Effect of Actinomycin D on Virus-induced Ribonucleic Acid Polymerase Formation in Foot-and-Mouth Disease Virus-Infected Baby Hamster Kidney Cells

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Actinomycin D, at a concentration that inhibits cellular ribonucleic acid (RNA) synthesis, inhibited the production of foot-and-mouth disease virus-induced RNA polymerase in baby hamster kidney cells. Inhibition was proportional to exposure time and reached 85% when actinomycin D was added 90 min before infection. Polymerase production was inhibited to the same extent in growth and minimal media, and the kinetics of its appearance were slightly different than in untreated cells. Enzyme preparations from actinomycin-treated cells having one-third to one-tenth the activity of untreated samples gave products with RNA profiles similar to those of controls. The 37S viral peak, 20S ribonuclease-resistant peak, and 26 to 28S peaks were present in all cases. Actinomycin D did not consistently inhibit virus production in either medium. Insulin did not prevent the actinomycin induced inhibition of polymerase and virus production from occurring.

It has been established that actinomycin D inhibits deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis, probably by binding to DNA (10, 15, 21). Actinomycin D has also been reported to have varying effects on the replication of RNA animal viruses. Poliovirus replication in HeLa cells is unaffected (22), but is inhibited in HEp-2 cells (12) and human amnion cells (7). However, different strains of poliovirus are inhibited to different extents in the same strain of HeLa cells (F. L. Schaffer and M. B. Reaume, *Bacteriol. Proc.*, p. 118, 1965). Mengovirus multiplication is not changed by actinomycin D in L cells (9), but it and virus-induced RNA polymerase formation are inhibited in Novikoff hepatoma cells (16). Rous sarcoma virus does not grow in the presence of actinomycin D (3, 23), and the multiplication of reovirus (11), influenza virus (5), and fowl plague virus (4) is also inhibited. Newcastle disease virus is unaffected in HeLa cells (24), but is inhibited in chick embryo monolayers (13).

The present paper describes the effect of actinomycin D on foot-and-mouth disease virus (FMDV)-induced RNA polymerase formation in baby hamster kidney (BHK) cells and on virus production. Previous work established the

existence of such a polymerase in FMDV-infected BHK cells (18). Enzyme action in a cell-free assay was inhibited by antibody, but not by actinomycin D (18, 19). It will be shown here that actinomycin D strongly inhibits the formation of FMDV-RNA polymerase in infected cells, but inconsistently affects the production of infectious virus in cells incubated with either growth or minimal media.

MATERIALS AND METHODS

Cells, virus, and media. BHK 21, clone 13 cells (American Type Culture Collection) were grown for 6 to 7 days in 2-liter Baxter bottles in a medium consisting of a modified Eagle's salt solution, 0.02 M tris(hydroxymethyl)aminomethane (Tris), 10% tryptose phosphate, and 10% bovine serum (20). FMDV, type A, strain 119, passaged in calf kidney cultures 110 times, was concentrated and partially purified to an "aqueous phase" stage (2). Two media were used in experiments: cell growth medium described previously, and a minimal medium consisting of a modified Hanks' salt solution in 1% Tris buffer containing 0.2% glucose, 0.03% glutamine, and 10% dialyzed bovine serum (17). Cells were infected at a multiplicity of 10 plaque-forming units (PFU) per cell, and virus production was measured 5 hr postinfection (17).

Enzyme preparation, assay, and sucrose gradient

TABLE 1. *Effect of actinomycin (Act D) on the kinetics of polymerase production^a*

Postinfection time of cell harvest	Polymerase activity ^b		Inhibition caused by Act D
	No Act D	Act D	
<i>min</i>			%
150	1,470	1,180	20
180	4,800	3,440	28
210	14,900	7,800 ^c	48
240	13,200	6,760	49

^a Actinomycin D at a concentration of 5 μ g/ml was present from infection onward in the minimal medium used for incubation. Enzyme preparations were made from cell harvests, and their activity was determined as counts per minute of acid-insoluble radioactivity per milligram of protein.

^b Expressed as counts per minute per milligram of protein.

^c Actinomycin D at a concentration of 1 μ g/ml yielded a preparation with polymerase activity 8,670 counts per min per mg of protein.

analysis. A 100,000 $\times$ g cytoplasmic pellet from infected cells suspended in 0.25 M sucrose-0.001 M magnesium at a protein concentration of 5 to 10 mg/ml was prepared as previously described (18). Enzymatic activity of this suspension was measured by the incorporation of tritiated uridine into acid-insoluble products (18). RNA products from enzyme assay samples incubated in the presence of bentonite were spun through linear sucrose gradients (5 to 25%, w/v) in 0.01 M sodium acetate, pH 5.1 (1).

RESULTS

Initial experiments showed that, at a concentration of 5 μ g/ml, actinomycin D decreased RNA synthesis in normal BHK cells more than 90% when present for 15 min or more before labeled uridine was added. Maximal inhibition of RNA synthesis, about 97%, was reached within 30 min.

The effect on polymerase formation of this concentration of actinomycin D present from infection onward was studied next. Enzyme formation was always inhibited, apparently independently of the incubation medium. Inhibition ranged from 12.0 to 61.8% (average for four experiments was 34.9 ± 17.3) for treated cells in cell growth medium harvested at the usual 210 min postinfection. Inhibition values for treated cells in minimal medium ranged from 24.6 to 53.5% (average for seven experiments was 39.7 ± 8.2). The kinetics of polymerase formation were slightly modified by actinomycin D. From 150 to 210 min postinfection, inhibition of polymerase formation by actinomycin D increased from 20 to 48% in the experiment shown in Table 1. As previously shown in infected cells

TABLE 2. *Effect of actinomycin D addition at varying pre- and postinfection times on polymerase production^a*

Actinomycin D addition (min pre- and postinfection)	Polymerase activity (counts per min per mg of protein)	Inhibition of polymerase activity
		%
Preinfection		
90	8,040	85
0	20,100	62
Postinfection		
45	26,000	51
90	28,600	46
150	43,600	17
195	52,700	0
210	52,200	—
No addition	52,800	—

^a Actinomycin D at a final concentration of 5 μ g/ml was added to the cell growth medium used for incubation. All cells were harvested at 210 min postinfection, and enzyme preparations made from them. Activity was determined as counts per minute of acid-insoluble radioactivity per milligram of protein. Per cent inhibition values refer to 210-min activity as 100.

not treated with actinomycin D (18), polymerase activity was significant at 150 min postinfection, sharply reached a maximum at 210 min, and declined thereafter. This behavior also occurred in actinomycin D-treated cells, although the peak level of enzymatic activity was about half that of untreated cells. At 210 min, actinomycin D at 1 μ g/ml was as effective as at 5 μ g/ml.

The extent of inhibition of polymerase formation was directly related to the time actinomycin D was in contact with infected cells prior to their harvest for enzyme preparation (Table 2). Actinomycin D, present for only 15 min just prior to harvest, had no effect on polymerase production, whereas, when it was present for 2 hr before harvest, there was 46% inhibition. Actinomycin D present from 90 min preinfection onward caused the highest inhibition, 85%.

Enzyme preparations from cells exposed to actinomycin D were studied for possible changes in their RNA products. It was first necessary to determine the effect of actinomycin D present during the polymerase assay on the nature of the RNA formed. It was known previously that actinomycin D has no effect on the amount of acid-insoluble products formed (18). Results obtained in the present investigation (not shown here) established that the typical RNA sucrose density gradient profile of reaction products was qualitatively unchanged by actinomycin D in the cell-free assay when the polymerase used was from cells infected in the absence of actinomycin

FIG. 1. Sucrose gradient of RNA produced in cell-free polymerase system. Enzyme preparations for B and C are from actinomycin D-treated cells. (A) Control, no actinomycin D; (B) actinomycin D added to cells at infection; (C) actinomycin D added to cells 90 min before infection. V = 37S viral RNA; D = 26 to 28S noninfectious RNA; R = 20S partially ribonuclease-resistant RNA (1). Sedimentation is from right to left.

D. In addition, Fig. 1 shows RNA profiles obtained from three enzyme preparations with tritiated uridine incorporation varied over a 10-fold range because of varying times of exposure of their source of infected cells to actinomycin D. The 37S viral peak, 20S ribonuclease-resistant peak, and 26 to 28S peaks (1) were present in each of the three trials.

The effect of actinomycin D on virus production was determined in cells incubated with cell growth and minimal media. Table 3 shows that actinomycin D, at a concentration of 5 $\mu\text{g}/\text{ml}$, altered virus production to a variable extent in both media. Inhibition of virus production in cells incubated with growth and minimal media ranged from 0 to 0.3 and from 0 to 0.6 log units, respectively. Increasing the exposure of cells to actinomycin D by preincubation with the antibiotic before infection did not always further decrease virus production.

It has been reported that the inhibition of poliovirus growth by actinomycin D may be prevented by insulin (7). Experiments were,

TABLE 3. Effect of actinomycin D (Act D) on virus production in BHK cells incubated with cell growth and minimal media^a

Expt	Virus yield (log PFU/ml ^b)			
	Cell growth medium		Minimal medium	
	Control	Act D present	Control	Act D present
1	8.9	8.8	8.6	8.4
2	8.6	8.4	8.2	7.6
3	8.0	8.0	7.7	7.3
4	8.4	8.1 ^c	8.0	8.1 ^d

^a Actinomycin concentration was 5 $\mu\text{g}/\text{ml}$, present from infection onward, unless otherwise specified. Virus titer represents combined infectivity of cells and fluid.

^b The results for each experiment represent the average of two independent runs assayed in four prescription bottles per virus dilution.

^c Value was unchanged when actinomycin D was present from 1.5 hr before infection. In another experiment, there was a decrease of 0.3 log unit in cells preincubated with actinomycin D compared to cells only treated from infection onward.

^d Value was decreased to 7.7 when actinomycin D was present from 1.5 hr before infection. In another experiment, there was no difference between cells preincubated and regularly treated with actinomycin D.

therefore, run in the presence of 2 to 50 μg of insulin per ml with actinomycin D-treated, infected cells incubated in both growth and minimal media. The results showed no significant differences in actinomycin D inhibition of virus and enzyme production in the presence of insulin.

DISCUSSION

Our results have indicated that actinomycin D interferes with the kinetics and ultimate level of polymerase formation to the same extent in both cell growth and minimal media. The greater percentage of inhibition of polymerase preparations harvested at later times in the FMDV-polymerase kinetic cycle may be due to the longer time period that actinomycin D was present. Polymerase formation was also inhibited when actinomycin D was added as late as 150 min postinfection, when enzyme synthesis was already manifest. Data reported for the Mengovirus-Novikoff hepatoma cell system indicated that actinomycin D added after the production of viral polymerase had begun did not inhibit subsequent production of enzyme (17).

The sucrose density profiles of RNA molecules

produced in the cell-free RNA-synthesizing system were the same for polymerase isolated from cells infected in the presence or absence of actinomycin D. The inhibiting effect of actinomycin D on polymerase formation was, therefore, apparently on the whole replicating enzyme system and not on any one specific step. Actinomycin-treated *Escherichia coli* infected with the RNA bacteriophage MS2 has been reported to synthesize components not necessarily present in the infected, untreated cell (14). The yield of infectious MS2 was also reduced.

The apparent inhibition of infectious virus production by actinomycin D was more readily detectable when minimal medium was used as the incubating fluid rather than growth medium. This is consistent with the known decreased virus yields obtained from infected cells in the minimal medium (17). The extra nutrients of cell growth medium are apparently able to counteract partially the actinomycin D inhibition of virus production, but are not able to prevent the decrease in polymerase formation. It has been reported that 1 μ g of actinomycin D per ml has no effect on FMDV, type O, multiplication in cloned BHK cells (6), and preliminary experiments indicated that 5 μ g of actinomycin D per ml did not change the yield of FMDV, type A119, in uncloned BHK cells in complete growth medium (Polatnick and Bachrach, *unpublished data*). It has also been reported that only 20% of the total virus-specific RNA formed in encephalomyocarditis virus-infected ascites cells was eventually incorporated into mature particles (8). Thus, the 35 to 40% inhibition of viral RNA polymerase formation seen in the experiments here may not affect the amount of virus subsequently produced, particularly if some other process such as the synthesis of viral coat protein is the limiting factor. It should also be realized that the plaque assay is detecting infectious viral particles only. Since such particles constitute only a very small percentage of the total viral mass in the infected cell, an assay procedure for determining changes in viral mass would be preferable.

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