Enzymatic Construction and Selection of Bacteriophage G4 Mutants with Modifications of a DNA Secondary Structure in the J-F Intercistronic Region

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The J-F intercistronic region of bacteriophage G4 has the potential to form a perfectly base-paired hairpin structure, thought to act as a terminator of transcription. To investigate this proposed structure-function relationship, viable mutants were constructed by site-specific mutagenesis with small deletions of 2 to 4 base pairs in the center of the corresponding palindromic sequence. These sequence modifications had a small positive effect on the growth efficiency of the phage. The approach of biochemical rather than biological selection of these mutant phages is generally applicable to the construction of virus and plasmid vectors.

Formation of secondary structures (hairpins or cruciforms) has been proposed to be one of the means by which DNA sequences communicate with each other or with other macromolecules in the cell (reviewed in reference 21). Although experimental evidence for the physical existence of these structures has recently become available (10, 11, 16), correlation with known biological functions has been mostly speculative (21). One of the predominant problems associated with the study of DNA structure-function relationships is our ignorance as to which of the almost infinite number of potential hairpins contained in even a small genome may be biologically relevant. Müller and Fitch have recently demonstrated that the potential to form perfectly base-paired hairpins (no interruptions, no G-T pairing) with small loops and stem length of at least 6 base pairs (bp) is nonrandomly distributed in the genomes of several DNA viruses which infect bacteria, plants, and animals (12, 13). Hairpins are generally overrepresented in the intercistronic regions and frequently coincide with significant regulatory sequences. Hence, it appears that perfect hairpin structures may be biologically significant.

I used the closely related single-stranded DNA phages ϕ X174 and G4 as models to study this hypothetical structure-function relationship. Their genetic maps are virtually identical, and despite considerable sequence variation, their intercistronic regions contain similar perfect hairpin structures (5). The hairpin located between genes J and F has been proposed to act as a terminator of transcription in both phages (Fig. 1) (2-4, 8). It has been shown previously that this structure is not essential for the survival of

ϕ X174 phage (14, 15), although it appears to be involved in expression of the neighboring genes (see accompanying papers). The analogous structure in the G4 genome is slightly larger (5), although possibly less stable (20). To test the effect of hairpin size, stability, or both on its putative biological activity, bacteriophage G4 mutants were constructed with modifications of this hairpin structure. Reported here is the technique for site-specific mutagenesis and an approach to the chemical rather than biological selection of viable mutant genomes. The effect of such mutations on the overall growth efficiency of the phage was assessed.

MATERIALS AND METHODS

Bacterial and phage strains. Bacteriophage G4 was originally obtained from N. Godson (Yale University, New Haven, Conn.), and the host *Escherichia coli* C was obtained from R. D. Wells (University of Alabama, Birmingham).

Enzymes. All restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories. Enzymatic reactions were performed under the conditions suggested by the manufacturer. Snake venom phosphodiesterase was from Worthington Diagnostics.

Growth of phage. Plaque-purified phages were prepared by cutting a well-isolated plaque from a tryptone-yeast extract (TYE) plate (14) with a sterile Pasteur pipette and transferring it to a 1-ml *E. coli* C culture grown to an optical density of 0.2 absorbance units at 550 nm (A_{550}) in TYE broth (14). Shortly before addition of phage, the cultures were made 1 mM in CaCl_2 . Lysis generally occurred after shaking for 40 min at 37°C. *E. coli* C (5 ml) in TYE (1 mM CaCl_2 ; A_{550} = 0.5) was then added and incubation continued until cultures cleared. These starter lysates were used for production of higher titer phage stocks

or production of replicative form (RF) DNA by the "miniprep" procedure as described below.

High-titer lysates. Cultures (20 ml) of *E. coli* C (TYE; $A_{550} = 0.5$) received CaCl_2 (1 mM) and 1 min later were infected with 6 ml of starter lysates. The infected cultures were incubated at 37°C until lysis and then mixed with 1 ml of chloroform and titers were determined. These lysates provided enough phage for production of high-titer lysates by the delayed lysis procedure described below (provided by N. Godson). A 1-liter *E. coli* C culture was grown in TPG minimal medium (19) containing 10% sucrose at 37°C to a density of 2×10^8 to 5×10^8 cells per ml. Phage was added at a multiplicity of infection of 10, and incubation was continued for at least 4 h or overnight. The incompletely lysed culture was then mixed with 112 g of polyethylene glycol 6000 and 21 g of NaCl and chilled on ice for at least 1 h. The precipitate was spun down, resuspended as well as possible in 1 ml of 50 mM tetraborate (pH not adjusted), and centrifuged again. This step was repeated three times, and the supernatants were kept separate. The highest titer was generally found in the second and third supernatant.

Production of RF DNA in minipreps. A 6-ml starter lysate was added to 20 ml of *E. coli* C culture (TYE; $A_{550} = 0.5$) after addition of CaCl_2 (1 mM). At 6 to 8 min after infection, protein synthesis was stopped with chloramphenicol (100 $\mu\text{g}/\text{ml}$), and incubation was continued at 37°C for 90 min. Cells were then pelleted and RF DNA was prepared according to the miniprep procedure described by Klein et al. (9) for preparation of plasmid DNA. The only modification was an additional phenol extraction step. RF DNA obtained in this rapid small-scale purification procedure generally weighed about 10 μg total, with small variations from sample to sample, and could be digested with all restriction enzymes used in this work. After completion of the restriction digest, 1/4 volume of bromophenol blue solution (1) containing RNase A (500 $\mu\text{g}/\text{ml}$) was added and samples were incubated for 30 min (37°C) before loading onto the gel. RNase A was heated (100°C) for 10 min before use.

Competitive growth experiment. An actively growing culture of *E. coli* C in TYE broth (5×10^7 to 8×10^7 cells per ml) was mixed with CaCl_2 (1 mM). Portions (10 ml) were infected with either wild-type (wt) G4 or a mutant strain (multiplicity of infection of 10) and incubated for 5 min at 37°C . Cells were then pelleted and resuspended in 10 ml of precooled 50 mM sodium tetraborate (0°C , pH not adjusted). Portions were later plated for survivors and titers were determined for infective centers to confirm all calculations. Portions (0.1 ml) of each culture were then mixed in various ratios (two phage types per mixture) and, based on the assumption that all cells were infected, diluted in 50 mM sodium tetraborate (pH not adjusted) to 5×10^2 infective centers per ml. One milliliter of each mixture was added to 10 ml of *E. coli* C culture (TYE; $A_{550} = 0.2$), and after incubation for 2 h, 20 ml of TYE medium was added. Cultures cleared completely after approximately 45 min and were then mixed with 1 ml of chloroform and titers were determined. This growth cycle was repeated with 5×10^2 PFUs of each lysate. Titters for the second lysates were also determined, and portions were then used to prepare RF DNA by the miniprep procedure.

Other methods. Production of purified RF DNA,

transfection of CaCl_2 -shocked cells, and electrophoresis conditions were essentially as described before for ϕX174 DNA (14).

RESULTS

Construction and isolation of G4 mutants. The function of the J-F intercistronic region and its role in efficient growth of bacteriophage G4 were unknown at the onset of this study. To be able to correlate a potential failure in isolation of viable mutants with an essential function of this region, it was necessary to construct mutants by site-specific mutagenesis and produce physical evidence of successful construction of such mutant genomes.

The fortuitous location of the single *SsrII* site of the G4 genome in the J-F intercistronic region (Fig. 1) allows for a simple and rapid approach to the construction of mutants with modifications of this sequence and the putative hairpin structure. Figure 2 shows a simplified scheme summarizing all steps in the construction of these genomes and the procedure used to efficiently render all nonmodified (wt) genomes inactive for transfection. In short, circular supercoiled G4 RF DNA was isolated from infected *E. coli* C cells and linearized by treatment with restriction endonuclease *SsrII*. This enzyme recognizes the hexanucleotide CCGCGG, and cleaves by leaving the central dinucleotide GC protruding unpaired at the 3' end. The single-strand-specific phosphodiesterase from snake venom was used to "nibble off" these 3' sticky ends, leaving blunt-ended genomes which can be religated into circular or multimeric forms. This resulted in generation of a new *HpaII* site (CCGG) characteristic of mutants. Since the desired genomes and multimers thereof were then devoid of a functional *SsrII* site, they could be enzymatically selected for by a double digest with *SsrII* and *PstI* (also cleaves at only one site in G4 RF DNA). Full-length linear RF DNA molecules with internal mutated *SsrII* sites were liberated from both multimeric molecules and circular forms. Thus, if the digests are driven to completion, only the desired mutant molecules are left in the linear form, whereas full-length wt DNAs are clipped into two fragments. Since linear full-length RF DNA is active in transfection, albeit at a much lower efficiency than supercoiled or circular DNA (approximately 10^4 PFUs per μg of linear RF as compared to 10^6 PFUs per μg of supercoiled RF), transfection of host cells yields plaques originating from viable mutant phages.

The efficiencies of all reactions described in this scheme were monitored by agarose gel electrophoresis (Fig. 3a). Supercoiled G4 RF DNA (lane A) was linearized with *SsrII* to near completion (lane B). Upon treatment with T4 DNA ligase, mostly linear multimeric products

FIG. 1. J-F intercistronic region of bacteriophage θ X174 and G4. The sequences are shown with their potential hairpin structures and the ribosome binding site (RBS) for gene F. The consequence of deleting 2 or 4 bp from the *SsrII* site in the G4 genome is illustrated to show how the size of these deletions can be measured by *HpaII*, *AvaII*, or *Sau3A-AvaII* digests. The middle *AvaII* site (bottom left) appears to remain uncut, since the 50-bp wt *AvaII* fragment cannot be detected in *AvaII* digests of mutant genomes.

were obtained, as well as some in the circular form (lane D). Treatment with snake venom phosphodiesterase damaged the sticky ends and substantially reduced the ligation efficiency (lane C). This was due not only to the inherently lower efficiency of blunt-end ligations, but also due to removal of nucleotides beyond the blunt end, as discussed below.

After addition of more ligase to samples C and D, the ligation reaction was continued. Sample D was then split into two portions, one of which

was applied to the gel without further treatment, showing the increased ligation (Fig. 3b, lane E). Samples C and D were treated with *SsrII* before gel electrophoresis (Fig. 3b, lanes C and D). As expected, the ligation products shown in lane E before *SsrII* treatment were almost completely converted to monomeric linear RF DNA by *SsrII* digestion (lane D). DNAs ligated after phosphodiesterase treatment, however, could not be cleaved with *SsrII*, indicating the presence of mutant DNAs (Fig. 3b, lane C). Further

FIG. 2. Strategy for site-specific mutagenesis and biochemical selection of mutant genomes. The results of deleting 0, 1, 2, or more nucleotides from the 3' sticky end of the *Sst*II linearized G4 RF DNA are illustrated by examples.

addition of *Sst*II to those samples shown in lanes C and D (Fig. 3b) and prolonged incubation to drive the reactions to completion was followed by digestion of all samples with *Pst*I (Fig. 3c). The expected products of G4 RF DNA when double digested with *Sst*II and *Pst*I are a large fragment of 4,670 bp (f_1) and a small fragment of 907 bp (f_2). These were obtained in sample D (Fig. 3c, lane D) with only a faint band of linear RF DNA left. *Pst*I digest of sample E (Fig. 3c, lane E) showed, in addition to fragments f_1 and f_2 , the dimeric forms df_1 and df_2 as well as linear RF, resulting from digestion of the multimers (Fig. 3b, lane E). Although digested with both

*Sst*II and *Pst*I, the products obtained with sample C (Fig. 3c, lane C) were similar to those in sample E, indicating again the loss of the *Sst*II site(s). Also in this sample, a substantial amount of DNA remained in the linear position, which upon transfection gave rise to plaques, originating mostly from mutant genomes.

A transfection of CaCl_2 -shocked *E. coli* C cells was performed with approximately 1 to 3 μg of the DNA shown in Fig. 3c, lane B to E. As expected, the stock of *Sst*II-linearized RF DNA (lane B) gave approximately 5×10^3 PFUs. The same DNA after religation and cleavage with *Pst*I (lane E) gave only five plaques, indicating

FIG. 3. Monitoring the construction and selection of mutant genomes by 1.4% agarose gel chromatography. Circular supercoiled G4 RF DNA was linearized

substantial damage to the DNA during the ligation or *Pst*I reaction (or both). No plaques were obtained with the control DNA, which was religated and then fragmented by treatment with *Pst*I and *Sst*II (lane D). The DNA in lane C, which differed from that in lane D only by a phosphodiesterase treatment before ligation, gave six plaques. High-titer phage stocks of each of these six plaques were prepared and used for the production of RF DNA. These DNAs were then checked for the presence or absence of the *Sst*II site by digestion with *Sst*II. Five of the six isolates obtained were found to be devoid of the *Sst*II site and thus were the expected mutants (data not shown); one of them still contained the *Sst*II site (wt) and was omitted from all further analyses.

Characterization of mutants obtained. As outlined in Fig. 1, deletion of the central GC dinucleotide from the *Sst*II site should result in formation of a new *Hpa*II site (CCGG) at this location. The mutant RF DNAs were therefore tested by *Hpa*II digestion for the presence of this new site (Fig. 4). Digestion of G4 wt RF DNA (lane A) resulted in seven fragments, and the smallest (51 bp) could not be detected on a

by treatment with *Sst*II. Approximately 1 µg of the DNA before (lane A) and after (lane B) *Sst*II treatment was run on each gel to serve as a marker for the positions of supercoiled (s), linear (l), and circular (c) forms. *Sst*II-linearized G4 RF DNA (15 µg) was treated with 10 µl of snake venom phosphodiesterase (1 mg/ml) for 30 min at 37°C (sample C). After removal of this enzyme by one phenol and three ether extractions, the DNA was ethanol precipitated (two precipitations). An equivalent amount of *Sst*II-linearized RF DNA received identical treatment, except for digestion with the phosphodiesterase, to serve as control (sample D). Both samples were ligated with T4 DNA ligase (1 U) at 15°C for 20 h. Portions (1 µg) of the phosphodiesterase-treated (lane C) and untreated (lane D) sample were run on a 1.4% agarose gel in TAE buffer (panel a) (14). Samples C and D received more ligase (0.5 U), and ligation was continued for 24 h, after which both samples were mixed with an equal volume of double-strength *Sst*II restriction buffer. Sample D was split into two portions, D and E, and 10 U of *Sst*II was then added to samples C and D. The reaction was carried out for 16 h at 37°C, after which portions (1 µg) were run on an agarose gel (panel b). To ensure completion of the *Sst*II digest, an additional 10 U of *Sst*II was added to samples C and D. *Pst*I (1 to 2 U) was then added to samples C, D, and E and digestion was continued for 16 h at 37°C. Portions (1 µg) were run on an agarose gel (panel c). The positions of monomeric and dimeric fragments f₁ and f₂ are indicated. More *Pst*I (1 to 2 U) was then added to samples C, D, and E, and digestion was continued for 6 h. Approximately 3-µg portions were used for transfection of CaCl₂-shocked *E. coli* cells.

FIG. 4. *HpaII* and *SalI* digest of purified mutant RF DNAs. *HpaII*-digested RF DNAs (approximately 4 μ g) were run on 4% polyacrylamide gels. Wt G4 RF DNA digested with *HpaII* (lane A) or *HpaII* and *SstII* (lane B) was run as a control to show the appearance of fragments 3a and 3b in the double digest. The digest of isolate 2 (lane C) is representative of those of isolates 4', 5, and 6'. The digest of isolate 3 (lane D) is representative of those of isolates 4, 5', and 6. A *SalI* digest of wt G4 RF DNA is shown in lane E; that of isolate 2 (lane F) is characteristic for all mutants obtained.

4% acrylamide gel. Fragment 3, which contained the *SstII* site, comigrated with fragment 4. The faint band between fragment 1 and 2 is a partial, which remained to a limited extent even after prolonged digestion. Replacement of the *SstII* site in fragment 3 by an *HpaII* site resulted in the appearance of two new bands, one of which (3a) was larger than fragment 5 and the other (3b) was smaller than fragment 6. As a control, bands 3a and 3b could be obtained by double digestion of wt G4 RF DNA with *HpaII* and *SstII* (lane B). The appearance of these characteristic bands was observed in the *HpaII* digest of all five mutant isolates and is represented here by a digest of isolate no. 2 (lane C). However, smaller amounts of these fragments than expected (as judged by comparison of band 5 with band 3a) were obtained in isolates 4, 5, 6, and especially isolate 3, where only a very faint band 3a could be observed on the original picture (data not shown). The most obvious explanation of this finding is the presence of at least two types of mutant DNAs in each of these preparations, both of which are devoid of the *SstII* site but only one with a new *HpaII* site.

This explanation proved correct, since both types of mutants could be readily isolated by plaque purification from isolates 4, 5, and 6. Purified isolates 4' (subisolate from 4), 5, and 6' (subisolate from 6) showed the appearance of fragments 3a and 3b upon *HpaII* digestion (data not shown). A wt-like *HpaII* pattern was obtained with purified isolates 4, 5' (subisolate

from 5), and 6. A representative *HpaII* digest of these strains is shown in Fig. 4, lane D. The finding that each plaque resulting from the initial transfection contained at least two types of phages will be discussed below.

Although DNA sequencing as a final and unambiguous characterization of these mutants is desirable, a more rapid preliminary characterization was carried out. The reason for this is that many more mutants were produced by deviations of the technique described above (see below), and sequencing will be reserved for those that prove to be biologically most interesting. In the following biochemical characterization, the size of the deletion was estimated.

Restriction endonuclease *SalI* recognizes the sequence CGCG and is therefore contained within the *SstII* site. Consequently, both of these sites should be lost in all mutants, if in fact the central dinucleotide of the *SstII* site has been deleted. A *SalI* digest confirmed this by the appearance of a fusion fragment in all mutant digests, resulting from loss of the site between *SalI* fragments 7 and 9. A *SalI* digest of G4 wt RF DNA is shown in Fig. 4, lane E, and that of isolate 2, which is representative for all mutants, is shown in lane F. This digest also demonstrates that in all mutants the characteristic fusion fragment was of similar length, i.e., the deletion was of similar size (data not shown). Since the sequence of these fusion fragments was basically the same with the exception of a few missing nucleotides, their migration on the gel was a sensitive assay for their length, which on this gel could be determined with an accuracy of ± 5 bp. This sets the upper deletion size at 7 bp, since a maximum of 2 bp was deleted in those mutants with a new *HpaII* site (Fig. 1).

However, generation of a new *HpaII* site does not imply that in each case the central GC dinucleotide of the *SstII* site has been deleted. In fact, removal of the phosphodiesterase of just the C rather than the GC would result in a new *HpaII* site as well (Fig. 2), but would simultaneously generate a new *NciI* site, as this enzyme recognizes the sequence CCGGG as well as CCCGG. Restriction of all RF DNAs with *NciI* was carried out but revealed no additional *NciI* sites (data not shown); thus, the possibility of deletion of only one nucleotide from the *SstII* site can be eliminated. Consequently, at least two types of mutants were obtained, those with deletion of a GC dinucleotide and generation of a new *HpaII* site and those with deletion of more than two nucleotides (no new *HpaII* site).

A more accurate estimate of the number of nucleotides deleted in those mutants without a new *HpaII* site was obtained by digestion with *AvaII* or double digestion with *AvaII* and *Sau3A*, respectively. The stem of the hairpin in

the G4 J-F intercistronic region contains two *Ava*II sites (GGACC and GGTCG), composing part of the *Sst*II recognition sequence, but separated by the central GC dinucleotide of the *Sst*II site (Fig. 1). Therefore, deletion of more than these two nucleotides should destroy one or both of the adjacent *Ava*II sites. Loss of the right-hand site can be easily measured if the total deletion does not exceed 5 bp, since the adjacent *Ava*II fragment is only 50 bp long and addition of as few as 2 bp can be detected on a 10% acrylamide gel. A destruction of the left *Ava*II site and addition of nucleotides to the adjoining fragment could only be measured in a *Sau*3A-*Ava*II double digest if not more than 5 bp were deleted, e.g., at least 2 bp added to the respective 194-bp-long *Sau*3A-*Ava*II fragment (Fig. 1). The *Ava*II digest (Fig. 5, lanes E and F) and an *Ava*II-*Sau*3A double digest (Fig. 5, lanes B, C, and D) confirmed the presence of at least two types of mutants. Those with an additional *Hpa*II site (deletion of 2 bp) revealed an unchanged 194-bp *Sau*3A-*Ava*II fragment (Fig. 5, lane C) but with an addition of approximately 5 bp to the 50-bp *Ava*II fragment (Fig. 5, lane F). Since in this case the two *Ava*II sites are fused, but theoretically intact (Fig. 1), the left site must be cut preferentially over the right, or two bands of 50 and 55 bp would be expected in the *Ava*II digest. The *Sau*3A-*Ava*II double digest showed a small increase in length of the 194-bp fragment in the case of those mutants without an additional *Hpa*II site (Fig. 5, lane D). Since the 50-bp *Ava*II fragment (and consequently the right *Ava*II site) was left intact in these mutants (Fig. 5, lane E), the deletion extends from the central GC into the left *Ava*II site. The total deletion must be ≤ 5 bp, however, or an increase in size of the *Sau*3A-*Ava*II fragment could not have been detected. These digests suggest that the deletion size of any of these mutants was a minimum of 2 bp (new *Hpa*II site) and a maximum of 4 to 5 bp.

Biological implications. The successful isolation and preparation of bacteriophage G4 mutants with small deletions in the J-F intercistronic region proves that conservation of the primary structure in this region is not essential for growth of the phage. To test whether these mutations had any effect on the growth efficiency, two mutant isolates (no. 2, with a 2-bp deletion [new *Hpa*II site] and no. 5', a subisolate of 5 [no new *Hpa*II site; approximately 4 bp deleted]) were compared with the parental G4 strain. Competitive growth experiments in which an *E. coli* culture is infected at very low multiplicity of infection simultaneously with a mutant and the parental wt strain were previously shown to be a sensitive measure for small differences in growth efficiencies (15).

As described above, mixtures of bacterio-

phage G4 and either mutant isolates 2 or 5', as well as a mixture of just the two mutants, were grown competitively for approximately 10 generations within the same host culture. The phage mixture obtained after complete clearing of the culture was analyzed for distribution of parental and mutant phage by preparation of RF DNA and subsequent restriction digests. Parental genomes were distinguished from mutants by presence of the *Sst*II site. The mutants were distinguished from each other by the presence (mutant no. 2) or absence (mutant no. 5') of an additional *Hpa*II site. Figure 6 shows the result of the *Hpa*II or *Hpa*II-*Sst*II double digest.

In each digest, the relative intensity (as judged by eye) of the band containing *Hpa*II fragment 3a (band 3a) as compared to that of the band containing *Hpa*II fragment 5 (band 5) gave a

FIG. 5. *Ava*II digest and *Ava*II-*Sau*3A double digest of mutant RF DNAs. G4 wt DNA and all mutant RF DNAs were analyzed by *Ava*II digests (10 μ g of RF DNA) or *Ava*II-*Sau*3A double digests (3 μ g of RF DNA) to estimate the deletion size. The *Ava*II digests were run on 10% polyacrylamide gels (lanes E to G), and the double digests were run on 4% polyacrylamide gels (lanes A to D). *Hae*III-digested pBR322 DNA (20 μ g) was run in lanes A and G to serve as an approximate size marker, and the 193-, 57-, and 51-bp fragments are identified by arrows. Only two representative mutant strains are shown for each digest. Lanes C and F are characteristic for mutants with a new *Hpa*II site (isolates 2, 4', 5, and 6'), whereas lanes D and E are characteristic for the remaining mutants (isolates 3, 4, 5', and 6). As shown in Fig. 1, one of the two *Sau*3A sites in the G4 genome is located 194 bp away from one of the two *Ava*II sites in the J-F intercistronic region. Loss of this *Ava*II site by the procedure used increases this fragment by maximally 4 bp. For the mutants represented by lane D, this fragment migrates somewhat slower than the analogous fragment of wt G4 (lane B). The increase in size was estimated to be 3 bp. For mutants represented in lane C, the 194-bp fragment migrated identically to that of wt G4; however, their 50-bp *Ava*II fragment increased in size by approximately 3 to 4 bp (lane F). No such increase in the length of this fragment was observed for the other mutant strains (lane E).

FIG. 6. Competitive growth experiment. Mutant isolates 2 and 5' were grown in mixed cultures with wt G4 for approximately 10 generations as described in the text. The distribution of phage in the final lysate was measured by preparation of RF DNA and restriction with either *HpaII* (H) or *HpaII* and *SstII* (H & S). The appearance of fragment 3a in either digest is characteristic for isolate 2 (lanes B and E). It appears only in a double digest in the case of wt G4 (lane F) but in neither digest for isolate 5' (lanes A and D). The intensity of band 3a as compared to band 5 (as judged by eye) was used as a measure to determine the distribution of phage RF DNA in the mixture.

rough estimate of the relative amounts of the two RF DNAs present in this mixture. For comparison, almost equal intensities of bands 3a and 5 would be expected when pure RF DNA from wt G4 is digested simultaneously with *HpaII* and *SstII* or when mutant 2 RF DNA is digested with *HpaII* alone, whereas no 3a band can be expected in either digest of mutant 5' RF DNA (lanes A to F). As described below, these concepts were applied to the interpretation of the other digests in Fig. 6 with the surprising finding that both mutants grow more efficiently than the parental strain under these competitive conditions. This interpretation is derived as follows. Growth of wt and mutant 2 with 1:1 starting ratios should result in approximately a 1:2 intensity ratio of band 3a to band 5 in an *HpaII* digest, if both strains grow and produce RF DNA equally well (Fig. 6, lane O). The same is true for lane L, where wt and mutant 5' were compared by an *HpaII*-*SstII* double digest. Yet, in lane O the 3a band (characteristic for mutant 2) appears as strong as band 5, whereas in lane L the 3a band (characteristic for wt) is much weaker than band 5. When wt G4 DNA was given a 10-fold advantage over either of these mutants at the onset of the experiment, this ratio changed in favor of both mutants at the end of the experiment. A 10:1 starting ratio of wt to mutant DNA should result in an approximate

1:11 intensity ratio of band 3a to band 5 in lane M and a 1:1 intensity ratio in lane J, if all strains grow equally well. However, the intensity ratio of band 3a to band 5 appeared to be about 1:3 in lane M and 1:2 in lane J. In the reverse situation where mutants were given a 10-fold advantage, the wt seemed to be completely outgrown, since no trace of band 3a was left in lane K, whereas in lane N it was as intense as band 5. Competitive growth between the two mutants indicated that they grow with approximately equal efficiency (lanes G, H, and I). Almost identical results were obtained in similar experiments (not shown) where mutant isolate 5' was replaced by isolate 3, which also lacks approximately 4 bp in the center of the hairpin sequence.

DISCUSSION

Over the past few years, studies of the molecular mechanisms of gene regulation have revealed elaborate mechanisms which allow a cell to respond quickly to various metabolic signals by influencing the efficiency with which an initiated message is completed. All of these mechanisms, attenuation, termination, polarity, and antitermination, involve formation of hairpin-like secondary structures in the transcript or cruciforms in the template (17, 21). Processing of a message and the efficiency with which it is translated also appear to be influenced by hair-

pin-like RNA conformations (6, 7). Since the regulatory signals are located with high frequency in intercistronic regions (12, 13), it is not surprising that only in a few instances have mutants become available to study these *cis*-acting control elements. Fewer models yet are available to study their role in viral gene regulation due to the difficulty in isolating appropriate virus mutants by traditional techniques.

Described here is a mechanically simple and rapid technique for site-specific mutagenesis and efficient enzymatic selection of such mutants. Although it can theoretically be applied to any circular double-stranded DNA, it is limited by the requirement that the site to be mutated (or used for cloning) occurs not more than twice in the genome. If the site occurs twice, partially digested DNA can be mutated, ligated, and then digested to completion with the respective restriction endonuclease, avoiding the necessity of a second unique site for enzymatic destruction of nonmutated genomes. I have used these techniques successfully to construct other deletion mutants of θ X174 or G4 (U. Müller and R. Stanzak, unpublished data) or for cloning of small DNA fragments (W. Russell and U. Müller, unpublished data), and I repeatedly obtain mutants with $\geq 80\%$ efficiency. This technique might be particularly useful for the cloning of small regulatory DNA sequences into eucaryotic or procaryotic virus and plasmid vectors, and it eliminates the need for biological selective markers. The yields that can be obtained by transfection of CaCl_2 -shocked *E. coli* cells with linear double-stranded G4 or θ X174 RF DNA are on the order of 10^4 plaques per μg (Fig. 3, lane B) if the DNA has not been damaged in an essential region of the genome. Such damage may occur, for example, by linearization of G4 RF DNA with *Pst*I and removal of the protruding ends by nucleases *in vivo* or *in vitro*.

In the experiment described herein, the enzymatic selections of mutant genomes (e.g., fragmentation of wt genomes by double digests with *Ssr*II and *Pst*I) were carried out with excess of enzymes and much increased incubation times to drive the reactions to completion. Under those conditions, even minute nuclease contaminations may cause enough damage to seriously reduce the yield of viable genomes. This explains the low yield of PFUs obtained after transfection with samples C, D, and E (Fig. 3c).

It has to be pointed out, however, that transfections with *Pst*I-linearized G4 and θ X174 RF DNAs are routinely done in my laboratory with high yields if the DNAs are not treated excessively with this enzyme.

Although a substantial amount of the DNA in sample E was full-length linear RF DNA, trans-

fection of sample C resulted in five mutant plaques only, at least four of which contained more than one type of phage. This finding indirectly supports the explanation given above and further leads me to hypothesize that all plaques arose from cells which were transfected with multimeric rather than monomeric RF DNA molecules. These concatemers were joined at their mutated *Ssr*II site. In most cases, however, the mutations were not just due to removal of the protruding 3' *Ssr*II ends but due to removal of nucleotides beyond those ends. Snake venom phosphodiesterase is known to degrade single-stranded DNA from the 5' end also (18); thus, it could have generated blunt-ended molecules with deletions of more than 2 bp per genome, and construction during the ligation of concatemers consisting of two or more types of molecules would be feasible.

A terminator function has been proposed for the J-F intercistronic region in both the θ X174 and the G4 genome (4), and we have obtained evidence to support this hypothesis (see accompanying papers). Furthermore, preliminary evidence suggests an involvement of this sequence in the phage eclipse reaction (A. Incardona and U. Müller, unpublished data). In addition, this region contains the ribosome binding site for gene F, and it is tempting to speculate that at least two of these functions are mediated or at least influenced by the potential hairpin structure therein. My previous finding that 70% of this region, including the hairpin but not the ribosome binding site for gene F, could be deleted in θ X174 suggests that any function of this region (except for ribosome binding) is nonessential. The G4 deletion mutants described in this report lack either 2 bp but in no case more than 4 to 5 bp in the loop region of the hairpin. Although removal of two to five nucleotides shortens the stem of the putative hairpin by 1 to 2 bp (the minimum loop size is three nucleotides), thermodynamic considerations would still favor the resulting structures, containing 5 or 6 bp in the stem, with a free energy of formation of approximately -10 Kcal/mol (20). Therefore, it can only be argued that the totality of the primary structure in the J-F intercistronic region is not essential in G4, as was previously shown for θ X174. Whether the residual secondary structure is essential and still functional in the modified form remains to be determined. The finding that mutants with deletions of as few as 2 bp in a nontranslated region grow more efficiently than the wt phage supports the hypothesis of a regulatory role of the J-F intercistronic region. This finding is difficult to interpret from an evolutionary standpoint, however, and may hint at distorted laboratory growing conditions.

ACKNOWLEDGMENTS

This work was supported in part by grant 2029 from the North Carolina Board of Science and Technology and Public Health Service grant GM 31993 from the National Institutes of Health.

I thank Richard Stanzak for his excellent technical help.

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