Acknowledgments

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References

Blattner, F., & Dahlberg, J. (1972) *Nature (London), New Biol. 237,* 227-232.
Burd, J., & Wells, R. (1974) *J. Biol. Chem. 249,* 7094-7101.
Burgess, R., & Jendrisak, J. (1975) *Biochemistry 14,* 4634-4638.
Chamberlin, M. (1974) *Annu. Rev. Biochem. 43,* 721-775.
Dahlberg, J., & Blattner, F. (1973) *Virus Research,* pp 533-543, Academic Press, New York, N.Y.
Dunn, J., & Studier, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 1559-1563.
Grohmann, K., Smith, L. H., & Sinsheimer, R. L. (1975) *Biochemistry 14,* 1951-1955.
Johnston, D., & McClure, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 413-428, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Konrad, M. (1973) *Anal. Biochem. 54,* 213-222.
Lebowitz, P., Weissman, S., & Radding, S. (1971) *J. Biol. Chem. 246,* 5120-5139.
Lebowitz, P., Stern, R., Ghosh, P. K., & Weissman, S. (1977) *J. Virol. 22,* 430-445.
Maitra, U., Nakata, Y., & Hurwitz, J. (1967) *J. Biol. Chem. 242,* 4908-4918.
Meyer, B. J., Kleid, D., & Ptashne, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4785-4789.
Miller, J. S., & Burgess, R. R. (1978a) *Biochemistry 17* (2nd of series of three papers in this issue).
Miller, J. S., & Burgess, R. R. (1978b) *Biochemistry 17* (3rd of series of three papers in this issue).
Minkley, E. G., & Pribnow, D. (1973) *J. Mol. Biol. 77,* 255-277.
Pettersson, U., & Sambrook, J. (1973) *J. Mol. Biol. 73,* 125-130.
Randerath, K., & Randerath, E. (1964) *J. Chromatogr. 16* 111-125.
Reiner, A. M. (1969) *J. Bacteriol. 97,* 1437-1443.
Reznikoff, W. (1972) *Annu. Rev. Genet. 6,* 133-156.
Schendel, P, & Wells, R. (1974) *J. Biol. Chem. 248,* 8319-8321.
Smith, L. H., Grohmann, K., & Sinsheimer, R. L. (1974) *Nucleic Acid Res. 1,* 1521-1529.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112,* 577-601.
Studier, F. W. (1969) *Virology 39,* 562-574.
Sugiura, M., Okamoto, T., & Takanami, M. (1969) *J. Mol. Biol. 43,* 299-315.
Surzycki, S. J., Surzycki, J. A., DeLorbe, W., & Gussin, G. N. (1976) *Mol. Gen. Genet. 143,* 177-184.
Takeya, T., & Fugisawa, H. (1974) *Biochim. Biophys. Acta 324,* 110-119.
Terzaghi, E., Okada, Y., Streisinger, G., Emrich, J., Inouye, M., & Tsugita, A. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56,* 500-507.
Thomas, C., & Abelson, J. (1961) *Proc. Nucleic Acid Res. 1,* 553-561.

Selectivity of RNA Chain Initiation in Vitro. 2. Correlation of 5′-Triphosphate-Labeled Oligonucleotides on Polyethyleniminecellulose Thin-Layer Chromatography with RNA Transcripts of Bacteriophage λcb2 and T7†

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ABSTRACT: Methods are described for the correlation of 5′-terminal oligonucleotides separated by two-dimensional polyethyleniminecellulose thin-layer chromatography with specific RNA transcripts made in vitro from DNA of phages T7 and λcb2. The 5′-terminal oligonucleotides transcribed from DNA containing mutations and alterations which affect the RNA transcripts from specific promoters were compared with fingerprints of RNA from wild type DNA. Specific RNAs were purified on polyacrylamide gels and digested and their 5′-terminal oligonucleotides subjected to chromatography. Transcription of DNA fragments containing specific promoters was carried out and the 5′-oligonucleotide fingerprints of the RNA products compared with fingerprints of the RNA from the whole DNA. Using these methods and information known about T7 and λcb2 RNA 5′-terminal sequences, it was possible to identify many of the oligonucleotides separated on the polyethyleniminecellulose chromatography system.

The *E. coli* RNA polymerase can selectively initiate and transcribe in vitro specific RNA transcripts on DNA templates from many bacterial and viral sources. To determine the parameters influencing selectivity, one would like to measure initiations of specific RNAs rapidly and quantitatively.

A thin-layer chromatography method has been described which allows the rapid and quantitative analysis of 5′-triphosphate oligonucleotides labeled in the γ position and generated by RNase digestion of transcripts synthesized in vitro (Miller & Burgess, 1978a). This procedure results in a fin-

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gerprint of the 5′-terminal oligonucleotides initiated with either ATP or GTP. We have demonstrated that fingerprints of RNAs from different DNA templates showed characteristic patterns. In this paper it will be demonstrated that many of these spots correlate to the 5′ ends of previously characterized transcripts.

The templates chosen for this study were phages λ and T7 since the transcription of these phages both in vivo and in vitro has been studied extensively. We have used three methods to correlate oligonucleotides we observe on the chromatograms with the 5′-terminal ends of known phage RNAs. (1) Mutations or DNA substitutions in λcb2 and T7 which were known to result in the decrease of a certain RNA transcript, the deletion of a certain transcript, or a change in the 5′ sequence of an RNA were examined. The 5′ ends of the RNA transcribed from mutant DNA templates were isolated and fractionated, and these fingerprints were quantitatively compared with fingerprints from the wild type RNA transcripts. (2) Total RNA populations from in vitro transcriptions were fractionated on the basis of size by electrophoresis on polyacrylamide gels. Specific bands were eluted from the gels and the 5′-terminal ends were isolated and analyzed. (3) DNA fragments containing certain promoters were generated by restriction enzymes and transcribed. The 5′-terminal ends of the transcripts from these fragments were fractionated on PEI[1]-cellulose and these fingerprints were compared with fingerprints of 5′ ends of RNA transcripts made from unfragmented DNA.

Experimental Procedures

Generation of 5′-Terminal Oligonucleotide Fingerprints. RNA synthesis, isolation, and digestion were carried out as described in the preceding paper (Miller & Burgess, 1978a). Fractionation of 5′-terminal oligonucleotides on polyethylenimine cellulose thin-layer chromatograms, autoradiography, and quantitation of the separated oligonucleotides were also performed as described in the preceding paper. In these experiments only the RNA from the void peak of the Sephadex G-50 column (corresponding to fractions 4–7 of Figure 1, Miller & Burgess, 1978a) was analyzed.

Phage Strains. λ phages λ$^+$, λcb2, λcb2*imm*21, λsexI, and λb2*imm*434 were the gift of W. Szybalski and Lois Farmer. T7 mutant D111 was the gift of J. Dunn (Dunn & Studier, 1973). The DNA was isolated as described (Miller & Burgess, 1978a).

DNA Fragments. The 9.8% and 3.7% DNA fragments from λKH100*nin*5 were generated using restriction enzyme *Eco*RI (Thomas & Davis, 1975), were isolated either by polyacrylamide gel electrophoresis or by column chromatography, and were the gift of K. Krueger. The 9.8% fragment extends from 44.5% to 54.3% on the λ map and contains most of the b2 region. The 3.7% fragment contains promoters P_R, P_{rm}, and P_O. A fragment produced by digesting λcb2 DNA with restriction enzyme *Hin*dIII containing only the P_L promoter and extending from 0% to 76.5% on the λ map was the gift of E. Rosenvold.

ρ Factor. ρ factor was purified according to the method of Roberts (1969) and was the gift of M. Rosenberg.

RNA Gels. RNA gels were run according to the procedure of Rosenberg et al. (1975). The gels contained 3.5% acrylamide, 0.175% bisacrylamide, 7 M urea, and a 0.08 M Tris-borate, 0.002 M EDTA buffer (pH 8.5). The RNA transcripts were eluted from the gels by cutting out the band localized by autoradiography and crushing the gel slice in 0.5 mL of buffer (0.01 M Tris-HCl (pH 7.9), 0.2 M NaCl, and 10 μg of carrier yeast tRNA) in a siliconized tube. The crushed gel was vortexed for an hour and incubated at 5 °C overnight. The crushed gel was then filtered through a Whatman No. 1 filter to remove the gel material and the gel was rinsed with 0.5 mL of sterile water. The RNA was precipitated by adding 2.5 volumes of ice-cold ethanol and incubating either at −70 °C for 60 min or at −20 °C overnight.

The ethanol precipitated RNA eluted from the gels was subjected to centrifugation at 10 000 rpm for 20 min in a Sorvall SS34 rotor. The precipitated RNA was dissolved in 10 μL of sterile water, digested with RNase, and subjected to chromatography and autoradiography as described in the preceding paper.

TABLE I: Comparison of Amount of Label Present in Spots of [γ-^{32}P]ATP-Initiated 5′-Terminal End Fingerprints of T7 and T7 Mutant D111.

	Spot number						
Template	1	2	3	4	5	(1a)[b]	(5a)[b]
T7	23.0[a]	41.0	5.7	21.0	2.0	1.0	6.0
T7 D111	4.5	35.0	3.4	27.0	13.0	10.0	7.0

[a] Data are given in terms of % of total radioactivity recovered and are the average of two experiments. Total recovery of radioactivity averaged 5000 dpm. [b] These spots appeared on the chromatograms inconsistently.

Results

Correlation of Spots with Known 5′-Terminal Sequences. *T7* has three major promoters capable of being transcribed by *E. coli* RNA polymerase (Dunn & Studier, 1973). These promoters have been designated A1, A2, and A3 (Pribnow, 1975; Kramer et al., 1974). When sufficient RNA polymerase is added to saturate the A promoter sites, the excess enzyme binds and initiates at four additional sites in the early region. These minor promoter sites have been designated B, C, D, and E (Minkley & Pribnow, 1973; Stahl & Chamberlin, 1977). A mutation in the early region, mutant D111, has been shown to be a deletion of two of the three major promoters, A2 and A3, leaving promoter A1 as the only major promoter (Studier, 1975). Mutant D111 DNA was used as a template for transcription with [γ-^{32}P]ATP and the 5′-terminal oligonucleotides from RNA transcripts of D111 were compared with those of wild type T7. Figure 1A shows a schematic of a fingerprint of T7 5′-oligonucleotides initiated with ATP. Five discrete spots are seen. Spots 1 and 2 exhibit the greatest intensity of label and are therefore considered major starts, whereas spots 3, 4, and 5 appear to be minor initiating oligonucleotides or due to abortive initiation. Table I compares the incorporation of label into each of the spots of T7 and D111. Quantitative determinations were made by counting the five regions of the chromatogram whether or not a spot was visible on the autoradiogram. A marked decrease in spot 1 is seen in D111 suggesting that this 5′-oligonucleotide is from the RNA transcript initiating at the A3 promoter which is deleted in D111. We have therefore assigned spot 1 the sequence pppApUpGp, the 5′ end of the RNA initiating at promoter A3 (Pribnow, 1975). The label remaining in spot 1 may be the result of background smearing. Spot 2, containing the largest percent of label, we have assigned the sequence pppApUpCpGp, the 5′ terminal sequence of the RNA from the A1 promoter (Kramer et al., 1974), known to remain on D111.

Transcripts from the A2 promoter initiate with GTP and

[1] Abbreviation used: PEI, polyethylenimine.

TABLE II: Comparison of Amount of Label Present in Spots of [γ-^{32}P]ATP-Initiated 5′-Terminal End Fingerprints of λcb2, λb2*imm*434, λ*sex*1, and λb2*imm*21.

Template	Spot number									
	1	2	3	4	5	6	7	8	(3a)[b]	(6a)[c]
λcb2	25.2[a]	10.1	11.8	12.8	14.8	8.1	4.3	5.2	5.4	
λb2*imm*434	4.6	10.2	14.1	11.4	17.4	15.5	6.3	9.9	10.0	
λ*sex*1	4.5	9.2	15.7	11.0	16.3	13.0	11.5	12.0	8.4	
λb2*imm*21	2.5	6.0	5.8	18.1	27.9	3.2	4.0	26.7		6.7

[a] Data are given in terms of % of total radioactivity recovered. Total recovery of radioactivity averaged 3000 dpm. Data for λcb2 and λb2*imm*434 are an average of five experiments. Data for λ*sex*1 and λb2*imm*21 are an average of two experiments. [b] Spot appeared on chromatograms inconsistently. [c] Spot appeared only on chromatograms of λb2*imm*21 RNA.

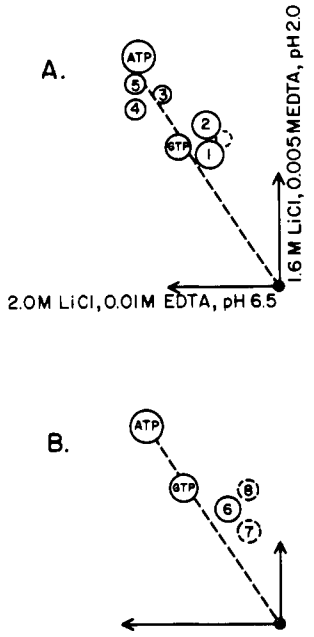

FIGURE 1: Schematic of two-dimensional fingerprint of the 5′-triphosphate terminal oligonucleotides of RNAs made from T7 DNA. RNAs were synthesized, isolated, and digested and the 5′-terminal oligonucleotides fractionated as previously described. ATP and GTP markers were run with the samples. A line from ATP to origin divides the chromatogram in half as a reference for migration of the spots. Dotted circles indicate spots of weak intensity. Numbers indicate regions of discrete spots. (A) The 5′ ends of RNAs initiated with ATP on T7; (B) 5′ ends of RNAs initiated with GTP on T7.

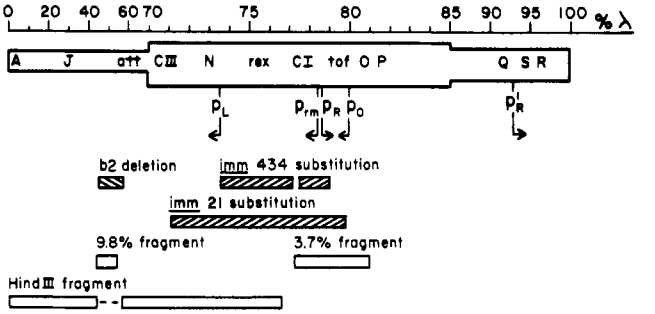

FIGURE 2: Schematic diagram of λ. The positions of certain genes, promoters, the b2 deletion (▨), the *imm*343 and *imm*21 substitutions (▩), and the restriction fragments utilized (□) are indicated. The arrows from the promoters indicate direction of transcription. The distances are given as a percent of the total λ length (Blattner et al., 1974).

have the 5′-terminal sequence pppGpCpUpApGp (Pribnow, 1975). Figure 1B shows the fingerprint of the 5′-terminal ends of T7 RNAs initiated with GTP. One major start, spot 6, is seen. This spot has been assigned the sequence of the 5′ terminus of the A2 promoter, pppGpCp. The nature of spots 7 and 8 is not clear. A summary of the T7 5′-terminal oligonucleotides and corresponding sequences is given in Table III.

λ*cb2*. λ DNA with the b2 deletion was analyzed in an attempt to produce a simpler fingerprint containing initiating sequences from the immunity region only. The elimination of the b2 region removes five promoters (E. Rosenvold, personal communication) which otherwise might complicate the analysis. Figure 2 shows a map of λ on which is indicated the positions of the known promoters, certain genes, the b2 deletion, and the *imm*434 and *imm*21 substitutions. In the experiment in Table II, several mutated or altered λ DNAs were transcribed and the 5′ sequences of the transcripts were examined quantitatively and compared with the 5′ ends of λcb2 RNA. The DNAs examined were all affected in the P_L promoter region. λb2*imm*434 contains both the b2 deletion and a substitution, in the immunity region, of phage 434 DNA for λ DNA which extends from just before the start site of P_L to the right of P_R. The start site for the P_L RNA is λ DNA but the substitution results in a large reduction of P_L transcription (Blattner & Dahlberg, 1972). λ*sex*1 is a point mutation in the P_L promoter region at 73.57% on the λ map which gives a 90% reduction of P_L RNA relative to P_R RNA (Blattner & Dahlberg, 1972). λb2*imm*21 contains the b2 deletion and a substitution of phage 21 DNA for λ DNA in the immunity region from the left of the P_L promoter to the left of P_O. Figure 3A shows a schematic of a typical fingerprint of λcb2 ATP-initiated terminal oligonucleotides produced by digestion with RNase T1. Eight discrete spots are seen. When a comparison is made between the 5′ ends of RNA transcripts made from λcb2 DNA and those made from altered DNAs, a dramatic reduction in spot 1 is observed in all the altered DNAs (Table II). λb2*imm*434 and λ*sex*1 show an 82% reduction in spot 1. The reduction in λ*sex*1 spot 1 is similar but not identical to 90% reduction seen by Blattner & Dahlberg (1972) but this may be the result of differences in conditions of synthesis of RNA. Spot 1 of λb2*imm*21 is reduced 90%. Label appearing in the spot 1 region of the chromatograms may be in part the result of some streaking by spot 2 and therefore the actual amount of label in spot 1 may be lower.

Spot 1 was further correlated with the RNA transcripts from the P_L promoter by the use of RNA gels. When transcription is carried out on λcb2 DNA in the presence of termination factor ρ, several discrete RNAs are synthesized (Roberts, 1969). When the RNA was analyzed on 7 M urea, 3.5% polyacrylamide gels, the following bands were seen: 12 S, corresponding to the RNA initiated at P_L; 9 S, corresponding to P_R RNA; 6 S, corresponding to P_R' RNA; and 4 S, corresponding to the P_O RNA (Rosenberg et al., 1975). Transcription of λcb2 was carried out at 0.1 M KCl in the presence of ρ factor. The 12S band was eluted, digested with T1 RNase and subjected to two-dimensional chromatography.

TABLE III: Summary of T7 and λcb2 5′-Terminal Oligonucleotide Spots and Sequences.

Template	Spot no.	Label	Sequence inferred from known sequence: T_1 or pancreatic RNase digested	Promoter	How identified
T7	1	ATP	pppApUpGp	A3	Absent in D111 deletion
	2	ATP	pppApUpCpGp	A1	Present in D111 deletion
	6	GTP	pppGpCp	A2	Major GTP start
λcb2	1	ATP	pppApUpCpApGp	P_L	Decreased by *sex*1 mutation and *imm*434 substitution, 12S band from RNA gel, restriction fragment carrying P_L
	2	ATP	pppApUpGp	P_R, P_{rm}	9S band from RNA gel
	5	ATP	pppAp(Ap)CpGp	P_R'	6S band from RNA gel
	9	GTP	pppGpUp	P_O	Major GTP start on 3.7% *Eco*R1 fragment
	11	GTP	pppGpCp	?	Minor GTP start with same R_f as T7 pppGpCp start

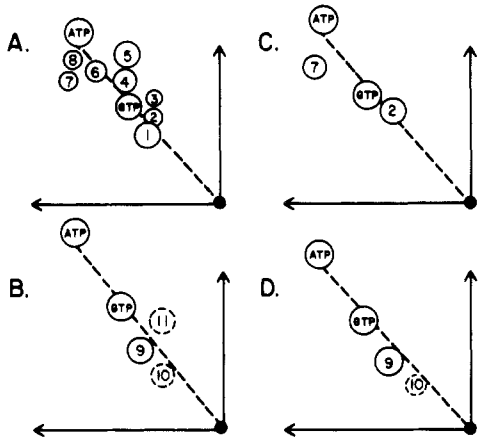

FIGURE 3: Schematic of two-dimensional fingerprints of the 5′-triphosphate terminal oligonucleotides of RNAs made from λcb2 (A and B) and the 9.8% fragment of λKH100*nin*5 DNA cut with *Eco*R1 (C and D). (A) The 5′ ends of RNAs initiated with ATP on λcb2; (B) 5′ ends of RNAs initiated with GTP on λcb2; (C) 5′ ends of RNAs initiated with ATP on 9.8% fragment; (D) 5′ ends of RNAs initiated with GTP on 9.8% fragment.

A single spot was obtained which migrated in the same position as spot 1 of Figure 3A (data not shown).

When a *Hin*dIII fragment of λcb2 DNA containing the P_L promoter but not P_{rm}, P_R, P_O, and P_R' was transcribed, the same single spot was obtained (data not shown). On the basis of these experiments, spot 1 has been assigned the sequence of the 5′ end of the RNA initiated at the P_L promoter, pppApUpCpApGp (Blattner & Dahlberg, 1972).

The 6S band seen on gels is made in large quantities in the presence or absence of ρ. This RNA was eluted and its 5′-end analyzed. The single oligonucleotide obtained from the 6S RNA migrated in the position of spot 5 of Figure 3A (data not shown). Therefore spot 5 is the 5′-terminal oligonucleotide of the RNA made from the P_R' promoter. The 5′ terminus of the RNA initiated at P_R' has been reported to have the sequence pppApCpGp by Lebowitz et al. (1971). However, recent evidence (E. Calva & R. Burgess, unpublished data; W. McClure, personal communication) suggests that this sequence may be pppApApCpGp.

The major λ GTP-initiated RNA, from the P_O promoter, has the terminal sequence pppGpUpUpGp (Dahlberg & Blattner, 1973). When transcription of λcb2 was carried out in the presence of [γ-^{32}P]GTP and the RNA was isolated, digested with pancreatic RNase A, and fractionated, most of the radioactivity appeared in a single spot in the position of spot 9 of Figure 3B. Spot 9 therefore is the 5′-terminal end of the P_O RNA and has been assigned the sequence pppGpUp. This spot was also identified by using a 3.7% fragment of λKH100*nin*5 produced by cleavage with *Eco*R1. This purified fragment contains the P_R, P_{rm}, and P_O promoters. When transcription was carried out in the presence of [γ-^{32}P]GTP, only the P_O RNA should be labeled. The 5′ end of this RNA migrated in the same position as spot 9 of Figure 3B (data not shown). The identity of the spot 10 is not known. Spot 11 migrated identically with the major T7 [γ-^{32}P]GTP 5′-oligonucleotide and thus was assigned the sequence pppGpCp.

The correlation of a spot to the 5′-terminal sequence of RNA initiated at the P_R promoter was made by eluting from a 3.5% polyacrylamide gel the 9S RNA which is made in the presence of ρ factor and subjecting it to digestion and chromatography as described. The 5′-oligonucleotide obtained migrated in the position of spot 2. This spot is assigned the sequence pppApUpGp corresponding to the 5′ end of the RNA initiated at P_R (Dahlberg & Blattner, 1973).

Comparison of the 5′-terminal fingerprints of the ATP starts and GTP starts in λ wild type and λcb2 showed qualitatively identical fingerprint patterns (data not shown). This was unexpected in light of evidence of several strong promoters in the b2 region (E. Rosenvold, personal communication). Transcription from the purified b2 fragment (the 9.8% fragment produced by cleavage of λKH100*nin*5 DNA with *Eco*R1) and subsequent fractionation of the 5′-terminal oligonucleotides suggest that the 5′-terminal sequences may be identical with two of the 5′ ends of RNAs transcribed from the immunity region, at least through the first G base in the case of ATP-initiated RNAs and through the first pyrimidine with GTP-initiated RNAs. Oligonucleotides initiating with ATP migrated in the positions of spot 2 and spot 7 (Figure 3C). A strong GTP start migrated in the position of spot 9 (Figure 3D).

A summary of the λcb2 5′-oligonucleotides and corresponding sequences is given in Table III.

Identification of Other Spots on the Chromatogram. Several spots on the chromatograms have not been correlated with known promoters of λcb2 and T7. Some may be products of minor promoters while others appear to be the products of abortive initiations (Johnston & McClure, 1976) which result in the formation of dinucleoside tetraphosphates with 3′-OH termini (see Discussion). The lack of a 3′-terminal phosphate

would allow the dinucleotide to migrate ahead of most oligonucleotides produced by T1 RNase treatment. Fingerprint patterns of both T7 and λcb2 RNAs show similar fast migrating spots (T7 spots 4 and 5, Figure 1A, and λcb2 spots 7 and 8, Figure 3A, respectively). These spots may correspond to pppApU and pppApC. To examine this possibility, poly[d(A-T)] was transcribed and the RNA products were isolated as described in the Experimental Procedures. The RNA was subjected to thin-layer chromatography without first being digested with pancreatic RNase. The pattern obtained showed that the majority of the label remained at the origin, presumably associated with long poly(AU) transcripts. In addition, a spot migrating in the position of λcb2 spot 7 and T7 spot 4 was seen. This suggests that this spot is the result of an aborted initiation, pppApU, which occurs in the course of transcription. λcb2 spot 4 is observed when transcripts of λcb2 are analyzed by thin-layer chromatography without prior RNase T1 treatment and may correspond to an aborted start having the sequence pppApUpG.

Discussion

Several methods have been used to correlate spots on the 5′-terminal oligonucleotide fingerprints described in the preceding paper with the 5′-terminal sequences of RNAs from T7 and λcb2. Table III gives a summary of the spots identified, their sequences, the promoter from which the RNA originated, and the method(s) by which correlation of a spot with the 5′ end of a specific RNA was made.

Some oligonucleotides appearing in the fingerprints of λcb2 and T7 RNAs remain to be correlated with known promoters. They may be products of other promoters which only initiate RNA synthesis under certain in vitro conditions. Minor promoters seem to exist on many DNA templates (Heyden et al., 1972; Minkley & Pribnow, 1973; Meyer et al., 1975; Walz et al., 1976; Stahl & Chamberlin, 1977). These promoters may require additional factors to initiate efficiently or may require specific conditions of salt or temperature. Such minor promoters may be biologically important and control the initiation of operons from which only low levels of transcription are required. Unidentified spots seen on the chromatograms may represent starts from minor promoters on T7 and λcb2 which have not been previously investigated.

Minor spots also would be obtained if certain major promoters initiate RNA chains with occasional ambiguity in the precise start site. Ambiguity of starting has been demonstrated in the initiation of several RNAs studied. Transcription at the *lac* promoter gives RNA chains initiated with pppApApU and, less frequently, with pppGpApApU (Gilbert et al., 1974). Smith & Sinsheimer (1976) have demonstrated a 5′-triphosphate oligonucleotide, pppCpGp(A), initiating on bacteriophage φX174. However, hybridization data suggest that this sequence could share a common initiation point with, but start one base before, another 5′-terminal oligonucleotide pppGpApUp(G). Analysis of initiations at the *E. coli tyr* tRNA promoter suggests that the initiating nucleotide may be either GTP or CTP and that this is dependent on the relative concentrations of the two nucleotides in the reaction (Küpper et al., 1976). These data suggest that, in vitro, RNA polymerase shows some limited ambiguity in the start point.

A third possible source of minor spots may involve the phenomenon termed "abortive initiation" (Johnston & McClure, 1976). During RNA synthesis on both λ and T7 DNA, the production of a dinucleotide tetraphosphate was observed. If abortive initiations occurred infrequently, they would appear as minor spots. An aborted dinucleotide tetraphosphate would lack the 3′-phosphate normally found on oligonucleotides formed as the result of cleavage by T1 RNase. These 3′-OH terminated oligonucleotides would migrate with a greater R_f than the equivalent oligonucleotide with a 3′-phosphate. In the presence of all four nucleotides, longer 3′-OH terminated oligonucleotides might be formed in addition to the dinucleotide tetraphosphates observed (Johnston & McClure, 1976) and these might appear as more slowly migrating minor spots.

A limitation of this system is the inability to distinguish between promoters which initiate RNA transcripts with the same 5′-oligonucleotide. An example of this is the λ promoters P_R and P_{rm}, both of which initiate with pppApUpGp (Blattner & Dahlberg, 1972; Walz et al., 1976). Also analysis of RNA transcribed from the b2 region of λ suggests that several transcripts share 5′-terminal oligonucleotide sequences with transcripts from other λ promoters. If it is desired to examine these starts independently, the RNAs first must be separated by polyacrylamide gel electrophoresis. Then they may be eluted from the gel, digested, and subjected to two-dimensional chromatography individually.

The identification of certain spots as the 5′-terminal ends of specific RNAs serves two functions. It offers evidence that the 5′-terminal oligonucleotides of specific RNAs transcribed from a given DNA template are actually being examined in the described system. Secondly, it enables initiation at a specific promoter to be examined under a variety of reaction conditions. It is possible to vary reaction conditions such as ionic strength, temperature, protein factors, DNA destabilizing agents, enzyme concentration, and so forth, and to study the effects of these changes on a specific set of promoters by examining changes in the 5′ ends initiated. Such a study is described in the following paper (Miller & Burgess, 1978b).

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References

Blattner, F., & Dahlberg, J. (1972) *Nature (London), New Biol. 237,* 227–232.

Blattner, F., Fiandt, M., Hass, K., Twose, P., & Szybalski, W. (1974) *Virology 62,* 458–471.

Dahlberg, J., & Blattner, F. (1973) in *Virus Research,* pp 533–543, Academic Press, New York, N.Y.

Dunn, J., & Studier, F. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 1559–1563.

Gilbert, W., Maizels, N., & Maxam, A. (1974) *Cold Spring Harbor Symp. Quant. Biol. 38,* 845–855.

Heyden, B., Nüsslein, C., & Schaller, H. (1972) *Nature (London), New Biol. 240,* 9–12.

Johnston, D., & McClure, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 413–428, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Kramer, R., Rosenberg, M., & Steitz, J. (1974) *J. Mol. Biol. 89,* 767–776.

Küpper, H., Contreras, R., Khorana, H. G., & Landy, A. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 473–484, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Lebowitz, P., Weissman, S., & Radding, S. (1971) *J. Biol. Chem. 246,* 5120–5139.

Meyer, B., Kleid, D., & Ptashne, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4785–4789.

Miller, J. S., & Burgess, R. R. (1978a) *Biochemistry 17* (1st in series of three papers in this issue).
Miller, J. S., & Burgess, R. R. (1978b) *Biochemistry 17* (3rd in series of three papers in this issue).
Minkley, E., & Pribnow, D. (1973) *J. Mol. Biol. 77*, 255-277.
Pribnow, D. (1975) *J. Mol. Biol. 99*, 419-443.
Roberts, J. W. (1969) *Nature (London) 224*, 1168-1174.
Rosenberg, M., Weissman, S., & deCrombrugghe, B. (1975) *J. Biol. Chem. 250*, 4755-4764.
Smith, L. H., & Sinsheimer, R. L. (1976) *J. Mol. Biol. 103*, 711-735.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112*, 577-601.
Studier, F. W. (1975) *J. Mol. Biol. 94*, 283-295.
Thomas, M., & Davis, R. (1975) *J. Mol. Biol. 91*, 315-328.
Walz, A., Pirrotta, V., & Ineichen, K. (1976) *Nature (London) 262*, 665-669.

Selectivity of RNA Chain Initiation in Vitro. 3. Variables Affecting Initiation of Transcription†

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ABSTRACT: The effects of salt, temperature, enzyme to DNA ratio, and heparin challenge on both total RNA synthesis and synthesis from specific promoters are examined using DNA from bacteriophages λcb2 and T7. Determination of synthesis from specific promoters is carried out by the fractionation and quantitation on polyethylenimine-cellulose thin-layer chromatograms of the 5′-terminal oligonucleotides produced by digestion of the RNA products with T1 RNase. The major findings of this work are that (1) λcb2 promoters are more salt sensitive than T7 promoters and the salt concentration affects individual promoters differently, (2) T7 promoters initiate maximally at 37 °C but the transition temperatures of promoters vary and may be dependent on the salt concentration, (3) increasing the enzyme to DNA ratio results in increasing initiations at the promoters on T7 DNA without causing measurable initiation at non-promoters, and (4) T7 and λcb2 promoters show differences in stability when challenged with heparin.

An important controlling point in regulation of gene expression is the selectivity of transcription at the level of initiation. *E. coli* RNA polymerase is capable of selectively initiating RNA synthesis in vitro at many promoters on bacterial and phage DNA. Selective initiation involves recognition by holoenzyme of a promoter region of the DNA, tight binding of the polymerase to form a stable, binary complex with the DNA, and initiation of RNA chain synthesis whereby the initiating ribonucleoside triphosphate binds to the polymerase and forms a phosphodiester bond with a second bound nucleotide (see Chamberlin, 1974).

Many factors influence the selectivity of initiation of transcription on a DNA template. Altering reaction conditions may cause either the suppression or enhancement of biologically incorrect transcription or the suppression or enhancement of a particular set of biologically correct transcripts.

Variables which have been implicated in altering the selectivity of transcription at the level of initiation include protein factors (Scaife, 1973; Losick & Pero, 1976), temperature (Walter et al., 1967; Chamberlin & Ring, 1972; Mangel & Chamberlin, 1974c), monovalent cations (Millette et al., 1970; Schäfer & Zillig, 1973; Mangel & Chamberlin, 1974b; Matsuge, 1972; Dausse et al., 1976), divalent cations (Nakanishi et al., 1975; Chamberlin & Ring, 1972), enzyme concentration (Takanami et al., 1970; Brody et al., 1970; Chamberlin & Ring, 1972; Dausse et al., 1972), organic solvents (Nakanishi et al., 1974; Küpper et al., 1976; Brody & Leutey, 1973), and the configuration of the DNA (Hayashi & Hayashi, 1971; Botchan et al., 1973; Wang, 1974; Richardson, 1975).

In this paper, the effects of several of these variables on both total RNA synthesis and synthesis from specific promoters are examined. DNAs from bacteriophage λcb2 and T7 were used as templates since their in vitro transcription patterns have been studied extensively and characterized. The method used for determining synthesis from specific promoters involves fractionation and quantitation of the 5′-triphosphate ends of the RNA following digestion by T1 RNase, as described in the preceding papers (Miller & Burgess, 1978a,b). The usefulness of this method in the rapid and quantitative analysis of changes in initiation of transcription is demonstrated.

Experimental Procedure

Preparation of the DNA, RNA Polymerase, and [γ-^{32}P]-ATP and GTP. DNA from λcb2 and T7 was isolated as previously described (Miller & Burgess, 1978a). RNA polymerase was purified by the method of Burgess & Jendrisak (1975). [γ-^{32}P]ATP and GTP were prepared as described by Miller & Burgess (1978a).

Synthesis of RNA. A standard reaction mix for RNA synthesis (0.25 mL) contained the following: 0.03 M Tris-HCl (pH 7.9 at 25 °C), 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM KPO_4, 0.12 mM of [γ-^{32}P]ATP and GTP, 0.24 mM of the remaining nucleoside triphosphates, 0.15 M KCl, 30 μg of DNA, and 30 μg of RNA polymerase. The specific activity of the [γ-^{32}P]NTP was 1000 to 30 000 dpm/pmol depending

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