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Fluorinated Pyrimidines

XLII. Effect of 5-Trifluoromethyl-1-2′-deoxyuridine on Transcription of Vaccinia Viral Messenger Ribonucleic Acid

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SUMMARY

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The effect of 5-trifluoromethyl-2′-dexoyuridine (F_3dThd) on transcription in vaccinia virus-infected HeLa cells was studied. The sedimentation profile of the RNA in the cytoplasmic fraction extracted from HeLa cells infected with vaccinia virus in the presence of the analogue shows that no detectable normal late viral mRNA was produced; only 4 S RNA was detected. Hybridization competition experiments, using purified total viral RNA preparations and the isolated 12 S early and 16–28 S late viral mRNA peaks, show that the viral RNA transcribed late in the presence of the analogue lacks 30% of the sequences found in normal late viral RNA. The 4 S RNA transcribed late in the presence of F_3dThd contains both small mRNA sequences and 4 S RNA sequences that are presumably due to tRNA. The presence of F_3dThd has no effect on the size or sequences of viral RNA transcribed from the parental viral genomes.

INTRODUCTION

5-Trifluoromethyl-2′-deoxyuridine was first synthesized in this laboratory (1) and has shown biological activity in a number of systems. The 5′-monophosphate of F_3dThd[4] is a potent inhibitor of thymidylate synthetase (2). F_3dThd has shown tumor-inhibitory activity against Adenocarcinoma 755 and L1210 leukemia in mice (3), and is undergoing clinical evaluation in adult (4) and pediatric (5) cancer patients. On a molar basis, F_3dThd is the most active chemotherapeutic agent known against herpes simplex keratitis in the rabbit's eye (6, 7), and a clinical double-blind comparison of F_3dThd and 5-iodo-2′-deoxyuridine has shown that F_3dThd is also the more effective drug in the treatment of patients with herpes simplex keratitis (8). It was shown in our laboratory that F_3dThd inhibits vaccinia viral replication in HeLa cells at the lowest concentration of any of the pyrimidine

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[4] The abbreviation used is: F_3dThd, 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine, F_3TdR).

nucleoside analogues tested (9), and that F_3dThd is incorporated into the DNA of L-5178Y and HeLa cells in culture (10). It was further shown that addition of labeled 1 μM F_3dThd to HeLa cells infected with vaccinia virus resulted in a 2–8% replacement of thymine by trifluorothymine in the progeny viral DNA (11). Moreover, this viral DNA containing trifluorothymine is smaller than normal vaccinia viral DNA (11). Although the incorporation of F_3dThd into vaccinia viral DNA is much less than the incorporation of 5-bromo- and 5-iodo-2′-deoxyuridine into vaccinia viral DNA (12–14), this 2–8% replacement of thymine by the trifluoro derivative is sufficient to render the progeny virions noninfective (11). It has recently been shown in our laboratory that the sedimentation properties of rapidly labeled RNA species transcribed 4.5–5 hr after infection of HeLa cells by vaccinia virus in the presence of F_3dThd are considerably different from those observed when the analogue is not present (15).

This paper reports on studies of DNA-RNA hybridization carried out on the fractions obtained from the sedimentation analyses of cytoplasmic extracts of HeLa cells infected with vaccinia virus under various conditions, and also on purified vaccinia viral RNA preparations transcribed in the presence and absence of F_3dThd. The timing and mode of transcription of vaccinia viral mRNA, the sedimentation properties of these messengers, and the hybridization procedures as applied to the analysis of the vaccinia transcription process are primarily those developed by Joklik and Becker (16) (16) and by Oda and Joklik (17). The replicative cycle of vaccinia virus, as reviewed by Woodson (18), is shown in Fig. 1.

MATERIALS AND METHODS

Cell culture and virus preparation. HeLa S3 cells were maintained in continuous logarithmic growth in Eagle's minimal essential medium for suspension (Gibco) supplemented with 10% calf serum (Gibco), 0.1% Pluronic F68, and antibiotics (1% of a combination of 5000 units of penicillin and 5 mg of streptomycin per milliliter). Stock suspensions of vaccinia virus, strain WR, were prepared and then purified by sedimentation in 35% sucrose solutions followed by sedimentation in linear 25–45% sucrose gradients as described previously (9, 11). Virus titer is expressed as plaque-forming units per milliliter; the plaque assay was performed as described previously (9).

Infection. HeLa cells at a density of

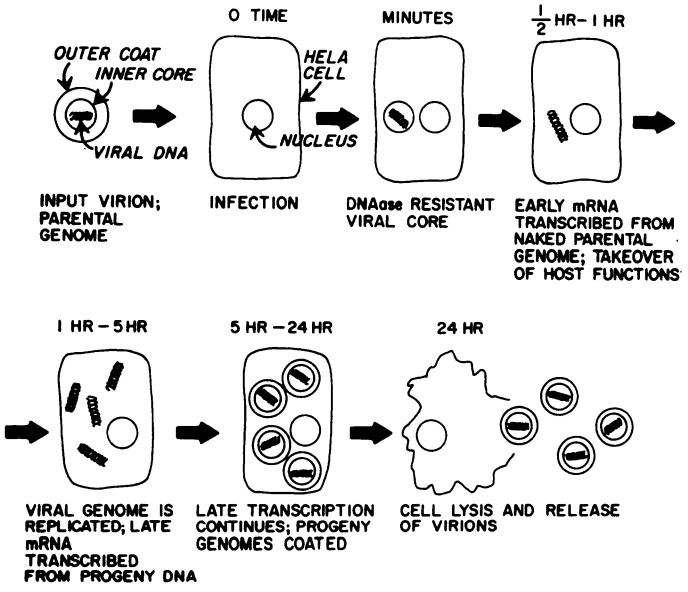

FIG. 1. *Replicative cycle of vaccinia virus*

3×10^5 cells/ml were concentrated to 5×10^6 cells/ml in Eagle's suspension medium supplemented with 1% calf serum, 0.1% Pluronic F68, antibiotics, and 10 mM Mg^{++} (adsorption medium). For purified viral RNA preparations, a multiplicity of infection of 20 plaque-forming units/cell was used; for sedimentation analysis of viral RNA, a value of 50 plaque-forming units/cell was employed. Thirty minutes after infection with the proper virus inoculum, the cells were diluted to 5×10^5 cells/ml in Eagle's suspension medium supplemented with 5% calf serum, 0.1% Pluronic F68, and antibiotics (virus growth medium). When analogue addition was required, the virus growth medium was made 1 μM in F_3dThd. Incubations at 37° were carried out for a period of 1 hr for early and 5 hr for late viral mRNA preparations.

[^{3}H]Uridine pulse-labeling. [5-^{3}H]Uridine (Schwarz/Mann; 4.0 Ci/mmole) was added to infected cells for 30 min at 0.5 or 4.5 hr after infection. For purified total viral RNA preparations, 16 μCi/10^7 cells were added; for viral RNA sedimentation studies, 40 μCi/10^7 cells were used. Incorporation was stopped by immersion of flasks into an ice–water–methanol bath followed by centrifugation at $800 \times g$.

Preparation of cytoplasmic fractions. The harvested cells were washed with phosphate-buffered 0.15 M NaCl and then treated with hypotonic solution [10 mM Tris (pH 8.0), 10 mM KCl, and 5 mM $MgCl_2$] at a density of 2×10^7 cells/ml. Thirty minutes after chilling at 4°, the cells were disrupted with a Dounce homogenizer, the homogenate was centrifuged at $800 \times g$, and the supernatant cytoplasmic fraction was removed.

Centrifugal analysis of rapidly labeled cytoplasmic RNA. The methods used were those previously described (15). Aliquots of cytoplasmic fractions were layered on 15–30% sucrose density gradients and centrifuged at 23,000 rpm for 18 hr at 20° in an SW 25.1 rotor in a Beckman model L ultracentrifuge.

Preparation of purified total viral mRNA. The method used was essentially that of Oda and Joklik (17). The RNA and protein were precipitated from the cytoplasmic fractions with ethanol, and the precipitates were dissolved in 0.05 M sodium acetate buffer (pH 5.0). Sodium dodecyl sulfate and sodium perchlorate were then added to the solutions, and deproteinization was achieved by four extractions with chloroform–isoamyl alcohol. This method produced purified total RNA preparations with an $A_{260}:A_{280}$ ratio of 2.10 or higher. Vaccinia viral DNA replication commences 1.5–2 hr after infection and is complete by 5 hr after infection (18). Therefore incubations at 37° were carried out for a period of 1 hr for early and 5 hr for late viral mRNA preparations. Viral RNA labeled by a pulse of [^{3}H]uridine 0.5–1 hr after infection (early viral RNA) is transcribed from the input viral genomes. Viral RNA labeled by a pulse of [^{3}H]uridine 4.5–5 hr after infection (late viral RNA) is transcribed from progeny viral genomes. Figure 1 illustrates these operational definitions of early and late transcription.

Molecular DNA-RNA hybridization. The methods used were those reported by Oda and Joklik (17), based on the techniques of Gillespie and Spiegelman (19) as reviewed and expanded by Green *et al.* (20). Vaccinia viral DNA was prepared as described by Oda and Joklik (17). Preparation of 25-mm nitrocellulose filters (Schleicher and Schull, Inc., Bac-T-Flex B-6) and immobilization of denatured DNA on the filters were done according to the method of Green *et al.* (20). Noncompetition and competition studies were carried out as reported by Oda and Joklik (17), except that 6 times the concentration of 0.15 M sodium chloride–0.015 M trisodium citrate (pH 7.0), which was 0.1% in sodium dodecyl sulfate, was used in the incubation mixture (final volume, 5 ml). The filters were processed exactly as described by Green *et al.* (20), and then counted in toluene–2,5-diphenyloxazole to determine the counts per minute of ^{3}H bound to each filter. In the analysis of fractions obtained by sedimentation of cytoplasmic extracts, aliquots of the fractions were pipetted directly into scintillation vials containing filters with immobilized denatured DNA and enough of the 6-fold concentration of 0.15 M sodium chloride–0.015 M trisodium citrate solution was added to give a final volume of 5 ml. Hybridization incubations were then carried out for 24 hr at 66° as described by Green *et al.* (20). In

competition studies, a relatively large amount of an unlabeled RNA was incubated for 24 hr with a filter containing 1 μg of denatured vaccinia DNA. Then a small amount of a labeled RNA was added, and the incubation was continued for another 24 hr.

Radioactivity determinations. All vials were counted in the same Packard Tri-Carb model 3360 liquid scintillation spectrometer sufficiently long to obtain the precision shown in the tables.

RESULTS

Figure 2 shows the results of the time course of vaccinia viral DNA-RNA hybridization on nitrocellulose filters. A plateau was reached at 16–18 hr, and all subsequent incubations were carried out for 24 hr.

A number of control experiments necessary for the correct interpretation of the data obtained from subsequent hybridization experiments were performed. The results are shown in Table 1. When 100 μg of purified total labeled late[5] viral RNA were incubated with filters containing 1 μg of denatured DNA from the bacterium *Cytophaga johnsonii*, 22 cpm/filter annealed to the DNA. This radioactivity is approximately the instrument background, but also would include any small amount of nonspecific binding of purified RNA to a nonhomologous DNA. This bacterium was chosen because its GC content is 42%, which is the same as that of mammalian cells. The 22-cpm value was used as the background for all experiments. Another important control was the incubation of 100 μg of purified total labeled late viral RNA with filters containing 1 μg of denatured HeLa cell DNA. In this case about 28 cpm above background annealed per filter, compared to 447 cpm that annealed to viral DNA. The annealing to HeLa cell DNA suggests that during infection the host cell DNA continues to code for tRNA. A similar control performed with purified total labeled early viral RNA gave the results shown in Table 1. With viral DNA on filters, 62 cpm above background annealed; with HeLa cell DNA on filters, only 3 cpm above background were bound. Thus there is insignificant annealing of this RNA to HeLa cell DNA, suggesting that if any tRNA is coded for by the HeLa cell genome early in infection the method was not sensitive enough to detect it. Since the multiplicity of infection was 50, it seems very unlikely that infection was incomplete.

The saturation levels of early and late purified total vaccinia RNA obtained from cells infected with vaccinia virus in the absence of F_3dThd were determined, and the results are shown in Figs. 3 and 4. In all the competition studies in which purified total RNA preparations were employed, 1000 μg of either purified total early or late RNA were used. One thousand micrograms of purified total early or late RNA obtained from HeLa cells infected with vaccinia virus in the presence of F_3dThd also were sufficient to prevent the annealing of labeled purified total early or late RNA transcribed in the presence of F_3dThd to 1 μg of denatured vaccinia DNA (Table 2). The efficiency of the hybridization shown in Fig. 3 is 0.4%. Oda and Joklik (17), in similar experiments, obtained a hybridization efficiency of 1.2%, and Jungwirth *et al.* (21) obtained comparable hybridization efficiencies. Although our efficiencies are somewhat lower than those obtained by these other workers, they are of the same order of magnitude. Since the results we have obtained from hybridization of vaccinia viral RNA transcribed in the absence of F_3dThd with vaccinia DNA agree very well with those obtained by Oda and Joklik (17), we believe that our lower efficiencies of hybridization do not detract from the accuracy or interpretation of our data.

Table 2 shows the results of the hybridization competition studies with purified total RNA preparations. Prior incubation of filters containing vaccinia DNA with 1000 μg of total cytoplasmic HeLa cell RNA did not reduce the extent of hybridization of purified viral RNA. One thousand micrograms of the appropriate unlabeled RNA was incubated for 24 hr per filter containing 1 μg of denatured vaccinia DNA. Then the indicated amount ot a labeled RNA preparation was added to each filter, and the incubation was continued for 24 hr. Any radioactivity re-

[5] Early and late functions are defined in Fig. 1 and MATERIALS AND METHODS.

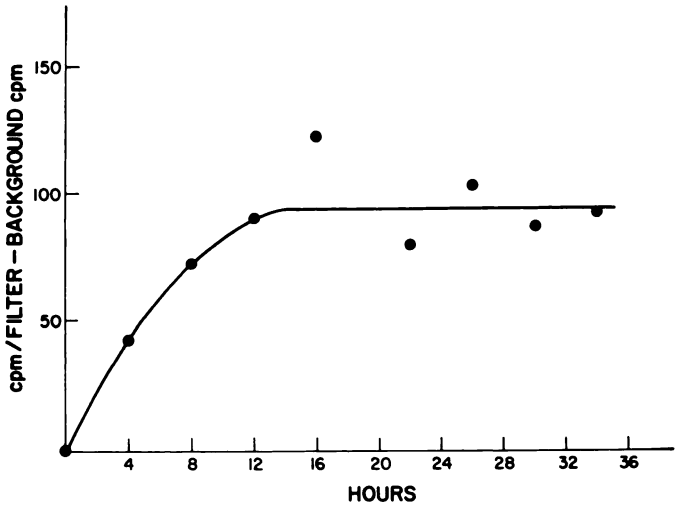

FIG. 2. *Time course of vaccinia DNA-RNA hybridization*

Aliquots containing 150 μg of purified total labeled late vaccinia RNA were incubated for various lengths of time with filters containing 1 μg of denatured vaccinia DNA. The incubation conditions and methods of processing are described in MATERIALS AND METHODS. Infected cells were pulse-labeled with [^{3}H]uridine 4.5–5 hr after infection. The specific activity of the RNA preparation was 150 cpm/μg.

TABLE 1

Summary of hybridization experiments

All samples were prepared as described in MATERIALS AND METHODS. All results are the means ± standard errors of duplicate experiments and have been corrected for background.

RNA and specific activity	Source of RNA from HeLa cells	RNA added	Vaccinia DNA	HeLa DNA	*C. johnsonii* DNA
		μg	*cpm/μg*	*cpm/μg*	*cpm/μg*
Purified total normal late viral RNA, 1100 cpm/μg	Infected; no F_3dThd	100	447 ± 10	28 ± 2	0
16–28 S late viral mRNA, 360 cpm/μg	Infected; no F_3dThd	9	45 ± 3	6 ± 1	2 ± 1
12 S early viral mRNA, 740 cpm/μg	Infected; no F_3dThd	7	26 ± 2	3 ± 1	1 ± 1
Normal 4 S late RNA, 6100 cpm/μg	Infected; no F_3dThd	10	50 ± 3	71 ± 4 (15 μg)	38 ± 2
4 S late RNA transcribed in presence of F_3dThd, 6400 cpm/μg	Infected; in presence of F_3dThd	22	53 ± 3	49 ± 3	60 ± 3
4 S RNA from noninfected cells, 9600 cpm/μg	Noninfected, pulse-labeled 0.5 hr	15	84 ± 4	70 ± 4	65 ± 4
Purified total normal early viral RNA, 370 cpm/μg	Infected, no F_3dThd	100	62 ± 3	3 ± 1	1 ± 1

maining on the filters after processing resulted from sequences of the labeled RNA that differ from sequences in the unlabeled competing RNA. The controls for this study were values representing both labeled and unlabeled RNA of the same type in competition; these controls were always 5% or less. All experiments were done in duplicate, and

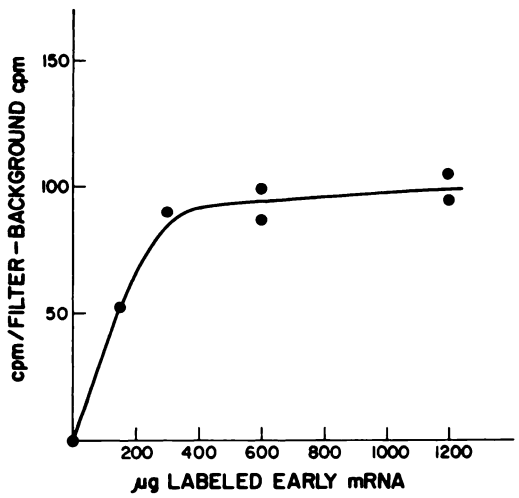

FIG. 3. *Saturation level of purified total early vaccinia RNA*

Increasing amounts of purified total labeled vaccinia early RNA were incubated with filters containing 1 μg of denatured vaccinia DNA. Incubation conditions and methods of processing are described in MATERIALS AND METHODS. Infected cells were pulse-labeled with [^{3}H]uridine 0.5–1 hr after infection. The specific activity of the RNA preparation was 85 cpm/μg.

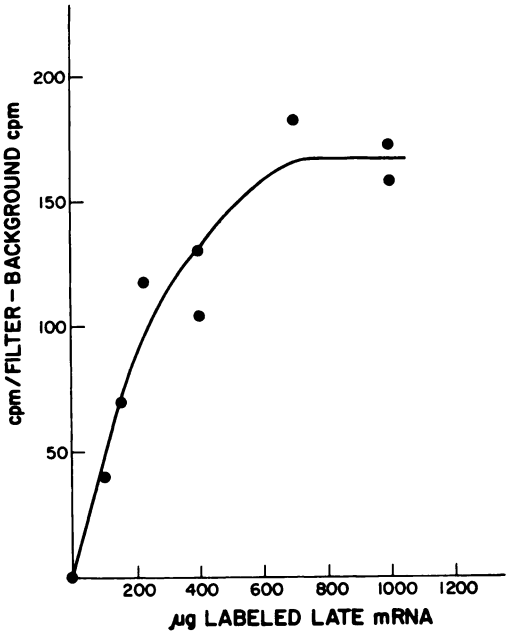

FIG. 4. *Saturation level of purified total late vaccinia RNA*

Increasing amounts of purified total labeled vaccinia late RNA were incubated with filters containing 1 μg of denatured vaccinia DNA. Incubation conditions and methods of processing are described in MATERIALS AND METHODS. Infected cells were pulse-labeled with [^{3}H]uridine 4.5–5 hr after infection. The specific activity of the RNA preparation was 150 cpm/μg.

processed filters containing low radioactivity were counted as long as 50 min to reduce the standard deviation to the level indicated in Table 2. Agreement between duplicate determinations was always good.

Our results with the competitions of the various RNAs transcribed in the absence of F_3dThd are in good agreement with those obtained previously by Oda and Joklik (17) and show that the sequences are identical whether or not early purified total RNA is transcribed in the presence or absence of F_3dThd. Thus, in all cases except one (Table 2), in which the two early RNAs were compared directly or with a third RNA, there is excellent agreement as to the percentage of hybridization. There is a difference only in the case when the two early unlabeled RNA preparations were each used in competition with late labeled normal viral RNA (40% vs. 27%). This difference of 13% is comparable to the variations reported by Oda and Joklik (17), and therefore may not be significant. This result may also suggest that transcription of late RNA is delayed in the presence of the analogue. There is, however, a significant difference in the sequences present in late RNA transcribed in the absence and presence of F_3dThd; 30% of the sequences in purified total normal late viral RNA are missing in purified total late viral RNA transcribed in the presence of the analogue. Our data also show, as did those of Oda and Joklik (17), that all sequences contained in purified early vaccinia RNA are also contained in purified total normal late vaccinia RNA. In addition, the purified late vaccinia RNA contains an average of an additional 33% of sequences not contained in the purified total early viral RNA. This value of 33% is obtained by averaging results from the experiments in which unlabeled early RNA transcribed in the presence or absence of F_3dThd competed with purified

TABLE 2

Results of hybridization competition studies with various purified total viral RNA preparations

In every case RNA was incubated with filters containing 1 μg of denatured vaccinia DNA. E is RNA transcribed early; L is RNA transcribed late (see Fig. 1 for definitions of early and late). N (normal) refers to RNA transcribed in the absence of F_3dThd. F refers to RNA transcribed in the presence of 1 μM F_3dThd. The meaning and method of calculation of the percentage values are explained in the text. Preparation of purified total RNA and hybridization techniques are described in MATERIALS AND METHODS. Percentages of 10% or less are considered to represent no significant annealing of the labeled RNA. All counts per minute are corrected for background. All experiments were done at least in duplicate, and values are means ± standard errors.

Conditions	Radioactivity			
	E_N, 150 μg (90 cpm/μg)	E_F, 100 μg (530 cpm/μg)	L_N, 100 μg (1100 cpm/μg)	L_F, 150 μg (400 cpm/μg)
	cpm/filter			
No added RNA	36 ± 2 (100%)[a]	203 ± 10 (100%)	444 ± 20 (100%)	37 ± 1 (100%)
E_N, 1000 μg	2 ± 1 (5%)	12 ± 2 (6%)	119 ± 6 (27%)	2 ± 1 (5%)
E_F, 1000 μg	1 ± 0.5 (3%)	5 ± 1 (2%)	176 ± 10 (40%)	5 ± 1 (13%)
L_N, 1000 μg	4 ± 1 (10%)	14 ± 2 (7%)	40 ± 2 (1%)	2 ± 1 (5%)
L_F, 1000 μg	8 ± 2 (22%)	41 ± 2 (20%)	134 ± 5 (30%)	2 ± 1 (5%)

[a] Numbers in parentheses are percentages of noncompetition controls.

labeled total normal late viral RNA (40% and 27%, respectively).

Oki and Heidelberger (15) had previously determined the sedimentation profile for the cytoplasmic fraction of noninfected cells pulse-labeled for 30 min and processed and centrifuged as described in MATERIALS AND METHODS. The profile for this control shows a peak of trichloracetic acid-precipitable radioactivity only in the 4 S region. We have repeated this control and confirmed this result.

The sedimentation profiles obtained when 1-ml aliquots of cytoplasmic fractions from F_3dThd- treated and -nontreated, pulse-labeled, infected cells were layered on sucrose gradients and centrifuged are shown in Figs. 5–7. The presence of the 16–28 S peak, representing the heterogeneous late normal vaccinia mRNA population (Fig. 6), and the presence of the 12 S peak of early vaccinia mRNA (Fig. 5) confirm the results obtained originally by Oda and Joklik (17) and by Oki and Heidelberger (15). Oki and Heidelberger showed in the same study that there was no detectable trichloracetic acid-precipitable labeled RNA in the 16–28 S region when cells were infected in the presence of the analogue and pulse-labeled 4.5–5 hr later (15). We have obtained similar results (Fig. 7), and in addition have shown that for all three profiles the peaks of trichloroacetic acid-precipitable radioactivity coincide with peaks representing ^{3}H radioactivity hybridized with denatured vaccinia viral DNA on filters. In the case of the 12 S peak in Fig. 5, the coincidence of acid-precipitable radioactivity and radioactivity bound to DNA on filters is not as close as with the other peaks of radioactivity shown in Figs. 5 and 6. In Fig. 7, where there is no detectable trichloroacetic acid-precipitable radioactivity in the 16–28 S region. there is also no radioactivity from fractions comprising this region bound to viral DNA on filters. There is, therefore, within the limitations of our methods, no detectable transcription of any 16–28 S late viral mRNA in the presence of F_3dThd.

Because we found that only 4 S viral RNA was transcribed late in the presence of the analogue, we thought it necessary to compare this RNA with the 4 S RNA transcribed late in infection without F_3dThd, as well as with 16–28 S late viral mRNA. This would provide more information on the nature of the sequences contained in the late 4 S RNA transcribed in the presence of F_3dThd than could be obtained by using purified total RNA preparations. We wished to determine

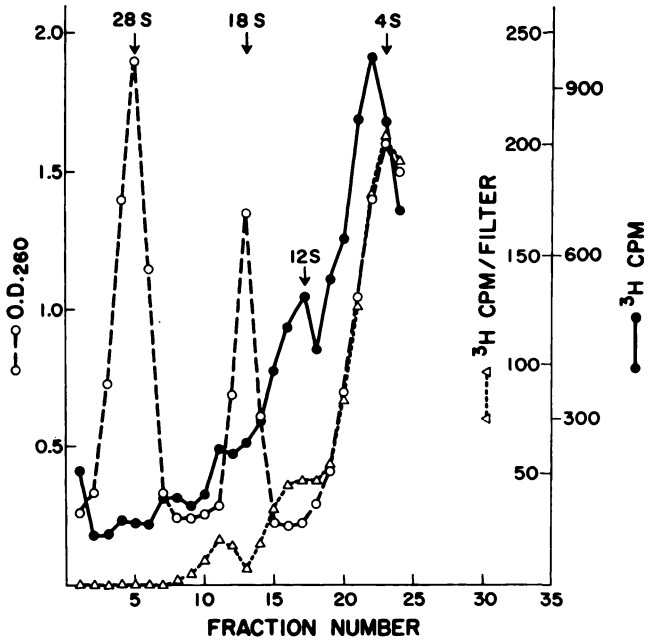

FIG. 5. *Sedimentation profile of normal early vaccinia viral RNA*

Infected cells were pulse-labeled with [^{3}H]uridine 0.5–1 hr after infection. Infection conditions, extraction of the cytoplasmic fraction, centrifugation conditions, and analytical methods are described in MATERIALS AND METHODS. An equal volume of each fraction was used in the hybridization experiments. Radioactivity bound to filters is in all cases counts per minute minus a background of 22 cpm.

whether this latter RNA contains normal or abnormal mRNA or tRNA sequences, or some combination of both, by comparison of the 16–28 S and 4 S peaks shown in Fig. 6 (normal late viral RNAs) with the late 4 S RNA transcribed in the presence of the analogue. A hybridization competition study using unlabeled 12 S early viral RNA and labeled 16–28 S late viral RNA compared with a competition of labeled late and unlabeled early purified total viral RNAs would determine whether the 4 S RNA present in both purified total RNAs affects the interpretation of hybridization competition experiments.

Several hybridization experiments were carried out with the fractions constituting the various peaks of radioactivity as determined by the sedimentation analyses. The fractions comprising each of the peaks shown in Figs. 5–7 and the fractions constituting the 4 S peak obtained from sedimentation of the cytoplasmic fraction of the noninfected, pulse-labeled host cell control were pooled as the individual peaks. Fraction 12–18 and 20–24 (Fig. 5) were pooled to obtain 12 S early viral mRNA and 4 S early RNA, respectively. Fractions 5–20 and 26–31 (Fig. 6) were pooled to obtain 16–28 S late viral mRNA and 4 S late RNA, respectively. Fractions 21–27 (Fig. 7) were pooled to obtain 4 S late RNA transcribed in the presence of the analogue. A similar range of fractions was pooled to obtain 4 S RNA from noninfected cells. The A_{260}:A_{280} ratio for the pooled 16–28 S and 12 S peaks was 2.0; the ratio for the 4 S peaks was around 1.70. In these hybridization incubations, summarized in Table 1, a large amount of annealing was obtained in all cases when a preparation of 4 S RNA isolated from either infected or noninfected cells was incubated with filters containing denatured vaccinia, HeLa cell, or *Cytophaga johnsonii* DNAs. All these 4 S RNAs annealed to about the same extent to all types of DNA tested; hence this technique of hybridization can give little information about the source of the DNA template(s) from which these 4 S RNAs were transcribed.

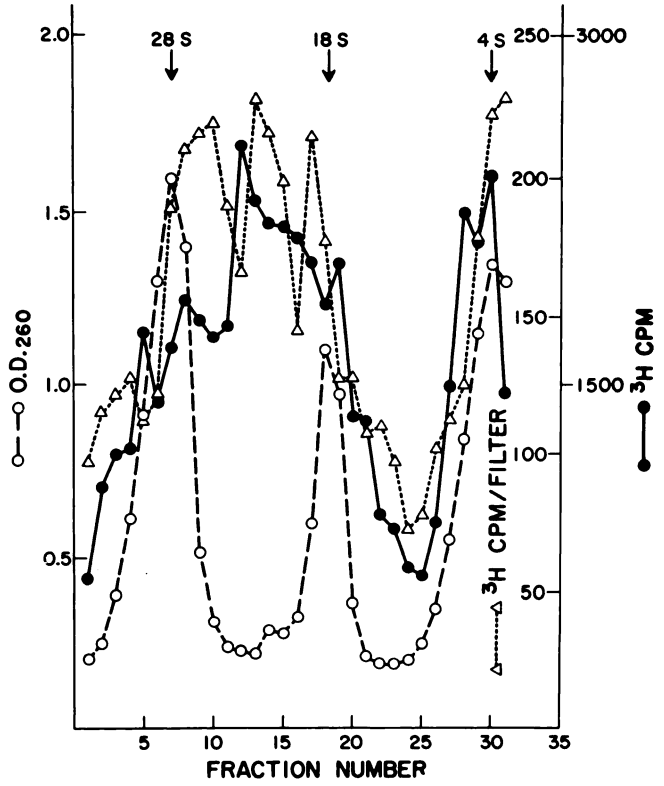

FIG. 6. *Sedimentation profile of normal late vaccinia viral RNA*

Infected cells were pulse-labeled with [^{3}H]uridine 4.5–5 hr after infection. Infection conditions, extraction of the cytoplasmic fraction, centrifugation conditions, and analytical methods are described in MATERIALS AND METHODS. An equal volume of each fraction was used in the hybridization experiments. Radioactivity bound to filters is in all cases counts per minute minus a background of 22 cpm.

There are at least two explanations for this equivalent amount of annealing of the 4 S types to all three DNAs on filters (see DISCUSSION). On the other hand, the 16–28 S late viral mRNA peak showed little significant binding to HeLa cell DNA, as did the 12 S early viral mRNA peak. With both these RNA preparations, approximately 10% of the radioactivity was bound to the HeLa cell DNA compared with that bound to viral DNA (Table 1); thus these two viral mRNA preparations have sequences that are homologous with viral DNA but are not significantly homologous to host cell DNA. The very small radioactivity above background obtained with the filters containing the HeLa cell DNA is probably not significant. We conclude that the 16–28 S and 12 S mRNA species are predominantly, if not entirely, coded by vaccinia virus.

The amount of unlabeled 16–28 S late viral mRNA that would prevent the annealing of almost all of the 9 μg of labeled 16–28 S mRNA was determined (Table 3). As much as 60 μg of late viral 16–28 S mRNA failed to prevent the annealing of radioactivity from an aliquot of the 4 S RNA peak isolated from cells infected in the presence of F_3dThd and pulse-labeled late in infection. These results suggest that no normal late mRNA sequences are present in this 4 S region, but the annealing of this 4 S RNA to bacterial DNA makes it impossible to draw any definite conclusions from this result. Next, aliquots of labeled 4 S RNA isolated from the infected cells not treated with F_3dThd but pulse-labeled 4.5–5 hr after infection were incubated with filters containing 1 μg of denatured vaccinia DNA and 60 μg of unlabeled 16–28 S mRNA. Approximately

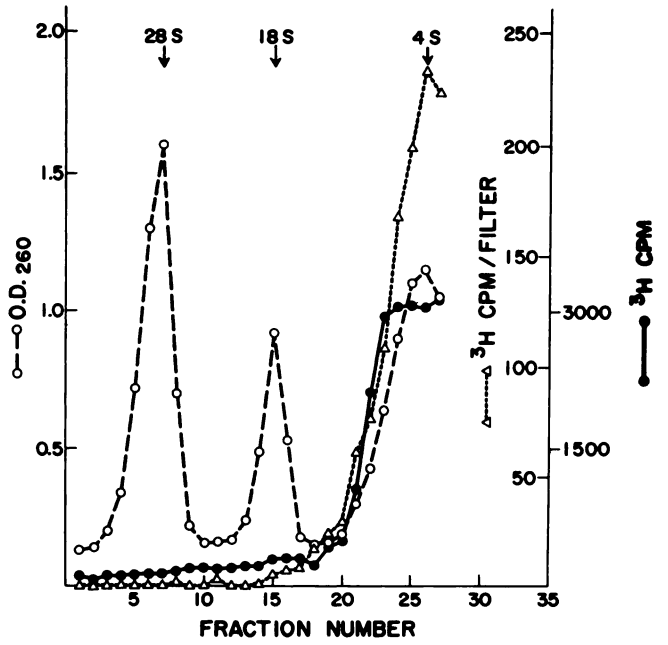

FIG. 7. *Sedimentation profile of late vaccinia viral RNA transcribed in the presence of F_3dThd*

Cells infected in the presence of 1 μM F_3dThd were pulse-labeled with [^{3}H]uridine 4.5–5 hr after infection. Infection conditions, extraction of the cytoplasmic fraction, centrifugation conditions, and analytical methods are described in MATERIALS AND METHODS. An equal volume of each fraction was used in the hybridization experiments. Radioactivity bound to filters is in all cases counts per minute minus a background of 22 cpm.

28% of the radioactivity annealed in the absence of unlabeled 16–28 S mRNA was not annealed in this competition experiment. This 28% could represent sequences of late viral mRNA that had been degraded to smaller 4 S molecules. The possibility of rapid degradation of normal-sized late viral mRNA molecules to smaller molecules of the 4–5 S size range must be considered in attempting to analyze results obtained in hybridization experiments involving various 4 S RNA species, and this possibility is considered under DISCUSSION. If all or some of the sequences of this 4 S RNA were bound nonspecifically to the viral DNA, which is one of the possibilities suggested by the results obtained with bacterial DNA on filters, this interpretation would have to be modified considerably.

The amount of unlabeled 12 S early viral mRNA that would prevent the annealing of 5 μg of labeled 12 S early viral mRNA was also determined (Table 3). When 45 μg of unlabeled 12 S mRNA were incubated with 1 μg of denatured viral DNA and 24 hr later 6 μg of labeled late 16–28 S viral mRNA were added, and the incubation was continued for another 24 hr, 11 ± 1 cpm were bound to the DNA on the filters. When 6 μg of labeled late 16–28 S viral mRNA were incubated with filters containing 1 μg of denatured viral DNA, 23 ± 1 cpm annealed. Early 12 S viral mRNA therefore contains about 50% of the sequences in late 16–28 S viral mRNA.

DISCUSSION

Oki and Heidelberger (15) previously showed that the sedimentation profiles of the cytoplasmic fraction of HeLa cells infected with vaccinia virus and pulse-labeled 0.5–1 hr after infection were the same whether or not F_3dThd was added. Our results, moreover, show that the sequences in purified total early viral RNA transcribed in the presence and absence of F_3dThd are also the same. Thus the analogue has no effect on either the size or sequences of RNA transcribed from

TABLE 3

Summary of hybridization competition experiments with isolated peaks of various RNA preparations

The preparation and isolation of the RNAs, as well as the hybridization techniques, are described in MATERIALS AND METHODS. In each case 1 μg of denatured vaccinia DNA was used. All results are averages of duplicate experiments (± standard deviation) and have been corrected for background (22 cpm).

Labeled RNA	Unlabeled RNA	Radioactivity
		cpm/filter
16–28 S late viral mRNA, 9 μg		45 ± 2
16–28 S late viral mRNA, 9 μg	16–28 S late viral mRNA, 30 μg	20 ± 2
16–28 S late viral mRNA, 9 μg	16–28 S late viral mRNA, 45 μg	14 ± 2
16–28 S late viral mRNA, 9 μg	16–28 S late viral mRNA, 60 or 70 μg	6 ± 1
4 S late viral RNA, 10 μg		50 ± 3
4 S late viral RNA, 10 μg	16–28 S late viral mRNA, 60 μg	36 ± 2
4 S late RNA transcribed in presence of 1 μM F_3dThd, 22 μg		53 ± 3 53 ± 3
4 S late RNA transcribed in presence of 1 μM F_3dThd, 22 μg	16–28 S late viral mRNA, 60 μg	53 ± 3
12 S early viral mRNA, 5 μg		15 ± 1
12 S early viral mRNA, 5 μg	12 S early viral mRNA, 35 μg	2 ± 1
12 S early viral mRNA, 5 μg	12 S early viral mRNA, 58 μg	1 ± 1
16–28 S late viral mRNA, 6 μg		23 ± 1
16–28 S late viral mRNA, 6 μg	12 S early viral mRNA, 45 μg	11 ± 1

the parental viral genomes. This finding is compatible with the fact that F_3dThd present during vaccinia viral replication is incorporated into the DNA of progeny virions (11); only transcription from these progeny viral genomes should be affected by the analogue (see Fig. 1).

A comparison of Figs. 6 and 7 shows that no detectable normal 16–28 S late viral mRNA was transcribed in the presence of F_3dThd. Only 4 S RNA was transcribed during the 30-min pulse. Since noninfected cells and cells infected in the absence of the analogue (normal) both transcribe 4 S RNA during a 30-min pulse, we must examine the various possibilities as to the nature and origin of the 4 S RNA transcribed late in the presence of F_3dThd. This latter 4 S RNA could be tRNA coded for by either the host cell genome, the viral genome, or both. It could also contain degraded viral mRNA or viral mRNA that is transcribed to a smaller size than normal in the presence of the analogue.

The most direct way to examine the sequences of the 4 S RNA transcribed late in the presence of F_3dThd would be to compare them by hybridization with those of 16–28 S late normal viral mRNA and 4 S RNA. However, the results in Table 1 show that all 4 S RNAs studied were hybridized to an equal extent to DNAs of denatured vaccinia, HeLa cells, and *Cytophaga johnsonii*. These findings suggest either that the 4 S RNAs bind nonspecifically to the various DNAs or that evolution has led to conservation of tRNA sequences, and that we observed a real sequence homology among these 4 S RNAs and the DNAs.

An examination of the literature on the sequences of tRNA molecules from different organisms that code for the same amino acid, and possess the same anticodon bases, shows that the latter possibility is most unlikely. The nucleotide sequence of phenylalanine tRNA from *Escherichia coli* (22) is considerably different from the sequence of phenylalanine tRNA from wheat germ (23). Also, the sequences of the serine tRNAs isolated from rat liver *E. coli* and yeast are all very different from each other (24–26). Therefore the annealing that we found of 4 S RNAs to all three DNAs must be nonspecific. There are at least two explanations for this conclusion. First, we observed a stoichiometric effect when the concentration of the 4 S molecules in the peak was high enough to cause nonspecific annealing, as had been observed by Lozeron *et al.* (27) with a phage-transduced bacterial tRNA. Another explanation is that we used the 4 S fractions

isolated from sucrose gradients without further purification. In order to obtain bacteriophage T4 tRNA sufficiently pure for hybridization, it was necessary to employ phenol extraction, column chromatography, and dialysis (28–30). Our 4 S fractions were probably not pure enough to use in hybridization experiments; therefore data obtained only from hybridization of purified total viral RNAs, 12 S early, and 16–28 S late viral mRNAs will be utilized in examining the process of vaccinia viral transcription. The purified total RNAs annealed very specifically to viral DNA but not appreciably to HeLa cell DNA, even though they contained 4 S RNA. Figure 5 shows that the great majority of trichloroacetic acid-precipitable labeled early RNA is of the 4 S and not the 12 S variety. Yet a purified total early viral RNA preparation containing these two RNAs annealed almost exclusively to viral DNA; the amount of annealing to HeLa cell DNA was insignificantly above background (Table 1). Therefore some of the 4 S RNA may be coded by the vaccinia viral DNA.

We have shown (Table 2), by competition of purified unlabeled early viral RNA and labeled purified total normal late viral RNA for hybridization to denatured vaccinia viral DNA, that about two-thirds of the sequences in these two viral RNA preparations are the same. Therefore about 33% additional viral RNA sequences are transcribed late that are not transcribed early. This result confirms the report of Oda and Joklik (17). Some of the common sequences in these late and early purified total viral RNAs probably are those contained in the viral coded 4 S RNA molecules that were also labeled during the 30-min pulse. The use only of purified total labeled RNA preparations prevents calculation of the number of identical sequences present in early and late viral mRNA and in 4 S RNA.

However, the result we obtained using labeled 16–28 S late viral mRNA and unlabeled 12 S early viral mRNA in a hybridization competition experiment gives us a means to calculate the approximate percentage of sequences in 4 S RNA that are common on those of late and early purified viral RNA. We have shown (Table 3) that early 12 S viral mRNA has about half of the sequences of 16–28 S late viral mRNA, while early purified total viral RNA contains about two-thirds of the sequences of late purified total viral RNA (Table 2). We can reconcile these two findings by interpreting the data in the following way.

One can set up the following equation:

$$1/2 = \frac{2 - X}{3 - X}$$

where X represents identical sequences in early and late purified total RNAs that are contained in the 4 S RNA present in both these preparations. This number of identical sequences is "subtracted" from early and late purified viral RNAs by sedimentation in sucrose gradients to give early 12 S viral mRNA, which contains 50% of the sequences in late 16–28 S viral mRNA. X is 1 in this equation, which means that one-half of the total purified total early viral RNA and one-third of the total purified total late viral RNA consist of identical sequences that are present in 4 S RNA. There are other, more complicated ways of interpreting our data, but this calculation represents the simplest case, in which the major assumption is that the tRNA sequences do not change appreciably during viral replication. We believe that this assumption is reasonable. Our interpretation, based on the foregoing arguments, is that about 50% of the sequences in late viral mRNA are also present in early viral mRNA. This is a somewhat different proportion from that proposed by Oda and Joklik (17), and our calculations were based on the use only of purified RNAs.

These calculations suggest some further information concerning the nature of the sequences in purified total late viral RNA transcribed in the presence of F_3dThd. This RNA (Table 2) has 70% of its sequences in common with purified total late normal viral RNA. We have calculated that about 33% of the sequences in purified total normal late viral RNA are 4 S RNA sequences. We have also shown that all the viral RNA transcribed late in the presence of F_3dThd sediments sharply as 4 S RNA (Fig. 7). This observation, together with the fact that the purified total late viral RNA transcribed in the presence of the analogue contains 70% of the

sequences in purified total normal late viral RNA, means that 4 S mRNA sequences must be present in the former purified total viral RNA to account for this large number of common sequences. Even if transcription of tRNA continues normally late in infection in the presence of F_3dThd, it could not provide enough common sequences to account for the 70% value we have obtained. These 4 S mRNA molecules either could have been formed by degradation of 16–28 S late viral mRNA or could have been transcribed initially as small mRNA species. There is experimental support for both possibilities. Weiss *et al.* (29) reported that bacteriophage T4-coded 4 S RNA contains degraded T4 mRNA as well as T4 4 S tRNA. When vaccinia virus was replicated in the presence of [2-^{14}C]F_3dThd, the DNA of the progeny virions contained the analogue and was smaller in size than normal vaccinia viral DNA (11). This suggests that smaller mRNA molecules may be transcribed from this smaller DNA.

In Fig. 7 no residual trichloracetic acid-precipitable radioactivity was detected in the 16–28 S region, and the 4 S peak is sharp. It is difficult to envision a degradation process so rapid and complete that there would be no 16–28 S mRNA detected following a 30-min pulse. Moreover, if there were extensive degradation, one would expect to see RNAs of sizes intermediate between the 16–28 S and 4 S regions. This was not found. Thus we believe that there was little degradation of 16–28 S RNA.

When purified total unlabeled viral RNA transcribed in the presence of F_3dThd was used in competition with labeled purified total early viral RNA (Table 2), about 21% of the radioactivity annealed. When the same experiment was conducted with unlabeled normal purified total late viral RNA and labeled purified total early viral RNA, about 8% of the radioactivity annealed. Thus sequences are missing in the late mRNA transcribed in the presence of F_3dThd that are normally present in both early and late viral mRNA.

We conclude that F_3dThd has no effect on the transcription of early vaccinia RNA, but prevents the transcription of 30% of the sequences normally present in purified total late vaccinia viral RNA. Thus some of the sequences normally transcribed both early and late in infection are missing in purified total late viral RNA transcribed in the presence of the analogue. The purified total late viral RNA transcribed in the presence of F_3dThd has 70% of the sequences present in purified late normal viral RNA. The common sequences consist of both viral mRNA and normal 4 S RNA (presumably tRNA) sequences. Experiments to label, isolate, purify, and characterize components of the viral 4 S RNA transcribed late in the presence of F_3dThd are in progress.

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