THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 253, No. 18, Issue of September 25, pp. 6551-6560, 1978
Printed in U.S.A.

Mutagenesis at a Specific Position in a DNA Sequence*

(Received for publication, February 22, 1978)

Clyde A. Hutchison, III,‡ Sandra Phillips, and Marshall H. Edgell§

From the Department of Bacteriology and Immunology and Curriculum in Genetics, University of North Carolina, Chapel Hill, North Carolina 27514

Shirley Gillam, Patricia Jahnke, and Michael Smith¶

From the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

Predefined changes in a known DNA sequence were introduced by a general method. Oligodeoxyribonucleotides complementary to positions 582 to 593 of the viral DNA strand of the bacteriophage φX174 *am*3 mutant (pGTATCCTACAAA), and to the wild type sequence in this region (pGTATCCCACAAA), were synthesized and used as specific mutagens. Each of these oligonucleotides was incorporated into a complete circular complementary strand when used as primer on a genetically heterologous viral strand template, by the combined action of subtilisin-treated *Escherichia coli* DNA polymerase I and T4 DNA ligase. Incomplete duplexes were removed or were inactivated by nuclease S1 and the products were used to transfect spheroplasts of *E. coli*.

Both oligonucleotides induced specific mutations at high efficiency when used with heterologous template (15% mutants among progeny phage). The *am* phages isolated by this procedure are phenotypically gene E mutants, and contain A at position 587 of the viral strand. They thus appear identical with *am*3 and provide evidence that the change G → A at position 587 is sufficient to produce a defective E function.

Since the template for the induction of *am* mutants carried another genetic marker (sB1), the strains carrying the induced mutations have the new genotype *am*3 sB1. It should be possible to introduce the *am*3 mutation into any known mutant strain of φX174 using this same oligonucleotide.

Both possible transition mutations were induced in these experiments. In principle, the method could also induce transversions, insertions, and deletions.

The method should be applicable to other circular DNAs of similar size, for example recombinant DNA plasmids.

The advent of methods for the rapid determination of DNA sequence (Sanger and Coulson, 1975; Maxam and Gilbert, 1977) has made available the exact sequence of extensive regions of DNA from a variety of organisms. In particular, a complete nucleotide sequence has been established for the genome of bacteriophage φX174 (Sanger *et al.*, 1977). Comparison of mutant DNA sequences to that of the wild type has been of great value in localizing genetic functions on the nucleotide sequence (Barrell *et al.*, 1976; Brown and Smith, 1977a,b; Smith *et al.*, 1977). It appears to us that general methods for the construction of any desired mutant sequence would greatly increase the ability of sequencing methods to yield biologically relevant information. Such methods should also make it possible to construct DNAs with specific modified biological functions.

We, therefore, have designed such a method for changing a specific nucleotide in a DNA sequence with high efficiency. The first step is the synthesis of an oligodeoxyribonucleotide complementary to the desired mutant sequence. This oligonucleotide is complementary to the wild type sequence except for the altered nucleotide(s). It is used as a primer for DNA polymerase on a circular, single-stranded wild type DNA template. Closed circular heteroduplexes are formed by ligation and incomplete duplexes removed. The mutant is then isolated biologically; by transfection of spheroplasts in the case of φX174 (Fig. 1).

In our first experiments, we have specifically mutated position 587 of φX174 DNA (Sanger *et al.*, 1977). In the wild type DNA, G is present at this position and is replaced by A in the gene E lysis mutant *am*3 (Barrell *et al.*, 1976). Genes D and E overlap in this region of the DNA (Fig. 2). We have synthesized, and used as specific mutagens, two oligodeoxyribonucleotides which are complementary to this region of wild type and *am*3 viral DNAs (Fig. 2).

EXPERIMENTAL PROCEDURES

Synthesis of Oligodeoxyribonucleotides—Chemicals and enzymes were obtained as described previously (Gillam *et al.*, 1977). The pGTAT was synthesized by chemical condensation of suitably protected pGT and pAT using the methods of Khorana *et al.* (1972). The protected tetranucleotide was isolated chromatographically on DEAE-cellulose. After removal of protecting groups, the pGTAT was further purified by successive chromatography on DEAE-cellulose in the presence and absence of 7 M urea (Tomlinson and Tener, 1963). Analytical high pressure RPC[1]-5 chromatography (Gillam *et al.*, 1977) and nucleoside analysis (Table II) indicated the presence of a small amount of dG-rich contaminating oligomer, probably pGTGT resulting from dimerization of the protected pGT. The stepwise enzymatic synthesis of pGTATCCTACAAA was performed by standard procedures (Gillam *et al.*, 1977), using the empirically established optimal

* This work was supported by National Institutes of Health Research Grants AI 08998 and GM 21313 and by a grant from the Medical Research Council (Canada). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of National Institutes of Health Career Development Award AI 70604.

§ Recipient of National Institutes of Health Career Development Award GM 00126.

¶ Medical Research Associate of the Medical Research Council (Canada).

[1] The abbreviations used are: RPC, reverse phase column chromatography; RF, replicative form DNA.

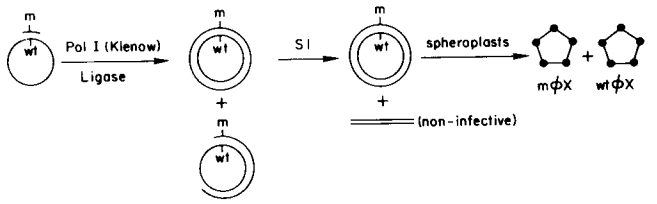

FIG. 1. Synthesis and isolation of a specific mutation (*m*) in φX174 (φ*X*) starting from a mutant oligonucleotide primer and a wild type (*wt*) viral strand template. The primer is extended by *E. coli* polymerase I (*Pol I*) which has been treated to destroy 5′-exonuclease activity (Klenow and Henningsen, 1970; Brutlag *et al.*, 1969; Klenow *et al.*, 1971). DNA ligase is present during the polymerase reaction to promote immediate conversion to closed circular duplex molecules. Such a heteroduplex molecule should be perfectly base-paired except at the site of the mutation. Because the conversion to complete duplex molecules is inefficient under our conditions, incomplete molecules are next removed or biologically inactivated. One simple procedure is treatment with nuclease S1 which cleaves any partially single-stranded structure to a linear noninfectious form. Transfection of *E. coli* spheroplasts then yields a phage population which contains a high proportion of the desired mutant.

conditions listed in Table I. Each preparative step was monitored by analytical high pressure RPC-5 chromatography. Reactions were stopped by addition of EDTA and the products isolated on DEAE-cellulose using a linear gradient of NaCl in 7 M urea and 0.05 M Tris·HCl, pH 7.5. Each oligonucleotide was desalted on a small DEAE-cellulose column (Gillam *et al.*, 1975a) and the purity was checked by analytical high pressure RPC-5 chromatography. At each stage, unreacted starting material was recovered and recycled one or more times (Table II). The products of the recycling steps were combined for nucleoside analysis (see below) before the next preparative step. Because the amount of pGTATCCTAC was small, the last step in the synthesis was carried out using [^{3}H]dADP as substrate to facilitate monitoring of the product (Fig. 3). Homogeneity of the product *am* 12-mer was examined after ^{32}P-labeling the 5′-terminus (van de Sande *et al.*, 1973) by homochromatography (Brownlee and Sanger, 1969) using elution mixture 5 of Jay *et al.* (1974). The pGTATCCCACAAA was synthesized by analogous procedures starting from a by-product of the above synthesis, pGTATCCC (Tables I and II).

Characterization of Oligodeoxyribonucleotides—Oligonucleotides were hydrolyzed with snake venom phosphodiesterase and *Escherichia coli* alkaline phosphatase and nucleoside compositions were quantitated by high pressure cation exchange chromatography on Aminex A5 (Pike and Rottman, 1974; Gillam *et al.*, 1977). The results are summarized in Table II. The two 12-mers, after ^{32}P-labeling of their 5′-termini, were characterized by two-dimensional sequence determination (Brownlee and Sanger, 1969) using the homochromatography mixture 5 of Jay *et al.* (1974). The mixture of partial digestion products required for this method of sequence determination was generated using a modification[2] of the endonuclease P_1 method (Lockard and RajBhandary, 1976). The oligonucleotide, ^{32}P-labeled at the 5′-terminus (0.03 μCi, at a specific activity of 1000 Ci/mmol), heat-denatured calf thymus DNA (1 μg), P_1 endonuclease (0.15 units), Tris·HCl, pH 7.5 (10 mM) in a volume of 10 μl was incubated at 20°C for 10 min and then heated at 100°C for 3 min to stop the reaction. The result of the sequence determination for pGTATCCTACAAA is shown in Fig. 4. The sequence is in agreement with that expected and there is only a small contamination of the 12-mer by a product of the same length separated electrophoretically. The sequence determination shows that the contaminant contains a dG at the 3rd residue from the 5′-terminus, a consequence of the pGTGT contaminant of the chemically synthesized pGTAT primer. The pGTATCCCACAAA was characterized in the same way and gave analogous results (not shown).

DNA Preparations—Viral strand DNA from φX174 *am*3 *cs*70 was prepared from phage purified by one equilibrium banding in CsCl, followed by sedimentation through a sucrose layer into a CsCl step gradient. Phages were dialyzed briefly against 0.05 M sodium tetraborate to remove CsCl, phenol-extracted twice, and dialyzed extensively against H_2O. This is the same preparation used in much of the sequence determination described by Sanger *et al.* (1977). Viral strand DNA from φX174 sB1 was a gift from J. A. Lautenberger and was prepared by phenol extraction of phage purified by two successive equilibrium bandings in CsCl.

Oligonucleotide Primed Synthesis of Covalently Closed Duplex DNA—φX174 viral strand (2 μl containing 1 μg of DNA) was mixed with 1.25 μl of oligonucleotide (A_{260} = 1) and 5.25 μl of "RF Synthesis Mix" (0.19 M KCl; 95 mM Tris·HCl, pH 7.8; 0.57 mM each of dATP, dTTP, dCTP, dGTP; 13 mM $MgCl_2$; 190 μM rATP; 19 mM NH_4Cl; in some experiments, one α-^{32}P-labeled deoxynucleoside triphosphate was included at approximately 0.08 μCi/μl). In some experiments, primer was preannealed by heating in a sealed capillary at 65°C for 2 min, followed by incubation for 30 min at 37°C. The sample was removed from the capillary to a polypropylene tube (Eppendorf). *E. coli* Pol I (0.5 μl of Boehringer Mannheim DNA polymerase (enzyme A according to Klenow *et al.*, 1971) at a nominal concentration of 892 units/ml) and T4 DNA ligase (0.4 to 0.5 units in 0.5 to 1.0 μl, obtained from Miles Research Products) were added and the reaction was incubated either at 0°C for 30 min followed by 2 h at 26°C, or simply at 26°C for 3 h with no noticeable difference in result. Reaction products were frozen and stored at −20°C for later analysis.

Enrichment for Closed Circular Duplex DNA—Incomplete RF and residual plus strand template were either destroyed by treatment with nuclease S1, or else removed by nitrocellulose filtration following alkaline denaturation. For S1 treatment, 1 μl of the Pol I plus ligase reaction product (this contains 0.1 μg of template strand) was mixed with 2.3 units of S1 in a total reaction volume of 25 μl containing 0.28 M NaCl, 4.5 mM $ZnCl_2$, 0.03 M sodium acetate, pH 4.0. Nuclease S1 prepared by a slight modification of the procedure of Vogt (1973) was a gift from Earl Ruley, a graduate student in the Department of Bacteriology, University of North Carolina, Chapel Hill. The reaction was incubated 30 min at 26°C, then 10 μl of 0.25 M Tris·HCl, pH 8.1, was added to stop the reaction. Samples were either used immediately for transfection or stored at −20°C.

In experiments employing the nitrocellulose filter method, 50 μl of 0.05 M Tris·Cl, pH 8.1, was added to the Pol I-ligase reaction mixture which was then extracted once with 50 μl of phenol:chloroform:isoamyl alcohol (25:25:1). The aqueous phase was extracted once with ether and then applied to a 1-ml column of Sephadex G-100 in 1 mM Tris·HCl, pH 7.5, 0.05 mM EDTA. The peak immediately after the void volume was collected and freeze-dried. Each sample was dissolved in 20 μl of H_2O and 200 μl of "denaturation buffer" (0.04 M K_2HPO_4, 0.05 M NaOH, 0.09 M NaCl, 0.01 M Tris·OH, 10^{-4} M EDTA), which has a pH of approximately 12.3, was added. After a few minutes at room temperature, 200 μl of 0.07 M KH_2PO_4 (the unadjusted pH of this solution was 4.8) was added, the solution was mixed and filtered slowly through a 13-mm diameter type HA Millipore filter which had been prewashed with 10 mM Tris·Cl, pH 8.1, 0.1 mM EDTA, 0.5 M NaCl. The filtrate was then filtered through a second Millipore filter. The filtrate was added to approximately 50 μl of packed nitrocellulose powder (RS 1/4 s, Hercules, Inc.) washed with 0.30 M NaCl, 0.03 M sodium citrate, pH 7, and the tube was mixed thoroughly. After centrifugation to pellet the nitrocellulose powder, the supernatant was either used immediately for transfection or stored at −20°C.

Transfection—Spheroplasts of *E. coli* strain W6 were prepared as previously described (Pagano and Hutchison, 1971) except that protamine sulfate (Benzinger *et al.*, 1971), was added to give a concentration of 25 μg/ml immediately following the termination of the lysozyme reaction with $MgSO_4$. The spheroplasts were stored at 0°C and could be used for at least 2 weeks after preparation. DNA samples were diluted to 0.4 ml with 0.05 M Tris·Cl, pH 8.1. Transfection was carried out as described (Pagano and Hutchison, 1971) except that all incubations were at 37°C. Phages were released by chloroform treatment and stored over chloroform at 4°C prior to plaque assay.

Genetic Analysis—Three φX174 mutations were used in these experiments: 1) *am*3 (Hutchison and Sinsheimer, 1966) is a suppressible gene E nonsense mutant resulting from a G → A change at position 587; 2) *cs*70 (Dowell, 1967) is a cold-sensitive mutant which has not been precisely localized but has been mapped in *Hin*dII fragment R1 (nucleotides 1295 to 2343); 3) sB1 (Schnegg and Hofschneider, 1969) is a mutation which confers sensitivity to *E. coli* B restriction and modification. This mutation has been localized to the vicinity of nucleotide 1650 by J. A. Lautenberger *et al.*(1978).

Total phage from transfections was plaque assayed on *E. coli* HF 4714 (su^+, r_km_k) indicator at 37°C, a condition which is permissive for all the mutations used. Phages which are wild type at the *am*3 site (am^+ phage) were selectively plaque assayed on *E. coli* C (su^-, r_om_o) at 37°C. Since there is no selective assay for *am* phage in the presence

[2] C. A. Astell, personal communication.

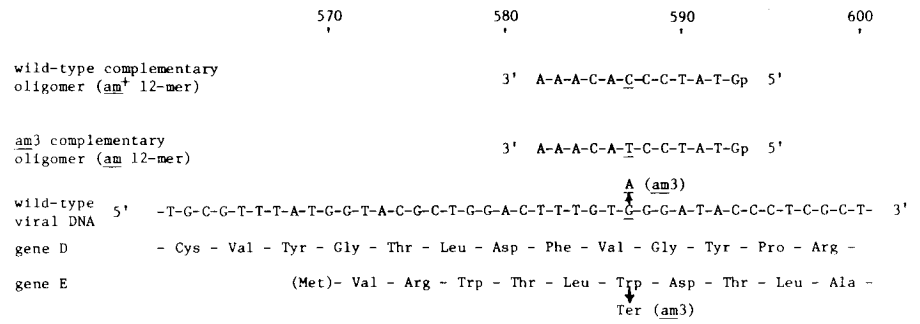

FIG. 2. Nucleotides 561 to 600 of wild type ϕX174 viral DNA and the synthetic oligodeoxyribonucleotides complementary to the region around nucleotide 587 in wild type and *am3* DNA. The mutant position 587 is *underlined* together with the corresponding nucleotide in the complementary oligodeoxyribonucleotides pGTATCCCACAAA (*am+ 12-mer*) and pGTATCCTACAAA (*am 12-mer*). The mutation of G to A at position 587 produces an *am* codon, TAG, in the gene E reading frame; the change from GTG to GTA in the gene D reading frame does not affect the protein sequence (Barrell *et al.*, 1976).

of *am+* phage, the frequency of induced *am* mutations was determined by first plating total progeny phage (HF 4714, 37°C), then spot testing individual plaques by stabbing with sterile toothpicks onto lawns of *su* (C) and *su+* (HF 4714). The presence of a clear spot on the *su+* indicator and no clearing of the *su* indicator demonstrates that the tested plaque is *am*. To test whether a plaque identified as *am* is mutant in gene E we used special media containing bile salts and lysozyme which in effect make E gene function nonessential. Gene E *am* mutants can make plaques on *su* cells (C) on these plates which were prepared as described previously (Barrell *et al.*, 1976). Plaques were tested for the presence or absence of *cs*70 by spot test on HF 4714 at 25°C and 37°C. Phages with the *cs*70 marker form plaques only at 37°C, whereas *cs+* phages form plaques at both temperatures. The presence of sB1 was demonstrated by a reduced efficiency of plaque formation of unmodified phage (grown in HF 4714) when plated on the r_Bm_B strain *E. coli* BC. Since this strain is *su* , it was necessary to perform tests for sB1 in the gene E *am* mutant strains on bile salts/lysozyme plates.

DNA Sequence Analysis—We used the rapid plus and minus method of Sanger and Coulson (1975) essentially as described by Air *et al.*, 1976 and by Brown and Smith, 1977b.

Fragment Z5 was used as primer on viral strand template to look at the sequence in the region of the *am3* site.

RESULTS

Synthesis and Characterization of pGTATCCTACAAA (am 12-mer) and pGTATCCCACAAA (am+ 12-mer)—The tetranucleotide pGTAT was synthesized using the chemical methods of Khorana *et al.* (1972). A small amount of contaminating oligonucleotide, detectable by high pressure analytical RPC-5 chromatography (Gillam *et al.*, 1977), persisted throughout purification of the tetranucleotide. Subsequent steps in the syntheses were carried out by the *E. coli* polynucleotide phosphorylase-catalyzed enzymatic method (Gillam and Smith, 1972, 1974; Gillam *et al.*, 1974; Gillam *et al.*, 1975a; Gillam *et al.*, 1977). Optimal conditions, as in earlier experiments, were achieved by varying $MnCl_2$, NaCl, and deoxynucleoside 5′-diphosphate concentrations and are listed in Table I. A notable observation was the difficulty in obtaining a good yield of the nonanucleotides, a consequence of the slow rate of addition of the pdC residues (Table I). Because of the small amounts of nonanucleotides which were available (Table II), the next step of the syntheses, addition of three pdA residues, was carried out with [^{3}H]dADP to facilitate product isolation (Fig. 3). At each stage of the syntheses, except the last, unreacted primer was recovered and recycled one or more times to increase the overall conversion (Table II).

Enzymatic conversion of oligonucleotides to their component nucleosides followed by quantitative high pressure cation exchange chromatography is a convenient method of characterization (Pike and Rottman, 1974; Gillam *et al.*, 1977) and was applied to all the oligonucleotide intermediates (Table II). The two dodecamer products were characterized using the two-dimensional sequencing method (Brownlee and Sanger, 1969; Jay *et al.*, 1974). Endonuclease P_1 has recently been used in RNA sequencing to prepare the complete set of partial digestion products required by the two-dimensional method (Lockard and RajBhandary, 1976). The method was used successfully in the present experiments with oligonucleotides (Fig. 4). In the case of pGTATCCTACAAA, the experiment gave the anticipated sequence and showed a contaminant of the same length with dG as the 3rd residue from the 5′-end. This was a consequence of the contaminant in the initial tetranucleotide primer pGTAT. Sequence determination of pGTATCCCACAAA gave analogous results (not shown).

Specific Priming by Synthetic Oligonucleotides on am3 and wt Templates—We have investigated the specificity of several of the synthetic oligonucleotides described above as primers for synthesis by *E. coli* Pol I using both *am3* and *wt* viral strands as templates. Synthesis in the presence of [α-^{32}P] dATP was cold-chased by the addition of excess nonradioactive dATP. The products were digested with *Hae* III, denatured, and analyzed as single strands by acrylamide gel electrophoresis in the presence of 7 M urea, as shown in Fig. 5.

The predicted product of these reactions extends from the 5′-terminal G (complementary to the C at position 593) to the *Hae* III cleavage site following residue 435, and therefore has an expected chain length of 158. Three specific priming products close to this expected size are observed, and calibration with *Hae* III fragments of ϕX174 RF indicates that *Band B* (Fig. 5) is the predicted product. Priming by the *am* 12-mer and the *am+* 12-mer is more efficient in each case with homologous template than with heterologous template, indicating that these 12-mer primers initiate synthesis at the *am3* site to produce *Band B*. Relative band intensities in the homologous and heterologous priming experiments also suggests that *Band A* (approximately 165 nucleotides) is also initiated by base-pairing in the vicinity of the *am3* site. The mechanism by which *Band A* arises is unknown.

The shorter *am* 7-mer primer also primes at the *am3* site to yield *Band B* when homologous (*am3*) template is used. This product is not produced on a *wt* template, presumably due to mispairing of the 3′-terminal T residue of the primer. However, both templates produce a new product (*Band C*, Fig. 5) with *am* 7-mer primer. We interpret this as the result of priming at the perfectly complementary sequence AGGATAC at residues 3265 to 3272 of the viral strand sequence. The synthesized fragment extending from this priming site to the *Hae* III site at residue 3121 would have a chain length of 151, in perfect agreement with the observed size of *Band C*. The sequence complementary to the *am* 7-mer does not occur elsewhere in the *wt* ϕX174 nucleotide sequence. We have no

TABLE I

Conditions for enzymatic stepwise synthesis of pGTATCCTACAAA and pGTATCCCACAAA catalyzed by E. coli polynucleotide phosphorylase

Incubations were at 37°C, [^{3}H]dADP had a specific activity of 2 mCi/mmol, and the enzyme had a specific activity of 16 units/mg as measured by the dADP polymerization assay.

Primer	μmol	dNDP	μmol	$MnCl_2$	NaCl	Enzyme	Volume	Time	Product	Yield
				μmol	*mmol*	*units*	*ml*	*h*		%
pGTAT	3.90	dCDP	24	160	2.4	56	8	5	pGTAT	34
									pGTATC	15
									pGTATCC	30
									pGTATCCC	12
pGTATC	0.81	dCDP	6	10	1.2	18	2	7	pGTATC	36
									pGTATCC	55
									pGTATCCC	9
pGTATCC	1.73	dTDP	20	100	0.5	60	5	16	pGTATCC	40
									pGTATCCT	51
pGTATCCT	1.40	dADP	5	100	3.0	60	5	7.5	pGTATCCT	81
									pGTATCCTA	14
pGTATCCTA	0.31	dCDP	1.8	6	0.7	20	1.2	6	pGTATCCTA	70
									pGTATCCTAC	15
pGTATCCTAC	0.054	[^{3}H]dADP	0.4	1	0.15	2	0.25	6.5	pGTATCCTAC	20
									pGTATCCTACA	31
									pGTATCCTACAA	15
									pGTATCCTACAAA	19
pGTATCCC	0.62	dADP	6	40	1.2	24	2	16	pGTATCCC	58
									pGTATCCCA	40
pGTATCCCA	0.30	dCDP	3.6	24	0.36	17	1.2	6	pGTATCCCA	87
									pGTATCCCAC	8
pGTATCCCAC	0.028	[^{3}H]dADP	0.15	0.4	0.06	0.7	0.1	6	pGTATCCCAC	35
									pGTATCCCACA	29
									pGTATCCCACAA	6
									pGTATCCCACAAA	12

TABLE II

Overall conversions at each step of the enzymatic synthesis and the nucleoside composition of the products

The amounts of product oligonucleotides are the sums of the material obtained after recycling the primer at each step. For other details see "Experimental Procedures."

Primer	μmol	Cycles	Product	μmol	Yield	dT	dG	dA	dC
					%				
pGTAT	3.90	5	pGTAT			1.84	1.25	1.00	0.00
			pGTATC	0.81	21	1.80	1.05	1.07	1.00
			pGTATCC	1.53	40	2.10	1.18	1.00	2.08
			pGTATCCC	0.62	16	2.07	1.33	1.00	3.08
pGTATC	0.81	2	pGTATCC	0.56	70				
			pGTATCCC	0.09	11				
pGTATCC	1.73	5	pGTATCCT	1.40	81	2.76	1.10	1.00	1.80
pGTATCCT	1.40	6	pGTATCCTA	0.31	22	3.04	1.14	1.96	2.00
pGTATCCTA	0.31	4	pGTATCCTAC	0.07	22	2.82	1.08	2.00	2.98
pGTATCCTAC	0.054	1	pGTATCCTACAAA	0.01	19				
pGTATCCC	0.62	3	pGTATCCCA	0.35	56	1.92	1.15	2.00	3.00
pGTATCCCA	0.30	5	pGTATCCCAC	0.028	9	1.98	1.17	2.00	4.18
pGTATCCCAC	0.028	1	pGTATCCCACAAA	0.003	12				

satisfactory explanation for the apparent decrease in priming at this site when using *wt* template. This could be partially due to a difference in quality of the template preparations used in these experiments. On the other hand, this effect might reflect a difference in secondary structure of the template resulting from the *am*3 mutation.

Taken together, these results indicate that both synthetic 12-mers prime in the regions which they were designed to

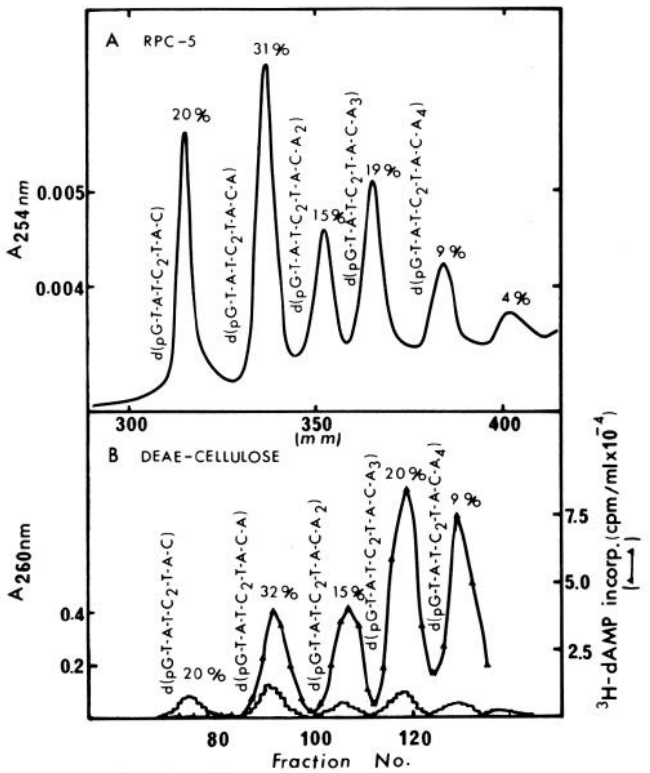

FIG. 3. Polynucleotide phosphorylase-catalyzed addition of [^{3}H]pdA residues to pGTATCCTAC. *A*, analytical separation of products by high pressure chromatography on RPC-5 (Gillam *et al.*, 1977). *B*, preparative separation of products on DEAE-cellulose (120 × 6 mm) using a gradient of NaCl (250 ml of 0.08 M and 250 ml of 0.2 M) in 7 M urea, 0.05 mM Tris·HCl, pH 7.5. Fractions (2 ml) were collected at 12-min intervals. Experimental details are in Table I.

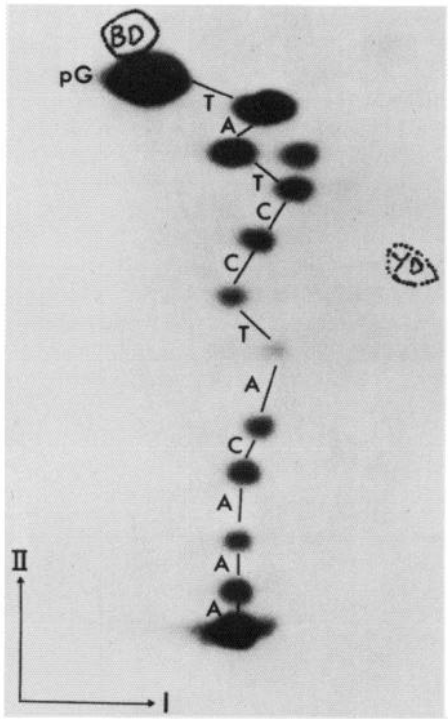

FIG. 4. Two-dimensional sequence determination for pGTATCCTACAAA. Details are under "Experimental Procedures." The trinucleotide spot corresponding to the contaminant with dG at the 3rd position from the 5′-terminus was produced in high yield due to preferential hydrolysis. The contamination is a much lower fraction of the total than is implied by the intensity of the spot. The letters *BD* and *YD* indicate the positions of "blue dye" (Xylene cyanol F.F.) and "yellow dye" (Orange G) markers.

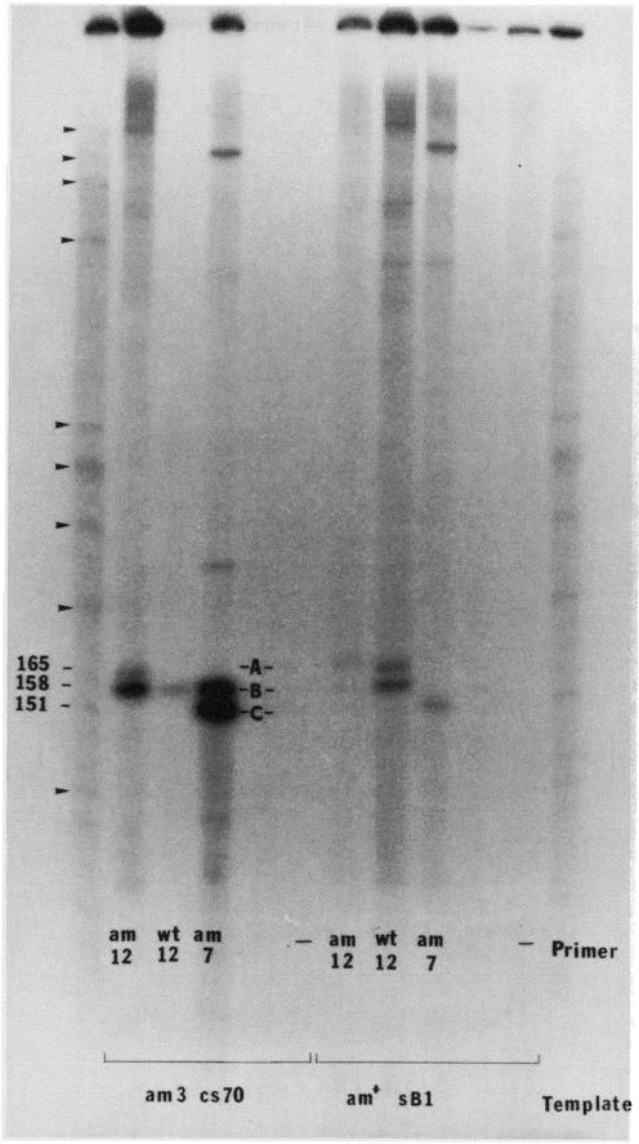

FIG. 5. Specific priming by synthetic oligonucleotides. Viral strand templates of genotype *am3cs70* and *am*$^+$sB1 were each primed with the *am* 12-mer (pGTATCCTACAAA), the *wt* 12-mer (pGTATCCCACAAA), the *am* 7-mer (pGTATCCT), or else were unprimed (indicated by ——), as indicated in the figure. The *leftmost* and *rightmost* channels contain *Hae* III digests of nick-translated *am3cs70* RF-DNA as markers. In the priming experiments, viral strand (1 μl of A_{260} = 15) and oligonucleotide (1 μl of A_{260} = 1) were mixed at room temperature to give a reaction volume of 10 μl containing 50 mM NaCl, 34 mM Tris·Cl, pH 7.4, 17 mM $MgCl_2$, 1.7 mM β-mercaptoethanol, 0.1 mM each of dCTP, dGTP, dTTP, and 3 μCi [α-^{32}P]dATP (approximately 100 Ci/mmol). The reaction mixture was chilled to 0°C and 0.5 μl (0.45 units) of *E. coli* Pol I (Klenow *et al.*, 1971) was added. After 15 min at 0°C, 0.5 μl of 10 mM dATP was added and incubation was continued for 5 min at room temperature. Polymerase was inactivated by heating the reaction to 70°C for 5 min. All samples were digested with excess *Hae* III at 37°C, then denatured by the addition of 25 μl of formamide followed by heating for 3 min at 100°C. Electrophoresis was performed in a 5% polyacrylamide gel containing 7 M urea and 90 mM Tris/borate, pH 8.3. The gel was fixed in 10% acetic acid and then autoradiographed. *Arrowheads* indicate the positions of single-stranded marker fragments Z1 (1342 n), Z2 (1078 n), Z3 (876 n), Z4 (606 n), Z5 (310 n), Z6a,b (271 n, 278 n), Z7 (230 n), Z8 (194 n), and Z9 (118 n). The letter n refers to nucleotides. Some reannealed fragments are also seen. Sizes of the fragments at positions *A* (165 n), *B* (158 n), and *C* (151 n), were estimated by interpolation from a plot of marker fragment mobility *versus* chain length.

specifically mutate. The *am* 7-mer shows an additional priming site which is interpretable in terms of the known φX174 sequence.

Specific Mutation—In preliminary experiments, we tested the ability of the *am* 12-mer to induce *am* mutants of φX174 using our standard marker rescue conditions which work with restriction fragments (Edgell *et al.*, 1972; Middleton *et al.*, 1972) and pancreatic DNase fragments larger than approximately 20 nucleotides in size (Hutchison and Edgell, 1971). A negligible level of mutation (one *am* mutant out of 600 plaques tested) was observed.

To raise the efficiency of mutations, we attempted reconstruction of an *am3* minus strand by using the *am* 12-mer as primer in a polymerase plus ligase reaction with an am^+ sB1 plus strand template. Initial experiments with this reaction indicated very inefficient conversion to covalently closed duplex DNA (less that 1%). We therefore incorporated a procedure for removing unused templates and incomplete duplex molecules by nitrocellulose filtration following alkaline denaturation and neutralization. The result of such an experiment is shown in Table III. A significant yield of *am* phage was found among the progeny when the *am* 12-mer was used as primer (19 *am* out of 225 plaques tested). No *am* mutants were found among the progeny when the am^+ 7-mer was used as primer, when primer was omitted, or when ligase was omitted from the reaction. Incorporation of ^{32}P into the synthesized DNA (data not shown) showed that the am^+ 7-mer primed essentially as well as the *am* 12-mer. The reaction with no added primer synthesized 50% as much DNA as the primed reactions, suggesting some extraneous source of priming. It seems likely that the efficiency of recovery of *am* mutants may be limited in part by the efficiency of the nitrocellulose enrichment procedure. If it worked perfectly, the nitrocellulose procedure should remove all infective DNA from the nonligated reaction. However, the total number of phage produced in spheroplasts was actually not dependent on the addition of ligase, presumably due to incomplete removal by nitrocellulose of single-stranded or reannealed material. Because all samples were denatured and filtered through nitrocellulose, we cannot draw any conclusion about the ability of a completely polymerized but nonligated duplex to produce mutants.

All 19 of the *am* mutants isolated in this experiment formed plaques on su^- *E. coli* in the presence of bile salts and lysozyme, indicating that they are all gene E mutants. We have tested 35 *am* mutants (from two experiments) induced by the *am* 12-mer and enriched by the nitrocellulose procedure for the ability to form plaques at 25°C. All were able to plate at this temperature and are therefore cs^+ and cannot be *am3cs70* which would be a possible contaminant due to its widespread use in the laboratory. Five of the *am* isolates from experiments using nitrocellulose enrichment have been tested for restriction by the *E. coli* B system. All five are restricted, indicating that they carry the sB1 marker from the template plus strand. No strains carrying *am* mutations and the sB1 mutation existed prior to these experiments.

The nitrocellulose enrichment procedure is cumbersome and inherently somewhat variable since its efficiency depends on the rate of filtration. We therefore have explored an alternative enrichment procedure which inactivates any φX genomes except those contained in completely duplex molecules by treatment with nuclease S1. Table IV shows the result of such an experiment. Under the S1 treatment conditions employed, infectious φX plus strand was inactivated by a factor of 10^3, while supercoiled RF (purified from infected cells) was inactivated less than 2-fold.

It is clear from the data of Table IV that the *am* 12-mer induces *am* mutants with high efficiency (16% of the progeny from transfection are *am*). Similarly the am^+ 12-mer specifically reverts *am3* to am^+ at the same rate (15%). Control experiments employing primers homologous to the target region of the template showed no detectable mutation or reversion rate (the *am3cs70* template DNA already contained one part in 10^4 am^+ revertants). In the reversion experiment it was possible to assay the induced revertants prior to the S1 enrichment step. S1 treatment did not reduce the absolute number of induced revertants, suggesting that only completed duplex molecules yield revertant progeny. The apparent lower susceptibility to S1 in the homologous *am3cs70* 12-mer primed reaction (Reaction 1, Table IV) is probably a consequence of a more efficient primed reaction with this combination.

TABLE III

pGTATCCTACAAA induces am mutants of ΦX174

The template plus strand in this experiment was wild type at the *am3* site and carried the sB1 mutation (am^+ cs^+ sB1). Primers were not preannealed. The polymerase plus ligase reactions were for 30 min at 0°C, then 2 h at 26°C. No ligase was added to Reaction 2. The products were enriched for closed circular duplex DNA by the nitrocellulose method. One hundred microliters of the nitrocellulose treated supernatant was used for transfection. Total progeny plaque-forming units in the 2-ml spheroplast reactions are tabulated.

Reaction	Primer		Ligase	Total progeny	*am* progeny
	Sequence	Genotype			Total progeny
1	GTATCCTACAAA	*am3*	+	1.6×10^4	19/225
2	GTATCCTACAAA	*am3*	−	1.6×10^4	0/225
3	GTATCCC	am^+	+	4×10^4	0/225
4	None		+	1.6×10^4	0/225

TABLE IV

Specific induction and reversion of am genotype by GTATCCTACAAA and GTATCCCACAAA

Primer and template were subjected to the preannealing procedure prior to polymerase plus ligase reactions for 3 h at 26°C. Products were enriched for closed circular duplex DNA by the S1 nuclease procedure. The entire S1 reaction product was used for transfection. Progeny phage yields are stated in terms of number of plaque-forming units in the 2-ml spheroplast reaction.

Reaction	Primer		Template genotype	Nonenriched progeny			S1 enriched progeny		
	Sequence	Genotype		Total	Mutant (*am*) or revertant (am^+)	Mutations Total	Total	Mutants (*am*) or revertants (am^+)	Mutations Total
1	pGTATCCTACAAA	*am3*	*am3cs70*	4×10^6	7.1×10^2 (am^+)	1.8×10^{-4}	8.8×10^5	2×10^1 (am^+)	2×10^{-5}
2	pGTATCCCACAAA	am^+	*am3cs70*	1×10^7	6.5×10^3 (am^+)	6.5×10^{-4}	8.2×10^4	1.3×10^4 (am^+)	0.16
3	pGTATCCTACAAA	*am3*	am^+sB1	8×10^5			1.8×10^4	2.7×10^3 (*am*)	0.15 (156/1021)
4	pGTATCCCACAAA	am^+	am^+sB1	1.6×10^6			4.3×10^4	$<\sim 9 \times 10^1$ (*am*)	$<\sim 2 \times 10^{-3}$ (0/500)

Ten *am* mutants induced in the experiment of Table IV were randomly selected for further study. All 10 were shown to form plaques on *su*⁻ *E. coli* in the presence of bile salts and lysozyme, and thus are gene E mutants. All 10 are *cs*70⁺ (form plaques at 25°C) and carry the sB1 marker (are restricted in *E. coli* BC). These isolates were further characterized by DNA nucleotide sequence analysis as described below.

Nucleotide Sequence of Induced Mutations—In order to

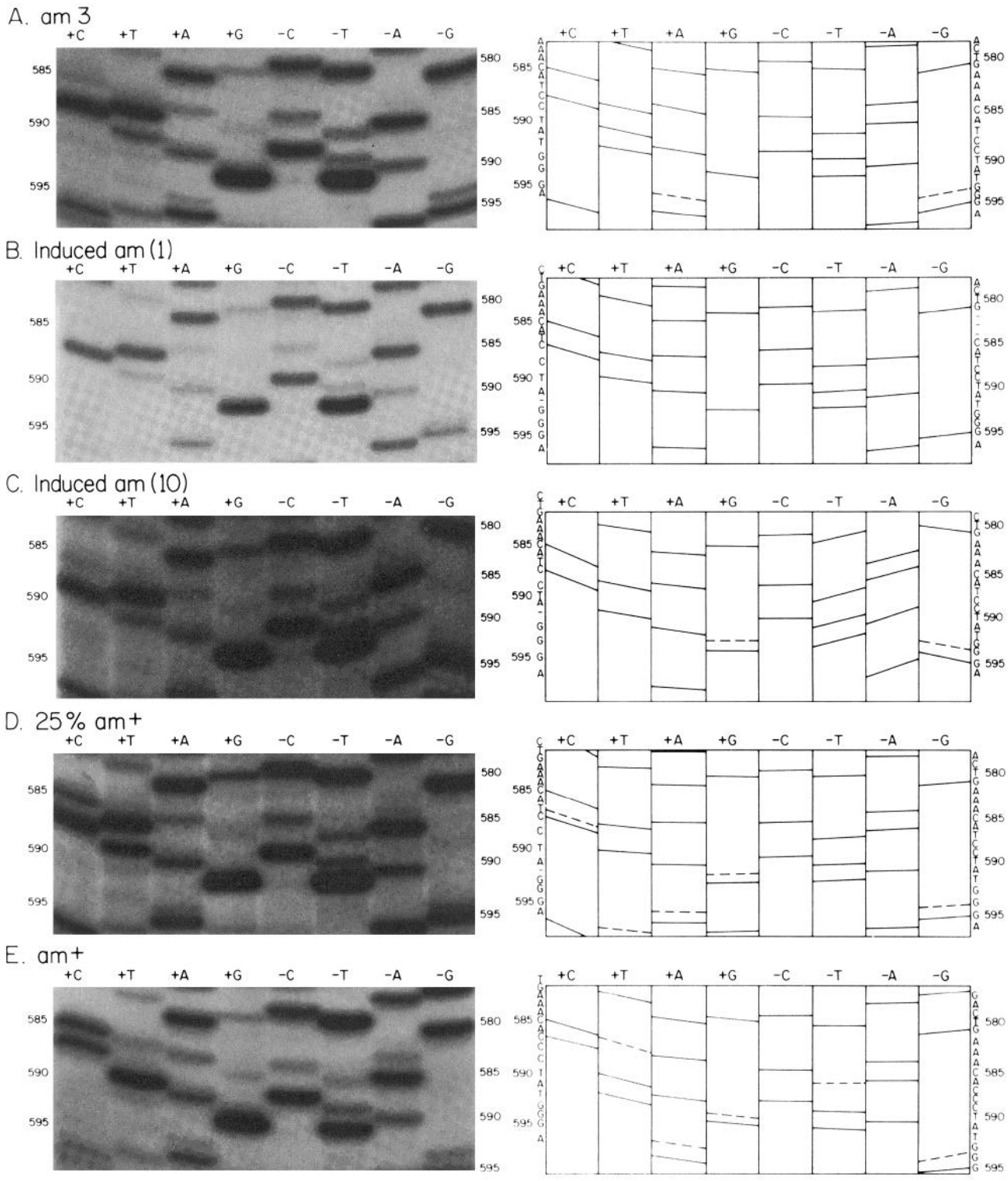

FIG. 6. Nucleotide sequencing of oligonucleotide induced *am* mutants. *A*, *am*3*cs*70 viral strand template; *B*, viral strand template from an *am* mutant induced by the *am* 12-mer (isolate *oam*6); *C*, viral strand template was prepared from a mixture of equal numbers of plaque-forming particles from 10 *am* isolates induced by the *am* 12-mer in the experiment of Table IV; *D*, viral strand template was a mixture of the pooled *am* mutants (*C*, above) and *am*⁺sB1 in the ratio 3:1, based on A_{260} measurements; *E*, *am*⁺sB1 viral strand template. A portion of each gel is shown along with a diagrammatic representation indicating the relevant bands. Deduced sequence is displayed beside the diagrams and is aligned with the outermost channels (bands are curved). *Dashed lines* indicate positions of artifact bands (internally inconsistent extra bands in either a plus or minus channel, but not appearing in both, see Sanger and Coulson, 1975). *Dashes* in the deduced sequences indicate bands which are missing in either the plus or minus channels. The apparent artifact in the +C channel of *Panel D* at position 587 is expected because of the *am*⁺ template deliberately added to this sample.

show conclusively that the method described here induces the intended mutation, we have determined the nucleotide sequence change in induced *am* mutants produced by the method. To determine whether all of the induced *am* mutants contain a G → A change at position 587 without sequencing a large number of individual isolates, we have performed some of the sequencing experiments with DNA prepared from a mixture of 10 different *am* clones. Fig. 6 displays plus and minus sequencing gels which compare the sequence of induced *am* mutants with those of *am3* and am^+ strains. A single *am* mutant induced by the *am* 12-mer and selected following nitrocellulose enrichment (Fig. 6*B*), and also an equal mixture of 10 *am* mutants selected following S1 enrichment (Fig. 6*C*), both show homogeneous sequence patterns identical with *am3* (Fig. 6*A*). These induced *am* mutants have an A at position 587 instead of G which is found in the am^+ strain (Fig. 6*E*). Reconstruction experiments in which am^+ sB1 DNA was added to the mixture of 10 induced *am* DNAs showed that 25% contamination with am^+ sequence is readily detectable (Fig. 6*D*) and that 10% am^+ sequence is at the limit of detectability by this method (data not shown). The only new band which would be expected to result from addition of am^+ DNA in such a reconstruction experiment would be a +C band at position 587. Because this band is not well resolved from the +C band at position 586 resulting from the *am* sequence, and because we sometimes see an artifact in the +C channel at approximately position 587, other criteria are more reliable for the estimation of am^+ contamination. Relative intensities of certain bands differ markedly in *am3* and am^+ sequence gels and can thus be used as markers. In particular, the +T band at position 589 and the +C band at position 585 have a much higher intensity in the am^+ pattern (Fig. 6*E*) than in the *am* patterns (Fig. 6, *A*, *B*, and *C*). The relative intensity of these bands is clearly increased by the addition of 25% am^+ DNA to the pool of 10 induced *am* isolates (Fig. 6*D*).

We conclude that at least 8, and probably all, of the 10 pooled *am* isolates are identical in sequence with *am3* in this region of the genome.

DISCUSSION

The need for precise control and targeting of mutagenic agents has long been evident. Chemical modification of a specific DNA fragment followed by use of that fragment to introduce mutations in a specific gene has been described (Borrias *et al.*, 1976). A mutagenic nucleoside analog, N^4-hydroxycytidine, has been enzymatically introduced at a specific site in bacteriophage Qβ RNA to produce a specific mutation (Flavell *et al.*, 1974, 1975; Sabo *et al.*, 1977), and this method should be adaptable to DNA mutagenesis. However, the ideal method would introduce all four classes of point mutation (transitions, transversions, deletions, and insertions) in a completely predictable manner at a specific site at an efficiency high enough to allow mutant isolation without biological selection. It seemed to us that the best prospects of success were offered by *in vitro* synthesis of an oligonucleotide for use as a specific mutagen.

To test the method, we decided to synthesize oligonucleotides designed to mutagenize position 587 in φX174 DNA. This site had a number of useful characteristics for a first test of the method. A well characterized gene E mutant *am3* had been shown to occur at this position. It is possible to screen for such *am* mutants by standard procedures, and to identify them as gene E mutants by phenotypic suppression on a special medium. This region may be readily sequenced to verify the structure of induced mutations (Barrell *et al.*, 1976).

Specific regions of φX174 DNA can be rescued from a small complementary wild type DNA fragment annealed to circular mutant viral single-stranded DNA (Weisbeek and van de Pol, 1970; Hutchison and Edgell, 1971). It has been suggested that this technique could be used to incorporate a synthetic oligonucleotide into an intact genome, although fragments less than about 20 nucleotides long were not rescued by this method (Hutchison and Edgell, 1971). Rescue was possible using a long fragment with the base pair mismatch eight nucleotides from its 5′-end, however, the same fragment with mismatches eight and six base pairs from the end would not rescue the mutations (Weisbeek *et al.*, 1976; Smith *et al.*, 1977).

The oligonucleotides were synthesized by the stepwise enzymatic method in order to minimize the possibility of low level chemical changes which would increase the mutagenic background. A length of 12 nucleotides was chosen on the basis of the following considerations. Firstly, in order to be unique in a genome of the size of φX174, the oligonucleotide should be at least a heptamer (Astell and Smith, 1972). Secondly, a mismatched base pair, in the center of an oligodeoxyribonucleotide paired with a complementary sequence, destabilizes the structure by an amount which is approximately equivalent to the removal of two base pairs (Gillam *et al.*, 1975b); much more extensive mismatching with a short oligonucleotide would probably not result in a stable structure (Astell *et al.*, 1973). It seemed that a decamer would be close to the minimal functional length and a dodecamer was closed to the maximum accessible by the synthetic method (Gillam *et al.*, 1977).

Oligodeoxyribonucleotides can be used as primers on circular single strand φX174 DNA for *E. coli* DNA polymerase I and the products can be converted to closed circular duplexes by ligation (Goulian *et al.*, 1967; Goulian, 1968a). At 20°C, oligonucleotides containing nine nucleotides and more are efficient primers (Goulian *et al.*, 1973), but most of the primer molecule is excised from the product, presumably due to the 5′-exonuclease activity in the enzyme (Goulian, 1968b). We therefore decided, because *E. coli* DNA polymerase I is so effective at DNA synthesis on a single-stranded template (Sherman and Gefter, 1976), to use the mismatched oligonucleotides as primers for *E. coli* DNA polymerase I in which the 5′-exonuclease had been inactivated by subtilisin (Brutlag *et al.*, 1969; Klenow and Henningsen, 1970; Klenow et al., 1971).

The synthesis and characterization of the *am* 12-mer and the am^+ 12-mer presented no major problems (Tables I and II, Figs. 3 and 4). A minor contaminant in both oligonucleotides did not produce any detectable problems. Both oligonucleotides primed DNA synthesis by subtilisin-treated *E. coli* DNA polymerase I at the desired site on *am* and am^+ DNAs in heterologous and homologous pairings.

Priming with a 12-mer homologous to the template was more efficient than heterologous priming in both cases (Fig. 5).

The products of synthesis by polymerase in the presence of ligase were tested for the induction of *am* mutants and for reversion of the *am3* mutation (Tables III and IV). Revertants could be selectively assayed and therefore could be readily detected among progeny phage from am^+ 12-mer primed reaction using *am3* plus strand template (Table IV). The frequency was low however, due to a high background of *am3* progeny arising from incomplete duplex molecules. This background could be reduced by removing incomplete molecules by nitrocellulose filtration after selective alkaline denaturation (Table III), or more simply by endonuclease S1 digestion

(Vogt, 1973). We employed digestion conditions where cleavage at a single mismatched base pair should be very infrequent (Shenk *et al.,* 1975; Dodgson and Wells, 1977).

Following the enrichment procedures, *am* mutants or revertants were produced at remarkably high efficiency by products of heterologously primed reactions. No detectable mutagenesis by homologous primers was observed. This indicates that mutations arise by incorporation of the primer into a complete minus strand as was intended and not as the result of poor synthetic fidelity during the *in vitro* polymerase reaction.

DNA sequence analysis of *am* mutants induced by the *am* 12-mer show an A at position 587, while G is present at this position in the am^+ sB1 template used in the mutagenesis experiment. This is the same mutational change which led to the formation of *am*3 from the wild type φX174 (Barrell *et al.,* 1976). In fact, the high yield of *am* mutants identical in phenotype with *am*3 which results from our procedure provides evidence that the observed mutation at position 587 is the only change necessary to inactivate gene E function. These experiments provide additional proof, by partial synthesis, for the overlapping of genes D and E, since this portion of the φX174 DNA is known to code for the D protein (Barrell *et al.,* 1976).

The am^+ template plus strand used for the induction of *am* mutants by the *am* 12-mer carried a mutation (sB1) at another site. Therefore, *am* mutants arising from the experiment were found to be *am*3 sB1. This suggests that this is a useful general method for the construction of double mutant strains carrying the *am*3 mutation.

In terms of the chemistry of mutation, the experiments described here have synthesized the two mismatched pairings, C,A and T,G, which are the intermediates for the two possible transition mutations, CG ↔ TA, and both mutations were produced. The stabilities of mismatched base pairs allows the prediction that the intermediates for all of the possible single base pair transversions can be produced equally easily (Gillam *et al.,* 1975b). It should be possible in principle to produce insertions and deletions by the strategy described here, although sufficient data is not available to estimate the chain length required for primers which are mispaired with the template strand in these ways.

An important feature of the method is its high efficiency. The experiment of Table IV yielded a phage population, 15% of which were *am* mutants at a single site. In isolation of φX174 *am* mutants by the traditional genetic approach, it is typical to screen populations containing one *am* mutant per 500 to 1000 phage. Such mutants are distributed among more than 20 mapped sites on the φX174 genome (Hutchison, 1969; Benbow *et al.,* 1971; Benbow *et al.,* 1974). The yields of mutants derived from 12-mer primers in these experiments are comparable to those obtained from full length minus strands annealed to a genetically distinguishable plus strand (Merriam *et al.,* 1971a; Merriam *et al.,* 1971b).

In the present experiments, it was convenient to screen for the biological phenotype of the induced mutations. However, in the general case, it is desirable to be able to isolate mutants in the absence of such a selection procedure. If mutants are obtainable at about a 10% frequency, then those in which a restriction endonuclease site is induced or deleted can be readily selected. If the only method for selection is DNA sequence determination, then a mutational efficiency of greater than 25% is desirable. It should be possible to obtain a frequency of 100% by strand separating the closed circular heteroduplex DNA and isolating the mutant strand. In the case of φX174, this can be used to infect directly (Rust and Sinsheimer, 1967; Siegel and Hayashi, 1967; Goulian *et al.,* 1967). Isolation of mutants without any selection based on phenotype should make it possible to isolate phenotypically silent mutants and mutants which would unconditionally inactivate gene function. The latter might be done, for example, by mutagenizing a phage gene which had been cloned onto a bacterial plasmid in order to make its replication independent of its function.

It will be possible to use the mutagenizing method with many DNAs because filamentous phage DNA can be used as a cloning vector (Messing *et al.,* 1977). With duplex DNAs, such as plasmids, the strands can be separated to permit use of the method. Alternatively, it may be possible to use the method via the intermediacy of displacement loop structures (Holloman and Radding, 1977).

This new method of mutagenesis has considerable potential in genetic studies. Thus, it will be possible to change and define the role of regions of DNA sequence whose function is as yet incompletely understood, for example, origins of DNA replication, promoters, and the sequences for ribosome-binding sites. In addition, specific mutation within protein structural genes will allow the production of proteins with specific amino acid changes. This will allow precise studies of structure-function relationships, for example the role of specific amino acids in enzyme active sites, and the more convenient production of proteins whose action is species specific, for example the conversion of a cloned gene coding for a lower vertebrate hormone to that for a human.

Acknowledgments—We thank Earl Ruley for a gift of endonuclease S1 used in this work. James A. Lautenberger generously provided us with φX174 sB1 viral strand and identified the sB1 mutation on an *am* mutant induced in our experiments. E. Beck and H. Schaller kindly communicated their procedures for *in vitro* synthesis of closed circular duplex DNA from single-stranded viral DNA.

REFERENCES

Air, G. M., Sanger, F., and Coulson, A. R. (1976) *J. Mol. Biol.* **108,** 519–533

Astell, C. R., Doel, M. T., Jahnke, P. A., and Smith, M. (1973) *Biochemistry* **12,** 5068–5074

Astell, C. R., and Smith, M. (1972) *Biochemistry* **11,** 4114–4120

Barrell, B. G., Air, G. M., and Hutchison, C. A., III (1976) *Nature* **264,** 34–41

Benbow, R. M., Hutchison, C. A., III, Fabricant, J. D., and Sinsheimer, R. L. (1971) *J. Virol.* **7,** 549–558

Benbow, R. M., Zuccarelli, A. J., Davis, G. C., and Sinsheimer, R. L. (1974) *J. Virol.* **13,** 898–907

Benzinger, R., Kleber, I., and Huskey, R. (1971) *J. Virol.* **7,** 646–650

Borrias, W. E., Wilschut, I. J. C., Vereijken, J. M., Weisbeek, P. J., and van Arkel, G. A. (1976) *Virology* **70,** 195–197

Brown, N. L., and Smith, M. (1977a) *Nature* **265,** 695–698

Brown, N. L., and Smith, M. (1977b) *J. Mol. Biol.* **116,** 1–30

Brownlee, G. G., and Sanger, F. (1969) *Eur. J. Biochem.* **11,** 395–399

Brutlag, D., Atkinson, M. R., Setlow, P., and Kornberg, A. (1969) *Biochem. Biophys. Res. Commun.* **37,** 982–989

Dodgson, J. B., and Wells, R. D. (1977) *Biochemistry* **16,** 2374–2379

Dowell, C. E. (1967). *Proc. Natl. Acad. Sci. U. S. A.* **58,** 958–961

Edgell, M. H., Hutchison, C. A., III, and Sclair, M. (1972) *J. Virol.* **9,** 574–582

Flavell, R. A., Sabo, D. L., Bandle, E. F., and Weissmann, C. (1974) *J. Mol. Biol.* **89,** 255–272

Flavell, R. A., Sabo, D. L. O., Bandle, E. F., and Weissmann, C. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 367–371

Gillam, S., Rottman, F., Jahnke, P., and Smith, M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 96–100

Gillam, S., and Smith, M. (1972) *Nature New Biol.* **238,** 233–234

Gillam, S., and Smith, M. (1974) *Nucleic Acids Res.* **1,** 1631–1647

Gillam, S., Waterman, K., Doel, M., and Smith, M. (1974) *Nucleic Acids Res.* **1,** 1649–1664

Gillam, S., Waterman, K., and Smith, M. (1975a) *Nucleic Acids Res.* **2,** 613–624

Gillam, S., Waterman, K., and Smith, M. (1975b) *Nucleic Acids Res.* **2,** 625–634

Goulian, M. (1968a) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 284–291
Goulian, M. (1968b) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 11–20
Goulian, M., Goulian, S. H., Codd, E. E., and Blumenfield, A. Z. (1973) *Biochemistry* **12,** 2893–2901
Goulian, M., Kornberg, A., and Sinsheimer, R. L. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **58,** 2321–2328
Holloman, W. K., and Radding, C. M. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3910–3914
Hutchison, C. A., III (1969) Ph.D. thesis, California Institute of Technology
Hutchison, C. A., III, and Edgell, M. H. (1971) *J. Virol.* **8,** 181–189
Hutchison, C. A., III, and Sinsheimer, R. L. (1966) *J. Mol. Biol.* **18,** 429–447
Jay, E., Bambara, R., Padmanabhan, R., and Wu, R. (1974) *Nucleic Acids Res.* **1,** 331–353
Khorana, H. G., Agarwal, K. L., Büchi, H., Caruthers, M. H., Gupta, N. K., Kleppe, K., Kumar, A., Ohtsuka, E., RajBhandary, U. L., van de Sande, J. H., Sgaramella, V., Terao, T., Weber, H., and Yamada, T. (1972) *J. Mol. Biol.* **72,** 209–217, 219–249, 251–288, 289–307, 309–327, 329–349, 351–373, 375–405, 407–426, 427–444, 445–456, 457–474, 475–492
Klenow, H., and Henningsen, I. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **65,** 168–175
Klenow, H., Overgaard-Hansen, K., and Patkar, S. A. (1971) *Eur. J. Biochem.* **22,** 371–381
Lautenberger, J. A., Kan, N. C., Lackey, D., Linn, S., Edgell, M. H., and Hutchison, C. A., III (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2271–2275
Lockard, R. E., and RajBhandary, U. L. (1976) *Cell* **9,** 747–760
Maxam, A. F., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 560–564
Merriam, V., Dumas, L. B., and Sinsheimer, R. L. (1971a) *J. Virol.* **7,** 603–611
Merriam, V., Funk, F., and Sinsheimer, R. L. (1971b) *Mutat. Res.* **12,** 206–210
Messing, J., Gronenborn, B., Muller-Hill, B., and Hofschneider, H. P. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 3642–3646
Middleton, J. H., Edgell, M. H., and Hutchison, C. A., III (1972) *J. Virol.* **10,** 42–50
Pagano, J. S., and Hutchison, C. A., III (1971) *Methods Virol.* **5,** 79–123
Pike, L. M., and Rottman, F. (1974) *Anal. Biochem.* **61,** 367–378
Rüst, P., and Sinsheimer, R. L. (1967) *J. Mol. Biol.* **23,** 545–552
Sabo, D. L., Domingo, E., Bandle, E. F., Flavell, R. A., and Weissmann, C. (1977) *J. Mol. Biol.* **112,** 235–252
Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M., and Smith, M. (1977) *Nature* **265,** 687–695
Sanger, F., and Coulson, A. R. (1975) *J. Mol. Biol.* **94,** 441–448
Schnegg, B., and Hofschneider, P. H. (1969) *J. Virol.* **3,** 541–542
Shenk, T. E., Rhodes, C., Rigby, P. W. J., and Berg, P. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 989–993
Sherman, L. A., and Gefter, M. L. (1976) *J. Mol. Biol.* **103,** 61–76
Siegel, J. E. D., and Hayashi, M. (1967) *J. Mol. Biol.* **27,** 443–451
Smith, M., Brown, N. L., Air, G. M., Barrell, B. G., Coulson, A. R., Hutchison, C. A., III, and Sanger, F. (1977) *Nature* **265,** 702–705
Tomlinson, R. V., and Tener, G. M. (1963) *Biochemistry* **2,** 697–702
van de Sande, J. H., Kleppe, K., and Khorana, H. G. (1973) *Biochemistry* **12,** 5050–5055
Vogt, V. M. (1973) *Eur. J. Biochem.* **33,** 192–200
Weisbeek, P. J., and van de Pol, J. H. (1970) *Biochim. Biophys. Acta* **224,** 328–338
Weisbeek, P. J., Vereijken, J. M., Baas, P. D., Jansz, H. S., and van Arkel, G. A. (1976) *Virology* **72,** 61–71