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Determination of nucleotide sequences beyond the sites of transcriptional termination

[DNA sequence/RNA-primed extension/oligo(A) additions]

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ABSTRACT **A procedure is described by which a discrete high-molecular-weight RNA transcription product can be used as a primer by DNA polymerase (DNA nucleotidyltransferase; EC 2.7.7.7; deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase) for determining nucleic acid sequence in the template DNA beyond the 3′-terminus of the transcript. This procedure is applied to two λ phage transcripts, the 4S "oop" RNA [short l-strand RNA transcript from the region of origin of replication (*ori*)] and the 6S RNA. Sequences of 35 and 19 nucleotides, respectively, following the sites at which these two transcripts terminate, are determined. Little structural homology is apparent in the template DNA beyond the 3′-ends of these two transcripts. The lack of homology suggests that this region might not be important to the termination process.**

We have been investigating primary structural information in those regions of a λ DNA template at which a number of *Escherichia coli* RNA polymerase-directed transcription products terminate, either in the presence or absence of the protein termination factor *rho* (Fig. 1). Two of these transcripts, the 4S "oop" RNA and the 6S RNA, have been shown to terminate (in the absence of *rho* factor) with the sequence purine$(U)_6A<^{OH}_{OH}$ (1, 2). Similar sequences have been found at the 3′-termini of a variety of bacterial RNA polymerase-mediated transcription products isolated from sources both *in vivo* and *in vitro* (3–5; M. Sogin, N. Pace, M. Rosenberg, and S. M. Weissman, submitted for publication). Our previous studies indicated that although the $(U)_6A<^{OH}_{OH}$ sequence predominates at the termini of both the λ 4S and 6S transcripts, these two RNAs actually possess similar degrees of 3′-terminal sequence heterogeneity which consists primarily of the addition of 1 to 5 adenylate residues to the 3′-ends of the transcripts (6). Similar oligoadenylate additions have been found on the λ 4S RNA isolated *in vivo* (7) as well as on the bacteriophage T7 "early" mRNAs isolated *in vivo* (8). We also found that *rho* factor strongly affected *in vitro* termination of the 4S RNA species; production of this RNA increased about 10-fold in the presence of *rho*.

The study presented here involves the determination of primary structure in the region of the λ DNA template distal to the 3′-termini of both the 4S and 6S transcription products. These sequences are determined by using the RNA transcripts, obtained *in vitro* and hybridized to the complementary single strand of λ template DNA, as primers for DNA polymerase I (DNA nucleotidyltransferase I) and extending them with deoxyribonucleotide substrates. The sequence information obtained will indicate whether the oligoadenylate additions and/or the structural effects resulting from *rho* action are template specified, and may define nucleic acid structure involved in the process of both independent and *rho*-dependent transcriptional termination.

Abbreviation: "oop" RNA, a short l-strand RNA transcript from the region of origin of replication (*ori*).

MATERIALS AND METHODS

Materials. *E. coli* RNA polymerase (RNA nucleotidyltransferase; EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase), *rho* factor, and separated strands of λ DNA were prepared by published procedures (9–11). Single strands were purified on alkaline sucrose gradients (12). Micrococcal nuclease and snake venom phosphodiesterase (Worthington Corp.), ribonucleoside α-^{32}P-labeled triphosphates (New England Nuclear Corp.), and DNA polymerase I (EC 2.7.7.7; nucleosidetriphosphate:DNA deoxynucleotidyltransferase) (Boehringer Corp.), were obtained commercially. Spleen phosphodiesterase was a gift from Dr. Edward Niles.

Preparative Synthesis of 4S and 6S RNAs. RNA synthesis was carried out as described (6) except for the following changes: reaction volume (1.0 ml); λ DNA concentration (100 μg/ml); ribonucleoside [α-^{32}P]triphosphate (specific activity about 0.5 Ci/mmol). Transcription reaction mixtures were incubated at 37° for 30 min. Reactions were terminated by the addition of 0.1 M Tris·HCl, pH 7.9, 0.1 M EDTA, 0.1% sodium dodecyl sulfate and rapid cooling to 0°. The reaction mixture was extracted with phenol and the RNA precipitated with 2.5 volumes of ethanol at −20°.

Specific RNA species were resolved on 4.5% polyacrylamide slab gels (8 M in urea) and eluted from the gel as described (6). The gel-purified transcripts were dialyzed extensively against distilled water and taken to dryness on a rotary evaporator at 35°. These transcripts were essentially pure and salt-free and could be used directly as primers for DNA extension. The yields of RNA obtained by these procedures are: 6S RNA, 8–10 pmol; 4S RNA, prepared without *rho*, 0.4–0.6 pmol; 4S RNA, prepared with *rho*, about 5 pmol.

Primed Extension by DNA Polymerase I. The purified RNA transcripts (0.5–1.0 pmol) were annealed to the corresponding complementary strand of λ*pgal*8 DNA (4S RNA to l strand, 6S RNA to h strand; 0.5–1.0 pmol) in 0.1 M Tris·HCl, pH 7.4, 0.1 M NaCl (200 μl). The mixture was intially heated to 95° for 2 min, followed by hybridization at 67° for 6–7 hr. The samples were then cooled to 4°.

For reactions in which only deoxynucleotide precursors were present, reaction conditions similar to those described by Loewen *et al.* (13) were used: dithiothreitol was added to 5 mM, $MgCl_2$ to 10 mM, the various deoxynucleoside triphosphates to 1–4 μM (one of which was labeled with α-^{32}P: specific activity 100–120 Ci/mmol), and DNA polymerase to 40 units/ml. The final volume of the reaction mixture was about 200 μl, and the reaction time (5–30 min) and temperature (5°–23°) varied for different reactions.

For reactions in which a ribonucleoside triphosphate was substituted for one of the deoxynucleotide precursors (i.e., "ribo-substitution" approach) (14–16), dithiothreitol was added to 5 mM, $MnCl_2$ to 0.67 mM, the unlabeled deoxytriphosphates to 50 μM, the labeled precursor (specific activity

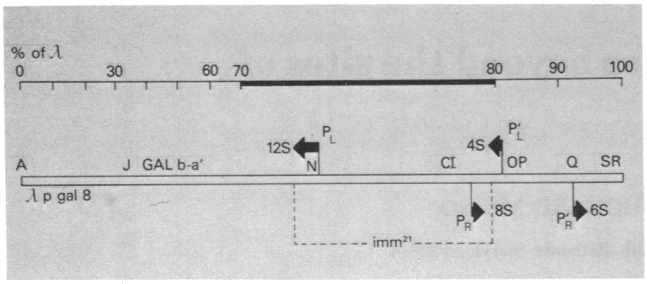

FIG. 1. Genetic map of λ*pgal*8. Locations and relative sizes of the major *in vitro* RNA transcripts and the boundaries of the λ*imm*21 immunity region are indicated.

100–120 Ci/mmol) to 5 μM, and the ribonucleotide to 1 mM. DNA polymerase was again used at a concentration of 40 units/ml. Reaction volumes were always about 220 μl; time (5–20 min) and temperature (15°–30°) again varied for different reactions.

The reactions were terminated by adding 0.2 M EDTA (20 μl) and tRNA carrier (100 μg), and subsequently extracted with phenol and precipitated with ethanol.

Isolation of the Extended RNAs. The precipitates obtained above were dissolved in 0.02 M Tris·HCl, 0.02 M EDTA (250 μl) and denatured at 100° for 2 min, followed by rapid cooling. This material was then chromatographed on a column (0.8 × 50 cm) of Sephadex G-50 to remove unincorporated precursor. Subsequent to elution the reaction products were reprecipitated with cold ethanol.

Sequence Determination. The elongated polynucleotide products were initially degraded enzymatically with either T1 or pancreatic RNase in order to remove all but the 3′-terminal oligoribonucleotide fragment of the RNA primer from the extended deoxynucleotide product. In this way, a product was obtained of appropriate size to be analyzed chromatographically by standard two-dimensional fractionation procedures. All further analyses performed on these products were by standard DNA sequencing techniques (16, 17). Homochromatography solutions were prepared similar to those described by Brownlee *et al.* (18).

RESULTS

Priming Ability of the 4S and 6S RNA Transcripts. The 4S and 6S RNA transcripts (chain lengths of about 80 and of 193 nucleotides, respectively), isolated *in vitro* and hybridized to the corresponding complementary single strand of λ DNA, serve as specific primers for the incorporation of deoxynucleotide precursors by DNA polymerase I. In the presence of the RNA primer, deoxynucleoside triphosphates are rapidly incorporated into high-molecular-weight material, whereas in the absence of RNA, little incorporation is observed. Even when all four deoxyprecursors are present, unprimed synthesis is ≤15% of that obtained with primer present. This primer-independent incorporation always remains associated with the template DNA and thus does not interfere with either the isolation or subsequent analysis of the primer extended products.

Characterization of Transcripts Extended Only with Deoxynucleotides. The 4S and 6S RNA transcripts prepared either in the presence or absence of *rho* factor were used in separate priming reactions (see *Materials and Methods*) in which various combinations of the four deoxynucleotide triphosphate precursors were present. The polynucleotide products of these reactions were digested with T1 RNase and characterized by two-dimensional "homochromatography" (18, 19) (Figs. 2 and 3). The RNA primers had also been labeled with ^{32}P, and thus both their characteristic T1 oligoribonucleotides and the newly synthesized deoxypolymers were detected by autoradiography. Identification of the various products by their relative chromatographic mobilities and subsequent analyses indicated that the deoxynucleotides were covalently linked to the characteristic 3′-terminal T1 oligonucleotide of the RNA primer [i.e., $A(U)_6A<^{OH}_{OH}$ for the 6S RNA and $(U)_6A(U)<^{OH}_{OH}$ for the 4S RNA]. The direct transfer of ^{32}P from the 5′ α-position of dTTP (the first deoxynucleotide to be incorporated) to the

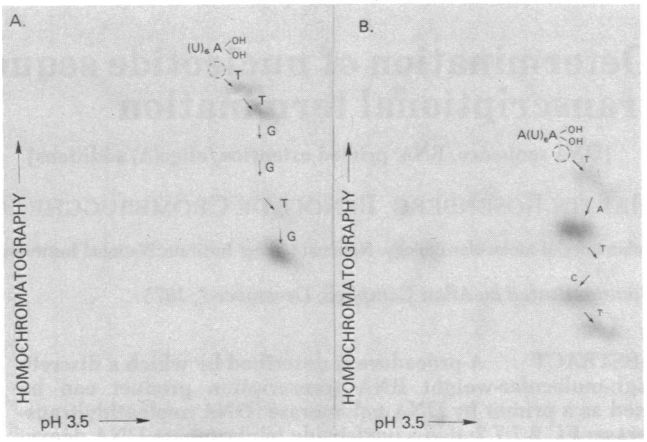

FIG. 2. Two-dimensional fractionation of T1 RNase digestion products resulting from 4S and 6S RNA-primed synthesis with DNA polymerase I. (A) 4S RNA extended in the presence of Mg^{++} with two deoxynucleotide triphosphates as substrates: [α-^{32}P]dTTP (2 μM; specific activity 100 mCi/μmol) and dGTP (2 μM). (B) 6S RNA extended in the presence of Mg^{++} with three deoxynucleoside triphosphates as substrates [α-^{32}P]dTTP (2 μM; specific activity 100 mCi/μmol), dATP (2 μM), and dCTP (2 μM). Reaction time and temperature for both reactions were 30 min at 10°. The products were purified as described in *Materials and Methods*, digested with T1 RNase, and fractionated by electrophoresis on Cellogel in 8.0 M urea at pH 3.5 (first dimension) and homochromatography (second dimension) on thin-layer plates of DEAE-cellulose (cellulose:DEAE, 9:1; 20 × 40 cm) using homomixture C (5% RNA hydrolyzed 30 min).

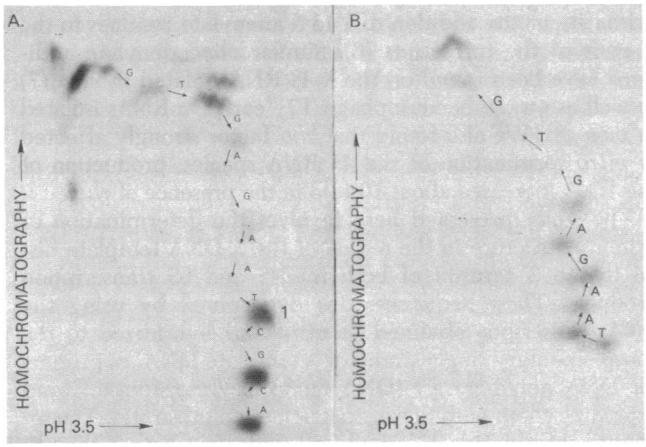

FIG. 3. (A) Two-dimensional fractionation (identical to that described in legend of Fig. 2) of a pancreatic RNase digest of the products resulting from 4S RNA-primed synthesis with DNA polymerase I in the presence of Mg^{++} and all four deoxynucleoside triphosphates: [α-^{32}P]dGTP (3 μM, specific activity 110 mCi/μmol), dATP (3 μM), dTTP (3 μM), and dCTP (1 μM). Incubation was for 10 min at 15°. (B) Two-dimensional fractionation (as in A) of the products resulting from partial digestion with snake venom exonuclease of the deoxy-extended oligonucleotide product designated 1 in Fig. 3A.

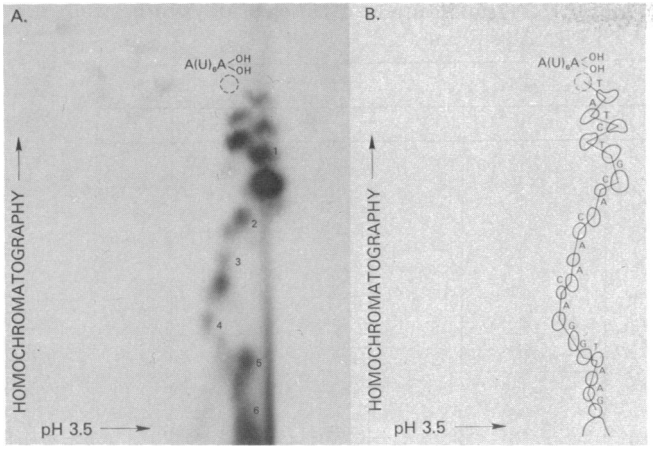

FIG. 4. Two-dimensional fractionation using homomixture B (5% RNA hydrolyzed 10 min) of a T1 RNase digest of the products resulting from a 6S RNA-primed synthesis with DNA polymerase I in the presence of Mn^{++} and rCTP (see *Materials and Methods*). Incubation was at 23° for 10 min. (A) Autoradiograph: numbers designate those products whose analyses are shown in Table 1. (B) Schematic sketch of A indicating the sequential order of single deoxynucleotide additions to the primer.

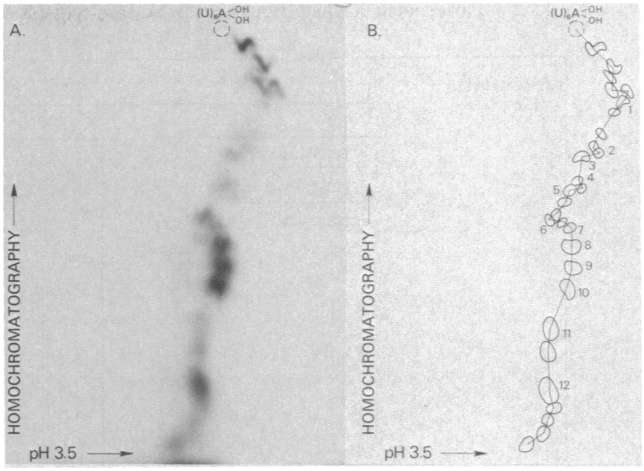

FIG. 5. Two-dimensional fractionation (identical to that shown in Fig. 4) of a T1 RNase digest of the products resulting from a 4S RNA-primed synthesis with DNA polymerase I in the presence of Mn^{++} and rCTP (see *Materials and Methods*). Incubation was at 15° for 12 min. (A) Autoradiograph; (B) schematic sketch of A: numbers designate those products whose analyses are shown in Table 2.

3′-position of the 3′-terminal ribonucleoside residue of the RNA was directly demonstrated for both the 4S and 6S species.

If one or more of the four deoxynucleotide triphosphates is omitted from the priming reaction, the size of the elongating product becomes limited (Fig. 2A and B). Increasing the reaction time or temperature does not increase the maximum chain-length of the synthetic products, but rather results only in the accumulation of the largest "limit" product. Elongation is impeded at a specific position due to the unavailability of the required incoming nucleotide. Only by the addition of the appropriate deoxynucleoside triphosphate could we obtain further extension of the product (as in Fig. 3A). These observations and subsequent analyses of the oligonucleotides obtained from the different reactions indicated that a unique sequence of deoxynucleotides was being added to the 3′-ends of both the 4S and 6S RNAs.

As demonstrated in Figs. 2–5, it is possible to control the conditions of a particular reaction (e.g., by variation of triphosphate concentration, reaction time, and temperature) so as to obtain a variety of products differing in the number of deoxynucleotide residues that have been added to the RNA primer. These oligomers of varying chain length represent a series of specific single-nucleotide additions to the 3′-terminus of the primer RNA. The relative mobility shift observed for each successively larger product is uniquely characteristic for the particular nucleotide that has been added to that product. Thus, the identity and, in turn, the sequential order of the deoxynucleotides being added to the primer are directly obtained. From this analysis it is possible to deduce an unambiguous sequence for some 15 to 20 nucleotide residues beyond the 3′-ends of both the 4S and 6S RNA transcripts. The sequences obtained in this manner were confirmed by: (*i*) nearest neighbor analysis of each synthetic product labeled separately with each of the four deoxynucleotide precursors (16); (*ii*) partial digestion of various products with snake venom exonuclease (17), successive exonucleolytic removal of single nucleotide residues from the 3′-end of the oligomer resulted in products that had relative chromatographic mobilities identical to the synthetic products obtained directly from the priming reactions (compare Fig. 3A and B); and (*iii*) the technique of "ribosubstitution" described in the following section (Figs. 4 and 5, Tables 1 and 2).

Characterization of Transcripts Extended by "Ribosubstitution". In order to both confirm and extend the sequences obtained in the previous section, the "ribosubstitution" approach (14–16) was adopted. Primed extension with DNA polymerase was carried out in the presence of Mn^{++}, rCTP, and the three other deoxynucleotide triphosphates (see *Materials and Methods*). The products, in which all the C residues were now present as ribonucleotides, were initially digested with T1 RNase to remove the RNA primer (except for the 3′-terminal oligonucleotide) from the elongated product and fractionated by two-dimensional "homochromatography" (Figs. 4 and 5). Homochromatography solu-

Table 1. Pancreatic RNase digestion products of successively longer rCTP-substituted polynucleotides synthesized by DNA polymerase I using the 6S RNA as primer

Polynucleotide (Figure 4)	Pancreatic RNase Digestion Products					
	PI	PII	PIII	PIV	PV_1	PV_2
1	rATATrC(T)					
2	"	TGrC(A)				
3	"	"	ArC(A)			
4	"	"	"	AArC(A)		
5	"	"	"	"	AGGT<OH,OH	
6	"	"	"	"	AGGTAAG<OH,OH	
Methods used to determine sequence*	a,b,c,d,e	a,b,c,d	a,b,d	a,b,d	a,b,c,d,e	

* Methods used: (*a*) nearest neighbor analysis of each oligonucleotide labeled separately with [α-^{32}P]dATP, dGTP, and dTTP (16); (*b*) electrophoretic mobility on DEAE-paper at pH 3.5 (19); (*c*) electrophoretic mobility on DEAE-paper at pH 1.7 (19); (*d*) characteristic mobility shifts on two-dimensional homochromatography resulting from the successive addition of this sequence of nucleotides to the extending polynucleotide chain (16, 17); (*e*) partial digestion with snake venom diesterase and characterization of products by two-dimensional homochromatography (17); (*f*) partial digestion with spleen acid phosphodiesterase and characterization of products by two-dimensional homochromatography (17).

Table 2. Pancreatic RNase digestion products of successively longer rCTP-substituted polynucleotides synthesized by DNA polymerase I using the 4S RNA as primer

Polynucleotide (Figure 5)	Pancreatic RNase Digestion Products											
	CI_1	CI_2	CI	CII	CIII	CIV	CV_1	CV	CVI	CVII †	CVIII	CIX
1	rATTGGTG<$^{OH}_{OH}$											
2	rATTGGTGAGAAT<$^{OH}_{OH}$											
3	rATTGGTGAGAATrC(G)											
4	"			GrC(A)								
5	"			"	AGrC(A)							
6	"			"	"	AArC(T)						
7	"			"	"	"	TTG<$^{OH}_{OH}$					
8	"			"	"	"	TTGTrC(G)					
9	"			"	"	"	"		GrC(G)			
10	"			"	"	"	"		"	GrC(rC)		
11	"			"	"	"	"		"	"	rC(A)	
12	"			"	"	"	"		"	"	"	AArC(G)
Methods used to determine sequence*	a,d,e,f			a,b,c	a,b,c	a,b,c	a,b,c,d,e		a,b,c,d	d	a,b,c	a,b,c,d

* See Table 1.

† This product is not labeled in these reactions. From the analysis of products CVI and CVIII, and from the mobility shifts observed for these polynucleotides in Fig. 5, product CVII was deduced to be $GrC_{\geq 1}(rC)$. Using rGTP substitution in a similar set of experiments, the T1 RNase product CCAATCrG was obtained, indicating the presence of *two* successive C residues at this position in the sequence.

tions were used that separated oligomers up to 40 residues in chain length. Thus, a series of products was obtained representing rC-substituted oligodeoxynucleotides of increasing size that had been added to the RNA primer (Figs. 4 and 5). Each of these was eluted from the thin-layer plate and digested under rather severe conditions (16) with pancreatic RNase. The resulting products, which had been cleaved specifically at the rC residues, were characterized as described in Tables 1 and 2. By simply comparing the various pancreatic RNase products obtained from the successively larger oligonucleotides, it was possible to deduce the relative order in which these products had been added to the primer.

A similar set of reactions was carried out using rGTP substitution. The products obtained after extensive T1 digestion (16) of the elongated primer were found to be consistent with the sequence derived above by rCTP substitution.

DISCUSSION

A procedure has been described by which a discrete high-molecular-weight RNA transcript obtainable in picomole quantities can be used as a primer by DNA polymerase for rapidly determining nucleotide sequences in the template DNA immediately adjacent to the 3′-terminus of the transcript. This method was applied to two well-characterized λ transcription products, the 4S and 6S RNAs. Sequences of 35 nucleotides beyond the $(U)_6A(U)<^{OH}_{OH}$ 3′-terminus of the 4S RNA and 19 nucleotides beyond the $A(U)_6A<^{OH}_{OH}$ 3′-terminus of the 6S RNA were determined (Fig. 6). The sequence obtained in the terminator region of the 6S RNA agrees with and removes the ambiguities associated with the sequence independently derived by Sklar *et al.* (20) using RNA sequencing techniques. After completion of our work, we learned that Kleid *et al.* (21) had obtained a sequence identical to ours for the region beyond the 3-′ end of the 4S RNA. This sequence was determined on a different λ strain (*c*I857S7) by direct DNA sequencing of restriction enzyme fragments derived from this region of the DNA.

Oligo(A) Additions and the Effect of *rho*. It has been previously demonstrated that both the 4S and 6S RNA transcripts terminate in identical sequences containing similar degrees of 3′-terminal heterogeneity (6). The 4S and 6S transcripts with 3′-terminal sequences $(U)_5U<^{OH}_{OH}$, $(U)_6A<^{OH}_{OH}$, and $(U)_6AU<^{OH}_{OH}$ all acted as efficient primers for DNA polymerase in our experiments. Extension of the minor $(U)_5U<^{OH}_{OH}$ terminated primer was established by detection and characterization of the products $(U)_6dA<^{H}_{OH}$, $(U)_6dAT<^{H}_{OH}$, etc. from reactions in which [α-^{32}P]dATP served as the labeled precursor. Thus, the terminal adenosine residue found on the 3′-end of both the 4S and 6S RNAs prepared in the absence of *rho* factor is encoded for in the template DNA. Furthermore, the additional uridylate residue found at the termini of some of the 6S RNA transcripts and the majority of 4S RNA transcripts prepared in the presence of *rho* is also template encoded and may be specified directly by transcription.

A second source of heterogeneity in the 4S and 6S transcripts is the presence of oligoadenylate additions at the 3′-termini (6). These transcripts with terminal sequences $(U)_6A(A)_{1-4}$ and $(U)_6AU(A)_{1-4}$ were not extended by DNA polymerase in our priming experiments. This suggests that the oligoadenylate residues are not template encoded and illustrates the sensitivity of the priming reaction to proper base-pairing between primer and template where primer extension is to occur. Examination of the sequences determined by extension of the $(U)_6A(U)$ terminated 4S and 6S RNAs (Fig. 6) confirms that the oligoadenylate additions are not specified by the template DNA. Apparently they represent a modification occurring subsequent to template specified transcription.

Termination and Anti-Termination. Comparison of the sequences in the template DNA beyond the ends of the 4S and 6S transcripts indicates that the 3′-terminal sequence homology, purine$(U)_6A<^{OH}_{OH}$, between these two RNAs extends for only a single additional residue. Beyond this position the two sequences diverge. The region of homology at the termination sites of these RNAs, however, occurs within a larger region of extensive A-T base-pairing: 11 consecutive A-T pairs for the 6S RNA and 9 consecutive A-T pairs for the 4S RNA (Fig. 6). The lack of any additional homology in

FIG. 6. Nucleotide sequences determined from the 4S and 6S RNA-primed syntheses on bacteriophage λ*pgal*8 DNA. DNA sequence actually deduced is indicated in **bold-face** type. (*a*) 3′-Terminal sequence of the RNA primer; (*l*) "light" strand of λ*pgal*8 DNA; (*h*) "heavy" strand of λ*pgal*8 DNA.

these regions suggests that most, if not all, of the nucleic acid structure specifying termination is transcribed. It is interesting to note that entry sites for RNA polymerase on DNA also appear to be rich in A-T base-pairs..

It has been suggested (22) that the termination sites of the 4S and 6S RNAs may be anti-terminated *in vivo* so as to allow expression of information distal to the 3′-ends of these transcripts. Evidence implicating the 4S RNA as a primer for λ DNA replication has also been presented (23). If transcriptional read-through occurred at either of these termination sites, our sequences would predict that the characteristic T1 oligonucleotides UUUUUUAUUG(G) for the 4S RNA and AUUUUUUAUAUCUG(C) for the 6S RNA would be generated as a direct result of read-through and could, in turn, be used to monitor the extent of anti-termination. Our own preliminary evidence indicates that the 4S RNA is not a completely self-terminating transcript. *In vitro* in the absence of *rho* factor, some "read-through" of the 4S RNA termination site occurs. In short kinetic transcription experiments much of the 4S RNA isolated by selective hybridization appeared to be stopped at the terminal $(U)_6A<^{OH}_{OH}$ position; however, the predicted T1 oligonucleotide $(U)_6$AUUG(G) was readily detected.

Terminal Region of 4S "oop" RNA from λ*imm*21 Is Different. The heteroimmune phage λ*imm*21, in which the immunity region of λ has been substituted by that of the lambdoid phage 21 (Fig. 1), also makes an "oop" RNA 4S in size. Comparison of T1 and pancreatic oligonucleotide "fingerprints" of the λ*imm*21 4S RNA with that made from λ*pgal*8 (with λ immunity) indicates that these two RNA species are similar in primary structure, but not identical. Furthermore, it appears that all of the sequence differences detected between the two RNA species occur in the 3′-terminal (about 20%) region of the molecule; their structures being identical for the first 80% of their chain length. Although these sequence differences do not affect the ability of the λ*pgal*8 4S RNA to efficiently hybridize to the l-strand DNA of λ*imm*21, we find that the resulting complex will not serve as template and primer in our reactions for extension by DNA polymerase I. However, when λ*imm*21 4S RNA is hybridized to λ*imm*21 l-strand DNA, the resulting complex readily serves as primer for extension by DNA polymerase. Preliminary examination of the products resulting from this reaction indicates that the sequence immediately distal to the 3′-terminus of the λ*imm*21 4S RNA is not identical to that determined above with the 4S RNA of λ*pgal*8. The extent of nonhomology apparently restricted to this region of the template suggests that the right-hand boundary of the immunity substitution in bacteriophage λ*imm*21 may occur within the 3′-terminal region of the "oop" gene (see Fig. 1). Thus, the 3′-terminal portion of λ*imm*21 4S RNA may originally derive from phage 21. Characterization and comparison of the nucleic acid sequences in the "terminator" regions of this and other similar transcripts should help define the structure that specifies transcriptional termination.

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