Mutations in the Elongation Factor 2 Gene Which Confer Resistance to Diphtheria Toxin and Pseudomonas Exotoxin A

GENETIC AND BIOCHEMICAL ANALYSES*

(Received for publication, January 27, 1995, and in revised form, July 31, 1995)

Brian T. Foley‡, Joan M. Moehring§, and Thomas J. Moehring§

From the University of Vermont, Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics and §Vermont Cancer Center, Burlington, Vermont 05405

Both diphtheria toxin and Pseudomonas exotoxin A inhibit eukaryotic protein synthesis by ADP-ribosylating diphthamide, a posttranslationally modified histidine residue present in the elongation factor 2 (EF-2) protein. Elongation factor 2 cannot be ADP-ribosylated by the toxins unless this histidine is modified. In this report we identify three new point mutations in toxin-resistant alleles of the Chinese hamster ovary cell elongation factor 2 gene. The mutations resulted in amino acid substitutions at positions 584 (serine to glycine), 714 (isoleucine to asparagine), and 719 (glycine to aspartic acid). All three amino acid substitutions prevented the biosynthesis of diphthamide. The amount by which the toxins reduced protein synthesis in each of these mutant cell strains suggested that all three mutations also either impaired the function of EF-2 or reduced its steady state level in the cytoplasm. Western blot analysis also showed that equal amounts of EF-2 were present in each of the cell strains, indicating that the mutations impaired the catalytic function of EF-2.

Elongation factor 2 (EF-2) is responsible for the GTP hydrolysis-dependent translocation of eukaryotic and archaebacterial ribosomes during polysynthetic translocation (1). Its counterpart in eubacteria, mitochondria, and chloroplasts is elongation factor G (EF-G). The primary distinction between EF-2 and EF-G is the presence in EF-2 of a unique posttranslationally modified histidine residue 2-

© 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license.

The abbreviations used are: EF-2, elongation factor 2; EF-G, elongation factor G; DT, diphtheria toxin; PEA, Pseudomonas exotoxin A; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

† This work was supported by National Institutes of Health Grant AI 09100 and the Lucille P. Markey Charitable Trust. 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.

‡ Supported by a Cancer Biology Training Grant T32CA-09286 from the National Cancer Institute.

§ To whom correspondence should be addressed: 316 Stafford Hall, University of Vermont, Burlington, VT 05405. Tel.: 802-656-1117; Fax: 802-656-8749.

PEA and DT catalyze transfer of the ADP-ribose moiety of NAD+ to N1 of the histidine imidazole ring of diphthamide (5). Toxin-resistant mutants lacking enzyme activities required for diphthamide synthesis have been isolated and studied in detail in both yeast and mammalian cells (6–9) and show no phenotypic alterations other than resistance to DT and PEA. Several mutations in the EF-2 structural gene can also result in the production of toxin-resistant EF-2 (10–13). Mutant cell lines with mutations in the EF-2 gene can be distinguished from those with mutations in genes encoding enzymes required for diphthamide synthesis by somatic cell dominance hybridization and complementation analyses, dose-response assays with DT and PEA, and in vitro ADP-ribosylation of EF-2 by DT (6, 8, 14–16).

In the present study we screened toxin-resistant mutant strains derived from CHO-K1 cells to identify EF-2 gene mutations. We used restriction fragment length polymorphism (RFLP) analysis and DNA sequencing to characterize point mutations in the EF-2 gene that are responsible for toxin resistance. We determined if these mutations disrupted the biosynthesis of diphthamide. Expression of cloned mutant and wild-type EF-2 in wild-type CHO-K1 cells confirmed that each point mutation was sufficient to account for the toxin-resistant phenotype.

EXPERIMENTAL PROCEDURES

Cells, Media, and Culture Conditions—CHO-K1 (Pro *) Chinese hamster ovary cells were obtained from the American Type Culture Collection. A hypoxanthine phosphoribosyltransferase negative strain, P1R2, was selected from CHO-K1. The procedure for isolation of toxin-resistant mutant strains has been described (8). The mutant strains used in these studies were selected by exposure of mutagenized cells to DT, with the exception of PPE.10, PPE.11a, and K1W1.P17, which were selected by exposure to PEA. Cell strains RE1.22 which has one wild-type and one toxin-resistant EF-2 allele, and RE1.22c (derived from RE1.122) in which both EF-2 alleles are toxin-resistant have been previously characterized in detail (11, 16). Cells were maintained in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (Sigma) supplemented with 5% fetal bovine serum, hereafter referred to as growth medium, at 37°C in an atmosphere of 5% CO2 in air.

RFLP analysis for an Mboll-site indicative of a mutation in codon 717. Genomic DNA was harvested from 4 × 10⁶ cells. A polymerase chain reaction (PCR) was used to amplify exon 11. Primers were 5’-cctgggtgttccttctc-3’ (bases 4812–4828; numbered as in GenBank U17362). The PCR reactions contained 0.75 μM of each primer, 200 μM of each dNTP, 3 mM MgCl2, 50 μM KCl, 10 μM Tris-HCl, pH 8.3, 0.1 μM bovine serum albumin, 0.05% Triton X-100, 0.1 μg of genomic DNA template and 1 unit of Taq polymerase (Life Technologies, Inc.) in a 100-μl reaction volume, overlaid with 50 μl of sterile mineral oil. Reaction mixtures were cycled between 94°C for 30 s, and 72°C for 90 s, for a total of 25 cycles, followed by a 7-min incubation at 72°C and storage at 4°C. The PCR product generated from a wild-type allele contains no Mbol recognition sequence. The product derived from an allele containing a G to A transition in codon 584 was digested with MboII, generating a 316-base pair product that was separated on a 3.5% agarose gel and visualized with ethidium bromide.
Ten primers of 180 and 136 base pairs. Ten 717 contains one mRNA using SuperScript RNase H (Life Technologies, Inc.). The first strand of cDNA was produced from 50 ng of poly(A) prepared from total RNA using an oligo(dT) column (Life Technologies, Inc.). The first strand of cDNA was produced from 50 ng of poly(A) prepared from total RNA using an oligo(dT) column (Life Technologies, Inc.).

In Vitro ADP-ribosylation of EF-2—EF-2 was prepared and ADP-ribosylated in vitro with [14C]NAD as described (8) except that cells were lysed with a rapid freeze-thaw cycle, rather than by Dounce homogenization.

Labeling and Enzymatic Digestion of the Tryptic Peptide Containing the His-715 Residue—Metabolic labeling of EF-2 with [3H]histidine was done as described (17). Five, 10, 15, and 20 min of each cell strain were allowed to recover for 24 h and then selected in growth medium containing 600 units of hygromycin B/ml or hygromycin B/ml of each PCR mixture were extracted twice with chloroform to remove the mineral oil and precipitated with ethanol. The 2.6-kilobase pair PCR product, containing the complete coding region of the EF-2 cDNA, was purified by agarose gel electrophoresis, digested with BamHI and EcoRI and ligated into pBlueScript KS+ (Fig. 1). The plasmid was not dephosphorylated. Several independent reverse transcription PCR reactions and ligations were performed on RNA from each cell strain, so that if misincorporation(s) occurred during either reverse transcription or PCR, the same one would not be found in clones isolated from different reactions.

Sequencing—All sequencing was done using either single-stranded DNA from M13 clones or double-stranded plasmid DNA as template and Sequenase-modified T7 DNA polymerase (U. S. Biochemical Corp.) following the manufacturer’s standard protocol. Template plasmid DNA was prepared by alkaline lysis, followed by a diatomaceous earth-based clean-up protocol as described in (20). The sequence of the entire BamHI to EcoRI insert of clone pBS-22c-EF2-A2 was determined on both strands. The sequences of the region, including the BamHI to EcoRI sites were determined on both strands for clones derived from both wild-type and mutant cDNAs from cell strains R1.41 and K1.W1.P17. The Apal to Apal region was sequenced on both strands for clones derived from RPE.11a.

Construction of Domain-swapped Plasmids—Having determined by sequencing that the plasmid pBS-22c-EF2-A2 had a single point mutation in codon 717, this clone was then used for domain swapping. The BamHI to BamHI insert of clone pBS-22c-EF2-A2 was determined on both strands. The 2.6-kilobase pair PCR product, containing the complete coding region of the EF-2 cDNA, and one from the multiple cloning site of the mammalian cell expression vectors pCEP4 (Invitrogen) and pMSG (Pharmacia Biotech Inc.), which had been digested with NheI and XhoI. Plasmid pCEP4 contains a hygromycin phosphotransferase gene conferring resistance to hygromycin as a selectable marker and the cytomegalovirus immediate early enhancer/promoter as a promoter for expression of the cDNA insert. Plasmid pMSG carries the Escherichia coli gpt gene conferring resistance to hygromycin-amino- nipterin-thymidine medium when transfected into hypoxanthine phosphoribosyltransferase negative cell strain (21), and the mouse mammary tumor virus long terminal repeat as a promoter for expression of the cDNA insert.

Transfections—50 x 105 cells were plated in 60-mm tissue culture dishes and incubated 18 to 24 h prior to transfection. Cells were transfected with Transfectam (Promega) according to the manufacturer’s recommended protocol for use with medium without serum. Trial transfections with varying ratios of Transfectam to DNA proved that 7 µg of plasmid DNA used with 7 µl of Transfectam solution gave the best results. After transfection with pCEP4 or pMSG plasmid constructs the cells were allowed to recover for 24 h and then selected in growth medium containing 600 units of hygromycin B (Calbiochem)/ml or hygromycin-amino-nipterin-thymidine medium, respectively.
RESULTS

Dose-response curves were generated for each mutant cell strain using an intact cell assay (8). With this assay, in which incorporation of radiolabeled amino acids into protein is measured after exposing cells to a range of concentrations of DT or PEA, it is possible to classify toxin-resistant mutant strains into three general categories: (i) class I mutants are defective in some aspect of the entry of toxins into the cytosol, and protein synthesis is completely inhibited by high concentrations of toxin; (ii) class IIa mutants either lack enzymes needed to modify histidine 715 to diphthamide (MOD) or have mutations in both copies of the elongation factor 2 gene, and protein synthesis is unaffected by any concentration of toxin; (iii) class IIb mutants have mutations in one of the two copies of the elongation factor 2 gene, and protein synthesis is typically reduced to 47–56% of control, by high concentrations of toxin (8, 14, 22). In this study, 12 mutant cell strains (Table I) produced typical class IIb dose-response curves as illustrated in Fig. 2. Incorporation of amino acids into protein in the presence of high doses of DT was reduced to between 47.5 and 56% of toxin-free controls. In the case of the mutant cell strains RPE.11a, R1.41, and K1W1.P17, protein synthesis was reduced well below 50%, but was never reduced to less than 15% (Table I and Fig. 2). Wild-type CHO-K1, and class IIa RE1.22c cell strains, were included as controls. Thus, the mutant strains which are the focus of this study were confirmed to be class IIb mutants.

Each class IIb mutant was screened for the presence of an MboI restriction endonuclease recognition sequence (GAGGA) indicative of a G to A transition in codon 717 of the EF-2 gene (GAGGA → GAAGA) (11, 13). This mutation, which was originally reported in four out of four independent isolates of mutagenized CHO-K1 cells selected for toxin resistance (13), prevents the first step in the biosynthesis of diphthamide (11). Fig. 3 presents results from a representative PCR RFLP analysis. Ten of the 13 class IIb mutant cell strains analyzed possessed an MboI site indicative of a G to A transition in codon 717, and three did not. Wild-type CHO-K1, heterozygous RE1.22 and homozygous RE1.22c (11) were included as controls. The three strains which did not possess an MboI site, were the same three that showed significantly less than 50% protein synthesis in the intact cell assay. This suggested either that their toxin-resistant EF-2 was functionally impaired or that it was less stable than wild-type EF-2.

After determining that DT-resistant cell strains RPE.11a, R1.41, and K1W1.P17 did not have a G to A transition in codon 717, we identified the mutations in their EF-2 alleles responsible for resistance to DT. Because the histidine precursor of diphthamide is encoded by exon 11 of the EF-2 gene, we first sequenced exons 11–13 from each of the mutants. PCR amplification of this region of genomic DNA using primers which hybridize to the previously published cDNA sequence (25) proved problematic. The PCR product produced by such primers always produced a product that was shorter in length than would be predicted for the gene, but identical in length to the product predicted for cDNA. Pretreatment of the template genomic DNA with RNase did not eliminate the artifact. The shorter product is most likely derived from an EF-2 pseudogene, based on partial sequencing of the artifact band (data not shown). Use of primers complementary to sequences in intron
10 and exon 13 enabled us to successfully amplify the correct region of the EF-2 gene. The intron 10-exon 13 PCR products from RPE.11a, R1.41, and K1W1.P17 were ligated into M13mp18 and M13mp19 and single-stranded DNA from several clones of each was harvested and sequenced. Roughly half of the R1.41 clones contained a T to A transition in the second position of codon 584, resulting in a change from isoleucine to asparagine in the predicted protein, the other half were derived from the wild-type allele. Roughly half of the R1.41 clones contained a G to A transition in the second position of codon 584, resulting in a serine to glycine substitution in the predicted protein. No mutation was found in the intron 10-exon 13 clones derived from RPE.11a. We then cloned full-length cDNA from RPE.11a, in order to avoid sequencing introns. RPE.11a cDNA contained an A to G transition in the first position of codon 584, resulting in a serine to glycine substitution in the predicted protein.

Having demonstrated that RPE.11a, R1.41, and K1W1.P17 each possessed a unique mutation resulting in an amino acid change in EF-2, we analyzed the EF-2 protein from each cell strain to determine the effect of these mutations on the post-translational biosynthesis of diphthamide. The post-translational modification of histidine 715 to diphthamide proceeds in several steps and four intermediate forms have been identified. The intermediates are 2- (3-carboxy-3-aminopropyl) histidine and the unamidated 1-, 2-, and 3-methylammonio forms of diphthamide. Each form can be resolved chromatographically, all eluting before histidine with the buffer system used (7, 16).

Amino acid analysis of EF-2 tryptic peptides, that contained the histidine 715 residue, was carried out for each mutant. Peptides from ADP-ribosylated and nonribosylated EF-2 from CHO-K1 cells were included as controls. Because each mutant cell strain is heterozygous at the EF-2 locus and produces both toxin-sensitive and toxin-resistant EF-2, the EF-2 in cellular extracts was first ADP-ribosylated to completion with an excess of NAD$^+$ and DT to ADP-ribosylate all of the wild-type EF-2. This enabled us to determine by chromatographic analysis if the product of the mutant allele possessed diphthamide that could not be ADP-ribosylated by toxin. Amino acid analysis revealed that only ribosyl-diphthamide and histidine were recovered from the EF-2 extracted from each mutant. No intermediate forms in the diphthamide biosynthetic pathway were detected (Fig. 4). A small but significant amount of ADP-ribosylated diphthamide was detected in extracts of CHO-K1 cells that had not been exposed to toxin. This is in agreement with other studies, which have shown that a small percentage of EF-2 in normal cells is ADP-ribosylated by an endogenous enzyme (23), apparently in a cell cycle-dependent manner (24).

Because the tryptic peptide contains one other histidine residue in addition to histidine 715, and the procedure for preparing the tryptic peptide does not eliminate all other histidine-containing peptides, a large histidine peak was always detected. Neither the size of the ribosyl-diphthamide peak, nor the ratio of histidine to ribosyl-diphthamide was used to calculate the amount of EF-2 present in cell extracts. Thus, these analyses did not prove that the mutant EF-2 contained only an unmodified histidine 715. The possibility remained that the mutant EF-2 contained diphthamide which had been ADP-ribosylated, but was still able to function in protein synthesis. Analysis of the amount of [14C]NAD$^+$ incorporated into EF-2 by DT in cell extracts from each of the mutants eliminated this possibility. Mutant cell strains RPE.11a, K1W1.P17, and R1.41 each possessed half as much ADP-ribosylatable EF-2 per mg of cellular protein as did CHO-K1 (Fig. 5).

In cell strains RPE.11a, R1.41, and K1W1.P17, protein synthesis was reduced to 20.8, 27.3, and 24% of control, respectively, by high concentrations of diphtheria toxin (Table I). In order to address the issue of whether the mutant, toxin-resistant EF-2 produced from the mutant allele in cell strains RPE.11a, R1.41, and K1W1.P17 was functionally impaired, or...
we had selected for hygromycin resistance, rather than any 10 µg DT/ml as a percentage of incorporation after incubation in toxin-free medium.

In order to select cells which stably expressed high levels of toxin-resistant EF-2, we exposed transfected populations, which had grown in hygromycin-containing medium for a period of 2 weeks, to PEA at a concentration of 100 ng/ml for 48 h. This exposure to PEA resulted in cessation of cell division or cell death in roughly 80–90% of the cells. The remaining cells grew into discrete colonies of varying sizes. One week after exposure to toxin, several individual colonies were picked and grown in toxin-free medium for 2 months. Intact cell assays were performed on these clonal lines, and their resistance to toxin was highly variable (Table III). The resistance correlated with the size of the colonies from which each clone was picked.

**Discussion**

We have characterized 13 mutant strains derived from CHO-K1 cells that are resistant to diphtheria toxin and Pseudomonas exotoxin A due to mutations in the elongation factor 2 gene. Ten of the strains have a G to A transition in the

| Donor DNA | Incorporation of 35S cpm per sample | % of control |
|-----------|-----------------------------------|-------------|
| None      | 414 ± 87                          | 0.4 ± 0.09  |
| Wild type | 654 ± 253                         | 0.6 ± 0.25  |
| Ile-714 → Asn | 4,567 ± 708                     | 4.6 ± 0.71  |
| Gly-717 → Arg | 8,996 ± 1,509                   | 9.0 ± 1.5   |
| Ser-584 → Gly | 3,950 ± 1,024                   | 4.0 ± 1.0   |

\[8\]

Incorporation of Tran35S-labeled cysteine and methionine into trichloroacetic acid-precipitable protein. Results are an average of four data points collected in two separate experiments, plus or minus the standard deviation.

Table II: Resistance of transfected cell populations to diphtheria toxin

| Resistance of transfected cell populations to diphtheria toxin |
|-------------------------------------------------------------|
| Replicate monolayers of CHO-K1 cells, either untransfected or transfected with the indicated plasmid and selected in 600 units of hygromycin/ml, were pulse with Tran35S-labeled cysteine and methionine after incubation for 24 h in either growth medium or medium containing 10 µg DT/ml. |
| | |
| **Table II** | Resistance of transfected cell populations to diphtheria toxin |
| Replicate monolayers of CHO-K1 cells, either untransfected or transfected with the indicated plasmid and selected in 600 units of hygromycin/ml, were pulse with Tran35S-labeled cysteine and methionine after incubation for 24 h in either growth medium or medium containing 10 µg DT/ml. |
| **Table II** | Resistance of transfected cell populations to diphtheria toxin |
| Replicate monolayers of CHO-K1 cells, either untransfected or transfected with the indicated plasmid and selected in 600 units of hygromycin/ml, were pulse with Tran35S-labeled cysteine and methionine after incubation for 24 h in either growth medium or medium containing 10 µg DT/ml. |
first position of codon 717 of the EF-2 gene, resulting in a glycine to arginine substitution in the protein. Three others have unique mutations: (i) a G to A transition in the second position of codon 719, resulting in a glycine to aspartic acid substitution in the predicted protein; (ii) a T to A transversion in the second position of codon 714, resulting in an isoleucine to asparagine change in the protein; (iii) an A to G transition in the first position of codon 584, resulting in a serine to glycine change in the protein. The mutant strains contained one-half as much ADP-ribosylatable EF-2 as parental cells and equal amounts of total EF-2. Only ribosylphosphoramidate, but not free diphthamide or intermediate forms of diphthamide, was detected following ADP-riboylation of the EF-2 in vitro. All four mutations prevent the addition of the backbone of methionine from S-adenosyl-methionine to the imidazole ring of histidine 715 in the posttranslational synthesis of diphthamide (2, 3).

Although it is tempting to label codon 717 of the EF-2 gene as a mutational "hot spot" (13) due to the high proportion of mutations to toxin resistance which occurred in this codon, our results indicate that this may be an artifact of the method of selection. While mutations at sites other than the first position of codon 717 result in EF-2 that is resistant to ADP-riboylation, they also may result in a rate of protein synthesis in the presence of toxin that is well below 50% of control. These cell strains grow much more slowly, if at all, in the presence of DT or PEA. Previous studies, using somatic cell fusions between wild-type and class IIb mutants, showed that the resulting hybrids could not survive in high levels of toxin, although they synthesized roughly 25% as much protein in the presence of toxin as they did in toxin-free medium (14). Thus, these mutant cell strains may be overlooked when picking clones of toxin-resistant cells selected in toxin. Other investigators, using site-directed mutagenesis of the Saccharomyces cerevisiae EF-2 gene, have also recently identified new toxin-resistance mutations, all of which result in an increased doubling time, compared with cells expressing only wild-type EF-2 (10, 28). In these studies Kimata and Kohno (28) recovered nine amino acid substitutions at codon 699 (equivalent to codon 715 in CHO-K1 EF-2) that were not recovered by Phan et al. (10) and postulated that the difference in growth rates led to this differential recovery (28).

A scan of the GenBank® nucleotide sequence data base release 84.0 (31) shows that the EF-2 or EF-G gene or cDNA has been sequenced from 27 species, including that from eukaryotic mitochondria and chloroplast organelles. A multiple sequence alignment of the protein translations of these sequences allowed us to identify many regions that are conserved in all 27 sequences (data available in computer-readable format upon request). Fig. 7 is a multiple sequence alignment of the two regions of EF-2/EF-G in which we found mutations. We chose sequences from five eukaryotes, two archaeabacteria, and two eubacteria as representative. The mutation in codon 584, identified in cell strain RPE.11a, lies at the carboxy-terminal end of one of the conserved regions of the protein (Fig. 7B). This mutation is of particular interest, because it is located far from the modified histidine. The three-dimensional structure of EF-G from Thermus thermophilus has recently been determined by crystallography (29, 30). The structure shows that amino acid 584 is located close to amino acid 715, on the surface of domain 4 of the protein. Thus, in the biosynthesis of diphthamide one or both of the enzymes (16) required for the addition of the backbone of methionine to the imidazole ring of histidine 715 in EF-2 may interact directly with amino acid 584. Alternatively, alteration of amino acid 584 could possibly disrupt the native conformation of domain 4 and prevent the enzymes(s) from modifying histidine 715.

The mutation in codon 584 results in EF-2, which apparently functions less well in protein synthesis than wild-type EF-2, as indicated by levels of protein synthesis that are 20% of control, while expressing equal amounts of wild-type and total EF-2 (Figs. 5 and 6), when cell strain RPE.11a is grown in the presence of DT (Fig. 2 and Table I). Two mutations that result in kanamycin resistance have been identified in the E. coli fusA gene that encodes EF-G (32). Both of the fusA mutations result in colonies that exhibit very slow growth at 42 °C. These mutations are both located within eight amino acids of the mutation we identified in cell strain RPE.11a (Fig. 7). The similar effects of mutations in this region of both EF-2 and EF-G suggest a common conserved functional role for this domain of the protein.

Given the mutations we have studied interfere with the biosynthesis of diphthamide, the location of mutations near histidine 715 is less surprising than the mutation in codon 584. A combination of multiple sequence alignments with the recently published three-dimensional x-ray crystallographic structure of EF-G (29, 30) shows that the diphthamide residue occurs in EF-2 at a position equivalent to the tip of domain four of the EF-G protein. Amino acids in this domain which are conserved between eukaryotic EF-2 and prokaryotic EF-G are primarily located on the surface of the three-dimensional structure. The multiple sequence alignments show that diphthamide occurs within a region of 100 amino acids near the carboxy-terminal end of EF-2 that, like the GTP-binding domain near the amino-terminal end of the protein, is highly conserved (Fig. 7B). The conservation of amino acid sequences in the region surrounding the site of diphthamide indicates that this region is important to the function of EF-2.

The results in Table III demonstrate that CHO-K1 cells can survive exposure to toxin even when protein synthesis is inhibited to less than 10% of control. Previous studies on cell-cell hybrids showed that cells could not survive in saturating doses (0.5 μg/ml) of DT if their level of protein synthesis was less than 25% of control (14). These results have important implications regarding strategies for recovering new mutations in the EF-2 gene. A brief exposure to toxin, followed by selecting clones that grow at different rates, may allow recovery of a wider variety of mutations than is possible by selecting clones
which grow when continuously exposed to toxin. With continuous exposure to toxin, colonies of class Ila mutants and class IIb mutants with mutations in codon 717 may overgrow the plate before other mutants can form colonies large enough to pick. Many of the clones of stable transfectants expressing EF-2 with a Gly to Arg mutation in codon 717 synthesized protein in the presence of toxin at a rate near 50% of toxin-free control (Table III). It is unlikely that this is due to allelic replacement of one of the two chromosomal copies of the EF-2 gene via homologous recombination, because homologous recombination has previously been shown to be an extremely rare event (33, 34).

Modified forms of DT and PEA are being tested for use in cancer therapy. These toxins are used because of their extreme toxicity to mammalian cells, and the ability to link the toxic domain to different ligand-binding domains, such as immunoglobulins specific for a certain cell type, either by chemical cross-linking or by genetic engineering (35). As with any chemotherapy, a major concern is the development of a toxin-resistant subset of target cells that escape the chemotherapy. The frequency of occurrence of class II toxin-resistant mutants varies widely from one cell line to another. Class II toxin-resistant cells arise at a frequency of less than $5.6 \times 10^{-8}$ in recently cloned CHO-K1 cells (14), but occur at a very high frequency (approximately $1 \times 10^{-3}$) in Vero monkey kidney cells. Within the class II mutants, the ratio of EF-2 gene mutations to mutations in genes responsible for the posttranslational modification of His-715 to diphthamide is also variable. In CHO-K1 cells, both MOD- and EF-2 mutants have been isolated. In S. cerevisiae only MOD- mutants have been described, but this is due to the selection criteria used (36), not an indication of a low rate of mutation in the EF-2 genes. In most cultured mammalian cell lines, only class IIb mutants have been isolated. Cancer cells are often found to be defective in one or more DNA damage and repair pathways, and this leads to the possibility that the frequency of occurrence of DT and PEA-resistant cells within a population of tumor cells will be highly variable from tumor to tumor. More studies with human tumor cell lines will be necessary to confirm this.

Despite extensive study, the role of diphthamide in eukaryotes is unknown. Also unknown is the extent to which EF-2 participates in the regulation of protein synthesis and the role that regulation of protein synthesis plays in growth, development, and cell cycle regulation. In this report we identify several new mutations which help map critical regions of the EF-2 protein and provide insight into modifications of selection procedures which will help to identify additional mutations that can be used to address these important issues. The mapping of bacterial EF-G mutations and eukaryotic EF-2 mutations to homologous regions of the protein sequence provides evidence that studies of the mechanism of ribosomal translational regulation in either system will be applicable to the other. The role of diphthamide, however, must be studied in archaeabacterial or eukaryotic cells. Our expression studies demonstrate that transfected cell strains expressing different levels of toxin-resistant EF-2 can be produced. The strains should prove valuable in studies to determine the role EF-2 plays in regulating protein synthesis.

Acknowledgments—We thank Dr. Douglas Johnson for helpful comments on the manuscript. We also thank Dr. Angus Nairn for providing rabbit anti-EF-2 antisera.

REFERENCES
1. Moldave, K. (1990) Methods Enzymol. 182, 809–818
2. Van Ness, B. G., Howard, J. B., and Bodley, J. W. (1980) J. Biol. Chem. 255, 10710–10716
3. Van Ness, B. G., Howard, J. B., and Bodley, J. W. (1980) J. Biol. Chem. 255, 10717–10720
4. Bodley, J. W., and Veldman, S. A. (1990) in ADP-Ribosylating Toxins and G Proteins (Moss, J., and Vaughan, M., eds) pp. 21–30, American Society for Microbiology, Washington, D.C.
5. Oppenheimer, N. J., and Bodley, J. W. (1981) J. Biol. Chem. 256, 8579–8581
6. Moehring, J. M., and Bodley, J. W. (1993) J. Biol. Chem. 268, 3840–3844
7. Moehring, J. M., and Moehring, T. J. (1988) J. Biol. Chem. 263, 857–860
8. Chen, J. Y., Bodley, J. W., and Livingston, D. M. (1985) Mol. Cell. Biol. 5, 453–468
9. Chen, J. Y., Bodley, J. W., and Livingston, D. M. (1985) Mol. Cell. Biol. 5, 1357–1360
10. Phan, L. D., Perentes, J. P., and Bodley, J. W. (1993) J. Biol. Chem. 268,
8665–8668
11. Foley, B. T., Moehring, J. M., and Moehring, T. J. (1992) Somat. Cell Mol. Genet. 18, 227–231
12. Omura, F., Kohno, K., and Uchida, T. (1989) Eur. J. Biochem. 180, 1–8
13. Kohno, K., and Uchida, T. (1987) J. Biol. Chem. 262, 12298–12305
14. Moehring, T. J., Danley, D. E., and Moehring, J. M. (1979) Somat. Cell Genet. 5, 469–480
15. Moehring, J. M., and Moehring, T. J. (1983) Infect. Immun. 41, 998–1009
16. Moehring, T. J., Danley, D. E., and Moehring, J. M. (1984) Mol. Cell. Biol. 4, 642–650
17. Popov, N., Schmitt, M., Schulzeck, S., and Mattheis, H. (1975) Acta Biol. Med. Ger. 34, 1441–1446
18. Nairn, A. C., Bhagat, B., and Palfrey, H. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7939–7943
19. Peppel, K., and Baglioni, C. (1990) BioTechniques 9, 711–713
20. Carter, M. J., and Milton, I. D. (1993) Nucleic Acids Res. 21, 1044
21. Mulligan, R. C., and Berg, P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2072–2076
22. Kohno, K., Uchida, T., Mekada, E., and Okada, Y. (1985) Somat. Cell Genet. 11, 421–431
23. Lee, H., and Iglewski, W. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2703–2707
24. Riis, B., Rattan, S. I. S., Cavallius, J., and Clark, B. F. C. (1989) Biochem. Biophys. Res. Commun. 159, 1141–1146
25. Kohno, K., Uchida, T., Ohkubo, H., Nakanishi, S., Nakanishi, T., Fukui, T., Ohtsuka, E., Ikehara, M., and Okada, Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4978–4982
26. Nakanishi, T., Kohno, K., Ishiura, M., Ohashi, H., and Uchida, T. (1988) J. Biol. Chem. 263, 6384–6391
27. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985) Cell 41, 521–530
28. Kimata, Y., and Kohno, K. (1994) J. Biol. Chem. 269, 13497–13501
29. Czwerdowski, J., Wang, J., Stetze, T. A., and Moore, P. B. (1994) EMBO J. 13, 3661–3668
30. Aeversson, A., Brashnikov, E., Garber, M., Zhetonosova, J., Chrgadze, Y., Al-Karadaghi, A., Svensson, L. A., and Liljas, A. (1994) EMBO J. 13, 3669–3677
31. Benson, D., Lipman, D. J., and Ostell, J. (1993) Nucleic Acids Res. 21, 2963–2965
32. Hou, Y., Lin, Y-P., Sharer, D., and March, P. E. (1994) J. Bacteriol. 176, 123–139
33. Scheer, J. B., and Adair, G. M. (1994) Mol. Cell. Biol. 14, 6663–6673
34. Kido, M., Miwatani, H., Kohno, K., Uchida, T., and Okada, Y. (1991) Cell. Struct. Funct. 16, 447–453
35. Roffler, S. R., Yu, M. H., Chen, B. M., Tung, E., and Yeh, M. Y. (1991) Cancer Res. 51, 4001–4007
36. Perentesis, J. P., Phan, L. D., Gleason, W. D., LaPorte, D. C., Livingston, D. M., and Bodley, J. W. (1992) J. Biol. Chem. 267, 1190–1197
37. Woese, C. R., Kandler, O., and Wheelis, M. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4576–4579
38. Schwartz, R. M., and Dayhoff, M. O. (1979) in Atlas of Protein Sequence and Structure (Dayhoff, M. O., ed) pp. 353–358, National Biomedical Research Foundation, Washington, D. C.
