

Determination of Genes, Restriction Sites, and DNA Sequences Surrounding the 6S RNA Template of Bacteriophage Lambda

(restriction enzymes/transcription termination/DNA sequences/lambda Q gene)

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ABSTRACT A major product of the transcription of bacteriophage lambda DNA *in vitro* is the 6S RNA. This article presents a detailed mapping of restriction endonuclease cleavage sites about the region of the 6S RNA template within the lambda genome. Restriction fragments defined by these sites have been used to localize the 6S RNA template within the physical and genetic maps of the lambda genome. Nucleotide sequence analysis of one of these fragments has largely confirmed the nucleotide sequence of the 6S RNA reported previously and has indicated the sequence of DNA that immediately follows the 6S RNA template. This article reports the nucleotide sequence following a known site of transcription termination by RNA polymerase of *Escherichia coli*.

Genetic analysis of bacteriophage lambda has been underway for a number of years and has revealed control mechanisms of potentially broad biological significance (1). In addition, the properties of lambda DNA as a template for transcription *in vitro* have come under intensive investigation in recent years (1-5).

One striking early finding concerning the template activity of lambda DNA was that purified *Escherichia coli* RNA polymerase, in the absence of rho termination factor, transcribes a discrete RNA species, the 6S RNA, from the lambda DNA template (2). The nucleotide sequence of the 6S RNA is now known (2). The transcription of this RNA continues to be of interest since its promoter initiates transcription more efficiently *in vitro* than any other promoter in lambda DNA (6) and termination of transcription occurs with precision, presumably determined by specific DNA sequences near the site of termination.

We are currently engaged in investigation of the 6S RNA to determine the nucleotide sequences within the DNA that control the transcription of this RNA and to determine what role the 6S RNA might play *in vivo* during the course of lambda infection. In this report we present a detailed mapping of restriction enzyme cleavage sites within the bacteriophage chromosome near the template for the 6S RNA. We have used the DNA fragments produced by these various cleavages to precisely locate the 6S RNA template both on the physical map of lambda DNA and with respect to known genetic markers deleted from certain defective lambda prophages. We have also used these fragments to confirm portions of the sequence of the 6S RNA and to analyze the sequence of the DNA beyond the 3'-end of this RNA.

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MATERIALS AND METHODS

Materials. ³²P-Labeled, radioactive lambda DNA was prepared from λgal8S7 bacteriophage (7) grown on *E. coli* strain C600 in medium containing 2-5 mCi of inorganic [³²P]-phosphate per liter. Phage were banded in CsCl density gradients, and DNA was extracted from these phage as described (2). Nonradioactive *E. coli* DNA bearing deletion mutants of the lambda prophage was prepared by phenol extraction from *E. coli* strains numbers 5061, 1153, 1118, and 509 (8), which were generously provided by Dr. I. Herskowitz. *E. coli* DNA-dependent RNA polymerase (EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase) was prepared by the method of Lebowitz *et al.* (2). *EcoRI* and *EcoRII* restriction endonucleases were prepared by the method of Yoshimori (9). Restriction endonucleases from *Hemophilus influenzae* D (*Hind*) (10), *H. influenzae* F (*Hinf*), *H. aegyptius* (*Hae*) (11), *H. parainfluenza* (*HpaII*) (12, 13), and *H. haemolyticus* (*Hha*) were prepared by the method of Smith and Wilcox (10). All other enzymes were obtained from commercial sources.

Preparation and Fractionation of DNA Fragments. DNA fragments of λgal8S7 [³²P]DNA produced by digestion with various restriction enzymes were fractionated by electrophoresis on slab gels of various percentages of polyacrylamide, as described (14). Fragment bands in the gels were visualized by autoradiography using Kodak RPR54 x-ray film. DNA fragments were eluted from excised gel segments by grinding and soaking the gel for several hours in 1.5 mM NaCl/1.5 mM Na citrate (pH 7.0) at 4°.

RNA-DNA Hybridizations. Procedures for the fixation of DNA to nitrocellulose filters and for the hybridization of RNA to filter-bound DNA are described in ref. 14.

Preparation of RNA and RNA Sequence Analysis. Transcription of lambda DNA for the preparation of ³²P-labeled 6S RNA is described by Lebowitz *et al.* (2). 6S RNA was purified from total RNA produced in lambda DNA transcription by electrophoresis at pH 8.3 on 4% polyacrylamide slab gels containing 8M urea. Transcription of *Hinf*-3 fragment (see *Results* and Fig. 1) was performed as described in ref. 14, except that the fragment was denatured by boiling in water for 5 min and cooled before being added to the reaction mix. α-³²P-Labeled nucleoside triphosphates (specific activity 50-150 Ci/mmol) used in all transcriptions were purchased from New England Nuclear Corp.

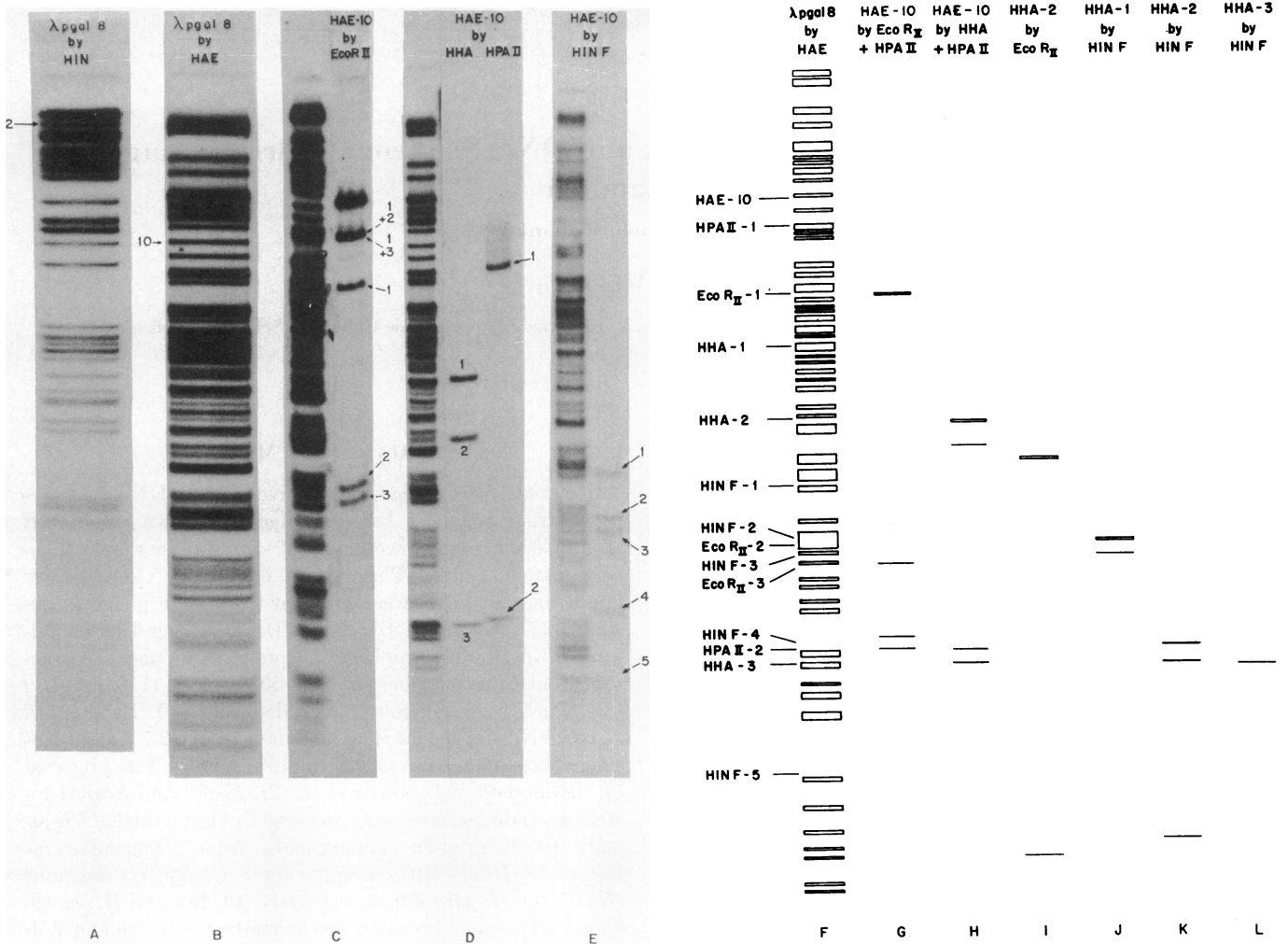


FIG. 1. Gel electrophoresis of restriction enzyme digests of λ pgal8S7 DNA. (A-E) Autoradiograms of restriction enzyme digests of 32 P-labeled λ pgal8S7 DNA and fragment *Hae*-10. Panel A is an autoradiogram of a 3.2% polyacrylamide gel; all other gels are 4% polyacrylamide. Panels D-F show digests of fragment *Hae*-10 alongside an *Hae* enzyme digest of whole λ pgal8S7 DNA coelectrophoresed in the same gel to indicate the position of *Hae*-10 subfragments with respect to a standard *Hae* fragment pattern of whole λ pgal8S7 DNA. (F-L) Schematic representation of autoradiograms of restriction enzyme digests of λ pgal8S7 DNA and subfragments of *Hae*-10. Panel F represents an *Hae* enzyme digest of λ pgal8S7 electrophoresed on a 4% polyacrylamide gel; the positions of cleavage products in all other panels are indicated with respect to the *Hae* digestion pattern in panel F.

Details of methods used for nucleotide sequence analysis of RNA are described in refs. 2, 15, and 16.

RESULTS

To locate the region of lambda DNA from which 6S RNA is transcribed, we prepared *Eco*RI, *Hae*, and *Hind* restriction endonuclease digests of λ pgal8S7 and lambda wild-type DNA. By hybridization, radioactive 6S RNA was found to be complementary to *Eco*RI fragment no. 3, the subterminal fragment from the right-hand half of the lambda chromosome (17, 18), and to fragments *Hind*-2 and *Hae*-10 (Fig. 1A and B). The two latter fragments also extended into the terminal *Eco*RI fragment, as verified by comparison of gel patterns produced by digesting *Hind*-2 and *Hae*-10 fragments with *Eco*RI and the subterminal and terminal *Eco*RI fragments with *Hind* and *Hae* enzymes.

A further series of restriction enzyme cleavage sites was localized within the *Hae*-10 fragment (Fig. 1). Cleavage of *Hae*-10 by *Hpa*II endonuclease produced two fragments (Fig. 1D). The smaller of these fragments was recleaved by *Eco*RI,

placing it on the right-hand end of *Hae*-10. Cleavage of *Hae*-10 by *Eco*RI gave two small fragments, one of which was recleaved by *Hpa*II, and one large fragment (Fig. 1G). Incomplete digestion by *Eco*RI enzyme gave partial digestion fragments whose lengths were equal to the length of the large plus one or the other of the small fragments (Fig. 1C), and which when recleaved by *Eco*RI produced the large and one of the small complete digestion fragments. Therefore, *Eco*RI enzyme cleaves *Hae*-10 twice, once near either end of the fragment. *Hha* restriction enzyme digestion produced three fragments from *Hae*-10 (Fig. 1D). The largest *Hha* fragment, *Hha*-1, was cleaved by *Hpa*II (Fig. 1H), and therefore lies at the right-hand end of *Hae*-10. When the second largest *Hha* fragment, *Hha*-2, was recut by *Eco*RI, a fragment was produced that was considerably smaller than any of the complete *Eco*RI digestion products and a large fragment that was larger than either of the two small *Eco*RI fragments (Fig. 1I). Therefore, the left-hand small *Eco*RI fragment must overlap *Hha*-2 and *Hha*-3, and *Hha*-3 must lie at the left-hand end of the *Hae*-10 fragment. Digestion of *Hae*-10 by *Hinf*

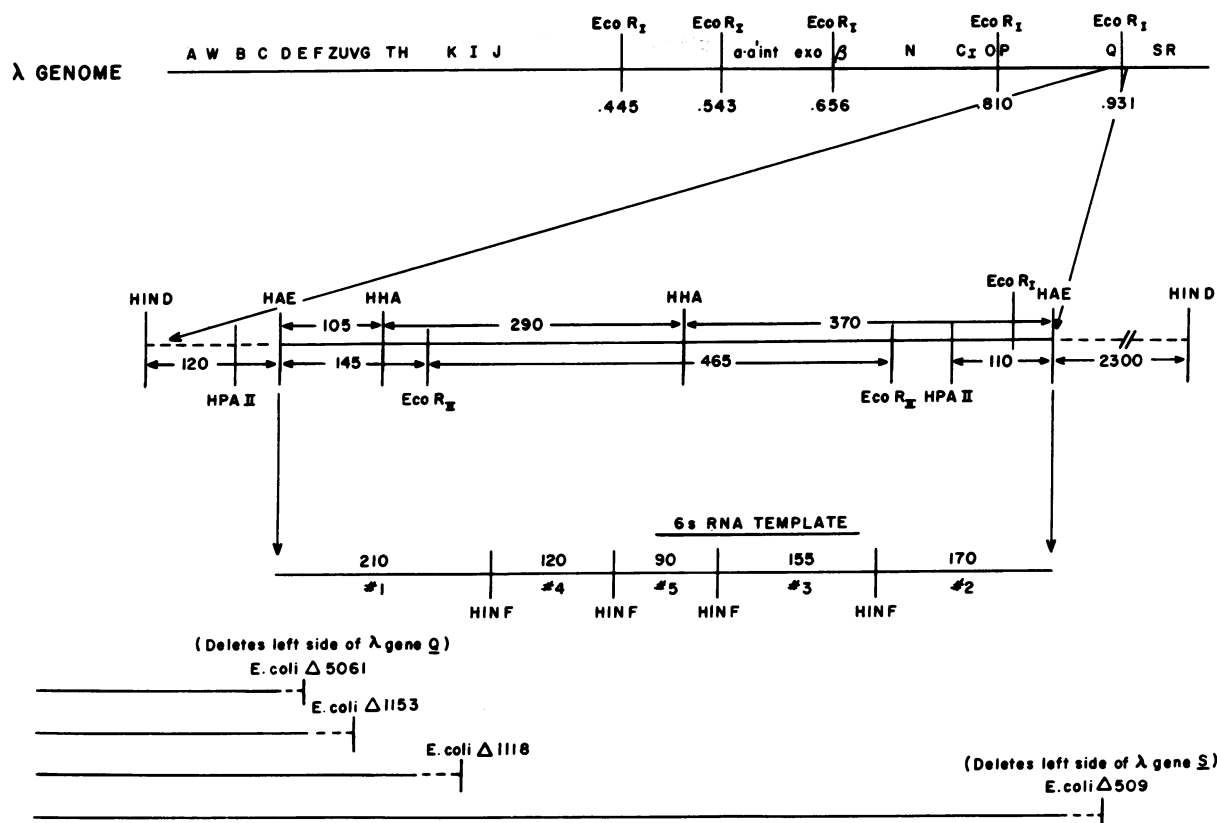


FIG. 2. Map of restriction enzyme cleavage sites within the region of the 6S RNA template of λ gal8S7 DNA. The upper line represents the linear λ genome, indicating the fractional distances of the *EcoRI* enzyme cleavage sites from the left-hand end of the wild-type λ chromosome. The region of the λ genome near the most rightward *EcoRI* site has been expanded on the second line of the figure to show the position of restriction enzyme sites within this region. Approximate distances between sites is shown in numbers of base pairs. The region between the two *Hae* sites, defining the *Hae-10* fragment, is redrawn in the third line of the figure, and the positions of the four *Hinf* sites and the 6S RNA template are shown. At the bottom of the figure is shown the approximate relative positions of the right-hand ends of four λ prophage deletions, as estimated from the data of Table 1. Although the precise positions of the deletion endpoints cannot be determined from these data, the ends of the three deletions at the left appear to lie within the DNA of fragment of *Hinf-1*, while the fourth deletion appears to remove all DNA corresponding to *Hae-10* from the deleted prophage.

enzyme gave five fragments (Fig. 1E). Fragment *Hinf-2* was recut by *HpaII*, so that it must lie at the right-hand end of *Hae-10*. The largest *Hha* fragment contained all of *Hinf-3* and -2, and the second largest *Hha* fragment contained *Hinf-4*, while the smallest *Hha* fragment was completely contained within *Hinf-1* (Fig. 1J-L). The only arrangement of cleavage sites consistent with these data is that shown in Fig. 2.

The localization of the 6S RNA template within various restriction enzyme DNA fragments was confirmed by transcribing the DNA fragments and mapping a T1-RNase digest of the RNA product by two-dimensional chromatography. When *Hinf-3* was transcribed and the resulting RNA mapped in this manner, a characteristic set of oligonucleotides was obtained (Fig. 3). Among these oligonucleotides were all those expected from approximately position 62 onward in the 6S RNA sequence (Fig. 4). Similarly, *Hinf-5* transcripts gave the T1 RNase products from the initial segment of the 6S RNA (data not shown; Sklar, Yot, and Weissman, manuscript in preparation). In addition to the oligonucleotides derived from the 6S RNA sequence, transcripts of *Hinf-3* gave a second set of oligonucleotides that could be identified as deriving from the DNA sequence complementary to the 6S RNA and a third set of oligonucleotides not contained in their entirety within either the 6S RNA or its complement. This third set must

contain transcripts of *Hinf-3* from beyond the 3'-terminus of the 6S RNA. Two large oligonucleotides from among this third set of T1 RNase products proved by sequence analysis to be complementary oligonucleotides which overlapped the site at which termination of the 6S RNA normally occurs when whole lambda DNA is transcribed. The sequence of these oligonucleotides establishes the sequence of the first seven bases beyond this point in the DNA. The remainder of the oligonucleotides from this set could be uniquely arranged into two antiparallel complementary sequences (Fig. 4, portion of sequence lying to the right of the empty parenthesis). The left-hand terminus of this segment could join directly to the extended 6S RNA sequence.

To locate the restriction endonuclease fragments with regard to known genetic markers, we prepared DNA from *E. coli* lysogenic for several prophages that carry deletions extending into or through the lambda *Q* gene and in one case into the lambda *S* gene (8). In experiments similar to those of Dr. J. Roberts, who has directly hybridized 6S RNA to the DNA of lambda prophage deletion mutants, transcripts of the *Hinf* restriction fragments were prepared and annealed to DNA that had been extracted from these bacteria and immobilized on nitrocellulose filters. DNA isolated from *E. coli* number 5061, in which the left end of the *Q* gene is

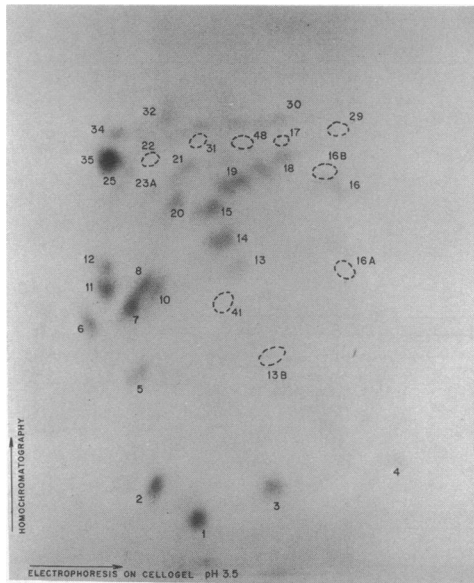


Fig. 3. Two-dimensional chromatography of T1 RNase digestion products of RNA transcribed from DNA fragment *Hinf-3*. The RNA was labeled with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Broken circles indicate positions of T1 oligonucleotides not labeled by $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ or not readily visible in the chromatogram shown.

deleted, hybridized RNA transcribed from fragment *Hinf-1* to a significant degree (Table 1). However, hybridization was less efficient than with control DNA from a nondeleted

lysogenic strain of *E. coli*, so that deletion 5061 probably extends a short distance into *Hinf-1* (Fig. 2). The deletions contained within *E. coli* strains numbers 1153 and 1118, which extend through all *Q* gene markers but which do not remove the site of action of the *Q* gene product, appear to delete DNA further into *Hinf-1* because RNA transcribed from *Hinf-1* hybridized to the DNA of these two strains at levels below that of strain 5061 (Table 1 and Fig. 2). The deletion that removes the site of *Q* gene action and extends into the *S* gene, contained in *E. coli* number 509, appears to remove all DNA complementary to *Hae-10* (Table 1 and Fig. 2). Therefore, within the limits of available deletion markers, the 6S RNA lies between genes *Q* and *S*, very close to the site of *Q* gene action.

DISCUSSION

The present results unequivocally locate the origin for the 6S RNA between the known markers for the *Q* and *S* genes of lambda, and within the DNA segment that bears the site at which *Q* gene product activates the expression of the late genes of the phage (8). Dr. J. Roberts has reached similar conclusions. Independent of other evidence our results demonstrate that the direction of transcription of the 6S RNA is from left to right, the same direction as that of the late genes. Several investigators, including Blattner and Dahlberg (4), J. Roberts, and ourselves, have independently considered that 6S RNA transcription may be involved in conjunction with the *Q* gene product in the initiation of transcription of the late genes, and that the *Q* gene product may act as an anti-terminator for the 6S RNA. This would make the action of the *Q* gene analogous

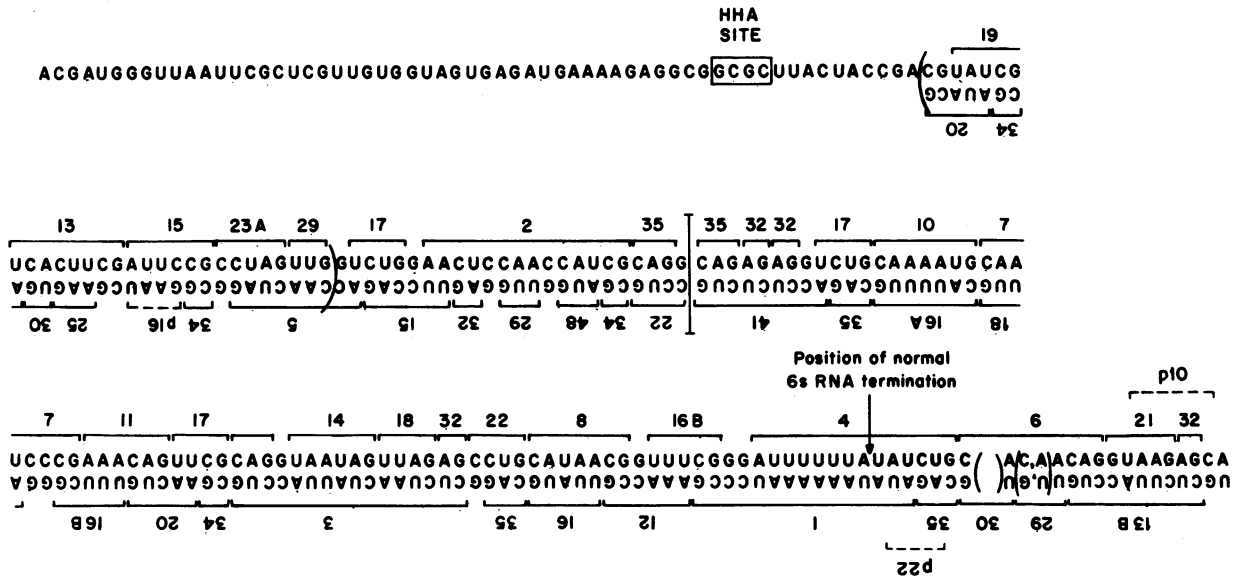


Fig. 4. Nucleotide sequence of RNA transcribed from DNA fragment *Hinf-3*. Hyphens have been omitted. The nucleotide sequence of the 6S RNA is presented in the upper line of the sequence. The left-hand end of the *Hinf-3* fragment transcript lies at the beginning of the double-stranded sequence in the top row of the figure. Brackets and numbers refer to the T1 oligonucleotides of Fig. 3. Several pancreatic RNase oligonucleotides that were helpful in confirming portions of the sequence are indicated by broken brackets. The large parentheses at the left-hand end of the fragment sequence enclose an area of sequence less certainly contained within the *Hinf-3* transcript, principally because the expected T1 oligonucleotide A-A-U-C-G complementary to oligonucleotides no. 13 and no. 15 could not be detected in any analysis of T1 RNase products. The vertical bar through the second row of the figure indicates the position where the sequence C-A-G-C-G had tentatively been placed in the original derivation of the 6S RNA sequence. This sequence has been removed from this figure because no C-A-G-(C) T1 oligonucleotide was detected, and inclusion of this sequence would predict an *Hha* enzyme site within the *Hinf-3* fragment. The empty parentheses after T1 oligonucleotide no. 4 represent a region of the sequence where no conclusive overlapping oligonucleotides were detected and where, consequently, additional sequence might be inserted. Assignment of oligonucleotides to the upper or lower lines of the sequence was confirmed by mapping RNA transcribed from *Hinf-3* which had first been hybridized to and eluted from separated strands of lambda DNA.

TABLE 1. Hybridization of ³²P-labeled transcripts of *Hinf* restriction fragments from the 6S RNA region of λ DNA to the DNA of λ prophage deletion mutants

Fragment transcript	Counts/10 min [³² P]RNA added per vial	Counts/10 min of [³² P]RNA hybridized					
		Control DNA		DNA of <i>E. coli</i> lysogen bearing λ prophage deletions			
		<i>E. coli</i> C600 (-λ)	<i>E. coli</i> 361 (+λ)	<i>E. coli</i> 5061	<i>E. coli</i> 1153	<i>E. coli</i> 1118	<i>E. coli</i> 509
<i>Hinf-1</i>	130,000	371	2,617	1,255	940	717	428
	500,000	1,601	8,888	5,983	5,342	3,319	2,501
<i>Hinf-4</i>	500,000	748	9,049	10,442	9,723	6,073	1,278
<i>Hinf-2</i>	500,000	1,003	15,923	19,194	19,105	20,635	1,457

DNA (50 μg) from six *E. coli* strains was fixed to separate nitrocellulose filters. In each experiment, each of the six filters was incubated in separate vials with identical numbers of cpm of [³²P]RNA transcribed from a *Hinf* DNA fragment. After incubation at 67° for 12 hr, T1 RNase treatment, and thorough rinsing, the amount of radioactive RNA annealed to each filter was determined.

to that of the *N* gene on early, immunity-regulated, leftward and rightward transcription of lambda (1, 3). Experiments designed to test this hypothesis are in progress in this laboratory.

In addition to rho factor-independent termination of transcription of 6S RNA at the sequence G-G-G-A-U-U-U-U-U-U-A, other RNA species have been observed either *in vivo* or *in vitro* with the terminus U-U-U-U-U-purine (19, 20), or, in the case of the *B. subtilis* 5S RNA precursor, U-G-G-G-G-U-U-U-U-U-U-G-U-U-U-U-U-G (M. Sogan, N. Pace, M. Rosenberg, and S. M. Weissman, in preparation). These findings seem beyond coincidence and might indicate that purine-U-U-U-U-U-purine is at least a part of a common termination signal. Other, untranscribed nucleotides may also be a part of the termination signal, although the sequence beyond the 3'-end of the lambda 4S RNA is not identical to that observed for the 6S RNA (Sklar and Weissman, unpublished observations). Comparison of DNA sequences known beyond the 3'-end of bacterial RNA or animal cell mRNA may be misleading because it is not clear whether the 3'-termini of these RNA arise through the action of a termination signal or through the post-transcriptional nucleolytic cleavage of a precursor (21, 22).

It was previously observed that, in the presence of rho factor, much of the 4S and some of the 6S RNA has the terminus U-U-U-U-U-A-U (5). In at least the case of the 6S RNA, the terminal uridine is also represented in the DNA and could have been added by transcription. In contrast, oligoadenylic acid, which is found attached to a portion of the molecules, is not due to the transcription of oligo(dT) sequences in the DNA.

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