ON THE STABILITY OF PHAGE MESSENGER RNA

Subir K. Bose and Richard J. Warren*
Department of Microbiology, St. Louis University School of Medicine
St. Louis, Missouri 63104

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During investigations on the regulation of protein synthesis in Escherichia coli infected with UV-irradiated and amber mutants of T4 phages, we became interested in examining the kinetics of messenger RNA (mRNA) synthesis in the above systems. The characteristic arrest of the synthesis of "early" enzymes is not observed in E. coli infected with UV-irradiated T-even phages (Dirksen et al., 1960). Similar lack of temporal regulation is obtained when amber mutants of T4, defective in the synthesis of viral DNA, are used to infect a nonpermissive strain of E. coli (Wiberg et al., 1962). The replication of viral DNA is clearly implicated in the overall regulation of phage-induced enzyme synthesis, i.e., arrest of "early" enzyme formation and the initiation of synthesis of "late" proteins. The mechanism of this regulation, however, remains a matter of considerable speculation. There seems little doubt that only "early" mRNA is detectable prior to the onset of phage DNA synthesis, while both "early" and "late" mRNA species are synthesized during the later periods of the phage replication cycle (Hall et al., 1964).

In this paper we present some preliminary data showing that the phage mRNA responsible for "early" enzyme synthesis is unstable and that contin-

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ued synthesis of this mRNA is required for the sustained formation of a phage-induced "early" enzyme, dTMP synthetase in E. coli infected with UV-irradiated T4.

MATERIALS AND METHODS

E. coli CR34 was obtained from Dr. R. Rolfe of this department and grown at 37° with aeration on a glycerol-salts medium containing: Na_2HPO_4, 8.4 g; KH_2PO_4, 4.5 g; NH_4Cl, 2.0 g; vitamin-free acid hydrolysate of casein (Nutritional Biochemicals, Cleveland, Ohio), 2.5 g; glycerol, 8.0 ml and $MgSO_4 \cdot 7H_2O$, 0.3 g, per liter of distilled water. After sterilization this medium was supplemented with 10 μg/ml each of thymine, uracil, and thiamine, and 100 μg/ml each of leucine and threonine. Bacteriophage T4D was kindly supplied by Dr. R. S. Edgar. Irradiated phage was prepared by exposing purified T4D to a 15 watt GE germicidal lamp (G15T8) for 120 seconds at a distance of 30 cm. The phage titer dropped from 10^{11}/ml to 10^6/ml.

Sensitization of the cells to Actinomycin D was accomplished by EDTA treatment (Leive, 1965). The cells in exponential phase ($2\text{-}3 \times 10^8$/ml) were harvested by centrifugation, washed once with 0.12 M Tris-HCl, pH 8.0, and resuspended in this buffer to 0.1 original volume ($2\text{-}3 \times 10^9$ cells/ml). EDTA (pH 8.0) was added to 10^{-3} M and the suspension incubated with shaking for 5 minutes at 37°. Two volumes of the supplemented growth medium was then added to terminate the EDTA treatment. The sensitized cells were infected immediately with UV-treated T4D at a multiplicity of 2 in the presence of 50 μg/ml L-tryptophan. Incubations were always carried out in Erlenmeyer flasks wrapped with aluminum foil to minimize both photoreactivation and the inactivation of Actinomycin D. Aliquots of cells were removed at desired times, chilled rapidly in centrifuge tubes, and the sedimented

cells washed once in 10^{-2} M Tris-acetate buffer, pH 7.4, which contained 10^{-2} M Mg-acetate and 5×10^{-3} M mercaptoacetate. Cell-free extracts were prepared by exposing the cell suspensions in the above buffer after one cycle of freezing and thawing to sonic oscillation (Biosonik II) for 1 minute. The latter treatment facilitated disruption without affecting a number of enzyme activities (e. g., DNases, dihydrofolate reductase, dTMP synthetase). Prior to enzymatic analyses, the disrupted cells were centrifuged at 15,000 g for 10 minutes to yield the "crude extract." All operations were performed at 0-4° C.

dTMP synthetase activity was measured by following the formation of dihydrofolate at 338 mμ in a Gilford Model 2000 spectrophotometer at 25° (Greenberg et al., 1962). A unit of activity is expressed as mμmoles product/min. Protein was measured by the method of Furth and Ho.

Incorporation of uridine-5-^{3}H into acid-insoluble material was followed by the adsorption of cells onto Whatman 3 MM filter paper discs which were immediately plunged into ice-cold 5% trichloroacetic acid (Horiuchi et al., 1966). After two washes with cold 5% TCA, the discs were rinsed with acetone, dried and then counted in a scintillation spectrometer (Nuclear Chicago Mark I).

Uridine-5-^{3}H (specific activity 14.3 c/mM) was purchased from Nuclear Chicago Corporation, Des Plaines, Illinois. Actinomycin D was a generous gift from Merck, Sharp and Dohme, Rahway, N. J.

RESULTS

Following the EDTA treatment, E. coli CR34 cells remain sensitive to Actinomycin D (as measured by inhibition of incorporation of ^{3}H-uridine into acid-insoluble material) for varying periods of time. We will present data

only from those experiments where the cells were sensitive to the antibiotic for the entire duration of the experiment. No dTMP synthetase activity is detected when an aliquot of sensitized CR34 cells infected with UV-irradiated T4 is introduced to a flask containing Actinomycin D (20 μg/ml) immediately after infection (Fig. 1). In the absence of the inhibitor, phage-induced dTMP synthetase is detectable in the sample removed 5 minutes after infection and this activity continues to increase. Cells treated with the antibiotic 10 minutes after infection show sustained enzyme synthesis for about 5 minutes. Samples removed 20 and 40 minutes after infection and placed with Actinomycin show similar kinetics of inhibition. None of the samples had any phage-induced lysozyme activity, indicating that "late" protein is not synthesized.

Uridine-5-^{3}H incorporation by these samples, however, shows a more immediate response to the inhibitor (Table I). Thus, mRNA synthesis is inhibited 90% in 1-2 minutes after the addition of the antibiotic although dTMP

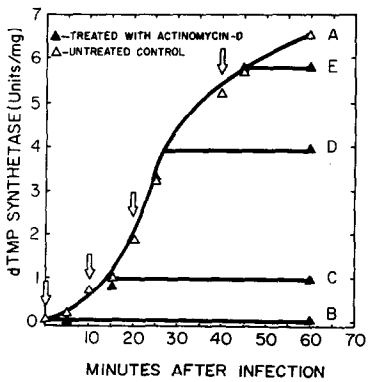

Fig. 1. Kinetics of inhibition of synthesis of phage-induced dTMP synthetase by Actinomycin D. 550 ml of "sensitized" E. coli CR34 cells were infected with UV-irradiated T4 as described in the text. Immediately after infection a 60 ml sample was pipetted into a flask containing 1.2 mg Actinomycin D: Zero time treatment (B). 10 (C), 20 (D), and 40 (E) min after infection (indicated by arrows) 60 ml aliquots were similarly treated. 30 ml aliquots were removed from control and treated samples at intervals shown. Enzymatic assays were performed as described in the text. A: control.

synthetase formation continues for 5-6 minutes and then ceases (Table II).

TABLE I

INHIBITION OF INCORPORATION OF ^{3}H-URIDINE BY ACTINOMYCIN (AM)

In an experiment identical to the one represented in Fig. 1, one ml aliquots of infected cells were removed, 100 mμc of uridine-5-^{3}H was added. At indicated times, 100 μl samples were removed for acid-insoluble radioactivity determinations. Numbers in parentheses show percent inhibition.

AM addition (Min after infection)	Uridine-5-^{3}H addition (min)	cpm incorporated			
		Control		+AM	
		0.5 min	2 min	0.5 min	2 min
0	1	54	652	0	13 (98)
10	11	152	748	12	30 (97)
20	21	47	287	27	43 (93)

Using un-irradiated T4D, similar kinetics of decay *in vivo* of functional phage mRNA have been obtained (manuscript in preparation).

TABLE II

STABILITY OF PHAGE mRNA

30 ml aliquots were removed from flasks used in the experiment summarized in Table I at indicated times. The values represent units of dTMP synthetase/mg protein.

Time after infection	-AM	AM added at 20 min
0	0	0*
20	1.55	
25	2.11	2.56
30	3.92	2.67
60	6.68	2.52

*AM added at 0 min and assayed after 60 min.

DISCUSSION

Actinomycin D probably inhibits protein synthesis by blocking transcription of the genome into mRNA (Hurwitz et al., 1962; Levinthal et al., 1963). It is also believed that mRNA is unstable (Jacob and Monod, 1961) and synthesis of mRNA and its decay have been documented in several instances of enzyme induction (Levinthal et al., 1962; Kepes, 1963). Actinomycin D, while inhibiting synthesis of RNA, often allows fairly extensive synthesis of protein (Pollock, 1963; Moses and Calvin, 1965), presumably as a result of the relative stability of many of the mRNA species existing in the cells. Our results bear on the problem of determining whether the continued synthesis of phage-induced enzymes in systems where viral DNA synthesis is blocked results from (a) stabilization of the "early" mRNA, or (b) continued synthesis and translation of the "early" mRNA. The data presented suggest that the alternative (b) is the correct explanation. In E. coli infected with UV-irradiated T4 and demonstrating sustained early enzyme synthesis the phage mRNA must be continuously formed during the infection period. Therefore the "early" mRNA transcribed on irradiated viral DNA, is also unstable and resembles in this respect other short-lived messengers, e.g., that of β-galactosidase. The kinetics of inhibition of dTMP synthetase when considered with the rate of inhibition of mRNA synthesis indicate that the phage mRNA, at least the species involved in the synthesis of dTMP synthetase, is functional for 5-6 minutes. Further experiments are in progress to determine the characteristics of phage-specific RNA in bacteria infected with amber and temperature-sensitive phages.

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