STIMULATION OF RNA SYNTHESIS BY DINUCLEOTIDES WITH EUKARYOTIC RNA POLYMERASE

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Received 20 June 1979
Revised version received 27 July 1979

1. Introduction

Regulation of gene expression occurs at many steps, among which are transcription, processing, modification, transport and translation of RNA. At the level of transcription, two major observations have suggested that RNA polymerase plays a critical role in the regulation of transcriptional activity: the discovery of sigma factor, a subunit of *Escherichia coli* DNA-dependent RNA polymerase, which is involved in the positive control of gene activity [1–4]; and the successful purification and identification of multiple forms of eukaryotic RNA polymerases [5]. Although the exact nature of the contribution of RNA polymerase in selection of specific classes of initiation is unresolved [6–8], it has been shown that the fidelity of in vitro transcription of chromatin is markedly affected by the concentration of RNA polymerase [9].

Transcription is influenced by a variety of factors: the search for promoter sites by DNA-dependent RNA polymerase; the formation of stable complexes between the enzyme and the template; the initiation of RNA synthesis; the rate of elongation of nascent RNA; and the termination of RNA synthesis. The initiation step in RNA synthesis is rate limiting and requires substantially higher nucleotide concentrations than does chain elongation [10–12]. Therefore at concentrations of nucleotides below the K_m for initiation of transcription, RNA polymerase can synthesize RNA by extending a dinucleotide primer that is complementary to sequences within an initiation site. Studies using bacteriophage T7 and the *E. coli* lactose and histidine operons have demonstrated that functional RNAs can be synthesized using specific dinucleotide primers. This system has permitted a closer analysis of the base sequence of the promotor for the *E. coli* lactose operon, as well as the turning on or off of three distinct promoters of phage T7 as a result of changes in ionic strength and temperature [13–16].

In this communication, we report experiments utilizing dinucleotide primers to probe the types of initiation sequences selected from human DNA by a prokaryotic and eukaryotic RNA polymerase. Although the two enzymes are stimulated by the same dinucleotides, they differ markedly in their efficiencies of utilization of these dinucleotides.

2. Materials and methods

2.1. *DNA purification*

DNA was purified from fresh human placenta by a modification of the method in [17]. All phenol steps were carried out using recently distilled phenol saturated with 0.1 × SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) with the aqueous phase adjusted to pH 7.0. The single- and double-strand molecular weights of the purified DNA were determined by boundary sedimentation analysis [18]. The

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mass ratio of double-strand to single-strand DNA was 1.97.

2.2. *Isolation of* E. coli *DNA-dependent RNA polymerase*

DNA-dependent RNA polymerase was purified according to the method in [19]. The enzyme preparation contained the sigma subunit, was 96% pure as demonstrated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and had spec. act. 270 units/mg protein. One unit of enzyme activity is equal to 1 nmol [^{3}H]UMP incorporated in 10 min at 37°C using equimolar concentrations of nucleotides. The polymerase had no detectable DNase contamination as judged by nicking of closed circular bacteriophage PM2 DNA (this assay can detect as little as 4 ng DNase I/ml) and no RNase activity on 18 S r [^{14}C]RNA as analyzed by agarose–urea polyacrylamide gels. The amount of *E. coli* RNA polymerase needed to saturate 5 μg human DNA was determined (fig.1A). Four units of *E. coli* RNA polymerase were used in the experiments that included rifampicin (10 μg/ml), 5.2 units in those without rifampicin.

2.3. *Wheat-germ RNA polymerase II extraction*

Wheat-germ DNA-dependent RNA polymerase II, prepared according to [20], was 90% pure polymerase II as determined by electrophoresis in 0.1% SDS, 12% polyacrylamide gels, and had spec. act. 188 units/mg protein. Saturating amounts (5.2 units) of wheat-germ RNA polymerase II were used in the subsequent experiments (fig.1B). Wheat-germ RNA polymerase was also free of RNase and DNase activities when assayed as described above.

2.4. *Characterization of the system of dinucleotide initiation of transcription*

2.4.1. Dinucleotide purity as assessed by thin layer chromatography

The dinucleotides GpA, GpC, GpU, GpG, ApG, ApA, ApC, ApU (Miles Res. Labs) were assayed for contaminating mononucleoside triphosphates by PEI-cellulose chromatography according to [21]. No mononucleoside triphosphate contaminants were revealed by this technique. The minimum level of contamination detectable by this system is ~2%,

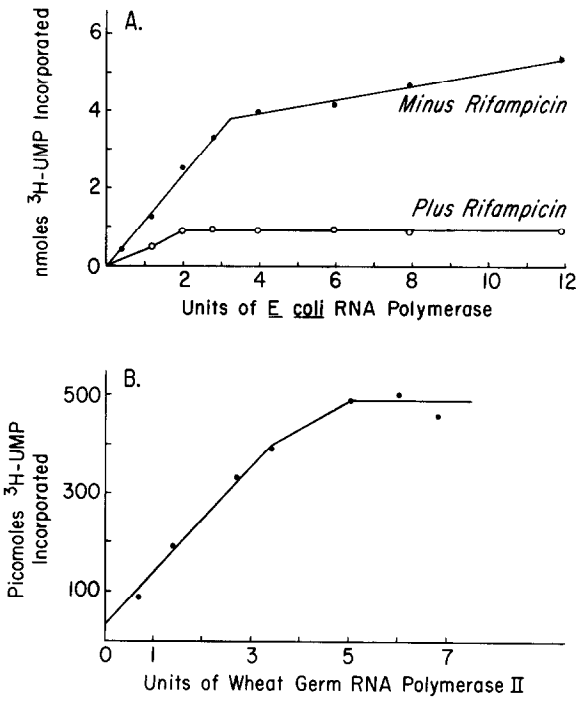

Fig.1. Effect of RNA polymerase concentration on RNA synthesis. See text for conditions. (A) *Escherichia coli* RNA polymerase saturation curve. (B) Wheat-germ RNA polymerase II saturation curve.

which would correspond to <4 μM in the usual dinucleotide initiation assay.

2.4.2. Effect of nucleotide concentration on dinucleotide initiation of transcription

The reaction mixture (0.25 ml) contained 80 mM Tris–HCl (pH 7.8), 50 mM KCl, 12 mM $MgCl_2$, 4.8 mM 2-mercaptoethanol, 100 μM GpA, 4 units *E. coli* RNA polymerase, 5 μg placental DNA, and varying concentrations of ATP, CTP, GTP, and [^{3}H]UTP (5.4×10^5 cpm/nmol). Rifampicin (10 μg/ml) was added after a 1 min incubation at 37°C to prevent reinitiation, and transcription continued for 10 min at 37°C. The reaction was stopped by quickly cooling to 0°C; 2 drops of 1 mg/ml bovine serum albumin and 2 ml 5% trichloroacetic acid containing 10 mM pyrophosphate were added and the precipitate was collected and washed onto glass fiber filters. The filters were dried and the radioactivity was counted in a liquid scintillation counter.

2.4.3. Determination of optimum dinucleotide concentration for maximal stimulation of RNA synthesis

The reaction mixtures were as described in section 2 with 2 μM ATP, CTP, GTP, and [^{3}H]UTP (5.4×10^6 cpm/nmol) and varying concentrations of GpA or GpC. Reactions mixtures were incubated at 37°C for 1 min, at which time 10 μg/ml rifampicin was added and incubation continued for 10 min. The samples were then trichloroacetic acid-precipitated and the radioactivity counted as described.

2.4.4. Effect of rifampicin on dinucleotide-initiated RNA synthesis

The reaction mixtures were as described in section 2 with 100 μM of either GpA or GpC, and 2 μM ATP, CTP, GTP and [^{3}H]UTP (5.4×10^6 cpm/nmol). Reaction mixtures were incubated for 0, 15, 30, 45 or 60 s before the addition of rifampicin (10 μg/ml); the reaction was allowed to proceed for 10 min at 37°C.

2.4.5. Insertion of the dinucleotide into the 5′-end of a growing RNA chain

The reaction mixtures were as described in section 2 with 200 μM [α-^{32}P]ATP (500 Ci/nmol), 2 μg poly d(A–T) and 4 units of *E. coli* RNA polymerase. Following incubation for 10 min at 37°C, the entire sample was spotted on a DEAE filter disc, and washed 3 times with 5% Na_2HPO_4, water, ethanol and ether. The dried disc was counted for ^{32}P by Cerenkoff radiation before eluting the labeled trinucleotide from the filter with 2 M pyridine adjusted to pH 4 with HCOOH. After flash evaporating 3 times and dissolving in water, the sample was again flash evaporated and then dissolved in 0.1 M KOH and incubated at 37°C for 2 h. The hydrolyzed trinucleotide was spotted on PEI cellulose and chromatographed in 0.25 M LiCl which permits migration of nucleoside monophosphates. Both Ap and Up standards were chromatographed on the same thin-layer sheet. The standards were located by an ultraviolet lamp and the migration distances measured. The radioactive sample was located by cutting the chromatogram in 1 cm slices, eluted with 0.5 ml 2 M pyridine adjusted to pH 4 with HCOOH, and counted in 10 ml Omnifluor (New England Nuclear Corp.).

2.5. *Preparation of RNA transcripts*

The reaction mixture (0.5 ml) containing 80 mM Tris–HCl (pH 7.8), 50 mM KCl, 4.8 mM 2-mercaptoethanol, 12 mM $MgCl_2$ or 12 mM $MnCl_2$, 10 μg placental DNA, 200 μM GpA, 2 μM ATP, CTP, GTP and [^{3}H]UTP (5.4×10^6 cpm/nmol) and 10.4 units of either *E. coli* RNA polymerase or wheat-germ RNA polymerase II was incubated for 10 min at 37°C. The mixture was then treated with DNase I (20 μg/ml) treatment for 30 min at room temperature. After 2 phenol and 2 chloroform–isoamyl alcohol (99:1) extractions, the transcripts were separated from free nucleotides by chromatography on Sephadex G-50 (Pharmacia). Transcripts were also prepared using 200 μM, ATP, CTP, GTP and [^{3}H]UTP and purified by the same procedure.

3. Results

The concentration of nucleoside triphosphates necessary for maximal dinucleotide stimulation of RNA synthesis was determined using various concentrations of ATP, CTP, GTP, and [^{3}H]UTP, 100 μM GpA, placental DNA, and *E. coli* RNA polymerase as described in section 2. A 3-fold stimulation of RNA synthesis was obtained at 2 μM nucleotide (fig.2A). This concentration of nucleoside triphosphates was used in the following experiments. The dinucleotide concentration (GpA or GpC) needed for stimulation of RNA synthesis in the presence of 2 μM nucleoside triphosphates was critical at <100 μM (fig.2B).

Two methods were used to determine whether or not a dinucleotide could be used to prime RNA synthesis using human DNA as a template:

(i) Rifampicin sensitivity of the dinucleotide, RNA polymerase–template complex;
(ii) The incorporation of [α-^{32}P]ATP into a nascent poly r (A–U) chain using poly d(A–T) as a template and ApU as primer.

The sensitivity of the dinucleotide-primed reaction to rifampicin was assessed in the following way. The reaction mixture was preincubated with 2 μg DNA and 1.6 units *E. coli* RNA polymerase for 1 min at 37°C before adding 10 μg/ml rifampicin. After rifampicin addition, incubation continued for 1 min at 37°C, then 100 μM NTPs including [^{3}H]UTP

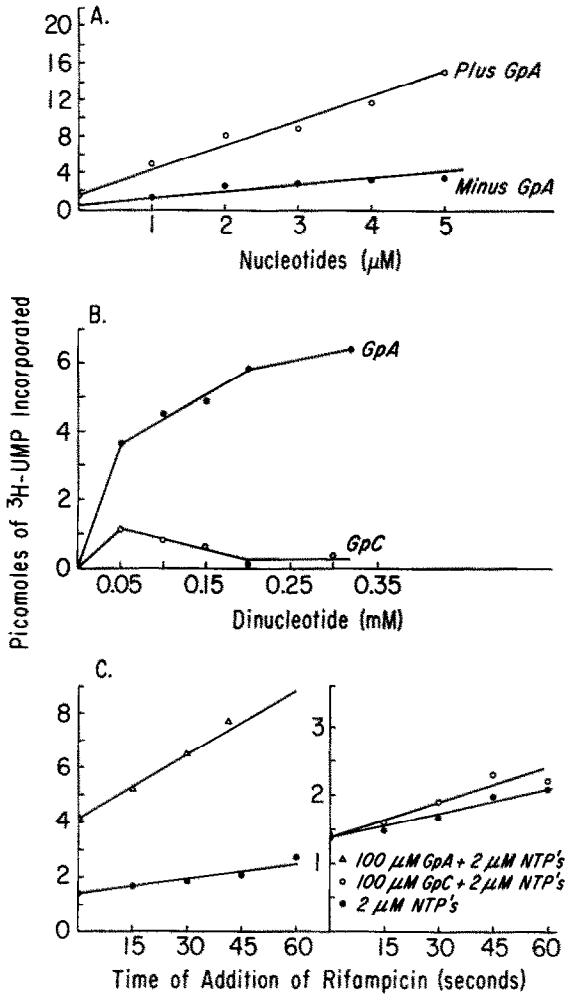

Fig.2. Characterization of dinucleotide initiation of transcription. See text for conditions. (A) Effect of nucleotide concentration upon initiation of transcription. (B) Effect of dinucleotide concentration upon initiation of transcription. (C) Effect of time of addition upon dinucleotide primed transcription.

(1.4×10^5 cpm/nmol) were added and incubation continued for 10 min (data not shown). The extent of RNA synthesis both with and without dinucleotide primers was only slightly above background, indicating that the complex formed by preincubation of enzyme, template and dinucleotide is still rifampicin sensitive. However, if rifampicin, dinucleotides and low levels of triphosphates are added simultaneously, a strong primer, GpA, stimulates RNA synthesis 3–4-fold (fig.2C). Insertion of a dinucleotide into the 5′-end of nascent poly r(A–U) was tested using ApU as a primer to synthesize the trinucleotide ApUpA from poly d(A–T) and [α-^{32}P]ATP by *E. coli* RNA polymerase. Alkaline hydrolysis of the resultant trinucleotide demonstrated that the ^{32}P-label resided in 3′-UMP, confirming dinucleotide insertion into the 5′-end of the transcript. Three types of control were analyzed in this experiment. The reaction mixture:

(1) In the absence of dinucleotide primer;
(2) In the absence of poly d(A–T);
(3) In the absence of *E. coli* RNA polymerase.

The first control showed the dinucleotide dependence of the initiation step and also suggested a lack of ligase activity in *E. coli* RNA polymerase. If [α-^{32}P]ATP were the substrate for a ligase reaction, ApApAp would be produced, and upon alkaline hydrolysis of the oligoandenylate, the ^{32}P-label would

Table 1
Dinucleotide initiation of transcription from DNA at enzyme excess

Dinucleotide added	[^{3}H]UMP (pmol) incorporated	Relative stimulation
GpA	115	2.0
GpC	22	0.4
GpU	82	1.5
GpG	78	1.4
ApA	90	1.6
ApC	77	1.4
ApU	91	1.6
ApG	126	2.2
CpA	150	2.7
UpA	161	2.9
CpC	93	1.7
UpG	137	2.4
CpG	95	1.7
–	56	1.0

The reaction mixture containing 80 mM Tris–HCl (pH 7.8), 50 mM KCl, 12 mM $MgCl_2$, 4.8 mM 2-mercaptoethanol, 2 μg DNA and 1.6 units *E. coli* RNA polymerase was incubated with the various dinucleotides (200 μM) as well as 2 μM each of ATP, CTP, GTP and [^{3}H]UTP (5.4×10^6 cpm/nmol) and the mixture incubated for 1 min at 37°C. Rifampicin (10 μg/ml) was added and the samples incubated for 1 min at 37°C, then 200 μM ATP, CTP, GTP and UTP were added and the samples incubated for 10 min at 37°C. The precision of these experiments was ±10%. The most efficient primers are indicated by underlining

reside with the 3′-AMP produced. Controls (2) and (3) demonstrated the template and RNA polymerase dependence of the initiation reaction; in both cases counts were not trapped on the DEAE filters.

Even though *E. coli* RNA polymerase preferentially initiates transcription from DNA with purine nucleoside triphosphates [22], CpC, CpU and UpC will also prime RNA synthesis from bacteriophage T7 at the same sites where synthesis is primed from purine-containing nucleotides [14]. To find the most efficient primers with human DNA, the experiments described here utilize dinucleotide primers with both purines and pyrimidines in the 5′-position of the dinucleotide. RNA synthesis from a DNA template can be primed by all of the dinucleotides tested (table 1); UpA, CpA, UpG, GpA and ApG are the most efficient primers. Dinucleotide stimulation of transcription from human DNA appears to be similar to phage systems, in that the pyrimidine dinucleotides will also prime RNA synthesis. The dinucleotide primers that have a purine at the 3′-position of the dinucleotide are more efficiently utilized by the RNA polymerase.

Since these results indicate that dinucleotide primers can be used by *E. coli* RNA polymerase in initiation of transcription of human DNA, a similar set of experiments was carried out with wheat-germ RNA polymerase II to ascertain whether or not the two enzymes select similar initiation sequences. These experiments were done in the absence of rifampicin as it does not prevent reinitiation with eukaryotic RNA polymerase. These data demonstrate that relative stimulation by dinucleotides on human DNA with *E. coli* RNA polymerase is affected by the presence of rifampicin (table 2). The dinucleotides giving greatest stimulation in the presence of rifampicin are: (1) UpA; (2) CpA; (3) UpG; (4) ApG (table 1), however in the absence of rifampicin the order is: (1) GpA; (2) ApG; (3) UpG; (4) GpG. Thus it is likely that the dinucleotides UpA and CpA initiate relatively inefficiently at a lot of sites, whereas GpA, ApG, UpG and GpG initiate at fewer sites, but more efficiently. Using wheat-germ RNA polymerase II,

Table 2
Comparison of wheat-germ RNA polymerase II and *E. coli* RNA polymerase using dinucleotide initiation of RNA synthesis

	E. coli RNA polymerase		Wheat germ RNA polymerase II	
Dinucleotide added	[^{3}H]UMP (pmol) incorporated	Relative stimulation	[^{3}H]UMP (pmol) incorporated	Relative stimulation
GpA	13.0	7.2	30.1	58.9
GpC	1.6	0.8	0.4	0.8
GpU	8.1	4.5	6.2	12.2
GpG	8.9	4.9	21.5	42.1
ApA	7.6	4.2	2.2	4.3
ApC	5.2	2.9	2.1	4.1
ApU	5.0	2.8	0.9	1.8
ApG	11.7	6.5	16.5	32.2
CpA	7.8	4.3	4.2	8.3
UpA	7.8	4.3	5.4	9.7
CpC	5.2	2.8	5.1	10.1
UpG	10.6	5.9	8.6	17.8
CpG	5.6	3.1	2.2	4.4
–	1.8	1.0	0.5	1.0

The reaction mixture containing 80 mM Tris–HCl (pH 7.8), 50 mM KCl, 12 mM $MgCl_2$ or 8 mM $MnCl_2$, 4.8 mM 2-mercaptoethanol, 200 μM dinucleotide, 2 μM each of ATP, CTP, GTP, [^{3}H]UTP (5.4 × 10^6 cpm/nmol), 2 μg DNA and 5.2 units *E. coli* RNA polymerase or 5.2 units wheat-germ RNA polymerase II was incubated for 10 min at 37°C. Relative stimulation by the most efficient primers is underlined

the dinucleotides giving the best stimulation are: (1) GpA; (2) GpG; (3) ApG; (4) UpG. These results are similar to the results obtained with *E. coli* RNA polymerase in the absence of rifampicin. However, the degree of stimulation over the control for the most efficient dinucleotides differs markedly between the two RNA polymerases. For example, there is a 58.9-fold stimulation of RNA synthesis using GpA as a primer and wheat-germ RNA polymerase, while GpA with *E. coli* RNA polymerase gives only a 7.2-fold stimulation. These data suggest several possibilities among which are:

(i) That wheat-germ RNA polymerase utilizes the primers more efficiently than the prokaryotic enzyme;

(ii) That the apparent K_m value for [NTP] for elongation may be different for the two enzymes and that at 2 μM NTPs, the rate of elongation with wheat-germ RNA polymerase II is less limited by the nucleotide concentration than is the case with *E. coli* RNA polymerase;

(iii) That the sizes of the transcripts are substantially different; or

(iv) That the two enzymes are transcribing different regions of the DNA.

In order to distinguish among these alternatives the following experiments were performed. GpA was used to initiate transcription of DNA in the presence of low nucleotides and either wheat-germ RNA polymerase II or *E. coli* RNA polymerase. The transcripts were purified as described in section 2 and were sized by denaturing agarose–urea gels using 28 S rRNA, 18 S rRNA, 5 S RNA and 4 S RNA as markers. Purified GpA-initiated transcripts made by wheat-germ RNA polymerase II and *E. coli* RNA polymerase in 2 μM nucleotides were not only very similar in size, ~100 nucleotides with a range from 80–150 nucleotides, but were also very homogeneous with respect to size distributions. Because the GpA-initiated transcripts synthesized by wheat-germ RNA polymerase II and *E. coli* RNA polymerase are nearly the same size, the dramatic difference between the two enzymes in the relative stimulation (table 2) of RNA synthesis by the GpA primer cannot be accounted for by transcript size. Similar transcript sizes were also obtained in the absence of the dinucleotide primer. Thus, the amount of RNA synthesis detected in this system reflects differences in the frequency of initiations by the enzymes.

Transcription of DNA using 200 μM nucleotides results in a difference in the sizes of transcripts generated by *E. coli* RNA polymerase compared to wheat-germ RNA polymerase. The size of the transcripts made from DNA by wheat-germ RNA polymerase at 200 μM nucleotides, shows a narrow distribution ranging from 100–750 nucleotides with a mean of 550 nucleotides. On the other hand, the transcripts synthesized by *E. coli* RNA polymerase are about twice as large, ranging in nucleotide length from 80–2500, with a mean length of 1000 nucleotides (data not shown). These data suggest that transcription with both the eukaryotic and prokaryotic enzymes is stimulated by the same dinucleotides, however, the degree of stimulation for the most efficient dinucleotides differs dramatically with the two enzymes. Whether this is due to efficiency of utilization of these dinucleotides or the rate of elongation has yet to be determined.

4. Discussion

The purpose of this investigation was to examine the role played by DNA-dependent RNA polymerase in sequence selection during the transcription process. The observations described here indicate that dinucleotide initiation of transcription can be effectively used to partially circumvent the problem of a vast heterogeneous population of initiation sites on eukaryotic DNA, and thus allow one to probe the role of sequence selection in transcription. Therefore, the dinucleotide system of initiation of transcription, in conjunction with a bacterial RNA polymerase, a eukaryotic polymerase and purified DNA, have been used to answer questions regarding sequence selectivity in initiation of transcription. It is evident from dinucleotide-primed transcription DNA, using wheat-germ RNA polymerase or *E. coli* RNA polymerase, that multiple initiation sites exist which are recognized with different affinities. The enzymes were stimulated to different extents by each of the 13 dinucleotides. Those which maximally stimulated the bacterial enzyme were somewhat different from those which stimulated the wheat germ enzyme. These data suggest that wheat-germ RNA polymerase prefers

those dinucleotide primers with purine ribonucleotides in the first and second position of the nascent RNA chains.

In conclusion, our data show that a prokaryotic and eukaryotic RNA polymerase differ markedly in their response to the same priming dinucleotides.

Acknowledgements

This investigation was supported by grants from the Robert A. Welch Foundation (G-267) and the National Institute of Health (CA 20124 and GM 23965). We thank Drs Roger Stone and Stan Blatti for the wheat-germ RNA polymerase. This work is taken from the dissertation of P.A.S. submitted to The University of Texas at Houston Graduate School of Biomedical Sciences in partial fulfillment of the requirement for the PhD degree.

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