Cloned DNA sequences that determine mRNA stability of bacteriophage ϕ X174 *in vivo* are functional

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ABSTRACT

The stability of two species of ϕ X174 polycistronic mRNA *in vivo* can be altered by mutating sequences existing immediately upstream of a termination site. The wild type phage contains an mRNA stabilizing sequence ((+) sequence), while the same sequence mutated by insertion ((-) sequence) reduces the stability of the mRNAs. These two sequences were cloned at the 3' ends of gene D or gene B of ϕ X174 in a pBR322 derivative plasmid. The cloned sequences were functional. The (+) sequence stabilized gene B or gene D mRNA; half-lives of these mRNAs were 7 to 8 min. When the (+) sequence is eliminated ((o) sequence) or replaced with the (-) sequence, the half-lives of the mRNA were reduced to about 1 to 2 min. The stabilization of mRNAs caused an increased production of these proteins.

INTRODUCTION

Most of the wild type bacteriophage ϕ X174 polycistronic mRNAs *in vivo* are structurally stable and decay with half-lives of 4.5 to 11 min at 37°C. (1). On the other hand, the half-lives of the host originated mRNAs in the infected cells are about 2 to 2.5 min (see ref. 2 for review). Previously we have shown that the stabilities of two species of ϕ X174 mRNAs starting at the promoter P_B or P_D and terminating at the common terminator T_J can be altered by mutating a sequence immediately upstream of the T_J . (3). This sequence is located between gene J and gene F (J-F intercistronic region) (Fig. 1). The two wild type mRNAs decayed with half-lives about 10 min. When the wild type sequence in the J-F intercistronic region was mutated by insertion or deletion (*ins* or *del* mutants), the two RNA species decayed faster than the wild type. We have cloned these mRNA stabilizing and destabilizing sequences in plasmids and compared their functionalities. The wild type sequence (mRNA stabilizing sequence) and one of the decay stimulating sequences isolated from the *ins* 2 mutant will be referred to as the (+) sequence and the (-) sequence in the text, respectively. The genes to be tested for their mRNA decay rates (ϕ X174 gene D or gene B) were cloned upstream of the (+) or (-) sequence. When the cloned genes were followed by neither sequence ((o) sequence), the mRNAs

decayed with the half-lives of about 1.5 to 2 min. The (+) sequence increased the half-lives to about 7 to 8 min. When the (-) sequence was inserted at the 3' end of the genes, the decay constant decreased to the level of the (o) sequence. Thus, the cloned (+) and (-) sequences are functional. The increased structural stabilities of gene D or gene B mRNA resulted in the increased synthesis of the gene D or gene B protein.

MATERIAL AND METHODS

Cloning the (+) or (-) sequence at the 3' end of gene D (Fig. 2).

RF (replicative form) DNA of ϕ X174 am3 (designated as wild type) or an insertion mutant of am3 (ins 2) was digested with Hae II and the fragment containing the J-F intercistronic region was isolated. The wild type sequence covers ϕ X174 (926 to 1014). The ins 2 fragment has an extra 46 bases derived from the Alu 9b fragment of pBR322 (2733 to 2778) inserted at the ϕ X174 979 position (a Hae III site) (See Fig. 1). The two Hae II fragments were treated with T_4 DNA polymerase to convert to flush ends. A plasmid pKJB824 X16 is a derivative of pBR322 containing the thermolabile λ repressor gene (cI), the λ P_R promoter and gene D of ϕ X174 (from ϕ X174 from 322 to 907) cloned immediately down stream of the λ P_R promoter. The ϕ X174 sequence had an incomplete -35 sequence of the P_D , thus the P_D is not functional. The Bam HI site at the 3' end of the gene D was cut, treated with DNA polymerase large fragment (Klenow fragment) and the (+) or (-) sequence was ligated and the clones containing the 5' to 3' direction identical to the gene D orientation were selected. The original pKJB824 X16 was used as the (o) sequence control plasmid. The prototypes of the pBR derivatives were constructed by K.J. Buckley in our laboratory and the construction procedures will be detailed in a separate paper.

Gene B with the (+) or (-) sequence (Fig. 2).

pMC-16 is identical to pKJB824 X16 except gene B (ϕ X174 from 4950 to 65) is present between Eco R₁ and Bam HI (Fig. 2). pMC-16 was digested with BamHI and PvuII and the larger fragment was isolated. The (+) or (-) sequence isolated by digestion with HaeII from the RFs was cloned in mp8 HincII site in both orientations. The clones marked (6) and (7) (Fig. 2) were cut with BamHI and PvuII and the (+) or (-) sequence containing fragment was ligated to the large fragment of pMC-16 to make pBAW (gene B with the (+) sequence) or pBAI (gene B with the (-) sequence). Mp8 was cut with BamHI and PvuII ((8) in Fig. 2) and fragment was ligated to the large fragment of pMC-16 to make pBAO (gene B with the (o) sequence). The (+) or (-) sequence inserted into mp8 with

Figure 1

A partial genetic map of ϕ X174. The nucleotide sequence number and the positions of A*, B, C, D, E, K, J and F genes (boxes) are taken from Sanger et al. (9). P_B and P_D are promoters. T_J is a terminator (3). Shaded boxes indicate gene B or gene D cloned into the plasmids. The original positions of (+) and (-) sequences are shown. The sequences are excised and cloned in plasmids. Thin lines ((1), (2) and (3)) are the positions of probes used for hybridization of ϕ X174 mRNAs.

orientations shown (4) or (5) (Fig. 2) was used for the probes of the 3' end of mRNA (see below). Plasmids are maintained in *E. coli* HF4740 (ϕ X^s. recA).

Induction of Cloned Genes

Plasmid containing bacteria were grown in 1X A synthetic medium (4) supplemented with 40 μ g/ml each of 20 amino acids and 0.4% glucose. Cells were grown at 30°C to OD₆₆₀ about 0.5, heat treated at 45°C for 5 min then transferred to 39°C.

Labeling mRNA and Protein

Cells were kept at 39°C for 10 min after heat-induction ($t=0$) 3 H uridine was added to the culture (10 to 30 μ Ci/ml, 1 μ g/ml) for 5 min. When the culture was pulse-labeled and chased, 3 H uridine was added for 4.5 min (gene D mRNA) or 3.5 min (gene B mRNA). At the end of the pulse labeling, 1 mg/ml of unlabeled uridine and 500 μ g/ml of rifampin were added. Samples (2-3 ml) were withdrawn at $t=5, 7, 9, 11$ and 13 min. and RNAs were isolated. When proteins were to be labeled, cells were grown in 1 x A medium supplemented with 40 μ g/ml each of 19 amino acids (minus methionine) and 0.4% glucose, heat treated as above and labeled with 2 μ Ci/ml and 1 μ g/ml of 35 S-methionine for 20 min (gene D containing clone) or 5 min (gene B containing clone).

Isolation of RNA and DNA-RNA Hybridization

Labeled cells were subjected to total RNA isolation as detailed in a previous paper (3). Isolated RNA preparations were hybridized to probes shown in

Fig. 1 and Fig. 2 as described previously (3). Hybridization probes: (1) gene D probe (ϕ X174, 322 to 907), (2) gene D 5' end probe (ϕ X174, 322 to 415), (3) gene B probe (ϕ X174, 5022 to 28) (Fig. 1), (4) and (5), the 3' end probes (Fig. 2) were cloned in mp8 or mp9* in appropriate orientations. The original mp9 contained a fragment of pBR322 (2352-2722) (11), a derivative of mp9, mp9*, that did not contain the fragment is a gift from K.J. Buckeley. Phages were isolated and the purified phage DNA was used for the hybridization probes.

Acrylamide Gel Electrophoresis of Proteins

³⁵S labeled proteins were run in 13.5% Laemmli gels (5). After impregnating with PPO, gels were autoradiographed. Marker ϕ X174 specific proteins were prepared as described (6).

RESULTS

The Cloned (+) or (-) sequence is functional

In vivo ϕ X174 mRNA contains the (+) or (-) sequence at the 3' end of polycistronic messages. In order to simplify the system and to determine whether or not the HaeII fragments containing J-F intercistronic region have sufficient information for determining the stability of mRNA, we cloned these two fragments after a single gene in a plasmid. The single gene we chose was gene D or gene B of ϕ X174. Because the expression of gene D or gene B inhibits cell growth (unpublished observation), the gene B or gene D sequence without a ϕ X174 promoter was cloned down stream of a heat-labile λ cI repressor and λ P_R promoter in a pBR322 derivative plasmid, and the expression of gene D or gene B was induced by heat inactivation of the cI repressor. The gene D of ϕ X174 contains an overlapping gene E (lysis) (7), which is expressed when gene D mRNA is synthesized. Therefore, the gene D sequence was isolated from RF DNA of an amber mutant of gene E (N11). The (+) or (-) sequence was inserted at the 3' end of gene D or gene B (Fig. 2).

Cells carrying the plasmid pDAW (gene D with (+) sequence) pDAI (gene D with (-) sequence) or pDAO (genes with (o) sequence) (Fig. 2) were grown and the rates of synthesis of various mRNA species were determined by pulse labeling the cells with ³H uridine and hybridizing the isolated bulk RNA to gene specific probes before and after heat treatment (Table 1). No messages corresponding to the gene D were detected at 30°C. On the other hand, RNA complementary to the pBR322 portion of the plasmid was detected in all three cells. When the repressor was heat inactivated, synthesis of gene D messenger RNA was observed indicating the expression of the gene D was under the control

Figure 2

The outline of cloning procedures. The probes (4) and (5) were used to measure the 3' end of messages.

TABLE 1 RNA synthesis in cells harboring different plasmids before and after induction.

plasmid	gene(s)	Hybridized counts/10 ⁵ cpm input	
		30°C	39°C
pDAW	D	0	4586
	pBR	321	774
pDAI	D	0	2667
	pBR	327	686
pDAO	D	0	2082
	pBR	434	909

The RNA samples were prepared as described in Materials and Methods. The ³H uridine pulse-labeling period was 5 min at 30°C or at 39°C. The probe (1) in Fig. 1 was used to detect gene D mRNA. Heat denatured pBR322 DNA was used for assay of pBR322 specific mRNA. The total incorporation of ³H uridine into the cells harboring these plasmids were very similar. The data were expressed hybridized counts per 10⁵ cpm of total input counts. In these conditions the background counts (hybridizable counts to mp8 or mp9* DNA) were about 50 cpm and have been subtracted.

Figure 3

The decay curves of gene D mRNA containing the (+), (-) or (o) sequence. The RNAs were prepared from ^3H uridine pulse-labeled and pulsed-chased cells and hybridized to the probes. A. Decay curves of gene D mRNA, the probe (1) of Fig. 1 was used. B. The pBR322 specific mRNA was determined by using heat denatured pBR322 plasmid DNA (prepared from form II DNA derived by mild DNase I treatment of form I (3)). C. Decay curves of the 5' end of gene D mRNA, the probe (2) of Fig. 1. D. Decay curves of the 3' end of gene D mRNA Probes (4) or (5) of Fig. 2. About 200 to 300 μl cell equivalent RNA was used for each time point (about 2×10^5 to 3×10^5 cpm of ^3H). The background counts (hybridized to mp8 or mp9* DNA) ranged from 80 to 120 cpm, which had been subtracted. The actual hybridized counts at $t=0$ (100%) are 4,000 to 10,000 cpm for gene D mRNA, 2000 to 2500 cpm for pBR322 specific mRNA, 1000 to 2500 cpm for 5' or 3' end mRNAs. —●— pDAW, —○— pDAI, —▲— pDAO.

TABLE 2 RNA synthesis in cells harboring different plasmids before and after induction and half-lives of gene B mRNA.

		Hybridized counts/ 10^5 cpm input ¹		half-life at
	gene	30°C	39°C	39°C min ²
pBAW	B	0	2316	7.2 ± 0.3
	amp	208	182	0.8 ± 0.1
pBAI	B	0	1592	2.0 ± 0.2
	amp	219	180	0.8 ± 0.1
pBAO	B	0	930	1.4 ± 0.2
	amp	249	227	0.8 ± 0.1

¹Cells containing these plasmids were pulse labeled with ^3H -uridine for 5 min and the total RNA was extracted and hybridized to the probe (3) in Fig. 1 for gene B mRNA or an mp9* probe containing pBR322 fragment PstI to EcoRI (covering 5' part of amp gene) for amp mRNA. Results were presented as hybridized counts of the 10^5 cpm input total RNA. The background counts were about 50 cpm (measured mp8 or mp9* DNA as the probe) and have been subtracted.

²Cells containing these plasmids were pulse labeled and pulsed-chased as described in Materials and Methods. The pulse-labeled cells were chased with unlabeled uridine in the presence of rifampin. Samples were taken at 2 min periods and total RNA was isolated. The decay curves similar to Fig. 3 were constructed and the half-lives were determined.

of the lambda repressor-promoter. pBR322 specific RNA was also detected at 39°C.

The decay rates of messages corresponding to the gene D and the pBR322 portion of the plasmids were determined at the high temperature. After the heat-treatment, the cultures were pulse-labeled with ^3H uridine, then chased with unlabeled uridine. Rifampin was added to prevent further RNA synthesis. Cell samples were withdrawn after the chase and RNA was extracted and hybridized to various probes or heat-denatured pBR322 DNA.

The results are shown in Fig. 3. In all strains, pBR322 specific mRNA decayed with half-lives of 2 min (Fig. 3B). When the decay of D message was compared, a significant difference was observed (Fig. 3A). In cells carrying DAW plasmid ((+) sequence), a half-life of D message was 8 min. D message from DAI plasmid ((-) sequence) has a half-life of 2.2 min, and DAO D message ((0) sequence) has a half-life of 2 min. The decay of the 5' or 3' portion of D message was examined using the probes covering these portions. As shown in Figs. 3C and D, the decay of these regions are very similar to the total decay of gene D message. The (+) sequence stabilized not only the total gene D mRNA decay but also the decay of the 5' and 3' termini sequences of the message.

This stabilizing effect disappeared when the (+) sequence is replaced with the (-) or (o) sequence.

The effect of (+) or (-) sequence on the stability of mRNA is not limited to gene D. When gene B of ϕ X174 was cloned onto the similar plasmid with these sequences, the decay rate of gene B mRNA was controlled by these sequences. Table 2 shows that (1) the expression of gene B is under the control of λ repressor- P_R promotor, while the ampicillin resistant gene is not, (2) at the high temperature gene B mRNA decayed with a half-life of 7 min in the presence of the (+) sequence at the 3' end, but mRNA of gene B with the (-) sequence had a half-life of 2 min and gene B mRNA with the (o) sequence decayed with a half-life of 1.4 min and (3) mRNA from the ampicillin resistant gene decayed at the same rate (about 1 min of half-life) in these three strains at 39°C. Thus, the (+) or (-) sequence functioned only in cis.

The Functionality of Stabilized mRNA

The functionality of the stabilized mRNA was examined by determining the ability to synthesize protein product from the mRNAs. (Fig. 4). When cells carrying pDAW, pDAI or pDAO plasmid were grown at 30°C and labeled with ^{35}S methionine for 20 min, no appreciable amount of the protein comigrating with the D protein marker was found (Fig. 4A) confirming the hybridization data (Table 1). When similar labeling procedure was performed after the thermal induction, pDAW containing cells produced larger amounts of gene D protein than the cells carrying pDAI or pDAO plasmid. The section of the gel containing gene D protein was excised and the radioactivity was determined. The ratio of D protein in pDAW, pDAI, and pDAO carrying cells was 3~4:1.3:1 indicating the stabilized gene D mRNA in pDAW containing cell produced three to four times more D protein than pDAO containing cells. When the protein labeling was performed after the cells were treated with rifampin, the ratio of gene D protein in pDAW, pDAI and pDAO cells was 11:1.6:1 indicating that a portion of the stabilized gene D mRNA is functional.

The effect of the (+), (-) or (o) sequence on the gene B expression was similar to that observed with the gene D protein synthesis. (Fig. 4B). Cells carrying plasmids containing gene B did not synthesize gene B proteins at 30°C (lane 1, 2 or 3). When the cells were heat treated, the gene B expression was observed. To assess the accumulated amount of gene B protein is difficult because gene B protein decayed in the cell containing plasmids with about 20 min of a half-life (data not shown) as reported in the infected cells (T. Pollock cited in ref. (10)). Therefore, the cells were labeled for a short period (5 min) to determine the rate of gene B protein synthesis. The rate of

Figure 4

The effects of the (+), (-) or (o) sequence on protein synthesis. A. gene D protein. pDAO (lanes 1,4,7), pDAW (lanes 2,5,8) or pDAI (lanes 3,6,9) containing cells were grown in different conditions and labeled with ^{35}S methionine. Total protein was isolated and run in 13.5% poly acrylamide gels. lanes 1,2,3. Cells were grown exponentially at 30°C and labeled with ^{35}S -methionine for 20 min. Lanes, 4,5,6. Cells were grown at 30°C and heat treated at 45°C then transferred to 39°C . 10 min after the transfer ^{35}S -methionine was added to the culture for 20 min. Lanes 7,8,9. Same as 4,5, or 6 except ^{35}S -methionine was added 2 min after rifampin addition at $t=10$ and labeling was continued for 20 min.

B. gene B protein. pBAO (lane 1,4,7) pBAW (lanes 2,5,8) or pBAI (lane 3,6,9). Lanes 1,2,3, at 30°C , lanes 4,5,6 after heat induction, lanes 7,8,9, after heat induction with rifampicin. all conditions were identical as in A except the labeling periods were for 5 min. Marker ϕX174 proteins are run at the right lane of each Fig. The positions of gene D protein and gene B protein are indicated.

gene B protein synthesis is highest with the plasmid containing the (+) sequence. The presence of the (-) or (o) sequence drastically decreased the amount of gene B protein. The ratio of gene B protein in pBAW, pBAI and pBAO

cells was 5~8:2:1. When the protein labeling was performed in the presence of rifampin, a considerable amount of gene B protein synthesis was observed when the gene B contained the (+) sequence at its 3' end, but very small amounts of gene B protein were synthesized from gene B mRNA with the (-) or (o) sequence. The ratio of B protein in pBAW, pBAI and pBAO was 10~15:2:1.

These results indicate that the (+) sequence stabilized gene B and gene D mRNAs structurally, and the stabilized mRNAs are functional. In these experiments, a higher rate of gene D or gene B protein synthesis was observed in the (-) sequence containing plasmids than in the (o) sequence containing plasmid. (Compare Figs. 4A and B lanes 4 and 6, and 7 and 9). It may be due to a slightly longer half-lives of the (-) sequence containing genes than the (o) sequence containing genes (Fig. 3A and Table 2).

DISCUSSION

The results described in this paper indicated that (1) the degree of the stability of gene D or gene B mRNA is solely determined by the sequence at their 3' end of the message, (2) the stability controlling sequences do not function in trans on the other genes in the plasmid (pBR322 coded genes) (3) the previously observed stability of two ϕ X174 mRNAs starting P_B or P_D and terminating at T_J in the infected cells is not due to physiological changes of the cells after infection and none of the ϕ X174 coded proteins are responsible for the stabilization, and (4) The stabilized mRNA is functional as well as structurally stable. The half-lives of the functional stability have not been determined in the clones described in this paper. Previously we have compared the functional and structural half-lives of gene B and gene D messages in the infected cells (2). The structural half-lives of gene B and gene D were 10.1 min and 8.8 min at 37°C respectively, and the functional half-lives of gene B and gene D were 6.5 min and 5.1 min respectively at 37°C. Thus, the functional half-lives are shorter than the structural half-lives. It remains to be determined if the similar relationship exists in the cloned sequences.

Previously, we have shown that the majority of pulse-labeled ϕ X174 mRNAs in the infected cells were found in the polyribosome region, and that when the pulse-labeled culture was chased with unlabeled uridine in the presence of rifampin, most of the ϕ X174 mRNAs remains bound to polyribosomes although the total amount of ϕ X174 mRNA in the polyribosome region decreased exponentially with time (1). No accumulation of ϕ X174 mRNA in the soluble region was observed. Evidently ϕ X174 mRNAs are continuously translated throughout their lifetimes. Once the degradation process is initiated on an RNA molecule it proceeds rapidly, resulting in complete decomposition of the entire message.

Thus, the degradation of ϕ X174 mRNA seems to involve a rate limiting step at the beginning of the entire degradation process.

The mechanism by which the (+) sequence stabilizes mRNA is unknown. Previously, we speculated that a hypothetical exonuclease (probably 3' to 5' direction) is responsible for the initial decay of mRNA and that the (+) sequence has a potentiality to slow down the initial degradation (3). The (+) sequence contains a six-base-paired hairpin structure upstream of the termination site. This hairpin structure was destroyed by the insertional mutation in the (-) sequence, thus the hairpin structure would be a candidate for the barrier of 3' to 5' hydrolysis of mRNA by the hypothetical mRNA nuclease. The involvement of the hairpin structure in stabilization of mRNA has been reported. (8).

However, more detailed investigation involving eight different deletion or insertion mutants revealed that the hairpin structure is not the primary requirement to form the (+) sequence (3). For example, one deletion mutant (del 2), although it can stabilize ϕ X174 mRNA as the wild type phage does, does not provide a stable hairpin structure. Another insertion mutant (ins 11) has the potential for small perfectly base paired secondary structure, but it is a (-) sequence mutant. Thus, the distinction of (+) or (-) sequences seems to demand more complicated structure requirements.

It should be noted that most of ϕ X174 mRNAs terminating after genes F, G and H (T_F , T_G and T_H) are also stable. These terminators may also contain the (+) sequences similar to that described in this paper. It will be interesting to clone these sequences to determine their functions. It may be possible to find (+) sequences that stabilize mRNA better than the J-F intercistronic region described in this paper.

The decay process of mRNA may involve several enzyme activities and interactions of mRNA sequences and these enzymes. Many endo- and exo ribonucleases have been found in *E. coli*, but no clear evidence has been provided concerning the mRNA degrading enzymes in vivo (12,13,14). The genes containing the (+) and (-) sequence described in this paper would be useful to identify the nucleases involved in mRNA metabolism.

Another interesting aspect of the present study is that mRNA stabilizing sequences are an important tool to maximize expression from cloned genes. Numerous genetic manipulations at the promoter or the ribosome binding site have been utilized to increase expression of a cloned gene. The present results indicate that it is also possible to improve gene expression by increasing the stability of the message.

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