JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 810–815
0021-9193/91/020810-06$02.00/0

Vol. 173, No. 2

Genetic Analysis of the *c*III Gene of Bacteriophage HK022

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Received 14 August 1990/Accepted 2 November 1990

The *c*III gene product of lambdoid bacteriophages promotes lysogeny by stabilizing the phage-encoded CII protein, a transcriptional activator of the repressor and integrase genes. Previous works showed that the synthesis of the bacteriophage λ CIII protein has specific translational requirements imposed by the structure of the mRNA. To gain insight into the mRNA structure and its role in regulating *c*III translation, we undertook a mutational analysis of the *c*III gene of the related bacteriophage HK022. Our data support the hypothesis that in HK022, as in λ, translation initiation requires a specific mRNA structure. In addition, we found that translation of HK022 *c*III, like that of λ, is strongly reduced in a host deficient in the endonuclease RNase III.

HK022 is a temperate bacteriophage of *Escherichia coli* (9). Its ability to form viable recombinants with bacteriophage λ shows that it is a member of the lambdoid phage family (10, 25). The lambdoid phages have a similar organization of the genes along the chromosome, which accounts for their ability to form functional hybrids through recombination; their primary sequences, however, are often very divergent (6). The *c*III gene of HK022 has been identified previously on the basis of its analogous location on the HK022 chromosome and its sequences (18).

The CIII protein of lambdoid bacteriophages is involved in the lysogenization process. Its role is to stabilize the CII transcriptional modulator (3, 13), which in turn stimulates the transcription of the repressor (*c*I) and integrase genes (20) and represses the expression of the late genes (12). Overproduction of the CIII protein was also shown to induce the heat shock response, probably through stabilization of the heat shock-specific subunit of RNA polymerase σ^{32} (4). The mechanism by which CIII stabilizes these proteins is unknown.

The λ *c*III gene has been found to have complex translational requirements. Previous experiments have shown that the host protein RNase III is required for efficient *c*III translation (2). In addition, genetic and biochemical evidence demonstrated that the region around the *c*III ribosome-binding site is found in two alternative conformations, only one of which is translated (1, 16). We suggested that these features are related to regulation of *c*III expression at the translational level.

In this work we tested whether the *c*III gene of bacteriophage HK022 presents similar features. Here we demonstrate that the HK022 *c*III gene is dependent on RNase III for its translation. We further describe the characterization of 10 mutations affecting the activity of the HK022 *c*III gene. Some of these mutations strongly reduce translation of the gene. These mutations support the hypothesis that in the HK022 *c*III gene, mRNA structure is involved in control of gene expression.

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MATERIALS AND METHODS

Plasmids and strains. Plasmid p*tac*λ*c*III, carrying the λ *c*III gene under *tac* promoter control, has been described previously (16). The plasmid used here is a mutant plasmid, obtained by site-directed mutagenesis, which yields a nonfunctional CIII protein (16a). Bacterial strain A5039 is *Escherichia coli* K37 *lacZ*::Tn*5* *lacI*^{q1} (D. Friedman). Strain A6008 is an *rnc* derivative of A5039, obtained by P1 transduction of the *rncA14* allele, which carries a mini-Tn*10* insertion in the *rnc* coding region (22). The phage strains used were HK022 (9) and λ *c*I857 *c*III *am*611.

Media. Bacteria were propagated in LB medium containing 40 μg of ampicillin per ml when appropriate. For protein labeling, the cells were grown at 37°C in M56 minimal medium, supplemented with 0.5% (vol/vol) glycerol and 20 μg of ampicillin per ml.

Plasmid construction. Standard cloning methods were described before (17). Plasmid vector pKK223-3 (5) was obtained from J. Brosius. The pGEM-3 vector was purchased from Promega Biotec. Plasmid p*tac*HK*c*III contains 196 bp of HK022 sequences, extending from a *Hae*III site (coordinate 377 [18]) to an *Fsp*I site (coordinate 182; see Fig. 2). The *Hae*III side of the fragment was fused to the *Sma*I site at coordinate 4581 of pKK223-3; the *Fsp*I side was fused to nucleotide 4454 of the vector, regenerating an *Fsp*I site. In this construct, the *c*III coding region lacks its last three codons; instead, 15 codons of vector sequences are added to the CIII reading frame, yielding a 56-amino-acid protein. Plasmid pT7HK was obtained by cloning the *Eco*RI-*Fsp*I fragment of p*tac*HK*c*III, containing the *c*III gene, into the *Eco*RI and *Sma*I sites of pGEM-3.

Hydroxylamine mutagenesis. In vitro mutagenesis of plasmid p*tac*HK*c*III was performed as described previously (14). From 3 to 5 μg of plasmid DNA was dissolved in 0.5 M sodium phosphate–0.4 M hydroxylamine–1 mM EDTA, pH 6. The mixture was incubated for 20 min at 65°C and then dialyzed five times against 2 liters of TE (10 mM Tris, 1 mM EDTA [pH 8]). The DNA was then used to transform A5039 cells.

DNA sequencing. DNA sequence analysis of the HK022 *c*III mutants was performed by the dideoxynucleotide chain termination method on supercoiled plasmid DNA prepared by the method of Chen and Seeburg (7).

Analysis of labeled proteins. Cells were grown in supple-

TABLE 1. Level of expression of the HK022 cIII mutants[a]

Mutant	λ cIII EOP	Protein band (% of control)	mRNA (% of control)
Wild type (control)	$<10^{-4}$	100	100
A(−10)	0.5	<5	50
A(−9)A(−7)	1	<5	35
T19	0.2	<5	65
T37	0.1	32	73
A39	0.5	<5	64
T40	0.2	6	36
T58	0.2	89	118
A60	0.05	90	96
A70	0.05	109	109
T81	1	<5	24

[a] The level of cIII function was quantitated by measuring the efficiency of plating (EOP) of a λ cIII *am*611 mutant on A5039 cells carrying the various p*tac*HK plasmids in the absence of IPTG relative to that of A5039 cells carrying the vector plasmid. In the presence of IPTG, the cells can form a lawn; under these conditions, efficiency of plating was somewhat reduced in all the mutants. For the protein band data, cells carrying the various p*tac*HK*c*III mutants were grown in minimal medium to mid-log phase, induced for 15 min with 1 mM IPTG, and pulse-labeled for 2 min with [^{3}H]proline. The proteins were then separated by gel electrophoresis and visualized by autoradiography (see Fig. 1). The radioactivity of the gel slice containing the CIII protein was measured by scintillation counting. The results presented in the table are normalized for each mutant to an apparently constant protein band (indicated by an arrow in Fig. 1). Amounts of CIII protein lower than 5% of the fully induced wild-type level could not be estimated owing to the high gel background levels. The mRNA was quantitated by binding equal amounts of total RNA from the various mutants to a nitrocellulose filter (dot blot), followed by hybridization to a cIII-specific probe. The amount of mRNA hybridized was measured by scintillation counting of the filter.

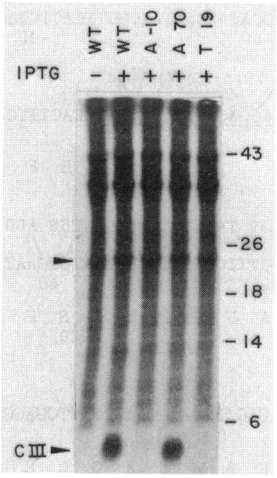

FIG. 1. CIII labeling in cells carrying the wild-type (WT) p*tac*HK*c*III plasmid before (lane —) and after (lanes +) 15 min of IPTG induction as well as in cells carrying some representative mutant p*tac*HK*c*III plasmids. Quantitation of the amount of CIII protein synthesized by all the isolated mutants is presented in Table 1. The arrow indicates the protein band used as a standard in Table 1.

mented minimal medium at 37°C to an OD_{600} of 0.4 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). At 15 min after the induction, 0.2-ml samples were pulse-labeled for 2 min with 10 μCi of [2,3-^{3}H]proline (31 Ci/mmol; Amersham Corp.). Samples were precipitated with 10% (wt/vol) trichloroacetic acid and subjected to gel electrophoresis on 15% acrylamide–6 M urea gels. The radioactive signal was enhanced by fluorography with Amplify (Amersham). The protein bands were quantitated by cutting out the relevant part from the dried gel, rehydrating it with 0.15 ml of water, and adding 0.85 ml of Soluene-350 (Packard) tissue solubilizer. After overnight incubation in the solubilizer, 4 ml of scintillation liquid was added to the samples, followed by scintillation counting.

Dot blot analysis of mRNA. Cells containing the various plasmids were grown and induced with IPTG as described above. At 15 min after induction, total RNA was extracted as described by Salser et al. (19). This method yields DNA-free RNA. The total RNA concentration was determined by measuring the OD_{260}, and decreasing amounts of RNA were bound to nitrocellulose filters (23). As a control, some samples were treated with DNase-free RNase before being loaded on the filters. The filters were probed with the labeled antisense cIII mRNA synthesized from the SP6 promoter of *Eco*RI-digested pT7HK or pHSY1.

Extension inhibition by 30S ribosomal subunit. The toeprint assay was done as described previously (1). Briefly, annealing mixtures contained RNA synthesized in vitro from plasmid pT7HK by T7 RNA polymerase and an oligonucleotide complementary to nucleotides 58 to 73 of the HK022 cIII gene (see Fig. 2). After the ^{32}P-end-labeled oligonucleotide was annealed to the RNA, 1 to 2 μM purified *E. coli* 30S ribosomal subunits (kindly provided by R. Traut) was added to the mixture, followed by formylmethionyl-tRNA (fMet-tRNA) and 0.5 U of reverse transcriptase. The reaction was terminated by the addition of loading dye; the reaction products were analyzed by electrophoresis on a sequencing gel.

Efficiency of plating. Approximately 100 PFU of the λ cIII mutant phage were added to 0.3 ml of overnight cultures of cells harboring the various p*tac*HK*c*III plasmids, to which Tris (pH 7.2) and $MgSO_4$ had been added to 10 mM. After 15 min of adsorption at 30°C, the cells were plated on TB plates and grown overnight at 30°C. The following day, the plaques were counted. The number of plaques on the cells containing the control pKK223-3 vector plasmid, approximately 100, was assigned an arbitrary value of 1.

RESULTS AND DISCUSSION

Functional identification of the HK022 cIII gene. Oberto et al. (18) identified the cIII gene of HK022 by its analogous location to λ cIII on the phage genome and by sequence similarities. A 180-bp DNA fragment carrying this putative cIII gene was cloned in the vector plasmid pKK223-3, yielding p*tac*HK*c*III. The cloned insert is under the control of the IPTG-inducible *tac* promoter. We tested for cIII function by complementation and found that the cloned HK022 cIII gene is able to complement a λ cIII mutant. Both the wild-type and the λ cIII phages as well as HK022 formed very turbid plaques when grown on a strain harboring p*tac*HK*c*III. When IPTG was added to the plate, the effect was stronger: no phage growth was visible at all with the λ phages, and the HK022 plaques were barely visible under these conditions. λ phages carrying mutations in the cI or cII gene, however, escaped this inhibition and formed clear plaques. This observation indicates that the inhibition of growth is not caused directly by CIII but indirectly, through its action on the CII protein. Presumably, in the absence of IPTG, the low level of constitutive transcription from the plasmid-borne *tac* promoter, about 3% of the fully induced

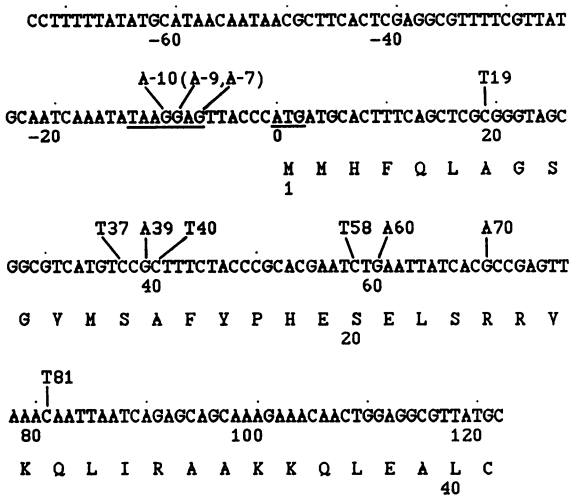

FIG. 2. DNA and derived protein sequence of *c*III, with the various mutations indicated above the wild-type sequence. The depicted sequence includes the whole HK022 DNA fragment (196 bp) that was subcloned in pKK233-3 to yield p*tac*HK*c*III (see Materials and Methods).

level (unpublished observations), yielded sufficient CIII protein to complement the phage *c*III mutation.

Isolation and characterization of mutants carrying the cloned *c*III gene. High levels of *c*III expression grossly impair cell growth, possibly because they lead to an uncontrolled expression of the heat shock genes (4). In the presence of the inducer IPTG, strains carrying the p*tac*HK*c*III plasmid form very small colonies. This phenotype allowed us to isolate mutants defective in *c*III activity.

The plasmid p*tac*HK*c*III was mutagenized in vitro with hydroxylamine and used to transform the *E. coli* host. Transformants that grew well in the presence of IPTG were tested for their ability to complement a λ *c*III mutant. In all the mutant plasmid clones, this ability was greatly reduced, as reflected in the lower turbidity of the phage plaques in the absence of IPTG. Furthermore, on these cells, the inhibition of plaque formation, as reflected in the efficiency of plating, was greatly reduced or completely eliminated (Table 1).

TABLE 2. Mutations in the HK022 *c*III gene[a]

Mutation	Base change	Amino acid change	Class
A(−10)	G→A		A
A(−9)A(−7)	G→A, G→A		A
T19	C→T	Ala-7→Val	B
T37	C→T	Ser-13→Phe	B, C
A39	G→A	Ala-14→Thr	B
T40	C→T	Ala-14→Val	B
T58	C→T	Ser-20→Phe	C
A60	G→A	Glu-21→Lys	C
A70	G→A	Arg-24→His	C
T81	C→T	Gln-28→stop	D

[a] Mutations are designated by their position in the *c*III gene. The mutations were divided into four classes by their effect on gene expression or function and their location in the gene. Class A includes the two mutations that affect the Shine-Dalgarno region, reducing translation; class B includes four mutations that reduce translation, possibly by affecting mRNA structure (see Fig. 4); class C includes three mutations that yield nonfunctional proteins; class D includes the nonsense mutation T81.

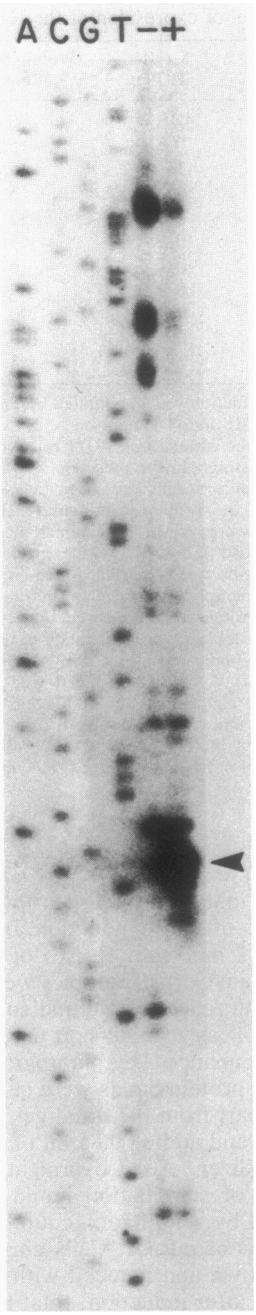

FIG. 3. Toeprint assay on the HK022 *c*III mRNA. The binding reactions were performed in the absence (−) or presence (+) of fMet-tRNA. The dideoxy DNA sequencing reaction was done with the same ^{32}P-5′-end-labeled oligonucleotide used in the toeprint assay. Lanes A, C, G, and T indicate the nucleotides complementary to the sequenced (antisense) strand. The arrow denotes the ribosome-induced termination site found 15 nucleotides downstream of the initiation codon.

The *c*III mutants were analyzed further to determine whether they are defective in transcription, translation, or protein function. The synthesis of CIII protein was assayed by labeling cells carrying the wild-type and mutant p*tac*HK*c*III plasmids after IPTG induction with tritiated proline. Figure 1 shows a protein gel of cells carrying some representative mutants. Of the 10 mutants assayed, six were

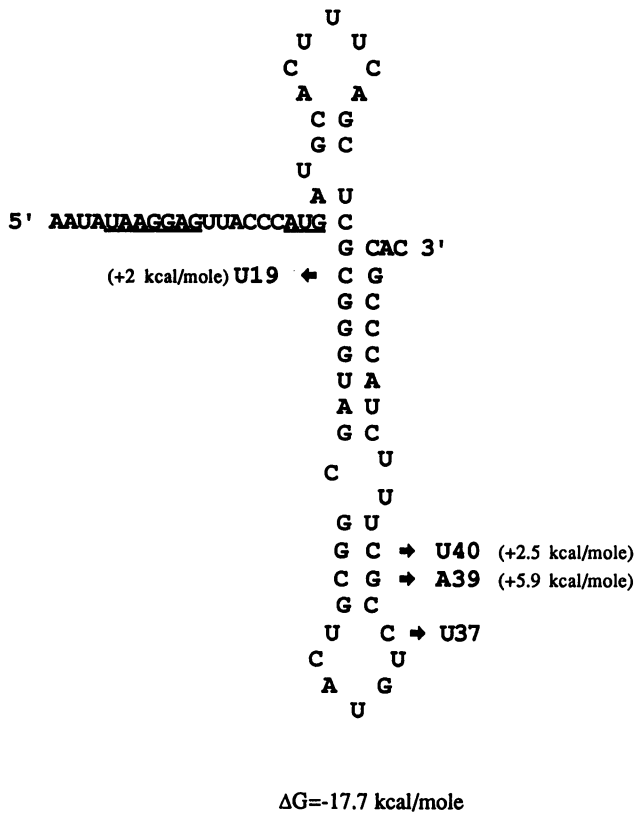

FIG. 4. Predicted structure of the HK022 *c*III mRNA, including 15 nucleotides upstream of the initiation codon and 54 nucleotides of the coding sequence. The predicted stability is indicated below the structure; the predicted change in stability is indicated next to each mutation.

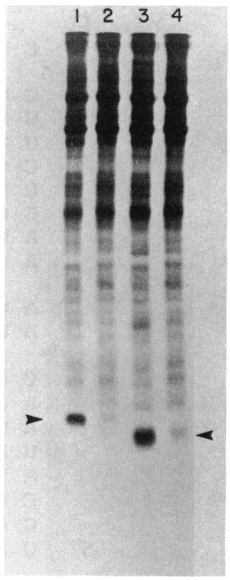

FIG. 5. Labeling of λ CIII (lanes 1 and 2) and HK022 CIII (lanes 3 and 4) in wild-type (lanes 1 and 3) or *rnc* (lanes 2 and 4) cells carrying the p*tac*λ*c*III plasmid and the p*tac*HK*c*III plasmid, respectively. The p*tac*λ*c*III plasmid used here carries a mutation yielding a nonfunctional protein (CIII48), since the *rnc* cells do not accept a wild-type p*tac*λ*c*III plasmid (unpublished observation). Cells were induced for 15 min with IPTG and then pulse-labeled for 2 min with [^{3}H]proline. The arrows indicate the CIII proteins.

clearly defective in CIII synthesis, whereas three displayed levels of CIII similar to the wild-type level. Mutant T37 displayed a moderate reduction in protein levels of approximately two-thirds. These results were quantitated by counting the radioactivity of the gel slices containing the CIII protein (Table 1). The mRNA level of the various mutants was assayed by dot blot hybridization, followed by counting of the radioactivity of the blot. The results (Table 1) show only a mild, two- to threefold reduction in mRNA levels of the mutants defective in CIII protein synthesis. We therefore conclude that these low-expression mutants are affected at some level of the translation process. The small reduction in mRNA levels is probably due to the relative instability of untranslated transcripts in procaryotes (15).

Some of the mutant mRNAs were subjected to primer elongation by reverse transcriptase (mutants T19, T37, A39, and T40). All the mutant RNAs, like the wild-type RNA, were found to extend up to nucleotide −27, indicating that the integrity of the coding region was maintained. This 5′ end was not detected in mRNA extracted from *rnc* mutant cells, indicating that it probably results from an endonucleolytic cleavage by RNase III (see below, Fig. 6).

The *c*III genes of the mutant plasmids were sequenced; all except one were found to carry single point mutations (Fig. 2). The mutations can be divided in four classes by their effect and their location in the gene (Table 2). The first two mutations, A(−10) and A(−9)A(−7), were located in the Shine-Dalgarno region (21). The next four mutations, T19, T37, A39, and T40, were located at the beginning of the coding sequence; they probably affect the initiation of translation. Since these mutations also affect the amino acid sequence of the protein, it is possible that the low levels of CIII protein, as assayed by pulse labeling, are the result of destabilization of the protein. However, we find this possibility unlikely, since this would require the mutant proteins to have a half-life of less than 5 s (for T19, A39, and T40). The third class includes the three mutants T58, A60, and A70 that synthesized nonfunctional proteins. These constitute the first available *c*III mutants expressing an inactive CIII protein. An analysis of these mutant proteins will be presented elsewhere (16a). The fact that mutant T37, although it synthesized a certain amount of CIII protein, still did not efficiently complement a λ *c*III mutant puts it in this class as well. Finally, mutation T81 introduces a termination codon at the 28th codon of the gene.

Determination of the translation start site. The HK022 *c*III gene has two consecutive AUG codons at the beginning of the coding region; each could serve as a translation start site (Fig. 2). Identification of the actual start site is important in understanding how the secondary structure of the mRNA affects the rate of translation initiation (see below). We therefore determined the precise location of a 30S ribosomal subunit bound to the mRNA by using the toeprint or extension inhibition assay (24). In this assay, the binding of a 30S ribosomal particle to the mRNA blocks the extension of a DNA primer by reverse transcriptase. Analysis of the reaction products on a sequencing gel allows determination of the location of the 3′ end of the RNA region covered by the ribosome, which is located 15 nucleotides downstream of the initiating AUG (1, 24). As shown in Fig. 3, according to this assay, the first AUG codon serves as the initiation codon of the *c*III gene.

mRNA structural requirements for translation initiation.

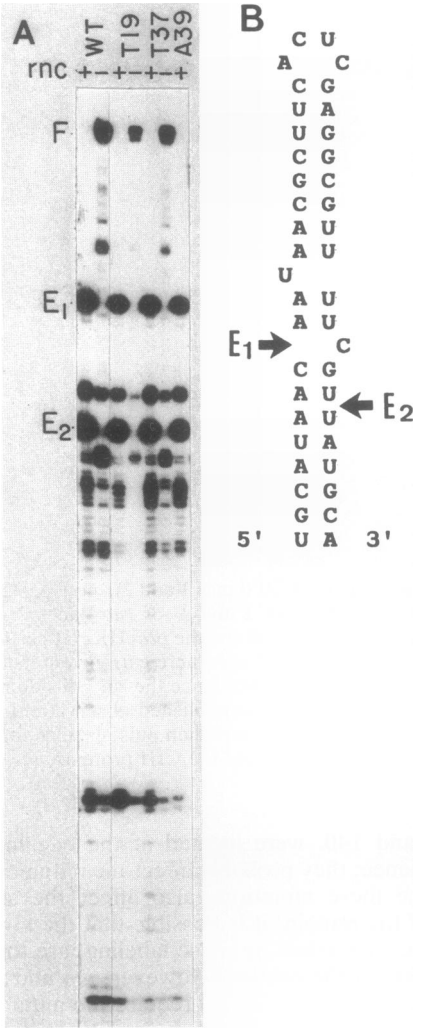

FIG. 6. HK022 *c*III mRNA processing by RNase III. (A) Primer elongation of *c*III mRNA in wild-type (WT) and *rnc* cells. RNA extracted from isogenic *rnc*$^+$ (lanes +) and *rncA14* (lanes —) cells containing wild-type and mutant HK022 *c*III genes was subjected to primer elongation with a *c*III-specific primer. F indicates full-length transcript; E1 and E2 indicate the two RNase III cleavage sites. (B) Secondary structure model of the RNA region cleaved by RNase III, with the cleavage sites indicated (E1 and E2).

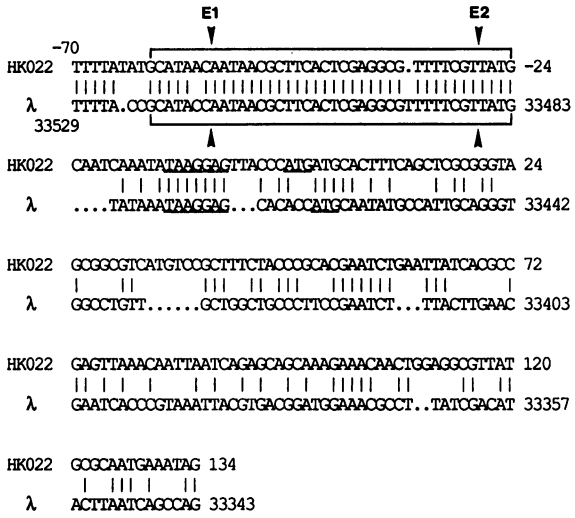

FIG. 7. Comparison of the primary sequences of the HK022 and λ *c*III genes. The Shine-Dalgarno sequence and ATG initiation codon are underlined. The region of strong homology between the two genes, which functions as an RNase III processing site, is boxed. E1 and E2 are the two conserved RNase III cleavage sites; the location of the λ sites presented here is slightly different from that in reference 2 and is presented according to the correction submitted in a separate paper (3a). The sequence coordinates are as in Fig. 2 for HK022 and according to the complete λ genome sequence (8) for λ. The sequences were aligned by computer; the alignment was edited so as to minimize changes that would modify the reading frame. Identical residues are joined by a vertical line; dots indicate gaps introduced for alignment.

The finding that single base changes in the first 40 nucleotides of the coding region can abolish *c*III translation led us to look at the effect of these changes on the predicted secondary structure of the *c*III mRNA. The computer prediction (26) of the wild-type mRNA structure is depicted in Fig. 4. Three of the four mutations affecting *c*III translation, T19, A39, and T40, destabilized this structure. The fourth mutation, T37, is not expected to affect the stability of the mRNA structure. Unlike the other mutations, however, T37 caused only a limited decrease in CIII levels (Table 1). Preliminary mRNA structure probing experiments (in vivo dimethyl sulfate modification; data not shown) support the structure depicted in Fig. 4. Mutations T19 and A39 led to large changes in the mRNA structure, whereas T37 and T40 seemed to induce subtler changes in the mRNA structure (not shown).

HK022 *c*III translation is reduced in a host defective in RNase III. Both HK022 and λ form clear plaques on an *rnc* host. In λ this has been shown to be due to a reduction in *c*III translation, as assayed with a *c*III-*lacZ* gene fusion (2). Here we tested the dependence of *c*III translation on RNase III by direct labeling of the HK022 and λ CIII proteins in an *rnc* host. The *rnc* allele used here is carried in a null mutant, in which the *rnc* gene is disrupted with a mini-Tn*10* transposon (*rncA14* [22]). Both a wild-type host and an isogenic host carrying the *rncA14* allele were transformed with p*tac*HK-*c*III or p*tac*λ*c*III (16), induced with IPTG, and labeled with [^{3}H]proline. The results (Fig. 5) show that CIII expression was reduced at least 10-fold in the *rnc* host. The mRNA levels, however, were reduced only two- to threefold, as assayed by Northern (RNA) blot and dot blot hybridization (not shown). These results support the hypothesis that HK022 forms clear plaques on an *rnc* host owing to a reduction in *c*III translation.

The RNase III dependence of *c*III translation prompted us to test whether the mRNA is processed by this enzyme. The results (Fig. 6) show that the HK022 mRNA was cleaved by RNase III within an upstream stem-and-loop structure. The processing sites were at precisely the same nucleotides as those within a conserved sequence upstream of the λ *c*III gene (Fig. 7). In HK022, however, in contrast to λ, no additional cleavage sites could be detected within the coding region (3a; data not shown).

Translational control of *c*III. The results presented above suggest that a large part of the coding sequence of the *c*III gene of bacteriophage HK022 is involved in its translation initiation and indicate that the *c*III translation is dependent on the presence of RNase III in the cells. These findings are similar to those obtained for the *c*III gene of bacteriophage λ

(1, 2, 16) in spite of extensive sequence dissimilarities between the HK022 and λ cIII coding regions (18) (Fig. 7). It should be noted that, for bacteriophage λ, our recent observations indicate that the conserved RNase III site upstream of the coding region (boxed in Fig. 7) is not required for the RNase III dependence. It appears rather that sequences located within the coding region are responsible for this dependence (3a).

The CIII protein is involved, through stabilization of the CII transcriptional modulator, in the control of the lysogenic pathway. It is known that the physiological state of the host cell at the time of infection partially determines which pathway, lytic or lysogenic, will be chosen (11). The conservation, in two divergent genes, of similar specific translational requirements led us to speculate that these features are of importance in the regulation of *c*III expression and therefore in the control of the frequency of lysogenization. Both binding of specific cellular factors, like RNase III, to the *c*III mRNA and modulation of the mRNA conformation according to the composition of the cytoplasm could influence the rate of *c*III translation, thereby allowing the infecting phage to sense the physiological state of the host cell.

ACKNOWLEDGMENTS

We thank Bob Weisberg for inviting D.K. to his lab, where this research was initiated, and for critical reading of the manuscript. We thank Don Court for sending us *rnc* strains, Jacques Oberto for sending us plasmids, and Ariella Oppenheim for critical reading of this manuscript.

This work was supported by a grant from the National Council for Research and Development, Israel, the Gesellschaft für Biotechnologische Forschung, mbH, Braunschweig, Federal Republic of Germany, and by grant GM38694 from the National Institutes of Health.

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