Nucleic Acids Research, Vol. 20, No. 4 711–717

Isolation of dominant negative mutants and inhibitory antisense RNA sequences by expression selection of random DNA fragments

Tatyana A.Holzmayer, Dmitry G.Pestov and Igor B.Roninson*
Department of Genetics, University of Illinois at Chicago, 808 South Wood St, Chicago, IL 60612, USA

Received December 3, 1991; Revised and Accepted January 15, 1992

ABSTRACT

Selective inhibition of specific genes can be accomplished using genetic suppressor elements (GSEs) that encode antisense RNA, dominant negative mutant proteins, or other regulatory products. GSEs may correspond to partial sequences of target genes, usually identified by trial and error. We have used bacteriophage lambda as a model system to test a concept that biologically active GSEs may be generated by random DNA fragmentation and identified by expression selection. Fragments from eleven different regions of lambda genome, encoding specific peptides or antisense RNA sequences, rendered *E. coli* resistant to the phage. Analysis of these GSEs revealed some previously unknown functions of phage lambda, including suppression of the cellular lambda receptor by an 'accessory' gene of the phage. The random fragment selection strategy provides a general approach to the generation of efficient GSEs and elucidation of novel gene functions.

INTRODUCTION

Selective suppression of a specific gene represents a general approach to the analysis of gene function. Such suppression can be accomplished either by gene disruption or by introduction of a genetic element that inhibits the function of the target gene. The antisense RNA approach to gene suppression involves production of RNA sequences complementary to mRNA of the target gene (1); antisense RNA sequences are utilized either alone or in combination with other sequences that promote the cleavage of the target RNA (ribozymes) (2). Another approach involves the use of protein mutants that interfere with the function of the wild-type protein in a dominant fashion (3). In some cases, expression of the target gene or virus can be inhibited by overexpression of natural regulatory products, such as the TAR RNA of HIV (4).

Despite the ease of expressing antisense RNA for any cloned gene, many antisense RNA constructs have little or no biological effect (5–8). Some of the more effective antisense RNAs comprise only a portion of cDNA targeted for suppression. This portion in some but not all cases corresponds to the 5′ end of the target gene; there are no clear rules to predict which part of the cDNA would make the most effective antisense inhibitor (8–10). Dominant negative mutants may be derived from normal proteins by truncation of domains involved in specific functional interactions (3). Unless the domain structure of the target protein is well understood, however, one cannot predict which if any segments of the protein would act as dominant negative mutants. Thus, biologically active dominant negative mutants and antisense RNA constructs are mostly designed by inefficient trial-and-error approaches.

We have hypothesized that GSEs, encoding dominant negative mutant proteins or inhibitory antisense RNA sequences, can be generated by random DNA fragmentation and identified by functional selection for the phenotype associated with suppression of the target. To test this hypothesis, we have analyzed the ability of randomly fragmented bacteriophage lambda DNA to protect *E. coli* cells from lambda-induced lysis. By this approach, multiple GSEs encoding either protein or antisense RNA fragments have been isolated. Sequence analysis revealed strong sequence selectivity for both peptide- and antisense RNA-expressing GSEs. Analysis of the biological effects of individual GSEs indicated some previously unknown functions of lambda, including suppression of the cellular lambda receptor by a previously uncharacterized gene of the phage.

MATERIALS AND METHODS

Library construction and GSE selection

Lambda (CI857 ind1 Sam7) DNA was partially digested with DNAseI in the presence of Mn^{++}, to maximize the generation of blunt ends (11). After filling in the termini with T4 DNA polymerase and Klenow fragment of DNA polymerase I, the fragments were ligated with NcoI linkers. After NcoI digestion, 300–500 bp fragments were selected by agarose gel electrophoresis. The fragments were ligated with

* To whom correspondence should be addressed

dephosphorylated pKK233-2 vector (12), that had been modified by the insertion of the oligonucleotide CATGGTGACTGACTG-AAGCT into the NcoI and HindIII restriction sites, to provide for translation termination of inserts in all three open reading frames. A library of approximately 80,000 recombinant plasmids was generated in *E. coli* strain K12 PLK-F′ and amplified by growth on agar plates prior to selection for lambda resistance.

For lambda infection, cells carrying either the library or the control pKK233-2 vector were grown in LB with 0.5% maltose in the presence of 50 μg/ml ampicillin to OD_{600} = 0.3−0.5. Expression of the *trc* promoter of pKK233-2 was induced with 1 mM IPTG for 1−1.5 hours. (IPTG induction was subsequently found to have only a marginal effect due to high basal levels of expression from *trc* promoter, and it was omitted in later assays). Aliquots of 10^6 cells were resuspended in 100 μl of 10 mM $MgSO_4$ and mixed with 10^6 pfu of lambda (low m.o.i.) or 10^9 pfu (high m.o.i.). After 20 minutes incubation at 37°C, cells were plated on L agar plates with ampicillin and grown overnight.

In the initial experiment, lambda-resistant clones were selected by infection at low m.o.i., to account for the possibility that the anticipated GSEs would be unable to protect cells from simultaneous infection with multiple phage particles. At low m.o.i., 1%-3% of both library-derived and control bacteria survived the infection. Selection at low m.o.i. was followed by plasmid extraction from the surviving cells, re-transformation and a second round of phage infection, carried out at either high or low m.o.i. At this stage, 10% of the library cells (corresponding to 0.3% of the starting clones) survived the infection at both high and low m.o.i., in contrast to only 0.02% (at high m.o.i.) or 3% (at low m.o.i.) of the control bacteria. In a subsequent experiment, lambda-resistant clones were isolated from the random fragment library by a single round of selection at high m.o.i., and by this procedure 0.13% of the clones were found to be resistant to the phage. Clones isolated after single-step or two-step selection at high m.o.i. were used for further studies.

GSE characterization

Individual phage-selected plasmids were transformed into *E. coli* K12 LE392 or DH5α. Plasmid preparations were tested for the absence of lambda *dv* plasmids (13) by PCR amplification of sequences adjacent to the origin of lambda DNA replication, using the following primers: GGAGCGTAATGTGGCAGA (sense) and GCCTGCCTGTTGCTTGTT (antisense). Lambda *dv* contaminant was detected in some of the plasmid DNA preparations, most of which lost their ability to confer phage resistance after re-transformation and amplification in uninfected bacteria. Sequencing was carried out by a standard dideoxy technique.

Modified versions of GSEs of the *Ea8.5*, *V* and *oop/ori* classes were prepared by PCR synthesis using either lambda DNA or the corresponding plasmids as templates. The coding sequence of *Ea8.5* was synthesized using PCR primers GGAT-CCATGGGTATCAATGAGTGA (sense) and GGATCTGC-AGTTAATCATCTATATGT (antisense); the resulting PCR product encodes the Ea8.5 protein with a *ser→gly* substitution in the second codon. The version with a frameshift in the second codon was constructed using the same antisense primer and GG-ATCCATGGATGAGTATCAATGAGTTA as the sense primer. Other modified GSEs were synthesized and subcloned using the primers corresponding to the desired sequences and restriction sites.

Assays for GSE activity were carried out in *E. coli* strains K12 PLK-F′, LE392, NM-522 or DH5α. Lysogen induction was performed on *E. coli* K12 C600 (lambda cI857 N7 N53 S7) transformed with different GSEs. The prophage was induced by temperature shift to 42°C for 20 minutes followed by 1.5 hour at 37°C, and its titer was determined on *E. coli* LE392.

Analysis of *lamB* expression

Maltose utilization by bacteria transformed with *Ea8.5*-containing GSEs was analyzed by the color of colonies plated on McConkey media with 1% maltose and 50 μg/ml ampicillin. Bacterial RNA from cells transformed with *Ea8.5* GSEs was isolated using a nucleic acid extractor (Applied Biosystems, Model 340A) under the conditions recommended by the manufacturer. The RNA concentration was measured by the orcinol reaction (14). *malK-lamB* RNA expression was analyzed by northern hybridization using a PCR-amplified segment of the *lamB* gene (positions 4514−5005) (15) as a probe. Even loading of RNA was confirmed by ethidium bromide staining after electrophoresis.

RESULTS

Isolation of lambda-derived GSEs

Lambda DNA was fragmented by partial digestion with DNaseI, and NcoI linkers carrying the ATG translation initiation codon were added to the termini of the resulting fragments. Fragments of 300−500 bp size were isolated after NcoI digestion. These size limits were selected from the following considerations: (a) 100-150 amino acids is believed to be the size of most protein structural domains (16), (b) nucleic acids >300 bp show the highest hybridization efficiency and therefore are likely to be the most efficient antisense GSEs, and (c) the resulting fragments would not include the full-length CI repressor gene (713 bp) likely to provide an efficient but not novel GSE. The fragment mixture was inserted into a multicopy plasmid expression vector pKK233-2 (12), modified to provide for termination of translation of the inserted fragments in all three open reading frames.

The ligated mixture was used to transform a lambda-sensitive strain of *E.coli*, and a library of approximately 80,000 clones was obtained. Clones resistant to lambda-mediated lysis were selected from this library by either one or two rounds of phage infection, as described in Materials and Methods. Plasmids from individual phage-selected clones were then tested for the ability to render *E.coli* resistant to lambda upon re-transformation, and >90% of the plasmids were found to confer resistance to both the wild-type and CI^- strains of lambda. This property was maintained upon several rounds of plasmid propagation in uninfected bacteria. No plasmids conferring lambda resistance could be isolated from control bacteria transformed with insert-free vector. Thus, the random fragment selection strategy has resulted in the isolation of *bona fide* GSEs from lambda DNA.

51 of the isolated GSE clones were characterized by DNA sequencing. Eight clones were found in two or more identical copies; these may represent either siblings or, more likely, independently derived identical clones whose sequences reflect the constraints of functional selection or preferential DNaseI digestion. The sequenced GSEs were derived from eleven different regions of the phage genome. Eight classes of GSE contained fragments of lambda genes inserted in the sense orientation relative to the promoter. These inserts encoded either partial or complete open reading frames of specific phage proteins, starting either from the native translation initiation

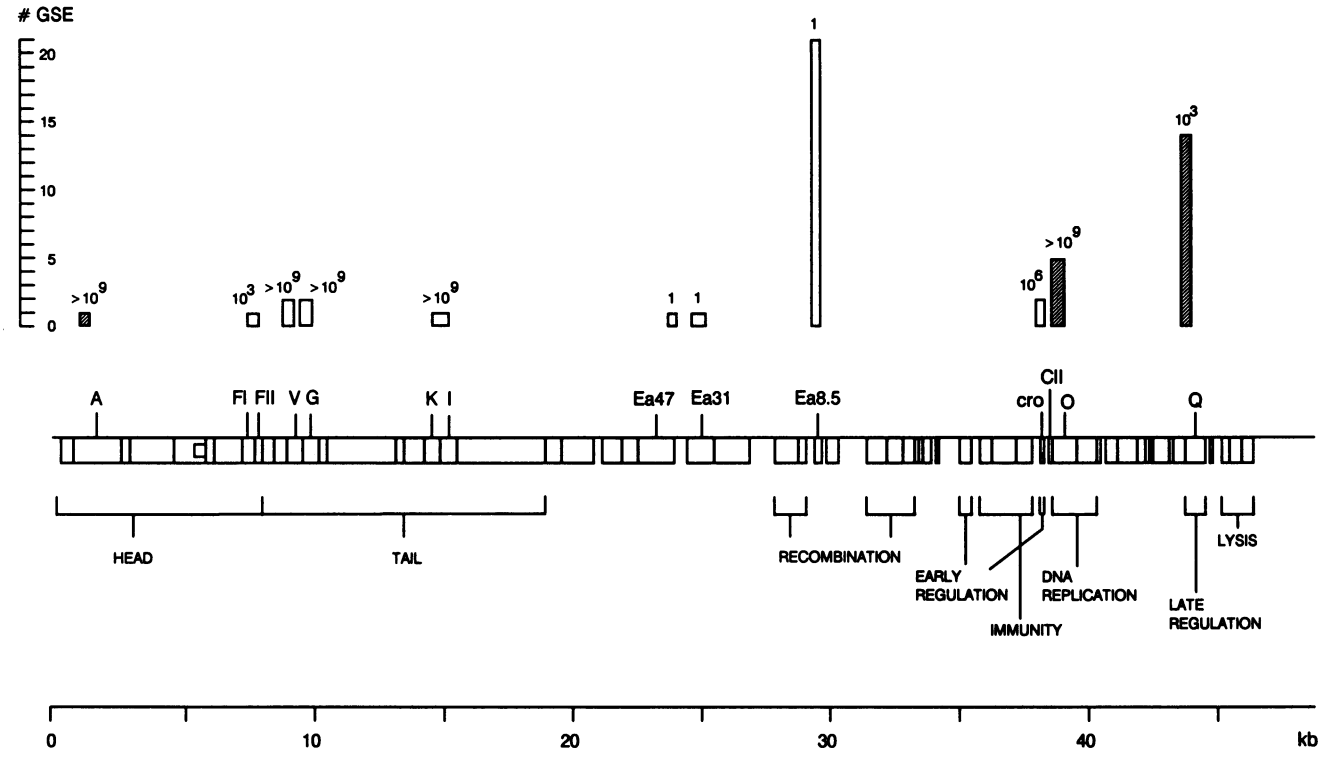

Figure 1. Distribution of GSEs in the lambda genome. Only the genes whose sequences were found in GSEs are indicated in the genetic map of lambda (30). Open bars: sense-oriented GSE. Hatched bars: antisense-oriented GSE. The height of the bars corresponds to the number of sequenced GSE clones for each class. The numbers on top of the bars indicate the extent of suppression of prophage induction by a representative clone of each class (the ratio of the phage titer obtained after induction of a control strain to the titer obtained from GSE-transformed clones).

codons of the corresponding genes, or from the linker-derived ATG codon joined in frame with the coding sequence, or (in one case) from an internal ATG codon located proximally to the linker. Three other classes contained lambda gene sequences inserted in antisense orientation relative to the promoter. The distribution of different classes of lambda-derived GSE is shown in Fig. 1.

The suppression efficiency of different classes of GSE was evaluated by the following tests. (a) Plating efficiency of transformed bacteria was measured after lambda infection at high m.o.i. Bacteria transformed with any of the GSE showed either none or a minor (<2-fold) decrease in the plating efficiency, in contrast to 0.02% of control bacteria surviving under the same conditions. All the GSE-transformed bacteria were equally resistant to CI^+ and CI^- strains of lambda, indicating that none of the GSEs exerted its effect by promoting lysogeny. (b) The decrease in phage titer was determined by plaque assay using the amounts of phage that produced 10^9 plaques in control bacteria. No plaques were discernible with most types of GSE, though some GSEs allowed for the formation of phage plaques at the incidence of 10^{-5} to 10^{-7}. These plaques reflected the appearance of GSE-insensitive mutant phage, since phage isolated from such plaques showed normal plating efficiency on the corresponding GSE transformants. (c) To determine the effect of GSEs on prophage induction, representative clones of each class were introduced into a strain of *E. coli* lysogenic for lambda, and the phage titer was determined after heat induction of the prophage. As indicated in Fig. 1, eight classes of GSE decreased the titer of the induced phage by three or more orders of magnitude, but GSEs of the other three classes had no effect on prophage induction.

Sense-oriented GSEs

The most abundant class of GSE carried sequences of the phage gene *Ea8.5*, inserted in the sense orientation. *Ea8.5* sequences were found in 21 (14 non-identical) of 51 sequenced GSEs. By colony hybridization, *Ea8.5* sequences were present in 40%-50% of all clones selected by either one or two rounds of phage infection. The *Ea8.5* gene, transcribed in the delayed early stage of lytic infection, is unnecessary for either lytic or lysogenic infection and encodes a 8.5 kD (93 amino acid) protein without a known function (17). Some of the GSE clones contained the entire *Ea8.5* gene (in the sense orientation), and other clones encoded truncated Ea8.5 proteins, missing 7 to 38 amino acids at the C-terminus or 3 to 10 amino acids at the N-terminus. The coding sequence of *Ea8.5*, free of flanking sequences, was synthesized by PCR and inserted into the pKK233-2 vector; the resulting clone protected *E. coli* from lysis by lambda. Introduction of a frameshift mutation into the second codon of *Ea8.5* abolished the GSE activity, thus indicating that this activity required expression of the Ea8.5 protein.

Ea8.5 expression in a lysogenic strain failed to suppress prophage induction, indicating that *Ea8.5* acted at the initial stages of phage infection, such as phage entry into the host cell, determined by the interaction of the phage with its cellular receptor. To test a role for the lambda receptor in *Ea8.5*-mediated resistance, we have infected *Ea8.5*-transformed cells with a recombinant phage $h^{80}imm\ \lambda$. This recombinant contains host-

range determinants of the phage ϕ80 (which enters the cell through a different receptor) replacing the corresponding portion of lambda DNA (18; N.C. Franklin, personal communication). The *Ea8.5* transfectants were sensitive to $h^{80}imm$ λ, suggesting that a change in the lambda receptor was responsible for the GSE activity of *Ea8.5*.

The cellular receptor recognized by lambda is the LamB protein that normally functions in the uptake of maltose (19); the *lamB* (or *malB*) gene is a part of *malK-lamB*, one of the three maltose operons of *E. coli* (15). We have found that bacteria expressing *Ea8.5* were deficient in maltose metabolism, judging by the white color of colonies grown on McConkey media with maltose (wild-type colonies appear red on this media). This effect was specific to maltose, and was not observed with galactose, lactose, mannose or arabinose. A deficiency in LamB alone would have been insufficient to produce white colonies on McConkey media with maltose (20 and our data not shown). We have hypothesized therefore that *Ea8.5* expression affected the entire maltose operon(s), most probably at the level of transcription. Northern hybridization with a *lamB* probe (Fig. 2) showed the disappearance of detectable *malK-lamB* RNA in cells transformed with full-length *Ea8.5* but not with its frameshift variant; these results were reproduced in two other bacterial strains (data not shown). Thus *Ea8.5* expression leads to the inhibition of the maltose operon encoding the lambda receptor.

Most of the isolated *Ea8.5* GSEs expressed truncated rather than full-length versions of the Ea8.5 protein. As shown in Table 1, increasing truncation of the protein at the N- or C-terminus was associated with a gradual change in the color of the colonies on McConkey media with maltose from white to pink to red; further truncation at the C-terminus, however, again rendered the colonies white. Transformation of three different strains of *E. coli* with truncated *Ea8.5* clones giving rise to pink or red colonies on McConkey media with maltose resulted in strongly decreased but detectable expression of *malK-lamB* RNA (Fig. 2 and data not shown), indicating that the phage receptor was only partially suppressed in the transformed clones.

The composition of the sense-oriented GSEs distinct from *Ea8.5* is shown in Table 2. Two classes of GSE, each represented by a single sequenced clone, contained portions of genes with unknown functions. These genes, *Ea31* and *Ea47*, belong to the same P_L operon that includes *Ea8.5* (17). Both GSEs were inserted in the sense orientation and encoded C-terminal segments of the corresponding proteins. Like *Ea8.5*, these GSEs failed to suppress lysogen induction and did not confer resistance to $h^{80}imm$ λ, suggesting that they could also act at the level of the phage entry. These clones, however, did not change the red color of colonies on McConkey media with maltose, indicating that they had no major effect on the maltose operon.

Another class of GSE, represented by two sequenced clones, contained the entire gene *cro* in the sense orientation. This gene encodes a regulatory protein known to suppress the expression of the early genes of lambda (21), and therefore its GSE effect was anticipated.

Four classes of GSE encoded truncated forms of structural proteins of the phage particle. One clone spanned the *FI* and *FII* genes of the phage head, encoding a C-terminal portion of the first and the N-terminal portion of the second protein. Another GSE encoded portions of two tail proteins, K and I. Interestingly, each of the two other classes of GSE, encoding C-truncated forms of tail proteins V or G, included two independent clones that encoded exactly the same segments of the corresponding proteins (Table 2), truncated at the C-terminus. In one case (G), the two GSEs differed at their 5′ ends but coincided at the 3′ ends. *V*-derived GSEs had different 5′ ends but terminated at different bases within the same codon at the 3′ end. The coincidence of the protein segments encoded by these non-identical GSE clones can be most redily explained by strong functional selection at the protein level. Protein-mediated action of the GSEs derived from the *V* gene was verified by preparing clones with PCR-synthesized inserts, encoding either the first 145 codons of *V*,

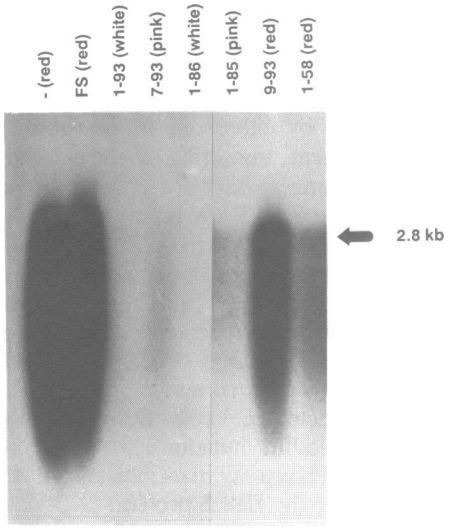

Figure 2. Effect of *Ea8.5* clones on *malK-lamB* RNA expression. Each lane contains 15 μg of total RNA from *E. coli* K12 LE392 transformed either with the insert-free plasmid vector (−) or with plasmids carrying *Ea8.5* sequences. The amino acids of the Ea8.5 protein encoded by the plasmids (1−93, full length; FS, frameshift) and the color of the corresponding colonies on McConkey media with maltose are indicated.

Table 1. Compositions and phenotypes of *Ea8.5*-derived GSEs

Position[a]	Amino acid residues of Ea8.5	Color on McConkey media with maltose
Full-length:		
29762−29269	1−93	White
29749−29353	1−93	White
29718−29243	1−93	White
29703−29300	1−93	White
N-truncated:		
29646−29084	4−93	White
29638−29217	7−93	Pink
29637−29155	7−93	Pink
29632−29242	9−93	Red
29626−29206	11−93	Red
C-truncated:		
29793−29398	1−86	White
29768−29398	1−86	White
29793−29400	1−85	Pink
29850−29480	1−58	Red
29931−29489	1−55	White
PCR-synthesized:		
29655−29374	1−93	White
Same, frameshift	−	Red

[a]Numbered according to ref. 30.

as found in the GSEs, or a variant of the same sequence with a nonsense mutation in the fourth codon. The clone encoding truncated V protected *E. coli* from lambda, but the nonsense mutation abolished its GSE activity.

Antisense-oriented GSEs

Among the GSEs encoding antisense RNA, one sequenced clone (positions 1050–1470) contained an internal segment of gene *A*, involved in DNA packaging. Two other classes of antisense GSE were represented by multiple clones. One of these classes included 12 non-identical clones (14 total) encoding RNA complementary to the 5′ portion of the gene *Q*, a positive regulator of late transcription (22). All the GSEs in this class (Fig. 3A) overlapped with a naturally occurring antisense transcript P_{aQ} (approximately 220 bases long), that normally downregulates *Q* expression (1,23). In addition to the complete or partial P_{aQ} sequence, the GSEs contained variable lengths of flanking sequences extending downstream from P_{aQ}. In contrast to the flexible composition of downstream sequences, none of the GSEs initiated more than 70 bp upstream from the P_{aQ} promoter, with seven of twelve different GSEs initiating within 16 bp from each other. The absence of the upstream sequences in the P_{aQ}-containing GSEs indicates functional selection against sequences complementary to the 3′ end of *Q*.

A particularly interesting group of four different GSEs (five total) encoded almost identical antisense RNA sequences, corresponding to the 3′ end of the gene *CII* (regulator of lysogeny) and the 5′ half of gene *O* that codes for a protein involved in lambda DNA replication; the origin of replication, located in the middle of *O*, was also present in these clones (Fig. 3B). The *CII* portion of the GSEs included a naturally occurring antisense transcript termed *oop* (77 bases long), which normally serves to suppress *CII* (1,24). Overexpression of the *oop* sequence has been shown to enhance lytic infection (25), an effect opposite to that of our GSEs. To explain this paradox, we hypothesized that the *oop* portion of the GSEs, complementary to *CII*, serves to promote a strong complementary interaction between the sense and the antisense RNAs, but the actual suppression is determined by another portion of the same GSE, derived from the *O* gene. To test this hypothesis, we have generated two truncated variants of these *oop/ori* GSEs (Fig. 3B). One variant was missing a 93 bp segment including most of the *oop* sequence, but still contained the 5′ portion of gene *O* including the origin of replication. The second variant had a deletion of a 158 bp segment of *O* comprising the origin of replication but still contained the *oop* sequence and the 5′ end of *O*. Cells transformed with either of the truncated plasmids showed no detectable resistance to lambda, indicating that both of the deleted segments were crucial for the GSE activity of *oop/ori*.

Table 2. Sense-oriented GSEs (other than *Ea8.5*)

Position in lambda DNA[a]	Gene(s)[b]	GSE peptide[c]
7317–7732	FI (117), FII (117)	38–117, 1–40
8800–9390	V (256)	1–145
8848–9391	V (256)	1–145
9650–10052	G (140)	1–113
9660–10052	G (140)	1–113
14397–15138	K (198), I (223)	40–198, 1–121
22997–22688[d]	Ea47 (410)	323–410
25224–24302[d]	Ea31 (296)	81–296
37939–38359	cro (66)	1–66
38029–38389	cro (66)	1–66

[a]Numbered according to ref. 30.
[b]The total number of amino acid residues in the corresponding protein is shown in parentheses.
[c]Position of the peptide in the corresponding protein.
[d]Transcribed in the direction opposite to that of numbering.

DISCUSSION

We have tested a concept that genetic suppressor elements (GSEs), encoding dominant negative mutant proteins or inhibitory antisense RNA sequences, can be generated by random DNA fragmentation and identified by expression selection. Using inhibition of lytic infection by bacteriophage lambda as a model system, we have found that DNaseI fragmentation of lambda DNA, followed by functional selection of fragments that render E. coli resistant to the virus, leads to the isolation of biologically active GSEs that inhibit viral infection by interfering with the functions of different viral or cellular genes. GSE derivation by our procedure requires no prior knowledge of the RNA or protein structure, or even the nature of the gene(s) to be suppressed.

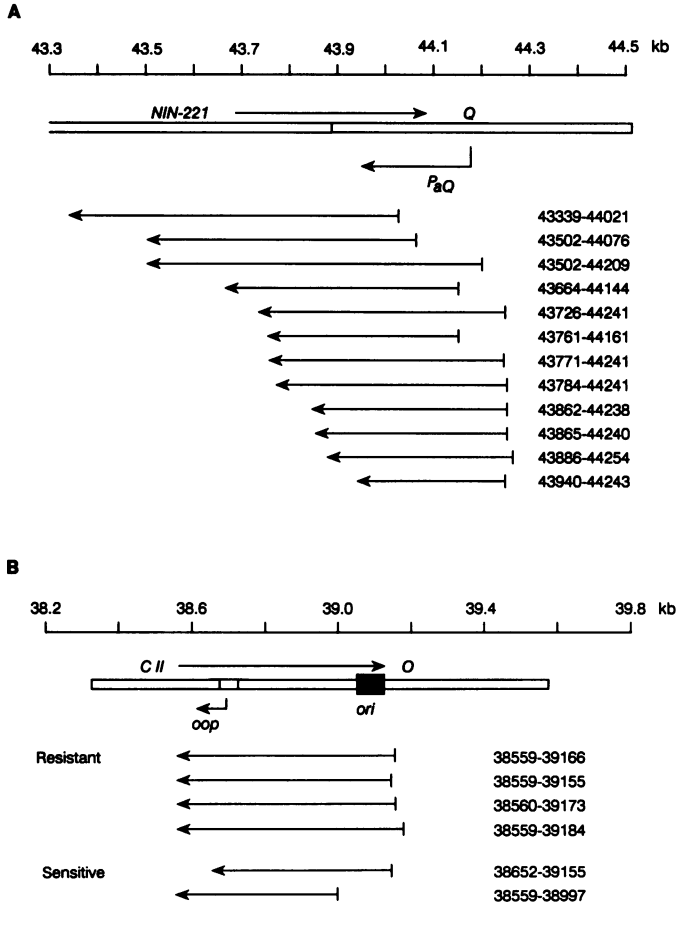

Figure 3. A. Distribution of the P_{aQ}-related GSEs. Arrows indicate the direction of transcription. Map position of the antisense P_{aQ} transcript is according to (23). B. Distribution of the *oop/ori* class of GSEs and the corresponding lambda resistance phenotypes. Arrows indicate the direction of transcription. Map position of the antisense *oop* transcript is according to (24). The four top clones were obtained by GSE selection. The two bottom clones were constructed by PCR synthesis using the corresponding primers.

The isolated lambda GSEs were derived from eleven different regions of the phage genome. Sequence analysis indicated that most classes of GSE acted by inhibiting the function of the corresponding genes of lambda, but at least one and possibly three classes exerted their effect by inhibiting the host gene(s) required for lambda infection. The latter GSE classes corresponded to early lambda genes with previously unknown functions. Our results suggest a biological role for these 'accessory' genes of lambda (17), that are unnecessary for either lytic or lysogenic infection. Specifically, we have demonstrated that the *Ea8.5* gene prevented phage infection by blocking the synthesis of the LamB phage receptor at the RNA level. Truncated forms of two other 'accessory' genes, *Ea31* and *Ea47*, are also likely to interfere with the phage entry, since these GSEs prevented infection with lambda but not with a recombinant phage carrying the host range determinant of ϕ80, and they failed to inhibit induction of the lambda lysogen. *Ea31* and *Ea47* sequences, however, did not inhibit maltose metabolism, indicating that these GSEs blocked the infection by a mechanism different from that of *Ea8.5*.

Suppression of the expression, processing or availability of the cellular receptor is a known evolutionary mechanism developed by different groups of viruses for preventing superinfection with a second virus (26,27), but this function has not been previously associated with the lytic cycle of lambda. It seems reasonable to suggest that the normal function of *Ea8.5* and other genes capable of inhibiting lambda infection is to prevent superinfection at an early stage of the lytic cycle. Inhibition of LamB synthesis at the RNA level by *Ea8.5* may be supplemented by the function of *Ea31*, *Ea47*, and possibly some other 'accessory' genes, whose products may interfere directly with either LamB or some other cellular proteins involved in phage adsorption or penetration.

Several other results of GSE analysis raise new questions concerning the biology of lambda infection. One unexpected observation was the effect of the GSEs derived from the structural genes of lambda, that should selectively interfere with the assembly of viral particles at the late stage of the infection. Nevertheless, these GSEs not only blocked the production of viral progeny but also prevented cell killing after phage infection at high m.o.i. Another paradoxical finding was the prevention of lysis by antisense GSEs containing a 'native' antisense RNA sequence, *oop*, that normally serves to enhance rather than suppress the lytic infection. This *oop/ori* class of antisense RNA, represented by several nearly identical clones, required both a segment corresponding to the origin of lambda DNA replication, and the *oop* sequence for its suppressive action. The strong sequence specificity and the very high efficiency of suppression by this class of GSE raise a question whether *oop/ori* antisense RNA may exist in nature as a presently unknown regulatory product of lambda. The specific function of the *ori* portion of these GSEs and its conceivable effect on DNA replication also represent an interesting issue for future studies.

A general question in the area of targeted gene suppression is whether antisense RNAs or dominant negative mutants constitute more efficient inhibitors. In the present study, we addressed this question in the lambda system by generating a representative GSE library, where both types of elements were placed into the same vector and selected for biological activity under the same conditions. In the starting library, antisense RNA-expressing clones for any given gene were more abundant than clones expressing truncated proteins with potential dominant negative activity, since most of the sense-oriented clones inserted out of frame with the ATG codon of the linker would not give rise to truncated normal proteins. Nevertheless, biologically active GSEs derived from the essential lambda genes encoded in most cases truncated proteins rather than antisense RNA. In fact, aside from the GSEs that incorporated evolutionarily selected 'native' antisense sequences, there was only one antisense GSE in the entire set. The observed limited efficiency of suppression by antisense constructs is in agreement with previous observations on phage lambda (7) and other organisms (5,6), and it underscores the importance of dominant negative mutant proteins as efficient tools for gene suppression (3).

The concept of using truncated proteins as dominant negative mutants (3) is based on the idea that proteins possessing domains involved in interactions with different molecules may form defective multimolecular aggregates if one of their domains is deleted or altered. In our analysis of sense-oriented GSEs, we have asked how precise should be the deletion that gives rise to a dominant negative mutant. For two structural genes of the phage (*V* and *G*), we have found two independent GSE clones encoding truncated forms of each of the corresponding proteins. Surprisingly, in both cases the truncated portions of the proteins coincided to a single amino acid between the two GSEs. This result indicates extremely strong structural limitations of truncated proteins that act as dominant negative mutants and shows that our use of a non-specific procedure for DNA fragmentation was essential for the isolation of this type of GSEs. Thus, selection of random fragments with biological activity provides a new approach to the delineation of functional protein domains.

Strong sequence selectivity was also associated with the two major classes of antisense RNA-expressing GSE. In particular, examination of the P_{aQ}-containing antisense GSEs indicates exclusion of sequences complementary to the 3′ portion of *Q* RNA and located more than 70 bp upstream from the P_{aQ} promoter. The apparent negative effect of specific RNA sequences on antisense inhibition may explain the failure of some full-length antisense constructs to inhibit the expression of corresponding genes. In contrast, the example of the *oop/ori* class of GSEs shows cooperative interaction of two antisense RNA elements, derived from different cistrons of a polycistronic message.

Identification of antisense RNA sequences capable of suppressing a target gene is a major goal in designing antisense RNA vectors for gene therapy and oligonucleotides that would have the desired suppressive effect in a chemically synthesized form. There are as yet no reliable procedures for predicting which antisense RNA sequences would be active in biological assays. The results of our study indicate that random fragment selection provides such a procedure. In this regard, it would be important to determine the minimum length of antisense GSEs that can be isolated by random fragment selection.

Our work with bacteriophage lambda may serve as a prototype for similar studies aimed at the derivation of GSEs providing the resistance to various pathogens (28). Furthermore, the same strategy is applicable to many other selectable phenotypes associated with decreased gene function. Such phenotypes may include, for example, changes in the expression of specific antigens, neoplastic transformation associated with inhibition of tumor suppressor genes, and resistance to drugs whose cytotoxic action is potentiated by a target enzyme. Thus, we have used the latter selection strategy to obtain a series of sense- and antisense-oriented GSEs suppressing the function of human topoisomerase II, a common target for many anti-cancer drugs (A.V. Gudkov, C. Zelnick, A. Kazarov, D.P. Suttle, W.T. Beck

and I.B.R., manuscript in preparation). We have also used random fragment libraries to suppress several other cellular and viral targets in mammalian cells (T.A.H., E.B. Mechetner, A.V. Gudkov and I.B.R., unpublished data).

The results of the present study show that the random fragment selection strategy not only allows one to derive efficient GSEs, but also leads to identification of novel genes associated with specific phenotypes. Expression selection of random DNA fragments, derived from total cDNA or genomic DNA, would provide a general approach to cloning of recessive genes. Antisense cDNA libraries (29) provide an alternative route to the same goal, but their efficiency is limited by the fact that not all the genes can be adequately inhibited by antisense RNA (8). Furthermore, random fragment selection allows one to identify not only the relevant genes, but also specific functional domains of the corresponding proteins.

ACKNOWLEDGEMENTS

We would like to thank N.C. Franklin for the gift of $h^{80}imm$ λ, J. W. Roberts for *E. coli* K12 C600 lysogen, T. Kolesnikova and D. Tarassiewicz for assistance with some experiments, and A. Dayn, A. Gudkov, S. Mirkin and W. Szybalski for helpful discussions. Supported by grants CA39365 and CA40333 from the National Cancer Institute and a Faculty Research Award from the American Cancer Society, Inc. (I.B.R.).

REFERENCES

1. Takayama, K.M. and Inouye, M. (1990). Crit. Rev. Biochem. Mol. Biol., **25**, 155–184.
2. Cameron, F.H. and Jennings, P.A. (1989). Proc. Natl. Acad. Sci. USA., **86**, 9139–9143.
3. Herskowitz, I. (1987). Nature, **329**, 219–222.
4. Sullenger, B.A., Gallardo, H.F., Ungers, G.E. and Gilboa, E. (1990). Cell **63**, 601–698.
5. Kerr, S.M., Stark, G.R. and Kerr, I.M. (1988). Eur. J. Biochem., **175**, 65–73.
6. Leiter, J.M., Krystal, M. and Palese, P. (1989). Virus Res., **14**, 141–159.
7. Hasan, N., Somasekhar, G. and Szybalski, W. (1988). Gene, **72**, 247–252.
8. Van der Krol, A.R., Mol, J.N. and Stuitje, A.R. (1988). Biotechniques, **6**, 958–976.
9. Daugherty, B.L., Hotta, K., Kumar, C., Ahn, Y.H., Zhu, J. and Pestka, S. (1989). Gene Anal. Tech., **6**, 1–16.
10. Rhodes, A. and James, W. J. (1990). J. Gen. Virol., **71**, 1965–1974.
11. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
12. Amann, E. and Brosius, J. (1985). Gene, **40**, 183–190.
13. Berg, D.E. (1971). In Hershey, A.D. (ed.), The Bacteriophage Lambda. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 667–678.
14. Lin, R.I.-s. and Schjeide, O.A. (1969). Anal. Biochem., **27**, 473–483.
15. Debarbouille, M., Cossart, P. and Raibaud, O. (1982). Mol. Gen. Genet., **185**, 88–92.
16. Janin, J. and Wodak, S.J. (1983). Mol. Biol., **42**, 21–78.
17. Court, D. and Oppenheim, A. (1983). In Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (eds.), Lambda II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 251–278.
18. Franklin, N.C. (1967). Genetics, **57**, 301–318.
19. Randall-Hazelbauer, L. and Schwartz, M. (1973). J. Bacteriol., **116**, 1436–1446.
20. Boulain, J.C., Charbit, A. and Hofnung, M. (1986). Mol. Gen. Genet., **205**, 339–348.
21. Gussin, G., Johnson, A., Pabo, C. and Sauer, R. (1983). In Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (eds.), Lambda II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 93–122.
22. Friedman, D. and Gottesman, M. (1983). In Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (eds.), Lambda II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 21–52.
23. Hoopes, B.C. and McClure, W.R. (1985). Proc. Natl. Acad. Sci. USA, **82**, 3134–3138.
24. Krinke, L. and Wulff, D.L. (1987). Genes Dev., **1**, 1005–1013.
25. Takayama, K.M., Houba-Herin, N and Inouye, M. (1987). Mol. Gen. Genet., **210**, 184–186.
26. Simon, K.O., Cardamone, J.J., Whitaker-Dowling, P.A., Youngner, J.S. and Widnell, C.C. (1990). Virology, **177**, 375–379.
27. Garcia, J.V. and Miller, A.D. (1991). Nature, **350**, 508–511.
28. Baltimore, D. (1988). Nature, **335**, 395–396.
29. Deiss, L.P. and Kimchi, A. (1991). Science, **252**, 117–120.
30. Daniels, D., Schroeder, J., Szybalski, W., Sanger, F. and Blattner, F. (1983). In Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (eds.), Lambda II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 467–518.