Cobaltous chloride-induced mutagenesis in the supF tRNA gene of Escherichia coli

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Introduction

The cytotoxicity and carcinogenicity of metals and their compounds have attracted attention and been studied for a long time. Of special interest among metal compounds is cobalt compounds have shown carcinogenic effects in animals (Heath, 1956; Heath and Webb, 1967). It has also been reported that CoCl2 exhibited DNA binding activity (Eichorn and Shin, 1968; Zimmer et al., 1974), caused DNA strand breaks (Robinson et al., 1982; Hamilton-Koch et al., 1986; Hartwig et al., 1990) and reduced the fidelity of DNA synthesis (Sirover and Loeb, 1976).

We have previously established the supF system for studying forward mutation in Escherichia coli (Uematsu et al., 1997). Using this system, the spectrum of mutations induced by CoCl2 in an E.coli supF suppressor tRNA gene was determined, as part of a program devoted to studies on metal ion mutagenesis (Ogawa et al., 1986, 1987a, 1987b, 1988, 1994; Morita et al., 1991).

In the present report, we found that CoCl2-induced mutations in the supF gene were primarily deletions and frameshifts, which occurred mostly at the sites of short direct repeats; the others were base substitutions and guanine residues are the suggested target for 90% of CoCl2-induced frameshifts and base substitutions.

Materials and methods

Plasmids, bacterial strains and bacteriophage

Vector pUB3, described by Rodriguez et al. (1992), contains the tyrosine amber suppressor tRNA gene (supF) gene, lacZA gene and ampicillin resistance (Amp') gene used for mutagenesis studies. Escherichia coli K12 strains DL16 [leu64 lacZ5(am) recA1 rpsL1511 deoC26 tsx trx metE7070 rbs4 malB45 doeC2] and KS40 [lacZ5(2am) CA7020 gyrA lacY1 sdsR hsdR hsdM araD319 Δ(arb-BC-Fe)7677 galU galK rpsL thi] were used to prepare plasmid pUB3 DNA and allow processing and replication of CoCl2-treated plasmid pUB3 (Akausa et al., 1992). The indicator host bacterial strain used to distinguish wild-type and mutant pUB3 was SY1032/pKY241 [gyrA96 Δ(pro-lac) supB (F' pro lacF lacZM15(sgyrAam) cml)] (Uematsu et al., 1997). Strain XL1-BlueMRF (Strategene) was used as a host for bacteriophage M13KO7, which was used to prepare single-stranded DNA for DNA sequencing.

Reagents and media

CoCl2 and other chemicals were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Enzymes and reagents used for DNA manipulation and DNA sequencing were purchased from Takara Shuzo Co. Ltd (Kyoto, Japan) and PE Applied Biosystems Inc. (Foster City, CA). Compositions of L, broth, L agar plates and M buffer were as described previously (Otsuji et al., 1988, 1987a, 1987b, 1988, 1994; Morita et al., 1997). The indicator host bacterial strain used to distinguish wild-type and mutant pUB3 was SY1032/pKY241 [gyrA96 Δ(pro-lac) supB (F' pro lacF lacZM15(sgyrAam) cml)] (Uematsu et al., 1997). Strain XL1-BlueMRF (Strategene) was used as a host for bacteriophage M13KO7, which was used to prepare single-stranded DNA for DNA sequencing.

The cytotoxicity and carcinogenicity of metals and their compounds have attracted attention and been studied for a long time. Of special interest among metal compounds is cobalt. In previous studies, cobalt(II) chloride (CoCl2) was propagated after transfection into Escherichia coli SY1032/pKY241 host cells. The vector plasmid carried an Escherichia coli supF suppressor tRNA gene as a target for mutations. After CoCl2 treatment, 64 independent nalidixic acid-resistant, ampicillin-resistant and Lac- transfectants were isolated from strain DL16/pUB3 according to the ‘midi’ protocol of the Qiagen kit (Qiagen Inc., Chatsworth, CA) (Lutze and Winegar, 1990; Kimura et al., 1993), was exposed to freshly prepared solutions of 20 μM CoCl2 for 2 h at 37°C in 0.1 M Tris–HCl buffer (pH 7.2). CoCl2-treated pUB3 DNA was immediately transfected into competent KS40 cells which were induced by the CaCl2/RbCl method (Maniatis et al., 1982). They were incubated overnight to attain plasmid replication and then the progeny plasmids were extracted from the cells by the alkaline lysis method (Birnboim and Doly, 1979). Progeny plasmids from individual transfections were assayed separately for the mutant supF gene to distinguish mutants that were derived from siblings. Competent SY1032/pKY241 cells were transfected with proton plasmids and the transformants with supF mutant plasmids were plated on M lactose agar plates containing 50 μg/ml nalidixic acid (Nal), 50 μg/ml ampicillin (Amp) and 30 μg/ml chloramphenicol (Cml). To collect supF mutants, only one nalidixic acid-resistant (Nal') and Lac+ colony was chosen from each transformation experiment. This approach ensured that all mutants were of independent origin.

DNA sequencing

Single-strand DNA was prepared by infecting putative mutants [SY1032/pKY241(pUB3[supF-])] with M13KO7 phage and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using an ABI automated sequencer model 373A, performed in exactly the same way as described previously (Uematsu et al., 1997). The polymerization reaction was primed

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Results and discussion

**CoCl₂-induced SupF⁻ mutations**

Using a vector pUB3 carrying the *E.coli* supF gene and the *E.coli* SY1032/pKY241 host cells, we analyzed the spectrum of CoCl₂-induced mutations. Initially, 700 ng of plasmid pUB3 DNA was exposed to 20 μM CoCl₂ for 2 h and then transfected into competent SY1032/pKY241 cells. The transformation frequency was ~9.7 × 10⁻⁴, which was the same as that of untreated DNA. The mutation frequency of induced Nal⁻ Lac⁰ (Sup⁻) transformants on the selective medium comprised of M lactose agar plates supplemented with Nal, Amp and Cml, averaged over eight experiments, was 2.5 × 10⁻⁶. This value was ~8-fold higher than that of spontaneous mutations (3.1 × 10⁻⁷). This fact indicated that 90% of the isolated mutant clones were probably induced by CoCl₂. Furthermore, from the treated cells, 64 Nal⁻ Lac⁺ (Amp⁺) mutant clones of presumably independent origin were isolated from selective medium and vector DNA molecules containing the expected mutated supF gene were recovered from all mutant cell clones. DNA sequences of the mutant supF genes were determined as ~150 bp from the Pre-tRNA to the End-tRNA region, by the dideoxy chain termination method, and altered sequences were found in all 64 mutant genes. While 63 mutants carried one mutation each, one mutant, CC19, carried two base substitutions 6 bases apart. Thus we identified 65 mutations. The distribution of mutants in the supF gene is shown in Figure 1.

**Spectrum of CoCl₂-induced mutations**

Table I lists CoCl₂-induced DNA sequence alterations in the supF gene. Forty six percent of mutational events were deletions involving loss of 8–85 bp, whereas 29% were base substitutions. They differed from the spontaneous mutations, which occurred rather non-specifically (Akasaka *et al.*, 1992). The remaining mutations were frameshifts (15%), insertion sequences (8%) and duplications (2%). Each class of mutations is hereby described in detail.

Deletions and duplication. The location and DNA sequence context at the sites of duplication and deletion mutations are presented in Table II. One duplication was recovered at site 66–73; this mutation was caused by the addition of 8 bp. Mutation at five of seven sites (55–111, 60–112, 68–129, 77–119 and 124–212) were large deletions (38–85 bp) and those at the remaining two sites (95–109 and 153–166) were deletions of 13 and 8 bp, respectively. One of these sites, 68–129, was found to be a hot-spot for CoCl₂ mutagenesis, since 20 mutations were recovered at this site. There were direct repeats of 10 bases (AAGGGAGCAG) flanking the fragment of 52 nucleotides to be deleted and one of the repeats was lost in the mutant sequences. This mutation was similar to spontaneous mutations in the same gene, as previously reported (Akasaka *et al.*, 1992). A common sequence resulting from the deletion of 8–85 bp was also seen at the sites of other deletions. They had two short direct repeats of 2–7 bases and one of the repeats was missing in the mutant sequences. This unique sequence feature at the sites of deletions was frequently observed in spontaneous and topB mutant-induced mutations (Uematsu *et al.*, 1997; Akasaka *et al.*, 1992). We suggest slippage–misalignment (Ikehata *et al.*, 1989; Akagi *et al.*, 1990; Ogawa *et al.*, 1993) to be the mechanism for CoCl₂ mutagenesis at the supF system.

Eichhorn and Shin (1968) and Zimmer *et al.* (1974) reported that CoCl₂ reacts with DNA producing two major adducts [the N7G–Co(II) and -O₃PO–Co(II) adducts] and a chelate [N7G–Co(II)–OPO₃] at the N7 position of guanine (N7G) or/and the phosphate residue (O₃PO⁻). It has also been shown that induction of DNA strand breaks generated by oxygen free radicals arose in vivo by reaction of Co(II) ions with superoxide radicals.

Statistical analysis

To decide if there was a difference in the mutation frequency between CoCl₂-induced and spontaneous mutations, the χ² test was used. P < 0.01 was regarded as significant.
CoCl₂ mutagenesis in E.coli supF

Table I. Classes of CoCl₂-induced and spontaneous mutations in an E.coli supF tRNA gene

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Spontaneous mutations</th>
<th>CoCl₂-induced mutations</th>
<th>χ² value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base substitution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition</td>
<td>33 (63%)</td>
<td>19° (29%)</td>
<td>7.52</td>
</tr>
<tr>
<td>G→C→A: T</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A:T→G: C</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Transversion</td>
<td>28</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>G:C→T:A</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>G:C→C: G</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>A:T→T:A</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A:T→C: G</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Frameshift</td>
<td>1 (2%)</td>
<td>10 (15%)</td>
<td>31.0</td>
</tr>
<tr>
<td>Deletion</td>
<td>5 (10%)</td>
<td>30 (46%)</td>
<td>76.4</td>
</tr>
<tr>
<td>Duplication</td>
<td>0</td>
<td>1 (2%)</td>
<td>~</td>
</tr>
<tr>
<td>Insertion sequence</td>
<td>13 (25%)</td>
<td>5 (8%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Mutation frequency</td>
<td>3.1×10⁻⁷</td>
<td>2.5×10⁻⁶</td>
<td></td>
</tr>
</tbody>
</table>

Data from Akasaka et al. (1992).
Based on experiments performed 398 times, 52 spontaneous mutants were isolated (Akasaka et al., 1992). In the case of CoCl₂-induced mutagenesis, we performed experiments >75 and <100 times and obtained 64 mutants. Thus, for calculating χ² values, we assumed that we performed experiments 100 times. χ² (0.01) is 6.63.
Included one double mutation (CC19).
Frameshift mutation here means the deletion or addition of a single nucleotide, shifting the coding frame.

Table II. Deletions and duplication

<table>
<thead>
<tr>
<th>Site</th>
<th>Incidence</th>
<th>Sequence context</th>
<th>Nucleotide change</th>
<th>Mutant gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>55-111</td>
<td>1</td>
<td>GUGCCUGUGAGCC→GUGCCUGAGC</td>
<td>-53 bp</td>
<td>CC6</td>
</tr>
<tr>
<td>60-112</td>
<td>1</td>
<td>GUGCCUGUGAGCC→GUGCCUGAGC</td>
<td>-46 bp</td>
<td>CC4</td>
</tr>
<tr>
<td>66-73</td>
<td>1</td>
<td>CCGGTAAGGGAGCG→</td>
<td>+8 bp</td>
<td>CC63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGGTAAGGGAGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68-129</td>
<td>20</td>
<td>GATAGGGAGCCAGCT→</td>
<td>-52 bp</td>
<td>CC1, CC3, CC5, CC11, CC14, CC17, CC18, CC22, CC23, CC24, CC28, CC30, CC34, CC35, CC39, CC41, CC42, CC48, CC51, CC66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATAGGGAGCCAGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>77-119</td>
<td>2</td>
<td>GCACGCUGUGAGCC→</td>
<td>-38 bp</td>
<td>CC15, CC38</td>
</tr>
<tr>
<td>95-109</td>
<td>1</td>
<td>TTACUUUGUGAG→</td>
<td>-13 bp (C→T)</td>
<td>CC53</td>
</tr>
<tr>
<td>124-212</td>
<td>2</td>
<td>AGGGAGCCAG→</td>
<td>-85 bp</td>
<td>CC50, CC52</td>
</tr>
<tr>
<td>133-166</td>
<td>3</td>
<td>GAATTCUGUGAGCC→</td>
<td>-8 bp</td>
<td>CC27, CC32, CC60</td>
</tr>
</tbody>
</table>

Numbers indicated are the nucleotide number of E.coli supF DNA; numbering is in accordance with Hauser et al. (1986).
Arrows below and bars above the sequences indicate direct repeats and deleted nucleotides, respectively. Boxed nucleotides are those duplicated in the mutant gene.
One base change shown in parentheses is the suggested nucleotide change in addition to those predicted by the slippage-misalignment mechanism.

or hydrogen peroxide (Moorhouse et al., 1985; Yamamoto et al., 1989; Kadiiska et al., 1989). These studies suggest that the premutagenic lesions of the deletions (Table II) may be due to cleavage at either the 5′- or 3′-position of phosphoester bonding of the DNA strand by the action of oxygen free radicals, which will probably be generated in cells by the reaction of the -O₃ PO–Co(II) adduct or/and the N7G–Co(II)N-OPO₃ chelate with superoxide or hydrogen peroxide.

Base substitutions. Base substitution mutations were found in 19 of 65 mutations (29%) in the supF gene, of which four were transitions (G→C→A: T) and 15 were transversions (eight G→C→T: A, six G→C→C: G and one A: T→C: G) (Tables I and III). CoCl₂-induced base substitution mutation frequency was 7.3×10⁻⁷ (= 2.5×10⁻⁶×29%), which is 3.6-fold higher than the frequency of spontaneous base substitution, i.e.
3.1×10^{-7}×63\% = 2.0×10^{-7}. Thus, CoCl_2 can induce base substitution weakly but significantly (Table I). The distribution of CoCl_2-induced base substitution sites was similar to that of spontaneous base substitution sites (Figure 1). There was an 18:1 bias in favor of the G:C base pair as the site of base substitutions, while transitions were predominant over transversions. The strong preference for CoCl_2-induced base substitutions at G:C sites suggests that the N7G–Co(II) adduct is the lesions responsible for inducing these mutations.

In contrast, Tkeshelashvili et al. (1991) demonstrated that Cu(I) and/or Cu(II) ions induced C→T transitions caused by oxygen free radicals and G→T transversions mediated by binding of copper ions to the N7G in 6X174 am3 DNA. A different result was observed with FeSO_4-induced base substitutions in the same supF gene of E.coli (Akasaka and Yamamoto, 1995). The mutation spectra induced by Fe(II) ion-generated oxygen free radicals suggested that 8-hydroxydeoxyguanosine (8-OHdG) is responsible for G:C→T:A transversions, whereas neither 8-OHdG nor 2,6-diamino-4-hydroxy-5-formamidopyrimidine can be the lesions responsible for G:C→C:G transversions. However, as the premutagenic lesions are not clearly documented, further research and development in this area is required. Finally, one site of base substitutions, 133, was found to be a warm-spot which was a part of the anticodon in the supF RNA gene, as four mutations were recovered. In addition, the observed mutational events were single base changes with one exception, which was a tandem double base substitution found at site 108–113 (CC19, Table III).

### Frameshifts

The sites and sequence contexts of frameshift mutations are presented in Table IV. Sequences with a run of a few identical residues were found at four of five sites (70–72, 102–105, 115–116 and 172–176) and mutations at these sites involved deletion or addition of 1 bp. One of these sites, 70–72, could be a warm-spot, as four mutations were recovered. Since the site was not a hot-spot for spontaneous mutations (Akasaka et al., 1992), it probably reflected specific interaction of CoCl_2 with the site. On estimating slippage events which were triggered by damaged bases using each of those sequence contexts, eight of 10 (80%) frameshifts occurred at G:C sites, while the remainder were found at A:T sites. These results indicate that the N7G–Co(II) adduct is the lesion responsible for inducing these mutations. Furthermore, this phenomenon also seems to support our previous study (Ogawa et al., 1986, 1988), where CoCl_2 induced specific frameshift mutations in the Ames Salmonella tester strains TA1537 and TA2637, but not in strains TA98 and TA100. Specifically, the hisC3706 frameshift mutant had an additional G base in the -GGG- site of the hisC gene, where reverse mutations were detectable depending on the deletion of one G (Ames et al., 1973, 1975).

#### Insertion sequences

Five mutants, CC31, CC56, CC57, CC59 and CC64, were found to contain the sequences of IS insertion elements; the frequency was 2.5×10^{-4}×8\% = 2.0×10^{-7}. They may be of spontaneous origin, since IS elements are very common among spontaneous mutations (the frequency is 3.1×10^{-7}×25\% = 7.75×10^{-8}; Table I). Four of five were IS50R insertions and one was an IS186 insertion. IS50R and IS186 were inserted into sites 124–125 and 114–115, respectively. IS50R was probably derived from the Tn5 in strain DL16 (Akasaka et al., 1992; Berg and Howe, 1989).

We conclude that exposure of plasmid pUB3 DNA to CoCl_2 primarily induced deletions and frameshifts followed by base substitutions, although very weakly, in the supF gene of E.coli. Eight of 10 frameshifts and 18 of 19 base substitutions occurred in the supF gene of E.coli.
at G:C sites. Thus, N7G–Co(II) adds contributed to 40% (26 of 65) of CoCl₂ mutagenesis. Slippage–misalignment was the suggested mechanism for generation of deletions, including CoCl₂-induced frameshifts. Furthermore, use of the SY1032/pKY241 system may provide an easy approach to DNA sequence analysis of metal ion mutagenesis in E.coli.

Acknowledgements

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