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# Specific tandem GG to TT base substitutions induced by acetaldehyde are due to intra-strand crosslinks between adjacent guanine bases

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## ABSTRACT

**Acetaldehyde is present in tobacco smoke and automotive exhaust gases, is produced by the oxidation of ethanol, and causes respiratory organ cancers in animals. We show both the types and spectra of acetaldehyde-induced mutations in *supF* genes in double- and single-stranded shuttle vector plasmids replicated in human cells. Of the 101 mutants obtained from the double-stranded plasmids, 63% had tandem base substitutions, of which the predominant type is GG to TT transversions. Of the 44 mutants obtained from the single-stranded plasmids, 39% had tandem mutations that are of a different type than the double-stranded ones. The GG to TT tandem substitutions could arise from intra-strand crosslinks. Our data indicate that acetaldehyde forms intra- as well as inter-strand crosslinks between adjacent two-guanine bases. Based upon the following observations: XP-A protein binds to acetaldehyde-treated DNA, DNA excision repair-deficient xeroderma pigmentosum (XP) cells were more sensitive to acetaldehyde than the repair-proficient normal cells, and a higher frequency of acetaldehyde-induced mutations of the shuttle vectors was found in XP cells than in normal cells, we propose that the DNA damage caused by acetaldehyde is removed by the nucleotide excision repair pathway. Since treatment with acetaldehyde yields very specific GG to TT tandem base substitutions in DNA, such changes can be used as a probe to identify acetaldehyde as the causal agent in human tumors.**

## INTRODUCTION

Acetaldehyde, one of the most common organic substances used in industry, is the raw material for such products as acetic acid, dyes, photographic chemicals, antioxidants, plastics and synthetic rubber (1). Acetaldehyde has been detected in many foods and in automotive exhaust gases (2). It is present in cigarette smoke, 0.8–1.4 mg acetaldehyde being produced per cigarette (3). Human exposure to acetaldehyde also is common due to the consumption of alcoholic beverages, because it is produced by the oxidation of ethanol. Acetaldehyde produces inter-strand cross-links in calf-thymus DNA *in vitro* (4). It also induces sister-chromatid exchanges in bone-marrow cells of rodents (5,6) exposed *in vivo* and in cultured human lymphocytes (7), as well as chromosomal aberrations in rat embryos (8) and mutations in cultured human skin fibroblasts (9). Inhalation of acetaldehyde causes adenocarcinomas and squamous-cell carcinomas in the nasal mucosa of rats, and laryngeal carcinomas in hamsters (2). Recently, some DNA adducts induced by acetaldehyde were identified. Acetaldehyde reacts with guanine, cytosine and adenine, but not with thymine. Acetaldehyde induces Schiff's base at exocyclic aminogroups of the guanine. The Schiff's base can be reduced by glutathione and ascorbic acid in cells, to form the stable $N^2$-ethyl-guanine (10). The level of this adduct in lymphocyte DNA of alcoholic patients was 2.1 ± 0.8 adducts/$10^7$ nucleotides (11). Although acetaldehyde seems to be related to the development of the human tumor, its mutation spectrum is not well known. In this report we present data showing that the predominant types of mutations induced in acetaldehyde-treated shuttle vector plasmids are tandem mutations. We will attempt to elucidate the mechanism for the production of such mutations and its possible biological significance.

## MATERIALS AND METHODS

### Cells

SV-40 transformed human fibroblast cell lines WI38-VA13 and XP2OS(SV) were used. All cells were cultured in Dulbecco's modified minimum essential medium supplemented with 10% fetal bovine serum.

### Chemicals

Biochemicals were obtained from Wako (Kyoto, Japan) except where stated otherwise. Oligodeoxyribonucleotides were synthesized by Nippon Seihun Co. (Tokyo, Japan). pBluescript KS(–) were purchased from Toyobo Co. (Tokyo, Japan). Dynabeads M-280 streptavidin were obtained from DYNAL A.S. (Oslo, Norway).

### Shuttle vector plasmid

The shuttle vector plasmid pMY189 was constructed previously by us (12). This plasmid has the *supF* suppressor tRNA gene as

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an indicator of mutation and replicates in both human and *Escherichia coli* cells. Single-stranded pMY189 was prepared using M13-derived helper phage VCSM13. *Escherichia coli* JM105 containing the pMY189 was infected with the phage in 2× TY medium. After an 8 h incubation at 37°C, the phage was harvested and the single-stranded pMY189 plasmids were purified with QIAGEN M13 Kit (QIAGEN Inc, Chatsworth, CA).

### Treatment of plasmids with acetaldehyde and transfection to human cells

Double- and single-stranded pMY189 plasmids (80 µg/ml in TE buffer) were treated with various concentrations of acetaldehyde at 37°C for 1 h. The plasmids were precipitated with ethanol to remove non-reacted excess acetaldehyde, and then redissolved in TE buffer (pH 8). WI38-VA13 cells ($2 \times 10^7$) transfected with 14.4 µg of the acetaldehyde-treated pMY189 by electric pulses were incubated at 37°C for 72 h in a $CO_2$ incubator.

### Plasmid recovery, selection of mutated supF and DNA sequencing

The plasmids were extracted from the cells and digested with the restriction endonuclease *Dpn*I to eliminate non-replicated input plasmids with the bacterial methylation pattern. Plasmid DNA was introduced into the indicator bacteria *E.coli* KS40/pKY241 (12,13) by electro-transformation. The bacteria were spread on LB agar plates containing 50 µg/ml nalidixic acid, 150 µg/ml ampicillin and 30 µg/ml chloramphenicol, together with IPTG and X-gal. Plasmids with mutated *supF* genes made *E.coli* cells resistant to nalidixic acid, whereas cells carrying plasmids with unmutated *supF* genes could not grow in the presence of nalidixic acid. IPTG and X-gal were added to confirm selection of the mutated *supF* gene by the color of the colonies. A portion of the bacteria was spread on plates containing ampicillin and chloramphenicol to measure the transformant fraction and plasmid survival.

Mutated plasmids were extracted and purified from the overnight culture and the base sequences of the *supF* gene of the plasmids were determined with the –21M13 primer and Dye-Primer Cycle Sequencing reagent Kit by the 370A automatic DNA sequencer (Applied Biosystems Foster, CA).

### Intra-strand DNA crosslink assay

The sequences of primers and oligonucleotides used in this assay and the scheme of the assay are shown in Figure 3A. The 175 bp region of pBluescript KS(–) was amplified by PCR using primers 1 and 2 (Fig. 3A). Primer 1 was 5′-biotinylated. Approximately 14 pmol of PCR products were bound to the Dynabeads M-280 streptavidin by conjugation between biotin and streptavidin. The PCR products bound to the Dynabeads were denatured with 0.1 M NaOH, collected using a magnet, washed several times with 0.1 M NaOH and then washed twice with TE buffer containing 1 M NaCl, resulting in single-stranded 175mer DNA bound to the Dynabeads. Three kinds of oligonucleotides, oligo 1, oligo 3 and oligo 5, were 5′-labeled with $^{32}P$. The labeled oligo 1 and unlabeled oligo 2 were annealed to the single-stranded 175mer DNA by heating and cooling in 1 M NaCl, to detect intra-strand crosslinks between adjacent guanine bases. Oligo 3 and oligo 4, or oligo 5 and oligo 6 were also annealed to the single-stranded 175mer DNA to detect CC- or GC-intra-strand crosslinks, respectively. The annealed DNA (1 pmol of each) was treated with 2 M of acetaldehyde in 0.1 M NaCl for 48 h at 4°C. After collecting the DNA with a magnet, the DNA was dissolved in 10 µl of 0.1 M NaCl. Then, 2 µl of loading buffer consisting of 50% urea, 15% glycerol, 0.25% bromophenol blue (BPB) and 0.25% xylenecyanol (XC) was added to 2 µl of the DNA solution and the samples were subjected to electrophoresis on 12% polyacrylamide gel (50% urea, 1× TBE) for 45 min. at 500 V, and the gel was analyzed by autoradiography.

### Cell survival assay

Sensitivity of human cells to acetaldehyde was assayed by colony-forming ability after treatment. An appropriate number of the cells were seeded onto 100 mm Petri dishes and incubated. The next day, the medium was removed and cells were washed with phosphate-buffered saline. After the cells were treated with various concentrations of acetaldehyde for 1 h in a $CO_2$ incubator, the dishes were washed and refilled with the medium and incubated further until colonies appeared. After fixing and staining, colonies were scored.

### XP-A protein binding assay

Nitrocellulose filter-binding assays were performed as described by Robins *et al*. (14) with some modifications. The purified XP-A protein was a kind gift of Dr Kiyoji Tanaka, Osaka University. A *Not*I-digested pBluescript SK(–)-based plasmid (4 kb), labeled with [α-$^{32}P$]dCTP by Klenow fragment, was used as a DNA probe. The DNA was extracted with phenol, precipitated with ethanol and resuspended in water. The labeled DNA was treated with acetaldehyde (0, 0.5 and 1 M, respectively) for 1 h at 37°C, then precipitated with ethanol and resuspended in water. Binding reaction mixture contained 1 ng of DNA (10 000 c.p.m.) in 50 µl of 20 mM HEPES–KOH pH 7.7, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml bovine serum albumin (BSA), 10% glycerol and XP-A protein. After the mixture was incubated for 30 min at 4°C, a 50 µl aliquot was applied to alkali-pretreated nitrocellulose filters (Millipore HAWP 02500, 0.45 µm pore size) and rinsed twice with 0.5 ml of binding buffer without BSA. The residual radioactivity of the filters was counted using a liquid scintillation counter.

### Statistics

Statistical comparisons were performed with Fisher's extract test for differences in proportions (15). The *P* values for a one-tailed test are presented.

## RESULTS

### Mutation induction by acetaldehyde in shuttle vectors replicated in human cells

Acetaldehyde treatment of both the single- and double-stranded pMY189 plasmids increased the frequency of mutation in the *supF* gene on shuttle vectors propagated in normal human cells (Table 1). The background plasmid mutation frequencies were 79 and $1.3 \times 10^{-4}$ with the single- and double-stranded plasmids, respectively. At 1 M of acetaldehyde treatment, the mutation frequencies of single- and double-stranded DNA were increased to 986 and $54 \times 10^{-4}$, respectively.

**Table 1.** Mutation frequency of acetaldehyde-treated single- and double-stranded plasmids pMY189 propagated in human fibroblasts

| Concentration of acetaldehyde (M) | Single-stranded DNA No. of colonies Mutant | Total | Mutation frequency ($\times10^{-3}$) | Double-stranded DNA No. of colonies Mutant | Total | Mutation frequency ($\times10^{-4}$) |
|---|---|---|---|---|---|---|
| 0 | 159 | 20 212 | 7.9 | 33 | 264 000 | 1.3 |
| 0.25 | 199 | 11 391 | 17.5 | | | |
| 0.5 | 77 | 2262 | 34.0 | 65 | 48 000 | 13.6 |
| 1.0 | 35 | 355 | 98.6 | 16 | 2961 | 53.7 |
| 2.0 | 21 | 777 | 270.3 | | | |

**Table 2.** Type of mutations in the *supF* gene in acetaldehyde-treated shuttle vector plasmids pMY189

| Type of mutation | No. of plasmids with base changes (%) double strand | single-stranded |
|---|---|---|
| Single base substitution | 24 (24) | 20 (45) |
| Base changes including tandem base substitution | 63 (62)[a] | 17 (39) |
| Deletion | 4 (4) | 2 (5) |
| Insertion | 1 (1) | 0 (0) |
| Others | 9 (9) | 5 (11) |
| Total | 101 (100) | 44 (100) |

[a]$P < 0.02$ versus single-stranded.

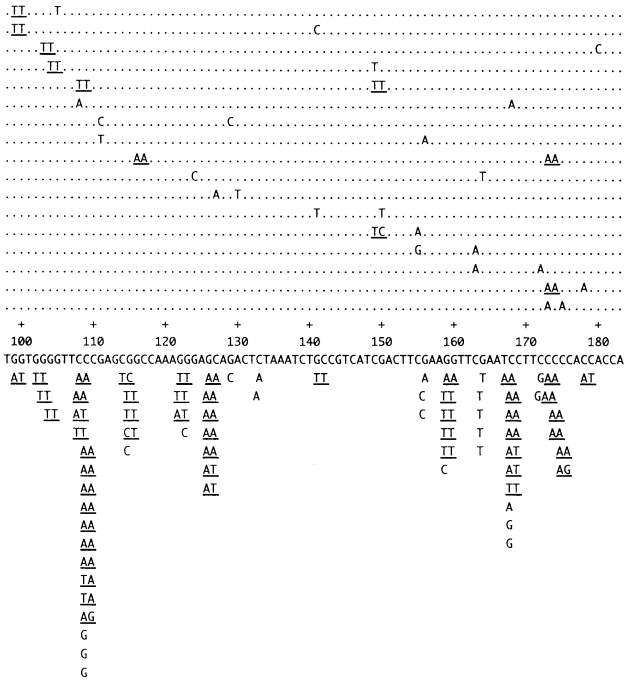


**Figure 1.** Mutation spectrum of acetaldehyde-treated double-stranded pMY189 replicated in VA13 cells. The location and nature of independent single and tandem (underlined) mutations are indicated below the sequence. The dashed lines above the *supF* sequence represent independent mutants carrying multiple mutations. Several tandem substitutions, present in these multiple mutants, are underlined. Other mutations found in the other region of *supF* gene were as follows; single mutations, G(65) to T and G(72) to A; multiple mutations, large deletion and tandem CC(142, 143) to AA, tandem CC(55, 56) to AA and tandem CC(109, 110) to AA, tandem CC(175, 176) to AA and C(199) to T.

## Types and spectra of mutations induced by acetaldehyde in the *supF* gene

Base sequence analysis of mutant plasmids (Table 2) showed that 62% (double-stranded) and 39% (single-stranded) of the mutated plasmids had tandem base substitutions. Tandem mutations were significantly more frequent with double-stranded ($P = 0.007$) than with single-stranded. The distribution of the base substitutions in the *supF* gene is shown in Figure 1 (double-stranded). Tandem base substitutions are present at various sites throughout the gene. While the spectrum of base substitution of single-stranded *supF* gene (Fig. 2) is quite different from that of the double-stranded gene, there is a clear hot-spot involving bases 126–127 in the single-stranded *supF* gene. The types of single base substitutions are listed in Table 3. The predominant types of the mutations are G:C to C:G (54.2%) and G:C to T:A (37.5%) transversions in double-stranded shuttle vectors, while in the single-stranded shuttle vector, G to T (40%) and C to G (20%) mutations are predominant, whereas C to A (15%) and G to C (0%) mutations are minorities. These results suggest that DNA damage induced by acetaldehyde in guanine bases may contribute to the G:C to T:A mutations and damage induced in cytosine bases may contribute to the G:C to C:G transversions. Table 4 shows the types of tandem base substitutions (double strand). Eighty percent of the tandem base substitutions are located at CC (GG) sites, 14% at 5′-GC-3′ sites, and 5% at 5′-CG-3′ sites. The predominant type of mutation is GG to TT (CC to AA) (61%) transversion, which has been reported only in *cis*-diamminedichloroplatinum(II)-induced mutations (16). While almost all the tandem base substitutions of single-stranded shuttle vectors are located at 5′-GC-3′ sites (94%), the predominant type of mutation, GC to TA (47%), is not found in double-stranded shuttle vectors (Table 5).

## Intra-strand crosslinks induced by acetaldehyde

GG to TT (or CC to AA) tandem base substitutions induced by acetaldehyde may arise from intra-strand crosslinks. To test this hypothesis, the experiment shown in Figure 3 was carried out. If acetaldehyde forms an intra-strand crosslink between $^{32}$P-labeled 20mer DNA and non-labeled 20mer DNA, which were annealed to 175mer DNA, a new 40mer band should be detected on the denaturing gel. Figure 3 showed that the 40mer product was detected on the lane where acetaldehyde-treated substrates designated for detection of a GG intra-strand crosslink were loaded, but were not detected on the other lanes. This result indicates that acetaldehyde forms a molecular bridge between adjacent guanine bases and forms little or no intra-strand crosslink between adjacent cytosines nor adjacent guanine and cytosine. Inter-strand crosslinks were also found, which is consistent with earlier findings.

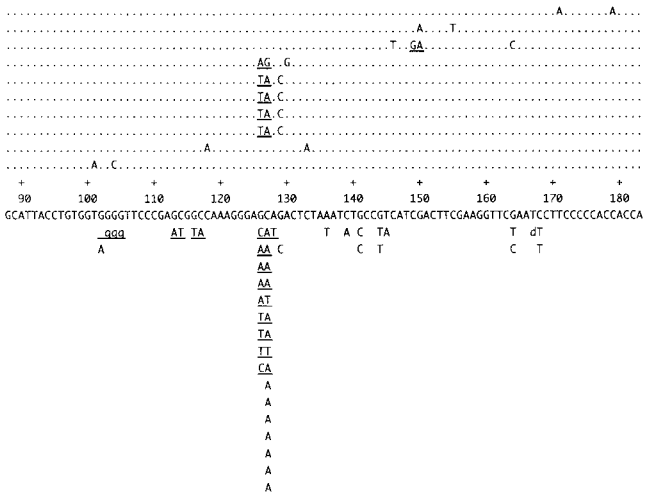


**Figure 2.** Mutation spectrum of acetaldehyde-treated single-stranded pMY189 replicated in VA13 cells. The location and nature of independent single and tandem (underlined) mutations are indicated below the sequence. The dashed lines above the *supF* sequence represent independent mutants carrying multiple mutations. Several tandem substitutions, present in these multiple mutants, are underlined. The 'd' denotes deletion. The 'ggg' at the region from 102 to 105 bp represents a GGGG to GGG base change. Another multiple mutation found in the other region of *supF* gene was tandem GC(78, 79) to TA and C(127) to A.

**Table 3.** Types of single-base substitution in the *supF* gene in acetaldehyde-treated shuttle vector

| Double-stranded | | Single-stranded | |
|---|---|---|---|
| Type of mutation | No. of mutations (%) | Type of mutation | No. of mutations (%) |
| G:C to T:A | 9 (37.5) | G to T | 8 (40) |
| | | C to A | 3 (15) |
| G:C to C:G | 13 (54.2) | G to C | 0 (0) |
| | | C to G | 4 (20) |
| G:C to A:T | 2 (8.3) | G to A | 2 (10) |
| | | C to T | 1 (5) |
| A:T to T:A | 0 (0) | A to T | 1 (5) |
| | | T to A | 1 (5) |
| Total | 24 (100) | Total | 20 (100) |

## High sensitivity of nucleotide-excision repair deficient cells to acetaldehyde

We compared the acetaldehyde sensitivity between normal (WI38-VA13) and nucleotide excision repair-deficient [XP2OS(SV)] cell lines (Fig. 4). XP2OS(SV) cells were slightly sensitive to acetaldehyde compared with the normal cells. To eliminate those effects of metabolism and cell toxicity that were not due to DNA damage by acetaldehyde, we examined the survival and mutations of acetaldehyde-treated double-stranded shuttle vectors propagated in both WI38-VA13 and XP2OS(SV) cells (Fig. 5). Survival of the plasmids extracted from XP2OS(SV) was much lower than that of plasmids from WI38-VA13 cells, and the mutation frequency in XP2OS(SV) cells at 0.5 M was five times higher than in WI38-VA13 cells. These results suggest that the DNA damage induced by acetaldehyde can be repaired by the nucleotide excision repair pathway.

**Table 4.** Types of acetaldehyde-induced tandem base substitution in the *supF* gene in double-stranded shuttle vector plasmids pMY189 propagated in human fibroblast cells WI38-VA13

| Type of mutation | No. of mutations | % |
|---|---|---|
| GG to TT (CC to AA) | 40 | 61 |
| GC to AA (GC to TT) | 7 | 11 |
| GG to AT (CC to AT) | 6 | 9 |
| GG to CT (CC to AG) | 3 | 5 |
| GG to AA (CC to TT) | 2 | 3 |
| GG to TA (CC to TA) | 2 | 3 |
| GC to AT (GC to AT) | 2 | 3 |
| CG to GA (CG to TC) | 2 | 3 |
| CG to AA (CG to TT) | 1 | 2 |
| GA to TT (TC to AA) | 1 | 2 |
| Total | 66 | 100 |

**Table 5.** Types of acetaldehyde-induced tandem base substitution in the *supF* gene in single-stranded shuttle vector plasmids pMY189 propagated in human fibroblast cells WI38-VA13

| Type of mutation | No. of mutations | % |
|---|---|---|
| GC to TA | 8 | 47 |
| GC to TT | 3 | 18 |
| GC to AT | 2 | 12 |
| GC to AA | 1 | 6 |
| GC to TG | 1 | 6 |
| GC to CT | 1 | 6 |
| CG to TC | 1 | 6 |
| Total | 17 | 100 |

## Binding of XP-A protein to acetaldehyde-treated DNA

The $^{32}$P-labeled DNA was treated with acetaldehyde at a concentration of 0, 0.5 and 1 M for 1 h at 37°C and incubated with various amounts of XP-A protein. If the DNA binds to the XP-A protein, radioactivity remains the on filter. As indicated in Figure 6, binding of the XP-A protein to the acetaldehyde treated DNA is dependent on the amount of XP-A protein and the acetaldehyde concentration. When no XP-A protein was added to the reaction mixture, the radioactivity retained by the filter was 0.6, 2.9 and 14.6% for acetaldehyde concentrations of 0, 0.5 and 1 M, respectively. When 10 and 20 ng XP-A protein were added, this was increased to 2.7, 8.6 and 50.1%, and 3.7, 12.6 and 56.4%, respectively. This result shows that the XP-A protein recognizes the DNA damage induced by acetaldehyde.

## DISCUSSION

Acetaldehyde induces GG to TT (or CC to AA) tandem mutations in double-stranded shuttle vectors. It is difficult to imagine DNA lesions that induce tandem mutations except by way of intra-strand crosslinks. UV light induces CC to TT (17,18), and *cis*-diamminedichloroplatinum(II) induces GG to TT tandem base substitutions (16). It is well known that UV light induces cytosine dimers and *cis*-diamminedichloroplatinum(II) induces G–G intra-strand crosslinks (19). Metal-induced oxygen radicals also induce GG to AA tandem mutations (20) and are believed to induce intra-strand crosslinks (21). Our results show that acetaldehyde forms intra-strand crosslinks in adjacent guanine bases. Adenines would be incorporated at the opposite sites of

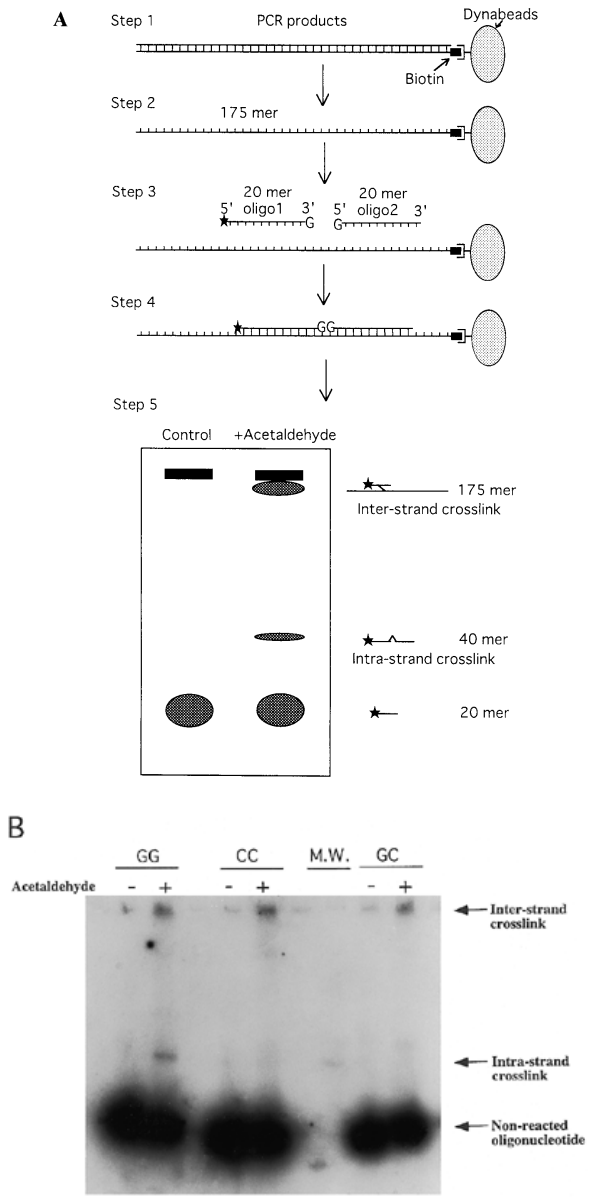


**Figure 3.** (**A**) Scheme of crosslink assay. The sequences of oligonucleotides used in this assay are as follows: primer 1, d(TTAACGCGAATTTTAACAAA) (biotinylated); primer 2, d(TCGTGACTGGGAAAACCCTG); oligo 1, d(TCGTGACTGGGAAAACCCTG); oligo 2, d(GCGTTACCCAACTTAATCGC); oligo 3, d(GCGTTACCCAACTTAATCGC); oligo 4, d(CCTTGCAGCACATCCCCCTT); oligo 5, d(GGCGTTACCCAACTTAATCG); oligo 6, d(CTTGCAGCACATCCCCCTTT). Step 1: the 175 bp region of pBluescript KS(–) was amplified by PCR using primer 1 and 2, and the PCR products were bound to Dynabeads. Step 2: the Dynabeads were washed with NaOH to make the 175mer single-stranded DNA. Step 3: the $^{32}$P-labeled oligo 1 and non-labeled oligo 2 were annealed with the 175mer single-stranded DNA for detecting intra-strand crosslinks between adjacent guanine bases. Alternatively, the $^{32}$P-labeled oligo 3 and non-labeled oligo 4, or the $^{32}$P-labeled oligo 5 and non-labeled oligo 6, were also annealed to detect CC- or GC-intra-strand crosslinks, respectively. Step 4: the substrates were treated with acetaldehyde. Step 5: the treated substrates were subjected to electrophoresis on 12% polyacrylamide gel (50% urea, 1× TBE) and the gel was autoradiographed. (**B**) Result of the crosslink assay. The substrates that were designated to detect intra-strand crosslinks between adjacent guanine bases (GG), adjacent cytosine bases (CC)and adjacent guanine and cytosine bases (GC) were treated with or without 2 M acetaldehyde for 48 h at 4°C and were subjected to the denatured gel electrophoresis. $^{32}$P-labeled 40mer oligonucleotide was also used as a molecular weight maker (M.W.).

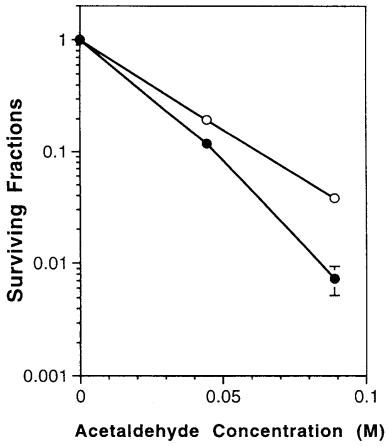


**Figure 4.** Survival curves of normal (VA13 ) (○) and XP-A [XP2OS(SV)] (●) cells treated with acetaldehyde. Three plates were used for each data point and the error bar represents standard deviation. Except for one point, the error bars were too small to show.

crosslinked guanines at DNA replication, which resulted in GG to TT tandem base substitutions. The chemical structure of this crosslink is still unknown. Chaw *et al*. showed that formaldehyde forms (-NH-$CH_2$-NH-) crosslinks between G–G, G–C, A–C, A–G and A–A (22). Acetaldehyde should form DNA crosslinks by a similar mechanism to formaldehyde, i.e. it should form covalent bridges between amino groups of DNA bases. Cytosine and adenine, as well as guanine, have amino groups in their molecules. Although we could not detect crosslinks at a 5′-GC-3′ site in our assay system, a little crosslink must be formed at this site, which leads to tandem mutations at 5′-GC-3′ sites. In single-stranded shuttle vectors, different from in double-stranded vectors, acetaldehyde does not induce GG tandem mutations, but induces GC tandem mutations. The types of GC tandem mutation are also different. We have no clear explanation of these observations, but it may depend on the difference of (i) the three-dimensional structure, (ii) DNA replication mechanisms and (iii) DNA repair mechanisms, between single- and double-stranded shuttle vectors.

Acetaldehyde is commonly present in many organisms. Therefore, it is not surprising that organisms have repair pathways for acetaldehyde-induced DNA damage. Results from the cell survival, shuttle vector mutagenesis and XP-A protein-binding assays strongly suggest that the human nucleotide excision repair system is involved in the repair of the acetaldehyde-induced DNA damage. In the cell survival assay, XP cells were slightly sensitive to acetaldehyde compared with normal cells, but the difference of sensitivity was not as distinct between the two cell lines. The reason should be that DNA damage is not the only biological lesion induced by acetaldehyde. Since acetaldehyde is reactive with the amino residue, it may react with proteins, amino acids and RNA molecules, as well as DNA molecules.

Since treatment with acetaldehyde produces very specific base changes in DNA, these changes can be used as a probe to show acetaldehyde as a causal agent in human tumors. The majority of human cancers have point mutations in the *p53* tumor suppressor gene (23), and the specificity in the types of mutations in this gene may be related to causal environmental agents; e.g. the CC to TT tandem double mutations in skin tumors to UV sunlight (17,18,24,25), and the G to T transversions in liver tumors to aflatoxinB1 (26,27). The database of *p53* gene somatic mutations

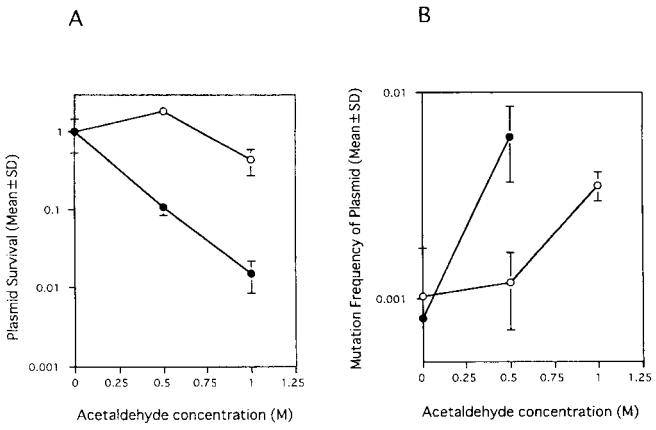


**Figure 5.** (**A**) Survival of acetaldehyde-treated pMY189 replicated in normal (VA13) (○) and XP-A [XP2OS(SV)] (●) cell lines. The relative number of ampicillin-resistant bacterial colonies obtained after repair and replication of acetaldehyde-treated pMY189 in the cells followed by transformation of the indicator *E.coli* is shown. (**B**) Mutation of acetaldehyde-treated pMY189 replicated in normal (VA13; ○) and XP-A [XP2OS(SV); ●] cell lines. The ratio of nalidixic acid-resistant bacterial colonies versus total bacterial colonies, obtained after repair and replication of acetaldehyde-treated pMY189 in the cells followed by transformation of the indicator *E.coli*, is shown. The data show mean values from three independent transfection experiments for each cell line, and the error bar represents the standard deviation.

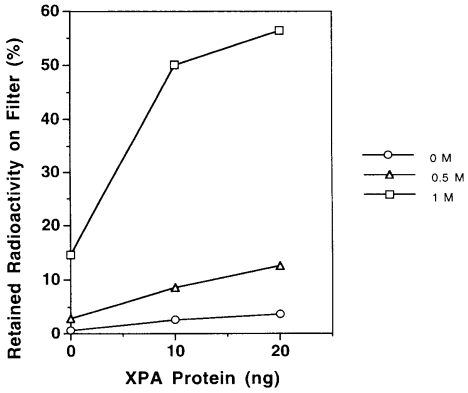


**Figure 6.** XP-A protein binding assay to acetaldehyde treated DNA. One ng of $^{32}$P-labeled DNA, either untreated (○), acetaldehyde-treated at 37°C for 1 h with a concentration of 0.5 M (Δ) or 1 M (□), was incubated with purified XP-A protein in the presence of excess amount of BSA (5 µg). DNA bound to protein was collected on nitrocellulose filters.

in human tumors and tumor cell lines (28) was searched. Of 4496 mutations in the *p53* gene in the database, only three GG to TT (or CC to AA) tandem base substitutions were found. Five GC to TT (or GC to AA) and three GG to AT (or CC to AT) tandem base substitutions were also found in 4496 mutations. Five of these tandem base substitutions were found in the respiratory tract cancers and one was found in a stomach cancer. Although no details of the smoking and drinking histories of the subjects concerned were given, these mutations may be related to smoking or alcohol consumption. A recent finding indicates that human aldehyde dehydrogenase gene (*ALDH2*) genotype is related to esophageal cancer risk (29). A study of the relationship between the tandem base substitutions in esophageal tumors and epidemiological and genetic properties of the patients, such as smoking and drinking habits and *ALDH2* genotypes, may reveal the significance of acetaldehyde on the development of human tumors.

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