Volume 6 Number 9 1979 Nucleic Acids Research

Defined transversion mutations at a specific position in DNA using synthetic oligodeoxyribonucleotides as mutagens

Shirley Gillam*, Patricia Jahnke*, Caroline Astell*, Sandra Phillips†, Clyde A.Hutchison III† and Michael Smith*

*Department of Biochemistry, Faculty of Medicine, University of British Columbia, 2075 Wesbrook Place, Vancouver, B.C., V6T 1W5, Canada, and †Department of Bacteriology and Immunology, University of North Carolina, Chapel Hill, NC 27514, USA

Received 11 May 1979

ABSTRACT

The oligodeoxyribonucleotides, pCCCAGCCTCAA, which is complementary to nucleotides 5274-4284 of bacteriophage ØX174 viral DNA, and pCCCAGCCTAAA, which corresponds to the same sequence with a C → A change at the ninth nucleotide, were synthesized enzymatically. The second of these oligonucleotides was used as a primer for E. coli DNA polymerase I, from which the 5'-exonuclease has been removed by proteolysis (Klenow enzyme), on wild-type ØX174 viral DNA template. After ligation, this yielded closed circular heteroduplex DNA with a G, A mismatch at nucleotide 5276. Transfection of E. coli spheroplasts with the heteroduplex DNA produced phage mutated at this nucleotide (G → T in the viral DNA) with high efficiency (13%). The mutant DNA, which corresponds to the gene B mutant am16, was reverted (T → G) by the wild type oligonucleotide with an efficiency of 19%. The nucleotide changes were established by sequence determination of the mutated viral DNA using the enzymatic terminator method. The production of specific transversion mutations, together with a previous demonstration of specific transition mutations (1), established that short enzymatically synthesized oligodeoxyribonucleotides can be used to induce any class of single nucleotide replacement with high efficiency and thus provide a powerful tool for specific genetic manipulations in circular genomes like that of ØX174.

INTRODUCTION

Recently, we have shown that the two transition mutations, AT ⟷ GC, can be induced with high efficiency at a specific nucleotide in ØX174 DNA by using short synthetic oligodeoxyribonucleotides as mutagens (1). The transition mutations were effected at nucleotide 587 of the ØX174 genome in the region where genes D and E are superimposed (1-3). (All numbering of the ØX174 genome in this paper refers to the sequence reported in reference 3). The general strategy was to use the mutating oligodeoxyribonucleotide as a primer for E. coli DNA polymerase I from which the 5'-exonuclease had been removed by proteolysis (4-6) with wild-type viral DNA as template. After ligation using T4-DNA ligase, the closed circular heteroduplex DNA was used

to transfect E. coli spheroplasts followed by the isolation of mutant phage (1).

The success in producing specific transition mutations encouraged us to investigate the use of short synthetic oligodeoxyribonucleotides to induce specific transversion mutations, there being no methods available for programming such changes. The target for these experiments was nucleotide 5276 of bacteriophage ØX174 DNA, which in wild-type DNA is G (3,7). Replacement of this G by T results in a well-characterized mutant in gene B; am16 (7). The specific details of the nucleotide sequences involved in these experiments are shown in Figure 1. Production of the mutant involves a G,A mismatch and its reversion a T,C mismatch at nucleotide 5276, thus providing examples of the two structural types of transversion-inducing nucleotide pairings (purine-purine or pyrimidine-pyrimidine). The success of these experiments described in this paper, in conjunction with the previous production of transition mutations (1) demonstrates the generality of the oligodeoxyribonucleotide method for achieving specific nucleotide replacements.

EXPERIMENTAL PROCEDURES

T4-DNA ligase was a gift from Dr. R.C. Miller. One unit of ligase catalyzes the conversion of 1 pmol of ^{32}P-labeled 5'-terminus of pT_{10} on polydeoxyadenylate template to a form resistant to bacterial alkaline phosphatase in 1 min at 20° (8). Endonuclease S_1 was purified as described by Vogt

Fig. 1

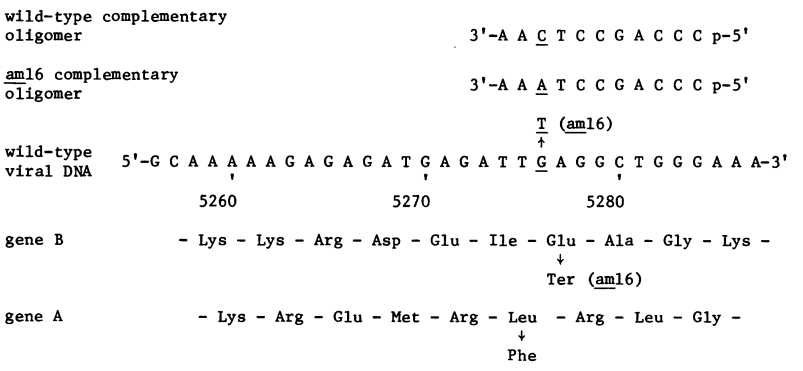

Fig. 1. Nucleotide sequences of wild-type ØX174 viral DNA in nucleotide complementary to this region. The mutation of G → T at position 5265 produces an am codon. TAG, in the gene B reading frame. The change from TGA to TTA in the gene A reading frame infers a Leu → Phe amino acid change in the A and A^+ proteins (7).

(9). One unit of S_1 is the amount of enzyme which converts to acid solubility 10 µg of sonicated, denatured calf thymus DNA in 10 min at 45° (9). Other chemicals and enzyme preparations have been described previously (1).

Synthesis and Characterization of Oligodeoxyribonucleotides

Synthesis was by the stepwise enzymatic method, using E. coli polynucleotide phosphorylase (10,11), with the oligodeoxyribonucleotide pCCC (12) as the initial primer. Conditions used for each step of the synthesis are summarized in Table 1. At each step unreacted primer was recycled; details and overall yields are listed in Table 2. The purity of each oligodeoxyribonucleotide was established by high pressure chromatography on RPC-5 (10,11) and its composition by quantitative deoxyribonucleoside analysis (Table 2) using high pressure chromatography on Aminex A-5 (10,11). The final oligodeoxyribonucleotide products, pCCCAGCCTCAA and pCCCAGCCTAAA were ^{32}P-phosphate labeled at their 5'-termini using T4-polynucleotide kinase (13) and their sequences confirmed by two-dimensional electrophoresis-homochromatography (14,15) after partial digestion with endonuclease P_1 (1,16). The characterization of pCCCAGCCTCAA is shown in Figure 2.

DNA Preparations

Viral DNAs were isolated as described previously (1).

Specificity of Oligodeoxyribonucleotide Priming of E. coli DNA Polymerase I (Klenow) on Viral DNA Template

The oligodeoxyribonucleotide (5-20 pmol) and ØX174 am3cs70 viral DNA (0.3 pmol) in a volume of 15 µl containing 50 mM NaCl, 20 mM Tris-HCl, pH

Table 1. Conditions for enzymatic stepwise synthesis of the oligodeoxyribonucleotides pCCCAGCCTAAA and pCCCAGCCTCAA catalyzed by E. coli polynucleotide phosphorylase (specific activity 16 dADP units/mg at 37° (10,11).

Primer	(µmoles)	dNDP	(µmoles)	$MnCl_2$ (µmoles)	NaCl (mmoles)	Enzyme (units)	Volume (ml)	Time (hrs)	Product	(µmoles)	Yield (%)
pCCC	8.3	dADP	21	70	1.05	50	7	16	pCCCA	2.9	35
pCCCA	5.5	dGDP	9	30	3.0	32	6	5	pCCCAG	0.76	14
pCCCAG	1.63	dCDP	7.5	50	0.75	7.5	2.5	4	pCCCAGC pCCCAGCC	0.99 0.17	60 11
pCCCAGC	1.8	dCDP	8	20	2.0	20	4	9	pCCCAGCC	0.4	22
pCCCAGCC	0.62	dTDP	6	40	0.6	15	2	14	pCCCAGCCT	0.3	49
pCCCAGCCT	0.14	dADP	1	10	0.3	3.6	0.5	5	pCCCAGCCTA pCCCAGCCTAA pCCCAGCCTAAA	0.034 0.003 0.004	24 2 3
pCCCAGCCT	0.43	dCDP	3	7.5	0.9	10	1.5	16	pCCCAGCCTC	0.03	7
pCCCAGCCTC	0.07	dADP	0.75	2.5	0.3	3.6	0.5	6	pCCCAGCCTCA pCCCAGCCTCAA	0.03 0.006	41 9

Table 2. Overall yield of oligodeoxyribonucleotides at each step of enzymatic synthesis and the deoxyribonucleoside composition of the products (see Experimental Procedures for analytical methods).

Product	(μmoles)	Cycles	Product	(μmoles)	Yield (%)	Molar ratio of nucleosides found			
						dT	dG	dA	dC
pCCC	13.3	4	pCCCA	7.6	59			1.0	2.83
pCCCA	7.6	6	pCCCAG	2.75	38		1.06	1.0	2.96
pCCCAG	2.5	4	pCCCAGC	1.87	75		1.06	1.0	3.8
			pCCCAGCC	0.43	17		1.09	1.0	4.87
pCCCAGC	1.8	3	pCCCAGCC	0.77	43		1.09	1.0	4.87
pCCCAGCC	1.16	3	pCCCAGCCT	0.76	66	1.0	1.0	1.09	4.87
pCCCAGCCCT	0.14	1	pCCCAGCCTA	0.034	24	0.84	1.0	2.08	4.74
			pCCCAGCCTAA	0.003	2				
			pCCCAGCCTAAA	0.004	3				
pCCCAGCCT	0.68	3	pCCCAGCCTC	0.072	11	1.0	1.0	1.01	5.88
pCCCAGCCTC	0.07	1	pCCCAGCCTCA	0.029	41	0.94	1.0	2.0	5.86
			pCCCAGCCTCAA	0.006	9				

7.5, 11 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.05 mM each of dCTP, dTTP and dGTP and 10 μCi of α-^{32}P dATP (300 Ci/mmol) were mixed with 1 μl (1 unit) of E. coli DNA polymerase I (Klenow) and incubated at 23° for 1 min. dATP (1.5 pmol) was then added (cold chase) and the solution incubated at 23° for 5 min. The enzyme was inactivated with phenol and the deoxyribonucleoside-5' triphosphates removed on Sephadex G-100 (7). The resultant polynucleotide, after lyophilization, was dissolved in 10 μl of buffer containing 50 mM NaCl, 6.6 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$ and 6.6 mM 2-mercaptoethanol and digested with Hae III (1 unit) for 30 min at 37°. The product was fractionated under denaturing conditions on a 5% polyacrylamide gel (7) with Hae III fragments of denatured nick-translated ØX174 am3cs70 RF DNA (17) as size markers. The gel was fixed and radioautographed under standard conditions (7); the results of this experiment are shown in Figure 3.

Oligodeoxyribonucleotide Primed Synthesis of Covalently Closed Heteroduplex DNA

(a) The mutant oligodeoxyribonucleotide, pCCCAGCCTAAA (27 pmol) and viral DNA (0.33 pmol), from ØX174sB1 which is wild-type in genes A and B (18), in 5 μl of buffer containing 100 mM NaCl, 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM 2-mercaptoethanol, were heated in a seated capillary tube for 30 min at 80° and then incubated for 1 hr at 0°. An aliquot (2 μl) was mixed with 3 μl of buffer containing 22 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.83 mM each of dATP, dTTP, dCTP and dGTP, 0.4 mM ATP, 0.45 units of

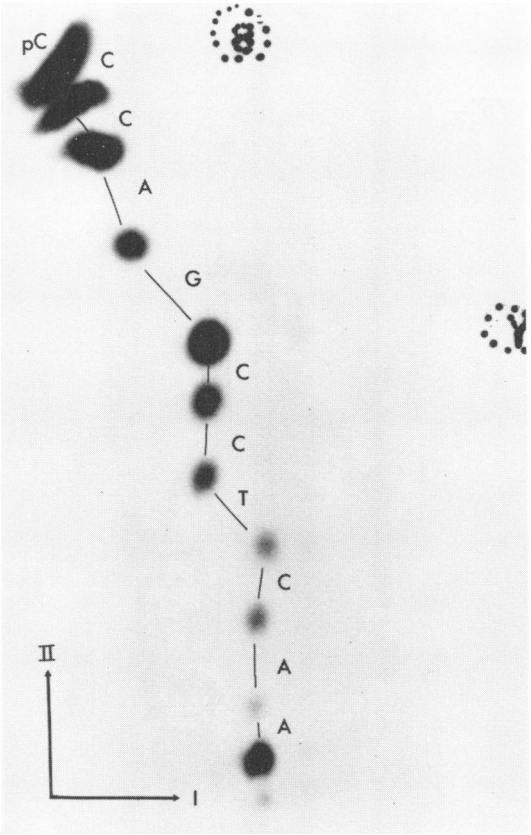

Fig. 2. Two-dimensional sequence determination of pCCCAGCCTCAA; 1, electrophoresis; 2, homochromatography (14,15). Other details in Experimental Procedures. The letters B and Y indicate the position of blue dye (Xylene cyanol F.F.) and yellow dye (orange G) markers.

E. coli DNA polymerase I (Klenow) and 4.8 units of T4-DNA ligase and incubated, first for 30 min at 0°, and then for 5 hr at 23°.

(b) The wild-type oligodeoxyribonucleotide, pCCCAGCCTCAA (27 pmol) and mutant, am16, viral DNA (0.33 pmol) in 5 µl of buffer as described in (a) were heated in a sealed capillary tube for 3 min at 80° and then incubated for 1 hr at 10°. An aliquot (2 µl) was mixed with 2 µl of buffer containing 30 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 1.5 mM 2-mercaptoethanol, 1.4 mM each of dATP, dTTP and dCTP, 0.55 mM ATP and 0.4 units of E. coli DNA polymerase I (Klenow) and incubated for 30 min at 10°. The temperature was then increased

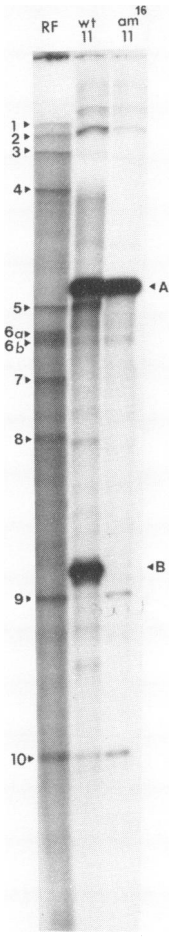

Fig. 3. Gel electrophoresis of products which demonstrate specific priming by synthetic oligodeoxyribonucleotides. The pulse chase experiments (Experimental Procedures) followed by Hae III cleavage and denaturation of the DNA generated fragments A by priming at the desired site. The origin of fragment B is described in Results and Discussion. The template was viral DNA from ØX174 am3cs70. The left channel (RF) contains a denatured Hae III digest of nicktranslated ØX174 am3cs70 RF DNA to provide size markers; arrowheads indicate the positions of single strand marker fragments (nucleotide residues in brackets) 1 (1353), 2 (1078), 3 (872), 4 (603), 5 (310), 6a (271), 6b (281), 7 (234), 8 (194), 9 (118) and 10 (72).

to 37° and after 5 min 0.3 µl of 10 mM dGTP was added and the reaction continued for 5 min at 37°. The solution was then cooled to 23° and E. coli DNA

polymerase I (Klenow), 0.2 units, and T4-DNA ligase, 5 units, were added and the incubation continued for 5 hrs.

Transfection and Genetic Analysis

The same procedures were used as described previously (1) with the following changes. Spheroplasts were prepared from E. coli C600.5 rather than from E. coli W6 and 0.1 ml of lysozyme (Seikagaka Kogyo Co., Tokyo, Japan, 20 mg/ml) per ml of cell suspension was used. Transfections were carried out for 5 hr at 30°.

Total phage from transfections were assayed on E. coli CQ_2 (su^+) at 30°, the host and condition being permissive for wild-type and am16 phage, and wild-type phage were assayed on E. coli C (su^-) at 30° (Table 3).

Isolation of Mutant Phage

(a) Presumptive am16 mutants, produced by the mutant oligodeoxyribonucleotide, pCCCAGCCTAAA. Phage from six plaques which did not grow on E. coli C (su^-) were grown on E. coli HF4714 (su^+) or E. coli CQ_2 (su^+) as described previously (1). 200 mM $MgSO_4$ was added 8 min after infection to inhibit cell lysis (19).

(b) Presumptive wild-type revertants, produced by the wild-type oligodeoxyribonucleotide, pCCCAGCCTCAA. Phage from four plaques which grew on E. coli C (su^-) were combined and grown on E. coli C, $MgSO_4$ again being used to inhibit cell lysis.

When am16 phage were purified by buoyant density centrifugation in CsCl, two bands of phage were obtained, the lighter band containing about 75% of

Table 3. Mutation at nucleotide 5276 of the ØX174 genome: G(am^+) ↔ T(am^{16}). Details are under Experimental Procedures.

Primer Oligodeoxyribonucleotide		Template ØX174 Viral DNA	Progeny Phage (% Mutants)*	
Sequence	Genotype		No S_1 endonuclease	S_1 endonuclease
pCCCAGCCTAAA**	am16	sB1 (am^+)	am 7 (20/300)	13 (52/400)
pCCCAGCCTCAA	am^+	sB1 (am^+)	am 0 (0/300)	0 (0/300)
pCCCAGCCTCAA	am^+	am16	22	19
pCCCAGCCTAAA	am16	am16	5×10^{-3}	6×10^{-3}

* The total yields of progeny ranged from 4×10^5 to 4×10^6 pfu/µg of am^+ template DNA and from 2.5×10^7 to 5×10^7 pfu/µg of am16 template DNA. Treatment with S_1 endonuclease reduced the yields by two- to four-fold. The numbers in parentheses for the experiments with am^+ template DNA indicate the numbers of plaques tested for am phenotype.

**The nucleotide pairing with nucleotide 5276 of ØX174 DNA is underlined.

the phage. Phage from both bands were isolated and found to have identical DNA sequences in the region of interest. Phage from both bands were equally infective in su$^+$ host and both reverted to wild-type with identical frequencies (2×10^{-5}).

Sequence of Phage DNA

The enzymatic terminator method (20) was used with analysis on thin 12% polyacrylamide gels (21). The fragment of ØX174 RF DNA used as primer was Hha I fragment 15 (7); Figures 4 and 5.

RESULTS AND DISCUSSION

Nucleotide 5276 of the ØX174 genome (1) was selected as the target for studies directed at the production of specific transversion mutations because it has been established that a G → T change at this position in viral DNA produces a suppressible mutation, am16 (7). Previous physical studies have established that the stability of a Watson-Crick duplex involving an oligodeoxyribonucleotide with one mismatched base-pair is similar irrespective of the nucleotides in the mismatch (22). Since oligodeoxyribonucleotides containing twelve nucleotides have proved very effective in producing specific transition mutations (1), similar sizes of oligodeoxyribonucleotides

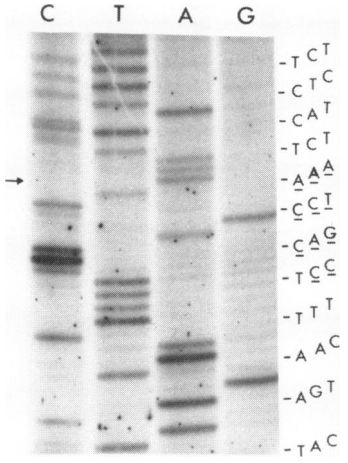

Fig. 4. Nucleotide sequence (Sanger enzymatic terminator method, 19) of the mutant phage induced by pCCCAGCCTAAA (details under Experimental Procedures). Since viral DNA is the template, the sequence is that of the complementary strand. The sequence corresponding to the mutagenic oligodeoxyribonucleotide is underlined and the mutant nucleotide is indicated by the arrow.

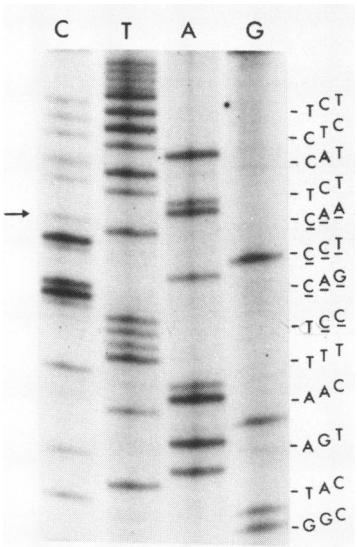

Fig. 5. Nucleotide sequence (Sanger enzymatic terminator method, 19) of the revertant phage induced by pCCCAGCCTCAA (details under Experimental Procedures). Since viral DNA is the template, the sequence is that of the complementary strand. The sequence corresponding to the mutagenic oligodeoxyribonucleotide is underlined and the mutant nucleotide is indicated by the arrow.

were used in the present studies. The two oligodeoxyribonucleotides which were synthesized each contain eleven nucleotides because they were most accessible to the method of synthesis, namely step-wise enzymatic synthesis catalysed by E. coli polynucleotide phosphorylase in the presence of Mn^{+2} ions (10,11). The syntheses were routine; only two steps resulted in low yields. These were the addition of G to pCCCA and the addition of C to pCCCAGCCT (Tables 1 and 2). On the small scale on which these syntheses were carried out, the final products were obtained in nmol amounts. These were ample for the present studies since the mutation experiments are on a pmol scale.

The specificity of two synthetic oligonucleotides for the desired site on the ØX174 wild-type viral DNA template was demonstrated in a pulse-chase experiment which measures the distance of the priming site(s) from the first Hae III cleavage site downstream (Fig. 3). The experiments show that the mutant oligodeoxyribonucleotide, pCCCAGCCTAAA, primed at a unique site on wild-type template, 325 nucleotides from the first down-stream Hae III cleavage. The ØX174 genome contains a cleavage site at nucleotide 4949, and thus

a fragment containing 325 nucleotides is predicted. The wild-type oligodeoxyribonucleotide, pCCCAGCCTCAA, on wild-type template, also produced the same 325 nucleotide fragment after Hae III cleavage (Band A, Fig. 3). However, an additional fragment containing 125 nucleotides was produced (Band B, Fig. 3), indicating priming at a second site. Inspection of the sequence of ØX174 DNA (3) shows that the sequence TTGAGGCT, which is complementary to the last eight nucleotides in the synthetic wild-type oligodeoxyribonucleotide, occurs at nucleotides 553-560 with an appropriately placed Hae III cleavage at position 435. Presumably the mutant oligodeoxyribonucleotide, pCCCAGCCTAAA, does not prime at the second site because there is a mismatch with nucleotide 555, which destabilizes the short duplex.

The replacement of G by T at nucleotide 5276 of viral DNA, using pCCCAGCCTAAA as the mutagenic primer for E. coli DNA polymerase I (Klenow) followed by ligation to obtain closed circular heteroduplex DNA and transfection of E. coli spheroplasts proceeded efficiently (Table 3). As indicated earlier, the nucleotide is expected to introduce an amber mutation in the reading frame of gene B (7,23). The sequence of the DNA of the am mutants confirms that the change in DNA sequence has been induced at the desired site (Fig. 4).

Because pCCCAGCCTCAA has a second priming site on viral DNA template (see above), a modified protocol for synthesis of heteroduplex DNA was used to induce preferential priming at the desired mutant site on am16 viral DNA (see Experimental Procedures). The conditions resulted in the efficient replacement of T by G as judged by the induction of reversion to wild-type (Table 3). DNA sequence determination established that the desired nucleotide replacement had been achieved (Fig. 5).

The efficiency of production of the two transversions, G → T (13%) and T → G (19%) using oligodeoxyribonucleotides containing eleven residues compares well with the G → A (15%) and A → G (15%) efficiencies at nucleotide 587 of ØX174 viral DNA achieved under similar conditions with oligodeoxyribonucleotides containing 12 residues (1). An essential factor in the success of all these experiments was the use of E. coli DNA polymerase I from which the 5'-exonuclease had been removed proteolytically (4-6). Predictably (1), attempts to use native E. coli DNA polymerase I with mutagenic oligodeoxyribonucleotides of similar length have been unsuccessful (24). Also it is possible that the 3'-exonuclease of E. coli DNA polymerase I could reduce the efficiency of a mutagenic oligodeoxyribonucleotide by editing out the mismatched nucleotide prior to elongation by the polymerase. It is encouraging

to find that the present experiments, where the mutated site is three nucleotides from the 3'-end of the oligodeoxyribonucleotide, were as successful as the experiments at nucleotide 587 of the ØX174 genome where the changed residue was six nucleotides from the 3'-ends of the mutagenic oligodeoxyribonucleotide (1). Clearly, it is not essential to have a long 3'-terminus on the mutagenic oligodeoxyribonucleotide to protect it from editing by the 3'-exonuclease of E. coli DNA polymerase I.

In the experiments which produced transition mutations at nucleotide 587, the relative numbers of desired mutant progeny were increased significantly by treatment of the synthetic heteroduplex with the single-strand specific endonuclease S_1 (1). Presumably, the enzyme selectivity destroys incomplete duplexes and single-stranded circles which are known to transfect more efficiently than duplex DNA (25). In the present experiments, endonuclease S_1 had little or no effect on the efficiency of production of mutant progeny (Table 3). This may indicate more efficient duplex synthesis in the present experiments, but it could also be an indication of the presence of a single-strand specific endonuclease contaminant in the enzymes used to produce heteroduplex DNA. It is probably not profitable to try to interpret quantitative differences in these experiments in terms of the in vitro priming efficiency of the different oligodeoxyribonucleotides; variations in burst size for different mutants and the dependency of the efficiency of in vivo heteroduplex repair on the position of the mismatch in the ØX174 genome (26) are likely to be significant factors affecting the efficiency of mutant production. It is, however, quite clear from the experiments with homologous pairs of primer oligodeoxyribonucleotide and template viral DNA (Table 3) that errors by the DNA polymerase or the DNA ligase play no role in the production of the mutations at nucleotide 5276 (27).

In conclusion, the present experiments have established the general principle that short synthetic oligodeoxyribonucleotides can be used to produce specific transversion mutations with high efficiency. This complements the already established procedure for transition mutations (1). In that the oligodeoxyribonucleotides are short and they can be synthesized by a simple enzymatic method (10,11), the procedure is generally accessible to molecular biologists. The production of an amber codon, starting at nucleotide 5276, which is lethal in su^- hosts, constitutes a formal synthetic confirmation that this reading frame, previously assigned to gene B (7,23) does define the gene of an essential protein. This illustrates one of the many potential biological applications of this specific mutagenic method.

ACKNOWLEDGEMENTS

We thank Dr. R.C. Miller for a gift of T4-DNA ligase and Dr. J.A. Lautenberger for ØX174 sB1 viral DNA. This research was supported by grants from the National Institutes of Health and the Medical Research Council of Canada. C.A.H. is a recipient of a National Institutes of Health Career Development Award and M.S. is a Research Associate of the Medical Research Council of Canada.

Abbreviations: RPC, reverse phase chromatography; A, G, C and T refer to deoxyribonucleotides; pfu, plaque forming units.

REFERENCES

1. Hutchison, C.A. III, Phillips, S., Edgell, M.H., Gillam, S., Jahnke, P. and Smith, M. (1978) J. Biol. Chem. 254, 6551-6560.
2. Barrell, B.G., Air, G.M. and Hutchison, C.A. III (1976) Nature 264, 34-41.
3. Sanger, F., Coulson, A.R., Friedmann, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchison, C.A. III, Slocombe, P.M. and Smith, M. (1978) J. Mol. Biol. 125, 225-246.
4. Brutlag, D., Atkinson, M.R., Setlow, P. and Kornberg, A. (1969) Biochem. Biophys. Res. Commun. 37, 982-989.
5. Klenow, H. and Henningsen, I. (1970) Proc. Nat. Acad. Sci. U.S.A. 65, 168-175.
6. Klenow, H. and Overgaard-Hansen, K. and Patkar, S.S. (1971) Eur. J. Biochem. 22, 371-381.
7. Brown, N.L. and Smith, M. (1977) J. Mol. Biol. 116, 1-28.
8. Panet, A., van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J. Lillehang, J.R. and Kleppe, K. (1973) Biochemistry 12, 5045-5050.
9. Vogt, V.M. (1973) Eur. J. Biochem. 33, 192-200.
10. Gillam, S., Rottman, F., Jahnke, P. and Smith, M. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 96-100.
11. Gillam, S., Jahnke, P. and Smith, M. (1978) J. Biol. Chem. 253, 2532-2539.
12. Khorana, H.G., Turner, A.F. and Vizsolyi, J.P. (1961) J. Am. Chem. Soc. 83, 686-698.
13. van de Sande, J.H., Kleppe, K. and Khorana, H.G. (1973) Biochemistry 12, 5050-5055.
14. Brownlee, G.G. and Sanger, F. (1969) Eur. J. Biochem. 11, 395-399.
15. Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1974) Nucleic Acids Res. 1, 331-353.
16. Astell, C.A., Smith, M., Chow, M. and Ward, D. (1979) in preparation.
17. Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 1184-1188.
18. Lautenberger, J.A., Kan, N.C., Lackey, D., Linn, S., Edgell, M.H. and Hutchison, C.A. III (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 2271-2275.
19. Gschwender, H.H. and Hofschneider, P.H. (1969) Biochim. Biophys. Acta 190, 454-459.
20. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 5463-5467.
21. Sanger, F. and Coulson, A.R. (1978) FEBS Letters 87, 107-110.

22. Gillam, S., Waterman, K. and Smith, M. (1975) Nucleic Acids Res. 2, 625-634.
23. Brown, N.L. and Smith, M. (1977) Nature 265, 695-698.
24. Razin, A., Hirose, T., Itakara, K. and Riggs, A.D. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 4268-4270.
25. Goulian, M., Kornberg, A. and Sinsheimer, R.L. (1967) Proc. Nat. Acad. Sci. U.S.A. 58, 2321-2328.
26. Weisbeek, P.J. and Van Arkel, G.A. (1976) Virology 72, 72-79.
27. Weymouth, L.A. and Loeb, L.A. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 1924-1928.