Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3158–3162, May 1984
Genetics

Chromosomal assignment of the gene for human elongation factor 2

(diphtheria toxin-resistant mutants/somatic cell hybrids/gene mapping/poliovirus sensitivity)

YASUFUMI KANEDA*, MICHIHIRO C. YOSHIDA†, KENJI KOHNO‡, TSUYOSHI UCHIDA*, AND YOSHIO OKADA*‡

*Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan; †Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060, Japan; and ‡Department of Cell Fusion, National Institute for Basic Biology, Okazaki, Aichi 444, Japan

Communicated by Frank H. Ruddle, February 2, 1984

ABSTRACT **Elongation factor 2 (EF-2), polypeptidyl-tRNA translocase, is an essential factor for protein synthesis in eukaryotic cells and Archebacteria. We isolated diphtheria toxin-resistant human primary embryo cells that contain EF-2 that cannot be ADP-ribosylated by diphtheria toxin and *Pseudomonas* exotoxin A (PA toxin). Somatic cell hybrids were constructed from mouse L cells and toxin-resistant human embryo cell mutants. Forty-one hybrid clones were isolated, of which 15 clones were resistant to PA toxin. Karyotypic analysis and isozyme studies revealed that there was an absolute correlation between human chromosome 19 and resistance to PA toxin in the hybrids. On subcloning of PA toxin-resistant hybrid cells, we obtained one PA toxin-resistant hybrid subclone containing human chromosome 19 as the only human chromosome. Furthermore, the resistance to PA toxin of hybrid cell strains was lost after infection with poliovirus, for which sensitivity is conferred by human chromosome 19. It was confirmed by using two-dimensional gel electrophoresis that PA toxin resistance in hybrid cells was caused by the presence of EF-2 resistant to ADP-ribosylation by fragment A of diphtheria toxin. These facts suggest that the gene encoding EF-2 is located on human chromosome 19.**

It is now well-established that both diphtheria toxin (1, 2) and *Pseudomonas* exotoxin A (PA toxin) (3) inhibit protein synthesis by catalyzing specific covalent binding of the ADP-ribose moiety of NAD to elongation factor 2 (EF-2) in eukaryotes and Archebacteria. EF-2 (M_r 100,000) is required for the translocation step in protein synthesis (4, 5), where peptidyl-tRNA is moved to the next codon on mRNA from the acceptor site on the ribosome at the expense of the energy provided by hydrolysis of GTP bound to EF-2. Diphtheria toxin sensitivity is mapped to human chromosome 5 (6), but the chromosomal location of EF-2 has not been determined.

In this investigation, we tried to map EF-2 using toxin-resistant mutants. The toxin-resistant cells that have been isolated are divided into two main classes (7–9). Class I mutants have a defect in the binding of transport of the toxin. Class II mutants have a defect in their protein synthesis machinery and their EF-2 is not ADP-ribosylated by diphtheria toxin or PA toxin. Cells of class II are much more resistant to the toxin than those of class I. Class II mutants include at least two subclasses; one could have a mutation in a structural gene for EF-2 and the other in genes for components of the post-translational modification system of EF-2 that direct the biosynthesis of diphthamide, which is the unique attachment site for ADP-ribose (10, 11). In somatic cell hybrids, the former subclass has a codominant phenotype, while the latter subclass is recessive (12). Cells of the first subclass that contain 50% resistant EF-2 and 50% sensitive EF-2 are the most frequently isolated type of class II mutants in several kinds of cell lines. We isolated diphtheria toxin-resistant mutants that had 50% toxin-resistant EF-2 from primary human embryo cells. Then somatic cell hybrids were formed between human primary cells possessing 50% toxin-resistant EF-2 and toxin-sensitive mouse cells. Hybrid clones were divided into PA toxin-resistant and toxin-sensitive groups. We determined the correlation between resistance to PA toxin and the presence of specific human chromosomes in hybrid clones and report here that the gene encoding EF-2 is assigned to human chromosome 19.

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MATERIALS AND METHODS

Human and Mouse Cells. Human primary cells were derived from various tissues of human embryo and cultured in Dulbecco's modified Eagle's minimal essential medium (ME medium) (Flow Laboratories) supplemented with 10% newborn calf serum. The cells were mutagenized by exposing them to 200 μg of ethylmethane sulfonate per ml for 20 hr. After the removal of ethylmethane sulfonate, cells were passaged and maintained in normal medium for 2 days. Then the cells were treated with high concentrations of diphtheria toxin (at first 20 ng/ml for 3 days, then 20 μg/ml for 3 days). Two batches of cells were independently treated with toxin, and the diphtheria toxin-resistant human cells were combined. About 50% of the EF-2 in these cells was not ADP-ribosylated by diphtheria toxin or PA toxin. The cells had a normal intact human chromosome count, 46 XY. The mouse parental cell lines used were L cells (resistant to bromodeoxyuridine) maintained on Eagle's ME medium with 10% calf serum. Mouse L cells resistant to PA toxin were also isolated by the exposure to PA toxin.

Human–Mouse Somatic Cell Hybrids. PA toxin-sensitive mouse L cells and diphtheria toxin-resistant human embryo cells were fused in suspension with UV-inactivated HVJ (Sendai virus) (13). Hybrid formation was carried out twice independently. Hybrid cells were cultured in HAT selection medium (ME medium with hypoxanthine/aminopterin/thymidine) containing 10% newborn calf serum for 14 days. Well-growing colonies were isolated with cloning cylinders and cloned by limiting dilution. Human–mouse hybrid cells were subcloned by using the limiting dilution method, as occasion demanded.

Toxins. Diphtheria toxin was purified by chromatography on DEAE-cellulose (14). Fragment A of diphtheria toxin was prepared as described (15). PA toxin was kindly provided by B. H. Iglewski (University of Oregon Medical School).

Assay of the Rate of Protein Synthesis in Cells Cultured with Toxin. This was measured by a slight modification of the method described previously (16). In brief, cells were exposed to various concentrations of PA toxin for 24 hr in normal medium. The medium was changed to assay medium containing 1/10th of the normal concentration of leucine and 1 μCi (1 Ci = 37 GBq) of [^{3}H]leucine was added. After incu-

Abbreviations: EF-2, elongation factor 2; PA toxin, *Pseudomonas* exotoxin A.

bation for 1 hr at 37°C, the radioactivity incorporated into cells was counted in a liquid scintillation system.

ADP-Ribosylation of EF-2 in Cell Extracts. ADP-ribosylation of EF-2 (1, 2) was carried out as described elsewhere (17).

Karyotypic Analysis. Chromosomes of hybrid clones were identified after 33258-Hoechst/quinacrine mustard staining as described by Yoshida *et al.* (18). At least 12 metaphase spreads in each hybrid were examined for chromosomal analysis.

Isozyme Analysis. Cell extracts were prepared as described by Khan and co-workers (19, 20). The following enzyme markers for each human chromosome (chr) were assayed in hybrid cells by using cellulose acetate gel electrophoresis: phosphoglucomutase 1 (EC 2.7.5.1) and adenylate kinase 2 (EC 2.7.4.3) (chr 1); malate dehydrogenase 1 (EC 1.1.1.37) (chr 2); β-galactosidase 1 (EC 3.2.1.23) (chr 3); phosphoglucomutase 2 (EC 2.7.5.1) (chr 4); hexosaminidase B (EC 3.2.1.30) and arylsulfatase B (EC 3.1.6.1) (chr 5); malic enzyme (EC 1.1.1.40) (chr 6); β-glucuronidase (EC 3.2.1.31) (chr 7); adenylate kinase 1 (EC 2.7.4.3) and aconitase 1 (EC 4.2.1.3) (chr 9); glutamate oxaloacetate transaminase (EC 2.6.1.1) (chr 10); lactate dehydrogenase A (EC 1.1.1.27) (chr 11); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (chr 12); esterase D (EC 3.1.1.1) (chr 13); nucleoside phosphorylase (EC 2.4.2.1) (chr 14); mannose phosphate isomerase (EC 5.3.1.8) and hexosaminidase A (EC 3.2.1.30) (chr 15); α-glucosidase (EC 3.2.1.20) (chr 17); glucose phosphate isomerase (EC 5.3.1.9) and α-mannosidase B (EC 3.2.1.24) (chr 19); adenosine deaminase (EC 3.5.4.4) (chr 20); superoxide dismutase 1 (EC 1.15.1.1) (chr 21); arylsulfatase A (EC 3.1.6.1) (chr 22); glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (X chromosome).

Poliovirus Infection. Poliovirus (3×10^8 plaque-forming units/ml) was diluted 1:1,000 with serum-free ME medium and added to cells grown to confluency in a 60-mm plastic Petri dish. Cells were incubated with the virus at 37°C for 30 min in serum-free ME medium. Then the virus was removed and cells were cultured in normal medium containing 10% newborn calf serum.

Two-Dimensional Gel Electrophoresis. Crude EF-2 fractions extracted from hybrid cells were prepared as described elsewhere (21). The standard reaction mixture for ADP-ribosylation of EF-2 contained 20 μl of crude EF-2 fraction, 6.7 mM dithiothreitol, 0.6% octylglucoside, 6.7 mM thymidine, and 30 μM NAD in a total volume of 26 μl. Reaction mixtures were incubated at 37°C in the presence or absence of 4 μl of fragment A (0.8 μg) of diphtheria toxin. The mixtures were analyzed by a slight modification of the two-dimensional gel electrophoresis technique of O'Farrell (22). The first dimension was isoelectric focusing in gels containing 3.5% acrylamide, 0.19% bisacrylamide, 8 M urea, 2% Nonidet P-40, and 2% Ampholines (pH 3.5–10). Isoelectric focusing was carried out at 400 V for 12 hr and then at 800 V for 1 hr. Second-dimension electrophoresis was performed employing a 5% stacking gel and an 8% separating gel. Gels were stained by the highly sensitive silver staining method (23). The identity of EF-2 or ADP-ribosylated EF-2 spots was determined by coelectrophoresis with purified rat EF-2 or [^{32}P]ADP-ribosylated EF-2, respectively.

RESULTS

Protein synthesis in mouse L cells and human embryo cells was almost completely inhibited after 24 hr of exposure to 50 ng of PA toxin per ml. The rate of protein synthesis in diphtheria toxin-resistant human embryo cells was similar at 50, 150, or 500 ng of PA toxin per ml and was >70% of the control rate (Fig. 1 *Upper*). We also tested that EF-2 of mouse L cells and normal human embryo cells was completely ADP-

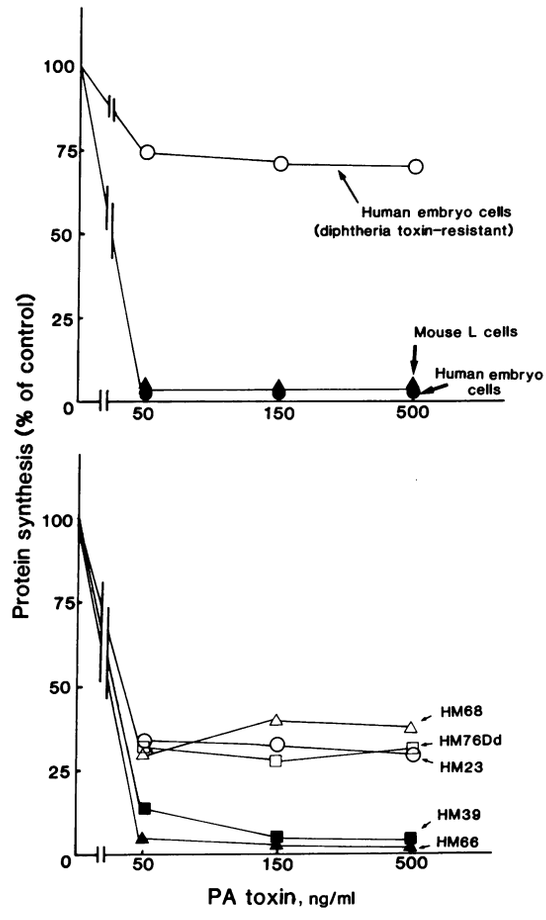

FIG. 1. Effect of PA toxin on the rate of protein synthesis of parental cells (*Upper*) and human–mouse hybrids (*Lower*). The rate of protein synthesis in each culture is expressed as a percentage of the value obtained in control cultures without toxin. (*Upper*) ○, human embryo cells (diphtheria toxin-resistant mutants); ●, human embryo cells; ▲, mouse L cells. (*Lower*) ○, HM23; △, HM68; □, HM76Dd; ▲, HM66; ■, HM39.

ribosylated by PA toxin but a part of EF-2 of diphtheria toxin-resistant mutants was not ADP-ribosylated by the toxin (data not shown). By fusion of mouse L cells and diphtheria toxin-resistant human embryo cells, 41 hybrid clones were isolated. The rate of protein synthesis of 15 of those hybrid clones was similar at 50, 150, or 500 ng of PA toxin per ml and was >25% of the control rate (PA toxin-resistant hybrid clones), whereas that of residual 26 clones was <10% under the identical condition (PA toxin-sensitive hybrid clones) (Fig. 1 *Lower*). In the presence of toxin the rate of protein synthesis of PA toxin-resistant hybrid clones was less than that of diphtheria toxin-resistant human embryo cells. This is because the proportion of toxin-resistant EF-2 per cell was diminished due to an increase of toxin-sensitive EF-2 as a result of the formation of human–mouse hybrids. The resistance of the hybrids suggests that the parental human embryo mutants had a mutation in a structural gene for EF-2, not in genes for components of the post-translational modification system of EF-2.

Next we examined the ADP-ribosylation of EF-2 in hybrid cell extracts. The values expressed as ^{14}C radioactivity per μg of cellular protein were as follows: PA toxin-sensitive human–mouse hybrid HM61, 10.6 cpm/μg of protein; PA toxin-resistant HM66A, 7.3 cpm/μg of protein; PA toxin-resistant HM39Dk, 6.3 cpm/μg of protein. As the ADP-ribosylation of EF-2 was carried out in the presence of excess fragment A of diphtheria toxin and NAD, all of the suscepti-

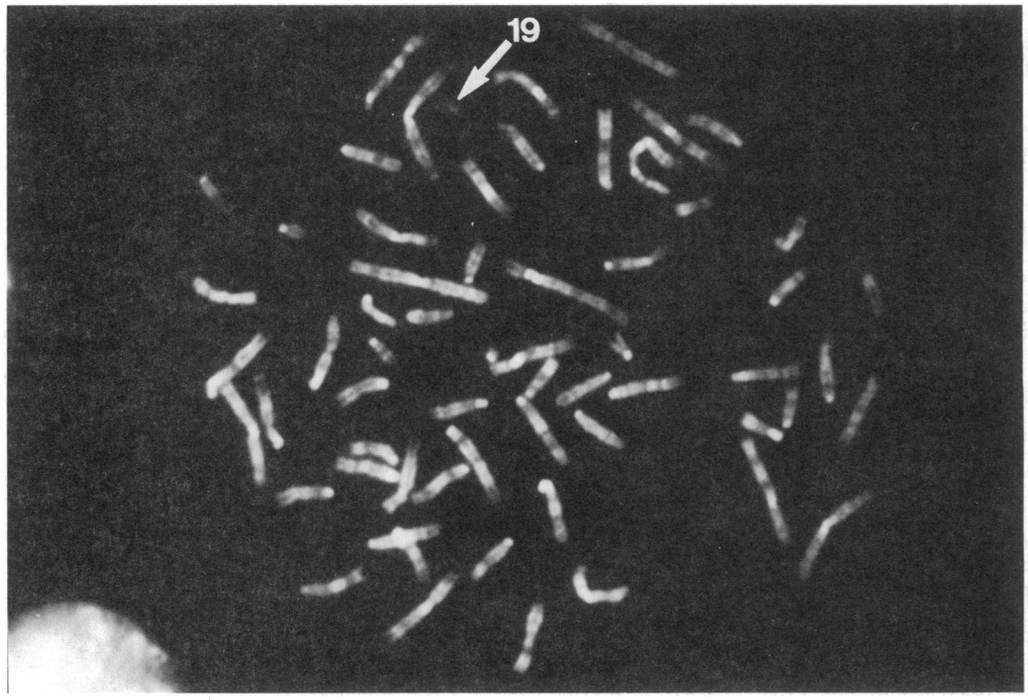

FIG. 2. Metaphase of human–mouse hybrid HM76Dd, which is resistant to PA toxin and has retained human chromosome 19 as the only human chromosome, stained by the 33258-Hoechst/quinacrine mustard method. The arrow indicates human chromosome 19.

ble EF-2 should be fully ADP-ribosylated. The value of ADP-ribosylation in PA toxin-resistant hybrid clones was 60–70% of the value in PA toxin-sensitive hybrid clones. This demonstrates that PA toxin-resistant clones possessed EF-2 that was not ADP-ribosylated by fragment A of diphtheria toxin.

On subcloning of PA toxin-resistant hybrid clones, we obtained 55 subclones resistant to PA toxin and 68 subclones sensitive to the toxin. Finally, 1 toxin-resistant clone, HM76Dd, was isolated that retained human chromosome 19 as the only human chromosome in addition to a full complement of mouse chromosomes (Fig. 2). All of the subclones of HM76Dd that lost human chromosome 19 became sensitive to PA toxin. Fig. 3 shows a panel of hybrid clones that were analyzed by karyotyping or enzyme markers or both. Concordant segregation of the resistance to PA toxin was observed only for human chromosome 19.

We investigated the correlation of human glucose phosphate isomerase, an enzyme marker for human chromosome 19 (24), and the sensitivity to PA toxin in hybrid clones. As shown in Table 1, the rate of concordancy was >98%, and only one clone sensitive to PA toxin expressed glucose phosphate isomerase of human origin.

The resistance of hybrid cells to PA toxin was probably

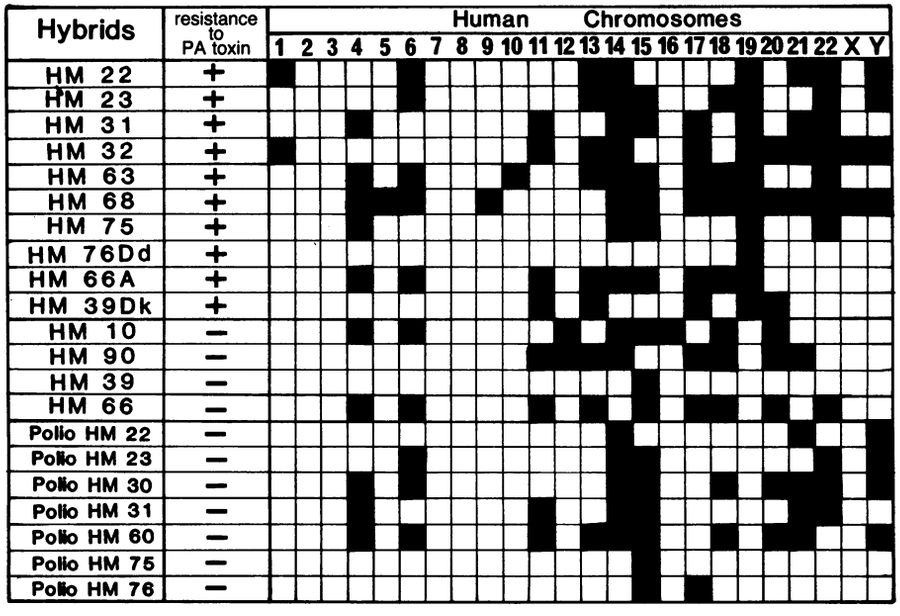

FIG. 3. Comparison of human chromosome complement and sensitivity to PA toxin in human–mouse hybrid cells. Individual chromosomes present in each hybrid as determined by isozyme analysis or karyotype analysis or both are indicated by black squares. Empty squares indicate that the hybrids have lost the human chromosomes shown in the upper row.

Table 1. Segregation of glucose phosphate isomerase with PA toxin resistance in human–mouse hybrid clones

PA toxin sensitivity	Glucose phosphate isomerase +	−
Resistant	43	0
Sensitive	1	11

Values are the numbers of hybrid clones.

due to the expression of human gene for EF-2 that cannot be ADP-ribosylated by the toxin. However, we must exclude the possibility of resistant EF-2 of mouse origin. PA toxin-resistant hybrids were infected with poliovirus and the surviving cells were characterized on the basis of sensitivity to PA toxin. Mouse cells are not susceptible to poliovirus, whereas human cells are susceptible. Poliovirus sensitivity has been mapped to human chromosome 19 (25). Twenty-one hybrids resistant to PA toxin were infected with poliovirus. Three of them died out completely and formed no colonies. Eighteen of them formed some colonies. All of the surviving cells were sensitive to PA toxin, but PA toxin-resistant mouse L cells were still resistant to the toxin after infection with poliovirus (Fig. 4).

We investigated the chromosomes and isozymes of seven hybrids selected by poliovirus. As shown in the lower seven rows of Fig. 3, the hybrids still contained some human chromosomes other than chromosome 19. These hybrids were found to retain all of the mouse chromosomes. These facts indicate that the resistance to PA toxin was not due to the expression of a mouse gene, but due to the expression of a human gene located on chromosome 19. We suppose that this resistance was probably caused by the expression of human EF-2 gene resistant to ADP-ribosylation by the toxin.

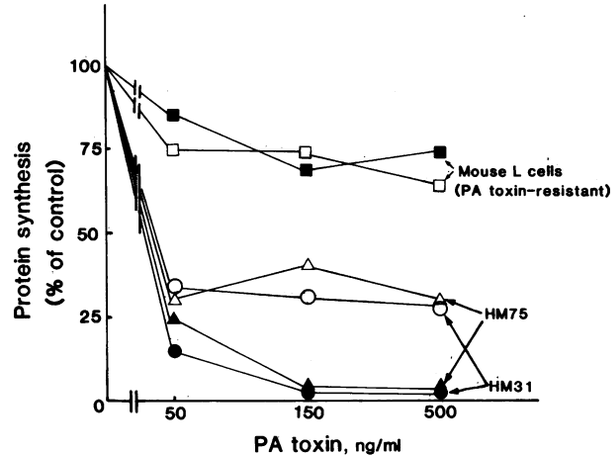

FIG. 4. Effect of poliovirus infection on sensitivity to PA toxin of mouse L cells and human–mouse hybrid clones. The rate of protein synthesis is expressed as a percentage of the value obtained in control cultures without toxin. ○, HM31 (mock-infected); ●, HM31 (poliovirus-infected); △, HM75 (mock-infected); ▲, HM75 (poliovirus-infected); □, mouse L cells resistant to PA toxin (mock-infected); ■, mouse L cells resistant to PA toxin (poliovirus-infected). Mock-infected cells were subjected to the same treatment without poliovirus.

To confirm this, we carried out two-dimensional gel electrophoresis. As EF-2 is a highly conserved protein, human EF-2 and mouse EF-2 are electrophoretically identical. But, by using two-dimensional gel electrophoresis, ADP-ribosylated EF-2 can be separated from EF-2 that is not ADP-ribosylated (21). The spot corresponding to EF-2 that was not treated with fragment A of diphtheria toxin is identified in Fig. 5 *A* and *C*. When EF-2 of the PA toxin-sensitive hybrid

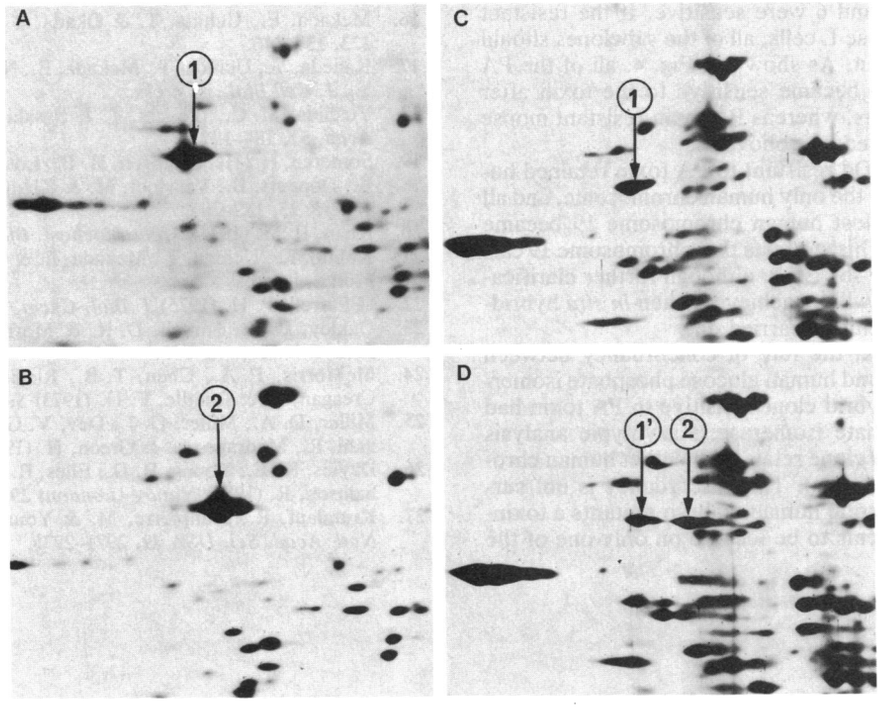

FIG. 5. Two-dimensional gel electrophoresis of crude EF-2 preparations extracted from toxin-sensitive and -resistant human–mouse hybrid cells. EF-2 preparations were incubated with NAD in the absence (*A* and *C*) or presence (*B* and *D*) of fragment A of diphtheria toxin and ≈30 μg of protein was loaded on isoelectric focusing gels. A part of each slab gel is shown. (*A* and *B*) HM61 (PA toxin-sensitive); (*C* and *D*) HM76Dd (PA toxin-resistant). Arrow 1 indicates EF-2 that was not treated with fragment A of diphtheria toxin. Arrow 1′ indicates mutant EF-2. Arrow 2 indicates EF-2 ADP-ribosylated by fragment A. The left side of each gel is basic and the right side is acidic. The pI values of arrow 1 (1′) and 2 were 6.6 and 6.4, respectively. The M_r of the spots corresponding to EF-2 was about 100,000.

HM61 was ADP-ribosylated by fragment A of diphtheria toxin, the spot of EF-2 was shifted to the acidic side (Fig. 5*B*). This suggests that all of the EF-2 of HM61 can be ADP-ribosylated. In the case of the PA toxin-resistant hybrid HM76Dd that retained chromosome 19 as the only human chromosome, a part of EF-2 was shifted to the acidic side, but residual EF-2 is also visible (Fig. 5*D*). When HM76Dd cells were cultured after treatment with poliovirus, chromosome 19 was lost and the resulting cells became sensitive to PA toxin. Nonribosylatable EF-2 was not observed in two-dimensional electrophoresis (data not shown). This suggests that HM76Dd possessed EF-2 resistant to ADP-ribosylation by fragment A of diphtheria toxin. These results confirm that the resistance to PA toxin in hybrid cells was due to EF-2 resistant to ADP-ribosylation by the toxin. We conclude that the structural gene for EF-2 is located on human chromosome 19.

DISCUSSION

We tried to determine the chromosomal location of EF-2 using human parental cells resistant to both diphtheria toxin and PA toxin, because normal human EF-2 and mouse EF-2 are indistinguishable. The action of diphtheria toxin and the characteristics of diphtheria toxin-resistant mutants are well established (1, 2, 7–12). Thus, we could assume that PA toxin-resistant hybrid clones derived from diphtheria toxin-resistant human cells and toxin-sensitive mouse cells possessed human EF-2 resistant to ADP-ribosylation by the toxin. We could confirm this using two-dimensional gel electrophoresis (Fig. 5). Although human EF-2 and mouse EF-2 cannot be separated by gel electrophoresis, it is clear that the resistant EF-2 in PA toxin-resistant hybrids derives from human embryo cells, not from mouse L cells. On subcloning of PA toxin-resistant hybrid cells, both PA toxin-resistant and -sensitive subclones were isolated; for example, in the case of HM03, 2 subclones, HM31 and HM32, were resistant and 11 subclones were sensitive to PA toxin, and 2 subclones of HM31 were resistant and 6 were sensitive. If the resistant EF-2 derives from mouse L cells, all of the subclones should be resistant to the toxin. As shown in Fig. 4, all of the PA toxin-resistant hybrids became sensitive to the toxin after infection with poliovirus, whereas PA toxin-resistant mouse L cells were not affected by poliovirus.

Furthermore, HM76Dd resistant to PA toxin retained human chromosome 19 as the only human chromosome, and all of the subclones that lost human chromosome 19 became sensitive to the toxin. This suggests that chromosome 19 carries the structural gene for EF-2, although further clarification of this conclusion will be achieved when *in situ* hybridization for EF-2 gene can be carried out.

Table 1 indicates that the rate of concordancy between resistance to PA toxin and human glucose phosphate isomerase was >98%. One hybrid clone sensitive to PA toxin had human glucose phosphate isomerase. Karyotypic analysis showed that this hybrid clone retained an intact human chromosome 19 (data not shown). This discordancy is not surprising, because in parental human embryo mutants a toxin-resistant EF-2 gene seems to be located on only one of the two homologous chromosomes, and this sensitive hybrid clone may have contained one human chromosome 19 that carries a toxin-sensitive EF-2 gene.

Some human chromosomes can be isolated by using a fluorescence-activated cell sorter (26, 27). Chromosome 19 will be also isolated before long and the genomic library from human chromosome 19 will be constructed. This mapping study may be useful in the isolation of not only the structural gene for EF-2 but also other genes located on chromosome 19.

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