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In vitro generation of specific deletions in DNA cloned in M13 vectors using synthetic oligodeoxyribonucleotides: mutants in the 5'-flanking region of the yeast alcohol dehydrogenase II gene

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ABSTRACT

Deletion mutants are particularly useful in defining the boundaries of noncoding genetic functions. Such mutants can be precisely generated using synthetic oligodeoxyribonucleotides as mutagens. In this paper we describe the application of this method to recombinant DNA cloned in a phage M13-derived vector. The mutagenic oligodeoxyribonucleotides, 20 and 21 nucleotides in length, were used to delete a tract of 20 dA-dT base-pairs and an adjacent 22 base-pair perfect dyad from the ADR3 locus, the 5'-flanking regulatory region of the ADR2 gene, of Saccharomyces cerevisiae with high efficiency.

INTRODUCTION

The structural gene, ADR2, coding for the alcohol dehydrogenase II (ADHII in S. cerevisiae) has been identified and analyzed genetically (1), cloned (2) and the DNA sequence determined recently (3). Genetic and biochemical characterization and DNA sequence determination of the cis-dominant constitutive (4-7) and in vitro constructed large deletion mutants (8) lead to the identification of the adjacent regulatory region (the ADR3 locus). This region is located between 200 and 1000 base pairs upstream of the structural coding sequences of ADHII. An examination of the sequence in this region reveals two distinctive structural features. The first is a stretch of 20 consecutive A-T base pairs (bp) located between nucleotides -242 to -223 and the second is a 22 bp sequence of perfect dyad symmetry located at nucleotides -292 to -271 with respect to the initiation codon (3, 6). These sequences present two extremes of DNA structure and provide excellent models for the development of procedures for construction of deletions using M13 vector recombinants and oligodeoxyribonucleotide mutagenesis.

We report here an in vitro construction of specific deletion mutants of the two unique sequences of the regulatory region. The procedure involves synthesizing oligodeoxyribonucleotides to flank the specific sequences to be

deleted from the gene followed by their *in vitro* incorporation into a heteroduplex DNA using the M13 clone as template. The proficiency of two oligodeoxyribonucleotides as mutagens and their specificity in the mutagenic processes are discussed.

MATERIALS AND METHODS

The source of *ADR2* gene was the BamHl fragment isolated from plasmid pADR2 (8). An *E. coli* strain W/RRl, containing pADR2 was a kind gift of Dr. David R. Beier (Department of Biochemistry, University of Washington, Seattle, Washington). An M13 phage vector, M13mp8 (9) and its *E. coli* permissive host strain JM103 (10) were originally obtained from Dr. J. Messing (Department of Biochemistry, University of Minnesota, St. Paul, Minnesota). Three oligodeoxyribonucleotides: an 18-mer, d(GATCTCCTCTGCCGGAAC), nucleotides -318 to -301 of the upper strand of the *ADR3* locus (Figure 1); a 20-mer, d(TGAGAGAATGGGCAGAGGAG) which corresponds to nucleotides -252 to -243 and -222 to -213 of the upper strand on either side of the A_{20} tract; a 21-mer, d(TCTCTTATTTGCCCGGTGTTC), which corresponds to nucleotides -262 to -270 and -293 to -304 of the lower strand spanning the dyad were synthesized using the phosphoramidite method (11) with the modifications of Adams *et al.* (12). The oligodeoxyribonucleotide d(pT_8CA), nucleotides -235 to -244, lower strand, was previously synthesized in this laboratory (13). BamHl, T4 DNA ligase and *E. coli* DNA polymerase (large fragment) were obtained from Bethesda Research Laboratories. T4 polynucleotide kinase was purchased from New England Biolabs. Enzyme reaction conditions were those specified by the supplier. α-^{32}P-dATP and α-^{32}P-ATP were obtained from New England Nuclear. Deoxyribonucleoside triphosphates (dNTPs) and 2', 3'-dideoxyribonucleoside triphosphates (ddNTPs) were purchased from PL-Biochemicals. Nitrocellulose filters (BA85) were obtained from Schleicher and Schuell.

Cloning of ADHII gene in M13mp8

10 µg of plasmid pADR2 (10.4 kb) was cleaved with BamHl (25 u) for 2 hours at 37°C. Incubation was terminated by heating at 65°C for 5 min. DNA was precipitated with 0.3 N sodium acetate and 3 volumes of 95% ethanol, washed once with ethanol, dried and redissolved in 25 µl 10 mM Tris-HCl pH 8.0 and 1 mM EDTA. An aliquot (0.5 µg) was analyzed in 0.7% agarose gel electrophoresis in 50 mM Tris-borate, pH 8.3, 1 mM EDTA containing 0.5 µg/ml ethidium bromide to confirm the presence of a 2.8 kb fragment (containing the 2.6 kb BamHl/SphI fragment of *ADR2* and 0.2 kb of pBR322

sequence) and a 7.6 kb fragment (containing the yeast centromere from chromosome 3 (CEN3), the TRP1 gene and the remaining pBR322 sequence). 1.6 µg of the mixed BamHl fragments were ligated to 200 ng of BamHl cleaved RF of M13mp8, using 1 unit of T4 DNA ligase, and incubated at 15°C for 13 hours. Aliquots of the ligation mixture were used to transform $CaCl_2$ treated E. coli JM103 cells as described (14). Approximately 10% of the plaques were those of recombinants (white). M13mp8 ADR2 recombinants (10.0 kb) were identified by direct 0.7% agarose gel electrophoresis with appropriate single-stranded M13 variant DNAs as markers. Recombinant M13mp8 phages with a 2.8 kb insert were classified into the two orientation groups using agarose gel electrophoresis analysis of different combinaations of two phage DNAs which had been annealed at 65°C for 1 hour and fast-cooled in ice. When two phage DNAs containing complementary sequences form a hybrid complex, a slower migrating band can be seen. The orientation of the ADR2 gene inserted in the two classes was determined by DNA sequence analysis using the chain terminator method (15) and a standard M13 primer to determine the sequence at the insert boundary (10). The recombinant containing the lower strand of the target DNA (Figure 1) is M13mp8 ADR2-2B and that of the upper strand is M13mp8 ADR2-2A.

5'-phosphorylation of the oligodeoxyribonucleotides

Oligonucleotides (200 pmole) to be used for mutagenesis were phosphorylated with T4 polynucleotide kinase as described before (16). For use as a hybridization probe, the oligodeoxyribonucleotide (20 pmoles) was phosphorylated as above but 20 µCi of high specific activity γ-^{32}P-ATP (2000 Ci/nmol) was used as the only source of ATP.

Oligodeoxyribonucleotide-directed synthesis of covalently closed double-stranded DNA

The conditions for annealing 5' phosphorylated oligodeoxyribonucleotide (20 pmole) and single-stranded M13mp8-recombinant DNA (1 pmole), extension with E. coli DNA polymerase I (large fragment) and ligation with T4 DNA ligase (Figure 2) have been previously described (16). The recombinant used as target for deletion of the dyad contained the upper ADR3 strand and that for deletion of the dA-dT tract contained the lower stand.

Isolation of covalently closed double stranded DNA

DNA synthesized by extension of the oligodeoxyribonucleotide were precipitated with polyethyleneglycol-NaCl to remove γ-^{32}P-dATP and closed circular double stranded M13-recombinant DNA was separated from single stranded DNA by density gradient centrifugation through a 5-20% linear

alkaline sucrose gradient as described (16).

Transformation with closed circle DNA

Aliquots (1-10 µl) of the pooled and neutralized fractions containing closed circle double stranded DNA were used to transform $CaCl_2$-treated E. coli JM103 cells (14).

Mutant screening procedures

Phage plaques formed in DNA transformation plates were picked and 1 ml phage infected cell cultures were grown and phages were isolated as described (16). The phages were screened for mutants using a dot hybridization procedure described (16) with the ^{32}P-labelled mutagenic oligodeoxyribonucleotides as probes.

DNA sequence determination

Putative phage mutants as determined by the dot hybridization method were confirmed by the dideoxy-DNA sequence determination (10, 15) using an oligodeoxyribonucleotide complementary to an adjacent sequence downstream from the 3' end of the two unique regions; d(pT$_8$CA) was the primer for the dyad deletions and d(GATCTCCTCTGCCGGAAC) for the dA-dT tract deletions.

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        HpaII                                          HphI                EcoRI*          MboII
TCCGTCTCTCCGGTTACAGCCTGTGTAACTGATTAATCCTGCCTTTCTAATCACCATTCTAATGTTTTAATTAAGGGATTTTGTCTTCAT
AGGCAGAGAGGCCAATGTCGGACACATTGACTAATTAGGACGGAAAGATTAGTGGTAAGATTACAAAATTAATTCCCTAAAACAGAAGTA
          -465           -450           -435           -420           -405           -390

                                                      FokI            FnuEIMnlI  HpaII
TAACGGCTTTCGCTCATAAAAATGTTATGACGTTTTGCCCGCAGGCGGGAAACCATCCACTTCACGAGACTGATCTCCTCTGCCGGAACA
ATTGCCGAAAGCGAGTATTTTTACAATACTGCAAAACGGGCGTCCGCCCTTTGGTAGGTGAAGTGCTCTGACTAGAGGAGACGGCCTTGT
          -375           -360           -345           -330           -315           -300

ScrFISfaNIHpaIICauII                EcoRI*                                          MnlI
CCGGGCATCTCCAACTTATAAGTTGGAGAAATAAGAGAATTTCAGATTGAGAGAATGAAAAAAAAAAAAAAAAAAAAGGCAGAGGAGAGC
GGCCCGTAGAGGTTGAATATTCAACCTCTTTATTCTCTTAAAGTCTAACTCTCTTACTTTTTTTTTTTTTTTTTTTCCGTCTCCTCTCG
          -285           -270           -255           -240           -225           -210

                    AluI  Nsp(7524)ISphI                                              TaqI
ATAGAAATGGGGTTCACTTTTTGGTAAAGCTATAGCATGCCTATCACATATAAATAGAGTGCCAGTAGCGACTTTTTTCACACTCGAAAT
TATCTTTACCCCAAGTGAAAAACCATTTCGATATCGTACGGATAGTGTATATTTATCTCACGGTCATCGCTGAAAAAAGTGTGAGCTTTA
          -195           -180           -165           -150           -135           -120

                                    MboII                    AluI
ACTCTTACTACTGCTCTCTTGTTGTTTTTATCACTTCTTGTTTCTTCTTGGTAAATAGAATATCAAGCTACAAAAAGCATACAATCAACT
TGAGAATGATGACGAGAGAACAACAAAAATAGTGAAGAACAAAGAAGAACCATTTATCTTATAGTTCGATGTTTTTCGTATGTTAGTTGA
          -105           -90            -75            -60 mRNA Start -45             -30

                          M  S  I  P  E  T  Q  K  A  I  I  F  Y  E  S  N  G  K  L  E
                                                      MboII  HinfIIHinfI
ATCAACTATTAACTATATCGTAATACACAATGTCTATTCCAGAAACTCAAAAAGCCATTATCTTCTACGAATCCAACGGCAAGTTGGAGC
TAGTTGATAATTGATATAGCATTATGTGTTACAGATAAGGTCTTTGAGTTTTTCGGTAATAGAAGATGCTTAGGTTGCCGTTCAACCTCG
          -15            1              15             30             45             60
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Figure 1. The DNA sequence of the 5'-flanking region of the ADHII gene. This represents a portion of the published ADR3 sequence (6) with the two target regions. The 20 bp dA,dT tract and the 22 bp perfect dyad underlined. In addition, the TATA box and the transcript start point are interlined.

RESULTS

Oligodeoxyribonucleotide mutagenesis

The DNA sequence 5' upstream from the initiation codon of the ADR2 locus is shown in Fig. 1. The two deletion targets, the 20 consecutive dA-dT residues (-242 to -223) and the 22 bp with perfect dyad symmetry (-292 to -271) are underlined. The mutagenic oligodeoxyribonucleotide d(TGAGAGAATGGGCAGAGGAG) contains ten nucleotides flanking the 5'-side of the dA_{20} tract and ten nucleotides flanking the 3'-side and is designed to delete the dT_{20} tract from the complementary lower strand of the ADR3 locus (Figure 3a). Each decamer was shown, by computer search, to be unique in the recombinant DNA target. The deletion of the dT_{20} tract rather than the dA_{20} tract was chosen because the former should have less structure as a consequence of base stacking. The seccond mutagenic oligodeoxyribonucleotide, d(TCTCTTATTTGCCCGGTGTTC), contains 9 nucleotides that flank the 5'-side of the 22 bp dyad, in the lower strand of the ADR3 locus (Figure 3b) and 12 nucleotides on the 3'-side and was designed to delete the dyad from the complementary upper strand.

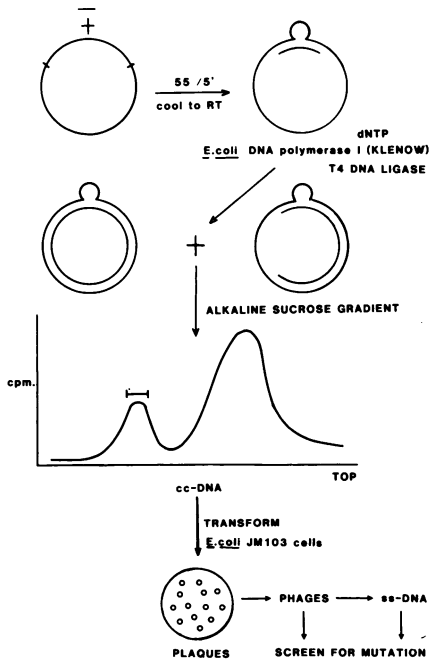

Figure 2. General scheme for generating specific deletions with oligodeoxyribonucleotides and M13 vector clones of target DNA.

Table 1
Mutants Detected by Dot Blot Hybridization of Phage

mutagenic oligodeoxyribo-nucleotide	site to be deleted	number of isolates tested	number of mutants	mutants obtained %
d(TGAGAGAATGGGCAGAGGAG)	$(dA\text{-}dT)_{20}$	60	10	17
d(TCTCTTATTTGCCCGGTGTTC)	22 bp dyad	20	11	55

The two oligodeoxyribonucleotides (20- and 21-mer) were found to interact specifically and efficiently with the desired targets by using them as primers for dideoxy DNA sequence determination (16). When used as primers for the production of double-stranded closed circular DNA (Figure 2), they yielded the desired products with 5% and 13% efficiency (data not shown), respectively. Mutant production in E. coli transformed by these DNAs was estimated by using the oligodeoxyribonucleotides to screen for recombinant phage DNA which formed stable duplexes by dot blot hybridization at successively higher temperatures (16). The yields of mutant recombinant phage were 17% and 55%, respectively (Table 1).

Characterization of mutants

It was important to evaluate the accuracy and specificity of the oligodeoxyribonucleotide-directed deletion mutagenesis. Therefore, the DNA sequences were determined for 10 mutants resulting from attempted deletion

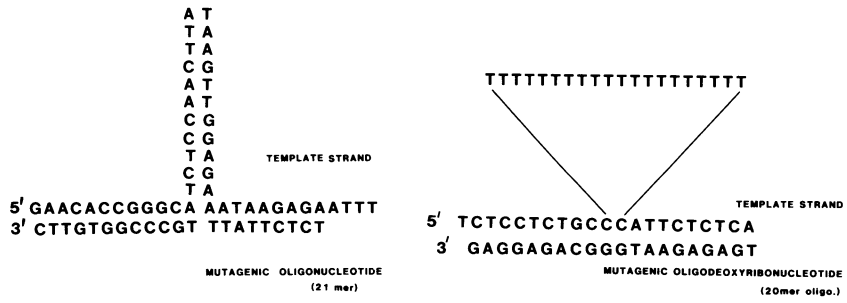

Figure 3. The heteroduplexes of mutagenic oligodeoxyribonucleotide and its target DNA strand. (a) 20-mer with the dT_{20} region and (b) 21-mer with the 22 bp dyad symmetry region.

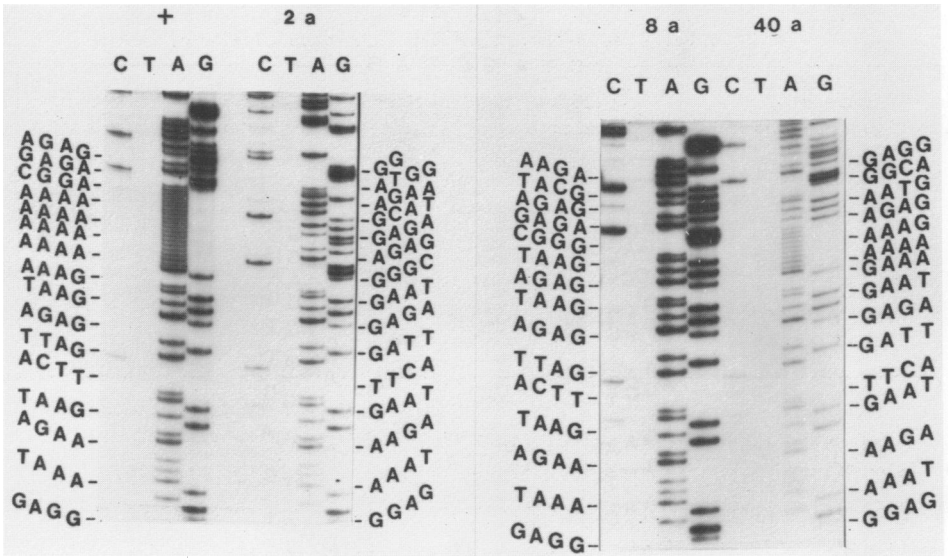

Figure 4. Autoradiogram of DNA sequencing gel of wild type (+) and mutants (2a, 8a and 40a) generated by the 20-mer oligodeoxyribonucleotide. The DNA sequences were determined using the chain termination method (15) primed by the 18-mer oligodeoxyribonucleotide and analyzed with a 10% polyacrylamide gel. The bands on the T tracks were light and do not show up well on the photograph but were clearly visible on the original autoradiogram.

of the $(dA\text{-}dT)_{20}$ tract and for 6 mutants from attempted deleton of the 22 bp dyad. Eight of the 10 mutant phage from the $(dA\text{-}dT)_{20}$ deletion had DNA with the expected sequence (Figures 4 and 6). However, the other two mutants contained unanticipated sequences. In one of these variants, 8a, the $(dA\text{-}dT)_{20}$ tract is deleted, but the first eight nucleotides of the mutagenic oligodeoxyribonucleotide are duplicated in a tandem repeat (Figures 4 and 6). In the second variant, 40a, $(dA\text{-}dT)_{10}$ is deleted and the residual $(dA\text{-}dT)_{10}$ tract is flanked by a 9 bp repeat which corresponds to the second to the tenth nucleotides of the mutagenic 21-mer (Figures 4 and 6).

The 6 mutants derived from the attempted deletion of the 22 bp dyad all had the anticipated sequence (Figures 5 and 6), indicating that precise and reproducible excision of the dyad had been achieved.

Oligodeoxyribonucleotide-DNA duplex stability

When the desired deletions of the dT_{20} and the dyad had been generated it was possible to define the relative stability of the duplexes

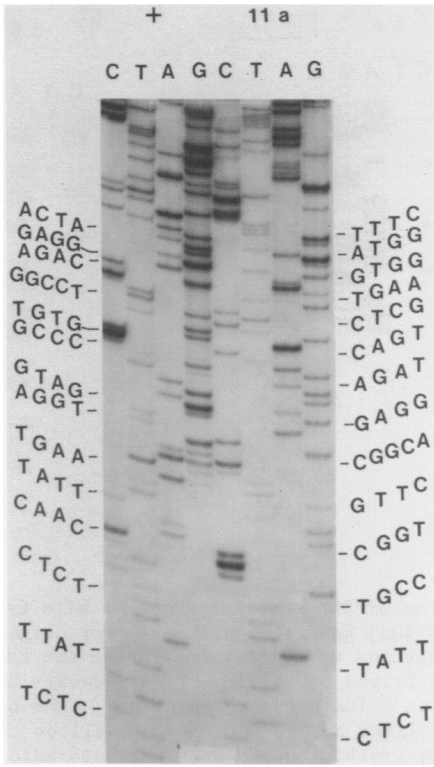

Figure 5. Autoradiogram of DNA sequencing gel wild type (+) and a mutant (11a) generated by the 21-mer oligodeoxyribonucleotide. The DNA sequences were determined by the chain termination methed (15) using d(pT$_8$CA) as a primer and analyzed with a 12% polyacrylamide gel.

formed by the mutagenic oligodeoxyribonucleotides with wild-type and mutant phage DNA. These experiments, carried out by dot blot hybridization followed by washing at successively higher temperatures are shown in Figure 7. In duplex with their perfectly matched, mutant, DNA complements, d(TGAGAGAATGGGCAGAGGAG) and d(TCTCTTATTTGCCCGGTGTTC) had similar stabilities, with melting temperatures of 70° and 65-70°, respectively. However, in duplex with wild-type DNA, their melting temperatures were 50-55° and 55-60°, respectively. While these data are fairly qualitative, they suggest that a dyad loopout is less destabilizing than a loop-out of a sequence with no potential for formation of a stable hairpin duplex. In both cases the oligodeoxyribonucleotide probes have a clearcut ability to recognize the desired mutant.

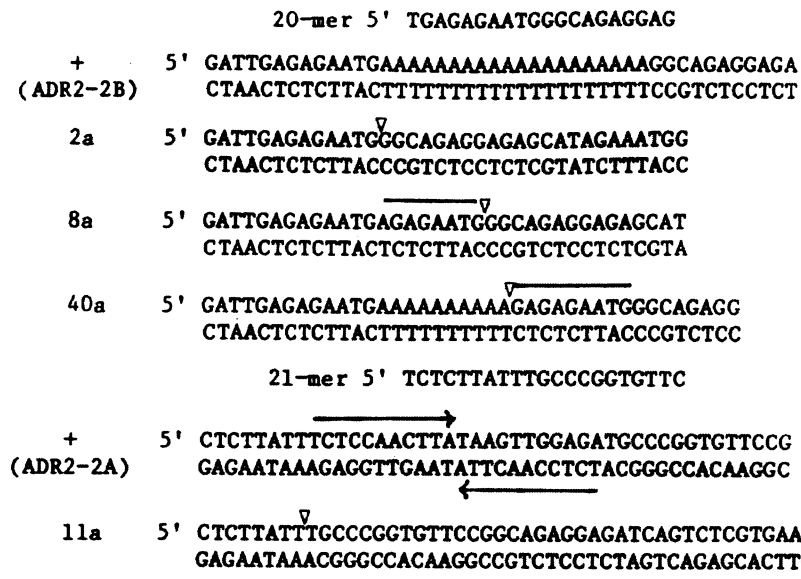

Figure 6. Summary of the DNA sequences of wild type and deletion mutants of ADR2 generated by the two oligodeoxyribonucleotides, 20 mer and 21 mer. (∇) denotes site of deletion, (-) denotes the sequence duplicated, the two arrows mark the 22 bp dyad symmetry. The ADR2-2A and 11a DNA sequences are of opposite orientation to that presented in Figure 1.

DISCUSSION

Use of oligodeoxyribonucleotides to generate a single nucleotide deletion in phage ϕX174 and a 14 bp deletion in a yeast tRNA gene cloned in pBR322 has been reported previously (17, 18). The objective of the experiments described in this paper was to develop an efficient procedure for generating specific deletions in DNA cloned in a M13 vector using oligodeoxyribonucleotide mutagenesis. In particular, the deletion of a sequence with a perfect dyad, which might be expected to be facile because it should form a stable hairpin, was compared with the deletion of a sequence, dT_{20}, in which no secondary stable structure would be anticipated.

An oligodeoxyribonucleotide of 21 nucleotides was successful in generating the deletion of the 14 bp tract from a tRNA gene (18). Consequently, a 20 nucleotide oligomer was chosen for deletion of the dT_{20} tract and a 21 nucleotide oligomer for deletion of the 22 bp dyad (Figure 3). The base compositions of the oligomer, 11 dG,dC residues in the 20-mer and 10 dG,dC residues in the 21-mer suggested that their duplexes with complementary DNA would have similar stability.

Both oligonucleotides were efficient and specific primers of DNA

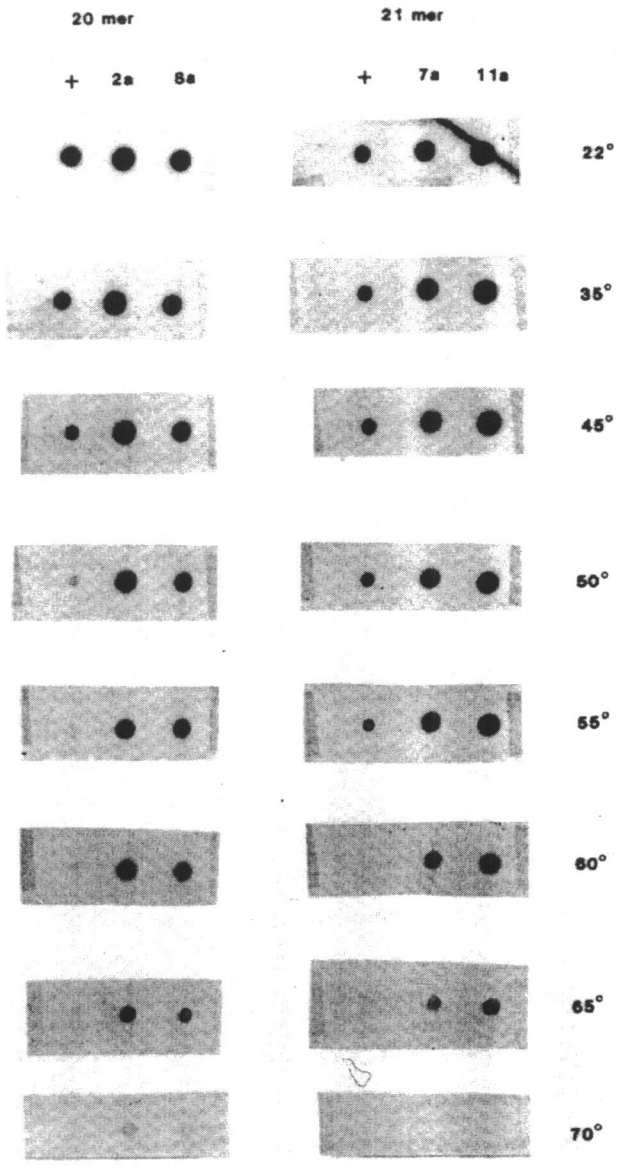

Figure 7. Dot hybridization analyses of the stability of the heteroduplexes formed between the mutagenic oligodeoxyribonucleotide and the wild type or mutant strand. Single stranded DNA from various phage strains were bound to nitrocellulose (16) and hybridized to 5'-^{32}P-labelled oligodeoxyribonucleotides (20 mer and 21 mer). Hybridization was carried out at 22°C for 1 hr. The filters were washed in 6 x SSC at 22°, 35°, 45°, 50°, 55° 60°, 65° and 70°C for 5 min and autoradiographed for 22-25 hours using Kodak NS-5T film. 2a and 8a are mutants of the dA-dT tract (see Fig. 6) and 7a and 11a are independent mutants of the dyad region with identical alterations (Fig. 6).

synthesis on their target DNA and, after ligation, produced closed circular duplex DNA with efficiencies, 5% for the 20-mer and 13% for the 21-mer; yields in the range usually obtaining for *in vitro* synthesis of double-

stranded M13-derived DNAs (16).

The heteroduplex DNAs, when used to transform JM103 cells, gave rise to mutant recombinant phage with excellent efficiency; 17% in deletion of dT_{20} by the 20-mer and 55% in deletion of the dyad by the 21-mer. This establishes the important point that the biological systems used in this study, E. coli JM103 and recombinant DNA in the vector M13mp8, are not subject to mismatch repair which prevents or greatly reduces generation of deletion mutants.

Mutants were identified by hybridization of the mutagenic ^{32}P-oligodeoxyribonucleotides to phage which were dot-blotted onto nitrocellulose followed by cycles of washing at successively higher temperature and radioautography (16). This technique, which makes no assumptions about oligonucleotide duplex stability, clearly identified mutant phage DNAs, by the greater stability of their interaction with the probes. However, it was desirable to establish the DNA sequence of a number of the mutants to determine the fidelity of mutagenesis. In the case of the attempted deletion of the dT_{20} tract, eight out of ten mutants examined had precisely the desired sequence (Figures 4 and 6) as did all six of the mutants examined in the attempted 22 bp dyad deletion (Figures 5 and 6). The two aberrant mutants obtained in the dT_{20} deletion (Figures 4 and 6) contain duplications of sequences corresponding to the 5'-end of the mutagenic oligodeoxyribonucleotide. They are explicable by a model in which the 3'-end of the oligodeoxyribonucleotide, with 7/10 dG,dC residues, pairs precisely with its target, but where the 5'-end, with 4/10 dG,dC residues, does not always pair precisely. Thus one variant mutant, 8a (Figures 4 and 6), in which the dT_{20} is completely deleted, contains a duplication of the first 8 nucleotides at the 5'-end of the mutagenic primer. This is the result expected if the first three nucleotides at the 5'-end of the primer, TGA, pair with their complement, nucleotides -244 to -242 (Figures 1 and 3) rather than with the desired sequence nucleotides -252 to -250. The second variant, 40a (Figures 4 and 6), has 10 nucleotides of the dT_{20} deleted and replaced by a duplication corresponding to nucleotides 2 to 10 of the primer. This mutant would result if the 5'-end of the primer aligned itself with the tract of dT residues immediately adjacent to the target of the 3'-end of the primer. In addition, this model requires that the 5'-dT residue of the primer be removed by an exonuclease action and that one dT residue in the target is looped-out. This suggests that DNA ligase will join DNA even though it is part of a very imperfect duplex.

A study of the melting temperatures of the duplexes of the mutagenic oligodeoxyribonucleotides with wild-type and mutant DNAs was carried out (Figure 7). This shows that the two oligodeoxyribonucleotides form perfect duplexes of similar stability. This probably should be expected with oligonucleotides of similar length, base composition and asymmetry of purine-pyrimidine composition. However, the oligonucleotide duplex spanning the dT_{20} tract is noticeably less stable than that spanning the 22 bp dyad (Figure 7). Presumably, the hairpin structure, which the dyad can form, facilitates binding of the oligonucleotide spanning it.

A comprehensive study of the properties of the mutants generated in the present study is in progress (D. Cox, E.T. Young, V.L. Chan and M. Smith). However it is of interest that, when the mutant genes are incorporated in a yeast CEN vector, the mutant in which the 20 bp dA,dT tract is missing has properties identical with wild-type ADR2 whereas the mutant in which the dyad is deleted behaves like the ADR3-4^c and ADR3-5^c mutants (7) and is a promoter-up mutant. This suggests that the dyad is the target of, or is a component of the target of the negative effector of ADR2 expression. In conclusion, this study has demonstrated that synthetic oligodeoxyribonucleotides can be used to delete specific sequences of DNA from M13-vector clones of recombinant DNA. The deleted segments can be of the two types of extreme structure encompassed by a dT_{20} tract or a 22 bp perfect dyad. The anticipated mutants are produced with high efficiency and fidelity. However, two examples of variant mutants were detected. These result from alternate mismatch pairing of the 5'-end of the mutagenic oligodeoxyribonucleotide. The mutants obtained in the present study will be useful in determining the biological function(s) of the two deleted regions. Cis-dominant constitutive mutants with an increased length of the dT:dA track had been isolated (7) but none of the regulatory mutants of the ADR3 locus reported to-date have been shown to be affected in the dyad symmetry region. The presence of dyad symmetries located upstream from the initiation codon of a number of eukaryotic genes have been reported but their function(s) are yet to be identified (19-21). The dyad deletion mutants constructed in this study would also aid in the identification and isolation of putative protein(s) which specifically interacts with this unique site of the ADR2 regulatory region, ADR3.

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