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Termination of transcription by bacteriophage T3 RNA polymerase: Homogeneous 3′-terminal oligonucleotide sequence of *in vitro* T3 RNA polymerase transcripts

(T3 late RNA synthesis/specific stop signals/dihydroxyboryl-cellulose chromatography)

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ABSTRACT **RNA was synthesized *in vitro* from a T3 DNA template by T3 RNA polymerase and subsequently separated into seven discrete size classes (molecular weights ranging between 0.21×10^6 and 6.2×10^6) by electrophoresis in polyacrylamide slab gels. RNase T1-generated 3′-terminal oligonucleotide fragments were then selectively isolated from either the unfractionated total RNA or the gel-purified specific transcripts by chromatography on columns of dihydroxyboryl-cellulose. Sequence analysis of these oligonucleotide products indicated that the unfractionated transcripts as well as all the individual major RNA species examined had a unique sequence, (Gp)UpUpUpUpUpG$_{OH}$, at their 3′ termini. The specificity of this sequence, as well as the total lack of any sequence heterogeneity at the ends of these transcripts, indicates a high degree of specificity of termination during transcription in this system.**

Bacteriophage T3 RNA polymerase is a single-chain protein, of molecular weight 105,000, that recognizes specific transcription regulatory sites on T3 DNA for initiation of RNA synthesis (1–7). Under standard conditions of *in vitro* transcription with T3 DNA as template, the T3 RNA polymerase selectively initiates, elongates, and terminates several discrete RNA species (3–7). Analysis of the products by electrophoresis on polyacrylamide gels indicates that there are six to eight major transcripts which vary in size from 0.21 to 6.2×10^6 daltons (8, 9). These results are all consistent with the existence of well-defined start and stop signals on T3 DNA that govern transcription *in vitro* by T3 RNA polymerase.

In order to examine the sites at which T3 RNA polymerase terminates transcription, we isolated the 3′-terminal oligonucleotide fragments from the various T3 RNA polymerase-directed RNA transcripts and determined their nucleotide sequence. Our results indicate that all of the *in vitro* T3 RNA polymerase transcription products terminate with an identical nucleotide sequence. Moreover, the absence of any heterogeneity at the 3′ ends of these transcripts suggests that the termination event is precise, occurring at a single nucleotide position.

EXPERIMENTAL PROCEDURE

Materials. T3 RNA polymerase was purified and characterized as described (10). α-^{32}P-Labeled ribonucleoside triphosphates were obtained from ICN Corp and from Amersham. The *N*-(*N*′-*m*-dihydroxyboryl phenyl)succinamyl aminoethyl-cellulose was prepared as described (11).

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Preparation of Labeled *In Vitro* T3 RNA Polymerase Transcripts. RNA synthesis was carried out in standard reaction mixtures (0.5 ml) which contained 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 50 μg of T3 DNA, and GTP, ATP, UTP, and CTP each at 400 μM; one of the triphosphates was labeled with ^{32}P in the α-phosphate position (2–5 $\times 10^6$ cpm/nmol). The polymerase reaction was initiated by the addition of 0.7 μg, equivalent to 8 pmol (3, 10), of T3 RNA polymerase. After the mixtures were incubated at 37°C for 15 min, reactions were terminated by the addition of pancreatic DNase (10 μg). Following incubation at 0°C for 20 min, EDTA was added to a final concentration of 50 mM and the reaction mixture was extracted with phenol. The aqueous phase containing the labeled RNA was chromatographed on a column (0.9 × 3 cm) of cellulose CF-11 according to the method of Franklin (12) to remove unincorporated precursor. The RNA was then precipitated with 2.5 vol of ethanol at −20°C.

RNase T1 Digestion of T3 RNA Polymerase Transcripts and Isolation of 3′-Terminal Oligonucleotides. The ^{32}P-labeled RNA transcription products were digested in 0.1 ml of 10 mM Tris-HCl, pH 7.5/10 mM EDTA at a RNase T1-to-substrate ratio of 1:10 (wt/wt). Incubation at 37°C for 1 hr gave complete digestion. The resulting 3′-terminal oligonucleotides isolated from the RNA species were selectively isolated by chromatography on columns (30 × 0.5 cm) of dihydroxyboryl-cellulose (13). Oligonucleotide fragments that contained 3′-terminal phosphate moieties were first eluted from the column at pH 8.8 with solvent A (50 mM morpholinium chloride/100 mM $MgCl_2$/1 M NaCl/20% dimethyl sulfoxide, pH 8.8). Under these conditions, oligonucleotides that contained a nucleoside residue at their 3′ end (the 3′ terminus of the RNA molecule) were selectively retained on the column. These oligonucleotides were subsequently eluted in highly purified form by using solvent A containing 100 mM sorbitol (14). The 3′-oligonucleotides were desalted by dialysis against several changes of distilled deionized water and were then lyophilized to dryness.

Sequence Determination. The isolated 3′-terminal oligonucleotides were characterized by standard two-dimensional fractionation procedures (15, 16). All other analyses were performed by techniques as described (17).

RESULTS

Characterization of 3′-Terminal Oligonucleotides. T3 RNA polymerase transcription products were synthesized from T3 DNA templates in four separate reaction mixtures, each containing a different α-^{32}P-labeled nucleoside triphosphate. The

RNA products from each reaction were subjected to complete digestion with RNase T1, and the resulting 3′-terminal oligonucleotide fragments were isolated on dihydroxyboryl-cellulose columns. The products obtained were initially characterized by standard two-dimensional RNA fractionation procedures. The results (Fig. 1) indicated that reaction mixtures containing [α-^{32}P]UTP or [α-^{32}P]GTP yielded one major labeled 3′-terminal oligonucleotide fragment.‡ No labeled 3′-terminal oligonucleotide fragments were obtained when either CTP or ATP was the labeled substrate (data not shown). The chromatographic mobilities of the 3′-terminal fragment derived from the [α-^{32}P]UTP and [α^{32}P]GTP-labeled RNA products were identical in the two-dimensional fractionation system (Fig. 1). These results indicate that all (or a large majority) of the RNA chains formed in the T3 RNA polymerase reaction terminate with the same oligonucleotide sequence.

Nucleotide Sequence of the 3′-Terminal Oligonucleotide Fragment. An unambiguous nucleotide sequence was determined for the 3′-terminal oligonucleotide isolated by borate chromatography. Because this RNase T1 oligonucleotide was labeled by [α-^{32}P]GTP, it must contain a single guanosine nucleoside residue at its 3′ end. Further characterization of this nucleotide fragment was achieved by enzymatic digestion with pancreatic RNase and electrophoretic analysis of the products on DEAE-paper (Whatman DE-81) at pH 3.5 (16). Separate analysis of the [α-^{32}P]UTP- and [α-^{32}P]GTP-labeled oligonucleotide fragments by this procedure indicated that (2′,3′)UMP was the only product obtained by pancreatic RNase digestion. Thus, the 3′-terminal oligonucleotide must have the sequence $(Up)_nG_{OH}$.

‡ The major oligonucleotide constituted >90% of the radioactivity present in the homochromatogram. The minor spots seen in the radioautogram constituted background of unknown origin and were only observed when total T3 RNA transcripts were analyzed without prior purification of individual transcripts by gel electrophoresis. These spots are presumably background of T1 oligonucleotides from various transcripts.

The chain length of this oligonucleotide was determined by chromatography on DEAE-cellulose in 7 M urea. Under the conditions of chromatography, fractionation occurs on the basis of number of phosphate residues per oligonucleotide having free 5′- and 3′-hydroxyl groups. As shown in Fig. 2, the labeled fragment eluted from the column as a single symmetrical peak between the marker compounds $(Up)_5U_{OH}$ and $(Up)_6U_{OH}$. This elution position is characteristic of an oligonucleotide having the sequence $(Up)_5G_{OH}$. The chromatographic behavior of the oligonucleotide in both the two-dimensional system and on DEAE-paper at pH 1.7 (electrophoresis at 200 mA for 24 hr) was also consistent with the $(Up)_5G_{OH}$ sequence.

Characterization of 3′-Terminal Oligonucleotides of Individual T3 RNA Polymerase Transcripts. Although $(Up)_5G_{OH}$ is the major 3′-terminal oligonucleotide resulting from transcription termination by T3 RNA polymerase, it was unclear whether each of the *in vitro* synthesized RNA species terminated exclusively in this sequence. In order to examine the 3′-terminal sequence of the individual RNA transcripts, T3 DNA was transcribed and the resulting products (labeled with [α-^{32}P]UTP) were fractionated by electrophoresis on 2% polyacrylamide/0.5% agarose gel slabs. Under these conditions, seven discrete size classes of RNA, ranging from 0.21×10^6 to 6.2×10^6 in molecular weight, were obtained (Fig. 3; refs. 9 and 10). These RNAs were eluted separately from the gel, enzymatically digested to completion with RNase T1, and chromatographed on columns of dehydroxyboryl-cellulose. The 3′-terminal oligonucleotide fragments obtained were then characterized by standard two-dimensional fractionation procedures as before. Each major transcript (data for transcripts VI and VII are only shown in Fig. 4) yielded a single 3′-terminal oligonucleotide fragment identical to the 3′-terminal fragment obtained from total T3 RNA polymerase products (see Fig. 1). Thus, all transcripts share the common 3′-terminal sequence $(Up)_5G_{OH}$.

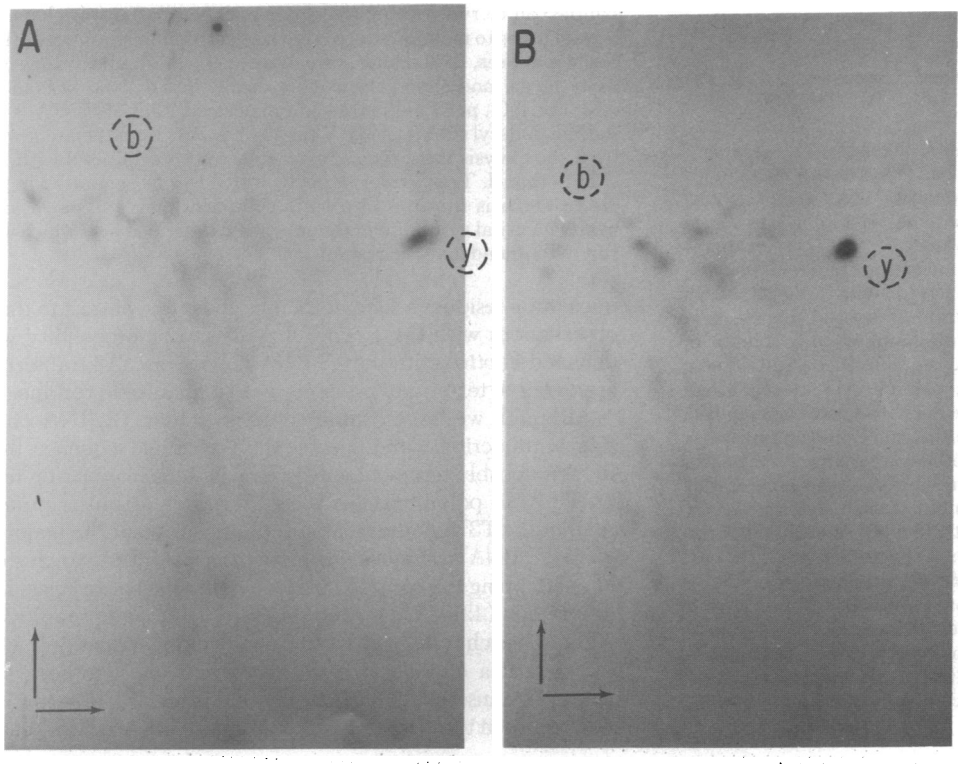

FIG. 1. Two-dimensional fractionation of the 3′-terminal oligonucleotides isolated on dihydroxyboryl-cellulose resulting from a complete RNase T1 digestion of total ^{32}P-labeled T3 RNA polymerase products of T3 DNA. First dimension: electrophoresis on cellulose acetate strips in 8.0 M urea at pH 3.5. Second dimension: ascending thin-layer chromatography on plates of DEAE-cellulose (40 × 20 cm) using homochromatography in solvent B (14–16). Marker dyes xylene cyanol (b) and methyl orange (y) are indicated. (*A*) Autoradiograph with [α-^{32}P]UMP-labeled RNA products; (*B*) autoradiograph with [α-^{32}P]-GMP-labeled RNA products.

DISCUSSION

In the present communication we report the 3′-terminal nucleotide sequences of RNAs transcribed by T3 RNA polymerase from a T3 DNA template. Analysis of both total unfractionated T3 RNA transcripts and separated major RNA species indicates that all the T3 transcription products terminate in the identical 3′-sequence—(Gp)UpUpUpUpUpG$_{OH}$. Apparently, transcription termination by the T3 RNA polymerase occurs at sequence-specific sites on phage T3 DNA.

Several features of the 3′ termini of T3 RNA polymerase transcripts are noteworthy. The uridine-rich sequences we find on the T3 RNAs bear striking similarity to transcriptionally terminated 3′ ends of bacterial and phage RNAs synthesized *in vivo* and *in vitro* by *Escherichia coli* RNA polymerase in the absence of ρ protein (18–25). These stretches of consecutive uridylate residues appear to be necessary for termination by the *E. coli* enzyme and may be similarly important for termination by the T3 RNA polymerase. Thus, the nucleic acid structural requirements for transcription termination may be quite similar for the relatively simply structured phage polymerase and for the multisubunit *E. coli* enzyme. However, in contrast to the T3 RNA polymerase, in the absence of ρ the *E. coli* RNA polymerase tends to terminate somewhat heterogeneously over several nucleotide positions (14, 23, 24, 26). This results in specific transcripts exhibiting heterogeneity in their 3′-terminal sequence (i.e., observed as a group of closely related 3′-oligonucleotides that differ only by having single additional nucleotide residues added at their 3′ ends). In contrast to these observations with the *E. coli* enzyme, no heterogeneity was detected *in vitro* with the T3 RNA polymerase. All transcripts appeared to terminate precisely with a guanosine residue.

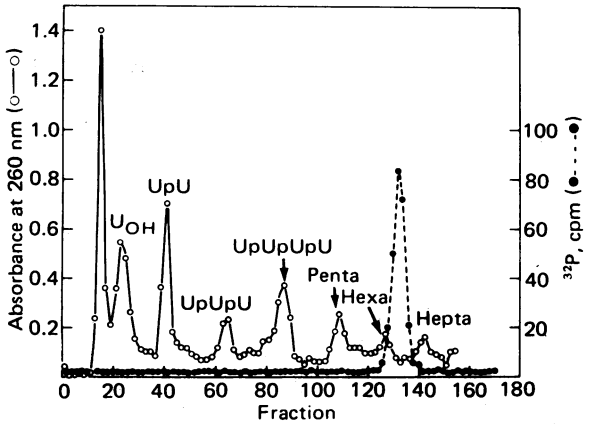

FIG. 2. Chromatography of 3′-terminal oligonucleotide fragment on DEAE-cellulose in 7 M urea at pH 7.4. The 3′-terminal oligonucleotide fragment (Fig. 1*A*) was eluted from the DEAE-cellulose plate with 2 M triethylammonium bicarbonate at pH 10.0, according to Brownlee (16). After removal of triethylamine by repeated evaporation to dryness, the ^{32}P-labeled oligonucleotide fragment was dissolved in 0.5 ml of 10 mM Tris·HCl, pH 7.4/7 M urea (buffer A) which also contained 20 A_{260} units of a mixture of oligo(U) fragments of varying chain lengths. The mixture was applied to a column of DEAE-cellulose (0.7 × 30 cm) previously equilibrated with buffer A. After the column was washed with 50 ml of buffer A, a linear gradient (300 ml total volume) of buffer A–buffer A + 0.4 M NaCl was applied. The flow rate was maintained at 12 ml/hr, and 2-ml fractions were collected. The elution positions of oligo(U)s of varying chain lengths from the column were determined by measuring absorbance of each eluted fraction at 260 nm; the ^{32}P content of each fraction was determined by assaying 1-ml aliquots with 10 ml of Aquasol (New England Nuclear). The mixture of oligo(U)s of varying chain lengths used in this experiment was prepared by carrying out partial digestion of 40 A_{260} units of poly(U) (obtained from Boehringer Mannheim) in 1 M KOH for 20 min at 25°C. After neutralization of the digest with $HClO_4$ and removal of the precipitate by centrifugation, the material was incubated with 250 μg of bacterial alkaline phosphatase to remove the terminal monoester phosphates from the oligonucleotide fragments.

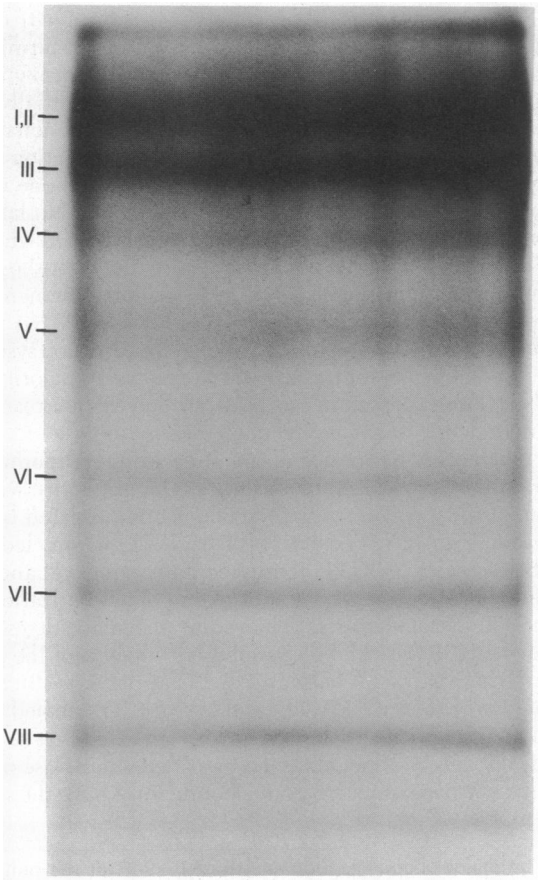

FIG. 3. Autoradiogram of a polyacrylamide/agarose gel of T3 RNA polymerase transcripts of T3 DNA synthesized under standard reaction conditions with 2 pmol of T3 RNA polymerase and [α-^{32}P]UTP (2 × 10^6 cpm/nmol) for 15 min at 37°C. After isolation of the labeled RNA, the pellet was suspended in 0.2 ml of gel buffer (90 mM Tris borate/2.7 mM EDTA, pH 8.5) containing bromophenol blue dye and glycerol. The RNA was then subjected to electrophoresis for 12 hr at 70 V on 2% polyacrylamide (acrylamide/bisacrylamide 20:1)/0.5% agarose slab gels (13.5 × 10 × 0.4 cm); in this system, only one sample can be run. During electrophoresis, gel buffer was changed several times to maintain the pH of the electrophoresis buffer. After electrophoresis, RNA bands were detected by autoradiography, cut from the gel, and eluted electrophoretically into dialysis bags in 20 mM Tris·HCl, pH 7.4/40 mM sodium acetate/1.0 mM EDTA/0.1% sodium dodecyl sulfate at 150 V for 10 hr. RNAs were then removed from the dialysis bags, phenol extracted, and precipitated with 2.5 vol of ethanol. Transcripts I and II, which can be separated in gel electrophoresis run in 6 M urea in cylindrical gels (8), move with nearly identical mobilities in the gel system described here which was run under nondenaturing conditions.

Although we have examined seven major T3 RNA polymerase transcripts with respect to the 3′-terminal sequence, they in fact probably represent only two major termination sites read by T3 RNA polymerase. A recently reported transcriptional map of the T3 genome clearly demonstrates that the major *in vitro* T3 RNA polymerase transcripts arise from two clusters of overlapping transcription units covering the entire "class III" late region (42–100% on the standard T3 genetic map) (27). Although each transcript arises from an independent promoter (located at a considerable distance from one another), one cluster of transcripts (T3 RNA species V, VI, and VII) terminate at a common terminator located at approximately 56% on the

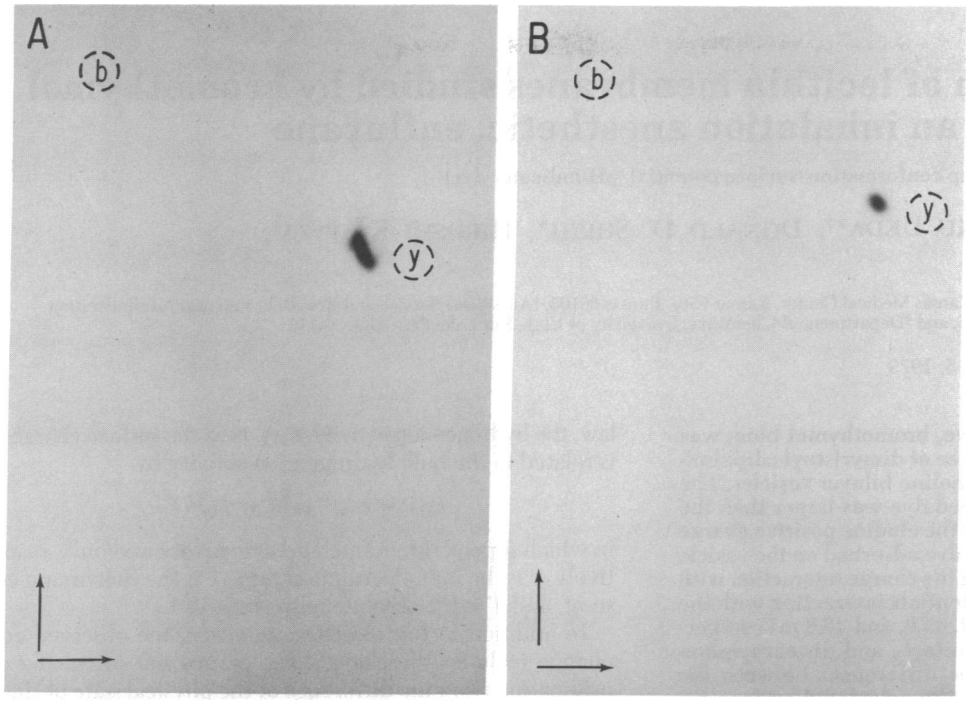

FIG. 4. Two-dimensional fractionation of the 3′-terminal oligonucleotides isolated on dihydroxyboryl-cellulose after complete RNase T1 digestion of the individual T3 RNA polymerase transcripts labeled with [α-^{32}P]UTP. The conditions used were identical to those in Fig. 1. The fingerprints were derived from individual T3 RNA polymerase transcripts: (*A*) species VI; (*B*) species VII.

standard T3 genetic map and the other cluster, representing species I, II, III, and VIII, terminates at a second terminator located at approximately 100% on the T3 genome (27).

Recent structural and biochemical studies indicate that in both ρ-independent and ρ-dependent termination catalyzed by *E. coli* RNA polymerase the secondary structure of the nascent transcript near its 3′ terminus, or the stability of the transient DNA·RNA hybrid formed during transcription, plays an important role (23, 25, 26, 28–30). In the light of these findings, and especially in view of the specificity of T3 RNA polymerase for transcription of T3 DNA, it will be interesting to determine the basic features of the structure of RNA and of DNA surrounding the termination sites for T3 RNA polymerase. Such work may shed further light on the mechanisms of the transcription termination reaction catalyzed by RNA polymerases.

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