A potential stem-loop structure and the sequence CAAUCAA in the transcript are insufficient to signal ρ -dependent transcription termination at λ tR1

Lester F.Lau*[§], Jeffrey W.Roberts*, Ray Wu*, Fawzy Georges⁺ and Saran A.Narang⁺

*Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA, and ⁺Division of Biological Sciences, National Research Council of Canada, Ottawa KIA OR6, Canada

Received 11 August 1983; Revised 2 November 1983; Accepted 16 November 1983

ABSTRACT

It has been suggested that a sequence in the RNA transcript that can form a stem and loop structure, followed by the sequence CAAUCAA, is the signal for ρ -dependent transcription termination. We tested this hypothesis by synthesizing a DNA duplex whose sequence corresponds to a region of the λ t_{R1} terminator that contains these structural features. We cloned this synthetic DNA fragment under the control of the lacUV5 promoter, and showed that it does not cause ρ -dependent termination in vitro. RNA polymerase pauses during in vitro transcription across the synthetic sequence, although less efficiently than at the corresponding sequence on the λ template. No ρ -mediated termination was detected even under conditions that prolonged transcriptional pausing at the synthetic site, indicating that the synthetic sequence is defective as a transcript release site. We suggest that unlike ρ -independent terminators, ρ -dependent terminators require sequences in addition to those immediately before the sites of termination.

INTRODUCTION

Like transcription initiation, transcription termination is a process important to the regulation of gene expression (1,2). Escherichia coli RNA polymerase responds to discrete sequences on the DNA template that direct transcription termination. Some such sequences cause transcription termination in vitro in the absence of additional protein factors. These sequences, commonly known as ρ -independent terminators, characteristically encode a stem and loop structure in the RNA transcript, followed by a 3'-terminal stretch of uridine residues (3,4). Extensive studies support the notion that these structural features are necessary and sufficient for ρ -independent termination (for reviews, see 4,5). Moreover, termination efficiencies are related to the stabilities of the associated stem and loop structures in the RNAs, as well as to the lengths of the stretches of uridine residues.

Another class of terminators, whose important structural features are obscure, requires the activities of the protein factor ρ (6). Our poor understanding of ρ -dependent terminators stems from having only a few well

characterized examples, and from the lack of consistent sequence homology among them. Structurally, these terminators fall in two groups. Members of one group share common features, including a consensus sequence, whereas terminators of the second group apparently do not. Among those that share common features are the terminator following the *E. coli* tRNA^{Tyr} gene (7,8) and the λt_{R1} terminator (9,10). Transcription terminates distal to the tRNA^{Tyr} gene at a sequence CAATCAA, upstream of which is a small dyad symmetry that might allow the formation of a weak stem and loop structure in the RNA transcript. Similar features are found at the λt_{R1} terminator, which lies within the intercistronic Y region between genes cro and cII (9), and is comprised of three termination clusters named sites I, II, and III (10; Fig. 1A). Termination at site II of λt_{R1} occurs at the sequence CAATCAA, upstream of which is also a region of dyad symmetry that may result in the formation of a stem and loop structure in the RNA transcript. A mutation (cin) that can strengthen the stem and loop also enhances termination (9), whereas a mutation (cnc) that can disrupt the stem and loop structure at site II abolishes termination at that site (10). Similar features have also been found at λt_{RO} , a ρ -dependent terminator reported to function in vitro (11), and at the ρ -dependent termination site downstream of the Colicin E1 gene (12). Termination occurs at the sequence CAACAA in the case of λt_{RO} and at the sequence CAAACAA in the case of the ColE1 terminator; both sequences are immediately downstream of a possible stem and loop structure in the RNA transcripts. The analogy of these features to those of ρ -independent terminators is clear: ρ -independent terminators encode a stem and loop structure followed by a stretch of uridine residue at the 3'-termini of the RNA transcripts, whereas the above examples of ρ -dependent terminators encode relatively weak stem and loop structures followed by the sequence CAAUCAA (or a variant of it) in the RNAs.

Other ρ -dependent terminators do not share these features. The trp t' terminator (13) and both sites I and III of λt_{R1} (10) have associated dyad symmetries but do not have the consensus sequence CAATCAA. Moreover, the dyad symmetry of λt_{R1} site I can be destroyed by mutations without affecting termination (10). Thus, neither a stem and loop structure nor the sequence CAAUCAA in the RNA appears to be obligatory for ρ -dependent termination in these cases, so that different structural features must signal ρ -dependent termination. Nevertheless, from their common occurrence in several terminators, it can be argued that a stem and loop structure in the RNA transcript followed by the sequence CAAUCAA may suffice to signal ρ -dependent

termination (3,9,11,12).

We have tested this possibility by synthesizing a DNA fragment which corresponds in sequence to the λt_{R1} terminator region that contains these structural features, and cloning this synthetic DNA fragment under the control of the *lacUV5* promoter. Surprisingly, we find that this sequence is insufficient to signal ρ -dependent termination. It has been shown that RNA polymerase pauses at ρ -dependent termination sites in the absence of ρ factor (9,14). We show here that transcription pauses, although inefficiently, at the synthetic sequence. Furthermore, we find no ρ -dependent termination even under conditions that accentuate the transcriptional pause. We suggest that there are additional sequence requirements for ρ action.

MATERIALS AND METHODS

Materials. [α - 32 P]UTP at about 400 Ci/mmol and [γ - 32 P]ATP at about 3000 Ci/mmol were purchased from Amersham. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, λ exonuclease, and DNA polymerase I Klenow fragment were obtained from New England Biolabs. *Escherichia coli* strain CGSC 5346 (F⁻ *thr*-1 *leu*B6 *thi*-1 *lac*Y1 *ton*A21 *Sup*E44 *hsd*5) was obtained from J. Calvo.

DNA Preparations. λ ci857Nam7Nam53Sam7 DNA was a generous gift from E.J. Grayhack. Plasmid DNA preparations were made using the procedure of Birnboim and Doly (15). Restriction fragments were isolated from 1% low melting agarose gels (Bethesda Research Laboratories) as described (14).

Purification of Proteins. RNA polymerase holoenzyme, ρ factor and NusA protein were purified from RNase I⁻ strain MRE600 (Grain Processing Corp., Muscatine, Iowa). RNA polymerase was purified according to the procedure of Burgess and Jendrisak (16) as modified by Lowe *et al.* (17), and was rendered at least 95% pure and 95% saturated with σ factor. ρ factor was purified to apparent homogeneity using the procedure of Finger and Richardson (18). NusA protein was purified as described (14).

Chemical Synthesis of Oligonucleotides and Enzymatic Construction of Synthetic DNA Duplex. Deoxyribooligonucleotides were chemically synthesized using the phosphotriester procedure of Narang *et al.* (19). Each oligonucleotide was 5'-phosphorylated and labeled using T4 polynucleotide kinase and [γ - 32 P]ATP, and purified to electrophoretic homogeneity on 15% polyacrylamide, 8 M urea slab gels (electrophoresis buffer: 50 mM Tris borate, pH 8.3, 10 mM EDTA). The nucleotide sequence of each synthetic oligonucleotide was confirmed by two dimensional mobility shift analysis (20).

The DNA duplex (Fig. 1B) was constructed by sequential ligation of seven oligonucleotides (fragment A to G) as follows. Oligonucleotide fragments A,B,C, and D were ligated together at 60 pmoles each in a reaction mixture of 75 μ l at 12°C for 10 hrs (ligation reaction mixtures contained 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM DTT, 1 mM ATP, 1 mM hexamine cobalt chloride, T4 DNA ligase at 500 units/ml, and indicated DNA fragments). The ligation mixture was then digested with EcoRI enzyme (50 units) for 2 hrs to eliminate end-to-end ligation products. The expected products were 29 nucleotides long, corresponding to fragments A-C and B-D (Fig. 1B). Since these two products were of the same size, they could not be separated electrophoretically. These products were purified as a mixture from polyacrylamide-urea gels from the unligated reactants. A total of 21 pmoles of ligated A-C and B-D fragments were recovered. Oligonucleotide fragments E,F, and G were ligated together similarly at 60 pmoles each in a separate reaction mixture, the products of which were digested with PstI enzyme (50 units) for 2 hrs. The desired products, 24 and 16 nucleotides in length, corresponded to fragments E-G and F, respectively. A total of 21 pmoles of fragment E-G and 50 pmoles of fragment F were recovered electrophoretically. Fragments A-C and B-D (20 pmoles), E-G (10 pmoles), and F (40 pmoles) were then ligated together at 12°C for 8 hrs. The ligation products were digested with an excess of EcoRI and PstI enzymes. A total of 1.0 pmole of the upper strand (53 nucleotides in length) and 0.4 pmole of the lower strand (45 nucleotides in length) were recovered electrophoretically.

Cloning of Synthetic Sequence Under lacUV5 Promoter Control. A plasmid vector, pLL1, containing the lacUV5 promoter suitable for cloning the synthetic putative terminator sequence, was constructed as follows. Fuller (21) has constructed a plasmid, pOP203-3, that contains the lacUV5 promoter in a pMB9 replicon with an EcoRI site located downstream of the promoter. The plasmid pOP203-3 was restricted with the enzyme HindIII, which cuts uniquely at a site upstream of the lacUV5 promoter, and the restriction cohesive ends were repaired using DNA polymerase I Klenow fragment. The plasmid was then digested with the enzyme EcoRI, which generated a fragment some 600 nucleotide pairs long containing the lacUV5 promoter. We purified this restricted fragment after agarose gel electrophoresis, and ligated this fragment to pBR322 (22) DNA digested with the enzyme EcoRI. The resulting ligation product was then subjected to a limit-digest with λ exonuclease to expose some portion of the 3'-termini of the DNA molecules. The reaction product then was used to transform competent E. coli 5346 cells using the procedure of Morrison

(23). Transformants were selected for both ampicillin and tetracycline resistances. A plasmid having the lacUV5 promoter containing insert oriented toward the β -lactamase structural gene was named pLL1 (Fig. 2A).

A preparation of pLL1 DNA was restricted with both EcoRI and PstI enzymes; the PstI site is within the β -lactamase gene downstream of the lacUV5 promoter. Two DNA fragments resulted from this digestion, and the large fragment (approximately 3500 nucleotide pairs) was purified after agarose gel electrophoresis for use as cloning vector. A ligation reaction mixture containing 0.06 pmoles of the purified vector, and 0.1 pmoles of the synthetic DNA duplex was incubated at 14°C for 8 hrs. The ligation mixture was used to transform competent E. coli 5346 cells (23). A clone that contains the expected restriction map, named pTR1 (Fig. 2B), was subjected to DNA sequence analysis using the method of Maxam and Gilbert (24). The nucleotide sequence of pTR1 at the lacUV5 promoter-insert region is as expected from the in vitro construction, and is shown in Fig. 2C.

In Vitro Transcription. In vitro transcription reaction mixtures containing 150 μ M ATP, 150 μ M GTP, 150 μ M CTP, 20 mM Tris-HCl, pH 7.9 (as measured at 23°C), 0.1 mM EDTA, 0.1 mM DTT, 50 mM KCl, 200 μ Ci/ml of [α -³²P]UTP at 400 Ci/mmol, 10 pmol/ml of DNA restriction fragment as template, 3 μ g/ml of RNA polymerase, and 5% glycerol were pre-incubated at 37°C for 10 minutes. Protein factors were added as indicated. Transcription limited to a single round was initiated synchronously by the addition of MgCl₂ to 5 mM, rifampicin to 10 μ g/ml, and UTP to the concentration indicated in figure legends. Reactions were incubated at 37°C for indicated times and stopped by immediate phenol extraction. Transcripts thus synthesized in vitro were analyzed by polyacrylamide gel electrophoresis as described (14).

RESULTS

Construction of DNA Templates Containing a Synthetic Putative Terminator

Sequence. Transcription of the λ rightward operon terminates in the presence of ρ factor at t_{R1} , a region that is comprised of three termination sites named I, II, and III (10). These sites are functionally independent by several criteria: termination at site I is specifically inhibited by NusA protein; termination at site II can be specifically abolished by mutation; and all three sites respond differently to varying concentrations of ρ factor (10). Immediately upstream of each termination site is a region of dyad symmetry. Termination at site II occurs at the sequence CAATCAA, and mutations (cin and cnc) that affect its associated dyad symmetry also affect

Figure 1: Nucleotide sequence of λt_{R1} and the synthetic putative terminator. (A) Nucleotide sequence of the λt_{R1} region as determined by Schwarz *et al.* (29) and Rosenberg *et al.* (9) is shown, numbered from the first nucleotide of the *cro* mRNA. Termination sites I, II, and III (10) are indicated, with their associated dyad symmetries represented by opposing arrows. The sequence CAATCAA is underlined. (B) Construction of a putative termination site using synthetic oligonucleotides. A synthetic DNA duplex that contains the sequence encoding nucleotides 276 to 315 of the λcro mRNA was constructed using seven overlapping synthetic oligonucleotides (A to G). The DNA duplex contains a 5'-protruding *EcoRI* restriction cohesive end and a 3'-protruding *PstI* restriction cohesive end. Internal to the synthetic sequence is a *BamHI* site, which lies close to the sequence CAATCAA.

the termination function (9,10).

We synthesized a DNA duplex which corresponded in sequence to termination site II of λt_{R1} , including the associated dyad symmetry and the sequence CAATCAA (Fig. 1B). The DNA duplex was constructed using seven overlapping oligonucleotides (A to G), which produced a DNA duplex of 53 nucleotides on the top strand and 45 nucleotides on the bottom strand. The DNA duplex contains an *EcoRI* restriction cohesive end on the 5'-protruding terminus and a *PstI* restriction cohesive end on the 3'-protruding terminus. Internal to the synthetic sequence and close to the expected termination site is a *BamHI* site. The DNA duplex was cloned into the *EcoRI* site and *PstI* site of the plasmid pLL1 (Fig. 2A) as described (MATERIALS AND METHODS), under the control of the *lacUV5* promoter. DNA sequence analysis showed that the cloned plasmid, pTR1, contains the expected sequence from the *in vitro* construction (Fig. 2B,C).

Figure 2: Cloning of synthetic putative terminator. (A) Physical map of the plasmid vector pLL1. The plasmid contains pBR322 (22) sequences except for the heavy line, which contains the LacUV5 promoter and is derived from the plasmid p203-3 (21). (B) Physical map of plasmid pTR1, which harbors the synthetic DNA sequence between the EcoRI and PstI sites. (C) Nucleotide sequence of cloned synthetic DNA encoding the putative termination site from plasmid pTR1. Cloned synthetic DNA encoding the putative terminator is under the control of the lacUV5 promoter. Nucleotide sequence of the cloned synthetic DNA and its flanking region is shown; nucleotides are numbered from the first nucleotide of the RNA transcript. Sequence analysis was carried out using the procedure of Maxam and Gilbert (24); DNA molecules were either labeled at the EcoRI site or the BamHI site for sequencing in either direction. Sequence between nucleotides -35 and 140 was determined by direct DNA sequence analysis; flanking nucleotide sequences were inferred from published data on the plasmid pOP203-3 (21) and pBR322 (30). DNA sequence that lies between the EcoRI and PstI sites is the synthetic putative terminator. The lac UV5 promoter that is insensitive to catabolite repression is the result of a double mutation involving nucleotides -8 and -9 (31).

1293

Figure 3: Restriction fragments used as templates for *in vitro* transcription. (A) The *Hind*III-*Bgl*I fragment of pTR1 shown is expected to produce a readthrough transcript (RT) of about 240 nucleotides. Transcripts terminating at the synthetic site are expected to be about 104 nucleotides long. (B) The *Hind*III-*Bam*HI fragment of pTR1 shown is expected to produce a readthrough transcript of about 107 nucleotides, very close to the expected length of transcripts terminating at the synthetic site. (C) λ restriction fragment produced by the enzyme *Hin*FI is about 560 nucleotides long, which contains the *P_R* promoter and the *cro* structural gene. Readthrough transcript from this template is expected to be 372 nucleotides long, and transcripts terminating at *t_{R1}* are expected to range from 290 to 350 nucleotides in lengths.

fragment (Fig. 3B) yielded a transcript about 110 nucleotides in length, as expected (Fig. 4, lane 2). Since the *Bam*HI site is located within a few nucleotides from the sequence CAATCAA where termination is expected to occur, this readthrough transcript provides a reliable size marker for transcripts terminating at the expected site within the synthetic sequence.

Surprisingly, only the readthrough transcript was made in the presence of ρ -factor (10 μ g/ml) in transcription of the *Hind*III-*Bgl*I fragment (Fig. 4, lane 3). Higher concentrations of ρ factor did not alter this result (data not shown). Thus ρ factor has no effect on transcription of the synthetic putative terminator sequence. Transcription of a restriction fragment from phage λ containing the *P_R*-*t_{R1}* region (λ *Hin*FI₅₆₀; Fig. 3C) yielded a readthrough transcript of the expected size (Fig. 4, lane 4) and three terminated transcripts in response to ρ factor (Fig. 4, lane 5; 10). In order to insure that the DNA preparation of the synthetic template was not inhibiting ρ action, we used a mixture of both the λ *Hin*FI₅₆₀ fragment and the pTR1 *Hind*III-*Bgl*I fragment as templates. In this reaction, the transcript

Figure 4: Cloned synthetic DNA encoding the putative terminator is insufficient for signaling ρ -dependent termination. In vitro transcription reactions were carried out at 40 μ M UTP, with 15 minutes of incubation at 37°C. The following restriction fragments were used as templates (Fig. 3): pTR1 HindIII-BglI fragment (lanes 1,3,6), pTR1 HindIII-BamHI fragment (lane 2), and λ HinfI fragment (lanes 4,5,6). Reaction products displayed in lanes 3,5, and 6 were made in the presence of ρ factor (10 μ g/ml). The sizes of the transcripts are indicated on the left.

from the λ restriction fragment terminated in response to ρ factor, but the pTR1 transcript did not (Fig. 4, lane 6). It is thus clear that the cloned synthetic sequence is not sufficient to signal ρ -dependent termination.

Synthetic Sequence is a Poor Transcriptional Pause Site and a Defective Transcript Release Site. We have analyzed the termination process at the λt_{R1} terminator in terms of two separately measurable events: RNA polymerase pausing and transcript release (14). In the absence of ρ factor, RNA polymerase recognizes certain sequences as transcriptional pause sites, including the ρ -dependent termination sites. While ρ factor does not affect transcriptional pausing, it serves to release transcripts at termination sites during transcriptional pauses. Given that the pause sites are also ρ -dependent transcript release sites, efficient termination occurs if the duration of the pause is kinetically compatible with the rate of transcript release (14).

We showed above that the synthetic sequence containing structural features thought to signal ρ -mediated termination is inactive in vitro. In order to understand the specific defects of this sequence as a terminator, we

Figure 5: Transcriptional pausing at the synthetic sequence. *In vitro* transcription reactions were carried out at 10 μ M UTP for indicated times. Transcription reactions of the pTR1 HindIII-BglI (Fig. 3A) fragment are shown in lanes 1-7 and 9-18; transcription reaction of the pTR1 HindIII-BamHI (Fig. 3B) fragment is shown in lane 8. Reactions displayed in lanes 9-18 contained NusA protein (10 μ g/ml). RT, readthrough transcript from the HindIII-BglI fragment. The position of paused RNA at the synthetic site is indicated by the arrow, as is the readthrough transcript from the HindIII-BamHI fragment.

examined its ability to signal either transcriptional pausing or transcript release. We performed a pausing experiment using the HindIII-BglI fragment (Fig. 3A) as template. Transcriptional pausing at the synthetic sequence at 25 μ M UTP was barely detectable (data not shown), although a slightly more prominent pause was observed when the UTP concentration was lowered to 10 μ M (Fig. 5). A far more substantial pause occurs at the corresponding site (site II of t_{R1}) on the λ genome at 25 μ M UTP (14). The synthetic sequence is thus a relatively poor pause site.

Since ρ -mediated transcript release at a pause may take 30-60 seconds *in vitro* (14,25), it might be that the pause is too short to allow any detectable termination under these conditions. We thus attempted to detect termination at the synthetic site under conditions that enhance pausing. By using a low

UTP concentration (10 μ M) and by the addition of NusA protein (10 μ M/ml), which prolongs transcriptional pausing at λt_{R1} (14), we increased the apparent half-life of the pause at the synthetic site to 2.8 minutes (data calculated from Fig. 5; for method of analysis, see ref. 14). This pause is long enough for ρ -mediated transcript release at the natural λt_{R1} site in the presence of NusA (14). However, even under this condition of enhanced pausing, no ρ -mediated termination was observed (data not shown). This result suggests that the synthetic sequence is defective as a transcript release site.

DISCUSSION

We have described the synthesis of a DNA fragment corresponding in sequence to that portion of the λt_{R1} region encoding a stem and loop structure in the RNA transcript, followed by the sequence CAAUCAA. We have shown that this synthetic sequence is insufficient to signal ρ -dependent termination when it is cloned under the control of the *lacUV5* promoter. Moreover, the synthetic sequence is a poor transcriptional pause site and is defective as a transcript release site.

One interpretation of these results is that the sequence environment into which the synthetic sequence was placed forbids the expression of terminator activity. This might occur, for example, through a fortuitous sequence homology between part of the pTR1 transcript and the synthetic terminator region that allows base-pairing of parts of the transcript to the terminator region and blocks formation of the stem and loop structure at the terminator. We cannot rule out this interpretation, although we find no extensive homology between the synthetic sequence and the preceding transcript.

It is more likely that sequences upstream of this 40 nucleotide portion of λt_{R1} are essential for the action of ρ . Our experiments give no information whether a specific sequence, or a minimum length of transcript preceding the release site, or both, are required for ρ to act. It is clear that a transcript of the length we expected from the synthetic sequence could be terminated at a ρ -independent terminator (3). However, the termination activity of ρ requires hydrolysis of ATP, and ATP hydrolysis requires in turn that ρ bind RNA. Presumably ρ must therefore bind the emerging transcript in order to release it (25). Studies of the stoichiometry of RNA binding to ρ indicate that a substantial length of RNA, at least 60 nucleotides and perhaps more, can bind. If an extensive stretch of RNA is required, the 100 nucleotides preceding the release site in our transcript may be insufficient for ρ to act. There are some indications to the contrary: thus when

guanosine is replaced by inosine in the λt_{R1} transcript, ρ produces transcripts as short as 100 nucleotides (27), and ρ may increase the termination efficiency at the (mostly) ρ -independent terminator of the 81 nucleotide loop transcript (28).

Since we can detect a pause at the expected site of the synthetic template, we suspect that this sequence is defective primarily in allowing ρ to release the transcript. However, the pause is less pronounced than we observed in the natural template. This result was somewhat unexpected, since it has been thought that only local sequence features such as a potential stem and loop in the transcript invoke pausing. Further investigation of the role of upstream sequences should reveal how they affect both pausing and ρ function at the actual region of termination.

ACKNOWLEDGEMENTS

This study was supported by a National Science Foundation Grant DAR-79-17310 to R.W. and National Institutes of Health Grant GM21941 to J.W.R.

§Present address: Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

REFERENCES

1. Adhya, S. and Gottesman, M. (1978) *Annu. Rev. Biochem.* **47**, 967-996.
2. Kolter, R. and Yanofsky, C. (1982) *Annu. Rev. Genet.* **16**, 113-134.
3. Rosenberg, M. and Court, D. (1979) *Annu. Rev. Genet.* **13**, 319-353.
4. Platt, T. (1981) *Cell* **24**, 10-23.
5. Platt, T. and Bear, D.G. (1983) In *Prokaryotic Gene Expression*, eds., J. Beckwith, J. Davis and J. Gallant (Cold Spring Harbor, NY). In press.
6. Roberts, J.W. (1969) *Nature (London)* **224**, 1168-1174.
7. Klüpper, H., Sekiya, T., Rosenberg, M., Egan, J. and Landy, A. (1978) *Nature (London)* **272**, 423-428.
8. Rossi, J., Egan, J., Hudson, L. and Landy, A. (1981) *Cell* **26**, 305-314.
9. Rosenberg, M., Court, D., Shimatake, H., Brady, C. and Wulff, D.L. (1978) *Nature (London)* **272**, 414-422.
10. Lau, L.F., Roberts, J.W. and Wu, R. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6171-6175.
11. Calva, E. and Burgess, R.R. (1980) *J. Biol. Chem.* **255**, 11017-11022.
12. Ebina, Y. and Nakazawa, A. (1983) *J. Biol. Chem.* **258**, 7072-7078.
13. Wu, A.M., Christie, G.E. and Platt, T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2913-2917.
14. Lau, L.F., Roberts, J.W. and Wu, R. (1983) *J. Biol. Chem.* **258**, 9391-9397.
15. Birnboim, H.C. and Doly, J. (1979) *Nucl. Acids Res.* **7**, 1513-1523.
16. Burgess, R.R. and Jendrisak, J.J. (1975) *Biochemistry* **14**, 4634-4638.
17. Lowe, P.A., Hager, D.A. and Burgess, R.R. (1979) *Biochemistry* **18**, 1344-1352.
18. Finger, L.R. and Richardson, J.P. (1981) *Biochemistry* **20**, 1640-1645.
19. Narang, S.A., Brousseau, R., Hsiung, H.M. and Michniewicz, J.J. (1980) *Methods in Enzymol.* **65**, 610-620.

20. Tu, C.-P.D., Jay, E., Bahl, C.P. and Wu, R. (1976) *Analy. Biochem.* 74, 73-93.
21. Fuller, F. (1982) *Gene* 19, 43-45.
22. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.D., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) *Gene* 2, 95-113.
23. Morrison, D.A. (1979) *Methods in Enzymol.* 68, 326-331.
24. Maxam, A.M. and Gilbert, W. (1980) *Methods in Enzymol.* 65, 499-560.
25. Richardson, J.P. and Conaway, R. (1980) *Biochemistry* 19, 4293-4299.
26. Galluppi, G.R. and Richardson, J.P. (1980) *J. Mol. Biol.* 138, 513-539.
27. Morgan, W.D., Bear, D.G. and von Hippel, P.H. (1983) *J. Biol. Chem.* 258, 9553-9564.
28. Howard, B.H., de Crombrughe, B. and Rosenberg, M. (1977) *Nuc. Acids Res.* 4, 927-842.
29. Schwarz, E., Scherer, G., Hobom, G. and Kössel, H. (1978) *Nature (London)* 272, 410-414.
30. Sutcliffe, J.G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* 43, 77-90.
31. Maizel, N. (1973) *Proc. Natl. Acad. Sci. USA* 70, 3585-3589.