Engineered Mutagenesis

Isolation of Mutants of an Animal Virus in Bacteria

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Summary. Mutants of animal viruses can be isolated in bacteria by recombinant DNA methods. Since no viral functions are required for propagation of recombinants in bacteria, viral mutants with lethal changes in *cis*- or *trans*-acting elements can be isolated, as well as partially or conditionally defective mutants. In the case of viruses with small DNA genomes, such as the tumorigenic simian virus 40 (SV40), the entire viral DNA can be inserted into the bacterial plasmid pBR322 and cloned in *Escherichia coli*. Recombinant plasmids with a single copy of SV40 DNA cause morphological transformation of mouse cells in culture with the same efficiency as SV40 DNA isolated from virus-infected monkey cells, but the recombinant DNA is noninfectious and replicates poorly in permissive cells. However, SV40 DNA excised from the plasmid replicates as well as authentic viral DNA and is fully infectious. SV40 mutants with small deletions or base substitutions have been isolated by in vitro site-specific or random local mutagenesis of recombinant DNA followed by cloning in *E. coli*. Many of the mutants thus isolated are defective in specific viral functions.

Genetic analysis of viruses involves the isolation and characterization of mutants, that is, viruses with changes in particular elements of the viral genome. The traditional way to isolate such mutants is to treat the virus or its nucleic acid with a mutagenic agent and to select from the resulting randomly mutagenized virus population mutants with some desired phenotype, for example, temperature sensitivity or modified host range. Sites of mutation would then be localized by complementation or recombination between pairs of different isolates. Eventually a detailed genetic map of the viral genome can be constructed whereby mutational sites are correlated with defects in virus development, changes in the effect of the virus on its host cell, or with structural and functional changes in specific gene products.

Recent developments in molecular genetics, notably the availability of restriction endonucleases, molecular cloning of DNA in bacteria, and local in vitro mutagenesis have provided a more direct and rapid way to construct and map mutants, including those with lethal mutations, in any biologically active molecule of DNA such as a viral genome or selected segments of a genome. In this article we describe the application of these methods to the isolation of mutants of the small tumor virus, simian virus 40 (SV40), which has served as a model for studies of DNA replication and gene expression in mammalian cells, and for probing the mechanisms of tumorigenesis.

SV40 replicates in the nuclei of infected monkey kidney cells and it transforms rodent cells to tumorigenicity in cell culture or in the living animal. The genome of the virus is a molecule of covalently closed, circular duplex DNA made up of 5243 nucleotide pairs whose entire sequence has been determined (*1*). The location of known genetic elements along the viral DNA is diagramed in Fig. 1, and a brief description of gene products is given in the legend. We call particular attention to the gene for T antigen, a protein involved in viral DNA replication, stimulation of cellular DNA synthesis, regulation of transcription, and transformation of cells to tumorigenicity. In an effort to define functional domains of the T antigen by genetic means, we set out to isolate in *Escherichia coli* a set of SV40 mutants with alterations in different parts of the T antigen gene that could be tested for various T antigen functions.

Outline of the procedure for isolating mutants. Figure 2 shows the scheme used in our laboratory for isolating SV40 mutants in *E. coli* (*2*). Similar procedures are in use in many other laboratories (*3*). The first step is the isolation of a pBR322/SV40 recombinant plasmid containing the entire SV40 genome with an intact T antigen gene. The recombined DNA is then modified in vitro by an efficient site-specific or random local mutagenesis procedure, resulting in deletions or small insertions of DNA or base substitutions. Individual mutant DNA's are next cloned by transformation of *E. coli* and isolation of recombinant-containing colonies, and the site of mutation in each cloned recombinant is determined by restriction analysis and nucleotide sequencing. The biological activities of mutant DNA's are then assessed by transfection of monkey cells or mouse cells in culture with recombinant DNA or with the excised viral DNA.

pBR322/SV40 Recombinants. To prepare a suitable recombinant plasmid con-

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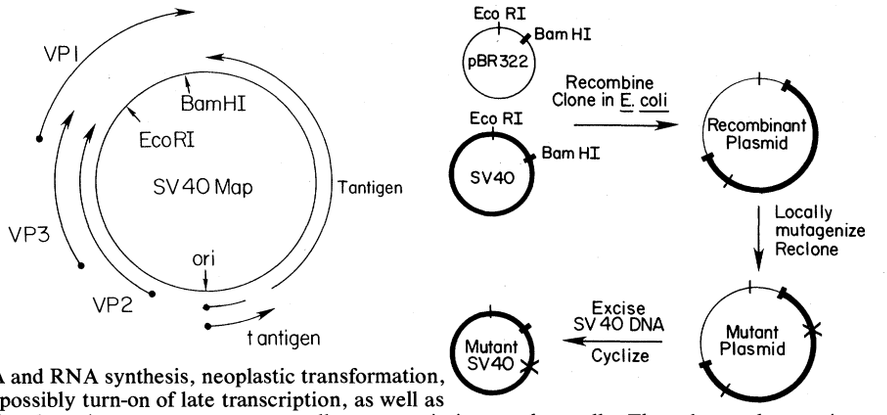


Fig. 1 (left). A functional map of the SV40 genome. The duplex circular genome is represented by the inner circle showing the unique origin of DNA replication *(ori)* and the positions of the single cleavage sites for Eco RI and Bam HI. Around the outside is depicted the approximate coding regions for the viral late proteins VP1, VP2, VP3, and the early gene products large T antigen and small t antigen; the direction of their transcription 5′ to 3′ is given by the arrows. The T antigens are synthesized early after infection of monkey cells and are found in SV40 transformed cells. T antigen, a multifunctional protein of about 94,000 daltons, is required for the initiation of viral DNA replication, stimulation of cellular DNA and RNA synthesis, neoplastic transformation, negative control of early region transcription and possibly turn-on of late transcription, as well as supplying an adenovirus helping function to enable adenoviruses to grow on normally nonpermissive monkey cells. The other early protein, t antigen, has a molecular weight of about 17,000, and its function is at present unclear since it is not required for lytic growth. The gene products VP1, VP2, and VP3 are the structural proteins of the virus capsid and are synthesized late in infection (*1*). Fig. 2 (right). Isolation of SV40 mutants in *E. coli*. Restriction endonuclease-digested viral DNA was ligated into pBR322 DNA at the corresponding restriction site with T4 polynucleotide ligase at a 2 to 1 molar ratio of ends (viral DNA/plasmid DNA) by means of standard procedures. After *E. coli* HB101 was transformed by the calcium chloride method (*17*), ampicillin-resistant, tetracycline-sensitive colonies (when cloning at the Bam HI site) or ampicillin-resistant, tetracycline-resistant colonies (when cloning at the Eco RI site) were picked and screened for the presence of SV40 DNA sequences by colony hybridization (*5*). Plasmid DNA was prepared from several colonies by the cleared lysate procedure (*18*) after overnight amplification in the presence of chloramphenicol (*19*). The structures of these plasmids were determined by restriction endonuclease mapping; the types of recombinants obtained via Bam HI linkage are shown in Fig. 3. Recombinant DNA from pSV-B3 was used as the starting material for the local mutagenesis described in the legend to Fig. 5. (For some experiments SV40 DNA was mutagenized prior to ligation into pBR322.) After recloning in *E. coli*, DNA prepared from ampicillin-resistant colonies was used for further study. For most biological experiments it was necessary to excise the mutant SV40 DNA from the recombinant plasmid with Bam HI and then cyclize the DNA with T4 polynucleotide ligase since the intact plasmid is not infectious (*2*).

taining the entire SV40 genome with an intact T antigen gene, SV40 DNA was recombined with pBR322 DNA via their respective Bam HI sites, thus disrupting the tetracycline resistance gene of pBR322 and leaving its ampicillin resistance gene intact (*4*). After transformation of *E. coli* and selection of ampicillin-resistant, tetracycline-sensitive colonies, those containing SV40 DNA were detected by the colony hybridization procedure of Grunstein and Hogness (*5*). Three classes of recombinant plasmids were isolated, distinguishable by restriction analysis (Fig. 3): class 1 (pSV-B3) had a single copy of SV40 DNA in the orientation shown; class 2 (pSV-B21) had a single copy of SV40 DNA with opposite orientation; and class 3 (pSV-B39) contained a head-to-tail dimer of SV40 DNA. Similar monomer-containing recombinants were also isolated by in vitro recombination at the Eco RI sites of pBR322 and SV40 DNA.

Infectivity of SV40 DNA propagated in E. coli. In order to use SV40 cloned in *E. coli* for biological studies we needed to demonstrate that SV40 propagated in *E. coli* was biologically active. We therefore tested recombinants or SV40 DNA excised therefrom for infectivity, that is, the ability to form plaques on a monolayer of monkey cells. As seen in Table 1, recombinants with a single copy of SV40 DNA were noninfectious, whereas pSV-B39 with two copies of SV40 DNA in tandem was infectious, presumably due to liberation of free SV40 DNA by cell-mediated homologous recombination (*6*). When SV40 DNA was excised from pSV-B3 by Bam HI digestion, the DNA was as infectious as comparable DNA (linear or cyclized forms) prepared in monkey cells, and virus from each of several plaques was found to produce SV40 DNA indistinguishable by restriction analysis from wild-type SV40 DNA. Therefore, SV40 DNA is fully infectious after propagation in *E. coli*. Similar results were reported previously for polyoma DNA (*7*).

Replication of recombinant DNA in monkey cells. Since we intended to screen SV40 mutants for the DNA replication function of mutant T antigens, we next investigated conditions for assessing viral DNA synthesis in monkey cells transfected with recombinant DNA or viral DNA excised from the recombinant plasmid. For this purpose we used the sensitive detection method of Southern (*8*) coupled with restriction of the viral or recombinant DNA with enzymes that distinguish between DNA propagated in *E. coli* HB101 and DNA propagated in monkey cells. The single Bcl I site in SV40 DNA (TGATCA) (T, thymine; G, guanine; A, adenine; C, cytosine) is methylated in *dam*$^+$ *E. coli* HB101 (TGmeATCA) (me, methyl), rendering the DNA resistant to Bcl I (*9*). On the other hand, SV40 DNA propagated in monkey cells contains no sites for Dpn I (GmeATC) (*10*), whereas SV40 DNA propagated in *E. coli* contains eight Dpn I sites. When monkey kidney cells were infected with pSV-B3 DNA and the low molecular weight DNA was extracted from the infected cell monolayer, re-

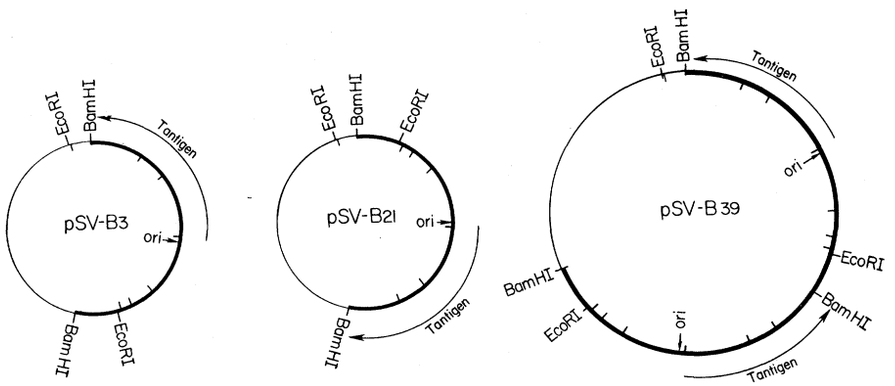
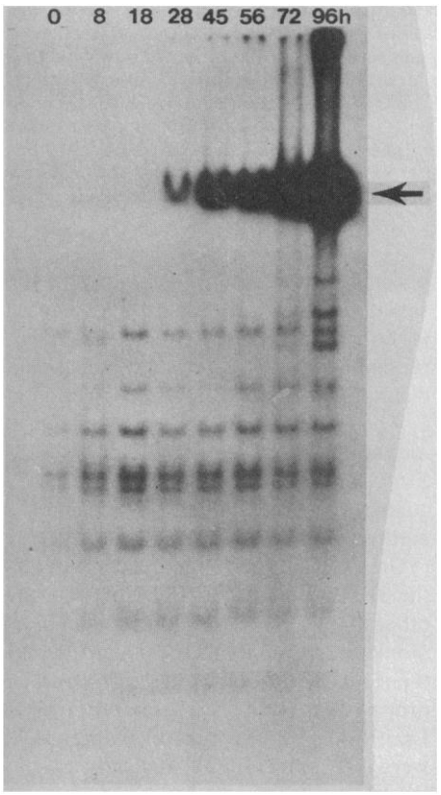


Fig. 3. Structures of three SV40/pBR322 recombinant plasmids based on restriction analysis. The thick lines represent SV40 DNA and the thin lines pBR322 DNA. The origin of replication of SV40 DNA is indicated *(ori)*, as is the T antigen gene. Marks inside the circle refer to the Hind III sites.

stricted with Bcl I, and analyzed by gel electrophoresis, Bcl I-sensitive recombinant DNA (that is, newly replicated DNA) was barely detectable. (Similar results were found with recombinants joined at the Eco RI site.) In contrast, when free SV40 DNA was generated from the recombinant prior to transfection, newly formed SV40 DNA was readily detectable (Fig. 4). In other experiments we found that cyclized SV40 DNA isolated from pSV-B3 replicated to about the same extent as circular SV40 DNA isolated from infected monkey cells, namely some 100 times better than did the intact recombinant plasmid (*11*). From this series of experiments it is evident that the replicative function of T antigen (and of the viral replication origin) can be readily assessed after enzymatic excision of SV40 DNA from the recombinant plasmid. It is also evident that the pBR322 component of the recombinant plasmid has an inhibitory effect on its replication in monkey cells. The mechanism of this effect remains to be determined.

Morphological transformation of mouse cells by recombinant DNA. The ability of recombinant SV40 DNA to transform mouse cells in culture was tested (Table 2). As is seen in the table, pSV-B3 DNA, pSV-B21 DNA, and pSV-B39 DNA were as active in transforming BALB/c-3T3 mouse cells as comparable forms of SV40 DNA isolated from infected monkey cells. Therefore, recombinant plasmids can be tested directly for the transforming activity of T antigen.

Isolation of deletion mutants. Figure 5 outlines some of the ways of constructing mutants of recombinant plasmids. Our initial experiments were designed to isolate a series of deletion mutants spanning the entire T antigen gene. For this purpose pSV-B3 DNA was randomly linearized by making one single-strand scission per molecule with pancreatic



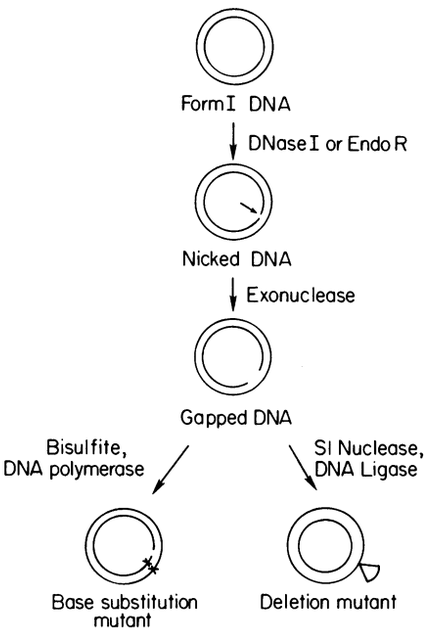


Fig. 4 (left). Replication of cloned SV40 DNA in monkey cells. Subconfluent BSC-40 monkey cells in 5-cm dishes were transfected by the DEAE-dextran method (*20*) with recombinant DNA (10 ng per dish) which had been restricted and cyclized to excise the SV40 DNA sequences from the vector. At various times after infection (noted above each slot) the cells were harvested, and the low molecular weight DNA was isolated (*21*). DNA was purified by proteinase K digestion, phenol extraction, and alcohol precipitation. Pellets were dissolved in 50 μl of 0.2 m*M* EDTA (*p*H 7.5), and 5 μl was used for digestion by Dpn I plus Bcl I. Digestion products were fractionated in 1.2 percent agarose gels and transferred to nitrocellulose filters by the Southern method (*8*). SV40 DNA sequences were detected by hybridization with ^{32}P-labeled SV40 DNA in vitro by nick translation (*22*) followed by autoradiography. The species of DNA noted by the arrow is replicated SV40 DNA linearized by Bcl I. The smaller fragments are Dpn I digestion productions of the input DNA. Fig. 5 (right). In vitro construction of mutants. Supercoiled DNA (form I DNA) is nicked once at a selected site with a restriction endonuclease or randomly with pancreatic deoxyribonuclease I, each in the presence of ethidium bromide (*23*). The nick is then extended into a small gap by means of the exonucleolytic activity of DNA polymerase I from *Micrococcus luteus*, which creates a gap of about 6 nucleotides (*13*). The gap creates a target for local mutagenesis with sodium bisulfite, which deaminates cytosine to uracil only in unpaired regions of the DNA (*24*). When the gap is subsequently filled in, an A · U base pair (XX) is substituted for an original G · C pair. To construct deletion mutants gapped DNA is linearized with a single-strand specific nuclease (S1 or BAL-31) (*25*) which digests at the gap, and the resulting shortened linear molecules are cyclized after 5′ phosphorylation of their ends with T4 polynucleotide kinase. Individual mutants of either class are then isolated by recloning in *E. coli*.

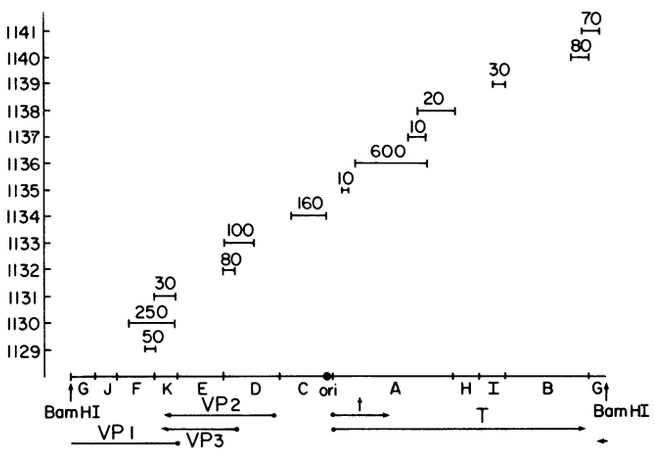


Fig. 6. Map positions of deletions in SV40 mutants isolated in *E. coli*. The extent and location of each deletion were inferred from restriction digest patterns. On the vertical axis is the mutant number and on the horizontal axis, the Hind II + Hind III restriction map of SV40 DNA opened at the Bam HI site, the origin of replication (*ori*), and the coding regions for known SV40 proteins. Numbers above the horizontal bars indicate the approximate sizes of the deleted segments in base pairs, and the left and right ends of the bars indicate the maximal limits of the deletion.

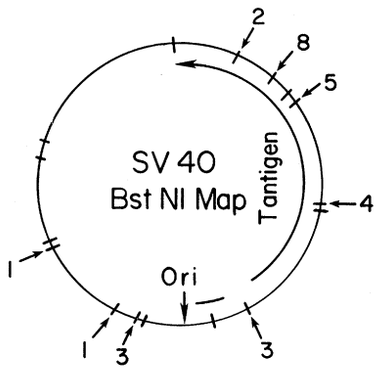


Fig. 7. Map position of base substitution mutants within Bst NI sites of SV40 DNA. The mutants were constructed as follows: SV40 form I DNA was nicked and gapped at a Bst NI site and incubated with sodium bisulfite for 9 hours [estimated to deaminate about 60 percent of all exposed cytosine residues (*13*)]. This DNA was joined to pBR322 DNA via their Bam HI sites and individual recombinants isolated and screened for missing Bst NI sites. The numbers outside the circle indicate the number of independent isolates with mutations in each of the Bst NI sequences noted. The clustering of mutations is presumably due to preferential nicking at particular Bst NI sites.

deoxyribonuclease in the presence of ethidium bromide, extending the nick to a small gap by means of the 5′ → 3′ exonucleolytic activity of DNA polymerase I of *Micrococcus luteus*, and excising the single-stranded segment thus exposed with a single-strand specific nuclease. The resulting shortened linear molecules were then cyclized by DNA ligase after phosphorylation with polynucleotide kinase. The ligation product was used to transform *E. coli* HB101, and ampicillin-resistant colonies were screened for the presence of recombinant plasmids with small deletions by restriction of isolated plasmid DNA with several different restriction endonucleases. From these results the approximate map positions and sizes of the deletions could be determined. Of the first 20 colonies screened in this way, 13 contained deletions entirely within the SV40 segment of pSV-B3, 5 in the pBR322 segment, and 2 at a pBR322/SV40 junction. Map positions of the SV40 deletions are shown in Fig. 6; seven are in the early segment of SV40 DNA covering most of the coding sequence for T antigen. The biological activities of these mutants will be reported elsewhere (*12*).

Isolation of mutants with base substitutions. To isolate mutants with base substitutions in different regions of the T antigen gene, we applied the local mutagenesis procedure of Shortle and Nathans (*13*) whereby a small gap in DNA is created at a desired restriction site, and exposed cytosine (C) residues are deaminated to uracil (U) by treatment with sodium bisulfite (Fig. 5). The resulting C to U change is equivalent to a base pair substitution at the selected site. For our initial experiments we constructed mutants with substitutions at Bst NI sites in SV40 DNA. Bst NI recognizes the sequence

$$\begin{array}{l} CC\downarrow T\ GG \\ GG\ A\uparrow CC \end{array}$$

cutting as noted by the arrow (*14*). Therefore, a 5′ to 3′ gap starting at Bst NI-nicked DNA exposes two C residues of the recognition sequence to deamination by bisulfite. Deamination would thus render that site resistant to Bst NI, allowing ready detection of mutants. (Note that six different mutations are possible at each site.) Of 45 colonies of *E. coli* HB101 transformed to ampicillin resistance by mutagenized recombinant DNA, 27 had detectable loss of a Bst NI site and one lost two sites; 21 had single mutations in the T antigen gene (Fig. 7). Some of these mutants were noninfectious, whereas others were partially or conditionally defective. Additional T antigen mutants have been isolated by variations of the local mutagenesis procedure (*15*).

Generality of the methods. It is evident that in vitro local mutagenesis of recombinant DNA, coupled with the isolation of mutants in *E. coli*, greatly simplifies the genetic analysis of a biologically active DNA molecule. Methods are now available for directing mutagenesis to any desired segment of DNA (*16*), and since propagation of a recombinant plasmid in *E. coli* is not dependent on the function of the DNA insert, mutants with totally defective *cis*- or *trans*-acting elements can be readily isolated. In the case of large viral genomes or functional segments of cellular DNA, individual restriction fragments can be mutagenized, cloned, and analyzed and then reinserted into the molecule of origin. Application of these methods should help define the nature of genetic regulatory elements and functional domains of proteins as well as aid in the production of proteins with altered properties.

Table 1. Infectivity of pBR322/SV40 recombinant DNA. Infectivity was assayed by transfection of monolayers of BSC-40 monkey cells with DNA-DEAE-dextran mixtures and counting the number of virus plaques formed after 12 days at 37°C. Infectivity is expressed as plaque-forming units (PFU) per nanogram of SV40 DNA present in each sample. Each value is the average of three dishes. Three different forms of DNA were tested: form I, DNA linearized by restriction with Bam HI (L_{Bam}), and DNA cut by Bam HI and cyclized with DNA ligase (L_{Bam}, cyclized). SV40 refers to DNA isolated from infected monkey cells. Each DNA sample was assessed by gel electrophoresis or electron microscopy.

DNA	Infectivity (PFU/ng)
SV40 I	237,248, 209,170
pSV-B3 I	< 0.006
pSV-B21 I	< 0.009
pSV-B39 I	32
SV40 L_{Bam}	22
pSV-B3 L_{Bam}	18
pSV-B21 L_{Bam}	30
pSV-B39 L_{Bam}	24
SV40 L_{Bam}, cyclized	183
pSV-B3 L_{Bam}, cyclized	202
pSV-B21 L_{Bam}, cyclized	248
pSV-B39 L_{Bam}, cyclized	237

Table 2. Transformation of mouse cells by recombinant DNA. BALB/c-3T3 cells growing in 5-cm dishes were transfected (*26*) with 2.5 μg of the DNA species indicated. Twenty-four hours after infection, 2.3×10^5 cells were transferred to duplicate dishes for focus assay (*27*), and 1.8×10^5 cells were transferred to soft agar dishes for the colony assay (*28*). Foci were scored after 4 weeks at 37°C, and colonies after 4.5 weeks. Each value is the average of four dishes.

DNA	Foci/μg	Colonies/μg
SV40	6.3	4.6
pSV-B3	7.9	5.5
pSV-B21	7.0	7.2
pSV-B39	6.9	7.8

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Directed Deletion of a Yeast Transfer RNA Intervening Sequence

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Perhaps the most surprising discovery in the initial studies of eukaryotic gene structure has been that many genes contain interruptions in the coding sequences. The origin and the function of these intervening sequences (IVS or introns) are not yet well understood but are the subject of intense investigation. In this article we test whether or not one of these introns, located in a yeast tRNA suppressor gene, is essential for the expression of the gene.

Summary. Many eukaryotic genes contain intervening sequences, segments of DNA that interrupt the continuity of the gene. They are removed from RNA transcripts of the gene by a process known as splicing. The intervening sequence in a yeast tyrosine transfer RNA ($tRNA^{Tyr}$) suppressor gene was deleted in order to test its role in the expression of the gene. The altered gene and its parent were introduced into yeast by transformation. Both genes exhibited suppressor function, showing that the intervening sequence is not absolutely essential for the expression of this gene.

In *Saccharomyces cerevisiae* there are eight tyrosine transfer RNA ($tRNA^{Tyr}$) genes (*1*) and each can be converted to either ochre or amber suppressor, a dominant phenotype (*2*). Each of these genes contains an intervening sequence of 14 base pairs (bp) located 1 bp to the 3′ side of the anticodon (*3*). In the expression of the tRNA gene the entire gene is transcribed to form a tRNA precursor (Fig. 1). Then in the enzymatic process of splicing the intervening sequence is removed and the half molecules are rejoined to form the mature tRNA molecule (*4*).

It is not yet known whether splicing is an essential step in the synthesis of yeast $tRNA^{Tyr}$. Not all tRNA genes contain introns (in yeast we estimate that perhaps one-tenth of the tRNA genes contain intervening sequences), so splicing is not an obligatory step in the synthesis of all tRNA. The presence of an intervening sequence in a precursor gives that RNA molecule a distinctive structure so that one could invoke an essential role for the intervening sequence in processing, base modification, or transport from nucleus to cytoplasm. Alternatively, the intron could be part of the transcriptional recognition site. This latter possibility is raised from the results of Brown and his colleagues who have shown that transcriptional initiation of the *Xenopus 5S* gene by RNA polymerase III requires sequences in the middle of the gene. Eukaryotic tRNA genes are also transcribed by RNA polymerase III.

To test whether the intervening sequence is essential, we have engineered the precise deletion of the intron from the $tRNA^{Tyr}$ SUP6 ochre suppressor gene. The ochre suppressor gene can be introduced into yeast via transformation (*6*) so the phenotype of the altered gene could be compared with that of the parent.

Technique for engineering specific deletions. The most precise and versatile way to introduce specific mutations in DNA is through the use of short synthetic oligonucleotides as site-specific mutagens. In this technique an oligonucleotide is synthesized which contains the desired mutation (point mutation, deletion or insertion). The oligonucleotide is used as a primer to direct the synthesis of DNA on a single-stranded circular DNA template. The oligonucleotide is thereby incorporated into the resulting circular DNA molecule forming a heteroduplex. Transformation into *Escherichia coli* followed by DNA replication resolves this heteroduplex into mutant and parental genes.

Until now this technique has only been used with ϕX174 DNA. Specific transition (*7*), transversion (*8*), and single-base deletion (*9*) mutations have been constructed.

We describe below a mutagenic strategy that is more general than previous strategies and differs from them in three ways:

1) The DNA to be mutagenized is double-stranded, allowing the use of recombinant plasmid DNA (in this case the yeast SUP6 gene cloned in the plasmid pBR322).

2) We demonstrate that large, specific deletions can be engineered. In this case

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