DNA-Directed Synthesis In Vitro of T4 Phage-Specific Enzymes
(deoxynucleotide kinase/α-glucosyl transferase/monovalent cations/initiation, completion, size of messenger RNA)

PETER J. NATALE AND JOHN M. BUCHANAN

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139

Contributed by John M. Buchanan, July 5, 1972

ABSTRACT The synthesis of deoxynucleotide kinase (EC 2.7.4.2) in vitro by a preparation consisting of T4 bacteriophage DNA and a cell-free extract of Escherichia coli has been reported. A study of the role of monovalent cations in the synthesis of this enzyme as well as α-glucosyl transferase (EC 2.4.1.2) shows that potassium ions are required for maximal enzyme production. Examination of the DNA-directed system indicates that potassium ions are more effective than ammonium ion in the translation of messenger RNA for the formation of the biologically active proteins studied. From a comparison of the magnitude of the effect of ions on both the DNA- and RNA-directed systems, we conclude that potassium ions may also have a marked stimulatory effect on transcription of the deoxynucleotide kinase gene of T4 DNA. The time required for messenger initiation and completion and the size distribution of messenger RNA formed in vitro were also examined.

Immediately after infection of Escherichia coli by bacteriophage T4, several phage-specific enzyme activities appear that are necessary for the synthesis of phage DNA. The individual species of messenger RNA concerned with production of these "early" enzymes have been divided into two classes (immediate-early and delayed-early), depending upon whether they can be formed in vitro in the presence of chloramphenicol (1–3). The separation of early enzymes into specific groups suggests the possibility of different control mechanisms for gene expression. In order to study these mechanisms, the synthesis in vitro of several phage-specific enzymes has been used as a probe (4, 5). With this goal in mind, we have succeeded in obtaining in vitro the DNA-directed synthesis of deoxynucleotide kinase (EC 2.7.4.2) with a crude cell-free extract of E. coli.* In this report, we have examined several parameters of the synthesis of both the kinase and another phage-specific enzyme, α-glucosyl transferase (EC 2.4.1.2).

MATERIALS AND METHODS

Chemicals. Uridine diphosphate-[14C]glucose and [14C]formaldehyde were purchased from New England Nuclear; DNase (electrophoretically purified) from Worthington Biochemical Corp.; and ATP, GTP, UTP, and CTP, phosphoenolpyruvate, pyruvate kinase, 2-mercaptoethanol, and dithiothreitol from Sigma. Polyethylene glycol (Carbowax 6000) was obtained from Union Carbide Corp.; L-amino acids and RNase-free sucrose from Schwarz–Mann; and Type B6 nitrocellulose membrane filters and no. 29 glass fiber filters from Schleicher and Schuell.

* The DNA-directed synthesis of deoxynucleotide kinase has been independently reported in preliminary form from this laboratory (6) and from that of Galivan, Trimble, and Maley (7).

Cell-Free Protein Synthesis. The S-30 extract was prepared from E. coli RNase 1 as described by Cappechi (8). Different cell-free preparations exhibited consistent results when used for RNA-directed enzyme synthesis, but significant variability was observed when similar extracts were used in DNA-directed systems. This variability may be attributed to the presence of DNase, added during preparation of the S-30 fraction by the Capecchi procedure. We have found, however, that this treatment with DNase is necessary for preparation of active extracts for DNA-directed enzyme synthesis. The reaction mixture for protein synthesis (usually 0.2 ml) contained 60 mM Tris-acetate (pH 7.8), 0.14 mM of 20 amino acids, 3.0 mM ATP, 0.2 mM GTP, 0.6 mM each of CTP and UTP (omitted in RNA-directed synthesis), 5.0 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, 0.25 mM dithiothreitol, 5.0 mM polyethylene glycol (average molecular weight, 6000) and 0.025 ml of S-30 extract [containing 0.9 mg of protein, 0.25 μmol Tris-acetate (pH 7.5), 0.25 μmol magnesium acetate, 0.75 μmol KCl, 0.15 μmol 2-mercaptoethanol]. The concentrations of magnesium acetate and monovalent cations used in the incubations are described in the legends of respective experiments. Protein synthesis was directed by either RNA (520 μg/ml) or DNA (25 μg/ml) at 25° for 30 min unless indicated otherwise.

Preparation of DNA and RNA. DNA was extracted by the procedure described by Thomas and Abelson (9) from phage stocks containing 1 to 2 × 10¹² phage per ml. Template RNA was prepared from cells infected with T4 as described by Sakiyama and Buchanan (10). RNA used in DNA–RNA hybridization experiments was similarly prepared except that after the separation of phenol–water phases the final aqueous layer was dialyzed at room temperature against a solution containing 0.3 M sodium chloride–30 mM sodium citrate (pH 7.0)–0.1% sodium lauryl sulphate to remove phenol and acid-soluble materials.

Enzyme Assays. Deoxynucleotide kinase was assayed with [2H]hydroxymethyl-dCMP (0.38 μCi/mmol) as substrate as described by Wiberg et al. (11), except that after incubation the reaction mixture was passed through a 6 × 40 mm Dowex-50 (H⁺) column and collected directly into scintillation vials. The eluent was then evaporated to a volume of 0.5 ml, cooled, and counted after addition of Bray’s solution (12). Reaction mixtures incubated without DNA or RNA yielded backgrounds of 600–900 cpm, which were subtracted from all experimental values.

The activity of α-glucosyl transferase was assayed (13) by addition of 0.1 ml of the protein-synthesis reaction mixture to 0.1 ml of reagents containing 200 mM Tris·HCl (pH 7.5),
200 mM 2-mercaptoethanol, 50 mM EDTA (pH 8.0) 50 μM uridine diphosphate-[14C]glucose (50 Ci/mol), 7 μg of actinomycin D, and 100 μg of T2 DNA (extracted from T2 gt am2 phage, a gift from Dr. Helen Revel). This assay mixture was incubated at 37° for 1.5 hr. The reaction was terminated by addition of 0.2 ml of cold 10% trichloroacetic acid, and the vessels were placed in ice for 10 min. The precipitate was collected by centrifugation, the supernatant was discarded, and the precipitate was dissolved in 0.5 ml of 0.3 N KOH. After addition of 1 ml of cold 10% trichloroacetic acid, the insoluble material was collected on glass fiber filters, dried, and counted by addition of 10 ml of Liquifluor. A background count of 200–300 cpm was subtracted from all experimental values. We have found that inclusion of actinomycin D in the assay system consistently improved the amount of glucosylated DNA formed per unit enzyme, possibly by inhibition of the degradation of the DNA substrate.

Sucrose Gradients. RNA synthesized in vitro was extracted with phenol and prepared as template RNA (see ref. 10). About 3 mg of RNA were then placed on 35-ml gradients of a solution of 5–20% sucrose containing 10 mM Tris-HCl (pH 7.5) and 100 mM KCl. The RNA was sedimented at 27,000 rpm for 16 hr at 4° with a Spinco SW 27 rotor. After centrifugation, 1.4-ml fractions were collected from the bottom of the tube, trNA at 100 μg/ml was added as carrier to each fraction, and RNA was isolated by alcohol precipitation for use as template for enzyme synthesis.

DNA–RNA Competition Hybridization. The DNA–RNA hybridization technique was essentially that of Gillespie and Spiegelman (14), as described by Grasso and Buchanan (1).

RESULTS

Effect of Monovalent Cations on DNA-Directed Enzyme Synthesis. The DNA-directed synthesis of deoxynucleotide kinase in vitro is influenced by the nature of the monovalent cations present in the incubation medium (Fig. 1A). Maximal synthesis of this enzyme occurs at 110 mM K+; much less synthesis occurs in the presence of NH4+ and none with Na+ throughout all concentrations of ions tested. K+ at its optimal concentration, yields 4–5 times the stimulation observed with NH4+ at its optimal concentration (50 mM). The effect of monovalent cations on the synthesis of α-glucosyltransferase (Fig. 1B) is similar but not identical to that observed for deoxynucleotide kinase. K+ likewise causes a marked stimulation (2–3 times) over that observed in the presence of NH4+, with Na+ again failing to support enzyme synthesis.

Effect of Monovalent Cations on RNA and Protein Synthesis. In an attempt to check whether the effect by monovalent cations could be attributed to altered amounts of RNA or protein synthesized in vitro, we have examined the incorporation of [3H]UTP and [14C]leucine into acid-precipitable material. The DNA-directed synthesis of RNA, assayed as [3H]UTP incorporated into acid-precipitable material (Fig. 2A), varies somewhat depending upon the nature of the monovalent cation present. Maximal RNA synthesis occurs in the presence of about 125 mM K+. NH4+ exhibits a similar concentration profile, but the amount of RNA formed is only 70% of that observed with K+. Na+ shows a linear response with respect to ion concentration in the amount of RNA formed. In the synthesis of general protein, directed by DNA and assayed by [14C]leucine incorporation into acid-precipitable material (Fig. 2B), K+ again produces the most significant response. About 25% more incorporation of [14C]leucine into protein is observed in the presence of K+ as compared to NH4+, with Na+ failing to support any protein synthesis throughout all concentrations tested. When extracted RNA is used to direct the synthesis of general protein (Fig. 2C), K+ and NH4+ both stimulate the reaction identically throughout the range of ion concentrations tested. Na+ did not support RNA-directed general protein synthesis at

Fig. 1. Effect of monovalent cations on DNA-directed enzyme synthesis. ●, K+; △, NH4+; ○, Na+. (A) Deoxynucleotide kinase synthesis in the presence of 9.0 mM Mg++. (B) α-Glucosyl transferase synthesis in the presence of 7.0 mM Mg++. All values of α-glucosyl transferase activity have been corrected to account for the inhibitory effect of ions upon the assay of this enzyme.

Fig. 2. Effect of monovalent cations on RNA and protein synthesis. ●, K+; △, NH4+; ○, Na+. (A) DNA-directed incorporation of [3H]UTP (41.7 Ci/mol) into acid-precipitable poly-nucleotides at varied ion concentrations. 7.0 mM Mg++, was present. (B) DNA-directed incorporation of [14C]leucine (2 Ci/mol) into acid-precipitable material. 7.0 mM Mg++, was present. (C) RNA-directed incorporation of [14C]leucine (2 Ci/mol) into acid-precipitable material. 7.0 mM Mg++, was again present.
any concentration tested. Similar observations have been reported by Lubin and Emnis (15).

**Effect of Monovalent Cations on RNA-Directed Enzyme Synthesis.** Monovalent cations also exhibit striking effects on RNA- as well as DNA-directed enzyme synthesis in vitro. In experiments done with RNA extracted 8 min after infection of *E. coli* by T4 phage, synthesis of deoxynucleotide kinase (Fig. 3A) and α-glucosyl transferase (Fig. 3B) at optimal concentrations of the respective ions was 2- to 3-fold greater in the presence of K⁺ as compared to NH₄⁺. Na⁺ again was unable to support the production of active protein. In contrast to the DNA-directed synthesis of deoxynucleotide kinase and α-glucosyl transferase, the magnitude of the effect of K⁺ over that of NH₄⁺ is similar in the RNA-directed synthesis for both of these enzymes.

**Kinetics of Enzyme Synthesis.** Since both of these enzymes have been classified as "delayed-early" or "Class II" (10, 16), it was of interest to investigate the time of their appearance (Fig. 4A). At 25° deoxynucleotide kinase activity first appears at about 6 min after the start of incubation, as compared to 12 min for α-glucosyl transferase. The time differential of 6 min was further examined in order to note whether the constraint on enzyme synthesis was transcriptional or translational. These experiments were performed by addition of an inhibitor of RNA synthesis at various times after the start of the reaction with continuation of the incubation for a total of 30 min to allow for complete translation of messenger RNA. The amount of enzyme formed is thus a measure of the quantity of messenger RNA present. Rifampicin, which blocks the initiation of RNA synthesis but does not affect the completion of started chains, failed to prevent the appearance of either deoxynucleotide kinase or α-glucosyl transferase messages when added as early as 2 min after the start of incubation. The experiment shown in Fig. 4B indicates that, for both enzymes, initiation of messenger RNA synthesis starts between 0 and 2 min and continues for several minutes thereafter. To measure the time for message completion, we likewise added actinomycin D, which blocks any elongation of incompletely messenger RNA chains, at various times after the start of incubation. The experiments with actinomycin D (Fig. 4C) thus permit an estimate of the time necessary for completion of kinase and α-glucosyl transferase mRNA. For deoxynucleotide kinase, message first appears at 3 min in contrast to 9 min for α-glucosyl transferase mRNA. Since both messenger RNAs are completed 2.5 min before the appearance of enzyme activity (noted by arrow positions, Fig. 4C), translation of these messages occurs with similar efficiencies.

**Characterization of T4-Specific mRNA Synthesized In Vitro.** The additional time necessary for completion of transcription of the α-glucosyl transferase gene over that required for transcription of the kinase gene suggested that transferase mRNA might be larger than kinase mRNA. Size analysis of mRNA synthesized in *vitro* was obtained by sucrose gradient separation of RNA (Fig. 5). For deoxynucleotide kinase mRNA, a pronounced peak at 15 S was observed with some messenger distributed into the higher regions of the gradient. The size of this mRNA synthesized in *vitro* is thus the same as that formed in *vivo* after T4 phage infection of *E. coli* (10). The mRNA for α-glucosyl transferase appeared to be somewhat larger in size but with a minimum value of 20 S. This minimum size of α-glucosyl transferase mRNA is considerably smaller than that anticipated from the time of messenger completion, if the rates of chain elongation for both kinase and α-glucosyl transferase mRNAs are considered to be equivalent.

![Fig. 3. Effect of monovalent cations on RNA-directed enzyme synthesis. □, K⁺; △, NH₄⁺; O, Na⁺. (A) Synthesis of deoxynucleotide kinase in the presence of 7.0 mM Mg++. (B) Synthesis of α-glucosyl transferase in the presence of 7.0 mM Mg++. All values for α-glucosyl transferase activity have been corrected to account for the inhibitory effect of ions upon the assay of this enzyme.](image)

![Fig. 4. Kinetics of enzyme and mRNA synthesis for DNA-directed system at 7.0 mM Mg++ and 125 mM K+. (A) A large reaction mixture was incubated at 25°, and at the times indicated, samples were removed for assay of deoxynucleotide kinase (●—●) and α-glucosyl transferase (O—O). (B) The time of initiation of mRNA synthesis was determined by addition of rifampicin (10 μg/ml) at the times indicated with continued incubation for a total of 30 min before assay for deoxynucleotide kinase (●—●) and α-glucosyl transferase (O—O). (C) The time required for completion of synthesis of mRNA was determined by addition of actinomycin D (35 μg/ml) at the times indicated with continued incubation for a total of 30 min to allow the translation of completed message before assay for deoxynucleotide kinase (●—●) and α-glucosyl transferase (O—O). Arrows, appearance of enzyme activity in the absence of inhibitors of mRNA synthesis.](image)
at 250 min of the presence of 7.0 mM MgCl₂ and 125 mM KCl. The reaction mixtures were then cooled. 0.2-ml aliquots were removed for assay of deoxynucleotide kinase (---) activity and 0.1-ml aliquots for assay of α-glucosyl transferase (O-O) activity. Incubations for enzyme determinations were performed at 37°C for 45 min and 1.5 hr, respectively.

**DNA–RNA Competition Hybridization.** In preliminary studies with RNA synthesized in vitro at 37°C and extracted shortly after the appearance of kinase activity, DNA–RNA competition-hybridization experiments indicated that this RNA was mostly of immediate-early class† (6). We have investigated this aspect of the problem with RNA produced at 25°C and in order to slow down the rate of chain elongation. When radioactive RNA synthesized in vitro for 10 min at 25°C was hybridized to T4 DNA, both CM-RNA and 0–5 min RNA effectively compete with more than 95% of hybridized transcripts (Fig. 6A). A more sensitive assay for delayed-early RNA was therefore performed. When unlabeled RNA synthesized for 10 or 30 min in vitro was used to compete with 0–5 min RNA labeled in vivo (Fig. 6B), a small amount of delayed-early species was detected by 10 min. As can be seen from Fig. 4C, RNA produced in vitro at 10 min would contain about 70% of the messenger capacity for kinase and 18% of the messenger capacity for transfase that one would expect if the incubation for RNA synthesis had been of 20 min duration. These results with specific species of mRNA confirm our estimate obtained by the DNA–RNA competition-hybridization technique that at 10 min of incubation at 25°C some delayed-early messages have been produced but that a significant portion is formed after this time. Continued incubation at 25°C increases the amount of delayed-

† “Immediate-early” or “Class I” RNA is defined as viral-specific transcripts synthesized in vivo in the presence of chloramphenicol (1, 2). Species representing this class are obtained by extraction of RNA 5 min after infection in the presence of chloramphenicol (CM-RNA). “Delayed-early” or “Class II” RNA consists of those viral-specific transcripts that are produced before the onset of DNA synthesis but are not formed in the presence of chloramphenicol. RNA extracted at 5 min is a composite of both “immediate-early” and “delayed-early” species (0–5 min RNA).

**DISCUSSION**

In this paper, we have reported the synthesis in vitro of deoxynucleotide kinase programmed by DNA extracted from T4 phage. For the DNA-directed synthesis of other enzymes (4, 5), usually a combination of both NH₄⁺ and K⁺ or NH₄⁺ alone has served as the requirement for monovalent cation. We have examined the effect of monovalent cations on the synthesis in vitro of deoxynucleotide kinase as well as a second delayed-early enzyme, α-glucosyl transferase.

The effect of monovalent cations on DNA-directed enzyme synthesis indicates an ability of K⁺ to promote selective transcription of specific segments of DNA for production of certain messages, for example, deoxynucleotide kinase mRNA. This conclusion is drawn from a comparison of the effect of monovalent cations on DNA- and RNA-directed enzyme synthesis in vitro. Stimulation by K⁺ is 2- to 3-fold higher than by NH₄⁺ when synthesis of either deoxynucleotide kinase or α-glucosyl transferase is directed by exogenous RNA (Fig. 3A and B). A similar difference in the stimulation by K⁺ and NH₄⁺ is observed for DNA-directed synthesis of α-glucosyl transferase (Fig. 1B). For the DNA-directed synthesis of deoxynucleotide kinase, however, there is a 4- to 5-fold greater synthesis of enzyme in the presence of K⁺ as compared to NH₄⁺ (Fig. 1A). These data suggest that K⁺ may have a pronounced effect on the synthesis of kinase mRNA.

Besides this transcriptional effect of K⁺, this cation also acts preferentially in the translation of mRNA for production of the biologically active proteins studied. This conclusion is evident from a comparison of the effects of monovalent cations...
on the synthesis of general proteins and the two phage-specific enzymes. In systems primed with extracted RNA, no distinctions are observed between K⁺ and NH₄⁺ for synthesis of general proteins (Fig. 2C), but for synthesis of either deoxynucleotide kinase or α-glucosyl transferase (Figs. 3A and B) incubation with K⁺ results in a 2- to 3-times higher yield than with NH₄⁺. These effects imply a preference for K⁺ during the translation of messenger RNA for synthesis of active enzymes in vitro.

In addition to these observations, we have noted that the size of the mRNA synthesized in vitro for α-glucosyl transferase does not agree with that anticipated from kinetic studies. This discrepancy arises if the rates of synthesis of both kinase and α-glucosyl transferase mRNA are assumed to be equivalent. The rate of chain elongation for deoxynucleotide kinase mRNA, when derived from the time for message completion and its minimal size of 15 S (molecular weight, 4.6 × 10⁶ (1)) calculated to be 7.2 nucleotides per sec at 35°C. This value is in good agreement with that determined for total RNA synthesis directed by T4 DNA and purified RNA polymerase (17). If the rates of chain elongation are considered to be equivalent for all messages synthesized in vitro, the size of α-glucosyl transferase mRNA would be expected to be much larger than the 20 S minimum value (molecular weight, 8.4 × 10⁶) observed from sucrose gradient analysis. When the rate of α-glucosyl transferase messenger synthesis was calculated in a manner similar to that used for kinase mRNA, a value of 4.4 nucleotides per sec was obtained. It is difficult to assess the significance of the differences in the calculated rates of synthesis of the mRNAs for the two enzymes in view of the assumption inherent in the estimations. However, our data may indicate that various segments of T4 DNA may be transcribed at different rates (an inference made from other data on the rate of T4 DNA transcription (18, 19).

Black and Gold (4) have summarized possible models to explain the discrepancies dealing with control of T4 DNA-directed RNA synthesis in vivo and in vitro. The effect of chloramphenicol noted by them for β-glucosyl transferase has been observed by us for deoxynucleotide kinase. Specifically, messenger RNA for both enzymes may be formed in vitro in the presence of chloramphenicol but not in vivo. Black and Gold (4) favor the hypothesis that chloramphenicol affects T4 DNA transcription by exerting a polarity effect rather than by blocking synthesis of a phage-specific protein needed for neutralization of a regulator of RNA synthesis (e.g., β factor) or for initiation of delayed-early RNA production.

1 The number of nucleotides was approximated from the formula derived by Spirin, A. S. (1961) Biochimiya 26, 511, and based on the assumption of an average molecular weight of 350 per nucleotide.

In this connection, evidence has been presented for the hypothesis that initiation of RNA synthesis occurs on immediate-early segments of T4 DNA and that synthesis of delayed-early RNA represents a passive extension of these immediate-early RNA strands (4, 20, 21). We would like to note here that the kinase mRNA, which is classified as delayed-early, is of relatively small size and is produced rather early in the incubation system in vitro. Galivan, Trimble, and Maley (7) have shown that it is formed first among the five species of mRNA examined by them and, in fact, appears earlier than the mRNA for dCMP hydroxymethylase, which is believed to be an example of immediate-early RNA (9). If one assumes that initiation occurs only on immediate-early promoter sites, then the transcription unit carrying the kinase gene may be a relatively small one.

This investigation was supported by a grant-in-aid (CA 02015) from the National Cancer Institute, National Institutes of Health.