A new system for studying the molecular mechanisms of mutation by carcinogens is described. The system involves (a) site-specific modification of the essential gene G in φX174 replicative form DNA by a combination of chemical and enzymatic steps; (b) production of mutant virus carrying a change at a single preselected site by transfection of spheroplasts with the site-modified φX174 DNA; (c) detection and propagation of mutants using a host carrying the plasmid, pαXG, that rescues all type of gene G mutants by complementation; (d) identification of the mutation in the progeny virus by isolating and sequencing mutant φX174 DNA in the region that carried the parental, site-specific change. To demonstrate that this system is operational, we have produced a previously unknown φX174 gene G mutant carrying a C → T base change at position 2401 of the viral (plus) strand. This preplanned, nonsense (amber) mutant was obtained by changing G to A at the appropriate position in a chemically synthesized, octadexynucleotide, minus strand primer; elongating this enzymatically with Escherichia coli DNA polymerase I (large fragment) (lacking 5' → 3' exonuclease activity) to a 17-mer; and repriming to obtain the site-modified φX174 replicative form DNA enzymatically with E. coli DNA polymerase I (large fragment) and T4 DNA ligase. After transfection of spheroplasts with the heteroduplex DNA, the lysate was screened for mutant virus with permissive (carrying pαXG) and nonpermissive (without pαXG) host cells. About 1% of the progeny virus were mutants. Out of 15 isolates, 11 were suppressible by an amber Su1* (serine) or an ochre Su8* (glutamine) suppressor strain, but not by an amber Su3* (tyrosine) suppressor. The other 4 isolates were not suppressed at all. Replicative form DNA produced from one of the suppressible mutants was shown (by sequencing) to contain the expected C → T change at the preselected site in the viral strand. Replicative form DNA from one of the nonsuppressible mutants was partially sequenced. No change was found at or around position 2401. The nature of the mutation(s) in these isolates is still unknown. The occurrence of mutations outside the preselected sites represents a potential problem for our projected studies, but additional data is required before the problem can be fully evaluated. In spite of this, it should be possible to study, in vivo, the biological effects of any site-specific modification (including covalent modifications by carcinogens) that can be introduced into gene G of φX174 DNA via a synthetic, oligonucleotide primer.

Most carcinogens are mutagens (2). They are also electrophilic reagents that react with DNA to form a variety of stable, covalent adducts (3). It is generally believed that these covalent adducts cause mutations, but the details of this process are poorly understood at the molecular level. With this fundamental problem in mind, we have developed a system to explore the following questions: (a) Which of the different carcinogen-induced covalent modifications of DNA produce mutations? (b) What kind of mutation(s) does each different kind of premutational lesion produce? (c) What role do the various DNA repair systems play in this mutation process?

Briefly, the system consists of four parts: (a) introduction of the site-specific, covalent modification to be studied at a preselected site in gene G of bacteriophage φX174 replicative form DNA by a combination of chemical and enzymatic steps; (b) expression of the modification in vivo by transfection of spheroplasts carrying different DNA repair backgrounds; (c) identification of any mutants that are produced; (d) characterization of the mutations by isolating mutant DNA and sequencing it in the region that carried the site-specific modification in the parental DNA.

While this approach is general, in principle, some important technical requirements must be satisfied in order to have a practical system. For our initial studies, we selected the viral bacteriophage, φX174, because of its relative simplicity (4, 5). We have focused on an essential gene (gene G) in order to maximize the biological effects of mutations produced by site-specific modifications.

Gene G codes for a viral spike protein (6). This protein is required for assembly of an infectious virus particle (5). In addition, gene G is necessary for production of a single-stranded viral DNA from RF (7). Thus, we can anticipate that many gene G mutations will be lethal. Since we will never know ahead of time what kind of mutation a given site-specific modification will produce, we need a system that is permissive for all kinds of mutation at the preselected site in this gene.

Previously, we have described a plasmid system that is permissive for gene G mutations (8, 9). The plasmid, pαXG, carries a functional copy of gene G that is expressed in cells carrying the plasmid, even without virus infection. This provides a source of normal gene G product that can rescue gene G mutants by complementation. We have demonstrated that

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1 The abbreviation used is: RF, replicative form.
the system is permissive for temperature-sensitive mutants at the nonpermissive temperature, for lethal missense mutants, for amber nonsense mutants, and for a lethal, site-specific deletion/frameshift mutant constructed in vitro. These experiments validate the biological part of the system.

This paper deals with the biochemical part of the system. Our objective is to show that a single, predetermined change can be introduced into infectious φX RF DNA via a small, synthetic, oligonucleotide primer, and that the biological expression of this change can be identified by isolating and sequencing the progeny DNA.

Our approach, summarized in Fig. 1, is patterned after the classical synthesis of infectious φX RF DNA (11, 12). At the time we began this work, only a short segment of the φX sequence was known (13). This segment residues before gene G and extended 27 residues into the translated portion of the gene (13, 14). Since it is much easier to make plus (viral) strands for template than minus strands, we chose to use a minus strand primer. Inspection of the plus strand sequence (Fig. 1) showed that a C → T transition in the 7th residue of the translated portion of gene G changes a glutamine codon to nonsense. Since we could be virtually certain that this change would be conditionally lethal, it seemed a good choice for working out the biochemistry and testing the entire system.

To produce, ultimately, a site-specific C → T change in the viral DNA, we introduced a G → A change into the 4th residue of a minus strand, octadeoxynucleotide primer (Fig. 1). This corresponds to a change at position 2401 in the current φX sequence (16) and creates a mismatch between the primer and the template at this position. In order to achieve specific priming at this preselected region of the genome, the octanucleotide primer was elongated enzymatically to a 17-mer. The 17-mer was isolated, sequenced, and used to reprogram the synthesis of infectious, site-altered φX RF DNA. After transcription of spheroplasts, mutant phage were used as a screen that employed cells carrying a functional copy of φX RF DNA. This DNA was sequenced and shown to contain a C → T transition at position 2401, exactly as predicted.

To produce ultimately, a site-specific G → A change at position 2401 (gene G) of φX174 RF DNA and the detection of mutant phage, the primer was prepared by the phosphodiester method (10). It is underlined on the figure; the arrow indicates the direction of elongation. The mismatch at the preselected mutation site in gene G is boxed. Escherichia coli DNA polymerase lacking the 5′ → 3′ exonuclease activity was used throughout. See text for further discussion and "Experimental Procedures" for details.

RESULTS

The Priming Reaction

Choice of Primer—For the reasons described above, we selected the minus strand sequence, d-(pT-C-T-A-A-A-A-C), as the initial primer. This octanucleotide, containing a site-specific change (G → A) at the 4th nucleotide, was synthesized by a modification of the diester route. The purity of the product was established by chromatography on DEAE-cellulose in the presence of 7 M urea, nucleoside composition analysis, and sequencing (10).

Specificity of Priming—Preliminary experiments with Escherichia coli DNA polymerase I (large fragment) (16) in the presence of dATP, dGTP, and dTTP. The expected products are shown in Fig. 2. Note that priming at the preselected mutation site in gene G gives a 17-mer. Priming at other sites is limited to the desired site in gene G, and 5 possible sites in other genes. In order to see which of these sites are actually primed in the enzymatic synthesis, we elongated the 5′-labeled octanucleotide primer with DNA polymerase I (large fragment) (16) in the presence of dATP, dGTP, and dTTP. The expected products are shown in Fig. 2. Note that priming at the preselected mutation site in gene G gives a 17-mer. Priming at other sites gives shorter products that are easily distinguished from the desired product by gel electrophoresis. One of the possible priming sites cannot be measured in this particular experiment since it does not elongate in the absence of dCTP. Two products from different sites are the same length (10-mer).

Fig. 3 shows the products formed by the elongation reaction in the absence of dCTP. Lane 1 shows the purity of the 8-mer primer by itself. Lane 2 shows the degradation of the primer (in the absence of template) by the enzyme preparation we used. Lane 3 shows the elongation synthesis. Four bands running slower than the primer are clearly visible in this radiograph. The slowest (top) band corresponds to the expected 17-mer. The next two bands correspond to a 14-mer and a 13-mer, respectively. Another band, corresponding to a 10-mer is just ahead of the labeled primer (8-mer). Densitom-
eter tracings gave a ratio of 1:5:8 for the 17-mer, 14-mer, and 13-mer, respectively; the 10-mer could not be measured accurately because of interference from the excess primer (8-mer).

The 17-mer band was eluted from the gel and sequenced by the Maxam-Gilbert procedure (18). The results are shown in Fig. 4. The first 3 nucleotides (T-C-T) at the 5'-end of the elongated primer do not show up on the radioautograph. The cleavage reactions are not as specific as is usually found with this method, perhaps because we were working with a single-stranded oligonucleotide rather than a duplex. Nevertheless, the sequence can be read without difficulty. The only possible ambiguities are at residues 9 and 10 (dashed lines in Fig. 4). Comparison of the intensities of bands 7, 9, and 11 indicates that priming and elongation could take place more efficiently. From inspection of sequence data, it has been suggested that this structure might be responsible for the low yield of 17-mer in the elongation reaction. Since ribosomal protein S1 is known to destabilize the ordered structure in +X174 DNA (19, 20), we hoped that this protein would bind asymmetrically to this ribosome binding site at the beginning of +X gene G, perhaps to the sequence shown by the boxed area in Fig. 5. This binding apparently destabilizes the ordered structure of the template molecule in such a manner that the 8-mer can bind and elongate leftward (counterclockwise) giving the 17-mer. Binding of ribosomal protein S1 at other priming sites is apparently nonspecific and inhibitory. At high concentrations of ribosomal protein S1, nonspecific binding occurs at the 17-mer priming site as well, and elongation of the 8-mer is inhibited at all priming sites.

This effect seems to be specific for ribosomal protein S1 since bacteriophage T4, gene 32 protein, a known DNA helix destabilizing protein (22) as well as E. coli DNA helix destabilizing protein (22), did not stimulate formation of the 17-mer with E. coli DNA polymerase I (large fragment) (19) in the absence of dCTP. The results (not shown) indicate that these proteins inhibited priming at all sites.

Synthesis of the Site-specific Gene G Mutant

Enzymatic Synthesis of Infectious øX174 RF DNA Containing a G → A Change at Position 2401 of the Minus Strand—In order to avoid the possible complication of multiple priming, we utilized the 17-mer described above to reprim the synthesis of RF DNA on a wild type, plus strand template in the presence of E. coli DNA polymerase I (large fragment), T4 DNA ligase, ATP, and all 4 dNTP's. After an appropriate incubation, the reaction mixture was treated with single-strand-specific nuclease S1 to destroy template DNA that remained and the incomplete duplex molecules (23). This mixture was used directly to transfect E. coli C600 Su2 spheroplasts.

The S1 nuclease step proved to be important since our initial attempts to find the mutant against a very high wild type background failed. The amount of enzyme used was determined by examining the number of virus particles produced by transfection after treating both viral and RF DNA with different amounts of S1 nuclease. Some representative
Molecular Mechanisms of Mutation

FIG. 3. Electrophoretic separation of the elongation products formed from an octanucleotide primer and a viral strand template in the absence of dCTP. Lane 1, the synthetic primer, d-(5)pT-C-T-A-A-A-A-C). Lane 2, primer + E. coli DNA polymerase I (large fragment). Lane 3, primer + φX174 DNA + dATP, dGTP, dTTP + DNA polymerase I (large fragment). The bands were located by radioautography.

Data are shown in Table I. Experiments 1 and 2 show that 58% of the RF DNA remained after treatment with 2.5 units of S1 nuclease. Experiments 3 and 4 show that this same amount of enzyme reduces template DNA to 0.3%. Thus, this step provides an RF DNA enrichment of almost 200-fold. However, these data provide only a rough guide since we have no way of knowing ahead of time what the actual concentrations of free template DNA and mutant RF DNA will be in the final reaction mixture. Furthermore, comparison of Experiments 1 and 3 shows that the efficiency of phage production from viral DNA is 4 times that from RF DNA under the conditions of transfection that were used. Thus, the S1 nuclease step gives an effective enrichment of only 50. Even this proved important in detecting the mutant.

Detection of φX Gene G Mutants—Phage produced by spheroplasts were screened in two different ways using our pφXG105 system (8). One method employed a double layer technique utilizing a soft agar layer of cells carrying the plasmid, pφXG, (HP4740 recA/pφXG105) to which an aliquot of phage had been added poured over a solidified layer of the same strain without pφXG (HP4740 recA). The results were never unequivocal, but any plaque with even a slightly turbid appearance was picked. Out of 855 plaques examined, 155 were suspected of being mutant. Of these, 12 were confirmed by replating on E. coli C and HP4740 recA/pφXG105.

The mutants were then tested on a variety of suppressor strains. The growth pattern showed that two classes of mutants were present. They were designated OBI and OBI1. The properties of these mutants on various host strains are summarized in Table II. OBI type mutants are suppressible by E. coli CR 63.1 and CQ3, both of which are Sul+ type amber suppressors. OBI1 type mutants are not suppressible by any of the amber, ochre, or opal suppressors we have on hand. It

The transfection was carried out in the presence of protamine sulfate to increase the efficiency of transfection by RF DNA (24). Without protamine sulfate, viral DNA is reported to be 60 times more efficient than RF (24). These differences may also be influenced by the host strain used for preparing the spheroplasts. In our hands, transfection of E. coli W 3350 (ATCC 27020) spheroplasts with viral DNA in the absence of protamine sulfate is only 10 times more efficient than RF DNA.
should be noted that Su1+ suppressors should insert serine instead of the wild type glutamine. The results indicate that this substitution is permissible at 32°C, but not at 38°C. Insertion of tyrosine for glutamine (Su3+) apparently inactivates the gene G protein. We did not have a φX-sensitive Su2+ (glutamine) strain (the "correct" suppressor) available. However, OBI mutants grow on the ochre suppressor, WWU Su2+. This is expected since this ochre suppressor suppresses both amber and ochre mutations and is thought to insert glutamine, the wild type amino acid (30).

Thus, the spheroplast lysate contains an amber mutant with the expected biological properties (OBI type) as well as a second, unexpected mutant (OBII type) that seems to contain a lethal mutation in gene G since it grows only by complementation in φpX-bearing strains.

The second screening method uses a gridding procedure that detects not only gene C mutants, but also differentiates types OBI and OBII in the same assay. Out of 500 plaques examined, 3 were found to be mutants. Two of these 3 mutants (OBI type) grew on appropriate nonsense suppressor hosts as well as on φpX-bearing hosts; the other (OBII type) grew only on the plasmid bearing host.

Out of 1355 plaques examined by these two assays, 15 confirmed mutants have been isolated. Of these, 11 are OBI type, 4 are OBII type. These data are summarized in Table I (Experiment 7).

The Nature of the Mutation in OBI—All 11 isolates designated as OBI type mutants give exactly the same suppression pattern (see Table II). We conclude from this and from the method used for their construction that they all contain the same mutation. One of these was investigated further. Mutant virus was purified from single plaques grown on the permissive strain HF4738 Su+, recA. Stocks containing 10^{-6} wild type φX174 were grown in liquid culture and used to infect either E. coli HF4738 or C in the presence of chloramphenicol to produce RF DNA. This DNA was sequenced by the Maxam-Gilbert procedure as described under "Experimental Procedures." The results are shown in Fig. 7a. Residue 2401 is clearly a T rather than the wild type C. The remaining sequence is wild type. Thus, the minus strand A at the position 2401, introduced into heteroduplex DNA via a short, synthetic primer of known sequence, gives rise in vivo to a T in the viral DNA, as expected, producing a nonsense mutation at the preselected site in gene G. This experiment demonstrates that all phases of the system are operational. It also constitutes

FIG. 5. The possible role of template ordered structure on the elongation of the octamer primer. a, a possible secondary structure for the site at the start of gene G that binds ribosomes (13). The synthetic minus strand primer is aligned with its complementary sequence. The boxed area is homologous to part of the strong Qβ ribosome binding site Sl (21) shown in c, b, opening the hairpin loop allowing the primer to bind more stably to the template. The direction and maximum extent of elongation in the absence of dCTP is shown by the curved arrow.

FIG. 6. The effect of ribosomal protein S1 on the priming reaction. Individual priming reactions were carried out with different amounts of ribosomal protein S1 and the products were separated by electrophoresis as shown in Fig. 3, lane 3. The radioautograms were scanned in a Beckman model 35 spectrophotometer using the gel scanning accessory. The peak area of the desired 17mer was estimated by planimetry and the results are plotted at the bottom of the figure.
TABLE I
Production of φX174 gene G mutants from a site-modified, minus strand primer.

| Experiment | Template | Primer | SI nuclease | Pfu/lysate | Plaques examined | Number of mutants found
|------------|----------|--------|-------------|------------|------------------|------------------------|
|            | pmol     | pmol   | units       |            |                  |                        |
| 1          | 0.2 (RF) | 0      | 0           | 2.5        | 1.2 × 10^8       |                        |
| 2          | 0.2 (RF) | 0      | 0           | 2.5        | 0.7 × 10^8       |                        |
| 3          | 0.2 (+ strand) | 0          | 0           | 2.5        | 4.8 × 10^8       |                        |
| 4          | 0.2 (+ strand) | 0          | 0           | 2.5        | 1.5 × 10^6       |                        |
| 5          | 0.2 (+ strand) | 0.3       | 2.5        | None Detected | 1.2 × 10^6       | 416                    |
| 6          | 0        | 0.3    | 2.5        | None Detected | 855 (500)       | 9 (2)                  |
| 7          | 0        | 0.3    | 2.5        | None Detected | 1.2 × 10^6       | 3 (1)                  |

* Experiments 1 to 4 were not carried through any of the steps used in the final enzymatic synthesis. Experiments 5 to 7 were carried through the entire synthesis protocol.

* A 17-mer (see Fig. 1).

* Based on data furnished by the supplier (Miles Laboratories).

* Total plaque forming units obtained by plating the spheroplast (C600 Su2*) lysate on HF4740 recA/pφXG105.

* OBI type mutants grow on Su1* and Su2* type suppressors. OBII type mutants grow only on pφXG bearing strains.

* Number in parentheses refers to plaque forming units examined by gridding procedure using HF4738 and HF4738/pφXG.

TABLE II
Plaque forming ability of φXG OB mutants on some φX-sensitive strains

| Class | Host Source | OBI | OBII |
|-------|-------------|-----|------|
| OBI   | R. C. Warner | +   | -    |
| OBII  | J. Hurwitz  | +   | -    |
| C (wild type)* | CR63.1 F* sup D 60r | +   | -    |
|        | M. Hayashi  | +   | -    |
| CQ2   | C. A. Hutchison, III | +   | -    |
|        | This laboratory | +   | +    |
|        | C. A. Hutchison, III | +   | -    |
|        | This laboratory | +   | +    |
|        | This laboratory | +   | +    |
|        | P. Howard-Flanders | +   | -    |
|        | P. Howard-Flanders | +   | +    |
|        | This laboratory | +   | +    |
|        | This laboratory | +   | +    |
|        | C. A. Hutchison, III | +   | -    |
|        | M. Hayashi  | +   | +    |

* ATCC 27020.

* The genetic markers are given in Ref. 25.

* CR63 is a K12 sup D strain (Ref. 26). It was made φX* and named CR63.1 by Tessman and Tessman (Ref. 27).

* CQ2 seems to be the same as C1792; CQ2 is C1757 (C. A. Hutchison, III, personal communication).

* Ref. 28.

* The genetic markers are given in Ref. 29.

* P. Howard-Flanders, personal communication.

* This strain was shown to be permissive for φX Gam9 by M. Z. Humayun (this laboratory).

* C. A. Hutchison, III, personal communication.

* Refs. 30 and 31; F. Funk, personal communication.

The Nature of the Mutation in OBII—The sequence of OBII type mutant DNA in the 2401 region was determined in the same manner as OBI. The results are shown in Fig. 7b. The sequence is wild type throughout the region complementary to the 17-mer used as a primer. The mutation must be in gene G because OBII grows only on pφXG-carrying strains. The position and the nature of the mutation are unknown. The DNA's from the other 3 OBII isolates have not yet been sequenced.
The purpose of this work was to establish the feasibility of using an essential gene of phage $\phi X174$ for studying the biological effects of site-specific, covalent modification of DNA by carcinogens. By producing a previously unknown, nonsense mutant ($\phi X$ Gam2401 OB1), carrying a single base change at a preselected position (2401) in gene G of $\phi X174$, we have shown that the $\phi XG$ system (Fig. 1) is technically operational. The use of a short, chemically synthesized primer for the enzymatic synthesis of infectious $\phi X$ RF DNA carrying a single base mismatch at position 2401 suggests that a wide variety of interesting covalent modifications can be introduced by this route. Our earlier work has demonstrated that host cells carrying the plasmid, $p\phi XG105$, are permissive for all types of mutation (misense, nonsense, frameshift, and large deletion) in gene G (8, 9). Thus, it should be possible to isolate any mutant phage derived from a site-specific, covalent, gene G modification of infectious RF DNA providing the mutation produces a recognizable biological effect. The successful isolation of $\phi X$ Gam2401 OB1 using a host carrying $p\phi XG105$ and the identification of the mutation by sequencing indicates that the $\phi XG$ system is ready for our projected studies on the molecular mechanisms of mutation by carcinogens.

Our results also have more general implications for the technology of site-specific mutagenesis that has been developed during the past 2 years. The particular synthesis described here utilized a chemically synthesized primer. Although not novel, this is important for our work because of the flexibility the chemical approach provides. Our results show that a very short primer (8 mer) can be used for the enzymatic synthesis of site-modified, infectious, RF DNA even when the initial priming reaction is nonspecific and inefficient. We have shown that the AT-rich primer, d-(pT-C-T-A-A-A-A-C), anneals with the viral strand template at several places, including the preselected site, with a single base mismatch (Fig. 2). Specificity for the preselected site was achieved by elongating the 8-mer using $E. coli$ DNA polymerase I (large fragment) (lacking 5' → 3' exonuclease activity) in the presence of dATP, dGTP, and dTTP. The elongated product (a 17-mer) was isolated and used to reprim the synthesis of RF DNA. This relay approach should be fairly general. Not only does it provide a means to achieve specific priming with a short obligomer, but it may provide a convenient way of cleaning up chemically synthesized primers. This is important because mistakes in the primer can lead to mutations that have nothing to do with the modification under investigation.

Our priming results can be compared with those reported recently by Hutchison et al. (23) since our synthesis of $\phi X$ Gam2401 OB1 was very similar to their site-specific synthesis of the well known $\phi X$ Eam3 mutant. These workers examined priming by a 7-mer and a 12-mer carrying the Eam3 nonsense mutation. The desired priming was achieved with the 12-mer on either a heterologous (wild type) or homologous (am3) template. The am3:7-mer, d-(pT-G-T-A-T-C-C-T), did not prime at the am3 site on the heterologous (wild type) template. The authors attribute this to the T,G mismatch between the 3' terminus of the primer and the template at the am3 site. The am3:7-mer did prime on the homologous template at a site in gene H. The authors attribute this to the perfect base pairing between the primer and the template that occurs fortuitously at the gene H site. Our data (Fig. 3) show that the 8-mer, d-(pT-C-T-A-A-A-A-C), primes at several sites, each having 7 base pairs and a single mismatch (Fig. 2). It may be significant that we found no priming in gene C (Fig. 2) where there is a purine-purine mismatch between the primer and the template. Priming in gene F (10-mer, Fig. 2) would also have a purine-purine mismatch. The data for this site are ambiguous. Thus, it appears that short primers with a purine-pyrimidine or a pyrimidine-pyrimidine mismatch will work providing the mismatch is not too close to the 3'-end where it can be edited by the 3' → 5' exonuclease activity of the polymerase (32).

The poor efficiency of priming by d-(pT-C-T-A-A-A-A-C) at a preselected site in gene G cannot be attributed solely to the primer sequence. The results with the helix destabilizing protein, ribosomal protein S1 (Figs. 5 and 6), show that ordered structure of the template in this region of $\phi X$ DNA plays an important role in inhibiting the priming reaction. Even in the presence of this helix destabilizing protein, the isolated yield of the desired 17-mer was only about 5% of the template input. This probably does reflect the length and the composition of the primer. Thus, it appears that both ordered structure of the template DNA and the primer sequence influence the priming reaction.

The poor yields, both in the elongation reaction and the RF synthesis from the 17-mer, caused considerable difficulty and made it impossible for us to characterize the RF DNA product biochemically. Assuming the data in Table I are representative and that the OBI mutants are all the same, we calculate that the yield of site altered RF DNA (before S1 nuclease treatment) was 0.03% based on template input. Goulian et al. reported a yield of about 30% in a similar synthesis using a boiled extract of $E. coli$ as a source of primers (12). Razin et al. report a yield of 0.1% using a synthetic 17-mer primer derived from a $\phi X$ gene E (lysis gene) sequence (33). We suspect our poor yield reflects not only inefficiency of priming with a 17-mer in this particular region of the $\phi X$ genome, but also the difficulty in copying through ordered structure in the template to complete the circle. In spite of this very low yield, we were able to isolate the desired mutant virus without difficulty because of the enrichment provided by the S1 nuclease step, the biological amplification provided by the spheroplasts, and the simplicity of the $p\phi XG$ screen for finding gene G mutants. Once this is done, the mutant DNA can be obtained in large amounts from the phage.

The most important feature of the $\phi XG$ system for site-specific mutagenesis is its generality. This is true not only for the biochemical part of the system, but also for the biological part. For example, the transfection experiments were carried out with spheroplasts derived from the $E. coli$ K12 derivative, C600 Su2'. This contains an amber suppressor that can read the site-specific amber mutation in the third codon of gene G and insert the correct amino acid, glutamine. However, this suppressor is probably unnecessary when a heteroduplex DNA containing a wild type template strand and a mutant complementary strand is used, as was the case in our experiments. Wild type RF DNA produced from the template strand should produce wild type gene G product so complementation should be set up automatically. Thus, infectious virus should be produced regardless of the nature of the modification in the RF DNA.

Rescue of mutant phage from spheroplast lysates with host cells carrying the plasmid $p\phi XG$ also seems to be general. The results reported here reinforce our earlier data (8, 9). They also demonstrate that one needs no prior knowledge concerning the nature of the gene G mutation in order to isolate it with $p\phi XG$-bearing hosts. For example, we had originally intended to use the suppressor approach to isolate the site-specific, amber mutant reported here since the appropriate $\phi X$-sensitive, Su2' host had been described (E. coli WWU Su2') (31). However, this particular mutant was not available. We did have $\phi X$-sensitive Su1' (amber → Ser) and Su3' (amber → Try) suppressor strains on hand, but we had no...
way of knowing ahead of time whether the missense substitutions that should result with these suppressors would give a functional gene G product. Therefore, we used a pXG-bearing host to detect and isolate the gene G mutants from the spheroplast lysate. Once the nonsense mutant ΔXG Gum2401 OBI was isolated, we were able to show that it was suppressed by Sua1, but not by Sua3. It could easily have turned out that neither of these suppressors worked, and without pXG we would not have been able to isolate the desired mutant even though it was present in the spheroplast lysate.

These results demonstrate another important use of site-specific nonsense mutations. The finding that ΔXG Gum2401 OBI is suppressed by Sua1, but not Sua3 hosts shows that the third amino acid (Gln) in the ΔX174 gene G spike protein can be replaced by serine, but not by tyrosine. Recent experiments (not reported here) suggest that the serine replacement produces a temperature-sensitive viral spike protein.

Another feature of the pXG-bearing system is its sensitivity and the ease with which it can be used as a mutant screen. The isolation of OBI type mutants illustrates this. Four isolates out of the 1355 plaques examined grow only on pXG-bearing hosts. One of the OBI mutants has been partially sequenced. It does not contain a mutation at the preselected site (position 2401). Since the nature and the origin of these mutants are unknown, it is possible that all four isolates are different mutants. Therefore, mutant frequency is between 7 × 10⁻⁴ and 2 × 10⁻³ yet there was no difficulty in detecting these mutants.

Assuming all OBI type mutants are the same, their apparent frequency compared to wild type phage was about 1% of the 1355 plaques examined. This is much less than the 15% reported by Hutchison et al. (23) for the ΔX Eam3 mutant synthesized from a 12-mer primer, but about the same as reported by Razin et al. (33) for the Eam3 revertant synthesized from a 17-mer primer by a procedure similar to that described by Hutchison et al. The data in Table I suggest that increased digestion with S1 nuclease would have raised the efficiency since we only reduced the template DNA by a factor of 320, Hutchison et al. reduced it by a factor of 1000. None of the data from the three site-specific syntheses reported so far (23, 33, this paper) should be regarded as true efficiencies of mutant production from site-modified ΔX RF DNA because in no case has the DNA actually used for transfection been isolated and characterized biochemically. Four yields in the primed, enzymatic synthesis made it impossible for us to do this. By gel electrophoresis, Razin et al. demonstrated the formation of RF DNA in their enzymatic synthesis, but they used a crude reaction mixture for transfection (33). So did Hutchison et al. (23).

After the classical enzymatic synthesis of ΔX174 Eam3 RF DNA in 1967 (11) and the demonstration that the DNA was infectious (12), Goulian and Kornberg pointed out, "The implication of studies of mutagenesis becomes clear at once, in as much as a variety of base analogs or ribonucleotides can now be incorporated into an infectious molecule" (11). Before meaningful studies of this kind could be attempted, however, three technical problems had to be solved: (1) development of techniques for obtaining sequence data from interesting regions of biologically active DNA; (2) development of methodology for introducing any desired site-specific modification at a preselected site in a suitable DNA; (3) development of methods for detecting and propagating any kind of mutation at the preselected site.

Through the efforts of several laboratories, all the necessary technology is now available and a new range of well controlled experiments bearing on important questions in molecular biology is possible. How covalent modifications of DNA by carcinogens produce mutations is one such question. Before we can use the XG system to its fullest potential some additional calibration studies are necessary since the biological properties of some missense mutant that may be produced are unknown at present. Even without this, it appears that considerable progress can be made with the information on hand.

A complete description of our strategy is beyond the scope of this paper, but the site-specific amber mutant described here plays a key role because it is unique. For example, the viral DNA from this mutant can be used as a template for the synthesis of RF DNA carrying a site-specific modification with a carcinogen in the third codon of the gene G minus strand. Any mutant produced in vivo from this RF DNA, regardless of the nature of the mutation, can be distinguished from amber mutant virus produced from the template strand. The new mutants can be grouped further as wild type and pseudo-wild type, temperature-sensitive, ochre, and lethal.

Out of nine possible transitions and transversions that can arise from site-specific modifications of the bases in this amber codon, the biological properties of five are already known (wild type, ochre, Ser = temperature-sensitive, Tyr = lethal). We expect frameshifts, large deletions, and rearrangements in this essential gene to be lethal. Therefore, only the properties of missense mutants carrying Trp, Leu, Gln, and Lys, instead of wild type Gln, are uncertain. These missense mutants can be constructed using the general approach described in this paper, and their biological properties can be determined. Even without this information it should be possible to identify biologically two of the three possible transitions arising from modifications of the three bases in this codon since they lead to wild type, ochre, and Trp codons, respectively. Similarly, three of the six possible transversions can be detected biologically since we know that Ser missense is temperature-sensitive and Tyr missense is lethal.

The lethal mutation group is expected to be the most complex. We already know it will contain two transversions leading to Tyr. We expect frameshifts, large deletions, and rearrangements to be lethal as well. At present these different mutations will have to be identified by sequencing. This presents a problem only if the mutation is a minor event compared to other lethal mutations.

Our approach is not limited to this amber codon, of course. As new mutants are isolated, either by synthesis of site-altered RF DNA or as a result of a site-specific mutation from a modified residue, the mutant DNA becomes easily available for use as a template. The only limitation that is clear at present is that virus from the template strand should be easily distinguished from the mutants one hopes to find. This is why nonsense codons in the template are useful.

From our results, as well as theoretical considerations, we are convinced that the questions posed at the beginning of this paper can be approached in a direct manner using the XG system described here.

**EXPERIMENTAL PROCEDURES**

**Enzymes**

Bacteriophage T₄ DNA ligase and polynucleotide kinase from T₄ am N98-infected E. coli B were purified by the method of Danet et al. (34). The latter enzyme was purified by Dr. M. Z. Humayun. E. coli polymerase I (large fragment) was from Boeringer Mannheim; the restriction endonucleases Hha I and Alu I were from Bethesda Research Laboratories. RNase A was from Worthington Biochemicals. The following proteins were gifts: ribosomal protein S1 from Dr. W. Szer; T₄ gene 32 protein from Dr. L. Gold; E. coli DNA helix destabilizing protein from Dr. J. Hurwitz.
Chemicals

All chemicals were analytical grade. Proline sulfate and ethidium bromide were from Sigma Chemical Co.; acrylamide (electrophoresis grade), bisacrylamide, N,N,N',N'-tetramethylenediamine, bis(N-trimethylammoniumethyl) ethane sulfonate (Eastman Kodak Co.); piperidine and bromphenol blue from Fischer Scientific Co.; dimethyl sulfoxide (gold label reagent) from Aldrich Chemical Co.; urea (ammonium-free) from Bethesda Research Laboratories, Inc.

Bacterial Strains and Virus

All the E. coli strains used are described in Table II. All E. coli strains carrying the plasmid pG0105 were constructed by Dr. M. Z. Humayun (8). Conditions for growth of bacteria and virus as well as titration procedures have been described previously (8).

Preparation of DNA's

Viral strand DNA from wild type φX174 was prepared from phage purified by two successive equilibrium bandings in CsCl followed by filtration through controlled pore glass (356 Å, Sigma Chemical Co.) column (1 × 100 cm). The phage was dialyzed against 50 mM sodium borate, pH 9.0, 1 mM EDTA, and phenol extracted three times. The aqueous layer containing the DNA was dialyzed against three changes of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE buffer).

The remainder of the preparation was carried out as described previously (9).

Synthesis of d(5'-pT-C-T-A-A-A-A-C-T-G-A-T-T-A-A-A)

The chemical synthesis of the octanucleotide primer, d(5'-pT-C-T-A-A-A-A-C-T-G-A-T-T-A-A-A) containing the φX174 minus strand sequence from residues 2397 to 2404 except for a G → A change at position 2401 was described previously (10). This primer was elongated on a viral strand template under the following conditions: 32P dATP (10 pmol) and the template (1 pmol) were dissolved in 0 μl of buffer containing 35 mM Tris-HCl + 10 mM MgCl₂ (final concentrations) and annealed in a sealed capillary tube by heating the contents at 90°C for 2 min. The tube was transferred to a 37°C bath where the contents were slowly cooled to 7-8°C over a period of about 2 h. The contents of the capillary tube were transferred to a 37°C bath where the contents were slowly cooled to 7-8°C over a period of about 2 h. The contents of the capillary tube were transferred to a 55°C bath. The contents were slowly cooled to 28°C over a period of 4 h. The contents were transferred to a polypropylene tube and the remaining mixture containing the triphosphates and 2-mercaptoethanol were added to it. After adding the polymerase and DNA ligase, the incubation was continued for 2 h at 37°C. The enzyme was then destroyed by treatment of the reaction mixtures (above) with N-lauroyl sarcosine.

Production and Detection of the Site-specific Gene G Non-sense Mutants—Spheroplasts of E. coli strain C600 (Su2*) were prepared as described by Guthrie and Sinsheimer (35) except that protease was added to the spheroplast preparation (3.8 X 10⁶ cells; the ratio of cells to DNA was -1) in a final volume of 4 ml (35). Phage was released by two successive cycles of freeze and thaw. The lysate from the complete synthesis (above) contained 3 x 10⁶ pfu/ml based on titers on Hφ4740 recA1/pXG105, a c感受性 host that is permissive for all types of gene G mutants (8, 9). This lysate, as well as those from controls (see above), were screened for mutants by a double layer technique.

An aliquot of the lysate was mixed with 0.2 ml of Hφ4740 recA1/pXG105 (fresh, mid-log culture) in 3 ml of soft agar and layered onto a prepped plate of Hφ4740 recA1. After 4 to 5 h at 37°C, the plaques revealed that some plaques were turbid and small to medium while the remaining plaques had the large, clear morphology of wild type phage. Out of a total of 855 plaques, 155 apparently turbid plaques were punched out with a capillary tube. Each plaque was resuspended in 1 ml of 0.2 M Tris-HCl, pH 8.0, buffer and plaque phage allowed to be extracted overnight at 4°C. Each extract was titrated at a suitable dilution on Hφ4740 recA1 and Hφ4740 recA1/pXG105, and 12 were found to produce plaques only on Hφ4740 recA1/pXG105. No mutants (out of 415 plaques examined) were found in the lysate from transfection of the reaction mixture from template DNA alone; no plaque forming units were found in lysates from reaction mixture containing primer alone (see Table I).

The lysate from the complete synthesis was also screened by a
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gridding procedure that detected all gene G mutants and partially classified them in the same assay. A suitable dilution of the above lysate was made so as to give about 100 pfu/plate on a lawn of HF4738 Su- recA1/paXG. Individual plaques were picked with a sterile toothpick and gridded sequentially on E. coli C, HF4738 Su- recA1 and HF4738 Su- recA1/paXG. Mutants were identified by comparing the plates. They were classified as OBI if they grew on plates 2 and 3 but not 1; as OBII if they grew only on plate 3. All mutants were confirmed by picking 10 plaques at random from plate 3 and restesting by gridding on fresh test plates. In each case, all 10 plaque grew on the permissive strain(s); none grew on the nonpermissive strain(s).

Partial Sequencing of RF DNA from OBI and OBII Type Mutants  The sequence around position 2401 of the plus strand, where a G -> A transition had been introduced in the minus strand primer, was determined by the method of Maxam and Gilbert (18). RF DNA was isolated from the mutant as described above (two 1-liter cultures). It was cleaved with the restriction endonuclease, Hha I. The reaction mixture (500 μl) contained 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 600 units of endonuclease Hha I, and 100 pmol of RF DNA. Incubation was at 37°C for 1 h. The reaction was terminated by two phenol extractions followed by two ether extractions. The fragments produced were fractionated on 5% polyacrylamide slab gel (0.3 X 20 x 40 cm) in 90 mM Tris-borate. PH 8.3. 2.5 μl of each fragment was excised and precipitated with radioactive phosphate. A portion of the restriction fragment (~5 pmol based on starting RF DNA) in 50 μl reaction mixture contained 10 mM Tris-HCl, pH 8.5; 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 5 μl [γ-³²P]ATP (specific activity 2700 Ci/mmol), and 5 units of polynucleotide kinase. After incubation for 40 min at 37°C 50 μl of H₂O was added and the reaction terminated by two phenol extractions followed by two ether extractions. After the removal of residual ether, 10 μl of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 100 mM 2-mercaptoethanol were added to it. An 80 base pair fragment with a single labeled 5'-end in the plus strand was generated by cleaving the doubly labeled H3 fragment with 10 units of restriction endonuclease AluI at 37°C for 40 min. This produces four fragments (25, 33, 80, 476 base pairs). Two labeled bands, 476 and 80 base pairs were obtained on a 15% polyacrylamide gel by electrophoresis at 400 V, 247, 224-231.

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