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Genetics

Multiple point mutations in a shuttle vector propagated in human cells: Evidence for an error-prone DNA polymerase activity

(mutagenesis/immunoglobulin diversity/suppressor tRNA/xeroderma pigmentosum/carcinogenesis)

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ABSTRACT **Mutagenesis was studied at the DNA-sequence level in human fibroblast and lymphoid cells by use of a shuttle vector plasmid, pZ189, containing a suppressor tRNA marker gene. In a series of experiments, 62 plasmids were recovered that had two to six base substitutions in the 160-base-pair marker gene. Approximately 20–30% of the mutant plasmids that were recovered after passing ultraviolet-treated pZ189 through a repair-proficient human fibroblast line contained these multiple mutations. In contrast, passage of ultraviolet-treated pZ189 through an excision–repair-deficient (xeroderma pigmentosum) line yielded only 2% multiple base substitution mutants. Introducing a single-strand nick in otherwise unmodified pZ189 adjacent to the marker, followed by passage through the xeroderma pigmentosum cells, resulted in about 66% multiple base substitution mutants. The multiple mutations were found in a 160-base-pair region containing the marker gene but were rarely found in an adjacent 170-base-pair region. Passing ultraviolet-treated or nicked pZ189 through a repair-proficient human B-cell line also yielded multiple base substitution mutations in 20–33% of the mutant plasmids. An explanation for these multiple mutations is that they were generated by an error-prone polymerase while filling gaps. These mutations share many of the properties displayed by mutations in the immunoglobulin hypervariable regions.**

Data on direct mutagenesis in mammalian cells have only recently become available, largely due to the development of shuttle vector plasmids designed for mutagenesis experiments (1, 2). Several recent publications (3–7) describe ultraviolet mutagenesis of different shuttle vectors in primate cells. The ultraviolet treatment resulted in single or tandem point mutations as have been seen with *Escherichia coli* (8). However, in some of the studies (4, 6, 7), plasmids with multiple point mutations in the marker gene were also found.

We studied ultraviolet mutagenesis of the pZ189 shuttle vector plasmid (2) in repair-proficient human fibroblast and lymphoid cell lines and in an excision–repair-deficient fibroblast line derived from an individual with xeroderma pigmentosum of complementation group A (9). We found that a substantial fraction of the mutant plasmids harvested from the normal cell lines carried multiple point mutations clustered in the marker gene. The frequency of this class of mutant plasmids was significantly reduced with the excision–repair-deficient cell line (6). In this report, we describe the nature and location of these mutations in the marker gene and adjacent sequences and generation of these mutations with xeroderma pigmentosum cells by introduction of a single-strand nick. These mutations share many of the properties displayed by those in the immunoglobulin hypervariable regions.

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MATERIALS AND METHODS

Plasmid and Cells. The shuttle vector plasmid pZ189 (2) carries a bacterial suppressor tRNA gene (*supF*) as a mutational target and sequences that permit replication in *E. coli* and in mammalian cells. Simian virus 40 (SV40)-transformed repair-proficient (GM0637B) and xeroderma pigmentosum complementation group A (XP12BE) cells and Epstein–Barr virus-transformed repair-proficient B-lymphoblastoid cell line GM606 (10) were obtained from the Institute for Medical Research (Camden, NJ). The plasmid, with or without ultraviolet treatment or with a single-strand nick at the *Eco*RI site (11), was transfected into the human cells by a calcium phosphate or a DEAE-dextran (lymphoblastoid cell line) technique. After a 2-day replication period, progeny plasmids were harvested and introduced into an indicator bacterial strain of *E. coli* that contains a suppressible (amber) mutation in the β-galactosidase gene. Colonies with plasmids having a mutated marker gene were white or light blue, in contrast to blue colonies with wild-type plasmid. Mutant plasmids were purified and nucleotide sequence was determined as described (2, 4, 6).

RESULTS

The shuttle vector plasmid pZ189 was passaged through the repair-proficient human fibroblast line GM0637. With untreated plasmid the spontaneous background mutation frequency in the GM0637 cells was 0.07% (6). About 30% of the spontaneous mutants contained deletions but the majority showed no change in the size of the marker. Sequence analysis of 29 independent mutants demonstrated that there were two classes of spontaneous mutant plasmids carrying point mutations. The first class had either one isolated or two or three adjacent (i.e., tandem) point mutations; these 22 plasmids contained transitions and transversions occurring at G·C base pairs (Table 1 and Fig. 1*a*). The second class of spontaneous mutant plasmids carried two to four nonadjacent point mutations and accounted for 23% (7 of 29) of the plasmids with point mutations (Figs. 1*b* and 2). These multiple changes were a mixture of transitions and transversions, largely (but not exclusively) at G·C pairs (Table 1). Despite the relatively small sample size, there appear to be hot spots at positions 123 and 129 (Figs. 1*b* and 3, part I). Mutant sites were found as much as 256 bases apart (plasmid number 0/169, Fig. 2).

When the plasmid was treated with ultraviolet radiation prior to transfection, the mutation frequency rose as high as 35-fold above background, in a dose-dependent manner (6). Sequence analysis showed that about one-quarter (24 of 89) of the mutant plasmids contained two to five nonadjacent

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Table 1. Types of base substitution mutations found in plasmids containing multiple mutations following propagation in human cells

Cell line	Plasmid treatment*	Mutant class†	Transitions, no.			Transversions, no.					No. of mutations/ no. of plasmids
			Total	G·C→A·T	A·T→G·C	Total	G·C→T·A	G·C→C·G	A·T→T·A	A·T→C·G	
GM0637‡	None	Single	9 (39%)	9 (39%)	0	14 (61%)	8 (35%)	6 (26%)	0	0	23/22
	None	Multiple	4 (22%)	3 (17%)	1 (5%)	14 (78%)	10 (55%)	3 (17%)	1 (6%)	0	18/7
	UV	Multiple	39 (53%)	37 (50%)	2 (3%)	35 (47%)	20 (27%)	9 (12%)	6 (8%)	0	74/24
XP12BE§	Nick	Multiple	13 (41%)	13 (41%)	0	19 (59%)	12 (37%)	7 (22%)	0	0	32/12
GM606¶	UV	Multiple	17 (65%)	15 (58%)	2 (8%)	9 (35%)	5 (19%)	3 (11%)	1 (4%)	0	26/13

*Treatment of plasmid prior to transfection and propagation: UV, ultraviolet; nick, single-strand nick at *Eco*RI site.
†Single, either single or tandem mutations; multiple, multiple base substitution mutations.
‡Simian virus 40-transformed, repair-proficient fibroblast line.
§Simian virus 40-transformed, repair-deficient (xeroderma pigmentosum) fibroblast line.
¶Epstein–Barr virus-transformed, repair-proficient lymphoblastoid cell line.

point mutations (Figs. 2 and 3, part II). Ultraviolet treatment increased the frequency of these multiple-mutant plasmids as well as increasing the frequency of plasmids with single point mutations. However, the proportion of mutant plasmids with multiple point mutations did not increase with dose (23% with no UV, 23% at 100 J·m^{-2}, 31% at 500 J·m^{-2}, 22% at 1000 J·m^{-2}). The mutants that contained single or tandem point mutations after ultraviolet treatment have been described (6).

The multiple mutations following ultraviolet treatment, like the spontaneous multiple mutations, occurred largely (but not exclusively) at G·C base pairs (Table 1 and Fig. 3). The pattern shown in Fig. 3 emphasizes the appearance of base changes at many of the same sites in different mutants. Hot spots were seen at base pair 129 as with the spontaneous multiple mutants and also at base pairs 65, 103, 139, 146, 155, and 185 (Fig. 3).

Mutagenesis in Xeroderma Pigmentosum Cells. Plasmid pZ189 with or without ultraviolet treatment was introduced into a cell line from an individual with the DNA-repair-deficiency disease xeroderma pigmentosum (complementation group A). We have characterized a few of the spontaneous mutant plasmids (the background mutation frequency was 5×10^{-5}) and found that 6 of 10 mutant plasmids contained point mutations; one of these had two point mutations, a transition (C→T) at base pair 146 and a transversion (C→G) at base pair 168.

Ultraviolet treatment of the plasmid increased the frequency of mutant plasmids by as much as 100-fold above the background. Sequence analysis of 61 independent mutant plasmids indicated that only one (2%) contained multiple base substitutions, in marked contrast to the 27% observed with the repair-proficient cells ($P < 0.00001$) (6). Only transitions (C→T and T→C) were observed in this mutant. The single point mutations are described elsewhere (6).

A Nick in the Plasmid Enhances Multiple Mutagenesis. The virtual absence of multiple base substitution mutations from the group of ultraviolet-induced mutants in the excision–repair-deficient xeroderma pigmentosum cells suggested that some aspect of excision–repair might be necessary for generation of multiple base substitution mutations. Since xeroderma pigmentosum cells are unable to make an endonucleolytic incision in DNA, we introduced a nick in the plasmid adjacent to the marker gene (at the *Eco*RI site) and transfected this construct into the xeroderma pigmentosum cells. Progeny plasmids were harvested and mutant plasmids were identified. We found a large increase in mutation frequency (up to 0.1–1% in several independent experiments). About two-thirds (12/19) of the mutant plasmids analyzed contained multiple base substitutions. There were as many as six base substitutions in a single plasmid (Fig. 2). All the mutations were located at G·C base pairs (Table 1 and Fig. 3, part III) and, if taken as changes in the same base (cytosine or guanine), all appeared to occur on the same strand and in the same direction (3′ from the cytosine at the nick, Fig. 2). Hot spots were seen at base pairs 12, 108, 133, 149, and 172, in addition to that seen at base pair 139 with the repair-proficient cells (Fig. 3).

The results of this experiment indicate that the xeroderma pigmentosum cells do have the capacity to generate mutant plasmids with multiple base substitution mutations if pre-

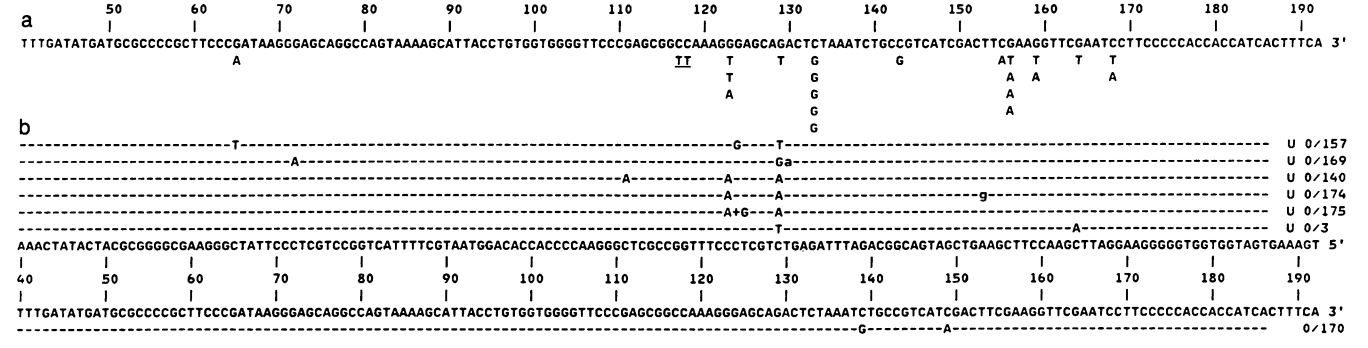

FIG. 1. Location of independent single, tandem, and multiple base substitution mutations found after passage of untreated pZ189 through the repair-proficient human fibroblast line GM0637. The 153-base-pair sequence shown contains the marker gene including the suppressor tRNA sequence (base pairs 99–183), the pre-tRNA sequence (base pairs 59–98), and a portion of the promoter (base pairs 24–58). (*a*) Single and tandem mutations with repair-proficient fibroblast line (22 mutant plasmids sequenced). Base substitutions are indicated below the mutated base. Each letter represents the single mutation found in a different plasmid. A tandem base substitution is indicated by underlining. (*b*) Multiple base substitution mutations with repair-proficient fibroblast line (7 mutant plasmids sequenced). Base substitutions are indicated below or above the mutated C in a G·C base pair in uppercase letters and below or above the mutated T of an A·T base pair in lowercase letters. Plus sign (+) indicates insertion of the following base. Each line represents the multiple mutations found in an individual mutant plasmid. Mutant plasmids are identified by the pretransfection ultraviolet dose/mutant number (all the plasmids represented in *a* and *b* were unirradiated). U indicates upper-strand cytosine mutation.

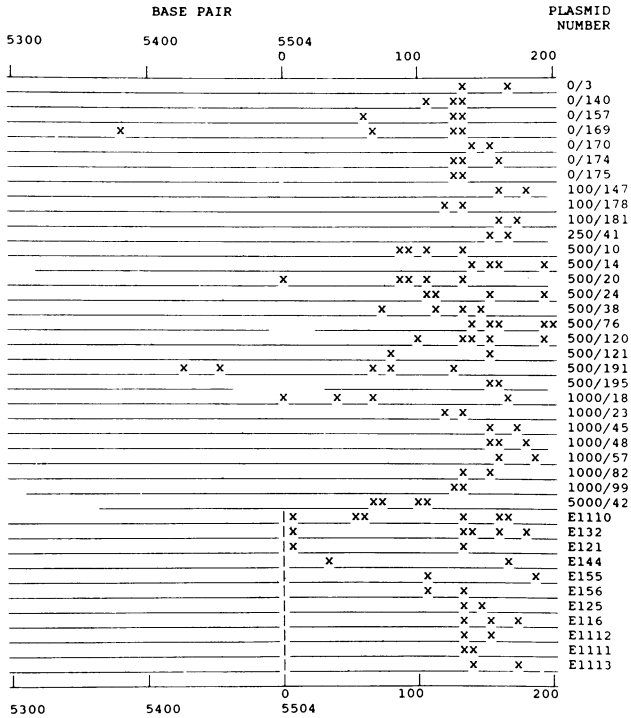

FIG. 2. Clustering of base substitution mutations in the marker gene. The portion of pZ189 from base pairs 5300 to 200 (2) is indicated. The marker gene includes base pairs 24–183. Lines indicate wild-type sequence; × indicates the position of a base substitution mutation; vertical bar indicates the location of the *Eco*RI nick. Each line represents an individual mutant plasmid sequenced. Mutant plasmids are identified by the pretransfection ultraviolet dose/mutant number, E indicates pretransfection *Eco*RI nick.

sented with an appropriate substrate. Interestingly, the introduction of the nick restored the two classes of mutations [transversions (Table 1) and multiple base substitutions] that were missing from the ultraviolet spectrum with these excision–repair-deficient cells (6).

Mutations Are Clustered. Our detection procedure requires that the tRNA gene be inactivated by the mutations, and so it is to be expected that the mutations would include the marker gene. Adjacent to the tRNA gene (counterclockwise from the *Eco*RI site) lies a region of ≈150 bases with no known function (2). Indeed, 168 bases (positions 5337–5504) can be deleted with no effect on plasmid viability or suppressor tRNA expression and function (M.M.S., unpublished data). It would appear then that mutations in this region would not interfere with plasmid survival. We determined the sequence of this region in 41 plasmids with multiple mutations from the experiments described above. A schematic diagram of the results (Fig. 2) shows that the mutations occur in clusters over as much as 256 bases, with a mean of 57 bases and a median of 35–40 bases. The majority of the clusters were contained within the tRNA gene; clusters only occasionally extended into the adjacent region. In particular, in the experiment with the nicked plasmid, mutations were not found in this region with the multiple mutants (Fig. 2) or with five additional plasmids containing single or tandem mutations (data not shown).

Mutagenesis in Lymphoblastoid Cell Lines. To determine if the multiple mutations could be generated in immunoglobulin-producing cells, we transfected ultraviolet-treated pZ189 into a B-cell line (GM606) from a normal individual. With the GM606 line, 22% (13 of 60) of the plasmids analyzed contained more than one base substitution. As with the fibroblasts, these occurred mainly at G·C base pairs (Table 1 and Fig. 3, part IV). With the lymphoblastoid cell line (and with the fibroblast line GM0637, Fig. 1*b*), if the mutations are arbitrarily designated as changes at cytosine, all appear to have occurred in one or the other strand in any given mutant plasmid (data not shown). Transfection of GM606 with pZ189 nicked at the *Eco*RI site resulted in 6 of 18 mutant plasmids having more than one base substitution mutation. Hot spots were seen at base pairs 108, 109, 127, 133, 139, 146, and 155 (Fig. 3, part IV).

DISCUSSION

In theory, the mutations in the shuttle vector described in this report could be caused by base damage (spontaneous deamination/depurination or other types of DNA modification) or by an error-prone polymerase copying undamaged DNA.

Deamination/Depurination. Spontaneous base-substitution mutagenesis of shuttle vector plasmids during passage through mammalian cells has been described by several groups (7, 12, 13). Miller *et al.* (13) suggested that some of the spontaneous mutations generated in these experiments arise from DNA damaged by depurination (at guanine) and deamination (of cytosine) during transfection. They proposed that these base modifications would occur during the exposure of DNA to an acidic environment in the cytoplasm during the transfection process (13). This hypothesis was based on studies of plasmids with G·C→T·A and G·C→A·T single point mutations: the depurinated G site would pair with A yielding a G·C→T·A transversion and the deaminated C would pair with A yielding a G·C→A·T transition, whereas G·C→C·G transversions would not be expected (13). For the following reasons this deamination/depurination hypothesis does not explain our data concerning the multiple base substitutions. (*i*) Lindahl and Nyberg (14, 15) studied depurination and deamination of single and double-stranded DNA under neutral and acidic conditions. Their results indicate that only 1% of the mutations could be explained by this mechanism of base damage. (*ii*) The majority of our multiple mutants can be read as having changes only at cytosine residues in one strand (or of course as changes of complementary guanine residues in the other strand). However, deamination of cytosine and depurination of guanine [and adenine (14)] would be expected to occur at cytosine, guanine, and adenine on a given strand and thus the sequence would be expected to display changes at guanine and adenine as well as at cytosine. (*iii*) A substantial portion of the multiple mutants we found had G·C→C·G transversions. (*iv*) There was a sharp reduction in the frequency of the multiple base substitution mutants in the xeroderma pigmentosum cells, although the transfection protocols for the normal and xeroderma pigmentosum cells were identical. These considerations suggest that depurination/deamination base modifications during transfection cannot explain the majority of the multiple mutations.

Ultraviolet Damage. The multiple base substitution mutations found in pZ189 were not the result of multiple random hits of ultraviolet-induced DNA damage. This conclusion is based on the following observations with the normal line GM0637. (*i*) The proportion of plasmids with multiple point mutations did not increase with ultraviolet dose. (*ii*) The average number of mutations per plasmid did not increase with dose. (*iii*) Based on the mutation frequency of 0.015 observed at 1000 $J \cdot m^{-2}$ (6), a truncated Poisson distribution of hits would predict only 0.3 mutant with two or more mutations per plasmid in the 36 mutants recovered. Nearly 27 times this number were found. (*iv*) The distribution of sites of multiple mutations after ultraviolet treatment was different from that of the ultraviolet-induced single and tandem mutations. With the multiple mutants (Fig. 3, part II) a major hot spot was at position 129 and few mutations were detected at position 156; the converse was found with the single and tandem mutations (6). (*v*) The distribution of types of base

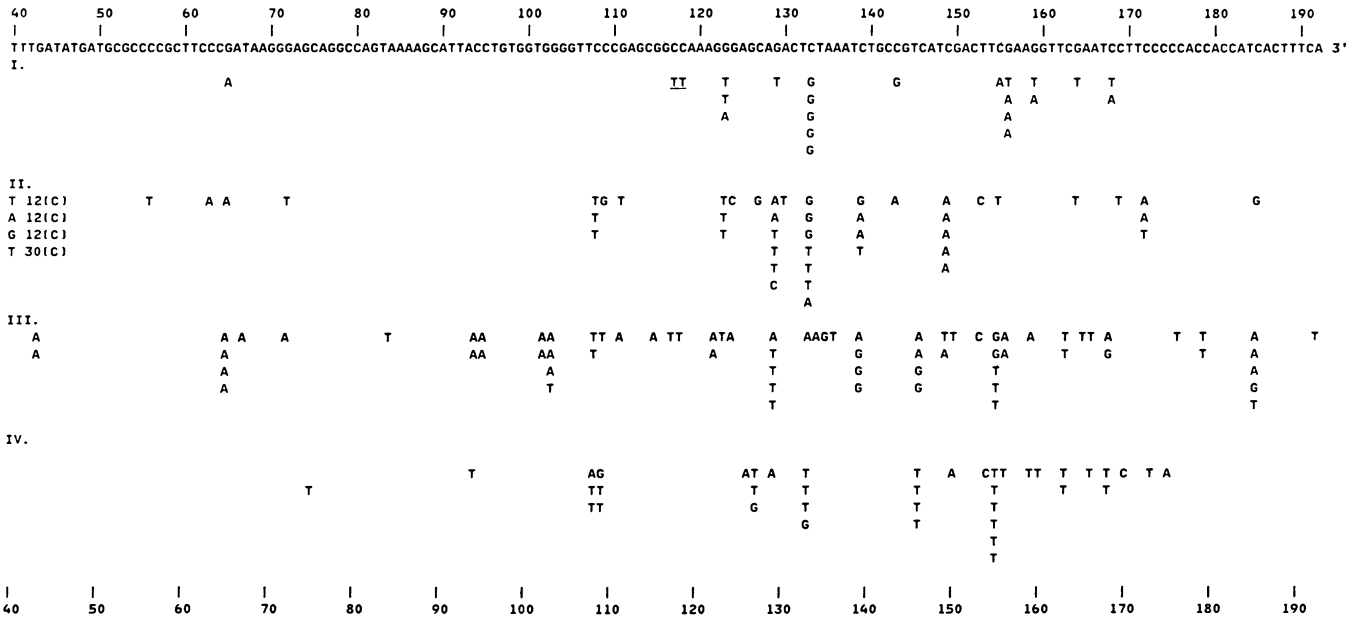

FIG. 3. Hot spots in plasmids with multiple mutations found after passage through fibroblast or lymphoblastoid cell lines. Base substitutions are indicated below the mutated base. Mutations at the same base pair in different plasmids are listed vertically under each other. Mutations are listed by type of plasmid pretreatment and by host cell: part I, untreated plasmid in repair-proficient fibroblast line (7 plasmids); part II, ultraviolet-treated plasmid in repair-proficient fibroblast line [24 plasmids; at left, T, A, or G plus a number followed by (C) indicates that cytosine was replaced by thymine, adenine, or guanine, respectively, at that position number]; part III, *Eco*RI-nicked plasmid in xeroderma pigmentosum fibroblast line (12 plasmids); part IV, ultraviolet-treated plasmid (13 plasmids) and *Eco*RI-nicked plasmid (6 plasmids) in repair-proficient lymphoblastoid cell line.

substitution mutations with the ultraviolet-treated plasmids with multiple mutations was different from that of the single and tandem mutations. There were significantly more G·C→T·A transversions ($P = 0.004$) and significantly fewer G·C→A·T transitions ($P = 0.005$) with the plasmids with multiple mutations (Table 1) than with the plasmids with single or tandem mutations (6).

Error-Prone Polymerase. The simplest explanation for the multiple base substitution mutations is that there is a causal link to the activity(ies) that is missing in the xeroderma pigmentosum cells—i.e., they require some aspect of excision–repair present in the normal cells. A highly error-prone polymerase has been proposed as an explanation for multiple mutations in studies of ultraviolet and immunoglobulin mutagenesis (4, 6, 7, 16–19) and warrants further consideration in view of the data presented here. A possible scenario is that an error-prone polymerase fills in gaps that appear in the DNA at sites of lesion excision. The gaps could be created by an exonuclease or by a polymerase with an associated exonuclease activity. In the experiments with ultraviolet-treated plasmid in repair-proficient cells (4, 6), the gaps would be generated during excision–repair [ultraviolet patch size has been estimated to be about 30–100 base pairs (20)]. The size of these gaps might reflect the properties of either the excision enzymes, the chromatin (e.g., nucleosome or linker size) (21), or those exonucleases without a specific repair function. The finding of multiple mutations in only one strand suggests a single mutagenic event. These gaps would be generated during the repair process in normal cells but would not appear in the xeroderma pigmentosum cells, since those cells are unable to perform the initial endonucleolytic incision step of excision–repair. Thus, as observed, the frequency of these mutants would rise as a consequence of ultraviolet dose to the plasmid with the wild-type cells because the frequency of gaps would be increased, but the gaps would not be present with the xeroderma pigmentosum cells. While the multiple mutations are a dramatic example of such activity at work, single mutations could also be generated by the same activity.

An alternative explanation of these data is that the xeroderma pigmentosum cells are deficient in the activity that generates these mutations, unrelated to the excision–repair defect. To distinguish between these possibilities, we examined the fate in the xeroderma pigmentosum cells of a plasmid with a single-strand nick in the vicinity of the marker gene. The multiple mutants produced in high frequency in the xeroderma pigmentosum cells only with the nicked plasmid are probably the result of extension of the nicks to gaps by cellular nucleases and filling-in of the gaps by an error-prone polymerase. The occurrence of multiple base substitution mutants in the nicked plasmid with the xeroderma pigmentosum line again implies that the multiple mutants are generated by a single event rather than by successive cycles of mutagenesis on the same plasmid or on progeny of a specific plasmid. In the xeroderma pigmentosum cells, the nick would be closed after the first cycle of replication and the frequency of multiple mutations in the un-nicked daughter plasmid would be expected to be extremely low (6).

If an error-prone polymerase is involved in the mutagenesis, it may randomly insert nucleotides across from positions at which it has paused. All possible base substitutions were observed at eight sites (base pairs 12, 129, 133, 139, 146, 155, 168, and 185) and seven of these sites showed more transversions than transitions (Fig. 3), as predicted by random insertion of the incorrect nucleotide. Pause sites in DNA have been described for all polymerases (21) and they presumably reflect some special feature of the primary sequence or secondary structure at which the polymerase stops. Strong pause sites might be revealed as hot spots in the mutagenesis pattern (Fig. 3). There appears to be a bias toward misincorporation at G·C base pairs by eukaryotic polymerases (22). Most sites at which mutagenesis can inactivate the marker gene are located in the mature tRNA gene (positions 99–183), and some of the present mutations must of necessity lie in this area. However, the reduced

frequency of mutations in the region from 1–98 and the virtual absence of changes in the region from 5350–5504 strongly suggest that the mutagenic process works over a limited distance from the site of entry. If an error-prone polymerase is involved in generating these mutations, then it may be an as yet uncharacterized enzyme or a known polymerase working with suboptimal template and conditions. [A highly error-prone polymerase has been described in *E. coli* (23).]

Immunoglobulin Mutagenesis. Somatic mutagenesis plays an important role in the generation of diversity of immunoglobulin genes (24–28). Sequence analysis of immunoglobulins and of immunoglobulin genes directed against particular antigens indicates that point mutations often occur in the hypervariable regions, whereas the constant regions are conserved (reviewed in refs. 27, 29, and 30). Possible mechanisms to explain this localized mutagenesis have been discussed for many years (16, 31, 32). Some of our results may be relevant to understanding the mutagenesis found in the hypervariable regions of certain antibody genes (16, 17, 24–29). It is often argued that mutations in hypervariable regions occur singly in sequential fashion. In this view, mutations are retained by selection for antibodies with higher affinity for antigen. Although this can explain the distribution of amino acid-replacement mutations (assuming all contribute to increased affinity), it cannot apply to silent coding-region mutations or to those in introns (16). Our suggestion that more than one mutation could occur in a single mutagenic episode would allow the introduction of some of the silent mutations at the same time as the replacement mutations. The same region might be exposed to more than one cycle of mutagenesis in which single or multiple mutations could be introduced. The appearance of silent mutations at the same site in the same hypervariable sequence in independent experiments (33) is comparable to our observation of hot spots.

The influence of an adjacent nick on mutagenesis of the marker gene suggests a molecular mechanism for localizing mutagenesis at a developmental stage. Our interpretation of this experiment is a virtual restatement of the proposal of Brenner and Milstein (17). They suggested that the process of immunoglobulin-gene mutagenesis would begin with recognition by a nicking enzyme of a specific sequence adjacent to the region in which the mutagenesis will occur. Regulation could be at the level of either the availability of the appropriate enzymes or the exposure of the specific sequence to these enzymes. Some features of the shuttle vector mutagenesis system might be usefully applied to this problem.

Note Added in Proof. A hypermutation mechanism associated with gene switching has been observed during the generation of immunological memory (34). These mutations might arise in a fashion similar to that discussed in this paper to account for the high frequency of multiple mutations generated in nicked plasmids replicated in xeroderma pigmentosum cells.

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