as mutants A3 and A5

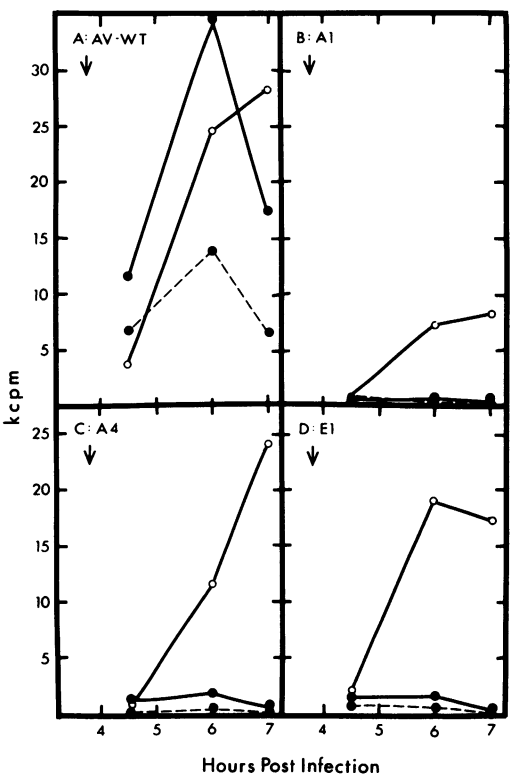

FIG. 3. Transcription of AV-WT and mutants A1, A4, and E1 at 41.8°C after preincubation at 37.5°C in the presence of cycloheximide. Cells were plated onto 35-mm plates in growth medium containing 1 mM $CaCl_2$. Cycloheximide (50 μg/ml) was present on all plates from 0.75 h before infection to 3.5 h postinfection, after which it was washed off (arrows). One set of plates was infected with each virus and maintained at 37.5°C (○), a second set was infected and maintained at 41.8°C (●—●), and a third set was infected and maintained at 37.5°C for 3.5 h and then shifted to 41.8°C (●- - -●). All cells were incubated in a 5% CO_2 atmosphere and labeled with [^{3}H]uridine in 1-h pulses in the presence of actinomycin D. The background value for uninfected cells (1,700 to 3,400 cpm) was subtracted.

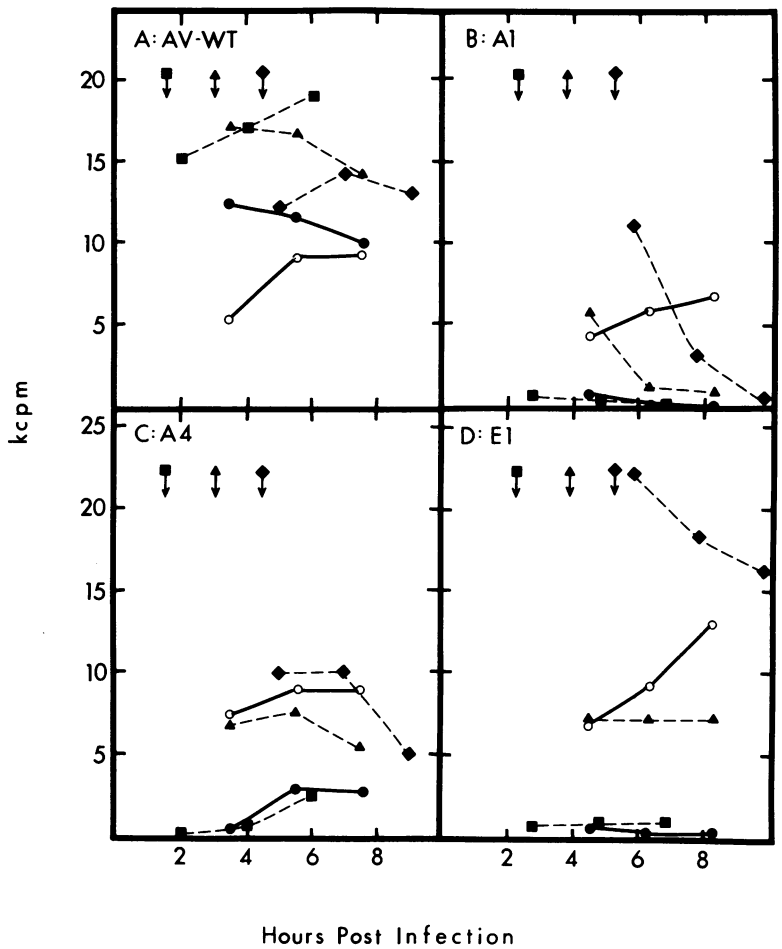

FIG. 4. Transcription of AV-WT and mutants A1, A4, and E1 after temperature shift-ups at different times postinfection. Cells were infected at 41.2°C (MOI, 10) and then either maintained (solid lines) at 37.5°C (○) or 41.2°C (●) or shifted (dashed lines) from 37.5 to 41.2°C at different times (arrows). Each point represents the average of duplicate 1-h pulses of [^{3}H]uridine in the presence of actinomycin D. The background value for uninfected CHO cells (1,000 to 2,000 cpm) was subtracted.

[data not shown]), the rate of virus-specific incorporation remained relatively constant for several hours after the shift, suggesting that stable but not amplifying RNA-synthesizing complexes were present. Similar results were obtained after the cultures were shifted up to 41.8°C (data not shown). In contrast, mutant A1-infected cultures rapidly lost the ability to synthesize virus-specific RNA. For this mutant, the activity present at the time of the shift was unstable.

For the mutants that were able to continue synthesizing virus-specific RNA after the shift to 41.2°C, it was of interest to determine whether such synthesis was limited to a particular size class or was distributed among the various NDV RNA size classes. The RNAs synthesized at 37.5°C or after a shift from 37.5 to 41.2°C were compared for AV-WT and mutants A4 and E1. Figure 5 shows the sedimentation patterns of the RNAs produced by AV-WT and mutants A4 and E1; the RNAs produced after a shift to 41.2°C were similar to those produced at 37.5°C. Most of the RNAs detected under these conditions are 18S to 35S RNAs, representing mRNA transcripts (7, 10, 27). Obviously then, mRNA transcription continued under these conditions. Because of the usual difficulties in visualizing 50S RNA, no conclusions about replication could be made.

To determine whether the loss of RNA-synthesizing ability of mutant A1 shifted to 41.2°C was reversible, another experiment was performed (Fig. 6). Cultures were incubated at 37.5°C for 4 h, then shifted to 41.2°C, and subsequently maintained at that temperature or

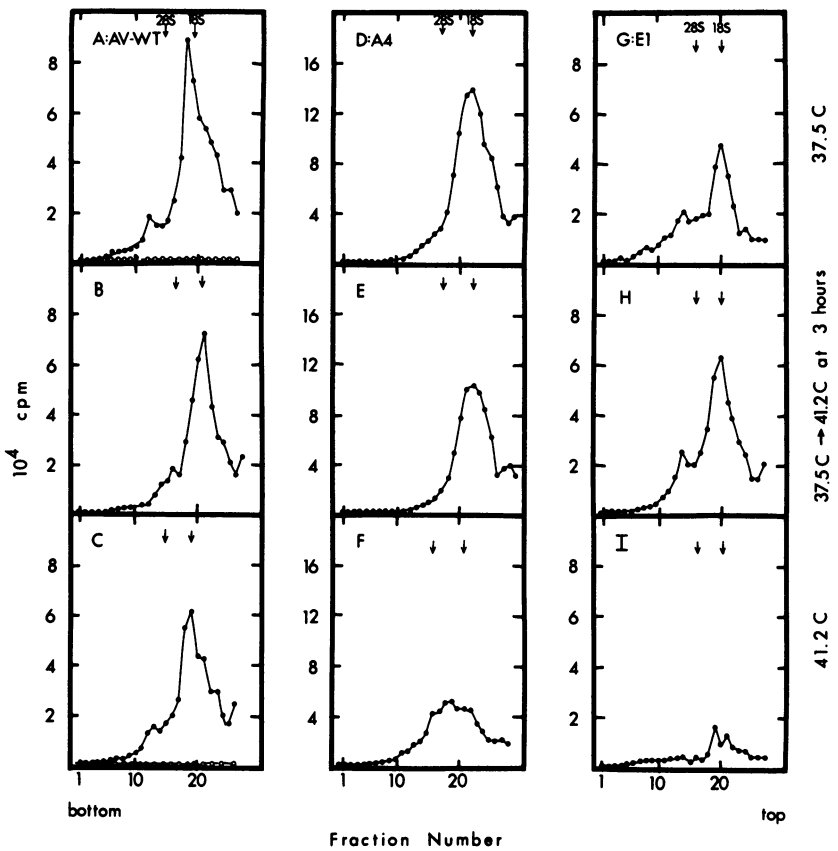

FIG. 5. Sucrose gradients of AV-WT, mutant A4, and mutant E1 virus-specific RNAs produced in infected cells at 37.5 and 41.2°C and after a shift from 37.5 to 41.2°C at 3 h. Cells were incubated in the presence of 10 μg of actinomycin D per ml for 0.5 h before they were labeled with 100 μCi of [^{3}H]uridine per ml for 3 h in the presence of actinomycin D (10 μg/ml). Cells were labeled from 4 to 7 h after infection. At the end of the labeling period, the RNA was phenol extracted and sedimented on a sucrose gradient. The TCA-precipitable radioactivity is plotted. Symbols: ●, virus-specific radioactivity; ○, CHO cell background radioactivity.

shifted back to 37.5°C at 6.5 h. Other cultures were maintained at 37.5 and 41.2°C throughout infection. The cultures that remained at 6.5 h received cycloheximide to prevent subsequent synthesis of new enzyme. (Under these conditions, RNA synthesis is limited to mRNA transcription because replication at all times requires protein synthesis.) The cells were labeled for 1 h at different times, beginning 4.5 h postinfection. Again, no [^{3}H]uridine incorporation was detected with continuous incubation at 41.2°C or after a shift-down from 41.2 to 37.5°C at 6.5 h. However, cells shifted up to 41.2°C at 4 h showed a decrease in RNA synthesis, as observed in the experiment shown in Fig. 4. Of interest was the fact that cells which had lost the ability to synthesize RNA after the shift-up to 41.2°C regained this ability when they were shifted back down to 37.5°C, even in the absence of additional protein synthesis. Thus, the thermolability of the ability of mutant A1 to synthesize RNA at 41.2°C after a shift-up represented a reversible loss of activity.

Revertants of the RNA$^-$ mutants. Having established that all group A and E mutants are *ts* for RNA synthesis, we wanted to determine whether this phenotype was causally related to the *ts* plaque-forming and virus-producing ability for which the mutants were selected originally (42). In addition, we wanted to determine whether the effects on primary transcription and on a postprimary transcriptive event were caused by a single lesion. Our approach was to isolate spontaneously occurring *ts*$^+$ plaque-forming revertants of each of the mutants. Coreversion of the unselected temperature sensitivity of RNA synthesis with plaque formation would support the notion that the former is indeed responsible for the latter. In addition, since RNA synthesis measured as [^{3}H]uridine incorporation between 3.5 and 7.5 h postinfection is dependent on primary transcription having already occurred,

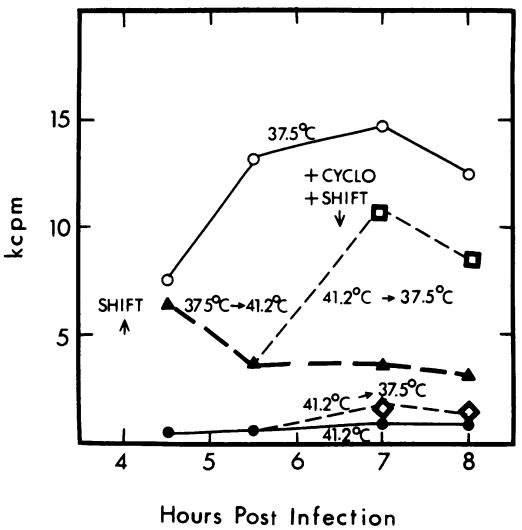

FIG. 6. Transcription of mutant A1 after a temperature shift up and back down. Cells were infected at 41.2°C (MOI, 10) for 0.5 h, diluted, and incubated at 37.5 or 41.2°C. At 6.5 h 50 μg of cycloheximide (CYCLO) per ml was added to each remaining culture. Each point represents a 1-h pulse of [^{3}H]uridine in the presence of actinomycin D. The experimental conditions were as follows: 37.5°C (○); 41.2°C (●); 37.5°C, shifted to 41.2°C at 4 h (▲); 37.5°C, shifted to 41.2°C at 4 h and back to 37.5°C at 6.5 h (□); and 41.2°C, shifted to 37.5°C at 6.5 h (◇). An uninfected cell background value (300 to 1,000 cpm) was subtracted from each point.

normal levels of incorporation for revertants would indicate that primary transcription had also reverted. A revertant for each mutant is shown in Table 2. In each case, reversion to plaque-forming ability at 41.8°C was accompanied by reversion of RNA-synthesizing ability at 41.8°C; thus, the *ts*RNA$^-$ lesion caused the *ts* plaquing phenotypes, and all of the RNA synthesis defects were the result of single lesions.

DISCUSSION

We examined the RNA-synthesizing activities of AV-WT and group A and E *ts* mutants derived from AV-WT. We found that the rate of AV-WT RNA synthesis was greater at temperatures above the permissive temperature (37.5°C). In contrast to AV-WT, the rates of virus-specific RNA synthesis in group A and E mutants were reduced at temperatures above 37.5°C. Revertants isolated for *ts*$^+$ plaque-forming phenotypes had also reverted for the RNA-synthesizing phenotype. Both of these findings confirmed the original assignment of RNA synthesis as the primary defect of the group A and E mutants.

At nonpermissive temperatures each of the *ts* mutants was defective in primary transcription compared with AV-WT, although the group A mutant defects were more pronounced than the defects of mutant E1. It was possible that at the nonpermissive temperature the defect in mutant E1 was that it produced genome-sized RNA instead of 18S mRNA. However, like AV-WT, mutant E1 continued to produce mainly 18S RNA under these conditions (Rasenas, unpublished data).

The *ts* defects of these mutants are also manifest at a level beyond primary transcription; for each mutant, allowing primary transcription to proceed (in the presence of cycloheximide) at the permissive temperature was not sufficient to permit subsequent RNA synthesis at the nonpermissive temperature. In addition, in experiments conducted without cycloheximide, shifts to nonpermissive temperatures earlier than 4 h postinfection did not allow amplified synthesis at the nonpermissive temperature. However, shifts-up later than 4 h did allow amplified RNA synthesis at the nonpermissive temperature. Although the RNA-synthetic abilities of mutants A4 and E1 did not increase, they were relatively stable at the nonpermissive temperature. This stability without an increase in activity suggests that although mutants A4 and E1 are not able to continue amplifying their RNA-synthetic abilities after a shift-up, they are not defective in late RNA synthesis. This pattern of a relatively early defect but not a late defect is similar to the

TABLE 2. Plaque formation and RNA synthesis by *ts*RNA$^-$ mutants and their revertants at permissive and nonpermissive temperatures

Virus[a]	Efficiency of plaque formation[b]	RNA synthesis (41.2/37.5°C)[c]
AV-WT	0.50	1.64 ± 0.20
A1	2.2×10^{-4}	0.08 ± 0.09
A1R1	0.76	1.36 ± 0.02
A3	1.5×10^{-5}	0.08 ± 0.06
A3R1	0.67	1.38 ± 0.23
A4	2.4×10^{-5}	0.24 ± 0.12
A4R1	0.97	1.50 ± 0.14
A5	2.0×10^{-5}	0.37 ± 0.09
A5R1	1.4	4.74 ± 1.38
E1	2.8×10^{-6}	0.04 ± 0.03
E1R1	0.98	1.16 ± 0.32

[a] -R1, Revertant.

[b] Number of plaques at 41.8°C/number of plaques at 37.5°C.

[c] Infected cells maintained at 37.5 or 41.2°C were pulsed with [^{3}H]uridine for 1 h beginning 3.5, 5.0, and 6.5 h postinfection. The TCA-precipitable radioactivity values from the three time points were averaged, and the ratio of radioactivity at 41.2°C to radioactivity at 37.5°C (mean ± standard deviation) was determined.

pattern observed with some *ts*RNA$^-$ mutants of Sendai virus (30) and VSV (34).

Possible postprimary transcriptional defects include processing of primary transcripts, protein function and stability, and replication. Processing of mRNA might be defective, resulting in replication instead of transcription. However, the RNAs produced by mutants A4 and E1 after a shift to the nonpermissive temperature are similar in size to those produced at the permissive temperature. VSV *ts*RNA$^-$ mutants also produced similar RNA species after a shift-up (22), with the exception of one mutant which replicated instead of transcribing mRNA after a shift-up (28). Defects in other RNA-processing functions, such as capping, methylation, or polyadenylation, might affect mRNA functioning. On the other hand, if the mRNA were functional, the defect might lie in a protein that it encodes which is labile or unable to associate with the transcribing cores. Finally, replication might be the defective process. This is difficult to determine since AV-WT normally produces so little 50S RNA, but in the future this possibility might be examined by hybridization methods.

Whereas the RNA-synthesizing capabilities of mutants A4 and E1 are stable after a shift to the nonpermissive temperature, the RNA-synthesizing capability of mutant A1 is thermolabile. Studies of *ts*RNA$^-$ mutants of Sendai virus (30) and NDV (32) have also found some mutants that were capable of continued RNA synthesis after a shift and others that were not. The thermolability of mutant A1 RNA synthesis suggests that the A gene product is involved in RNA synthesis from progeny genomes.

The loss of the mutant A1 transcriptase after a shift to the nonpermissive temperature was reversible upon a second shift back to permissive temperature. A similar conclusion was drawn about the thermal sensitivity of the mutant A1 virion transcriptase from shift-down experiments (Rasenas, unpublished data). In these experiments, cells infected with mutants A1, A3, A4, A5, and E1 were examined for the ability to make virus-specific RNA at 37.5°C after infection and maintenance at 41.2°C for as long as 4 h. None of the mutants produced much virus-specific RNA while they were maintained at 41.2°C, but all gained that synthetic ability when they were subsequently incubated at 37.5°C. Reversible defects have also been found for *ts*RNA$^-$ mutants of Sindbis virus (19) and VSV (39).

The following three proteins are associated with viral nucleocapsids: the nucleocapsid protein NP, the large protein L, and phosphoprotein P (11, 26, 37). These three proteins are probably involved in RNA synthesis (11, 23, 24, 27a, 36). The VSV nucleocapsid is also composed of three proteins (N, L, and a phosphoprotein, NS); all three of these proteins are required for in vitro RNA synthesis (13).

The A gene product seems to be involved in several phases of virus-specific RNA production, including primary transcription (all of the group A mutants are affected), RNA synthesis from progeny genomes (mutant A1 is affected), and, possibly, replication. Although no direct evidence links the A gene with protein L, this seems likely for a number of reasons. First, the L gene is the largest NDV gene (27) and according to target theory, might be expected to be represented by a large group of mutants. Group A is the largest *ts* group isolated from AV-WT (42). Similarily, *ts*RNA$^-$ group I is the largest group of the Indiana strain of VSV (33), and the defect in this group lies in the L gene (17, 38). Second, the noncytopathic mutants of AV-WT are uniformly deficient in both virus-specific RNA synthesis and accumulation of protein L in infected cells (23, 24), suggesting that protein L is involved in RNA synthesis. Furthermore, all of these noncytopathic mutants complement mutant E1 but not group A mutants for RNA synthesis (23). Finally, the results of recent studies of UV inactivation of the ability of mutant E1 to complement mutant A1 are consistent with the placement of the mutant A1 defect farthest from the promoter (27a), as the L gene is (9).

The E gene product may also be involved in primary transcription, although this defect is not as pronounced as the defect of the group A mutants. In addition, the E gene product is involved in a subsequent RNA-synthetic step(s), as previously discussed. The E gene product has not been linked to RNA synthesis from progeny genomes since it is able to synthesize RNA after a shift-up. Group E probably represents a lesion in protein P since mutant A1 complementation of mutant E1 is inactivated by UV irradiation at a rate (27a) similar to that of the P gene (9).

One major question in negative-strand virus RNA synthesis is the relationship between mRNA transcription and genome replication. One model suggests that when protein N of VSV is present in high enough concentrations, it may bind to nascent RNA, promoting readthrough of mRNA termination signals and resulting in replication (3, 20, 21). Another model suggests that protein NS in one of its phosphorylated forms may regulate the transcription to replication switch (8, 40). Protein P of NDV also exists in multiple forms, which differ in their phosphorylation states (37). How these multiple forms of protein P might be involved in transcription and replication is not known, but the *ts* defect in mutant E1 may prove to be a useful tool in examining this question for NDV.

In conclusion, it appears that the group A and E mutations and, therefore, the proteins which they represent (proteins L and P) are integrally involved in RNA synthesis. This involvement is in at least two separable steps, primary transcription and a postprimary transcriptive step(s). Both defects are before or during the assembly of progeny nucleocapsids in mutants A4 and E1. The temperature sensitivity of the transcribing nucleocapsids of mutant A1 indicates that the A gene product is also required for RNA synthesis from progeny genomes.

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