Abortive initiation by bacteriophage T3 and T7 RNA polymerases under conditions of limiting substrate

Mei-Ling Ling¹, Steven S.Risman, John F.Klement¹, Nancy McGraw¹ and William T.McAllister

Department of Microbiology and Immunology, Morse Institute for Molecular Genetics, New York State University, Brooklyn Health Science Center, 450 Clarkson Avenue, Box 44, Brooklyn, NY 11203-2098 and ¹Department of Microbiology, Rutgers Medical School, Piscataway, NJ 08854, USA

Received October 31, 1988; Revised and Accepted January 6, 1989

ABSTRACT

Initiation of RNA synthesis by the phage polymerases is abortive if the concentration of pyrimidine triphosphates is limiting. Under abortive initiation conditions the polymerases repeatedly initiate transcription but produce ribooligonucleotides that terminate just prior to the first occurrence of the limiting substrate. Abortive initiation is most severe if the limiting substrate occurs within the first 8-12 nucleotides of the nascent RNA chain and is particularly evident when UMP is limiting. The formation of stable elongation complexes (as determined by gel retardation experiments) occurs after the synthesis of an RNA product 8-12 nucleotides in length.

INTRODUCTION

Due to their simple structures, the RNA polymerases that are encoded by bacteriophages T3 and T7 are attractive models for studies of RNA polymerase:promoter interactions. The phage enzymes each consist of a single protein species of 98 kD which is active *in vitro* in the absence of additional transcription factors (1,2). The genes that encode the polymerases have been cloned and overexpressed in bacteria, making possible a variety of genetic and biochemical approaches towards understanding polymerase structure and function (3-5). The enzymes are highly specific in their template requirements, recognizing promoters that are related to a 23 bp consensus sequence that extends from -17 to +6 (summarized in 6,7). Footprinting experiments using MPE.Fe(II) (8,9) or DNase I (10) have shown that, in the absence of substrate, the polymerases protect a region from -22 to -3. After the addition of substrate, the transcription complexes undergo a series of alterations as detected by changes in the footprint, until an elongation complex is formed (see Fig. 1).

In previous work we had noted that initiation of the T7 RNA polymerase is inhibited at moderate salt concentrations (above 50mM KCl), but that once initiation has occurred the transcription complexes become resistant to

Figure 1. Structures of promoters for T7 RNA Polymerase
The nucleotide sequences of the non-template strands of seventeen T7 promoters are summarized. Capital letters in the top line indicate the consensus sequence; lower case letters indicate the sequence that is found outside of this region in the ø10 promoter in T7 DNA. Nucleotide positions within the promoter are numbered relative to the start site for RNA synthesis (+1); the direction of transcription is indicated by the arrow above the sequence. Brackets at the bottom of the figure indicate the "footprint" of the T7 RNA polymerase on the 10 promoter sequence under various conditions, as probed by resistance to cleavage by MPE.Fe (II) (8,9), and the RNA length of the product that could be formed under those conditions.

elevated levels of salt (>200mM KCl) (11). During the course of our experiments with various T3 and T7 promoters we observed that initiation of the phage polymerases at certain promoters is abortive if the concentration of either pyrimidine nucleoside triphosphate is limiting (12,13). Under abortive initiation conditions the polymerase repeatedly initiates transcription but produces oligonucleotides that terminate just before the first occurrence of the limiting nucleotide. In this report we have explored this phenomenon at a number of T3 and T7 promoters and have found that abortive initiation is most pronounced if the limiting substrate occurs within the first 10-12 nucleotides of the nascent RNA chain. We have also examined the stability of the transcription complexes as a function of RNA chain length by use of a gel retardation assay. The results of our experiments suggest that the formation of a stable elongation complex requires the synthesis of an 8-12 nucleotide-long product.

Recent reports by Martin *et al* (14) and Milligan *et al* (15) have demonstrated that even at high concentrations of substrate (twice the Km) there is a significant level of abortive initiation by the T7 RNA polymerase. These findings have important implications concerning practical

applications of the phage enzymes, as well as having significance for studies of the mechanisms of transcription initiation and elongation.

MATERIALS AND METHODS

Enzymes and DNA

T7 and T3 RNA polymerases were purified from induced cultures of BL21/pAR1219 (3) or BL21/pCM56 (5) as previously described.

Plasmids pAR436, pAR35, pAR41, pJFK1, pJB20, pJB23 and pJB30 have been previously described (16,17). Plasmids pJFK9 and pJFK16 are dual promoter vectors that contain synthetic T7 and T3 promoters flanking the polylinker sequence of pSP64 (18). Their construction has been described elsewhere (19). The backbone of pJFK9 consists of the 1.4 kb PvuII-PvuI fragment of pSP64 DNA joined to the 0.65 kb PvuI-ClaI fragment of pBR322 DNA (20). The backbone of pJFK16 consists of the 1.6 kb EcoRI-PvuI fragment of pSP64 joined to the 0.65 PvuI-ClaI fragment of pBR322. The EcoRI site within the pBR322 interval in both plasmids was converted to an XmnI site by repair with DNA polymerase and blunt end ligation. The sequences of the T3 and T7 promoters and the flanking DNA are given below. An (X) next to a restriction site indicates that the original cloning site in the vector has been inactivated as a consequence of the construction.

pJFK9

pJFK16

RNA Synthesis

Unless otherwise indicated, all reactions were carried out in a volume of 40 μ l that contained 2 μ g template DNA; 20 mM Tris-HCl, pH 7.9; 20 mM MgCl₂; 1 mM dithiothreitol (DTT); 2 mM spermidine-HCl; 0.4 mM GTP, CTP and UTP; 0.4 mM α -³²P-ATP (specific activity = 80 μ Ci/ μ mole) and 1-10 units of RNA polymerase. Ultrapure ribonucleoside triphosphates were purchased from Pharmacia. After incubation at 37° for 60 min., samples were prepared for electrophoresis in 5% or 20% polyacrylamide gels as previously described (5,21).

Gel permeation assays

Binding reactions (20 μ l) were carried out at room temperature in a buffer that contained 20 mM potassium phosphate (pH 7.7); 8 mM MgCl₂; 1 mM DTT; 2 mM spermidine-HCl; 50 mM NaCl; 5% glycerol; and RNA polymerase and ribonucleoside triphosphates as indicated. After incubation as indicated the samples were layered directly onto a 20 cm 5% polyacrylamide gel prepared in 0.04 M Tris, 0.02 M acetic acid, 2 mM EDTA. Electrophoresis was carried out at 200 V for 3 hrs.

RESULTS

Abortive Initiation of the T7 Polymerase

To examine the effects of limiting the concentration of substrate on RNA chain elongation, plasmids that contain a T3 promoter were digested with restriction endonucleases to produce templates that would yield runoff transcripts 60-200 nucleotides in length. The linearized templates were incubated with T3 RNA polymerase and varying mixtures of substrate, and the products were analyzed by electrophoresis in 20% polyacrylamide gels (Fig.2).

In the presence of template DNA and all four ribonucleoside triphosphates at a concentration of 400 μ M the reaction products consisted predominantly of the full-length transcripts. As the concentration of either CTP or UTP in the reaction decreased, products representing termination of transcription at the first occurrence of the limiting nucleotide were increasingly observed. The abundance of shortened transcripts produced under these conditions indicates that this is not simply a pause in the transcription reaction, but rather an abortive initiation process in which the polymerase repeatedly initiates transcription, releases the product, and reinitiates (12).

Although some abortive initiation is observed even at normal levels of

Figure 2. Effect of limiting concentrations of pyrimidine triphosphates on initiation and elongation.

Plasmid pJFK9 was digested with Hind III so as to give rise to a 57 nt runoff transcript from the T3 promoter. The expected sequence at the 5' end of this transcript is 5' GGGAGAUUCUG—. The linearized template was transcribed by T3 RNA polymerase in the presence of varying concentrations of UTP or CTP. In order, from left to right, the concentrations of these substrates were 400, 200, 100, 50, 25, and 12.5 μ M. The concentrations of all other substrates were maintained at 0.4 mM and α - 32 P-ATP was present as the labelled substrate. Products were analyzed by electrophoresis in 20% polyacrylamide gels. The position of the 57 nt runoff transcript is indicated by R; the positions of smaller transcription products are indicated by their length (in nt) in the margin. The relative production of abortive vs. full length products was determined by densitometric analysis of the films and is presented in Table 1; the data were normalized to take into account the number of AMP residues in each transcript.

substrate (400 μ M), the effect becomes increasingly pronounced until the abortive transcripts predominate at UTP concentrations less than 100 μ M or CTP concentrations less than 50 μ M (Table 1). Longer exposure of the gel shown in Fig. 2 reveals additional transcripts that correspond to termination at subsequent occurrences of the limiting substrate, but these are present at much lower levels among the reaction products (not shown).

Martin and Coleman recently reported that abortive initiation by the T7 RNA polymerase occurs even at high (>400 μ M) substrate concentrations, and that the polymerase preferentially aborts after synthesizing a product of 6

TABLE 1
RELATIVE PRODUCTION OF ABORTIVE vs FULL LENGTH PRODUCTS AS A FUNCTION
OF UTP OR CTP CONCENTRATION
Relative Production of Each Species as Fraction of Total¹

UTP concn (nM)	5nt	6nt	7nt	8nt	runoff
12.5	0.12	0.76	0.02	0.06	0.03
25	0.15	0.69	0.02	0.07	0.06
50	0.16	0.52	0.02	0.09	0.20
100	0.19	0.35	0.06	0.07	0.32
200	0.17	0.17	0.11	0.10	0.45
400	0.21	0.04	0.19	0.00	0.55
CTP concn (nM)					
12.5	0.30	0.18	0.40	-	0.12
25	0.23	0.17	0.39	-	0.21
50	0.32	0.16	0.22	-	0.30
100	0.33	0.17	0.09	-	0.40
200	0.35	0.20	0.04	-	0.42
400	0.37	0.16	0.05	-	0.41

¹Data from Figure 2.

nt (14). We have also observed this product and have found that its synthesis is largely independent of the concentration of UTP or CTP (see Fig. 2).

Because the transition of the polymerase from an unstable initiation complex to a stable elongation complex requires the synthesis of a short oligoribonucleotide (11), we expected that the effect of limiting CTP or UTP would depend upon the position at which the substrate is incorporated into the nascent chain. We have examined the consequences of limiting CTP or UTP on production of RNA at a number of T3 or T7 promoters and have found that abortive initiation is most pronounced when incorporation of the limiting substrate is required within the first 8-10 nucleotides of the nascent RNA chain (Fig. 3 and Table 2). Regardless of the position of the limiting substrate in the nascent chain, however, lowering the concentration of UTP has a more profound effect on abortive initiation than limiting the concentration of CTP.

When CTP is limiting (10 μ M) and the first site of incorporation is at +7 nearly all RNA products made by the T7 RNA polymerase are terminated at

TABLE 2
SEQUENCES OF TRANSCRIPTS FROM PROMOTERS TESTED

Plasmid	Promoter ¹	Transcript Sequence ²					Product Length ³			
		+1	5	10	15	20	-AUC	-UC	-U	-C
		.	.	.	.	.				
pAR35	T7 ϕ 6.5	GGGAGAU <u>AGGGGC</u>					3	6	6	<i>12</i>
pAR436	T7 ϕ 10	GGGAGAC <u>CCACAA</u> CGU					3	6	<i>15</i>	6
-	T7 ϕ 17	GGGAGAGGCGAA <u>U</u>					3	8	<i>13</i>	8
pAR41	T7 ϕ OR	GGGAGAGGAGGGA <u>CGAA</u> AGGU					3	<i>13</i>	<i>20</i>	<i>13</i>
pJFK9	T7	GGGAGAAG <u>CU</u>					3	8	9	<i>8</i>
pJFK9	T3	GGGAGA <u>UC</u>					3	6	<i>6</i>	7
pJFK16	T3	GGGAGAGA <u>CGAA</u> U					3	8	<i>12</i>	8
pJB20	T3 ϕ 2.5	GGGAAC <u>CA</u> CCCCAACCU					3	5	<i>15</i>	5
pJB23	T3 ϕ 6.5	GGGAAGAGGGAG <u>CCU</u>					3	12	<i>14</i>	<i>12</i>
pJFK1	T3 ϕ 9	GGGAGA <u>CCU</u>					3	6	8	6
pJB30	T3 ϕ 10	GGGAGAGA <u>CCA</u> U					3	8	11	<i>8</i>

1. pJFK9 and pJFK16 carry synthetic T3 and T7 class III promoters.
2. The sequence of the RNA to the first CMP and UMP residues (underlined) is presented. Positions in the transcript are numbered relative to the initiation site (+1).
3. The length (nt) of RNA product that could be made when the indicated substrate is missing from the reaction (Fig. 4) or is limiting (Fig. 3) is presented. (G = GTP, A = ATP, etc.). Conditions that result in the formation of stable elongation complexes as detected in Figure 4 are indicated by underlining the appropriate product length. Conditions that permit significant production of full length run-off transcripts, as described in Figure 3, are indicated by italicizing the appropriate product length.

this point and very few full-length products are observed (Fig. 3, lane 1c). When the first CMP incorporation site is at +9, strong termination is observed at this point but significant production of full length products is observed (Fig. 3, lane 2c). Finally, when the first CMP residues occur at +13 or +14 little premature termination is seen and near-normal levels of full length product are observed (Fig. 3 lane 3c and 4c).

Very strong termination is observed when UTP is the limiting substrate and the first occurrence of UMP in the RNA chain is at +7 or +10 (Fig. 3,

lane 3u and 2u). However, significant termination is observed even when the first UMP residue is at +16 or +21 (lanes 1u and 4u).

Similar effects are observed with the T3 RNA polymerase at various promoters (Fig. 3, lanes 5-10, and Table 2).

Stability of initiation and elongation complexes as measured by template competition experiments

To examine the binding of the T7 polymerase to promoters, a gel permeation assay was performed as described in Figure 4. In this assay a mixture of restriction fragments, some of which contain a phage promoter, is incubated with polymerase and the sample is then loaded onto a polyacrylamide gel. Restriction fragments that contain a T7 promoter are bound by the enzyme and migrate in an anomalous fashion, resulting in the loss of the corresponding band in the electrophoresis pattern. Restriction fragments that do not contain promoters are unaltered in mobility and appear at their normal position (Fig. 4a, left panel). The stability of these complexes in the absence of any substrate is rather low, and the addition of excess competitor DNA (such as intact T7 DNA) shortly before loading the sample onto the gel results in the reappearance of the promoter-containing restriction fragments at their normal positions (Fig. 4a, right panel). We interpret this result to mean that, prior to entering the gel, the polymerase dissociates from the promoter in the restriction fragment and becomes associated with the competitor DNA.

Figure 3. Abortive initiation at individual promoters. Plasmids that contain promoters for the T7 or T3 RNA polymerases were digested with restriction endonucleases such that run-off transcripts having a size of 60 to 200 nucleotides would be produced. The linearized templates were transcribed under standard conditions and the products were analyzed by electrophoresis in 20% polyacrylamide gels. Unless otherwise noted the concentration of all ribonucleoside triphosphates was 400 M; in lanes marked C or U the concentration of CTP or UTP was 10 M. The bands marked [+] were present in all reactions in this series of experiments but were not observed when other batches of labeled substrates were utilized (c.f. Fig. 2); it is likely that they represent a contaminant or breakdown product that was present in the labeling mixture.

The template and the RNA polymerase (RNAP) present in each reaction were as follows: Lane 1, pAR436, T7 RNAP; Lane 2, pJFK9, T7 RNAP; Lane 3, pAR35, T7 RNAP; Lane 4, pAR41, T7 RNAP; Lane 5, pJB20, T3 RNAP; Lane 6, pJFK1, T3 RNAP; Lane 7, pJB23, T3 RNAP; Lane 8, pJFK9, T3 RNAP; Lane 9, pJFK16, T3 RNAP; Lane 10, pJB30, T3 RNAP.

The positions of the runoff transcripts are indicated by R. The positions of products that would arise from termination at the first occurrence of the limiting substrate are indicated in the margin by noting the position of the base in the nascent chain followed by c for CTP or u for UTP. (See Table 2 for the initiation sequence found at each of these promoters).

Figure 4. Detection of RNA polymerase:DNA complexes by gel retardation.

Panel A. Hae II restriction fragments of T7 DNA (0.25 g) were incubated with a four-fold or eight-fold molar excess of RNA polymerase, as indicated by the numbers above the lane. After 15 min of incubation at room temperature a 10-fold molar excess of competitor DNA (genomic T7 DNA) was added to the samples marked with +, and incubation was continued for a further 5 min. All samples were then loaded onto a 5% polyacrylamide gel and analyzed by electrophoresis. The gels were stained with ethidium bromide and photographed. Note especially the disappearance of certain restriction fragments in the left panel and their persistence in the gel patterns in the right panel; all of these restriction fragments contain promoters for the T7 RNA Polymerase.

Panel B. Restriction fragments of T7 DNA generated by digestion with Hpa I and Cla I were incubated with an 8-fold molar excess of T7 RNA polymerase in the presence of varying mixtures of substrates, each at a concentration of 0.4 mM, as indicated above each lane. Where indicated, 3' dATP was present at a concentration of 10^{-3} M. After 15 min of incubation competitor DNA was added, and incubation was continued for a further 5 min. All samples were then loaded onto a polyacrylamide gel and analyzed as described above. Certain restriction fragments carry only a single promoter for the T7 RNA polymerase, and these fragments are identified in the left margin of the panel by the promoter which they contain.

When a mixture of nucleoside triphosphates is present and the RNA polymerase can synthesize an RNA chain of suitable length, a stable transcription complex that is resistant to the presence of competitor DNA is detected (see Fig. 4b). In the digest used in Figure 4b certain restriction fragments contain only a single T7 promoter. The conditions that were found to give rise to stable complexes at these promoters, and the length of the RNA products that would be formed under these conditions, are listed in Table 2. Note that at the $\phi 6.5$, $\phi 10$ and $\phi 17$ promoters a mixture of GTP and ATP (which would result in the synthesis of transcripts 6-8 nt in length) is not sufficient to form stable complexes.

From the data of Figure 3 and Table 2, we conclude that the transition from an unstable to a stable complex occurs at some point between the synthesis of an 8 and a 12 nt transcript. We do not know if this observation is true at all T3 and T7 promoters, or whether other factors might have an effect upon the stability of the complexes. The effects of supercoiling, ionic strength, temperature, etc., will need to be explored, as will the effects of changes in the sequences in which the promoter is embedded.

In previous work, we had demonstrated that the ribonucleotide analog 3'dATP acts as a chain terminator for the T7 and T3 RNA polymerases (21). As shown in the leftmost lane of Fig. 3b, inclusion of this inhibitor in a complete transcription reaction results in the formation of complexes that are stable in the presence of competitor DNA for at least 5 min. Thus, incorporation of the analog must result in the formation of a "stalled" complex that does not favor the release of the enzyme from the DNA template.

DISCUSSION

In this work, we have demonstrated that when the concentration of either pyrimidine triphosphate is limiting, the T3 and T7 RNA polymerases abortively initiate transcription, resulting in premature release of a product and subsequent reinitiation. The effects of limiting substrates are most severe if the site of incorporation occurs within the first ten nucleotides of the nascent transcript. Under these conditions, few full-length transcripts are produced when the concentration of UTP or CTP is less than 10 μ M. If the first occurrence of the limiting substrate is subsequent to +12, abortive initiation is less severe and significant levels of full-length product are produced. Abortive initiation appears to

be most severe when UTP is the limiting substrate. In related experiments, Martin *et al* had observed that, in the presence of normal levels of substrate, transcription by the T7 RNA polymerase was preferentially aborted after the incorporation of UMP (14). These authors proposed that the poor bonding of an rU.dA base pair weakens the forces that correctly orient the base, resulting in slower addition of the next nucleotide and in an increased probability of dissociation. The same explanation could account for the preferential abortion that we have observed when UTP is the incoming substrate. Lowering the concentration of UTP would exacerbate the delay in completing the catalytic event and further increase the probability of dissociation.

These findings have practical implications in the use of the phage polymerases for making highly-labelled RNA probes in which the label is introduced by incorporation of triphosphates that are present at low concentrations. For optimal synthesis of full length RNA products the concentration of triphosphates should not be as low as 50 μ M, and if possible UTP should not be utilized as the limiting substrate. Furthermore, promoter sequences should be chosen such that the first occurrence of the limiting substrate does not occur within the first 8-12 nucleotides of the nascent transcript.

If at each position on the template there is an equal probability of polymerase dissociation due to failure to incorporate a limiting substrate nucleotide, one would expect to see a ladder of paused or terminated transcripts. Although some evidence for the occurrence of these transcripts is apparent after prolonged exposure of the gel, most of the polymerase molecules that proceed past the initial pause site appear to reach the end of the template with relatively high frequency (see Figs. 2 and 3). One interpretation of this finding is that a subset of the enzyme population is particularly sensitive to limiting concentrations of nucleoside triphosphate, and that these enzymes are preferentially terminated at the first pause site; polymerases that transcribe past the initial blockage resist dissociation from the template at subsequent pause sites and proceed to the end of the template. Since it has been demonstrated that proteolytically nicked RNA polymerase has a less stable association with the template during elongation and is likely to terminate prematurely (22,23), it is possible that a small proportion of the enzyme used in these experiments is nicked or otherwise damaged, and that these enzyme molecules

preferentially abort upon encountering the first occurrence of the limiting triphosphate. Intact enzyme molecules are better able to remain in association with the template until the triphosphate can be incorporated, and do so at every subsequent occurrence of limiting triphosphate. This interpretation also has practical implications for the use of the phage polymerases in making synthetic probes in that the quality of the enzyme used for these purposes should be carefully monitored to ensure a high proportion of fully processive enzymes.

ACKNOWLEDGEMENTS

This work was supported by grant GM38147 from the National Institute of General Medical Sciences. We are grateful to Drs. Ellen Jorgensen and Russell Durbin for critical reading of the manuscript.

REFERENCES

1. Chamberlin, M., McGrath, J. and Waskell, L. (1970). *Nature* **228**:227-231.
2. Dunn, J.J., Bautz, F.A. and Bautz, E.K.F. (1971). *Nature (London)* **230**:94-96.
3. Davanloo, P., Rosenberg, A.R., Dunn, J.J. and Studier, F.W. (1984). *Proc. Natl. Acad. Sci., U.S.A.*, **81**:2035-2039.
4. Tabor, S. and Richardson, C.C. (1985). *Proc. Natl. Acad. Sci. U.S.A.*, **82**:1074-1078.
5. Morris, C.E., Klement, J.F. and McAllister, W.T. *Gene* **41**:193-200 (1986).
6. Dunn, J.J. and Studier, F.W. (1983). *J. Mol. Biol.* **166**:477-535.
7. Schmitt, M.P., Beck, P.J., Kearney, C.A., Spence, J.L., DiGiovanni, D., Condreay, J.P. and Molineux, I. (1987) *J. Mol. Biol.* **193**:479-495.
8. Ikeda, R. and Richardson, C.C. (1986). *Proc. Natl. Acad. Sci., U.S.A.*, **83**:3614-3618.
9. Gunderson, S.I., Chapman, K.A. and Burgess, R.R. (1987). *Biochem.* **26**:1539-1546.
10. Basu, S. and Maitra, U. (1986). *J. Mol. Biol.* **190**:425-437.
11. McAllister, W.T. and Carter, A.D. (1986). *Nucl. Acid. Res.* **8**:4821-4837.
12. McAllister, W.T., Kupper, H., and Bautz, E.K.F. (1973) *Eur. J. Biochem.* **34**:489-501.
13. Morris, C.E., McGraw, N.J., Joho, K., Brown, J.E., Klement, J.F., Ling, M.L. and McAllister, W.T. (1987). *In* *RNA Polymerase and the Regulation of Transcription*, ed. W.S. Reznikoff *et al*, Elsevier.
14. Martin, C.T., Muller, D.K. and Coleman, J.E. (1988). *Biochemistry* **27**:3966-3974.
15. Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987). *Nuc. Acids. Res.* **15**:8783-8798.
16. Bailey, J.N., Klement, J.F. and McAllister, W.T. (1983). *Proc. Natl. Acad. Sci., U.S.A.*, **80**:2814-2818.
17. Studier, F.W. and Rosenberg, A.H. (1981). *J. Mol. Biol.* **153**: 503-525.
18. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M. (1984). *Nuc. Acids. Res.* **12**: 7035-7056.

19. Klement, J.F. (1986). Ph.D. Thesis, Rutgers University.
20. Bolivar, F., Rodriguez, R.L., Greene, P.J. Betlach, M.C., Heynecker, H.L. and Boyer, H.W. (1977). *Gene* 2:95-113.
21. Klement, J.F., Ling, M.L. and McAllister, W.T. (1986). *Gene Anal. Tech.* 3:59-66.
22. Ikeda, R.A. and Richardson, C.C. (1987). *J. Biol. Chem.* 262:3800-3808.
23. Muller, D.K., Martin, C.T. and Coleman, J.E. (1988). *Biochem.*27:5763-5771.