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Posttranslational modification of elongation factor 2 in diphtheria-toxin-resistant mutants of CHO-K1 cells

(protein synthesis/recessive mutation/somatic cell hybrids)

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ABSTRACT We have identified two types of mutants of Chinese hamster ovary cells in which the unique ADP-ribose attachment site in elongation factor 2 (EF-2) is altered, thereby rendering them resistant to diphtheria and *Pseudomonas* toxins (TOX^R). The first is mutant in the gene for EF-2 and possesses a permanently altered, TOX^R gene product. The second lacks a component of a posttranslational modification system that converts TOX^R EF-2 to the toxin-sensitive (TOX^S) state. We postulate that this modification system is involved in the conversion of a single histidine residue in EF-2 to the specific target of toxin-catalyzed ADP-ribosylation, the novel amino acid X. We have designated the second type MOD$^-$ mutants. The missing or nonfunctional component in the MOD$^-$ mutants can be restored by hybridizing them with either normal TOX^S cells or with EF-2 structural gene mutants. The TOX^R EF-2 from MOD$^-$ mutants is also converted to toxin sensitivity *in vitro* by incubation with extracts of TOX^S or EF-2 gene mutant cells in the presence of an energy-generating system. Our results demonstrate that EF-2 can be synthesized and released from ribosomes in a toxin-resistant form and then converted to toxin sensitivity by posttranslational modification.

Diphtheria toxin and *Pseudomonas* exotoxin A inhibit protein synthesis in eukaryotic cells and cell-free systems by catalyzing the transfer of ADP-ribose from NAD$^+$ to elongation factor 2 (EF-2) (1, 2). The action of these toxins is the same, despite the fact that they are entirely distinct serologically and have different host ranges (1, 2). These toxins act specifically upon eukaryotic cells, attaching ADP-ribose covalently to a single unique basic amino acid that is found in EF-2. Recently, strong evidence has been presented that this amino acid, originally designated X by Robinson *et al.* (3) and presumed to be derived by posttranslational modification of a standard amino acid, is a C2-modified histidine residue (4, 5). All naturally occurring EF-2 is sensitive to the action of diphtheria and *Pseudomonas* toxins, and amino acid X has been demonstrated in a highly conserved sequence of amino acids in EF-2 from four unrelated eukaryotic species (i.e., bovine, rat, yeast, and wheat germ) (6).

Somatic cell mutations of at least two types might affect the sensitivity of EF-2 to ADP-ribosylation by diphtheria and *Pseudomonas* toxins. One would be a mutation in the structural gene for EF-2, and another would be a mutation altering a component of the posttranslational modification system that directs the biosynthesis of X. We (7–9) and others (10) have previously reported on the isolation and characterization of mutant cells possessing an altered form of EF-2 that could not be ADP-ribosylated. We designated such mutants class II strains, as opposed to class I strains in which resistance to toxin resides at the level of the cell membrane (9). Somatic cell hybridization studies showed that the mutation(s) in the previously reported EF-2 mutants was codominant in nature, and evidence was presented that it was in the structural gene for EF-2 (7).

We now report the isolation of cells that are mutant in the modification system that converts EF-2 from a toxin-resistant (TOX^R) to a toxin-sensitive (TOX^S) state. This mutation confers total resistance to both diphtheria and *Pseudomonas* toxins and behaves recessively in somatic cell hybrids. We demonstrate that a soluble factor required for this modification is missing or nonfunctional in the mutant cells, but present in wild-type cells and in the previously described structural gene mutants. Further, we show that this modification can be carried out *in vitro*.

MATERIALS AND METHODS

Cells, Media, and Culture Conditions. CHO-K1 Chinese hamster ovary cells, which are auxotrophic for proline (PRO$^-$), were obtained from the American Type Culture Collection (ATCC CCL 61). For hybridization studies, strains were selected that were prototrophic for proline (PRO$^+$), resistant to 8-azaguanine (AZA^R), and would not grow in hypoxanthine/amethopterin/thymidine medium (11). The cell lines used in these studies and their origins are listed in Table 1. All cell strains were maintained in Ham's nutrient mixture F12 containing 10% fetal bovine serum in an atmosphere of 5% CO_2 in air.

Toxins. Results obtained with partially purified diphtheria toxin (Connaught Medical Research Laboratories, Toronto, ON Canada) were verified by using column-purified toxin (gift of R. J. Collier, University of California, Los Angeles) (8). Purified *Pseudomonas* exotoxin A was the gift of S. H. Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) (12).

Intact Cell Assay for Inhibition of Protein Synthesis by Toxins. This assay, which involves the exposure of intact cells to various concentrations of toxins for 24 hr followed by pulse-labeling with medium containing ^{14}C-labeled amino acids, is detailed elsewhere (9).

Preparation of EF-2 and the ADP-Ribosylation Assay. The details of the preparation of EF-2 extracts from cultured cells and their assay by toxin-mediated ADP-ribosylation, involving transfer of one molecule of [*U*-^{14}C]NAD to toxin-sensitive EF-2, are presented elsewhere (9).

***In Vitro* Modification of EF-2.** Postmitochondrial cell extracts were prepared as described (13). Extracts were a product

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Abbreviations: EF-2, elongation factor 2; TOX^S, sensitive to diphtheria and *Pseudomonas* toxins; TOX^R, resistant to these toxins; PRO$^+$, not requiring proline for growth; PRO$^-$, requiring proline; AZA^S, sensitive to 8-azaguanine; AZA^R, resistant to 8-azaguanine; MOD$^-$, deficient in a component required for posttranslational modification of EF-2.

Table 1. Strains used and their origins

Strain	Phenotype and origin
CHO-K1c	TOX^S, PRO^-, AZA^S, sensitive EF-2, subclone of CHO-K1 (9)
CH-P1R2	TOX^S, PRO^+, AZA^R, sensitive EF-2, selected from CHO-K1c after EMS mutagenesis (7)
CH-RE1.22c	TOX^R, PRO^-, AZA^S, resistant EF-2 due to EF-2 structural gene alteration, selected from CHO-K1 after EMS mutagenesis (8, 9)
CH-RE1.22c-A3P2	TOX^R, PRO^+, AZA^R, resistant EF-2 due to EF-2 structural gene alteration, selected from CH-RE1.22c after EMS mutagenesis.
CH-RPE.3b	TOX^R, PRO^-, AZA^S, resistant EF-2 due to mutation in EF-2 modification system (MOD^-), selected from CHO-K1c after EMS mutagenesis
CH-P1R2.DS	TOX^R, PRO^+, AZA^R, resistant EF-2 due to mutation in EF-2 modification system (MOD^-), selected from CH-P1R2

EMS, ethyl methanesulfonate.

of 50 million cells per ml. Cell extracts, which comprised 40% of the complete reaction mixture, were incubated at 30°C in the presence of an energy-generating system, for up to 3 hr. Reaction mixtures contained 30 mM Tris·HCl (pH 7.5), 74 mM KCl, 4 mM magnesium acetate, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, and 0.16 mg of creatine phosphokinase per ml (components of the energy-generating system obtained from Calbiochem). For detection of *in vitro* modified EF-2, 50-μl samples were withdrawn at appropriate times and tested in the ADP-ribosylation assay.

To measure peptide synthesis in these mixtures, we added 20 amino acids, each at 50 μM, and L-[U-^{14}C]valine (100 μCi/ml, New England Nuclear) to give 1.6 μCi/ml (1 Ci = 3.7 $\times 10^{10}$ becquerels). Samples of 50 μl were withdrawn at appropriate times and precipitated in 10 vol of chilled 7% perchloric acid containing 20 mg of casamino acids per ml. Samples were heated at 90°C for 10 min, chilled at 4°C for 1 hr, collected on Whatman GF/C filters with washes of 5% trichloroacetic acid, and dried. Radioactivity was measured in Permafluor liquid scintillator in a Tri-Carb scintillation spectrometer (Packard Instrument).

Somatic Cell Hybridization. Details of our hybridization procedure have been presented elsewhere (7). In each hybridization, one of the two quasidiploid parental strains was PRO^- and AZA^S and the other PRO^+ and AZA^R. Parental cells could therefore be eliminated by the use of proline-free hypoxanthine/amethopterin/thymidine selective medium (11), allowing only true hybrids to develop into clones. These clones were then isolated. Appropriate controls were included in each hybridization experiment. The phenotype of the parental strains was verified at the time of the experiment. The hybrid nature of recovered clones was verified by their growth in the selective medium. Karyological analysis was performed to show that the hybrids possessed a quasitetraploid modal chromosome number and that marker chromosomes unique to each parent were present in the hybrid karyotype (7).

RESULTS

Intact Cell Assay. We refer to the toxin-resistant cell strains discussed in this report [CH-RPE.3b, CH-P1R2.DS, CH-RE1.22c, and CH-RE1.22cA3P2 (see Table 1)] as class IIa strains. That is, they possess no EF-2 able to be ADP-ribosylated and they synthesize protein at the same rate whether grown in

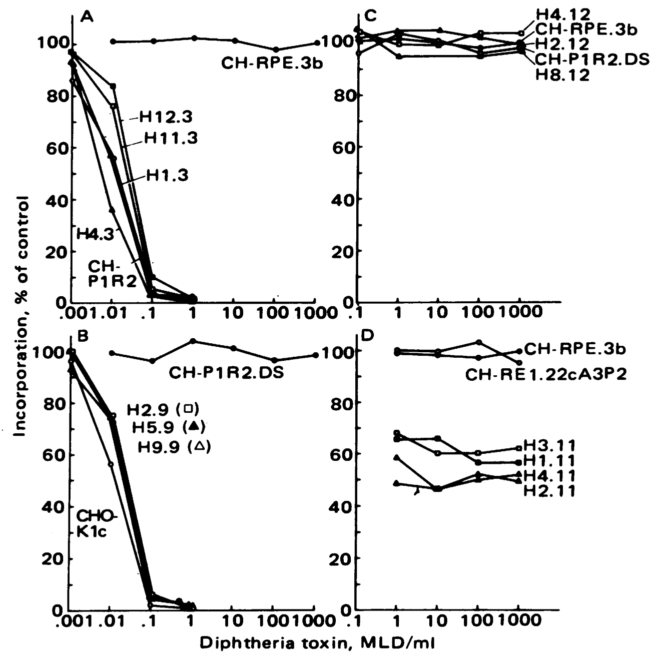

FIG. 1. Response to diphtheria toxin, in intact cell assays, of TOX^R and TOX^S Chinese hamster ovary cell strains and hybrids formed between them. MLD, minimal lethal dose, guinea pig. (*A*) TOX^S CH-P1R2, EF-2 MOD^- CH-RPE.3b, and hybrids H1.3, H11.3, and H12.3. (*B*) TOX^S CHO-K1c, EF-2 MOD^- CH-P1R2.DS, and hybrids H2.9, H5.9, and H9.9. (*C*) EF-2 MOD^- CH-RPE.3b and CH-P1R2.DS and hybrids H2.12, H4.12, and H8.12. (*D*) EF-2 gene mutant CH-RE1.22cA3P2, EF-2 MOD^- CH-RPE.3b, and hybrids H1.11, H2.11, H3.11, and H4.11.

the presence or absence of toxin.* This class of mutant occurs at a very low frequency ($<5 \times 10^{-7}$) even in mutagenized cell populations. The previously described strains CH-RE1.22c and CH-RE1.22cA3P2 will be referred to as EF-2 gene mutants. The two strains that are the subject of this report, CH-RPE.3b and CH-P1R2.DS, will be referred to as EF-2 MOD^- mutants.

To study the nature of the mutations in the MOD^- strains, we formed hybrid cells by fusion of the MOD^- cells with certain other cell strains. Fig. 1 shows the response of the MOD^- mutants and their hybrids to diphtheria toxin in an intact cell assay for inhibition of protein synthesis. Fig. 1 *A* and *B* shows the results when the MOD^- mutants were fused with cells possessing normal (wild-type) EF-2 (CH-RPE.3b × CH-P1R2 and CH-P1R2.DS × CHO-K1c). Even at the highest concentration of toxin tested (1000 MLD/ml), protein synthesis in the MOD^- mutants was unaffected. However, the hybrids (designated as H-strains) were equally as sensitive as their wild-type parents. These results demonstrate that the mutations causing toxin resistance in the MOD^- mutants are recessive in nature.

Fig. 1*C* shows the response of hybrids formed when the two MOD^- mutants were fused with each other (CH-RPE.3b × CH-P1R2.DS). These hybrids were as resistant to toxin as their parental strains, indicating that mutations in the parents are noncomplementary and, therefore, affect the same gene.

Toxin resistance in EF-2 gene mutants is codominant. Therefore, hybrids formed from strains of this type, which

* Class I strains are those in which resistance resides at the level of the cell membrane. Class IIb strains are those which contain 50% sensitive and 50% resistant EF-2 and are heterozygous for resistance at the EF-2 gene locus (7, 9).

Table 2. Content of ADP-ribosylatable EF-2 in extracts of parental and hybrid cells

Cell strain	ADP-ribose incorporated, pmol/mg protein*	% of WT†
CH-P1R2 (TOX^S, WT)	106 ± 2	–
CH-RE1.22cA3P2 (TOX^R, EF-2 gene)	0.01 ± 0.02	0
CH-RPE.3b (TOX^R, EF-2 MOD$^-$)	0	0
CH-P1R2.DS (TOX^R, EF-2 MOD$^-$)	0	0
Hybrids of CH-P1R2 × CH-RPE.3b:		
H4.3	105 ± 5	99
H9.3	115 ± 5	108
H11.3	126 ± 3	119
Hybrid of CH-RPE.3b × CH-RE1.22cA3P2:		
H3.11	50 ± 1	47

* pmol of ADP-ribose incorporated = pmol of TOX^S EF-2 (1, 2, 14).

† WT, wild type, or normal, with respect to EF-2.

contain 100% TOX^R, and normal cells containing 100% TOX^S EF-2 possess 50% TOX^S and 50% TOX^R EF-2, and protein synthesis in them can be inhibited to only 50% of control values. When a MOD$^-$ mutant was fused with such an EF-2 gene mutant (CH-RPE.3b × CH-RE1.22cA3P2), the resultant hybrids also were inhibited to only 50% of control values (see Fig. 1*D*). The EF-2 gene mutant, like the cells possessing normal TOX^S EF-2, could complement the mutation in the MOD$^-$ strain. This demonstrated that the EF-2 gene mutant possesses a normal EF-2 modification system.

ADP-Ribosylation of EF-2. The results of ADP-ribosylation assays to determine the amount of toxin-sensitive EF-2 in the MOD$^-$ mutant CH-RPE.3b and its hybrids are presented in Table 2. Extracts prepared from CH-RPE.3b contained no ADP-ribosylatable EF-2. Extracts of hybrids formed between CH-RPE.3b and the TOX^S strain CH-P1R2 (i.e., H4.3, H9.3, and H11.3) possessed an amount of ADP-ribosylatable EF-2 that approximated that found in the TOX^S parental cells. Extracts of H3.11, a hybrid formed by fusion of CH-RPE.3b with the EF-2 gene mutant CH-RE1.22cA3P2, contained approximately half as much ADP-ribosylatable EF-2 as wild-type cells.

Posttranslational Modification of EF-2 *In Vitro*. Extracts of CH-RPE.3b and CH-RE1.22c were mixed and incubated to determine if the EF-2 synthesized by CH-RPE.3b cells could be converted to toxin sensitivity *in vitro*, as it was in cell hybrids. When extracts were mixed in a ratio of 1:1 and incubated without further additions at 30°C for 150 min, the amount of ADP-ribosylatable EF-2 that could be detected was minimal (in four determinations, 0.50 ± 0.54 pmol of [^{14}C]ADP-ribose was bound per mg of CH-RPE.3b extract protein). However, when CH-RPE.3b and CH-RE1.22c extracts were mixed and incubated in the presence of an energy-generating system, ADP-ribosylatable EF-2 was generated at a linear rate over periods of 3 hr or more (Fig. 2*A*). In contrast, when CH-RPE.3b extracts were mixed 1:1 with extracts of CH-P1R2.DS, a second MOD$^-$ strain belonging to the same complementation group, no ADP-ribosylatable EF-2 was formed during the same period of incubation.

Although extracts were incubated under conditions that could support incorporation of amino acids into protein for 2 hr or more (Fig. 2*B*), protein synthesis was not required for the formation of toxin-sensitive EF-2. This was demonstrated by adding cycloheximide or RNase to incubation mixtures or by centrifuging extracts to remove ribosomes prior to incubation *in vitro*. These treatments, which completely inhibited incorporation of [^{14}C]valine into protein, did not significantly alter the formation of toxin-sensitive, ADP-ribosylatable EF-2.

The effect of dialysis of the extracts prior to incubation is shown in Table 3. When dialyzed extract from the EF-2 gene mutant was incubated in the complete system with undialyzed extract from a MOD$^-$ mutant, conversion of TOX^R to TOX^S EF-2 was almost equal to that obtained using undialyzed extracts. However, when both extracts were dialyzed before incubation, conversion of EF-2 in the complete system was minimal.

From the above studies we may conclude that it was toxin-resistant EF-2 extracted from the MOD$^-$ mutant that was converted to toxin sensitivity *in vitro*, that this conversion did not require protein synthesis or ribosomes, that a nondialyzable factor found in the EF-2 gene mutant was required for this conversion, and that a second, dialyzable factor was also required. Heating of the extract from the EF-2 gene mutant at 90°C for 10 min totally inactivated the nondialyzable factor.

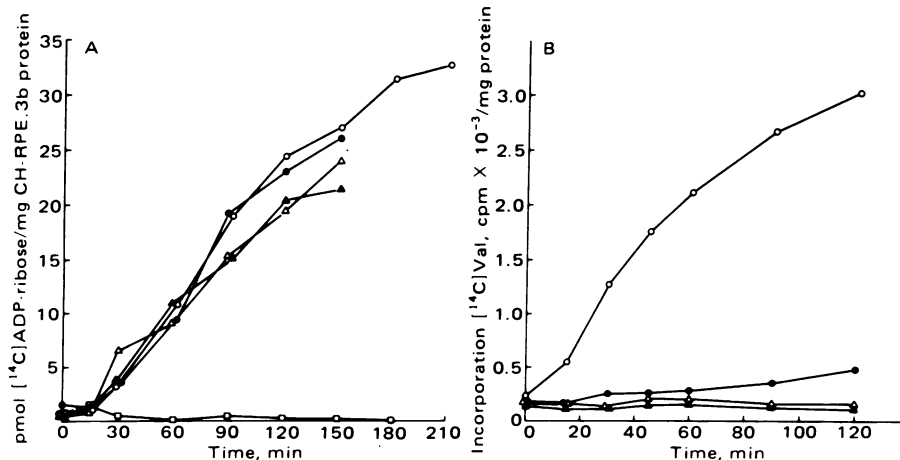

FIG. 2. *In vitro* conversion of EF-2 from CH-RPE.3b to an ADP-ribosylatable form. (*A*) Cell-free extracts of MOD$^-$ mutant CH-RPE.3b and EF-2 gene mutant CH-RE1.22c were mixed in equal volumes and incubated. At the indicated times, samples withdrawn from reaction mixtures were reacted in the ADP-ribosylation assay. The control curve (○) indicates the activity of the complete, standard system. The effect of centrifugation of extracts at 100,000 × *g* to remove ribosomes (▲) and the addition of 1 mM cycloheximide (●) and ribonuclease (10 μg/ml) (△) to the system are also shown. An incubation mixture of extract from MOD$^-$ CH-RPE.3b and MOD$^-$ CH-P1R2.DS serves as a negative control (□). The total protein concentration of these extracts was: CH-RPE.3b, 4.4 mg/ml; CH-RE1.22c, 5.8 mg/ml; and CH-P1R2.DS, 5.9 mg/ml. (*B*) Cell-free protein synthesis measured by incorporation of [^{14}C]valine into acid-precipitable material in mixtures similar to those shown in *A*.

Table 3. Effect of dialysis of extracts on conversion of TOX^R EF-2 to TOX^S EF-2

Cell extracts*			
CH-RPE.3b (MOD^-)	CH-RE1.22c (EF-2 gene)	Energy system	ADP-ribose incorporated, pmol/mg protein†
ND	ND	−	1.4
ND	ND	+	29.9
ND	D	+	23.4
D	D	+	0.4
D	D	−	0

* ND, not dialyzed; D, dialyzed.
† pmol of ADP-ribose incorporated = pmol of TOX^S EF-2 (1, 2, 14).

The extent to which TOX^R EF-2 from MOD^- mutant CH-RPE.3b could be converted to sensitivity *in vitro* was estimated. Cell extract from TOX^S CHO-K1c cells was incubated in the complete modification system and samples were withdrawn periodically for assay of ADP-ribosylatable EF-2. The amount of ADP-ribosylatable EF-2 in the samples was unchanged over an incubation period of 0–180 min. As shown in Table 4, 52 pmol of [^{14}C]ADP-ribose was incorporated per mg of CHO-K1c extract protein; 33 pmol of [^{14}C]ADP-ribose was incorporated per mg of CH-RPE.3b extract protein after its incubation with CH-RE1.22c extract for 210 min. This indicates that an amount of CH-RPE.3b EF-2 equal to 60% of the total EF-2 in TOX^S CHO-K1c cells was converted to toxin sensitivity *in vitro*.

DISCUSSION

We have now identified two types of mutants of Chinese hamster ovary cells in which the unique ADP-ribose attachment site in EF-2 is altered, rendering them resistant to diphtheria and *Pseudomonas* toxins. The site of specific ADP-ribosylation of EF-2 catalyzed by these toxins is a novel amino acid first reported by Robinson *et al.* and designated X (3). Amino acid X was presumed to be derived from posttranslational modification of a standard amino acid, and recently strong evidence has been presented that X is a C2-modified histidine residue bearing a complex side chain (4, 5). It is found in eukaryotic, but not prokaryotic, elongation factor in the ratio of one molecule of X per EF-2 molecule (3, 15).

We have previously reported on EF-2 structural gene mutants of CHO-K1 cells (7). These mutants appear to possess two functional alleles at the EF-2 gene locus and the toxin-resistance marker in this type of mutant behaves codominantly in cell hybrids. That is, the hybrid of an EF-2 gene mutant that contains 100% TOX^R EF-2 and a cell that contains 100% TOX^S EF-2 will have 50% TOX^R and 50% TOX^S EF-2. Based upon the unique structural nature of X and its presumed biosynthesis by posttranslational modification, we have postulated either that EF-2 structural gene mutants may have another amino acid substituted for the critical histidine residue or that there may be a substitution for an amino acid near the histidine residue which, if altered, prevents the modification of histidine to X (7).

We have now isolated two new TOX^R strains that are indistinguishable from EF-2 gene mutants either by intact cell assay for inhibition of protein synthesis or by assay of cell extracts for ADP-ribosylatable EF-2. Protein synthesis in these strains was not inhibited by high concentrations of diphtheria or *Pseudomonas* toxins and they possessed no ADP-ribosylatable EF-2. However, it became apparent that these strains were different from EF-2 structural gene mutants when hybrids were constructed between the new strains and normal, TOX^S cells. The

Table 4. Comparison of content of ADP-ribosylatable EF-2 in wild-type CHO-K1c extract and in *in vitro* modified CH-RPE.3b extract*

Cell extract	pmol ADP-ribose incorporated per mg extract protein†
CHO-K1c	52 ± 2
CH-RPE.3b	0
CH-RPE.3b (modified by incubation with CH-RE1.22c extract)	33

* Values given are all from extracts that were treated in the cell-free modification system prior to assay in the ADP-ribosylation system. The value of CHO-K1c is an average of 12 samples collected over 0–180 min. The value for CH-RPE.3b is an average of 5 samples collected over 0–180 min. The value for CH-RPE.3b (modified) is the 210-min point from the experiment described in Fig. 3.
† pmol of ADP-ribose incorporated = pmol of TOX^S EF-2 (1, 2, 14).

toxin resistance markers in these strains behaved recessively. The hybrids were as sensitive to toxin as was their normal parent, and they contained a comparable amount of ADP-ribosylatable EF-2. It was evident that the new strains were not mutant in the gene for EF-2 but rather at a second, unidentified locus, and the modification system that converts EF-2 to toxin sensitivity (i.e., converts the critical histidine residue to amino acid X) was altered in these strains. In the hybrids the component of the modification system that was altered in the mutants was supplied by the TOX^S parent, and all of the EF-2 produced in the hybrids was modified in the normal manner. We have designated these "modification deficient" strains MOD^- mutants.

When hybridizations were carried out between a MOD^- mutant and an EF-2 structural gene mutant, both of which contained no ADP-ribosylatable EF-2, the hybrids contained 50% TOX^S and 50% TOX^R EF-2. As expected, the modification system in the EF-2 gene mutant was functional and able to convert the EF-2 from the MOD^- mutant to toxin sensitivity, but the MOD^- mutant was not able to complement the lesion in the EF-2 gene mutant.

When hybrids were formed between the two MOD^- mutants, CH-RPE.3b and CH-P1R2.DS, the hybrids were as resistant as were the parents and possessed no ADP-ribosylatable EF-2. They therefore belong to the same complementation group and presumably contain the same genetic lesion.

From our hybridization data it could not be determined whether the MOD^- phenotype arose as the result of a single mutation in a gene that is either structurally or functionally haploid, the hemizygous state described by Siminovitch (16), or as the result of two mutations in a diploid gene. Because the frequency of recovery of this type of variant is very low ($<5 \times 10^{-7}$ even in mutagenized cell populations), it would appear that CH-RPE.3b and CH-P1R2.DS are homozygous recessive mutants.

Using extracts of the MOD^- and the EF-2 structural gene mutants, we have developed an *in vitro*, cell-free modification system for use in investigating further the biosynthesis of X. When cell-free extracts of either of the MOD^- strains were mixed with an extract from an EF-2 gene mutant and incubated in the presence of an energy-generating system, a product was produced that was ADP-ribosylatable in the standard toxin-catalyzed assay. Because of the specificity of this toxin-catalyzed reaction (1, 2, 14), this product is assuredly modified EF-2. In addition, this product co-electrophoreses with wild-type EF-2 in $NaDodSO_4$/polyacrylamide gels (unpublished data). The TOX^S EF-2 is produced at a linear rate for approximately 90

min and at a slower rate thereafter. The yield obtained after 210 min approached the amount of TOX^S EF-2 found in extracts of normal TOX^S cells. No TOX^S EF-2 was generated when extracts of the two MOD^- strains were incubated together. It was determined that neither protein synthesis nor ribosomes were necessary for the modification to take place, and that a heat-labile, nondialyzable factor present in the extract from the EF-2 gene mutant was required. It was also determined that a dialyzable factor present in both extracts was required for modification of EF-2 *in vitro*.

It therefore appears that an enzyme required for posttranslational modification of EF-2 may either be altered or missing in the MOD^- mutants reported here, and that one or more low molecular weight factors, which serve as donors or cofactors, are also required. Posttranslational modifications can occur at the level of tRNA, the nascent peptide, or the completed protein molecule (17). Our data on the MOD^- mutants discussed here rule out, in them, a mutation that affects modification at the level of tRNA.

EF-2 was shown to be synthesized and released from ribosomes in a toxin-resistant form which could subsequently be converted to toxin sensitivity. In normal cells this modification might occur either while the peptide is nascent on the ribosome or after release of the completed protein molecule.

Biochemical and genetic data indicate that the biosynthesis of X involves an elaborate process of posttranslational modification. The side chain that is attached to the C2 position of histidine is complex (5) and most likely synthesized in more than one step. Further, our recent identification of MOD^- mutants that fall into two complementation groups distinct from the group described here is in keeping with this concept and suggests that at least three enzymatic steps are involved in the biosynthesis of X.

Our results provide evidence for mutations outside the structural gene for EF-2 giving rise to an altered, functional EF-2 that is resistant to ADP-ribosylation. These mutants provide us with a natural substrate for investigation of the posttranslational modification process in eukaryotic cells. Further study of the MOD^- mutants and their EF-2 may contribute to an understanding of the biosynthesis of X and the biological function of the toxin-sensitive site in EF-2.

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