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Fluorinated Pyrimidines

XXXIX. Effects of 5-Trifluoromethyl-2′-deoxyuridine on the Replication of Vaccinia Viral Messenger Ribonucleic Acid and Proteins

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SUMMARY

The mechanism of the antiviral activity of 5-trifluoromethyl-2′-deoxyuridine (F_3TdR) has been studied in vaccinia virus-infected HeLa cells. When normal virions are used to infect the cells in the presence of the analogue, sucrose gradient sedimentation has shown that the early messenger RNA is normal and associates normally with polyribosomes. However, any late mRNA that may be produced under those conditions has abnormal sedimentation properties and does not associate normally with polyribosomes. When the cells are infected with purified virions containing F_3TdR in their DNA, they adsorb to the cells and are uncoated normally. However, early mRNA is not transcribed normally. Studies of viral protein synthesis with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate suggest that a major virus-induced protein is not synthesized in the presence of F_3TdR, and that another protein is formed instead.

INTRODUCTION

5-Trifluoromethyl-2′-deoxyuridine, which was first synthesized in this laboratory (1), is incorporated into the DNA of bacteriophage T4 (2) and into the DNA of HeLa and leukemia L5178Y cells in culture (3). Moreover, its nucleotide, 5-trifluoromethyl-2′-deoxyuridylate, irreversibly inhibits thymidylate synthetase (4). F_3TdR[3] currently shows activity in clinical trials in advanced cancer patients. Kaufman and Heidelberger (5, 6) have shown that on a molar basis F_3TdR is the most active chemotherapeutic compound known against herpes simplex keratitis in rabbit eyes and has utility in such infections in man. In this laboratory, the effects of F_3TdR and other pyrimidine nucleoside analogue have been studied on various cells in culture (7) and on the replication of vaccinia virus in HeLa cells (8). It was found that F_3TdR inhibited viral replication at the lowest concentration of any of the analogues tested, and that its inhibition was prevented by simultaneous addition of thymidine. However, when thymidine was added 1 day after the time of infection, virus production was not rescued. It was suggested that this irreversibility might be a consequence of the incorporation of the analogue into viral DNA (8). We then demon-

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[3] The abbreviations used are: F_3TdR, 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine); TdR, thymidine; FUdR, 5-fluoro-2′-deoxyuridine; SDS, sodium dodecyl sulfate.

strated that F_3TdR is incorporated into the DNA of purified vaccinia virions (9), but to a much lesser extent than had been found for 5-bromo- and 5-iodo-2′-deoxyuridine (10–12). Nevertheless, virions containing as little as 2–8% replacement of DNA thymidine by F_3TdR were completely noninfective. Sucrose density gradient centrifugation of the DNA isolated from L5178Y cells (3) and purified vaccinia virions (9) revealed that the molecules of DNA containing F_3TdR are smaller than normal; this suggests that the process of assembly of the DNA molecules is slower and less complete as a result of analogue incorporation.

The present paper reports studies on the transcription of early and late messenger RNA in vaccinia virus-infected HeLa cells in the presence and absence of F_3TdR. The methodology used in this work was developed by Oda and Joklik (13), who established precisely by sucrose gradient centrifugation the characteristics and properties of vaccinia mRNAs in HeLa cells.

MATERIALS AND METHODS

Cell culture and virus. HeLa S3 cells were maintained continuously in exponential growth in Eagle's suspension medium supplemented with 10% calf serum, 0.1% Pluronic F68 poloxalene polymer, and antibiotics. Shock suspensions of vaccinia virus, strain WR, were prepared and purified in sucrose density gradients as previously described (8, 9). The virus titer was determined by plaque assay on HeLa cell monolayers with a starch overlay (8).

Mode of infection. Cells, grown to a density of 3–4 $\times$ 10^5/ml, were harvested and concentrated to 5 $\times$ 10^6/ml in Eagle's suspension medium containing 1% calf serum, 0.1% Pluronic F68, antibiotics, and 10 mM $MgCl_2$ (adsorption medium). The cells were then incubated with an inoculum of 50 plaque-forming units/cell for 30 min. In the case of highly purified, F_3TdR-containing virions, 500 particles/cell were used.

After this period, the unadsorbed virions were washed off, and the cells were resuspended in growth medium (Eagle's suspension medium containing 5% calf serum, 0.1% Pluronic F68, and antibiotics) at a concentration of 5 $\times$ 10^5/ml and incubated for 20–24 hr.

Pulse-labeling with ^3H-uridine. Uridine-5-^3H (Schwartz/Mann, 27.2 Ci/mmole) was added for 30 min to the cells (50 μCi/10^7 cells) at 30 min or 4.5 hr after infection. The incorporation was stopped by pouring the labeled cells onto crushed, frozen phosphate-buffered 0.9% NaCl.

Preparation of cytoplasmic extracts. The harvested cells were washed twice with cold phosphate-buffered 0.9% NaCl and allowed to swell for 15 min in a hypotonic solution [10 mM Tris (ph 8.0), 10 mM KCl, and 5 mM $MgCl_2$]. The cells were homogenized in a Dounce glass homogenizer and were examined microscopically to determine the presence of unbroken cells. The cytoplasmic fraction was separated from nuclei by centrifugation for 5 min at 800 $\times$ g.

Analysis of cytoplasmic RNA. Polyribosomes, ribosomes, and their subunits were analyzed by centrifugation of the cytoplasmic extract in 15–30% (w/w) sucrose density gradients in a Spinco SW 25.1 rotor at 25,000 rpm for 2 hr in a Spinco model L ultracentrifuge. The rapidly labeled cytoplasmic RNA (mRNA) species was sedimented by making the cytoplasm 1% with respect to sodium dodecyl sulfate, followed by centrifugation in 15–30% (w/w) sucrose gradients that contained 0.5% SDS, 5 mM Tris (pH 7.3), and 0.1 M NaCl for 18 hr at 23,000 rpm in an SW 25.1 rotor. All gradients were collected from the bottom of tubes in 90- or 45-drop fractions, and the absorbance was measured at 260 mμ. After addition of carrier RNA and protein, the material precipitable with 10% trichloracetic acid was centrifuged and dissolved in 0.5 ml of formic acid, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer in Scintisol (Isolab, Inc.). The sedimentation coefficients were calculated as previously described (9).

Preparation of radioactive, highly purified virions. Radioactive, highly purified virions were prepared from sonicated cytoplasmic fractions of cells as previously described (9). Virus production proceeded for 24 hr in the presence of 0.5 μM 2-^{14}C-F_3TdR (7.7 mCi/mmole), 0.02 μCi/ml of TdR-2-^{14}C (59 mCi/

mmole; Amersham-Searle), or 0.05 μCi/ml of TdR-2-^{14}C 0.01 mM and 1 μM nonlabeled F_3TdR.

After centrifugation of the crude virus suspension through 35% sucrose, the virus was banded twice in 25–45% sucrose density gradients and centrifuged at 15,000 rpm for 45 min in a Spinco SW 39L rotor. The amount of virus was estimated by measurement of the absorption at 260 mμ, based on an absorbance of 1.00 = 1.2 × 10^{10} virus particles = 64 μg of viral protein = 3.3 μg of viral DNA (14).

Uncoating of highly purified, labeled virus. Following adsorption, 30-ml samples were taken for analysis at various times. After centrifugation of each sample at 500 × *g* for 5 min, the cell pellet was suspended in 2 ml of hypotonic solution and sonicated in an MSE disintegrator for 60 sec at 1.5 mamp and 125 V. Two 0.5-ml samples were removed. To one sample, trichloracetic acid was added to a final concentration of 10%, while the other sample was incubated for 60 min at 37° with 100 μg/ml of DNase (Worthington). Trichloracetic acid was then added, and the total acid-insoluble, cell-associated radioactivity was determined. The remainder of the sonic extract was used for determination of plaque-forming units.

Amino acid incorporation. Portions of infected (input multiplicity, 425 PFU/cell) or uninfected cells were resuspended in the virus growth medium in the presence of TdR or F_3TdR (1 μM). Tritiated leucine (10 Ci/mmole, Amersham-Searle) was added for 30 min to the cells (40 μCi/4 × 10^7 cells) at 90 min or 6 hr after infection. The incorporation was terminated by pouring the cell suspension into 2 volumes of frozen phosphate-buffered 0.9% NaCl. The cells were collected by centrifugation, washed twice, and resuspended in 2 ml of a hypotonic solution for 15 min. Then the cells were homogenized with a Dounce homogenizer and centrifuged at 800 × *g* for 5 min to remove nuclei. Particulate material was centrifuged at 100,000 × *g* for 2 hr, and the supernatant fraction was dialyzed against 0.01 M sodium phosphate (pH 7.2) for 48 hr and made 10% in glycerol.

The protein concentration of the post-ribosomal supernatant fluids was determined by the method of Lowry *et al.* (15).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The method was performed essentially as described by Summers *et al.* (16). The supernatant fraction was incubated in 2% SDS, 1% mercaptoethanol, and 0.01 M sodium phosphate, pH 7.2, for 1 hr at 37°. Polyacrylamide gels (7.5%, 10 × 0.6 cm) were prepared containing 0.1% SDS, 0.5% *N,N,N′,N′* - tetramethylethylenediamine, 0.1% ammonium persulfate, and 0.1 M sodium phosphate, pH 7.2. Between 75 and 100 μl (50 μg of protein) of the sample were applied to each gel.

Electrophoresis was carried out at 10 mamp/gel for 3.5 hr at room temperature. After electrophoresis, the gels were fixed in 20% sulfosalicylic acid overnight, followed by staining with 0.25% Coomassie blue for 8 hr; then the gels were destained with 7.5% acetic acid for 48 hr. The gels were scanned at an optical density of 550 mμ in a Gilford model 2400 spectrophotometer. Gel slices, 2 mm thick, were placed into individual scintillation vials and solubilized with 0.5 ml of 30% hydrogen peroxide at 55° overnight. The radioactivity was measured in 10 ml of Scintisol with a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

The effects of F_3TdR on the synthesis and fate of vaccinia virus-specific mRNA were studied under two different conditions. Early and late mRNA synthesis was determined in the presence of FUdR and F_3TdR with normal input vaccinia virions. The transcription of early mRNA from the F_3TdR-containing input viral genome in the absence of added F_3TdR was also studied.

In a cell infected with vaccinia virus, the genomes of the host cell and the virions both code for and control the synthesis of DNA, RNA, and protein (17). Host cell RNA, except for 4 S tRNA, is not released into the cytoplasm during a 30-min pulse of ^{3}H-uridine (18, 19), as shown for uninfected cells in Fig. 1. This provides the basis for the study of vaccinia viral mRNA, which is synthesized in the cytoplasm. TdR (1 μM), FUdR (10 μM), or F_3TdR (1 μM) was added at the beginning of the absorption period and was present continuously during virus multi-

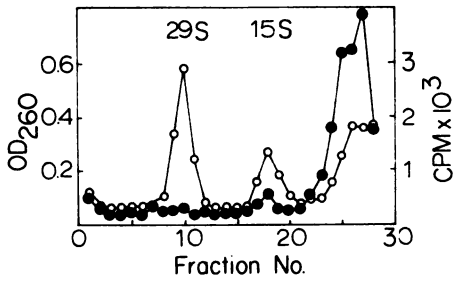

FIG. 1. *Sedimentation distribution of mRNA in cytoplasm of noninfected cells*

HeLa cells (2.5×10^7) in suspension in 50 ml of growth medium were incubated for 30 min with 25 μCi of ^{3}H-uridine. The cells were rapidly chilled, washed, and homogenized in hypotonic solution. The cytoplasmic fraction, obtained after centrifugation at $800 \times g$ for 5 min, was made 1% in SDS and centrifuged in a 25-ml linear gradient of 15–30% sucrose in SDS buffer for 18 hr at 15° and 23,000 rpm in the SW 25.1 rotor of a Spinco model L ultracentrifuge. The tubes were punctured through the bottom, 90-drop fractions were collected, and the optical density at 260 mμ and radioactivity in each tube were determined. O——O, absorbance at 260 mμ; ●——●, ^{3}H radioactivity (counts per minute).

plication. FUdR under these conditions completely blocks the replication of vaccinia viral DNA, so that mRNA synthesis is directed only by the parental input viral DNA (20). Furthermore, 1 μM F_3TdR completely inhibits the replication of normal virions (8).

The sedimentation distribution of cytoplasmic RNA labeled by a 30-min pulse of ^{3}H-uridine added 30 min after infection is shown in Fig. 2; this is the time when early mRNA synthesis is maximal (13). The ultraviolet absorption scan revealed three peaks: 4 S tRNA, and 16 S and 28 S ribosomal RNAs (the actual sedimentation coefficients obtained in the individual determinations are shown in the figure). The relative amounts of these RNAs did not vary under the different conditions used. During this 30–60 min period after infection, the sedimentation coefficient of the viral mRNA was approximately 12 S, in good agreement with the results of Joklik and co-workers (13, 18). This early mRNA synthesis was not affected by FUdR or F_3TdR, as expected, since early mRNA is transcribed from the input viral genome. The late mRNA, transcribed from the progeny viral DNA 4.5–5 hr after infection, had a sedimentation coefficient greater than that of the early mRNA (13, 18), as shown in Fig. 3. By contrast to the situation with early mRNA, FUdR and F_3TdR blocked the synthesis of viral 17 S late mRNA (Fig. 3). The lack of late mRNA synthesis in the presence of FUdR was expected, since the synthesis of progeny DNA was prevented. On the other hand, in the presence of F_3TdR, viral DNA was synthesized but was smaller in size than normal vaccinia DNA (9); yet no late mRNA sedimenting at 17 S could be detected.

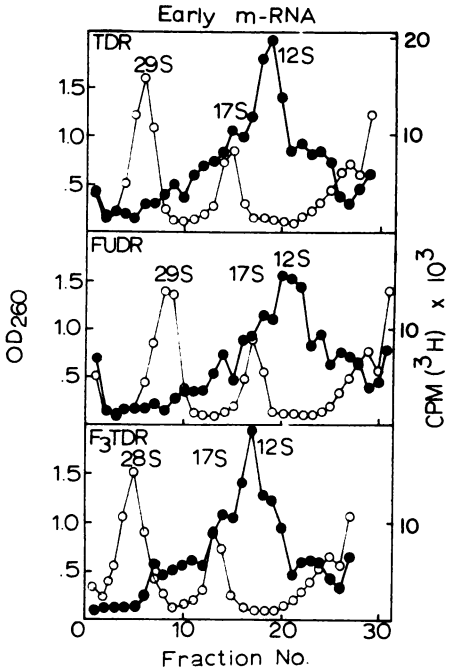

FIG. 2. *Sedimentation distribution of early mRNA synthesized in cytoplasm of infected cells in the presence of TdR, FUdR, or F_3TdR*

HeLa cells in suspension at a level of 5×10^6/ml were infected with purified vaccinia virions (50 PFU/cell) for 30 min at 37° in the presence of TdR (1 μM), FUdR (10 μM), or F_3TdR (1 μM). The cells were washed twice with fresh medium, resuspended in growth medium, and cultured at 37°. After 30 min, ^{3}H-uridine was added and incubation was carried out for an additional 30 min. The subsequent procedure was as described in Fig. 1. O——O, absorbance at 260 mμ; ●——●, ^{3}H radioactivity (counts per minute).

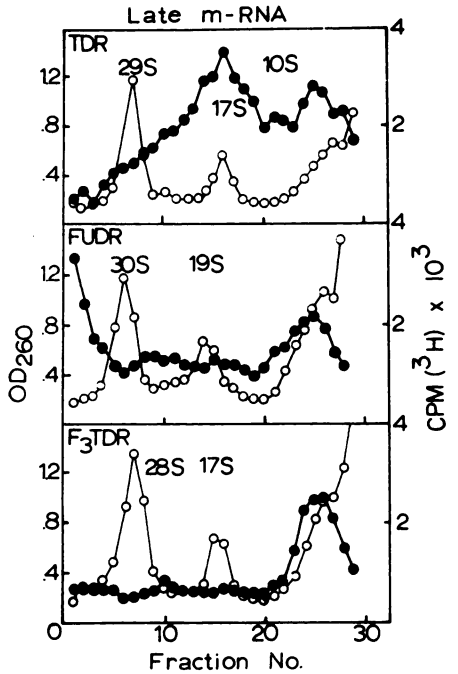

FIG. 3. *Sedimentation distribution of late mRNA synthesized in cytoplasm of infected cells in the presence of TdR, FUdR, or F_3TdR*

The experiment was done exactly as described for Fig. 2, except that the ^{3}H-uridine was added 4.5 hr after infection. O——O, absorbance at 260 mμ; ●——●, ^{3}H radioactivity (counts per minute).

These experiments were repeated with the procedure and centrifugation conditions altered to study the association of the mRNA with polyribosomes. The sedimentation profile from uninfected cells following a 30-min pulse of ^{3}H-uridine (Fig. 4) reveals ultraviolet absorption at 74 S, corresponding to ribosome monomers, and more rapidly sedimenting regions of polyribosomes, none of which contained radioactivity. This experiment confirms the one shown in Fig. 1 and shows that during the 30-min pulse no host cell mRNA was released into the cytoplasm and associated with polyribosomes. The results of a similar pulse in cells 30–60 min after infection (Fig. 5) clearly show the association of the early viral mRNA with polyribosomes; as expected and in agreement with the experiments shown in Fig. 2, the presence of F_3TdR did not affect this association. On the other hand, in the presence of both FUdR and F_3TdR, there was no association of late viral mRNA with polyribosomes (Fig. 6), probably because no late mRNA was available, as indicated in Fig. 3.

Vaccinia virions that had been replicated in the presence of 1 μM F_3TdR and ^{14}C-TdR

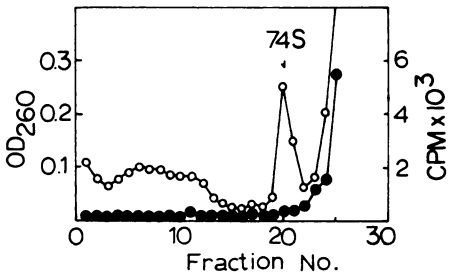

FIG. 4. *Association of mRNA with polyribosomes in cytoplasm of noninfected HeLa cells*

The cells were labeled with ^{3}H-uridine for 30 min as described in Fig. 1. The cytoplasmic fraction was centrifuged in a 15–30% (w/w) sucrose gradient in 10 mM Tris (pH 7.4), 10 mM KCl, and 1.5 mM $MgCl_2$ for 2 hr at 5° and 25,000 rpm in the SW 25.1 rotor of a Spinco Model L ultracentrifuge. The procedure was as described in Fig. 1. O——O, absorbance at 260 mμ; ●——●, ^{3}H radioactivity (counts per minute).

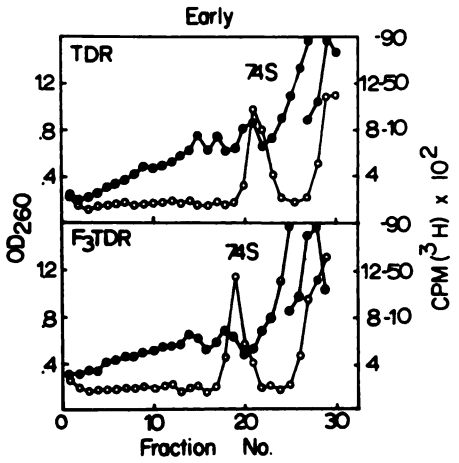

FIG. 5. *Association of viral early mRNA with polyribosomes in cytoplasm of Hela cells infected in the presence of TdR or F_3TdR*

The conditions of the infection and incubation were as described in Fig. 2. The centrifugation was performed as described under Fig. 4. O——O, absorbance at 260 mμ; ●——●, ^{3}H radioactivitv (counts per minute).

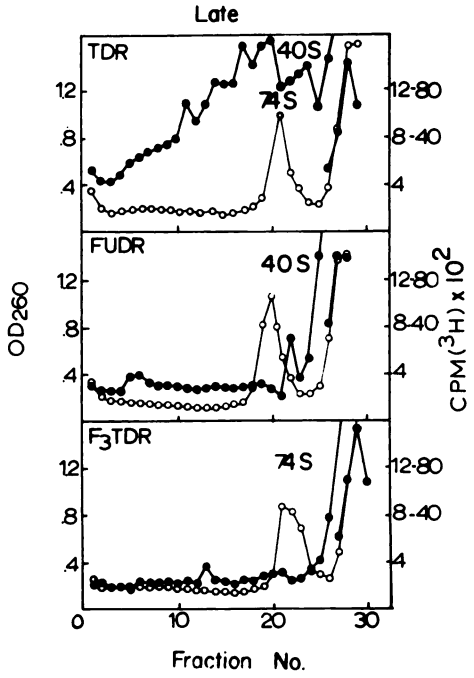

FIG. 6. *Association of viral late mRNA with polyribosomes in cytoplasm of HeLa cells infected in the presence of TdR, FUdR, or F_3TdR*

The conditions of the infection and incubation were as described in Fig. 3. The centrifugation was performed as described under Fig. 4. O——O, absorbance at 260 mμ; ●——●, ^3H radioactivity (counts per minute).

[which were shown previously to contain F_3TdR in the DNA (9)] were isolated from the cytoplasm of infected cells and purified by four sucrose gradient centrifugations. As shown in Fig. 7, these virions sedimented as a single, DNase-resistant peak, indicating that they were coated. The sedimentation coefficient coincided exactly with that of normal purified virions. Similarly, normal virions labeled with ^{14}C-TdR were purified, and the properties of the two types of virions are compared in Table 1. The 100–200-fold increase in the number of particles per plaque-forming unit confirms our earlier observation that purified F_3TdR-containing virions are essentially noninfective as compared with the normal ones (9).

The first step of vaccinia viral infection involves the adsorption of the virions to the host cells. Highly purified, labeled, normal and F_3TdR-containing virions were used to study the kinetics of this adsorption, which were very similar at two levels of input multiplicity (Fig. 8). Further data from such experiments show (Table 2) that the percentage adsorption of the normal and analogue-containing virions was comparable at 30 min. Following the adsorption of these labeled virions, the cells were washed and resuspended in growth medium, and samples were taken for analysis of the uncoating process at different times, as measured by DNA-resistant, trichloracetic acid-precipitable DNA. The kinetics of uncoating of the adsorbed virions is shown in Fig. 9. Within 1 hr DNase sensitivity was found, and by 4 hr the uncoating was complete. There was no significant difference between the uncoating of normal and F_3TdR-containing virions (Fig. 9B). In the same experiment (Fig. 9A), infectivity was measured, and although normal and analogue-containing virions were replicated at approximately the same rate, the latter were only 1% as infective. This probably reflects the further replication of the original parental virions that did not contain the analogue.

Since we had demonstrated the adsorption and uncoating of F_3TdR-containing virions,

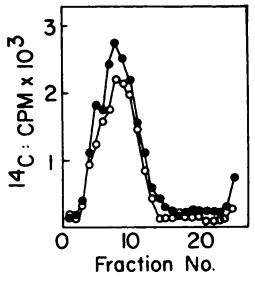

FIG. 7. *Sedimentation of vaccinia virions containing ^{14}C-F_3TdR*

Highly purified virions containing F_3TdR and ^{14}C-TdR were centrifuged in a 25–45% sucrose gradient for 45 min at 15,000 rpm in the SW 39L1 rotor of a Spinco model L ultracentrifuge. A 0.5-ml aliquot of each fraction was digested with DNase (100 μg/ml, Worthington) for 2 hr at 37° in 0.01 M $MgCl_2$. The acid-insoluble material was precipitated with trichloracetic acid, and the radioactivity was measured. Another aliquot was precipitated with trichloracetic acid without DNase treatment. ●——●, acid-precipitated; O——O, DNase-treated and acid-precipitated.

TABLE 1

Properties of purified virions

Property	Precursor		
	^{14}C-TdR (1 μM)	^{14}C-TdR (0.01 μM) + F_3TdR (1 μM)	^{14}C-F_3TdR (0.5 μM)
A_{260}/ml	0.382	1.25	0.43
Particles/ml	4.59×10^9	1.5×10^{10}	5.17×10^9
PFU/ml	2.30×10^8	6.40×10^6	4.10×10^6
PFU/A_{260}	6.02×10^9	5.12×10^6	9.55×10^6
Particles/PFU	20	2340	1260
Radioactivity, dpm/ml	6.9×10^3	5.92×10^4	176
DNA, μg/ml	1.25	4.12	1.42
Specific activity of DNA, dpm/μg	5.55×10^3	1.44×10^4	124

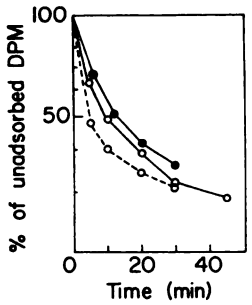

FIG. 8. *Adsorption of normal and F_3TdR-containing vaccinia virions to HeLa cells*

The virions, labeled from ^{3}H-TdR or ^{14}C-F_3TdR at the input multiplicities indicated, were adsorbed to HeLa cells (5×10^6 cells/ml) for various periods of time at 37°; they were washed twice, and the amount of labeled virus not adsorbed to the cells was calculated from the cell-associated radioactivity. ●, normal virions; ○, F_3TdR-containing virions; ——, input of 50 particles/cell; - - -, 500 particles/cell.

we could then attempt to determine whether normal early mRNA is transcribed from the F_3TdR-containing viral genome. Such an experiment (Fig. 10) showed only a small amount of radioactivity in the 12 S region of normal early mRNA; this is quite different from the normal transcription of early mRNA from normal virions in the presence of F_3TdR (Fig. 2).

Since the transcription of late mRNA in the presence of F_3TdR is abnormal (Fig. 3) and the morphology of analogue-containing virus particles is also abnormal (9), it is likely that different and/or fewer proteins are produced. This was studied by the incorporation of ^{3}H-leucine into the soluble proteins of the cells, which were separated by polyacrylamide gel electrophoresis in the presence of SDS. Figure 11 shows a tracing of the scan of the stained proteins in the gel and the radioactivity determined in individual slices following a 30-min pulse of ^{3}H-leucine in noninfected cells. The pattern is quite complicated, but the reproducibility is good, and there is no significant difference between the cells incubated with TdR and those with F_3TdR. When the leucine was added for 30 min, 2 hr after vaccinia infection, the scans shown in Fig. 12 were obtained. These patterns are also complicated and resemble those of Fig. 11. However, a peak of radioactivity is clearly evident in fraction 5 of the TdR-treated (control, viral-infected) cells that is not present in the noninfected cells (Fig. 11) or in those infected in the presence of F_3TdR. Scans of the proteins synthesized 6 hr after infection (Fig. 13) show that the radioactive peak at fraction 5 in the TdR is also detectable by staining and hence has increased in quantity; in the F_3TdR experiment it is still lacking. The radioactive peak at fraction 28 of the latter experiment is considerably higher than in the control. These observations suggest that in the analogue-treated group an abnormal protein is produced; this might explain the morphological changes seen in the electron micrographs of particles in F_3TdR-treated cells (9). Further work along these lines is in progress.

DISCUSSION

The studies presented here lead to the conclusion that when HeLa cells are infected

TABLE 2

Adsorption of vaccinia virus WR on HeLa cells

Incubation was carried out for 30 min at 37° at a cell density of 5 × 10^6/ml in Eagle's suspension medium containing 1% calf serum and 0.01 M $MgCl_2$.

Property	Normal virions		F_3TdR-containing virions	
Input multiplicity, particles/cell	50	50	50	500
Amount adsorbed, dpm/ml	229	153	614	5540
Radioactivity, dpm/particle	1.51×10^{-6}	1.10×10^{-6}	3.44×10^{-6}	3.95×10^{-6}
Radioactivity, dpm/cell	4.6×10^{-5}	3.1×10^{-5}	1.23×10^{-5}	1.11×10^{-4}
Particles/cell	31	28	36	281
Percentage adsorbed	62	56	72	56

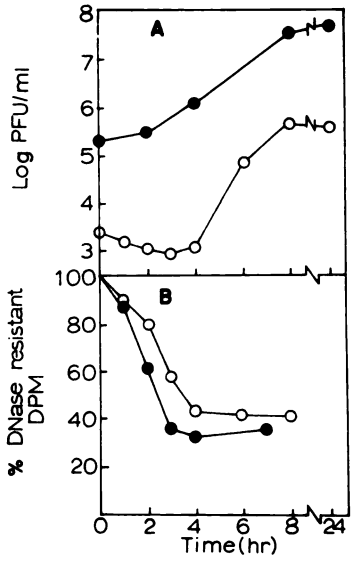

FIG. 9. *Uncoating and recoating of normal* (●——●) *and F_3TdR-containing* (○——○) *vaccinia virus WR*

Highly purified virions that had been labeled with 0.01 μM ^{14}C-thymidine in the presence of 1 μM F_3TdR were used to infect HeLa cells at an input multiplicity of 31 particles/cell in the normal and 281 particles/cell in the F_3TdR-containing virions. At various times thereafter the number of plaque-forming units per milliliter (A) was measured in the cell sonic extracts. The same sonic extracts were incubated with DNase (100 μg/ml) and 0.01 M Mg^{++} for 60 min at 37°. They were then precipitated with cold 10% trichloracetic acid and dissolved in formic acid. This DNase-stable radioactivity was compared with that of aliquots not treated with DNase. The results are expressed as percentage of DNase-resistant disintegrations per minute (B).

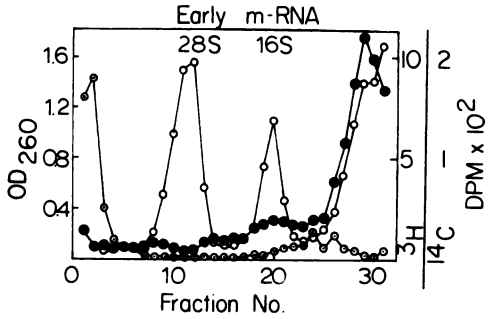

FIG. 10. *Size distribution of viral early mRNA prepared from HeLa cells infected with F_3TdR-containing virions in the absence of F_3TdR*

HeLa cells in suspension at a concentration of 5 × 10^6/ml were infected with highly purified F_3TdR-containing virions at a multiplicity of 500 particles/cell (about 5 PFU/cell) for 30 min at 37° in the presence of 1 μM thymidine. At 30 min after infection, the infected cells were pulse-labeled with 0.5 μCi/ml of ^{3}H-uridine for 30 min. Other conditions were the same as in Fig. 1. ○——○, absorbance at 260 mμ; ●——●, ^{3}H radioactivity (counts per minute); ⊙——⊙, ^{14}C radioactivity (counts per minute).

with vaccinia virus, its genome is transcribed into early mRNA, which is associated with polyribosomes. Such early mRNA is transcribed normally in the presence of quantities of FUdR and F_3TdR sufficient to block the synthesis of progeny viral DNA. This provides additional evidence to support the previous conclusions (13, 19) that early mRNA is transcribed from the input viral DNA. On the other hand, late mRNA is not transcribed normally when vaccinia virus repli-

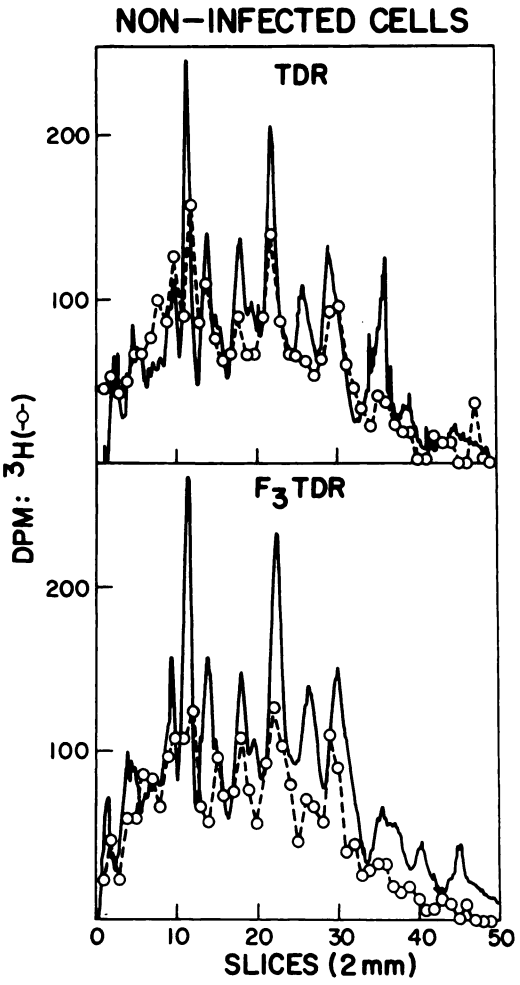

FIG. 11. *Protein synthesis in noninfected HeLa cells in the presence of 1 μM TdR or 1 μM F_3TdR.*

HeLa cells growing in suspension were labeled with ^{3}H-leucine (0.5 μCi/ml) for 30 min. The supernatant fluids obtained by centrifugation for 2 hr at 100,000 × *g* were fractionated by electrophoresis in SDS-polyacrylamide (7.5%) gels. The gels were fixed in 20% sulfosalicylic acid and stained with 2.5% Coomassie blue. A continuous trace of the optical density at 550 mμ was made in a Gilford model 2400 spectrophotometer. The gels were sliced into serial 2-mm sections. Each slice was dissolved in 0.5 ml of 30% H_2O_2 in scintillation vials and counted in Scintisol. ——, absorbance at 550 mμ; O- - -O, ^{3}H radioactivity (counts per minute).

cates in the presence of FUdR and F_3TdR, which also supports the earlier conclusions (13, 19) that late mRNA is transcribed from the progeny DNA. Nevertheless, it is clear from the finding of F_3TdR-containing virions resistant to DNase and sedimenting under the same conditions as normal virions that late mRNAs of some sort must be transcribed in order to code for coat proteins. Such transcription of abnormal late mRNA is currently being studied by DNA-RNA hybridization. These conclusions are further supported by the finding of the lack of a characteristic viral protein in the F_3TdR-treated infected cells and the appearance of a different viral protein in those cells. Since the DNA of F_3TdR-containing virions is smaller than normal (9), it is possible that it directs the transcription of late mRNA of a smaller size than normal; this would be compatible with the sedimentation patterns that we have

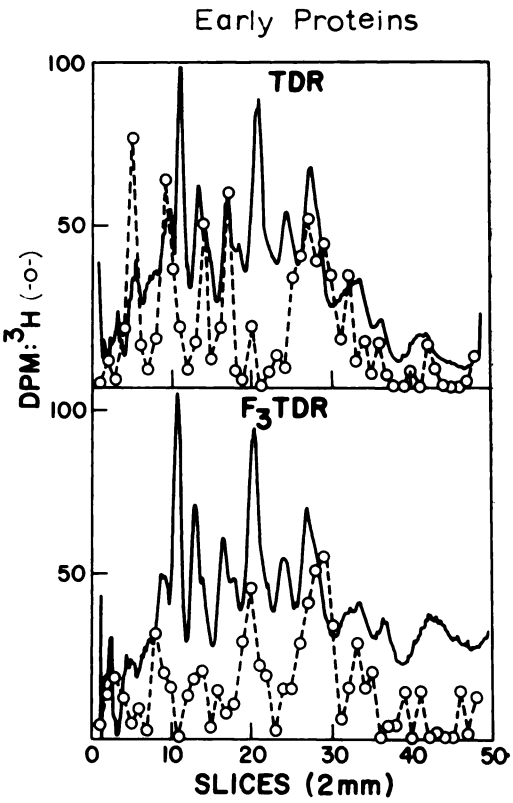

FIG. 12. *Synthesis of early proteins in vaccinia virus-infected cells*

HeLa cells were infected as described in Fig. 2. At 2 hr after infection, ^{3}H-leucine was added for 30 min. The rest of the procedure was as described under Fig. 11. ——, absorbance at 550 mμ; O- - -O, ^{3}H radioactivity (counts per minute).

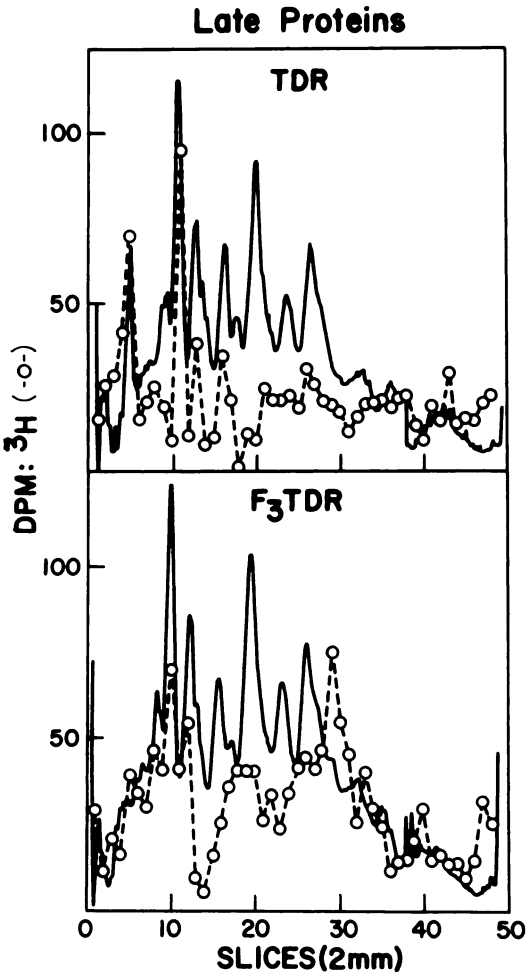

FIG. 13. *Synthesis of late proteins in vaccinia virus-infected HeLa cells*

The experiment was as described under Fig. 12, except that the ^{3}H-leucine was added 6 hr after infection and incubated for 30 min. ——, absorbance at 550 mμ; O- - -O, ^{3}H radioactivity (counts per minute).

obtained. We have also established that purified, F_3TdR-containing virions adsorb to cells and are uncoated normally, and that they are not transcribed into normal early mRNA.

Based on the results obtained previously (9) and reported here, it can be stated that F_3TdR exerts its inhibition of vaccinia viral replication as a consequence of its incorporation into DNA. Such DNA is smaller in size than normal (9), and it is not transcribed into normal messenger RNA. Further studies, currently under way, of early and late mRNA by DNA-RNA hybridization and of early and late proteins by immunoelectrophoretic methods should clarify the exact mechanism of the antiviral activity of F_3TdR.

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