annealing temperature of 57°C (3′-untranslated) or for 6 cycles at an annealing temperature of 60°C followed by 24 cycles with an annealing temperature of 57°C (exons 4 to 7).
9. The DNA probe and enzyme combinations used in addition to pCMM86 and Hinf I (D17S74) were as follows: pMMZ10 and Msp I (D9S11), and pMCT96.1 and Hinf I (D9S14) [M. Lathrop *et al.*, *Genomics* **3**, 361 (1988)].
10. PCR products were precipitated with ethanol, cleaved with Eco RI and Bam HI, and cloned into pBluescript (Stratagene Cloning Systems). Clones containing wild-type and mutant alleles were distinguished by Msp I digestion of amplified insert. Double-stranded DNA was isolated from two clones each of normal and mutant alleles and sequenced (Sequenase, U.S. Biochemical Corporation). Sequencing primers used were as follows: 5′-TTGAATTCCGGCCACACTCACTAATCG-3′ (bp 3401 to 3419), 5′-TTGAATTCAGACCAAAGGTCGCTACTG-3′ (bp 3473 to 3491), and 5′-TTGGATCCGGATCATTAGATACATGGTGG-3′ (bp 3738 to 3718).
11. A shave biopsy specimen was frozen in liquid nitrogen, and RNA was isolated with a Micro-Fast Track mRNA isolation kit (Invitrogen Corporation, San Diego). The cDNA was prepared with random hexamers and was amplified with oligonucleotides corresponding to the 5′ end of exon 5 [5′-TTGAATTCAGAGGAGCTGAACCGCG-3′ (bp 3225 to 3242)] and to the 3′-untranslated region (bp 4581 to 4564) (7).
12. J. M. Bonifas *et al.*, data not shown.
13. P. Steinert and D. Roop, *Annu. Rev. Biochem.* **57**, 593 (1988).
14. K. Albers and E. Fuchs, *J. Cell Biol.* **108**, 1477 (1989); S. S. M. Chin, P. Macioce, R. K. H. Liem, *ibid.* **111**, 176a (1990).
15. R. Vassar, P. A. Coulombe, L. Degenstein, K. Albers, E. Fuchs, *Cell* **64**, 365 (1991).
16. B. Sykes, D. Ogilvie, P. Wordsworth, J. Anderson, N. Jones, *Lancet* **ii**, 69 (1986).
17. I. Herskowitz, *Nature* **329**, 219 (1987).
18. B. Olaisen and T. Gedde-Dahl, *Hum. Hered.* **23**, 189 (1973); L.-C. Tsui, M. Farrall, H. Donnis-Keller, *Cytogenet. Cell Genet.* **51**, 166 (1989).
19. M. M. Humphries *et al.*, *Genomics* **7**, 377 (1990).
20. Genomic DNA was amplified with primers flanking exons 4 to 7 (*6*) and purified by gel electrophoresis. A 19-bp oligonucleotide primer [5′-GGCAGCGTGGAGGAGCAGC-3′ (bp 3523 to 3541)] was added with either [^{32}P]dCTP deoxycytidine triphosphate or [^{32}P]TTP thymidine triphosphate, extended with Taq polymerase for one cycle, separated by electrophoresis on a denaturing 20% polyacrylamide gel, and analyzed by autoradiography [M. Kuppuswamy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1143 (1991)].
21. P. O'Connell *et al.*, *Genomics* **1**, 93 (1987).
22. WEAV214 was a generous gift of R. Knowlton; all other probes were obtained from the American Type Culture Collection. We thank M. Lee for assistance in calculation of the lod scores; J. W. Bare, C. J. Epstein, and M.-C. King for discussions; M. Bogan and M. Williams for culturing keratinocytes; D. Cox, J. Gitschier, and Y. W. Kan for suggestions on the manuscript; C. McMonigle for preparation of the manuscript; and the Fo and Sc families for their generous donations of samples of blood and skin. Supported in part by USPHS grants R01-AR28069 and R01-AR39953.

29 May 1991; accepted 9 September 1991

Functional Importance of Sequence in the Stem-Loop of a Transcription Terminator

SHEAU-WEI C. CHENG, EILEEN C. LYNCH, KENNETH R. LEASON, DONALD L. COURT, BRUCE A. SHAPIRO, DAVID I. FRIEDMAN*

Intrinsic transcription terminators of prokaryotes are distinguished by a common RNA motif: a stem-loop structure high in guanine and cytosine content, followed by multiple uridine residues. Models explaining intrinsic terminators postulate that the stem-loop sequence is necessary only to form structure. In the tR2 terminator of coliphage λ, single-nucleotide changes reducing potential RNA stem stability eliminated tR2 activity, and a compensatory change that restored the stem structure restored terminator activity. However, multiple changes in the stem sequence that should have either maintained or increased stability reduced terminator activity. These results suggest that the ability of the stem-loop structure to signal transcription termination depends on sequence specificity and secondary structure.

EARLY WORK ON GENE REGULATION focused interest on promoters, the sites of transcription initiation; further work has expanded interest to the study of termination and the generation of the 3′ ends of transcripts (*1*). Two classes of terminators have been defined for *Escherichia coli* (*2, 3*), those that require ρ protein (ρ-dependent) (*4*) and those that do not (intrinsic or ρ-independent) (*5*). The hallmark features of the DNA sequence of an intrinsic terminator are a region of hyphenated dyad symmetry, followed by multiple T residues, yielding an RNA with a stem-loop structure followed by a run of U residues (*6–8*). Lytic growth of coliphage λ requires read-through of multiple transcription terminators, including the tR2 intrinsic terminator (Fig. 1A). To assess the role of the hyphenated dyad symmetry in signaling termination, we systematically changed nucleotides in the λ tR2 terminator (*9*) and measured the in vivo effectiveness of these mutated terminators by using plasmid vector pKL600, designed to quantitate terminator activity (*10*) (Fig. 1B), and λ constructs with mutated tR2 regions, designed to test in situ terminator activity.

We cloned the tR2 terminator into pKL600 on a 434-bp Sau 3AI fragment, generating pKL600tR2$^+$, which places tR2 between the *lac* promoter (P_{lac}) and the *galK* reporter gene. This allows us to use *galK* expression as a measure of terminator activity. Mutations were introduced only in the sequences encoding the stem-loop structure; therefore, differences in *galK* expression yielded a direct measure of the effects on termination of alterations in this region of hyphenated dyad symmetry. A derivative of pKL600tR2$^+$ with an 18-bp deletion that removes most of the stem-loop region (pKL600tR2$^{\Delta 18}$) (Fig. 2) served as the terminator-deficient control; the expression of *galK* from this plasmid was set as the 100% read-through transcription or as the 0% termination base line. On the basis of this comparison, tR2$^+$ allowed 15% read-through transcription.

We next assessed the importance of the RNA stem structure using derivatives of pKL600tR2$^+$ with mutations in tR2 (Fig. 2). Mutants tR2^1 and tR2^7, with single base changes that weakened the stem structure, eliminated terminator activity. A double mutation (tR2^2), which re-created a G:C base pair eliminated in tR2^7, restored terminator activity. Mutations that reduce the number of distal U residues or the strength of the stem-loop structure of an intrinsic terminator reduce termination (*2, 7, 11*). This offers strong evidence that RNA structure plays a role in intrinsic terminator action. Two other mutants, tR2^5 and tR2^6, had compensatory changes in the nucleotide sequence of the stem structure that made the sequences of their stems equivalent to the wild type in number and quality of paired residues. Unexpectedly, tR2^5 and tR2^6 exhibited little terminator activity. This suggests that the secondary structure is not the only characteristic of the tR2 stem sequence that is important for transcription termination. We introduced mutations in the loop of tR2 (tR2^4) to determine if the sequence of the loop influenced termination. Although the number of nucleotides that composed the loop remained the same, the se-

S.-W. C. Cheng, E. C. Lynch, K. R. Leason, D. I. Friedman, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109.
D. L. Court, Molecular Control and Genetics Section, Laboratory of Chromosome Biology, ABL–Basic Research Program, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702.
B. A. Shapiro, Imaging Processing Section, Laboratory of Mathematical Biology, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702.

*To whom correspondence should be addressed.

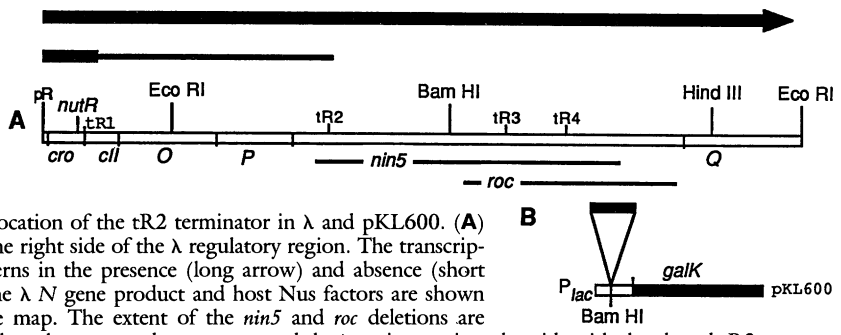

Fig. 1. Location of the tR2 terminator in λ and pKL600. (**A**) Map of the right side of the λ regulatory region. The transcription patterns in the presence (long arrow) and absence (short bar) of the λ *N* gene product and host Nus factors are shown above the map. The extent of the *nin5* and *roc* deletions are shown below the map, and we constructed the λ variants using plasmids with the cloned tR2 mutants (*29*). (**B**) Relevant features of the pKL600 terminator test vector. In addition to P_{lac}, the cloning site, and *galK*, the vector contains the amp^R gene and the replication machinery of pBR322. The amount of *galK* expression reflects the strength of the terminator within the fragment from (A) cloned in the polycloning site (indicated by the wedge). The stronger the terminator, the lower the amount of galactokinase activity (*10*). The tR2 variants were all contained in Sau 3AI fragments that extended from base pairs 40,235 to 40,669 of the standard λ sequence (*27*). We used the $galK^-$ bacterial strain K3443 as the host to generate galactokinase values (*30*).

quence was different. Read-through with $tR2^4$ was 20%, approximately the same as for wild-type tR2.

Lytic growth of λ requires read-through of all terminators in the *nin* region of the λ genome. These include tR2, as well as tR3 and tR4 (*12, 13*) (Fig. 1A). Read-through is mediated by the N antitermination system that acts at the *nutR* site upstream of *nin* to render RNA polymerase termination resistant (*14*). This modification, requiring the phage *N* and host *nus* gene products, produces a polymerase that can extend transcription past the *nin* terminators through the Q gene, whose product turns on essential downstream genes (*15*). Because N modification is defective in hosts that carry a *nus* mutation [such as *nusE71* (*16*)], λ will grow in *nus* mutants only if the function of all three *nin* terminators is eliminated. The λ*nin5* variant has a deletion of all three terminators (*12, 17*) and grows well in the *nusE71* mutant host, whereas λΔ*roc*, with a deletion that removes tR3 and tR4 and leaves tR2 intact, grows poorly in this *nus* mutant (*18*). We examined the in situ effect of tR2 mutations on tR2 terminator activity by replacing wild-type tR2 in λΔ*roc* with mutant forms of tR2 and determining the effect on phage growth in the *nusE71* mutant under conditions normally restrictive for λΔ*roc* growth. We found that all of the mutations that did not cause transcription termination ($tR2^1$, $tR2^5$, $tR2^6$, and $tR2^7$) (Fig. 2), allowed growth in the *nusE71* host under restrictive conditions where λΔ*roc* normally fails to grow. Moreover, those changes in tR2 that caused termination ($tR2^2$ and $tR2^4$) prevented λΔ*roc* from growing in *nusE71*. Thus, all of the tR2 variants functioned in situ as they did in the test vectors.

Models of termination focus on mechanisms for disrupting a hybrid formed between the coding strand of DNA and the 3′ end of the RNA within the RNA polymerase (*2, 6*). The DNA-RNA hybrid in the open DNA duplex (the transcription bubble) has been postulated to extend for 12 nucleotides (nts) [although studies suggest it might be shorter (*19*)]. One accepted model used to explain the action of intrinsic terminators, such as tR2, postulates that formation of the RNA stem-loop structure shortens the RNA-DNA hybrid (*6*), leaving only the run of U residues weakly paired to the coding strand (*20*). This facilitates release of the RNA, reformation of the DNA duplex, and termination of transcription. The effects of the $tR2^1$, $tR2^{\Delta 18}$, $tR2^7$, and $tR2^2$ mutations on RNA stem stability and termination are consistent with such a model. However, because the differences in the calculated free energies between wild-type tR2 and the $tR2^5$ and $tR2^6$ mutants are negligible, the failure of these mutants to terminate is unexplained by this model. Thus, contributing stem-loop stability per se may not be the only role for the nucleotide sequences that make up the tR2 dyad symmetry.

The structures of the eight tR2 sequences were computationally determined by means of an algorithm (*21*) that uses defined energy rules (*22*) and can generate many suboptimal solutions for a given sequence. For each tR2 variant, we analyzed a 130-nt region that included the version of tR2 in question and ~50 nt of upstream and downstream sequence. There was no significant difference in the structure or base-stacking energy of $tR2^+$, $tR2^2$, $tR2^5$, $tR2^6$ or $tR2^4$ ($tR2^4$ has the altered loop); the free energies of these structures varied between −17.7 and −17.9 kcal/mol. Moreover, in no case was an alternative structure favored over the terminator stem. Thus, either subtle differences in stem-loop structure not currently understood influence terminator action or the sequence per se plays a role in the signal to terminate transcription. There are precedents for postulating that both the sequence and structure of a region of dyad symmetry are important for single-stranded nucleic acid signals. One example is the φX174 origin of replication, which is required for conversion of single-stranded φX174 DNA to duplex replicative form. This origin of replication contains a region of dyad symmetry that can potentially form a stem-loop structure but is only active in one of its two possible orientations (*23*). A second exam-

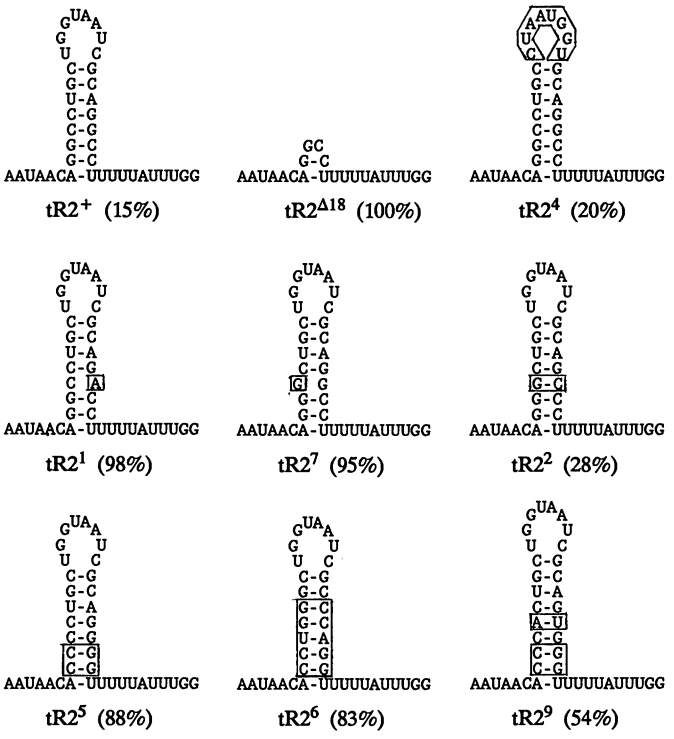

Fig. 2. RNA sequences of wild-type and mutant tR2 constructs with potential stem-loop structures displayed as duplexes. The $tR2^1$ mutation was genetically selected, whereas all of the other mutations were generated by site-directed mutagenesis (*28*). Boxed nucleotides denote changes from wild type. Numbers in parentheses represent read-through of the cloned fragments in pKL600, as measured by the galactokinase assay, relative to the read-through observed with $pKL600tR2^{\Delta 18}$. In a representative experiment, pKL600 expressed 464 units of galactokinase activity, whereas $pKL600tR2^{\Delta 18}$ expressed 404 units. A measurement of plasmid copy number revealed no significant differences between the pKL600 variants.

ple is a region of dyad symmetry that serves as substrate for *E. coli* ribonuclease III. Both the sequence and the structure of the target RNA appear to be important for enzyme action (*24*).

To further assess the role of stem structure in tR2 terminator activity, we constructed $tR2^9$, a variant of $tR2^5$ that has additional A and U nucleotides positioned so that they pair in the stem. The calculated free energy of this new stem structure, −18.7 kcal/mol, is less than that of the wild-type structure, −17.9 kcal/mol. Using the pKL600 test vector, we found that $tR2^9$ allowed 54% read-through. This indicates that although $tR2^9$ has a stronger stem structure than the wild-type tR2, it exhibits significantly less terminator activity and that the small increase in termination over that observed with $tR2^5$ presumably reflects the increase in stem stability.

The simple model of intrinsic terminator function based on destabilization of the RNA-DNA hybrid by an RNA stem-loop and a run of U residues has been challenged. Sequences both upstream and downstream of the stem-loop and run of U residues influence the action of intrinsic terminators (*25*). Algorithms derived from analysis of 148 proven and suggested terminator sequences, as well as similar sequences without terminator activity, suggest that some characteristic of the terminator in addition to its ability to form a stem-loop structure is necessary for efficient termination (*8*). A formal representation based on contributing factors that predicts efficiency of known intrinsic terminators indicates that terminator efficiency may be affected by proteins and other environmental factors (*26*).

Because sequences surrounding tR2 were not changed, our in vivo studies directly assess the effect on termination of variations in the region of dyad symmetry of tR2. The importance of structure is shown by the fact that termination can be restored by a compensating mutation that restores the dyad symmetry with a mutant sequence. Our studies also show, however, that not all mutations can be so compensated even if this stem structure is strengthened, suggesting that the sequence per se might also be important. We offer two plausible scenarios, both based on recognition by RNA polymerase, for action of a sequence-specific element. The signal could be a double-stranded sequence in the RNA stem, or it could be a sequence in one of the single strands of DNA formed by the open complex in the region of the hyphenated dyad symmetry. One function of the latter signal might be to influence the binding of RNA polymerase to the nontemplate strand in the open complex. Accordingly, more tightly bound sequences might be more effective at holding the DNA of the open complex apart, favoring elongation, whereas weaker polymerase interactions might allow renaturation of the DNA duplex, especially at stems in the RNA, favoring termination.

Terminator strength likely reflects the sum of the contributions of each of the components (*26*). One explanation for the sensitivity of tR2 to changes in stem sequence may be that the sum of the contributions of its components is just barely tilted toward termination and that a slight change in one of the elements could be sufficient to tilt activity away from termination. A similar change in a stronger terminator might not eliminate activity. Although we cannot rule out stem stability as the only role for the hyphenated dyad symmetry, our results suggest that models of termination based only on that idea warrant re-examination.

REFERENCES AND NOTES

1. D. I. Friedman, M. J. Imperiale, S. Adhya, *Annu. Rev. Genet.* **21**, 453 (1987); C. A. Spencer and M. Groudine, *Oncogene* **5**, 777 (1990); N. J. Proudfoot, *Trends Biochem. Sci.* **14**, 105 (1989).
2. T. Platt, *Annu. Rev. Biochem.* **55**, 339 (1986).
3. M. Rosenberg and D. Court, *Annu. Rev. Genet.* **13**, 319 (1979).
4. J. W. Roberts, *Nature* **224**, 1168 (1969); J. P. Richardson, *Cell* **64**, 1047 (1991).
5. R. Reynolds, R. M. Bermudez-Cruz, M. J. Chamberlin, *J. Mol. Biol.*, in press.
6. T. D. Yager and P. H. von Hippel, in Escherichia coli *and* Salmonella typhimurium; *Cellular and Molecular Biology*, F. C. Neidhardt *et al.*, Eds. (American Society for Microbiology, Washington, DC, 1987), pp. 1241–1275.
7. R. Landick and C. Yanofsky, in *ibid.*, pp. 1276–1301.
8. Y. d'A. Carafa, E. Brody, C. Thermes, *J. Mol. Biol.* **216**, 835 (1991).
9. M. Kroger and G. Hobom, *Gene* **20**, 25 (1982); M. C. Schmidt and M. J. Chamberlin, *J. Mol. Biol.* **195**, 809 (1987); J. Greenblatt, M. McLimont, S. Hanly, *Nature* **292**, 215 (1981).
10. K. McKenney *et al.*, in *Gene Amplification and Analysis*, J. G. Chirikjian and T. S. Papas, Eds. (Elsevier/North-Holland, Amsterdam, 1981), pp. 383–415.
11. S. P. Lynn, C. E. Bauer, K. Chapman, J. F. Gardner, *J. Mol. Biol.* **183**, 529 (1985); S. P. Lynn, L. M. Kasper, J. F. Gardner, *J. Biol. Chem.* **263**, 472 (1988); H. M. Johnston and J. R. Roth, *J. Mol. Biol.* **145**, 725 (1981); C. Yanofsky, *Nature* **289**, 751 (1981).
12. D. L. Court and K. Sato, *Virology* **39**, 348 (1969).
13. N. Costantino, M. Zuber, D. Court, *J. Bacteriol.* **172**, 4610 (1990); S.-W. C. Cheng, D. L. Court, D. I. Friedman, unpublished data showing two regions of ρ-dependent termination in the *roc* region of λ.
14. J. S. Salstrom and W. Szybalski, *J. Mol. Biol.* **124**, 195 (1978).
15. D. I. Friedman, in *The Bacteriophage*, R. Calendar, Ed. (Plenum, New York, 1988), pp. 263–319; J. W. Roberts, *Cell* **52**, 5 (1988).
16. D. I. Friedman, A. T. Schauer, M. R. Baumann, L. S. Baron, S. L. Adhya, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1115 (1981).
17. M. Fiandt, Z. Hradecna, H. A. Lozeron, W. Szybalski, in *The Bacteriophage Lambda*, A. D. Hershey, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1971), pp. 329–354.
18. K. R. Leason and D. I. Friedman, *J. Bacteriol.* **170**, 5051 (1988).
19. G. Rice, C. Kane, M. J. Chamberlin, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4245 (1991).
20. F. H. Martin and I. Tinoco, Jr., *Nucleic Acids Res.* **8**, 2295 (1980).
21. M. Zuker, *Science* **244**, 48 (1989).
22. S. M. Freier *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9373 (1986).
23. D. S. Ray *et al.*, in *The Initiation of DNA Replication*, D. S. Ray, Ed. (Academic Press, New York, 1981), pp. 169–193.
24. L. Krinke and D. L. Wulff, *Nucleic Acids Res.* **8**, 4809 (1990).
25. R. Reynolds and M. J. Chamberlin, *J. Mol. Biol.*, in press; J. A. Goliger, X. Yang, H. C. Guo, J. W. Roberts, *ibid.* **205**, 331 (1989); A. P. W. Telesnitsky and M. J. Chamberlin, *ibid.*, p. 315.
26. P. H. von Hippel and T. D. Yager, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2307 (1991).
27. D. L. Daniels, J. L. Schroder, W. Szybalski, F. Sanger, F. R. Blattner, in *Lambda II*, R. W. Hendrix, J. W. Roberts, F. W. Stahl, R. A. Weisberg, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1983), pp. 469–517.
28. We constructed pLR31Δ*roc* by inserting the Bam HI–Eco RI fragment from λΔ*roc* (nucleotides 41,732 to 44,972) into pBR325. [Numbers represent nucleotide positions according to the standard nucleotide sequence of λ (*27*)]. Site-directed mutagenesis on an M13mp18 derivative carrying the wild-type tR2 region on a Sau 3AI fragment that extends from base pairs 40,235 to 40,669 of λ ($pKL600tR2^+$) was essentially as described [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. Plasmids $pKL600tR2^2$, $pKL600tR2^4$, $pKL600tR2^5$, $pKL600tR2^6$, and $pKL600tR2^9$ were constructed by ligation of the small Sst I–Xba I fragments from the appropriate M13mp18tR2 derivatives into Sst I–Xba I digested pKL600. Plasmid $pKL600tR2^{\Delta 18}$ was generated by complete Stu I digestion and religation of $pKL600tR2^+$. The resulting 18-bp deletion was confirmed by DNA sequencing [M. D. Biggin, T. J. Gibson, G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983)]. We constructed $pKL600tR2^7$ by cloning a synthetic oligonucleotide with the altered sequence into the Stu I site of $pKL600tR2^{\Delta 18}$. We constructed pBR325 derivatives containing regions with tR2 mutations ($tR2^2$ and $tR2^4$) using the Eco RI–Bam HI fragment (nucleotides 39,168 to 41,732) from M13 derivatives with a cloned *nin* region, yielding pST4 and pST6, respectively.
29. The characteristics of plasmids used in the construction of these λ derivatives are listed in (*28*). $\lambda tR2^1\Delta roc$ was selected from λΔ*roc* as a spontaneous mutant that grows in the *nusE71* host at 42°C. $\lambda tR2^{\Delta 18}\Delta roc$ was obtained by homologous recombination between λΔ*roc* and $pKL600tR2^{\Delta 18}$. We constructed $\lambda tR2^2$ and $\lambda tR2^4$ using $\lambda tR2^{\Delta 18}$ *Pam3* to rescue the tR2 mutations $tR2^2$ and $tR2^4$ by selecting for the linked P^+ allele from plasmids pST4 and pST6, and appropriate clones were identified by plaque hybridization with DNA probes. We constructed $\lambda tR2^2\Delta roc$ and $\lambda tR2^4\Delta roc$ by crossing Δ*roc* into $\lambda tR2^2$ and $\lambda tR2^4$ from plasmid pLR31Δ*roc*. We obtained $\lambda tR2^5\Delta roc$, $\lambda tR2^6\Delta roc$, and $\lambda tR2^7\Delta roc$ by a similar marker rescue using λΔ*roc* and $pKL600tR2^5$, $pKL600tR2^6$, and $pKL600tR2^7$, respectively.
30. S. Adhya and W. Miller, *Nature* **279**, 492 (1979).

31. We thank J. Kilpatrick, C. Thompson, D. Engelke, and S. Brown for helpful suggestions on the manuscript; and R. Weisberg, M. Savageau, and A. Granston for helpful discussion. Supported by NIH grant AI1459-10 (to D.I.F.) and a contract from the National Cancer Institute (NO1-CO-74101) with Bionetics Research. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the United States government.

20 June 1991; accepted 15 August 1991