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Processing in the 5′ region of the *pnp* transcript facilitates the site-specific endonucleolytic cleavages of mRNA

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ABSTRACT

The primary transcript of *pnp*, the gene encoding polynucleotide phosphorylase in *Escherichia coli*, is processed in the 5′ end region by ribonuclease III (RNase III). The unprocessed transcript shows enhanced stability compared with the processed transcript. We report here that, unlike the processed transcript, the unprocessed *pnp* transcript did not accept endonucleolytic attack at, at least, five cleavage sites. Sequencing analysis of the four cleavage products shows no sequence specific to all these sites, but AU rich stretches were observed at three sites.

INTRODUCTION

In procaryotes, the stability of mRNA is an important factor which determines the level of gene expression. The half-lives of mRNA species varies at least by a factor of 50 (1) and the decay-rate of some mRNA is growth-rate-dependent (2). The differential expression of genes within an operon encoding a polycistronic transcript can be accomplished by the difference in mRNA stability (3–6). The difference in mRNA stability, in turn, could be accounted for by the difference in its susceptibility to degradation by cellular 3′-exo- and endonucleases (for a review, see 7).

The 3′-exoribonucleases RNase II and polynucleotide phosphorylase have been implicated in mRNA decay and appear to be the major enzymes involved in the degradation of mRNA (8). On the other hand, there have been many reports which showed that the endonucleolytic cleavage of the mRNA determines its decay rate. Decay of some mRNAs like those of *ompA, bla, LacZ, pnp* and *rnc* genes appear to be controlled by endonucleolytic attack near the 5′ end of the transcript (9–19), whereas the decay of *int* mRNA is initiated by endonucleolytic cleavage downstream of the gene (18). In spite of the importance of endonucleolytic cleavage in mRNA decay indicated in these reports, our knowledge about the endonucleases and the mechanism involved is still limited.

Endoribonuclease RNase III is responsible for the processing of *pnp, rnc* and *int* gene transcripts but it is probably only a small class of mRNAs that are inactivated by this enzyme (19). RNase M is involved in the degradation of *lacZ* mRNA (21) and RNase K plays a role in controlling the initial step in the degradation of *ompA* and *bla* mRNAs (17). RNase E is encoded by *ams* (altered mRNA stability) gene (22–24) and it is involved in the processing of 5S rRNA (25) and RNA1, the inhibitor of ColE1 plasmid replication (26). Moreover, in the RNase E-deficient cells, the chemical half-lives of the T4 and *E.coli* mRNA were increased several times (23–26), suggesting that this enzyme is essential for general mRNA decay in *E.coli*.

Previous studies have shown that the transcript of *pnp* is processed by RNase III in the 5′ end region and this processing dramatically affects the decay rate of *pnp* mRNA (14,15). Unprocessed transcript in the RNase III-defective strain is stabilized up to 10-fold compared with the processed transcript. Thus, the *pnp* transcript provides a model system for the elucidation of the relation between mRNA processing and its decay.

In this article, we analyzed the endonucleolytic cleavage of the *pnp* mRNA by S1 protection assay. Comparison of the cleavage products shows that the unprocessed transcript does not accept endonucleolytic cleavage at five sites compared with the processed transcript. The relation of the 5′ processing to the endonucleolytic cleavage is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmid and media

E.coli RNase III-defective strain N2077 (*rnc105 thi argH nadB lacY gal malA xyl ara mtl str^r tonA supE*) and its isogenic parent strain N2076 (*rnc$^+$*) were gifts from Dr. D.Apirion (29). Strain SK5003 (*thr leu pnp7 rnb500*) was supplied by Dr. S.Kushner (8). Plasmid pYN811 (30) was used as a source of DNA fragments for preparation of the end-labeled probes. For preparation of RNA, strains N2076 and N2077 were grown in LB medium at 37°C to a density of 2×10^8 cells /ml. Strain SK5003 was grown in LB medium at 32°C to a density of 1×10^8 cells /ml. The temperature was then shifted to 43°C and incubation was continued for 20 more minutes.

Preparation of the end-labeled probes

Plasmid DNA and DNA fragments were isolated as described (31). Restriction endonucleases and other enzymes were purchased from Takara Shuzo Co., Japan. DNA restriction fragments were 5′-end-labeled with polynucleotide kinase or 3′-end-labeled with Klenow fragment of *E.coli* DNA polymerase I as described (31). Then these ^{32}p-labeled fragments were digested with the second restriction nuclease to prepare the DNA probes which were labeled at one 5′ or 3′ end. The 5′-end-labeled

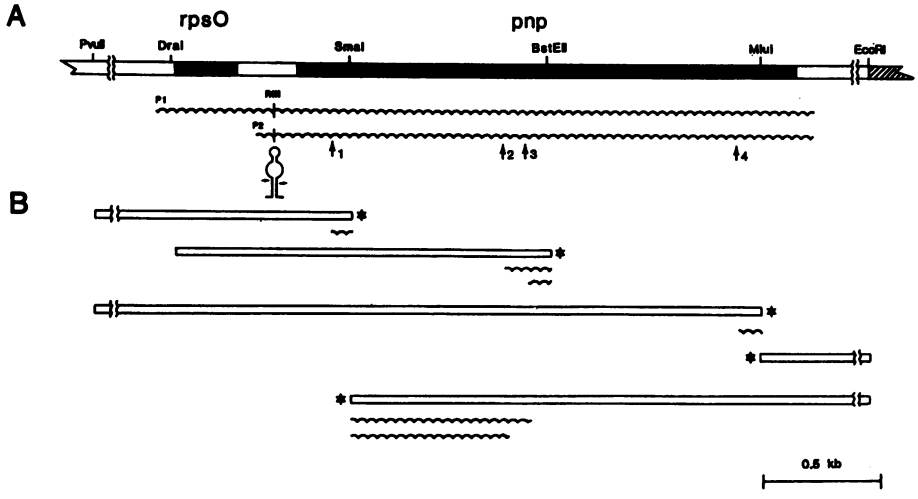

Fig. 1. Structural organization of the *rpsO-pnp* operon, the DNA probes used in the S1 nuclease analysis and summary of the results obtained in this experiment. Panel A: The genes for *rpsO*, which encodes ribosomal protein S15, and *pnp* are indicated by the solid line (22). Two promoters, p_1 and p_2, and the restriction sites are indicated. Below the map, the transcripts covering *pnp* are presented by wavy lines. The processing site by RNase III is indicated by RIII. The possible stem-loop structure around the processing site (indicated by the arrow) is also shown. Four cleavage sites, **1-4**, demonstrated in this experiment are indicated by arrows below the transcripts. Panel B: The structure of the DNA probes. 5'-end-labeled probes are *Sma*I-*Pvu*II, *Bst*EII-*Dra*I and *Mlu*I-*Pvu*II fragments. 3'-end-labeled probes are *Mlu*I-*Eco*RI and *Sma*I-*Eco*RI fragments. The labeled end is indicated by asterisks. The cleavage products detected by using these probes are also shown by wavy lines.

probes were the *Sma*I-*Pvu*II fragment of 1.5 kbp, the *Bst*EII-*Dra*I fragment of 1.6 kbp and the *Mlu*I-*Pvu*II fragment of 3.3 kbp. The 3'-end-labeled probes were the *Sma*I-*Eco*RI fragment of 2.9 kbp and the *Mlu*I-*Eco*RI fragment of 1.1 kbp (Fig. 1). The entire region of the *pnp* transcript was covered by these probes.

S1 nuclease protection assay

S1 nuclease assay were performed essentially as described (31). Total RNA was extracted from the cells by the hot phenol method of Salser et al. (32). In RNA-DNA hybridization reaction, 40 μg of RNA was mixed with 0.05−0.2 μg of end-labeled DNA probe in 30 μl of the hybridization buffer. After being denatured at 72°C, the reaction mixture was cooled gradually to 37°C. S1 nuclease digestion (300 units per ml) was carried out for 40 min at 37°C. After nuclease treatment, the hybrids were denatured and electrophoretically separated on 5% polyacrylamide gels or 8% polyacrylamide gels containing 8M urea with chemical cleavage ladders of the same DNA fragment. Chemical cleavage of the end-labeled DNA fragments was performed according to the method of Maxam and Gilbert (33).

RESULTS

For the identification of the endonucleolytic cleavage product of the transcript, 5'- or 3'-end-labeled probes were hybridized with total RNA extracted from rnc^+ or rnc^- strains. In addition to these strains, a pnp^-rnb^- (polynucleotide phosphorylase- and RNase II-defective) strain was also used because it was expected that the deficiency of the 3' exonucleases would make it easier to detect the cleavage product. The hybrids were treated with S1 nuclease and separated on polyacrylamide gels.

When a 5'-labeled *Sma*I-*Pvu*II fragment was used as a probe, a band of 300 nt length with the greatest intensity was observed in rnc^+ RNA (Fig. 2, lane 3,5). This band corresponds to the

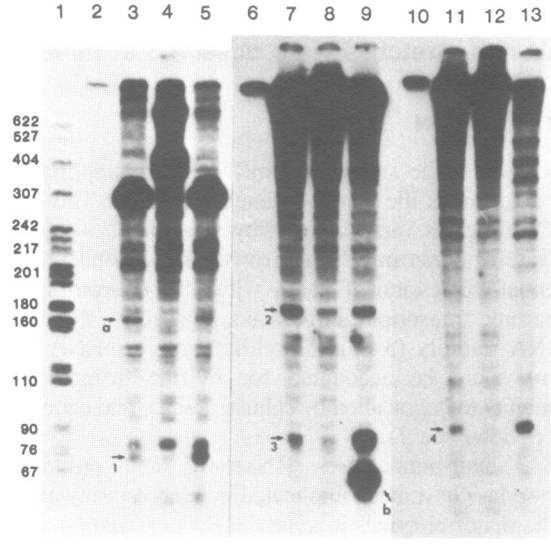

Fig. 2. SI nuclease analysis of the *pnp* transcript by using 5'-end-labeled probes. Lane 1; size marker (^{32}p-labeled *Hpa*II fragments of pBR322). Lanes 2,6,10; probes only. Lanes 2−5; *Sma*I-*Pvu*II fragment as a probe. Lanes 6−9; *Bst*EII-*Dra*I fragment as a probe. Lanes 10−13; *Mlu*I-*Pvu*II fragment as a probe. Lanes 3,7,11; hybrids with rnc^+ RNA. Lanes 4,8,12; hybrids with rnc^- RNA. Lanes 5,9,13; hybrids with pnp^-rnb^- RNA. Bands **a, 1, 2, 3** and **4** observed in rnc^+ RNA but not visible or greatly reduced in rnc^- RNA and band **b** observed in only pnp^-rnb^- RNA are indicated by arrows.

5' region of the processed *pnp* transcript (22, see Fig. 1). Similarly, three bands observed in rnc^- RNA with the length of 842 nt, 420 nt and 379 nt correspond to the 5' region of the unprocessed transcripts (lane 4). An autoradiogram of the gel also showed many bands with less intensity in addition to the main transcripts. Among these, two bands with the length of 165

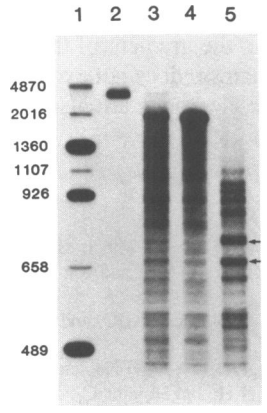

Fig. 3. S1 nuclease analysis by using a 3′-end-labeled *Sma*I-*Eco*RI fragment as a probe. Lane 1; size marker (^{32}p-labeled *Hin*dIII-*Hae*III fragments of pHY300 purchased from Takara Shuzo Co). Lane 2; probe only. Lane 3; hybrids with rnc^+ RNA. Lane 4; hybrids with rnc^- RNA. Lane 5; hybrids with pnp^-rnb^- RNA. Two bands which presumably originated from the cleavage at sites **2** and **3** are indicated by the arrows (see Fig. 1).

Table 1. Nucleotide sequence of the cleavage sites in the 5′-processed *pnp* transcript

Cleavage site	Sequence
Site 1	5′-CAGAA**AAAAG**↓C↓CAAACCAGG-3′
Site 2	5′-AACUGGGUGA↓**A**↓AUUCUGCAC-3′
Site 3	5′-CGUGA**AAAAG**↓A↓UA UGAUCC-3′
Site 4	5′-CUACACUGG↓U↓**AAAG**UGACCC-3′

Cleavage sites 1−4 are described in the text (see Fig. 1) and they are indicated by the arrows in the sequence. The sequence common to these sites is shown in bold letters. All nucleotide sequences are according to Régnier et al. (36).

nt and 72 nt, denoted as **a** and **1**, were visible in rnc^+ RNA, but not visible or reduced in rnc^- RNA. Two explanations are possible for the origin of these bands: one is that they originate from the endonucleolytic cleavage of the transcript and the other is that they are transcripts initiated from intragenic promoters. But it is unlikely that intragenic initiation products are different in two *pnp* genes with the same structure. Therefore we conclude that these two bands correspond to the cleavage products. Band **1** is amplified in pnp^-rnb^- RNA (lane 5) suggesting that 3′-exonucleases are involved in the degradation of this fragment.

The use of a 5′-end-labeled *Bst*EII-*Dra*I fragment revealed two bands, **2** and **3**, with lengths of 174 nt and 94 nt (lane 7,8), which are present in rnc^+ RNA but absent or greatly reduced in rnc^- RNA. The band **b** (lane 9) could be seen only in pnp^-rnb^- RNA and it might be caused by a base change (s) in the *pnp* mutation.

There is another band, **4**, with the length of 94 nt, which is observed only in rnc^+ RNA, when a 5′-end-labeled *Mlu*I-*Pvu*II fragment was used as a probe (lane 11). Two bands, **3** and **4**, are also amplified in pnp^-rnb^- RNA as band **1**.

The 3′-end-labeled probes were used to identify the cleavage products. When a 3′-end-labeled *Mlu*I-*Eco*RI fragment was used, no difference was found in the hybrids with rnc^+ and rnc^- RNAs (data not shown). When a 3′-end-labeled *Sma*I-*Eco*RI probe was used, two clear bands could be observed in pnp^-rnb^- RNA (indicated by arrows in lane 5, Fig. 3), which are absent in rnc^- RNA. The length of these bands

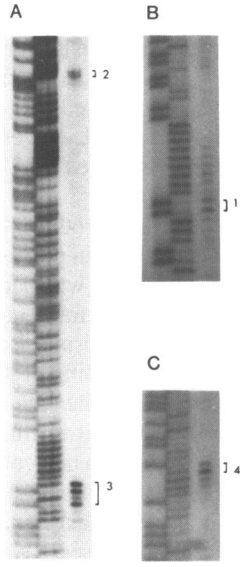

Fig. 4. Determination of the 5′ end of the cleavage products. Panel A; hybrids with a 5′-end-labeled *Bst*EII-*Dra*I probe. Panel B; hybrids with a 5′-end-labeled *Sma*I-*Pvu*II probe. Panel C; hybrids with a 5′-end-labeled *Mlu*I-*Pvu*II probe. The hybrid formed between end-labeled DNA probes and rnc^+ RNA were digested with S1 nuclease, denatured and separated on a 8% polyacrylamide gel with the chemical cleavage ladders. The ends of the protected fragments are indicated by arrows **1-4** (right lane in each panel). The arrow number corresponds to the band number in Fig. 2. Only G+A (left lane) and T+C (middle lane) sequencing reactions were performed with the end-labeled probe used in the hybridization experiments.

(approximately 670 nt and 750 nt) corresponds to that expected for the 5′-upstream cleavage products at sites **2** and **3** (the site number corresponds to the band number, see Fig. 1). The reduced amount of these bands in rnc^+ RNA (lane 3) is probably due to the degradation of the 3′ end by 3′-exonucleases. These results also indicate that bands **2** and **3** are originated by endonucleolytic cleavage and not by intragenic transcription initiation. Thus, at least five cleavage products were identified by using 5′- and 3′-end-labeled probes. All these results are summarized in Fig. 1.

In order to determine the 5′ ends of these cleavage products, S1 nuclease-protected fragments were sized on a sequencing gel next to DNA sequence ladders (Fig. 4). In these experiments, the bands with the highest intensity were assumed as the ends of the fragment. But this assignment is tentative until confirmed by mRNA sequence analysis. In addition to the main bands, numerous much less intense bands could be seen on the gel (Fig. 4, B,C). These minor bands might be generated by true 5′-termini of the mRNA present in low abundance or they might be caused by localized melting of heteroduplexes followed by S1 nuclease digestion. These cleavage sites and the flanking sequence are shown in Table 1. The result shows no sequence specific to all the sites, but AU rich stretches are observed at three sites (sites **2**, **3** and **4**). In addition, 5′-AAAG is observed on either side of three cleavage sites (site **1**,**3** and **4**). In site **2**, the sequence AAA could be seen instead of AAAG.

DISCUSSION

The data presented here shows that, unlike the processed transcripts, the unprocessed *pnp* transcripts do not accept endonucleolytic attack at several sites. These cleavage sites are

dispersed throughout the whole transcript. The results suggest that processing of the 5′ region in the *pnp* primary transcript leads to the endonucleolytic cleavage in, at least, five sites. The extended half-life of the unprocessed transcripts might be due, therefore, to the absence of these cleavages. In other words, the decay of the *pnp* mRNA is facilitated by 5′ processing followed by endonucleolytic cleavages.

In addition to the five cleavage products, there are other bands on the gel which were visible both in processed and unprocessed transcripts (Fig. 2,3). It is not clear whether they originated from the cleavage of the transcript or transcription initiation from promoters within the gene. If the former is true, these cleavages seem to be unaffected by 5′ processing of the transcript and they might be catalyzed by enzymes other than that involved in the cleavage at the five sites described in this experiment.

S1 protection analysis showed no nucleotide sequence specific to all of the cleavage sites. But it seems that AU rich stretches are vulnerable to the endonucleolytic attack. Similar observations were reported for endoribonucleases RNase K and RNase E. Cleavage sites by RNase K are predominantly located within AU rich stretches in *ompA* transcript (17), although there is no sequence specific to these sites. The sequence A/AU is conserved at most of the RNase E cleavage sites in 5S rRNA (25), RNA1 (26), T4 mRNAs (27, 28) and *rpsT* mRNA (34). These results suggest the possibility that the enzyme responsible for the endonucleolytic cleavage of *pnp* transcript described here corresponds to RNase K or RNase E. But, the presence of the sequence AAAG near the cleavage site has not been reported before with any endoribonucleases. Further investigation is necessary to identify the enzyme (s) involved in these cleavages. In addition to the AU rich stretches, sequence AAAG could be seen in three out of four sites although its relative position to the cleavage end is variable. It should also be noted that there are 19 AAAG sequences in total in the whole *pnp* mRNA. This might suggest that AAAG is only one of the factors necessary in determining the cleavage site.

Two models could possibly explain how the 5′ region of the unprocessed transcript prevents endonucleolytic cleavage of *pnp* mRNA. The first implies that the 5′ region of the unprocessed *pnp* transcript creates a secondary structure with the cleavage site leading to difficulty in attacking the site by endonuclease. But analysis of the nucleotide sequence did not show any potential secondary structure between the 5′ region and the cleavage sites as might be expected to order to prevent nuclease attack. The second, which is more likely, is that the entry of the endonuclease responsible for the cleavage at the five sites described here is limited only to the 5′ end of the mRNA and it proceeds along the transcript to meet the primary and/or secondary structure serving as a substrate. In the unprocessed transcript a large stem-loop structure, which is the target site for RNase III (see Fig. 1), in the 5′ region provides a barrier to the enzyme resulting in the absence or decrease of the cleavage. Studies with T4 gene 32 mRNA have shown that, consistent with the second model, a stem-loop structure in the 5′ end stabilizes the transcript (35).

We have previously shown that the 5′, middle and 3′ region of the processed *pnp* transcript are rapidly degraded with equal frequency, whereas the 5′ region of the unprocessed transcript is more stable than the 3′ region (30). All these results, including the data presented here, suggest that the cleavage at the five sites described is responsible for the rapid and uniform decay of the *pnp* mRNA. These cleavages generate short RNA fragments which are exposed to attack by 3′-exonucleases. In the absence of these cleavages, degradation from the 3′ end of the transcript is dominant leading to the gradient of the decay rate within the transcript. This mechanism does not exclude the possibility that other endonucleolytic cleavages are also involved in the decay of *pnp* mRNA.

REFERENCES

1. Perdersen,S.,Reeh,S., and Friesen,J. (1978) Mol.Gen.Genet. **166**, 329–336.
2. Nilsson,G.,Belasco,J.G.,Cohen,S.N. and von Gabain,A. (1984) Nature **312**, 75–77.
3. Burton,Z.F.,Gross,C.A.,Watanabe,K.K., and Burgess,R.R. (1983) Cell **32**, 335–349.
4. Blumer,K.J. and Steege,D.A. (1984) Nucleic Acids Res. **12**, 1847–1861.
5. Newbury,S.F.,Smith,N.H. and Higgins,C.F. (1987) Cell **51**, 1131–1143.
6. Baga,M.,Goransson,M.,Normark,S. and Uhlin,B,E. (1988) Cell **52**, 197–206.
7. Belasco,J.G. and Higgins,C.F. (1988) Gene **72**, 15–23.
8. Donovan,W.P. and Kushner,S.R. (1986) Proc.Natl.Acad.Sci.USA **83**, 120–124.
9. Belasco.J.G.,Nilsson,G.,von Gabain,A. and Cohen,S.N. (1986) Cell **46**, 245–251.
10. Cannistraro,V.J.,Subbarao,M.N. and Kennell,D. (1979) J.Mol.Biol. **192**, 257–274.
11. Melefors, Ö. and von Gabain,A. (1988) Cell **52**, 893–901.
12. Bardwell,J.C.A.,Rognier,P.,Chen,SM.,Nakamura,Y.,Grunberg-Manago,M. and Court,D.L. (1989) EMBO J. **8**, 3401–3407.
13. Nilsson,G.,Lundberg,U. and von Gabain,A. (1988) EMBO J. **7**, 2269–2275.
14. Portier,C.,Dondon,L.,Grunberg-Manago,M. and Régnier,P. (1987) EMBO J. **6**, 2165–2170.
15. Takata,R.,Mukai,T. and Hori,K. (1987) Mol.Gen.Genet. **209**, 28–32.
16. Emory,S.A. and Belasco,J.G. (1990) J.Bacteriol. **172**, 4472–4481.
17. Lundberg,U.,von Gabain,A. and Melefors, O. (1990) EMBO J. **9**, 2731–2741.
18. Petersen,C. (1991) J.Bacteriol. **173**, 2167–2172.
19. Schmeissner,U.,McKenney,K.,Rosenberg,M. and Court,D. (1984) J.Mol.Biol. **176**, 39–53.
20. Apirion,D. and Gitelman,D.R. (1980) Mol.Gen.Genet. **177**, 339–343.
21. Cannistraro,V.J. and Kennell,D. (1989) Eur.J.Biochem. **181**, 363–370.
22. Claverie-Martin,F.,Diaz-Torres,M.R.,Yancey,S.D. and Kushner,S.R. (1989) J.Bacteriol. **171**, 5479–5486.
23. Mudd,E.D.,Krisch,H.M. and Higgins,C.F. (1990) Mol.Microbiol. **4**, 2127–2135.
24. Babitzke,P. and Kushner,S.R. (1991) Proc.Natl.Acad.Sci.USA **88**, 1–5.
25. Roy,M.K.,Singh,B.,Ray,B.K. and Apirion,D. (1983) Eur.J.Biochem. **131**, 119–127.
26. Tomcsanyi,T. and Apirion,D. (1985) J.Mol.Biol. **185**, 713–720.
27. Mudd,E.A.,Prentki,P.,Belin,D. and Krisch,H.M. (1988) EMBO J. **7**, 3601–3607.
28. Mudd,E.A.,Carpousis,A.J. and Krisch,H.M. (1990) Genes Develop. **4**, 873–881.
29. Apirion,D. and Watson,N. (1975) J.Bacteriol. **124**, 317–324.
30. Takata,R.,Izuhara,M. and Hori,K. (1989) Nucleic Acids Res. **17**, 7441–7451.
31. Sambrook,J.,Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor.
32. Salser,W.,Gestland,R.F. and Bolle,A. (1967) Nature **215**, 588–591.
33. Maxam,A.M. and Gilbert,W. (1980) in Meth.Enzymol. **65**, 499–560. Academic Press, New York.
34. Mackie,G.A. (1991) J.Bacteriol. **173**, 2488–2497.
35. Gorski,K.,Roch,J.M.,Prentki,P. and Krisch,H.M. (1985) Cell **43**, 461–469.
36. Régnier,P.,Grunberg-Manago,M. and Portier,C. (1987) J.Biol.Chem. **262**, 63–68.