Highly Frequent Single Amino Acid Substitution in Mammalian Elongation Factor 2 (EF-2) Results in Expression of Resistance to EF-2-ADP-ribosylating Toxins*

Kenji Kohno and Tsuyoshi Uchida

From the Department of Cell Biology, National Institute for Basic Biology, Okazaki, Aichi 444, Japan and the Institute of Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan

Toxin-resistant polypeptide chain elongation factor 2 cDNA has been cloned from a mutant hamster cell line with only non-ADP-ribosylatable elongation factor 2. The mutation conferring resistance to diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A is a G-to-A transition in the first nucleotide of codon 717. Codon 715 encodes a histidine residue that is modified post-translationally to diphthamide, which is the target amino acid for ADP-ribosylation by both toxins. Transfection of mouse L cells with a recombinant elongation factor 2 cDNA differing from the wild-type only by this G-to-A transition confers resistance to *P. aeruginosa* exotoxin A. The degrees of toxin-resistant protein synthesis of stable transfectants are dependent on the ratio of non-ADP-ribosylated elongation factor 2 to wild-type elongation factor 2, not the amount of non-ADP-ribosylated elongation factor 2. The mutation creates a new MboII restriction site in the elongation factor 2 gene. Several independently isolated diphtheria toxin-resistant Chinese hamster ovary cell lines show the same alteration in the MboII restriction pattern.

Eukaryotic polypeptide chain elongation factor 2 (EF-2) is essential for protein synthesis in all eukaryotes. EF-2 catalyzes the translocation of peptidyl tRNA from the ribosome A site to the P site during peptide chain elongation (1, 2). It is a single polypeptide whose molecular weight lies in the range from 93,000 and 110,000 in most preparations (3-6). EF-2 from eukaryotes has one unique amino acid named diphthamide, 2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine, which is a post-translationally modified histidine residue (7, 8). Diphthamide has not been found in any eukaryotic proteins examined except EF-2, but archaeobacterial elongation factor also contains a diphthamide residue that can be ADP-ribosylated by fragment A of diphtheria toxin (DT) (9, 10). EF-2 can be specifically inactivated by fragment A of DT or *Pseudomonas aeruginosa* exotoxin A (PA), enzymes which catalyze the transfer of the ADP-ribose moiety of NAD+ to the N-1 nitrogen of the histidine imidazole ring of the diphthamide residue, and thereby inhibit protein synthesis (11-13). The diphthamide residue in EF-2 is essential for ADP-ribosylation by DT and PA, but its physiological role is unknown. The presence of a single molecule of fragment A of DT in the cytosol is sufficient to kill the cell (14). The enzymic activity of DT is essential for its lethal activity, because CRM197 lacking only the enzymic activity by missense mutation has no toxicity to susceptible cells and animals (15). The two toxins have the same enzymatic activity as NAD:EF-2-ADP-ribose transferase, but the sensitivities of different animal species to the two toxins differ greatly. Humans, monkeys, and hamsters are sensitive to DT, whereas mice and rats are insensitive to DT, but very sensitive to PA. All the EF-2 prepared from these animal cells can be ADP-ribosylated by DT fragment A or PA in the presence of NAD in cell-free system.

To obtain information on the structure and function of EF-2, the physiological roles of diphthamide, and ADP-ribosylation, DT-resistant cells have been isolated and characterized (16-20, 22-24). Among some of them, translational mutant contains EF-2 that cannot be ADP-ribosylated and is cross-resistant to PA. The mutation was found to be a codominant trait by cell hybridization analysis (17, 19, 24).

To determine the molecular basis of the alterations in the structure of EF-2 that render it resistant to ADP-riboseylation, we decided to isolate and sequence wild-type and mutant EF-2 cDNA. Recently, we constructed a plasmid, pHEW1, containing the complete hamster EF-2 cDNA and determined the primary structure of EF-2 deduced from the nucleotide sequence (26). Hamster EF-2 contains 857 amino acids and has a Mr of 96,192 without post-translational modification. The amino acid sequences near histidine residue 715 are well conserved in rat, bovine, yeast, and wheat germ EF-2 (27), and are also conserved in hamster EF-2. Thus the region around diphthamide seems to be important for the function of EF-2. In this work, we cloned EF-2 cDNA from DT-resistant hamster cells and identified its mutation conferring resistance to DT and PA by transient and long-term expression assay with a recombinant EF-2 cDNA. Furthermore, by restriction fragment length polymorphism (RFLP) we detected a “hot spot” in the EF-2 molecule for causing resistance to toxins.

* This work was supported in part by grants from the Ministry of Science, Education, and Culture of Japan, Toray Science Foundation, the Agricultural Chemical Research Foundation, and the Foundation for Promotion of Cancer Research backed by Japan Shipbuilding Industry Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1. Present address: Dept. of Biochemistry, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235.

2. To whom correspondence should be addressed: Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan.

3. The abbreviations used are: EF-2, elongation factor 2; DT, diphtheria toxin; PA, *Pseudomonas aeruginosa* exotoxin A; RFLP, restriction fragment length polymorphism; CHO, Chinese hamster ovary; MEM, minimal essential medium; SDS, sodium dodecyl sulfate; bp, base pair(s); kb, kilobase pair(s); EMS, ethyl methane sulfonate.
**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Chinese hamster ovary cells, CHO-K1 (Pro7), were obtained from the American Type Culture Collection. Cells (22) were cultured for glycine, adenosine, and thymidine, and transformed cells were provided by Dr. L. Simovich (University of Toronto) via Dr. T. Sudo (Kyoto University). Mouse L cells deficient in thymidine kinase activity (bromodeoxyuridine-resistant) were provided by Y. Okada (Institute for Molecular and Cellular Biology, Osaka University). CHO-K1 cells and L cells were routinely maintained in α-MEM without nucleosides, and CHO Gat− cells were maintained in α-MEM with nucleosides, supplemented with 8% fetal calf serum. KE1, KE1, KN2, and KN7 cells were isolated previously as DT- and PA-resistant cells from CHO-K1 cells (24, and GE) cells were also established as toxin-resistant cells from Gat− cells.2

**Cloning and Expression**—A cDNA library from KE1 cells, which are fully resistant to DT, was constructed by the method of Okayama and Berg (28) using a pcD vector that permits the expression of cDNA in mammalian cells, as described previously (26). Transformed Esherichia coli HB101 clones were replicated on nitrocellulose filters (HATF, Millipore). After baking, the filters were washed with 3 × SSC containing 0.1% SDS at 60 °C, and were prehybridized for 3 h at 60 °C in 5 × SSC, 10 × Denhardt's solution, 50 μg/ml denatured herring sperm DNA. The solution was then replaced by hybridization buffer consisting of 6 × SSC, 10 × Denhardt's solution, 50 μg/ml denatured herring sperm DNA. The filters were hybridized for 15 h at 60 °C and then washed in 6 × SSC, 0.1% SDS at 60 °C for 30 min. By screening of about 70,000 transformants, 55 clones were obtained that hybridized with two probes prepared from the rat phosphatid PRE2 (the 5′-76 bp RsaI fragment and the 161-bp ClaI-AuAII fragment) as described before (28). Of the 55 clones, the two carrying the largest inserts, named pD45 and pDS1, were isolated and analyzed. Appropriate restriction fragments were inserted into M13mp18 and M13mp19 vectors, and the DNA was sequenced by a slight modification of the dyeodeoxy chain termination method (29, 30).

**Transient Expression Assay**—Inocula of 8 × 10⁶ mouse L cells in 35-mm diameter plastic dishes were incubated for 24 h, and then appropriate DNA was coprecipitated with calcium phosphate (1.7 μg/35-mm dish) (31). After 24 h, DNA was removed from the cells and the medium was replaced by growth medium (α-MEM + 8% fetal calf serum). After additional incubation for 24 h, the medium was changed to selection medium containing 0.1 μg/ml PA (day 0). Usually after 48 h, the medium was replaced by Ham's F-12 medium containing 10% fetal calf serum and 2 μCi/ml [3H]leucine. The cultures were incubated for 2 h, the medium was removed, and the cells were washed with saline and treated with 0.5 ml of 0.1 N NaOH. The lysates were treated with trichloroacetic acid, acid-insoluble materials were subjected to electrophoresis in 1% agarose formaldehyde gel, transblotted to a nitrocellulose filter, and then treated with trichloroacetic acid, acid-insoluble fraction was measured. Protein synthesis was expressed as a percentage of the value in control cultures without toxin.

**Nothern Blot Analysis**—Total RNA was isolated from exponentially growing cells by their homogenization with 4 ml guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion. Poly(A)+ RNA was purified once by oligo(dT)-cellulose chromatography (P-L Biochemicals, Type 7). Samples (RNA) were treated with trichloroacetic acid, acid-insoluble materials were counted in a liquid scintillation counter.

**RESULTS**

**Cloning of Mutant EF-2 cDNA from Toxin-resistant Cells Carrying Non-ADP-ribosylatable EF-2**—A cDNA library derived from a DT-resistant CHO-K1 cell line (KE1) was prepared in the expression vector pcD (28). KE1 cells showed full resistance to DT and PA and produced only non-ribosylatable EF-2 (24). About 70,000 transformants were screened by colony hybridization with two probes prepared from the rat EF-2 cDNA as described under "Experimental Procedures." Of the 55 clones hybridized with these two cDNAs, 16 were subjected to ADP-ribosylation by using a high molecular weight hamster genomic DNA from wild-type and DT-resistant CHO cells. These DNAs were separated on agarose gel and transferred to a Zeta-Probe blotting membrane (Bio-Rad). Probes were labeled by a multiprime DNA labeling system (Amersham Corp., I-2 × 10⁶ cpm/μg) and blots were hybridized at 65 °C for 22 h in 10 × Denhardt's solution, 5 × SSC, 50 mM sodium phosphate (pH 7), 1% SDS, and 500 μCi/ml denatured herring sperm DNA. The filter was washed twice with 3 × SSC, 0.5% SDS at room temperature for 10 min, and then washed with 0.1 × SSC, 0.1% sodium pyrophosphate, and 1% SDS at 65 °C for 5 min.

Two-dimensional Gel Analysis—Inocula of 10⁶ mouse L cells or cells of each transfectant were seeded into 10 ml of α-MEM supplemented with 8% fetal calf serum in 90-mm diameter plastic dishes. After incubation for 12 h, the medium was replaced by Eagle's MEM (methionine-free) containing 50 μCi/ml [35S]methionine (1430 Ci/mmole, Amersham Corp.) and the cells were incubated for 4 h. Cell extracts were prepared as described previously without dialysis (24). The standard reaction mixture for ADP-ribosylation of EF-2 contained about 50 μg of protein of cell extract (about 2 × 10⁶ cpm), 6.9 mM dithiothreitol, 5.6 mM thymidine, 0.4% (v/v) n-acetylglucoside, and 75 μM NAD in a total volume of 26.5 μl. Reaction mixtures were incubated at 57 °C for 15 min in the presence or absence of fragment A or C of DT (22 μg). The reaction was stopped by addition of 30 μl of a Laetolyzed buffer and 35 μg of urea, and 40-μl samples were applied to gel (33). Separation in the first dimension was achieved by isoelectric focusing in gel containing 3.5% acrylamide, 0.19% bisacrylamide, 8% urea, 2% Nonidet P-40, and 2% Ampholines (1.6%, pH 3.5-10, 0.4%, pH 7–9), first at 400 V for 12 h and then at 800 V for 1 h. Separation in the second dimension was achieved in a vertical slab of sodium dodecyl sulfate-polyacrylamide gel as described previously (24). The gels were fixed and dried and then exposed to x-ray film at −80 °C for 4 days with an intensifying screen.

**FIG. 1. Schematic representation of hamster EF-2 cDNA from wild-type CHO-K1 and toxin-resistant KE1 cells.** Nucleotide residues are numbered from the beginning of the initiation codon as reported previously (26). The numbers at each restriction site in the hamster cDNA indicate which 5′-terminal nucleotide is generated by cleavage. The open box corresponds to the coding region of hamster EF-2 cDNA. GC and AT indicate a G-C stretch and poly(dA)-poly(dT) tract, respectively, which are connected with the pcD vector DNA sequence. The terminal site of the G-C stretch indicated by the arrow is redefined by Parm (28). The open triangle denotes the start position of nucleotide number of cDNA insert. ADP-R shows the site specifically ADP-ribosylated by DT and PA. M1 in pD61 and M2 in pD46, indicated by arrowheads, show the single point mutational sites in the coding region of EF-2 cDNA. M1 mutation is a G-to-A transition in the first nucleotide of codon 717, leading to amino acid substitution of arginine for glycine. M2 is a C-to-T transition in the second nucleotide of codon 207, leading to substitution of leucine for proline.

---

of these PstI-Sau3A fragments were the same as that of the wild-type pHEW1 sequence.

Expression of Toxin-resistant EF-2 cDNA in Mammalian Cells—To ascertain whether these clones express the resistance to DT and PA, we constructed plasmids in which the 940-960-bp segments from the ClaI to Sau3A site of the two clones were recombined with that of wild-type pHEW1 containing the 5' region of EF-2 cDNA (Fig. 2) to make full-length cDNA. When these recombinants, named pWD46 and pWD51, were transfected into mouse L cells by the conventional calcium phosphate method (31, 34), cells transfected with pWD51 DNA showed protein synthesis in the presence of PA, whereas those transfected with pWD46 did not (Table I). This result shows that pWD51 contains the nucleotide sequence conferring resistance to PA in the EF-2 cDNA coding region, which is limited to the downstream region from the Sau3A restriction site. Next we examined the sequences of the 529-bp fragments between the BglII and Sau3A restriction sites, which contain the coding region of diphthamide. Com-

Table I

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Incorporation of (^{3}H)leucine (cpm/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHEW1 (wild-type)</td>
<td>380</td>
</tr>
<tr>
<td>pHD1</td>
<td>7630</td>
</tr>
<tr>
<td>pD51</td>
<td>120</td>
</tr>
<tr>
<td>pWD51</td>
<td>8520</td>
</tr>
<tr>
<td>pD46</td>
<td>140</td>
</tr>
<tr>
<td>pWD46</td>
<td>150</td>
</tr>
<tr>
<td>Herring sperm</td>
<td>130</td>
</tr>
</tbody>
</table>

Transient expression of toxin resistance using recombinant plasmids

The details of plasmid constructions are described in the text. Protein syntheses of mouse L cells transfected with each plasmid and herring sperm DNA were measured as described under "Experimental Procedures." In this experiment, the DNA concentration used was 1.7 μg/0.5-mm culture dish without carrier DNA. The cells were labeled with \(^{3}H\)leucine after incubation for 2 days.

Fig. 3. Transient and long-term expression of hamster EF-2 cDNA. A, transient expression of hamster EF-2 cDNA in mouse L cells. Protein syntheses of mouse L cells transfected with wild-type EF-2 cDNA (pHEW1, ○), toxin-resistant EF-2 cDNA (pHEW1, •), and denatured herring sperm DNA (△) as a negative control were measured as described under "Experimental Procedures." Day 0 indicates the time when the medium was changed to selection medium containing 0.1 μg/ml PA. B, dose-response curves for transfection by hamster EF-2 cDNA. The DNA concentrations used were 0.17, 0.34, 0.85, 1.7, and 3.4 μg/0.5-mm culture dish without carrier DNA. In this experiment, the cells were labeled with \(^{3}H\)leucine after incubation for 3 days in the presence of 0.1 μg/0.5 ml PA; pHEW1, C; pHEW1, •; herring sperm DNA, △. C, long-term expression of the DT-resistant character. Inocula of 5 × 10⁴ L cells in 90-mm diameter plastic dishes were incubated for 24 h. Cells were then transfected with 10 μg of pHEW1 or pHD1 plasmid DNA coprecipitated with calcium phosphate. After adsorption of DNA for 24 h, DNA precipitates were removed. The cells were fixed with 10% formalin after incubation for 20 days in the presence of 50 μg/ml PA, and then stained with 0.1% crystal violet.
Cloning and Expression of Toxin-resistant EF-2 cDNA

Fig. 4. Effects of PA on cellular protein synthesis. Relative protein syntheses of seven stable transfectants treated with PA were measured as described under "Experimental Procedures." This experiment was done using transfectants within 1 month after cloning to avoid alteration in their toxin sensitivity.

Fig. 5. Expression of toxin-resistant EF-2 derived from pHED1. A, Northern blot analysis. Total cellular RNAs (30 μg) isolated from CHO-K1 cells, L cells, and seven transfectants (LpD53, LpD59, LpD10, LpD12, LpD11, LpD55, and LpD54) were analyzed with a 520-bp fragment from BglII to SmaI of hamster EF-2 cDNA as a probe. Hybridization conditions were as described under "Experimental Procedures." After hybridization, filters were washed with 0.1 × SSC, 0.1% SDS at 50°C. After exposures to x-ray film, the bands corresponding to EF-2 mRNA were cut out and their radioactivities were counted in a liquid scintillation counter. The mRNA ratio represents the radioactivity corresponding to EF-2 mRNA relative to that of hamster EF-2 mRNA in CHO-K1 cells. The amount of mRNA in each transfectant derived from pHED1 (DT-resistant EF-2 mRNA) was calculated by subtracting the amount of mRNA of L cells from that of each transfectant. The total amount of EF-2 mRNA in each transfectant was calculated as the amount of DT-resistant EF-2 mRNA plus that of CHO EF-2 mRNA which was assumed to be equal to that of mouse EF-2 mRNA. The ratio of the amount of DT-resistant EF-2 mRNA to the total EF-2 mRNA in each transfectant is shown. Protein synthesis in the presence of 0.1 μg/ml PA is shown as a percentage of that in control cultures without PA. B, detection of toxin-resistant EF-2 by twodimensional gel analysis. Extracts of LpD55, LpD10, and LpD11 cells were prepared and ADP-ribosylation of EF-2 was carried out at 37°C for 15 min in the absence (top) or presence (bottom) of fragment A (22 μg) of DT. Arrows 1 and 2 indicate EF-2 and ADP-ribosylated EF-2, respectively. Arrow 1′ shows nonribosylatable EF-2 (DT-resistant hamster EF-2). The intensities of the spots corresponding to 1′ and 2 were measured by microdensitometry. IEF, isoelectric focusing.
products were separated on blot analysis of CHO-K1 DNA. Hamster CHO-K1 DNA was com-

Toxin-resistant Protein Gene

![Fig. 6. Southern and Northern blot analyses. A, Southern blot analysis of CHO-K1 DNA. Hamster CHO-K1 DNA was com-
pletely digested with BamHI, EcoRI, KpnI, and HindIII, and the products were separated on 0.7% agarose gel. DNAs were transferred to a Zeta-Probe blotting membrane and hybridized with hamster whole EF-2 cDNA as a probe. Hybridization and washing conditions were as described under "Experimental Procedures." The closed arrowsheads denotes the bands hybridized with whole EF-2 cDNA and the 247-bp HaeII fragment (~48-+199). The open arrowsheads show the bands hybridized with only whole EF-2 cDNA. B, Northern blot analysis of CHO-K1 and KEE1 cells. Total RNA (20 μg) and poly(A)+ RNA (10 μg) were separated on 1% agarose gel and transferred to a nitrocellulose filter. The filter was hybridized with the 520-bp BglII-Smal fragment from pHED1 which was nick-translated to a specific activity of 1.5 × 10^6 cpm/μg DNA as described under "Experimental Procedures." After hybridization, the filter was washed with 0.1 × SSC, 0.1% SDS at 50 °C, and exposed to x-ray film at −80 °C for 3 h with an intensifying screen.

acid-precipitable material was 2300 cpm per plate for cells transfected with the plasmid containing the mutant EF-2 cDNA sequence versus 290 cpm per plate for cells transfected with the wild-type sequence. These results indicate that pHED1 encodes EF-2 cDNA which is resistant to both DT and PA.

On the other hand, cells transfected with the recombinant plasmid pWD46 did not show any protein synthesis in the presence of PA (Table I). Since cells transfected with pHEW1 showed slight incorporation of [3H]leucine in the presence of PA, as mentioned above, pD46 contains a nucleotide sequence that causes loss of the function of wild-type EF-2 as a trans-

case, such as, for example, a nonsense mutation. Thus we compared the nucleotide sequence of pD46 with that of wild-
type pHEW1. Only 1 base substitution in the coding region of pD46 was detected, as shown in Fig. 1. This point mutation is a C-to-T transition in the second nucleotide of codon 207, and caused amino acid substitution of leucine for proline (Fig. 1). We reported previously that ADP-ribosylatable EF-2 was not detected in KEE1 cells by two-dimensional gel electrophoresis following the cell extracts treated with [32P]NAD and fragment A of DT (24). This amino acid substitution may result in nonfunctional and non-ADP-ribosylatable EF-2 or very rapid degradation of EF-2 in cells.

Relationships between Ratio of Non-ADP-ribosylated EF-2 to Wild-type EF-2 and the Degrees of Toxin-resistant Protein Synthesis of Stable Transfectants—When mouse L cells transfected with pHED1 were incubated for 20 days in the presence of 50 μg/ml PA, resistant colonies appeared. There are various sizes of the colonies in the plates (Fig. 3C). On the other hand, in cells transfected with pHEW1, no colonies were formed in the presence of toxin (Fig. 3C). Seven PA-resistant colonies from L cells transfected with pHED1 were selected at random for further analysis.

We first studied the protein in synthesis of each transfectant in the presence of PA, using logarithmically growing cells treated with various concentrations of PA for 24 h. Wild-type L cells did not incorporate [3H]leucine into macromolecules in the presence of 0.01 μg/ml PA, whereas LpD53 and LpD59 cells showed 65% of the protein synthesis observed in the absence of PA. Similar resistance to toxin was seen at wide range doses of PA (Fig. 4). Similar results were obtained with other transfecants, even when they showed different degrees of resistance to PA. These results well reflected those obtained by analyses of mutant cells, which were classified according to their toxin resistance at the level of EF-2 (17, 19, 24).

Fig. 5A shows the results of Northern blot analysis of these transfecants and of wild-type CHO cells and L cells. Although the EF-2 mRNAs from the endogenous mouse and hamster genes and the introduced cDNA are all about 3 kb, hamster EF-2 mRNA can be distinguished from mouse EF-2 mRNA under highly stringent conditions, as described under "Experimental Procedures." Transfectant LpD53, which expressed the mutant EF-2 cDNA in pHED1, produced about twice as much hamster EF-2 mRNA as that produced by wild-type hamster cells (Fig. 5A). It was expected that if a sufficient amount of mRNA for non-ADP-ribosylatable EF-2 were transcribed, protein synthesis of transfectants would not be decreased by toxin. However, protein synthesis in the presence of toxin was only about 65% of that in the absence of toxin (Fig. 5A). Similar results were obtained in analyses of six other transfecants. In these transfecants, incorporation of [3H]leucine in the presence of PA depended on the ratio of their productions of toxin-resistant hamster EF-2 mRNA to that of total EF-2 mRNA. We interpreted this result as indicat-
ing that ADP-ribosylated EF-2 can compete with non-ribo-
sylatable EF-2 for binding to ribosomes and GTP under physiological condition in the presence of toxin. Thus the ratio of non-ADP-ribosylated EF-2 to total EF-2, not the amount of non-ADP-ribosylated EF-2, controls the rate of protein synthesis (see "Discussion").

We confirmed that the proportion of non-ADP-ribosylata-
ble EF-2 reflected the estimated ratios of mutant to total EF-
2 mRNA. Mouse and hamster EF-2s are electrophoretically identical, but ADP-ribosylated EF-2 can be separated from non-ADP-ribosylated EF-2 by two-dimensional gel electrophoresis (24, 37). As shown in Fig. 5B, after treatment with DT fragment A, all the EF-2 in control L cells can be ADP-
ribosylated, with an acidic shift of its isoelectric point. By contrast, after treatment with DT fragment A, EF-2 in the transfecants is separated into two spots corresponding to recipient mouse EF-2 (ADP-ribosylatable) and mutant ham-
ster EF-2 (non-ADP-ribosylatable) (Fig. 5B). The intensities of the spots were measured by microdensitometry. The ratios of non-ADP-ribosylatable to total EF-2 were 51, 40, and 31% for LpD53, LpD10, and LpD11, respectively, which agree fairly well with the percentages of mutant EF-2 mRNA and leucine incorporations in the presence of toxin (Figs. 4 and 5). This fact suggests that ADP-ribosylated EF-2 molecules temporarily bind to ribosomes and thus compete with non-
ADP-ribosylated EF-2 molecules.

Characterization of Toxin-resistant Cells at the EF-2 Gene Locus—Biochemical analyses using somatic cell mutants have indicated that the EF-2 gene in CHO cells is a single copy gene and that there are two functional copies of the gene per cell. For examination of whether the EF-2 genes in CHO-K1 cells is a single copy per haploid, high molecular genomic DNA was prepared from CHO-K1 cells and digested com-
pletely with several restriction endonucleases. Fig. 6A shows a HindIII digest. When the same filter was probed with the DNA digested with BamHI or EcoRI gave one major band and two or three faint bands. The major bands obtained with BamHI or EcoRI were about 8.7 and 23 kb, respectively, whereas the faint bands obtained with these enzymes were about 23.5 and 9 kb, respectively. The dense major bands obtained with BamHI or EcoRI were about 23.5 and 9 kb, whereas the faint minor bands were about 21 and 6.4 kb in the KpnI digest and about 5, 2.3, and 1.6 kb in the HindIII digest. When the same filter was probed with the 247-bp HaeII fragment (−48 to +199), which is located in the 5′ region of EF-2 cDNA, only the major bands were detected.

The same result was obtained by rehybridization analysis with the 154-bp PvuII (+350)−PstI (+504) fragment (data not shown), which is located in the GTP binding domain. Since EF-2 cDNA has no site for BamHI, EcoRI, or KpnI and has a single site for HindIII, these results indicate that CHO-K1 cells have a single copy gene for EF-2 per haploid genome and show that the EF-2-like gene that cross-hybridizes with the hamster EF-2 cDNA, only the major bands were detected.

As a G-to-A transition mutation in codon 717 creates a new MboII site. To determine whether a G-to-A transition in codon 717 is present in other independently isolated toxin-resistant mutants, we examined these mutants for the presence of a novel restriction fragment of the EF-2 gene produced by the transition. Probing of total genomic DNA from CHO-K1 cells completely digested with MboII with a 160-bp HinfI fragment from genomic EF-2 (see legend to Fig. 7A) generates a single fragment of 1297 bp (Fig. 7B). The mutant allele gives rise to two fragments of 1042 and 255 bp. We previously isolated and characterized several DT-resistant CHO cell lines that are EF-2 structural gene mutants: KE1, KN2, KN7, and KE1 from CHO-K1 cells (24) and GE1 from CHO Gat− cells. KE1 and GE1 were selected after EMS mutagenesis, whereas KN2 and KN7 were selected after N-methyl-N'-nitro-N-nitrosoguanidine treatment. Wild-type CHO-K1 and CHO Gat− gave a single band of about 1300 bp, but all the mutants gave a new 255-bp band derived from the mutated allele and the normal 1297 bp (Fig. 7B). All four independently isolated CHO DT-resistant cells showed the same RFLP. Consequently, the site presented here is a hot spot for causing resistance of hamster EF-2 molecules to DT.

**DISCUSSION**

We showed that the mutation conferring resistance to DT and PA is a G-to-A transition in the first nucleotide of codon 717. This transition mutation results in the substitution of arginine for glycine at a position 2 residues to the carboxyl-terminal side of diphthamide (Fig. 2). The same transition mutation frequently occurred in the hamster EF-2 gene locus. As a G-to-A transition mutation in codon 717 creates a new MboII restriction site (Fig. 2), we could examine the MboII RFLP of four independently isolated DT-resistant cells. Un-
expectedly, all four mutants showed the same MboII RFLP pattern as KEE1 cells (Fig. 7B), and thus they contain the same mutation in one allele of the EF-2 gene. For survival of cells in the presence of toxin, EF-2 must be non-ADP-ribosylatable, but must also function in protein synthesis. It is difficult to meet these two conditions simultaneously, so the potential sites in the EF-2 structural gene for mutation to DT and PA must be restricted. The mutation we describe here appears to be a hot spot for such resistance. The 5-amino acid sequence from Asp-712 to Arg-716 in ADP-ribosylated peptide of EF-2, which contains diphthamide (715), is perfectly conserved in four eukaryotes (8, 27, 35), and this sequence is also present in hamster EF-2 (26). Furthermore, ribosome-bound EF-2 did not show ADP-ribosylation catalyzed by DT (36). Probably the region around diphthamide is associated with ribosomes and is essential for the recognition of ribosomes.

Why could we not find the mutation of the His-715 residue? One would predict that the replacement of His-715 by another amino acid in EF-2 would result in toxin resistance, since another residue might not be ADP-ribosylated by toxins since it would not be modified post-translationally. One possible explanation is that the His-715 residue in EF-2 is essential in EF-2 for recognition of the appropriate ribosomal site for translocation during the polypeptide elongation step. Although the mutated EF-2 with a substitution for His-715 was not recognized by toxins, it might lose the function of EF-2. This possible explanation can be tested by the expression assay in our system using mutant EF-2 cDNA prepared by site-directed mutagenesis. It is not clear why substitution of arginine for Gly-717 makes EF-2 resistant to ADP-ribosylation by DT or PA. This substitution blocks the interaction of EF-2 with the enzymatic fragments of the toxins or prevents modification of His-715 to diphthamide, thus indirectly blocking ADP-ribosylation, or it has both effects.

ADP-ribosylated EF-2 can apparently compete with toxin-resistant EF-2 in physiological conditions in the presence of toxin. The ratios of EF-2 mRNA to total RNA in L cells and CHO cells are considered to be very similar. Assuming that the amount of hybridization of the hamster EF-2 probe to mouse EF-2 mRNA is the same in all transfectants as in untransfected cells, we can estimate the ratio of mutant hamster to recipient mouse (wild-type) mRNA (see legend to Fig. 5A). The percentage of total mutated EF-2 mRNA that encodes non-ribosylatable EF-2 is closely correlated with the proportion of protein synthesis resistant to treatment with a wide range of concentrations of PA (0.01–1 µg/ml) for 20 h, despite differences in the total amount of EF-2 message (Figs. 4 and 5A). This estimation is based on the following two conditions. One is that stable transfectants produce as much native mouse EF-2 as untransfected L cells, and the other is that mutated EF-2 shows the same activity as wild-type EF-2 as a translocase. The former condition is confirmed by ADP-ribosylation assay using extracts from transfectants (data not shown). Two-dimensional gel analysis also showed that transfectants and untransfected mouse L cells produced similar amounts of native mouse EF-2 (toxin-sensitive EF-2), measured as the densities of spots of EF-2 and other proteins produced in exponentially growing cells (Fig. 5B and data not shown). The second condition has not been confirmed, but mutant EF-2 may show normal activity as a translocase since KEE1 cells showed no growth retardation in the presence of toxin (24). Although ADP-ribosylated EF-2 cannot synthesize polypeptide, it can bind to ribosomes, GTP, and GDP, and interferes with the function of native EF-2 in a cell-free system (21, 38, 39) not so effectively as observed here in living cells.

KEE1 cells, which showed full resistance to DT and PA and produced only non-ribosylatable EF-2, were isolated from KE1 cells by two-step mutation. KE1 cells isolated by a single step mutation maintained about 50% of the normal level of cellular protein synthesis in the presence of DT or PA and contained equivalent amounts of ADP-ribosylatable and non-ribosylatable EF-2 molecules (24). Southern blot analysis of genomic DNA from these clones showed that KEE1 and KEE1 cells contained the same mutation in one allele (Fig. 7B). This result indicates that single point mutations in codon 717 in both cells were caused by the first EMS treatment, and that this mutation conferred resistance to DT and PA. The second mutation in KEE1 cells by EMS treatment was introduced in codon 207 of the other allele (Fig. 1), and this mutation conferred KEE1 cells with full toxin resistance. Transient expression assay using pWD46 showed that mutation in codon 207 resulted in loss of the native activity of EF-2 as a translocase (Table I). This result indicates that EF-2 synthesized by only one allele may be sufficient for normal protein synthesis without causing retardation of cell growth, since KEE1 cells grew normally with or without toxin. The reason why a type of toxin-resistant cell, represented by KEE1 cells, showed 50% decrease of protein synthesis in the presence of toxin (19, 20, 22, 24) may be that ADP-ribosylatable EF-2 synthesized from the wild-type allele interfered with the function of non-ribosylatable EF-2 in the presence of toxin although sufficient non-ribosylatable EF-2 was produced for protein synthesis. Further experiments are required to determine whether this explanation is correct.

The DT resistance produced by the missense mutation in EF-2 is expressed codominantly, and toxin-resistant EF-2 cDNA provides a good codominant transferable marker for selection in DNA transformation experiments. The cloned mutant cDNA may also be useful in studies on the structure and function of EF-2 and on somatic mutation of autosomal genes.

Acknowledgments—We thank Drs. Shigetada Nakanaishi and Yoshio Okada for valuable discussion and encouragement during this work. We also thank Drs. Hiroaki Okkubo and Ryoichiro Kageyama for helpful advice and discussion, Dr. Hiroto Ota for providing the vector plasmids, Dr. Saeko Mizusawa for information of DNA sequencing method and for providing deoxy-7-deazaguanosine triphosphate, Dr. Shinya Ohnishi for providing Pa53, Dr. Tomohisa Inoue and Shinya Ohnishi for DNA sequencing analysis, Drs. Masahiro Iiura and Hiroshi Ohashi for cloning of hamster EF-2 gene, and Yukiko Arakawa and Yuki Imai for technical assistance.

REFERENCES
Cloning and Expression of Toxin-resistant EF-2 cDNA