Eur. J. Biochem. *51*, 587–591 (1975)

Synthesis *in vitro* of Φ29-Specific Early Proteins Directed by Phage DNA

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(Received August 2/October 28, 1974)

The RNA and proteins synthesized in an *Escherichia coli* cell-free system of protein synthesis directed by *Bacillus subtilis* phage Φ29 DNA were studied. Hybridization-competition experiments showed that most of the RNA species synthesized *in vitro* are early RNAs. Many of the early proteins induced after phage infection were also synthesized in the *E. coli* cell-free system. None of the late proteins, structural or non-structural, were synthesized in the system *in vitro*.

The *Bacillus subtilis* phage Φ29 has a double-stranded DNA with a molecular weight of 11×10^6 [1], closed non-covalently by a protein [2]. Up to 21 proteins have been shown to be induced after phage infection [3–6], accounting for over 90% of the information content of Φ29 DNA.

The proteins induced by phage Φ29 can be classified in two main groups: those whose synthesis starts early after infection and those which are synthesized later [3–5]. Two classes of RNAs are also synthesized after Φ29 infection: the early RNAs produced in the presence of chloramphenicol, transcribed from the L strand, and the late RNAs, whose synthesis requires phage-specific protein(s), transcribed from the H strand [7–10]. To study the control of Φ29 DNA expression as well as to determine whether the phage-induced proteins are coded for by Φ29 DNA, we have studied the RNA and proteins synthesized in an *Escherichia coli* cell-free system under the direction of linear Φ29 DNA. In this system only early RNA species and early proteins are synthesized indicating that the mechanism(s) which control late gene expression *in vivo* are not operating in the system *in vitro*.

MATERIALS AND METHODS

Bacteria

E. coli K12, strain 7-Cavalli [11] was obtained from Dr García Ballesta and *B. subtilis* 110NA, an asporogenous mutant of *B. subtilis* 168 *try*$^-$, from Dr F. Moreno.

System of Protein Synthesis in vitro

The protein-synthesizing system *in vitro* was prepared from *E. coli* K12 as described by Gold and Schweiger [12] except that the ribosomes were incubated for 90 min at 37 °C in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 22 mM ammonium chloride, 1 mM dithiothreitol, 10 mM ATP and 0.1 M 3-phosphoglycerate, to degrade endogenous messenger RNA [13].

The incubation mixture for protein synthesis contained, in a final volume of 0.1 ml, the following components: 50 mM Tris-HCl, pH 8, 50 mM potassium acetate, 2 mM dithiothreitol, 2 mM ATP, 0.5 mM CTP, GTP and UTP, 20 mM phospho*enol*pyruvate, 0.31 mM calcium leucovorin (*i.e.* calcium 5-formyl-5,6,7,8-tetrahydrofolate), 0.2 mM each of 19 amino acids (except leucine which was as indicated in each case labeled with ^{14}C or ^3H), 100 μg of tRNA from *E. coli* B, 500 μg of ribosomes, 0.04 ml of supernatant protein (2–3 mg/ml) and 5 μg of linear Φ29 DNA. A control without DNA was also carried out. The incubation was at 37 °C for the times indicated in each figure. To follow the incorporation of radioactivity into proteins, 5% trichloroacetic acid was added; the samples were treated at 90 °C for 15 min and filtered through discs of glas fiber paper (Whatman GF/C, 2.4 cm diameter).

To follow RNA synthesis in the system *in vitro*, the same components were used as for protein synthesis except that 2 μCi of [^3H]UTP were also added and the cold 5% trichloroacetic acid-insoluble radioactivity determined.

Enzymes. Pancreatic deoxyribonuclease (EC 3.1.4.5); pancreatic ribonuclease (EC 3.1.4.23); lysozyme or mucopeptide *N*-acetylmuramylhydrolase (EC 3.2.1.17); trypsin (EC 3.4.21.4).

Preparation of the Proteins Synthesized in vitro *for Gel Electrophoresis*

The proteins synthesized in the presence of Φ29 DNA, labeled with [^{14}C]leucine (15 μCi/ml, 0.05 mM) and those synthesized in the absence of DNA, labeled with [^{3}H]leucine (50 μCi/ml, 0.05 mM) were treated with pancreatic RNase (10 μg/ml) at 0 °C for 1 h and precipitated with 10% trichloroacetic acid. The precipitate was washed once with ether–ethanol (1:1) and twice with ether–ethanol (3:1). The final precipitate was dried under a nitrogen stream and the proteins were dissociated by addition of 0.2–0.3 ml of a buffer containing 5 mM sodium phosphate pH 7.1, 1% (w/v) sodium dodecyl sulfate, 1% (v/v) 2-mercaptoethanol and 8 M urea and heating in a bath of boiling water for 5 min.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was carried out in 15-cm-long gels containing 12.5% acrylamide, 0.6% *N,N'*-methylenebisacrylamide and 8 M urea in the presence of 0.1% sodium dodecyl sulfate as described [5]. Electrophoresis was carried out at room temperature for 12 h, at a constant current of 5 mA per gel. The gels were cut in fractions of 1 mm with a Mickle gel slicer and counted as described [5].

RNA Isolation. Hybridization-Competition Experiments

The RNA synthesized in the *E. coli* cell-free system, labeled with [^{3}H]UTP (25 μCi/ml, 0.5 mM) during 10 min of incubation at 37 °C, was extracted with hot phenol [14] in the presence of 1% sodium dodecyl sulfate and 0.1 M sodium acetate, pH 5.2, after addition of yeast RNA (40 μg/ml) as carrier. The RNA in the aqueous phases was concentrated by precipitation with 3 volumes of ethanol in the presence of 0.3 M sodium acetate, pH 5.2. The precipitate was washed with 85% ethanol, dried under a nitrogen stream and dissolved in 1 mM EDTA, pH 7.

Unlabeled RNA was isolated from *B. subtilis* 110NA infected with phage Φ29 at a multiplicity of infection = 20 in the presence of chloramphenicol (400 μg/ml), added 5 min prior to infection (chloramphenicol RNA), or in its absence (late RNA). After 30 min of incubation at 37 °C, the infected cells were cooled down, centrifuged and lysed by treatment with lysozyme (500 μg/ml) at 0 °C for 1 h. The RNA was extracted by the hot-phenol method [14] in the presence of 1% sodium dodecyl sulfate.

Hybridization was carried out by incubating the RNA which had been synthesized and labeled with ^{3}H *in vitro* in a total volume of 1.5 ml 2× standard saline citrate with 0.5 μg of denatured DNA fixed to nitrocellulose filters (Millipore HAWP, 2.4 cm diameter) at 64 °C for 20 h [15]. Increasing amounts of unlabeled competitor RNA (chloramphenicol or late RNA, respectively) were added. Controls containing filters without DNA were also carried out. After hybridization, the filters were washed with 2× standard saline citrate, incubated with 4 ml of RNase in this solution (20 μg/ml) at room temperature for 1 h, washed with this solution, dried and counted.

Lysozyme Assay

Lysozyme activity was determined by measuring the release of radioactivity from Whatman 3 MM filter discs containing *E. coli* labeled with [^{3}H]diaminopimelic acid [12]. About 40% of the total radioactivity contained in the filters was released by incubation with pure egg-white lysozyme. A further 40% of the radioactivity remaining in the filters was released by treatment with trypsin. One unit of lysozyme activity is defined as the amount of enzyme releasing 1 count/min during one min of incubation at 37 °C [16].

RESULTS

Linear Φ29 DNA stimulates the incorporation of [^{3}H]UMP (A) and [^{3}H]leucine (B) into trichloroacetic acid-insoluble material in a protein-synthesizing cell-free system from *E. coli* (Fig. 1). In both cases, there is a 4–5-fold stimulation in the incorporation as compared to that obtained in the absence of DNA.

To study the RNA species synthesized in the *E. coli* cell-free system directed by linear Φ29 DNA, the RNA, labeled with [^{3}H]UTP and isolated as described in Materials and Methods, was hybridized to Φ29 DNA, which had been adsorbed to nitrocellulose filters. The hybridization was performed both in the absence and in the presence of increasing amounts of competitor RNA produced *in vivo* that had been isolated either from cells infected with phage Φ29 in the presence of chloramphenicol (chloramphenicol RNA) or from cells infected in the absence of chloramphenicol and harvested 30 min after infection (late RNA). As shown in Fig. 2, chloramphenicol RNA competes up to 75% with the RNA synthesized *in vitro* in the presence of Φ29 DNA. The percentage of competition does not increase essentially when late RNA is used as competitor, the value obtained in this case being 80% at saturation.

The proteins synthesized *in vitro* were examined in two ways: (a) by detection of specific enzyme

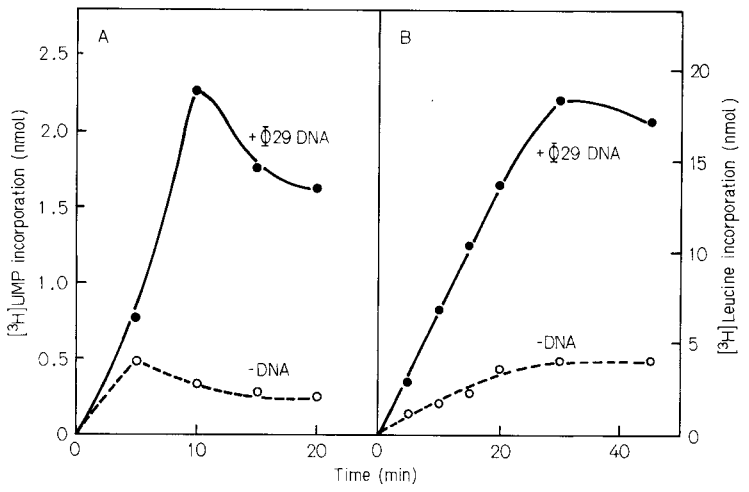

Fig. 1. *RNA and protein synthesis directed by Φ29 DNA in an* E. coli *cell-free system.* The incubation was carried out as described in Materials and Methods, with or without linear Φ29 DNA. At the times indicated aliquots of 25 µl were taken and the radioactivity determined. (A) 2 µCi of [^{3}H]UTP in a volume of 0.1 ml was added and the cold trichloroacetic acid-insoluble radioactivity determined. (B) 0.8 µCi of [^{3}H]-leucine in a volume of 0.2 ml was added and the hot 5% trichloroacetic acid-insoluble radioactivity determined

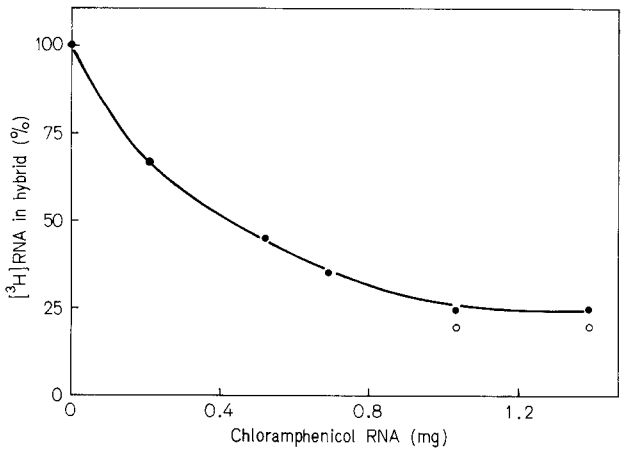

Fig. 2. *Competition of the RNA synthesized in an* E. coli *cell-free system directed by Φ29 DNA by unlabeled chloramphenicol Φ29-induced RNA synthesized* in vivo. The RNA produced *in vitro* and labeled with [^{3}H]UTP, and the unlabeled chloramphenicol RNA, induced by phage Φ29 *in vivo*, were prepared as indicated in Materials and Methods. To each filter, containing 0.5 µg of denatured Φ29 DNA, ^{3}H-labeled RNA synthesized *in vitro* (2500 counts/min) and the indicated amounts of chloramphenicol (●) or late (○) RNA produced *in vivo* were added. 40% of the input RNA hybridized to the DNA filters in the absence of competitor RNA

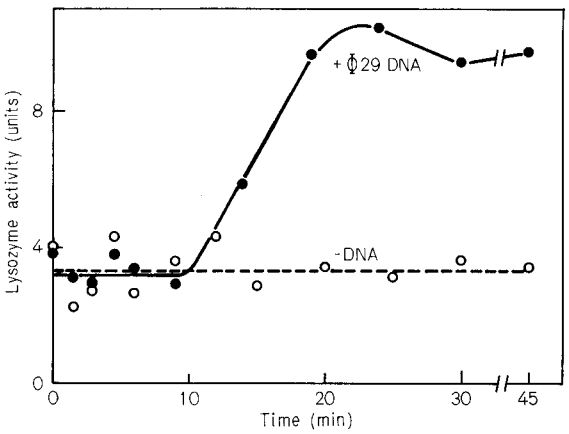

Fig. 3. *Lysozyme synthesis directed by Φ29 DNA in an* E. coli *cell-free system.* The incubation for protein synthesis was carried out in the presence and absence of linear Φ29 DNA, in a final volume of 0.6 ml, as described in Materials and Methods. At the times indicated, aliquots of 50 µl were removed, added to filters containing *E. coli* labeled with [^{3}H]diaminopimelic acid in 0.5 ml of 0.1 M ammonium acetate, incubated for 4 h at 37 °C and the radioactivity released from the filters determined. The radioactivity released from a control, without protein fraction added, was subtracted in all cases

activity; (b) by analysis of the proteins on polyacrylamide gels in the presence of sodium dodecylsulfate. Lysozyme-like activity is induced in *B. subtilis* infected with phage Φ29 [10]. This activity has been shown to be synthesized in *E. coli* cell-free systems directed by several phage DNAs included phage Φ29 [13]. As shown in Fig. 3 the synthesis of lysozyme directed by Φ29 DNA takes place after a 10-min lag and it is maximal at about 20 min. The analysis of labeled proteins on polyacrylamide gels in the presence of sodium dodecyl sulfate revealed the synthesis of several proteins which were absent from the control without DNA (Fig. 4A). Coelectrophoresis of the proteins synthesized *in vitro* with those induced in Φ29-infected cells *in vivo* late after infection (where all Φ29 proteins are labeled) [5] showed good correlation as

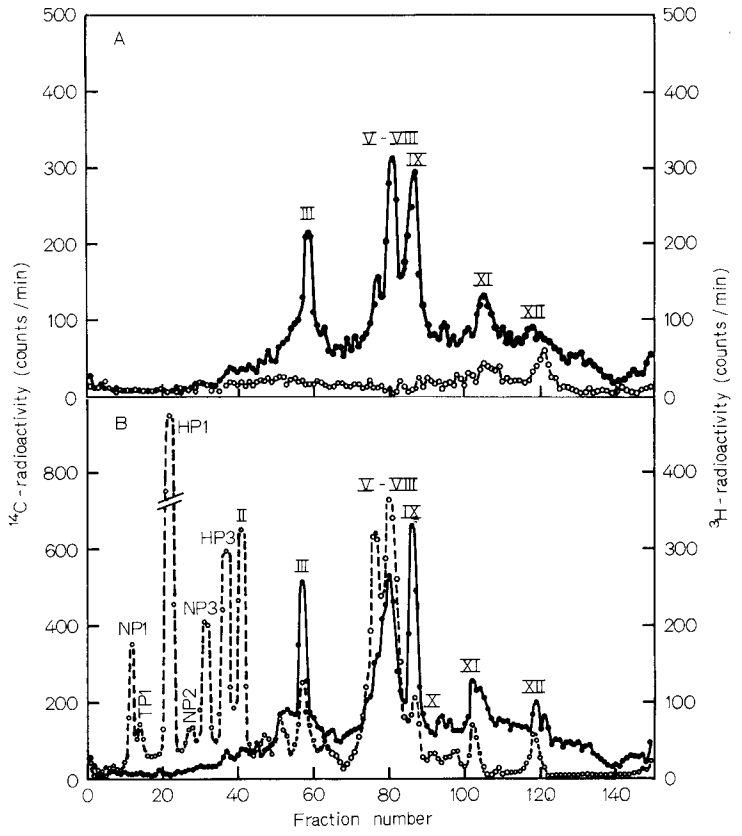

Fig. 4. *Polyacrylamide gel electrophoresis of the proteins synthesized in an* E. coli *cell-free system directed by Φ29 DNA.* (A) An aliquot of 50 µl containing the proteins synthesized *in vitro* in the presence of Φ29 DNA, labeled for 17.5 min with [^{14}C]leucine (15 µCi/ml, 312 µCi/µmol) and chased for 2.5 min with a 100-fold excess of unlabeled leucine, was mixed with the same aliquot of proteins synthesized in the absence of DNA pulse-labeled with [^{3}H]leucine (50 µCi/ml, 1000 µCi/µmol) as indicated above. The mixture was treated with RNase (1 µg) and DNase (1 µg) and prepared for electrophoresis as described in Materials and Methods. (B) A 50-µl aliquot of the proteins synthesized *in vitro* in the presence of Φ29 DNA as described in (A) labeled with [^{14}C]-leucine, was mixed with a sample containing proteins synthesized *in vivo*, after infection of *B. subtilis* 110 NA with phage Φ29, labeled with [^{3}H]leucine in a 20–23-min pulse as described [5]; the mixture was prepared for electrophoresis as indicated in (A). Electrophoresis was carried out in 15-cm-long gels containing 12.5% acrylamide, 8 M urea and 0.1% sodium dodecyl sulfate [5]. (●——●) [^{14}C]leucine; (○- - -○) [^{3}H]leucine

far as early proteins are concerned (Fig. 4B). Most early proteins (III, V–IX, XI and XII) were synthesized *in vitro*. The only protein missing was protein I, a minor protein which is normally synthesized early after infection and which moves like protein HP3 in the gel. No late proteins, either structural (NP1, TP1, HP1, NP2, NP3, HP3) or non-structural (II, IV and X), were synthesized in detectable amounts *in vitro*.

DISCUSSION

Phage Φ29 induces *in vivo* the synthesis of two main classes of phage-specific RNA: the early RNA which is synthesized in the presence of chloramphenicol, and the late RNA which requires the synthesis of phage-induced proteins [7–10]. The *E. coli* system *in vitro* synthesizes RNA which has the same sequences as has the chloramphenicol RNA (early RNA synthesized *in vivo* in the presence of chloramphenicol). The latter RNA competes up to 75% with that produced *in vitro* from *E. coli*. The percentage of competition does not increase essentially when late RNA, containing most of the early species, is used as the competitor (80% at saturation). These results indicate that most of the RNA species synthesized *in vitro* under the direction of Φ29 DNA are early RNAs. No late RNA species seem to be synthesized *in vitro* as suggested by the fact that no further competition is obtained when late RNA is used as competitor. The fact that only 75 to 80% of competition is obtained by using chloramphenicol or late RNA, respectively, can be explained from the fact that not all the early RNA species are present in the chloramphenicol or late RNAs [9].

Analysis of the proteins that had been synthesized *in vitro* with linear Φ29 DNA as template, demonstrates that all early proteins are synthesized except one minor protein. No late proteins can be detected on gels. In addition, Φ29 lysozyme synthesis *in vitro* was seen suggesting that it is an early protein. Experiments to be reported elsewhere indicate the existence of three promoters, located in different positions on Φ29 DNA, recognized by *B. subtilis* RNA polymerase and involved in the transcription of the early genes. The results presented suggest that *E. coli* RNA polymerase is recognizing the correct early promoters on Φ29 DNA. Furthermore, the mechanism(s) which controls late gene expression *in vivo* is not operating *in vitro*.

We have shown that Φ29 DNA can be isolated from the phage particle as a circle closed non-covalently by a protein [2]. On the other hand, it has been reported that the conformation of the DNA may play an important role in the control of transcription [17]. A possibility is that for the transcription of late genes in phage Φ29, the DNA has to circularize. We are presently isolating circular Φ29 DNA in amounts high enough to study its transcription and translation products in the *E. coli* cell-free system. The existence of other possible protein factor(s) which might control late gene expression in phage Φ29 using as an assay the synthesis of late RNA and/or protein in the cell-free system *in vitro* is also being studied.

This investigation has been aided by a grant from the *Comisión Asesora para el Desarrollo de la Investigación Científica*. J. L. C. and F. J. are Fellows of *Fondo Nacional para la Formación de Personal Investigador*. We are grateful to Drs P. Herrlich and M. Schweiger for their help in the initial experiments and for critically reading the manuscript, to J. de la Torre for the hybridization experiments and to J. López for the growth of the cells.

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