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A novel technique for the rapid preparation of mutant RNAs

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ABSTRACT

We have developed a novel *in vitro* mutagenesis technique that allows us to introduce mutations at the level of double-stranded DNA and then transcribe the mutant DNA directly. The technique is useful for those wishing to produce recombinant RNA, particularly if the desired recombinant is the result of an insertion or deletion. It is also useful for the preparation of 3'-truncated RNAs with a defined end. The technique is not dependent on the presence of a convenient restriction site within the target gene, and does not involve construction of a clone or amplification of the mutant DNA within a bacterial host. It is intended as a simple and rapid method for the preparation of roughly 100-200 pmol of mutant RNA, which would be sufficient for obtaining sequence information and assessing the functional consequences of the mutation.

INTRODUCTION

Studies concerning the molecular biology of RNA frequently make use of site-directed mutagenesis techniques. These techniques can be cumbersome and time consuming, particularly when one wishes to survey a number of mutations by examining their effect at the level of RNA. We have developed a novel mutagenesis technique that is performed entirely *in vitro*. The technique does not involve construction of a clone or amplification of the mutant DNA within a bacterial host. Beginning with wild type DNA and a mutagenic oligodeoxynucleotide, one can obtain purified mutant RNA in less than two working days. This compares with five or six days required to carry out oligonucleotide-directed mutagenesis using a single-stranded bacteriophage vector (1).

The novel mutagenesis technique is based on two useful properties of T7 RNA polymerase. This enzyme is able to produce several hundred moles of RNA transcript per mole of DNA template (2) and is able to operate on templates that are single-stranded (3). The enzyme requires a double-stranded promoter, but beyond position +2 (relative to the site of initiation) is essentially indifferent to the absence of the non-coding strand (4). We reasoned that if T7 RNA polymerase is able to make the transition from a double-stranded to a single-stranded template at position +2, then it might be able to make the transition from a single-stranded template back to a double

strand. This turned out to be the case. As a result, one is able to incorporate mutagenic oligonucleotides into the coding strand and transcribe the partially mismatched template directly.

We have used this technique to produce RNAs containing defined insertions, deletions, or substitutions of virtually any length. The mutagenesis procedure is performed in a single reaction vessel, beginning with 10-20 µg (~ 3-6 pmol) of plasmid DNA and ending with 100-200 pmol of a purified mutant RNA that is typically 300-500 nucleotides in length. The transcription products are loaded directly onto a polyacrylamide gel and are purified by electrophoresis and subsequent column chromatography. Since the mutant RNAs are distinguished from wild type by their electrophoretic mobility, the technique is best suited for mutations that result in a discernable size difference between mutant and wild type or involve the use of a mutagenic oligonucleotide that hybridizes very efficiently to the target DNA. The technique does not depend on the presence of a convenient restriction site within the target gene, and, except for the T7 promoter and a restriction site located somewhere downstream from the gene, does not place any limitations on the design of the plasmid DNA.

MATERIALS AND METHODS

Nucleotides and Enzymes

Unlabeled nucleoside triphosphates, deoxynucleoside triphosphates, and dideoxynucleoside triphosphates were purchased from Sigma. [α ^{32}P] GTP and [γ ^{32}P] ATP were from ICN Radiochemicals and [^{3}H] UTP was from New England Nuclear. Synthetic oligodeoxynucleotides were obtained from Operon Technologies and were purified by polyacrylamide gel electrophoresis and chromatography on Sephadex G-10. Restriction enzymes were from New England Biolabs, T4 polynucleotide kinase, T7 gene 6 exonuclease, T4 DNA polymerase, and T4 DNA ligase from U.S. Biochemical, and AMV reverse transcriptase from Life Sciences. T7 RNA polymerase was prepared as previously described (5), and purified according to a procedure originally developed for SP6 RNA polymerase (6). Plasmid pTT1A3, which contains a 533 base-pair fragment of *Tetrahymena* rDNA (7), and pTL-45, which contains a 5'-truncated 399 base-pair fragment of the same gene (8), were provided by T.R. Cech.

Preparation of Mutant RNAs

In a typical preparation, 10-20 µg of pTL-45 DNA was cleaved at a *Hin*dIII restriction site that lies immediately downstream from the gene for *Tetrahymena* rRNA. The cleaved DNA was added to a 100 µl volume containing 50 mM Tris (pH 8.1), 20 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 50 U T7 gene 6 exonuclease, which was incubated at 37° C for 30 min. The exonuclease was removed by three phenol extractions and the DNA was purified by ethanol precipitation. Two oligodeoxynucleotides were then hybridized to the single-stranded (minus strand) DNA; one oligonucleotide forming a perfect duplex at the 3' end of the target gene and the other forming a

partial duplex that introduces the desired mutation. Annealing was performed in a 300 µl volume containing 20 mM Tris (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, and a 5-fold molar excess of the two oligonucleotides, which was incubated at 70° C for 5 min and then steadily cooled to 30° C over 40 min. Synthesis of the mutant strand was completed by adding 40 U of T4 DNA ligase and 15 U of T4 DNA polymerase, and incubating at 37° C for 60 min in the presence of 20 mM Tris (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, and 0.5 mM (each) dNTPs. The resulting DNA was purified by ethanol precipitation, and then used to direct the transcription of mutant RNA.

Transcription took place either in a 10 µl volume containing 1 µg of mutant DNA, 2 µCi [α ^{32}P] GTP and 50 U T7 RNA polymerase or in a 400 µl volume containing 10 µg of mutant DNA, 40 µCi [^{3}H] UTP and 2,400 U T7 RNA polymerase. In either case, the transcription mixture also contained 40 mM Tris (pH 7.5), 15 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, and 1 mM (each) NTPs, and was incubated at 37° C for 90 min. T7 RNA polymerase was extracted with phenol and the transcription products were purified by ethanol precipitation. The mutant RNA was isolated by electrophoresis in a 5% polyacrylamide / 8 M urea gel, eluted from the gel, and purified by ethanol precipitation and chromatography on Sephadex G-50.

Sequencing of Mutant RNAs

The mutant RNAs were sequenced by primer extension analysis using reverse transcriptase in the presence of dideoxynucleotides (9). 1.0 pmol of [5'-^{32}P]-labeled synthetic DNA primer was annealed with 0.3 pmol of mutant RNA by incubating at 65° C for 5 min and then cooling to 30° C over 5 min. The primer-extended cDNA products were analyzed on a 10% polyacrylamide / 8 M urea sequencing gel.

RESULTS

Development of the Mutagenesis Procedure

The most widely used technique for site-directed mutagenesis involves hybridization of an oligodeoxynucleotide to single-stranded DNA, forming a partial duplex structure that contains a region of base mismatch. The oligomer strand is extended using a DNA-dependent DNA polymerase, and the resulting double-stranded DNA is used to transform bacterial cells (10,11). This technique is useful for producing a specific mutation at a defined location. However, it is awkward when one wishes to perform wholesale mutagenesis without taking the time to construct clones and harvest DNA from bacterial cells.

Introduction of the mutant DNA into a bacterial host serves two useful purposes. First, the mutation becomes fixed as a result of bacterial repair processes that resolve the region of base mismatch. Second, the mutant DNA becomes amplified as a consequence of bacterial growth, so that one can obtain an essentially unlimited supply of pure mutant DNA. Oftentimes, however, one only needs enough material to sequence the mutant and to conduct a simple assay to examine

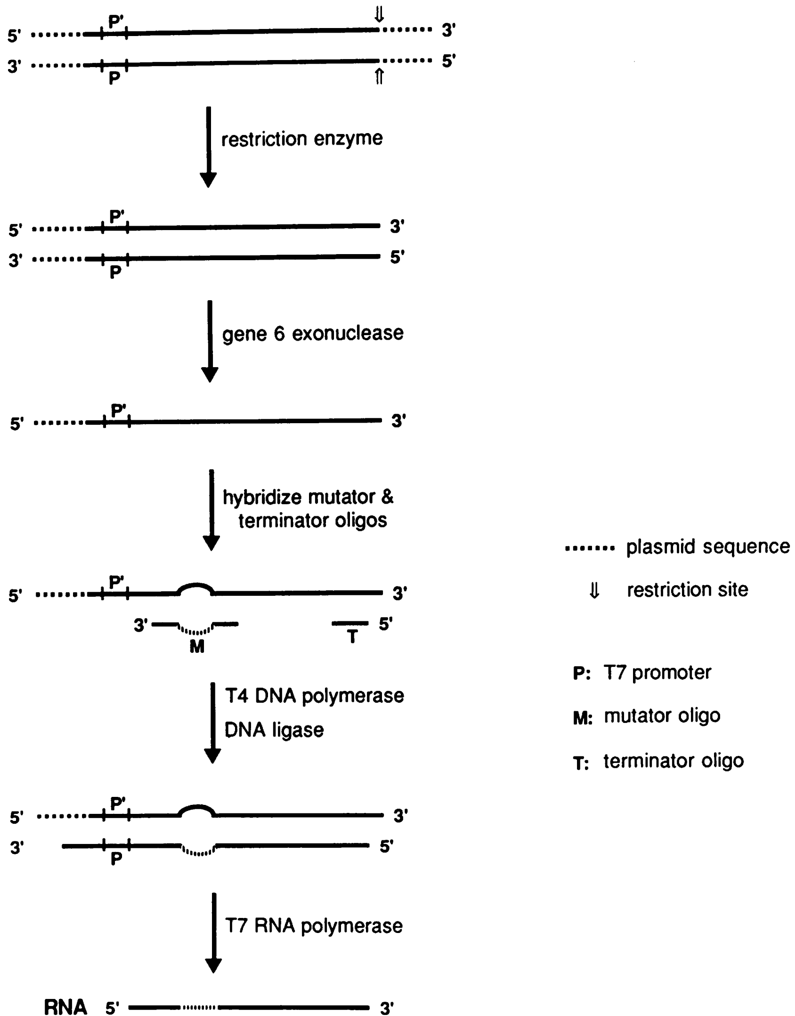

Figure 1: Outline of the mutagenesis procedure, beginning with plasmid DNA and ending with mutant RNA. The mutator oligo (M) directs an insertion, deletion, or substitution, as indicated by hatched lines within its central portion.

its functional consequences. In such instances, the time required to prepare the mutant becomes a critical factor.

We have found that the two useful aspects of bacterial transformation, fixation of the desired mutation and amplification of the mutant DNA within the bacterial host, can be met in an entirely *in vitro* reaction system that makes use of T7 RNA polymerase. This enzyme is able to transcribe

partially mismatched DNA, reading the template strand while ignoring the non-coding strand, and in doing so generates several hundred copies of RNA transcript per copy of DNA template. We have exploited these properties in order to develop a "mini-prep" method for the rapid production of mutant RNA. The method involves excising the coding strand of wild-type DNA and replacing it with a new strand that contains the desired mutation. The resulting partial duplex structure is then used to direct the transcription of mutant RNA.

In the most general form of the technique, plasmid DNA, containing a T7 promoter and the gene of interest, is cleaved at a site that lies downstream from the target gene (Fig. 1). The restriction site need not lie immediately downstream from the target gene; one can choose any unique restriction site that lies within a few hundred base pairs of the end of the gene. The cleaved plasmid is partially digested using a 5'→3' exonuclease to produce a stretch of single-stranded (minus strand) DNA. We prefer to use gene 6 exonuclease of T7 phage because of its distributive properties and because of its marked preference for duplex DNA (12). One can easily control the extent of the digestion to ensure complete removal of the coding strand of the gene as well as the plus strand of the adjacent T7 promoter. Disruption of the promoter region provides an internal selection mechanism since incomplete reconstructs will not obtain a functional promoter and will be inert in the subsequent transcription reaction.

T7 gene 6 exonuclease operates inefficiently at termini that have a 5' overhang. When using a restriction enzyme that leaves a 5' overhang, we found it necessary to increase the amount of exonuclease from 50 U to 100 U in order to ensure adequate digestion of the coding strand. Removal of a 5' overhang may, to some extent, be dependent on the sequence of the overhanging bases, so that somewhat different amounts of exonuclease may be required in certain cases.

After digestion of the coding strand, the exonuclease is removed by phenol extraction, and the DNA is purified by ethanol precipitation. Two oligodeoxynucleotides are then hybridized to the segment of single-stranded (minus strand) DNA. One, which we refer to as the "terminator oligo", forms a perfect duplex at a chosen location near the 3' end of the target gene. The other, which we refer to as the "mutator oligo", forms a partial duplex at a site of interest within the gene. The mutator oligo is designed such that it contains a central region of base mismatch flanked by two regions that form a perfect duplex. The mismatched region may be shorter or longer than the original complementary DNA, and may consist of a defined sequence or a mixture of random sequences. As in all oligonucleotide-directed mutagenesis techniques, the mutator oligo should be designed such that it can form a stable partial duplex structure at the desired location. The mutator oligo must be phosphorylated at its 5' end so that it can serve as a donor substrate for DNA ligase.

The two oligos are extended using T4 DNA polymerase and are ligated to form a template for transcription of the mutant RNA. T4 DNA polymerase is used because, unlike most other DNA-dependent DNA polymerases, it does not have strand displacement activity (13). We tested the

Klenow fragment of *E. coli* DNA polymerase I in this reaction and obtained very unsatisfactory results. We usually begin the reaction by incubating at 25° C for 5 min to give the polymerase a chance to extend the two oligos under conditions that enhance duplex stability. The reaction is completed by incubation at 37° C for 60 min, and the DNA is purified by ethanol precipitation. The precipitation step is not absolutely necessary, but tends to increase the yield in the subsequent transcription reaction.

Transcription is performed under conditions similar to those described by Milligan *et al.* (4), using a large amount of T7 RNA polymerase and high concentrations of $MgCl_2$ and the four NTPs. After phenol extraction and ethanol precipitation, the transcription products are loaded onto a polyacrylamide gel and the mutant RNA is isolated electrophoretically. Depending on how efficiently the mutator oligo hybridizes to the target DNA, there may be an appreciable amount of wild-type RNA included among the transcription products (for example, see below). For this reason, the mutagenesis technique is best suited for insertion or deletion mutations that result in a discernable size difference on the gel. In some cases, hybridization of the mutator oligo is very efficient and the amount of "revertant" wild-type RNA is negligible (again, see below). This is more likely to occur when the 5' portion of the mutator oligo forms a long stretch of stable duplex structure with the minus strand DNA. However, hybridization of the mutator oligo may also depend on features of secondary structure that are not possible to predict.

Application of the Mutagenesis Technique

We have applied the above-described mutagenesis technique to the study of a self-splicing group I intron. Working with the intervening sequence (IVS) of *Tetrahymena* pre-rRNA, we wished to produce sizeable internal deletions within the non-conserved portions of the molecule. In the present paper we focus on the mutagenesis technique itself, and present our data as an example of how the technique can be applied. In a subsequent paper (14) we will detail the effect that these and other internal deletions have on the catalytic activity of the *Tetrahymena* ribozyme.

We made two internal deletions within the *Tetrahymena* IVS (Fig. 2). M1 is a 38-nucleotide deletion from position 56 through 93 that removes structural elements P2.1 and L2.1. M2 is a 69-nucleotide deletion from position 127 through 195 that removes structural elements P5a, P5b, L5b, P5c, and L5c. The location of these deletions was chosen based on known features of group I secondary structure (15-17). The mutator oligos were hybridized to the minus strand DNA by flanking regions consisting of 11-14 complementary residues. Two terminator oligos were used, each containing 15 nucleotides. T1 hybridizes at positions 305 through 319 of the IVS and T2 hybridizes at positions +8 through +22 of the 3' exon.

The two terminator oligos were used either alone or in combination with one or both of the mutator oligos. All eight combinations were tested using pTL-45 DNA, which contains a portion of the *Tetrahymena* IVS (beginning at position 45) and 29 nucleotides of the 3' exon, inserted 6 nucleotides downstream from a T7 promoter (8). Figure 3 shows the direct transcription products

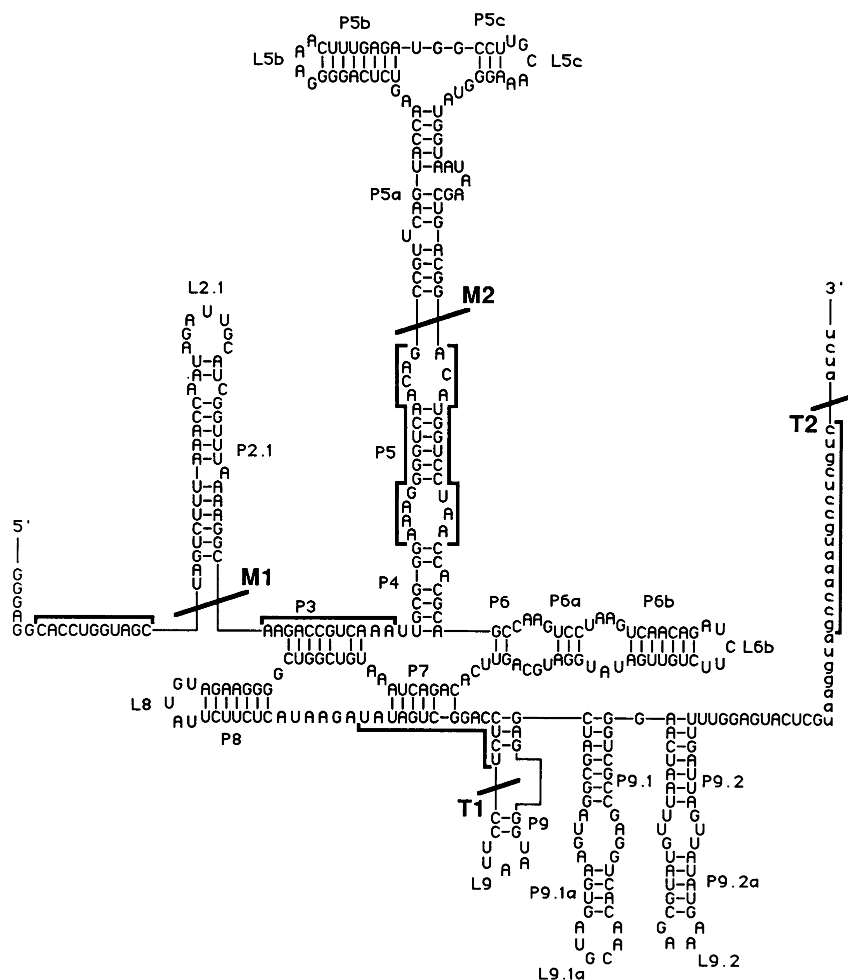

Figure 2: Sequence and secondary structure of the *Tetrahymena* IVS, showing the location of the two terminator and two mutator oligos. The RNA is truncated at its 5' end, corresponding to the direct transcription product of pTL-45 DNA. Structural elements within the IVS are labeled according to the standard nomenclature for group I introns (23). A portion of the 3' exon is shown in lower case letters. The location of the 3' terminus produced by T1 and T2 and the site of internal deletion produced by M1 and M2 are indicated using a heavy diagonal line. The extent of hybridization by the terminator and mutator oligos is indicated by a heavy bracketed line.

that were obtained. Comparable results were achieved using a different plasmid that contains the entire *Tetrahymena* IVS, although in that case the autoradiogram was much more complicated due to the catalytic activity of the precursor rRNA in the transcription buffer (data not shown).

Digestion of the coding strand with gene 6 exonuclease is essentially complete. Only a trace

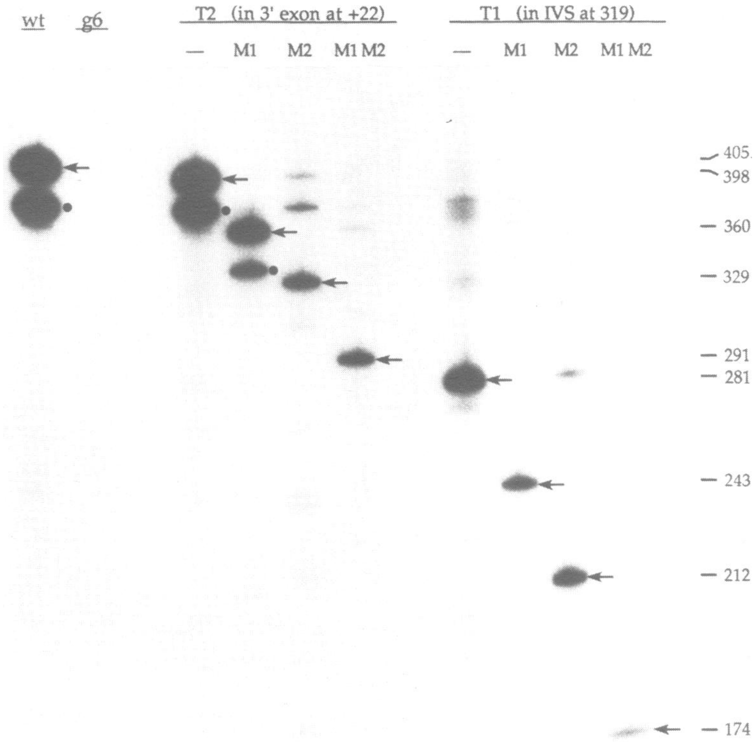

Figure 3: Autoradiogram of wild-type and mutant RNAs obtained by transcription in the presence of [α ^{32}P] GTP. The bands marked by an arrow correspond to the expected transcription product, the size of which is indicated at the right. Bands marked by a dot correspond to materials derived from the expected transcription product as a result of RNA-catalyzed cleavage at the 3' splice site that occurs during the transcription reaction (24). Unmarked bands correspond to wild-type RNA and its cleavage products that appear as a result of inefficient hybridization of the mutator oligo. *wt* is the transcription product obtained from intact pTL-45 DNA that has been cut with *Hin* dIII. *g6* is the transcription product obtained after digestion of the wild-type DNA with gene 6 exonuclease. The products were separated by electrophoresis in a 5% polyacrylamide / 8 M urea gel run in 90 mM Tris/borate buffer.

(< 1%) of the wild type is detected when DNA that has been treated with gene 6 exonuclease is transcribed directly. Hybridizing either T1 or T2 to the minus strand DNA and then extending with T4 DNA polymerase allows one to produce 3'-truncated RNAs with a defined end. Hybridizing one or two mutator oligos in addition to the terminator oligo allows one to control internal positions as well as the 3' terminus. The data presented in Figure 3 indicate that M2 does not hybridize as efficiently to the minus strand DNA as does M1. This is evidenced by material in the M2 lanes corresponding to transcripts whose 3' end is defined by the terminator oligo but

Figure 4: Sequence analysis of the M2 and M1 M2 mutants by the primer extension method. A deoxynucleotide complementary to positions 274-288 of the IVS was hybridized to the mutant RNA and extended using reverse transcriptase. Lane –, primer extension in the absence of dideoxynucleotides. Lanes C, U, G, or A, primer extension reactions in the presence of ddGTP, ddATP, ddCTP, or ddTTP, respectively. Angle brackets indicate the extent of hybridization by the mutator oligos. Arrows indicate the site of internal deletions.

whose internal positions are unchanged from the wild type. The M1 M2 double deletion mutant is accompanied by a smaller amount of the M1 single deletion mutant. For the most part, however, the desired single or double deletion mutant dominates the family of transcription products.

The identity of the mutants was confirmed by eluting the transcription products from the gel and determining their nucleotide sequence. Figure 4 shows the nucleotide sequence of the M2 and M1 M2 mutants as determined by primer extension analysis using reverse transcriptase in the presence of dideoxynucleotides (9). It is important to note that the transition from a double- to a single-stranded template and from a single-stranded template back to a double strand takes place without appreciable slippage of the polymerase enzyme. The transcription products obtained using a partially mismatched template do not appear to be any less accurate than one would obtain using a complete double strand.

The data presented in Figure 3 was prepared quantitatively, that is, differences in the amount of radioactivity reflect either a loss of material during the workup or differences in the efficiency of transcription. The lane corresponding to the T1 oligo alone demonstrates that in some cases the reconstruction of the template strand is nearly complete. The comparatively lower efficiency of template reconstruction with the T2 oligo alone is likely to be due to decreased hybridization efficiency of the T2 oligo. When a mutator oligo is used, the efficiency of template reconstruction is lowered even further. This is partly because the mutator oligo presents a more difficult hybridization task and partly because the extended terminator oligo must be ligated to the 5' end of the mutator oligo. Despite the loss of viable templates due to inefficient strand reconstruction, one can obtain an adequate amount of mutant RNA as a result of the high turnover of T7 RNA polymerase. In a large-scale preparation, we used 10 μg (~ 3 pmol) of plasmid DNA as starting material. The yield of mutant RNA, after elution from the gel, ethanol precipitation, and chromatography on Sephadex G-50, was 183 pmol for the 329-nucleotide M2 mutant and 106 pmol for 291-nucleotide M1 M2 mutant. The mutant RNA was found to exhibit catalytic activity *in vitro* (14), attesting to its purity and reasonable sequence homogeneity.

DISCUSSION

We hope that others will find our mutagenesis technique useful for the rapid preparation of mutant RNAs. We have been using the technique routinely for the past several months to produce a number of mutations within the *Tetrahymena* IVS. In addition to deletions, we have produced single base insertions, multiple base substitutions, and various combined insertions and deletions (data not shown). Because the mutant RNA is usually accompanied by a significant amount of "revertant" wild-type RNA, we prefer to include an insertion or deletion along with any substitution to produce a discernable size difference, allowing the mutant RNA to be separated from the wild type on a polyacrylamide gel. This would not be necessary if one used a mutator oligo that hybridizes very efficiently to the minus strand DNA or if one is willing to tolerate a

small amount of wild type included among the mutant RNAs.

The major advantage that the mutagenesis technique has to offer is its speed and simplicity. Producing a 3'-truncated RNA with a defined end is especially straightforward since it does not require the use of a mutator oligo and thus is not subject to contamination by wild-type RNA. The 3' end may be fixed at any point along the gene or may extend from any point into an extraneous sequence as determined by a terminator oligo with a dangling 5' end (14). There are established methods for cloning a defined region of DNA (18) and for the *in vitro* synthesis of RNAs with defined ends (19). The latter technique is similar to our own, except that it uses cloned single-stranded DNA (e.g. M13 phage DNA) and a "portable promoter" to define the transcription start site. One could combine the M13 technique with our own to produce RNAs that have internal mutations.

The site of an internal mutation may lie at any point along the gene, and need not be in proximity to a restriction site. The design of the mutator oligo must take into account three factors: the desired mutation, the need for efficient hybridization, and the cost. If one wishes to produce a radical alteration of the wild type, it is probably wise to design the mutator oligo with long flanking regions so that it will be able to bind tightly to the minus-strand DNA. This, of course, will increase the cost, but is likely to be economical in the long run. Similarly, if one plans to use two or more mutator oligos simultaneously, each should contain long flanking regions so as to maintain the combined efficiency of hybridization at an adequate level.

Our primary interest has been the construction of recombinant RNAs. However, the mutagenesis technique that we describe could also be used to generate recombinant DNAs. The mutant RNA could be reverse transcribed to cDNA using the terminator oligo as a primer for reverse transcriptase. Typically, the yield of full-length cDNAs is only about 20-30% relative to the input of RNA template (20), so that that the net yield of mutant DNA would be 10-15 times the input of wild-type DNA. Alternatively, after reconstruction of the template strand, one could excise the minus-strand DNA using exonuclease III, and then run the polymerase chain reaction (21,22) to amplify the mutant DNA. The terminator oligo and the minus strand of the T7 promoter could serve as the two primers for this reaction.

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