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Mutations reducing the activity of *c*17, a promoter of phage λ formed by a tandem duplication

(transcription/Pribnow Box/nucleotide sequence)

MARK A. MOZOLA*, DAVID I. FRIEDMAN*, CAROL L. CRAWFORD†, DANIEL L. WULFF†, HIROYUKI SHIMATAKE‡, AND MARTIN ROSENBERG‡

*Department of Microbiology, The University of Michigan, Ann Arbor, Michigan 48109; †Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717; and ‡Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **We report the isolation of four independently selected mutations (*scs*) in the *c*17 promoter of phage λ that reduce or eliminate the promoter activity. The *c*17 promoter is not normally present in λ, and has been shown to be generated by a tandem duplication which creates a "Pribnow Box," a heptamer sequence implicated in promoter activity. This sequence is located upstream from the site of transcription initiation and is present, with some variation, in all promoters whose sequences have been determined. Analysis of the *c*17 duplications carrying the *scs* mutations reveals that three of these mutants carry single base-pair changes in the most highly conserved base pairs of the Pribnow Box and that the other mutation is a reversion to the wild-type sequence in this region (i.e., a loss of the duplicated base pairs).**

Transcription of genes originates at promoters where RNA polymerase molecules form stable complexes with the DNA to begin the process of RNA synthesis (1). The formation of these complexes protects about 40 base pairs of DNA from digestion by DNase I, including the initiation point for mRNA synthesis (2). DNA sequence analysis of the protected promoter fragments from several sources (2–15) revealed a common sequence of seven base pairs that is located five to six base pairs upstream from the initiation site for transcription. This heptamer sequence, described by Pribnow (7, 8) to be of the general form

5′ T-A-T-Pu-A-T-G 3′

3′ A-T-A-Py-T-A-C 5′,

is referred to as a "Pribnow Box" and has been implicated as a critical binding sequence in the formation of the stable promoter–RNA polymerase complex (7–9). It is probably significant that this heptamer sequence is A·T-rich because the formation of such transcription complexes involves a partial unwinding or "melting out" of base pairs (16).

The *c*17 promoter, found in one type of virulent mutant of bacteriophage λ (17), maps within Y (Fig. 1), a region necessary for the establishment of repressor synthesis (18, 19). Sequence analysis suggests that the *c*17 promoter arose by an exact tandem duplication of nine base pairs in the Y region, between the transcription termination site t_{R1} and the *c*II gene (20). The promoter activity of the *c*17 mutation apparently results in part from the formation of a Pribnow Box created by the tandem duplication (20, 21). The nucleotide sequence of the *c*17 region is shown in Fig. 2.

For wild-type λ, early transcription of lytic functions is regulated from two promoters, P_R and P_L. These promoters are under the negative control of the λ repressor. Initial transcription from these promoters proceeds short distances through the *cro* gene on the right and the *N* gene on the left, terminating, respectively, at t_{R1} and t_{L1} (Fig. 1). In the presence of N function, the transcription proceeds through these and other termination barriers; e.g., t_{R2} (22, 23).

When the *c*17 promoter is present, initiation of transcription occurs distal to t_{R1}, and thus transcription of the *c*II-*O*-*P* operon is no longer under repressor control nor is it dependent on N function. This means that replication of the phage is constitutive, not turned off by repressor (17).

Although *c*17 permits N-independent expression of *c*II, *O*, and *P*, λ*c*17 derivatives fail to grow in a mutant *Escherichia coli* host, *nusA*, under conditions where wild-type λ grows. The *nusA* host reduces N activity (24). The paradoxical finding that a mutation conferring partial N-independence results in poor growth under conditions of reduced N activity where totally N-dependent phage can grow led to the proposal that transcription initiating only from specific promoters (P_R and P_L) could be modified by the N protein (25). According to this model, transcription initiating at *c*17 is not N-modified and thus cannot transcend the next termination signal, t_{R2}. Thus, λ*c*17 grows poorly in the *nusA* host, because under conditions of reduced N activity there are not enough N-modified transcription complexes initiating at P_R to override the inhibitory effect of the unmodified complexes initiating at *c*17. Consistent with this idea is the fact that recent studies have implicated a site for N-modification (nut_R) downstream from P_R and upstream from *c*17 (refs. 20 and 21; E. Flamm, personal communication).

It was postulated that variants of λ*c*17 with a nonfunctional *c*17 promoter should grow in the *nusA* host under conditions where λ itself grows. We report the isolation of four mutants of λ*c*17 selected for the ability to grow in the *nusA* host, all of which show alterations in the *c*17 promoter. These mutations are called *scs* for suppressor of *c*17.

MATERIALS AND METHODS

Media. Tryptone media used have been described (26).

Strains. All bacterial strains used are derivatives of *E. coli* K-12. K95 is a *nusA-1* derivative (24). λ*c*17 is described in the text. Bacteria and phage were cultivated and handled in the standard manner (26).

Nucleotide Sequence. Direct DNA sequence analysis was carried out by the chemical procedures described by Maxam and Gilbert (27). For each *scs* mutant, the sequencing methods were applied to a DNA restriction fragment identical to that used previously for sequence analysis of the λ*c*17 promoter region (20). All other procedures were as described (20, 21).

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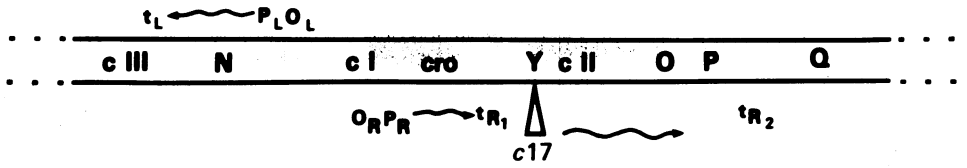

FIG. 1. Genetic map of the "early" region of λ, showing the location of the *Y* region and the *c*17 duplication. The arrows mark the origin and direction of transcription from the various promoters.

RESULTS AND DISCUSSION

Isolation of *scs* Mutants. Two variations of the same approach were used in isolating *scs* mutants, the only difference being that two different variants of λ*c*17 were used and in one case mutagenesis was used prior to selection. The mutants were isolated as phage capable of forming plaques on the *nusA* host, K95, at 30°C.

The mutations *scs*10 and *scs*15 were isolated spontaneously from λ*c*I$^+$*c*17, a phage capable of expressing the *c*I gene product (the repressor). These two mutants represent two phenotypically different classes of variants of λ*c*I$^+$*c*17 able to grow on *nusA* that were obtained at essentially the same frequency (10^{-6}). Members of the class represented by *scs*10 form plaques with lightly turbid centers, intermediate between the clear-plaque phenotype of λ*c*17 and the turbid phenotype of wild-type λ (λ$^+$). Mutants of the second class, represented by *scs*15, have a plaque morphology indistinguishable from that of λ$^+$.

The mutations *scs*20 and *scs*30 were isolated from λ*c*I$^-$*c*17, a variant unable to express λ repressor. Mutagenesis was accomplished by growing phage stocks in the *E. coli* strain *mutD5* (28). The two mutations arose from separate events since the mutants were isolated from two separate stocks grown from two different single plaques. Preliminary mapping studies (unpublished data) placed all four mutations in the region of the *c*17 duplication.

Characteristics of *scs* Mutants. The fact that the λ*c*17*scs* mutants were able to plate on *nusA* at 30°C was taken as presumptive evidence that the *c*17 promoter was inactive. For *scs*20 and *scs*30, an additional measure of the loss of the *c*17 promoter activity was available. When the *c*17 mutation is combined in *cis* with a *c*I$^-$ mutation, the phage becomes virulent (able to grow in a λ lysogen) because the many copies of the phage genome produced titrate the repressor produced by the prophage. This occurs only in hosts lysogenic for a single copy of λ. In hosts with multiple copies of λ, the concentration of repressor is too high to be titrated by the replicated λ*c*I$^-$*c*17 and the phage cannot form a plaque (17). Since *scs*20 and *scs*30 were isolated from a phage that carried a *c*I$^-$ mutation, the parental phage was virulent. If the *c*17 promoter is rendered inactive, the λ*c*17 derivative should lose its virulence. We find that both λ*c*I$^-$*c*17*scs*20 and λ*c*I$^-$*c*17*scs*30 are no longer virulent. Virulent derivatives of these *scs* mutants can be isolated at a frequency in excess of 1 in 10^7, a frequency far greater than that with which virulent mutants are usually obtained from a stock of λ (29). These virulent derivatives of the *scs* mutants are of the *c*17 type; they plate on single, but not on multiple, lysogens of λ. This suggests that these two *scs* mutants still carry a substantial portion of the *c*17 duplication. Moreover, we find that the virulent derivatives now fail to plate on the *nusA* host, confirming our assumption that they are authentic *c*17 virulents.

The fact that *scs*10 shows a plaque morphology unlike that of λ$^+$ suggested that this mutant, like *scs*20 and *scs*30, may still contain a substantial portion of the *c*17 duplication. The partially clear phenotype of *scs*10 could be due either to the physical presence of a portion of the *c*17 duplication or residual promoter activity from this region. It seemed likely that *scs*15, with a phenotype like that of λ$^+$, may be a revertant to wild-type λ. These conclusions on the nature of the various *scs* mutations were subsequently confirmed by direct base-sequence analysis.

Nucleotide Sequence Analysis of *scs* Mutants. The *c*17 region of the chromosomes of the four λ*c*17*scs* mutants were subjected to DNA base-sequence analysis. Three of these mutants, *scs*10, *scs*20, and *scs*30, carry single base-pair changes in the Pribnow Box generated by the *c*17 duplication. For the *scs*10 mutant isolated from λ*c*I$^+$*c*17 in the absence of any mutagenic agent, the resultant change is a T·A to A·T transversion in the sixth position of the *c*17 Pribnow Box (residue −7, Fig. 2). Both of the independently selected mutants of λ*c*I$^-$*c*17 isolated from mutagenized stocks (*scs*20 and *scs*30) also carry single base-pair changes in the Pribnow Box. These two mutants have an identical base-pair change, an A·T to G·C transition at the second position of the *c*17 Pribnow Box (residue −11, Fig. 2). Analysis of the *scs*15 mutation shows a loss of the nine duplicated base pairs of the *c*17 mutation and thus a reversion to the wild-type sequence of this region. Representative autoradiographs of sequencing gels for the *scs*10 and *scs*20 mutants are shown in Fig. 3.

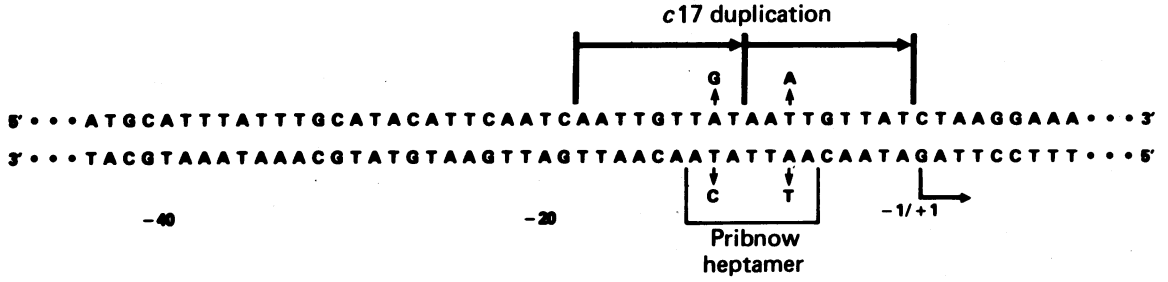

FIG. 2. DNA sequence of the *c*17 duplication and the surrounding region. The location of the "Pribnow" heptamer sequence implicated in promoter activity is marked. The base changes resulting from the *scs* point mutations are shown. *scs*10 is a T·A to A·T change at position −7 (sixth position within the heptamer) and *scs*20 and *scs*30 are A·T to G·C changes at position −11 (second position within the heptamer). Residue positions are numbered from the startpoint of transcription (+1), which is located at the designated site six nucleotides from the heptamer sequence (−12 to −6) (21).

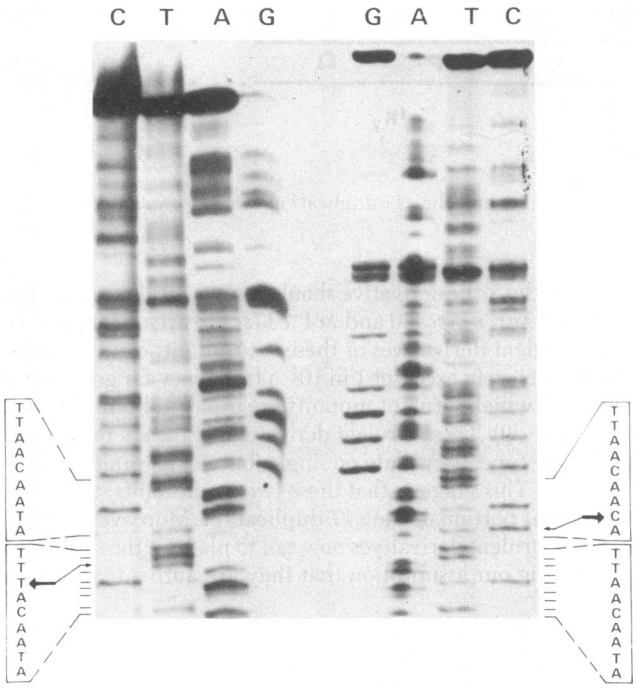

FIG. 3. Representative autoradiographs of DNA sequencing gels displaying the results of the chemical sequencing methods (27) as applied to the appropriate DNA restriction fragment obtained from two of the *scs* mutants. A complete description of the isolation, characterization, and labeling of the particular DNA restriction fragment used for sequence analysis of the c17 promoter region has been reported (20). (*Left*) Restriction fragment obtained from the *scs*10 mutant; the first discernible residue is an A, 57 nucleotides from the labeled end (position −1, Fig. 2). (*Right*) Identical fragment obtained from the *scs*20 mutant; the first discernible residue is an A, 57 nucleotides from the labeled end (position −1, Fig. 2). For comparison of the sequencing gels of these mutants with the original sequence determination of λc17, see figure 5 of ref. 20.

CONCLUSIONS

The procedure for selection and the subsequent demonstration of loss of virulence (for *scs*20 and *scs*30) are taken as evidence that the *scs* mutations eliminate or reduce the activity of the promoter generated by the c17 duplication. Biochemical studies support these conclusions. *In vitro* transcription and RNA polymerase binding assays show that c17 promoter activity is reduced more than 80% by the three *scs* point mutations (unpublished data).

The demonstration that three of these mutations are single base-pair changes in the c17 Pribnow Box clearly shows a role for this sequence in the transcription process. In the *scs* mutations, decreased promoter activity correlates with a reduction in similarity of the heptamer sequence to that of the general Pribnow Box sequence. Comparison of the known Pribnow Box sequences shows (2–15) that the *scs* mutations are located in the most highly conserved sequences of the heptamer; there is always an A·T base pair (A in the nontemplate strand) at position 2 and a T·A base pair (T in the nontemplate strand) (Fig. 2) at position 6. The nucleotide composition at these two positions obviously must be crucial to the proper functioning of the heptamer sequence in the λc17 promoter and presumably in all promoters. Consistent with this is the demonstration by Musso *et al.* (15) that an A·T to T·A transversion at position 2 of the Pribnow Box in the P1 promoter of the *E. coli gal* operon eliminates the activity of this promoter. In addition, Post *et al.* (30) have recently reported that a T·A to C·G change at position 6 of the Pribnow Box of the promoter of the *E. coli str* ribosomal protein operon abolishes the activity of this promoter.

Two obvious explanations for the effect of mutations that reduce promoter activity are that (*i*) the specific sequence for RNA polymerase recognition or binding or both is altered or (*ii*) the ability of the heptamer to "melt out" is reduced. The fact that the specific orientation (relative to nontemplate and template strands) of the A·T and T·A base pairs at positions 2 and 6 of the heptamer sequence are so stringently conserved, coupled with the observed T·A to A·T change in the *scs*10 mutant, strongly suggests that these two base pairs are important for polymerase recognition or binding or both. However, both *scs*20 and *scs*30 result in a substitution of a G·C base pair for an A·T base pair, which should cause a reduction of melting. It is unlikely that reduced melting is of primary importance, though, in light of the observation of Musso *et al.* (15) that an A·T to T·A change at this position also reduces the activity of the *gal* promoter.

The observation that the two classes of mutants derived from λcI$^+$c17 (represented by *scs*10 and *scs*15) were obtained at a similar frequency suggests that the rate of reversion to the wild-type sequence in the c17 region (loss of the nine duplicated base pairs) and the rate of single base-pair mutations in this region may be comparable. Although we have no evidence as to the molecular mechanism generating the loss of c17, we suggest that this small duplication could be lost by Rec-promoted recombination, a mechanism that operates in the loss of larger duplications (31).

In summary, we have demonstrated that single base-pair mutations in conserved regions of a heptamer sequence located upstream from the site of transcription initiation from the λc17 promoter reduce or eliminate the promoter activity associated with this sequence. We have also shown that the nine base pairs duplicated in the c17 mutation can be exactly deleted, resulting in a phage indistinguishable phenotypically and by base sequence in the c17 region from wild-type λ.

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