ESTABLISHMENT AND CHARACTERIZATION OF ETHIDIUM BROMIDE RESISTANCE IN SIMIAN VIRUS 40-TRANSFORMED HAMSTER CELLS

Effects on Mitochondria In Vivo

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ABSTRACT

This study describes the isolation and subsequent characterization of four mammalian cell lines resistant to ethidium bromide (EB). Treatment of the simian virus 40- (SV40) transformed hamster cell line F5-1 first led to the establishment of the F2 cell line, which is resistant to 2 μg EB/ml. At this concentration cytochromes *c* and *b* are present in almost normal or only slightly diminished amounts, whereas cytochromes $a + a_3$ show an obvious decrease. The mitochondria of the F2 cell show a normal ultrastructure, not distinct from the parental cell line F5-1, and contain closed circular DNA. The sensitive parental F5-1 cells, however, when exposed to the same dye concentration exhibit the typical EB-induced ultrastructural changes in the mitochondria, and no more component I mitochondrial DNA can be demonstrated. 1 yr after establishment we derived from the F2 cell three more cell lines, resistant against 4, 8, and 16 μg of EB/ml. These cell lines, termed F4, F8, and F16, respectively, also revealed relatively intact-appearing mitochondria, although distinguishable from F5-1 and F2 mitochondria by a more condensed or unorthodox cristae conformation. F4, F8, and F16 cell lines contained closed circular mitochondrial DNA in the same position as that of the parental F5-1 cells, when analyzed in an isopycnic CsCl-EB gradient. A small shoulder at the lower density side of the DNA I peaks was observed. The newly acquired drug resistance of the F cells is hereditarily transmitted to the progeny cells and retained even after a period of growth in EB-free medium.

INTRODUCTION

The acquisition of drug resistance of cells or subcellular organelles has been a most useful tool in the investigation of many genetic, biochemical, and pharmacological problems (1–10). One approach to the complex task of identifying which gene products are of nuclear and which are of mitochondrial origin has been to find altered mitochondrial traits that may be the consequence of nonlethal mutations in mitochondrial DNA. In yeast, resistance to erythromycin (7, 8), mika-

mycin, and chloramphenicol (9) appears to be mitochondrially coded. In *Chlamydomonas*, resistance to streptomycin appears to be the consequence of gene mutations in chloroplasts (6).

The phenanthridine dye ethidium bromide (EB) is of particular interest because the mitochondria are a prime target for this drug. Treatment of facultative anaerobic yeast with EB leads to a quantitative conversion from wild-type to respiratory-deficient mutants (11, 12). The mutation is genetically stable or may be reversible under certain conditions (13). EB acts as an intercalating agent for double-stranded DNA (14, 15). Its differential binding affinity for covalently closed circular and nicked circular or linear duplex DNA has been widely applied (16–18). In mammalian cells treatment of mouse L cells with EB leads to a decrease, first, of cytochromes $a + a_3$ and, then, of cytochrome b, accompanied by severe structural alterations of the mitochondria (19). A reduction of cytochrome oxidase activity (48) and cytochrome content (22) was shown in other types of mammalian cells at certain concentrations of EB. Furthermore, the drug was found to inhibit the incorporation of [^{3}H]thymidine into closed circular mitochondrial DNA but not into nuclear (20–22) or simian virus 40 (SV40) (23, 24) DNA in vivo. EB was also shown to inhibit preferentially the activity of partially purified mitochondrial DNA polymerase (25). Other effects of EB include the selective inhibition of mitochondrial RNA synthesis (26) and protein synthesis (27) in HeLa cells. Further references have been reviewed (21, 12).

None of the reported EB-induced effects on mammalian cells were genetically stable, but rather were dependent on the presence of the dye and reversible in its absence. Prolonged exposure to EB always resulted in cell death. Since no EB-resistant mutant of a mammalian cell has been described thus far, it was the aim of this study to induce or select such a mutant and to characterize some of the differences between the mutant and parental cell.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

All cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) and 100 μg/ml streptomycin and 100 IU/ml penicillin. Where indicated, EB was added from freshly made stock solutions of 500 μg/ml.

The cell line, F5-1, was derived from a primary tumor induced by inoculation of a newborn Syrian gold hamster (Lakeview Hamster Colony, Newfield, N. J.) with SV40 virus (28).

The F2 cell line was derived from the F5-1 cell line (29). Approximately 5×10^7 F5-1 cells of the 109th passage level were seeded in a Blake bottle and exposed with a culture medium containing 2 μg EB/ml. Thereafter the cells were maintained continuously in the presence of the drug for the 18 mo since then. 3 wk later, most of the cells had died, and the medium was changed. After another month, several colonies of cells had grown out. After repeated changes of medium, the cells resistant to treatment with EB appeared morphologically healthy and were cloned. They have been subcultivated for over 40 passages up to the present time. Cells of the 15–35th passage levels were used in the experiments carried out for the comparative characterization of the parental and EB-resistant cell lines. Repeated immunofluorescent tests have given positive results for the presence of the SV40-specific T antigen in both lines.

The Nil-2E cell line was kindly supplied by Dr. L. Diamond of The Wistar Institute, Philadelphia, Pa. Nil-2 is a line of spontaneously transformed hamster embryo cells that evolved without selective factors, chemical, physical, or viral (30). Nil-2E is a clone of the Nil-2 cell line isolated by McAllister and Macpherson (31).

HK15-SV (also kindly supplied by Dr. L. Diamond) is a line of SV40-transformed hamster kidney cells obtained by infecting kidney explants from newborn hamsters with the Rh911 strain of SV40. All the cells contain SV40 T antigen as demonstrated by immunofluorescence staining.

M-HeLa is a cell line of our laboratory stock; the line grows as a monolayer.

Colony Formation Assay

The colony-forming efficiency of cells from different cell lines was tested by seeding 10^3 cells in 50-mm Petri dishes. The cells were suspended in 5 ml of medium containing 2, 4, and 8 μg EB/ml; EB-free medium was used for the controls. The cultures were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air. The dishes were refed twice and kept in the dark. On day 12 they were fixed with methanol and the newly formed colonies were stained and scored for number and size. For HeLa cells, concentrations of 0.1, 0.5, and 1.0 μg EB/ml were used.

Electron Microscopy

For electron microscopy the cells were fixed at 0°C for 2 h in 3% glutaraldehyde adjusted to pH 7.2 with

0.1 M cacodylate buffer. They were then postfixed for 1 h with 2% OsO_4 in 0.1 M phosphate buffer and embedded in Epon 812. Sections were stained with uranyl acetate followed by lead citrate and examined with a Siemens Elmiskop IA electron microscope. Cells from at least two duplicate experiments were compared to verify the results.

DNA molecules were spread and prepared for electron microscopy by the formamide method as described (32), using 50% formamide in the spreading solution. The preparations were rotary shadowed with platinum.

Determination of Cytochromes

Absorption difference spectra between the fully reduced and fully oxidized state of cytochromes at the temperature of liquid nitrogen were determined with a split-beam spectrophotometer (33). For one experiment approximately 1×10^8 untreated or EB-treated cells suspended in phosphate-buffered saline (PBS) were used. Cytochromes were oxidized by adding 5 μM rotenone under aerobic conditions in the "reference" cuvette. The reduction of cytochromes in the "measure" cuvette was obtained by adding either sodium dithionite to the cell suspension to determine the total cytochrome content in cells or KCN to determine cytochrome content in the mitochondrial respiratory chain sensitive to cyanide. After 2 min the samples were frozen by the trapped steady-state method (34). The amount of cytochromes was calculated from the quotient of the cytochrome concentration and the cell number and cell protein content. The extinction coefficients used are given elsewhere (35). The absorbance changes were measured at the following wavelength pairs: for cytochrome $a + a_3$, 600 and 630 nm; for cytochrome *b*, 559 and 575 nm (cytochrome *b* "total," 557 and 575 nm); and for cytochrome *c*, 540 and 548 nm. The following intensification factors at low temperatures were used: for cyotchrome $a + a_3$, 7.0; cytochrome *b*, 7.0; and cytochrome *c*, 8.0. A value of 8 was assumed for "cytochrome *b*" of trace *B* in Fig. 5.

Isolation and Purification of Mitochondria and Mitochondrial DNA

Approximately 3–4 $\times 10^8$ cells were collected by the usual trypsinization procedure, washed twice with PBS minus calcium and magnesium, resuspended in hypotonic medium (0.1 M sucrose, 2 mM EDTA, 0.025 M Tris-HCl, pH 7.4), and disrupted in a Dounce homogenizer. After adjustment to 0.25 M sucrose and differential centrifugation procedures, the mitochondrial fraction was further purified in a sucrose gradient. The method is essentially as described (36). Mitochondrial fractions were not treated with DNase to assure relative intactness of the mitochondrial DNA.

The isolation of mitochondrial DNA has been described in detail (21). Further details for the centrifugations in CsCl-EB gradients are given in the legends to Figs. 10 and 11. It should be noted that only the covalently closed circular DNA (which normally comprises 60–90% of total mitochondrial DNA) was analyzed in this study.

Preparation of SV40-Marker DNA

SV40 virus, strain Rh911, was grown in African green monkey kidney cells. Infection was performed with 500 plaque-forming units (PFU) per cell. Labeling of the viral DNA was started 12 h postinfection for 48 h, using [^{14}C]methyl thymidine, 10 μCi/ml. The viral DNA was extracted as previously described (24), based on the method of Hirt (37). After isopycnic CsCl-EB gradient centrifugation the dye was removed by three serial extractions with isopropanol (38), and CsCl was removed by dialysis against 1/100 $\times$ standard saline citrate (SSC) (1 $\times$ SSC in 0.15 M NaCl, 0.015 M Na-citrate) and 10 mM EDTA buffer pH 7.5. The specific radioactivity of the closed circular DNA component I was determined

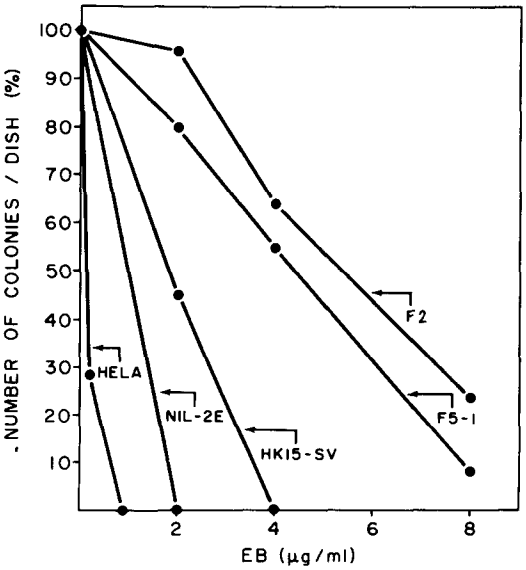


FIGURE 1 Effect of EB on colony-forming efficiency of different cell lines. For comparative purposes the number of colonies obtained for a given cell in normal medium lacking EB has been considered as 100% plating efficiency. The numbers of colonies scored in the presence of EB are plotted as a percentage of their respective controls.

as 21,000 cpm/μg DNA, that of component II as 9,000 cpm/μg DNA.

Source of Chemicals

EB (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) was purchased from Calbiochem, San Diego, Calif. [^{3}H]Methyl thymidine (sp act 47.2 Ci/mmol) and [^{14}C]methyl thymidine (sp act 54.7 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. Cesium chloride, optical grade, was purchased from Harshaw Chemical Co., Solon, Ohio.

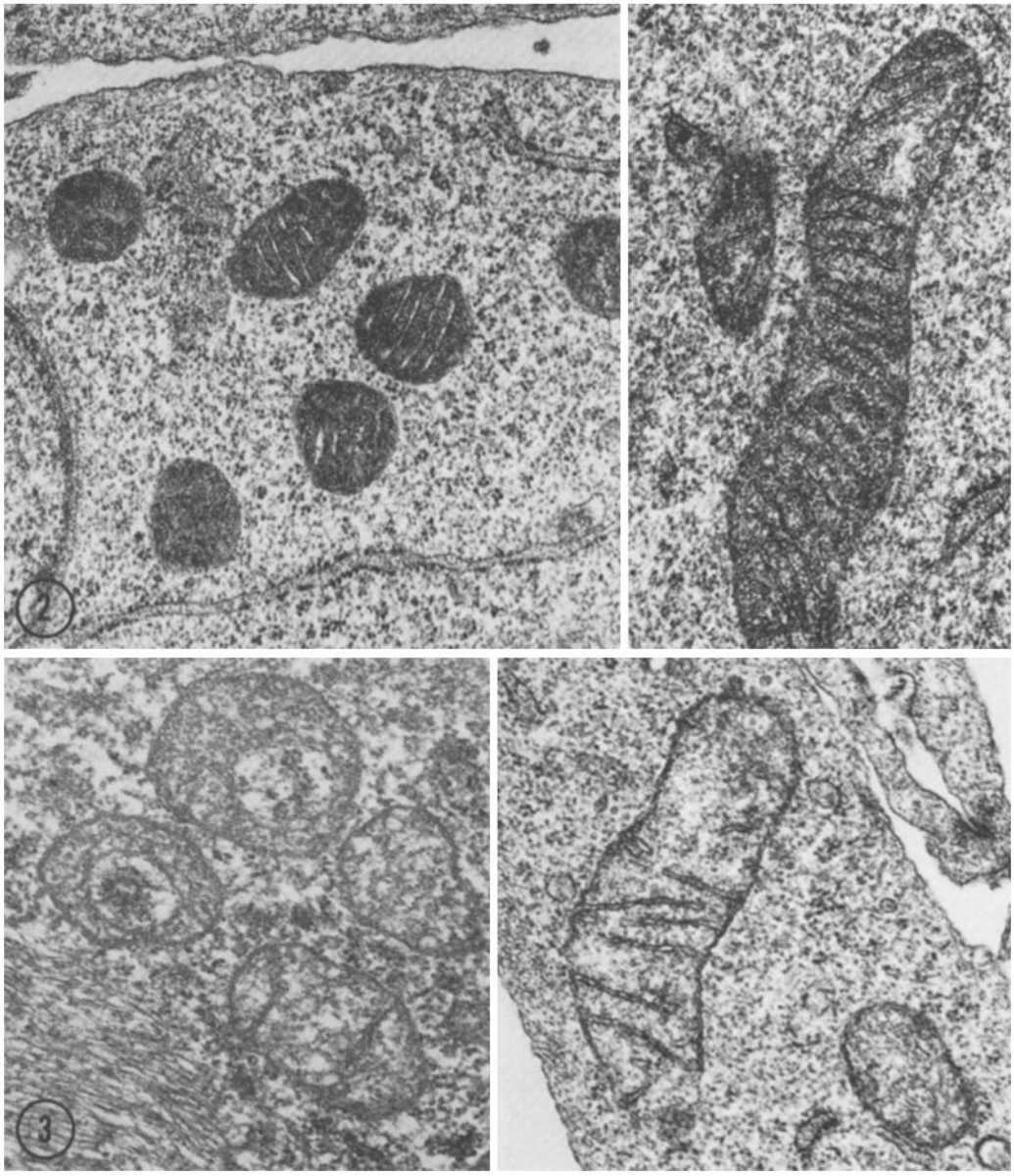


FIGURE 2 Electron micrograph of parental cell F5-1 showing typical profiles of mitochondria, both in the condensed (left) and expanded (right) configuration. × 35,000.

FIGURE 3 Parental cells F5-1 grown in the presence of 2 μg/ml EB for 4 days. Mitochondrial shapes appear irregular, cristae are relatively sparse and frequently concentrically arranged. × 35,000.

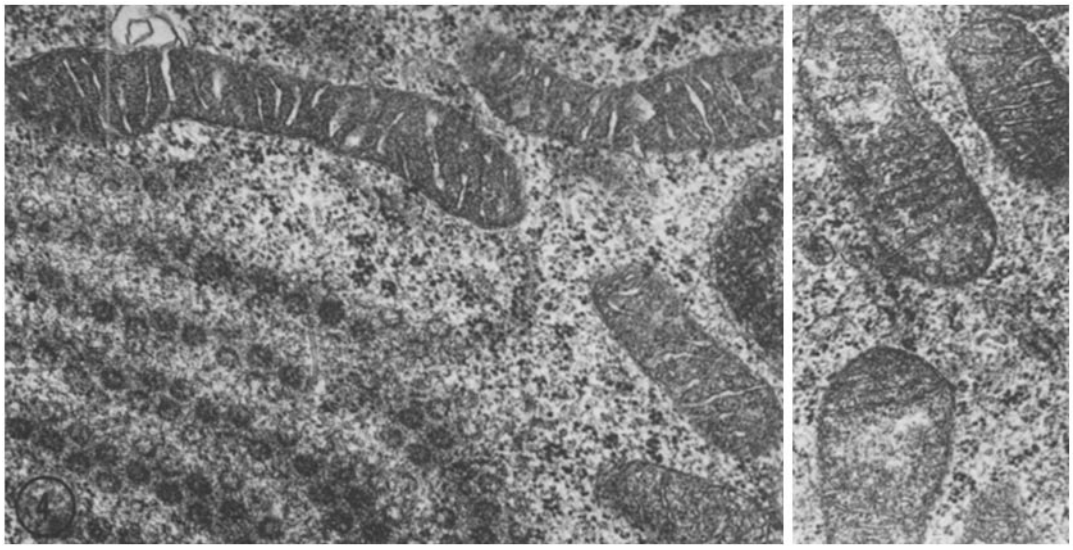


FIGURE 4 F2 cell resistant to the presence of 2 μg EB/ml (maintained for 2 yr). Mitochondria have an essentially normal ultrastructure. There appear to be relatively more mitochondria in the condensed than expanded configuration as compared to the parental line (Fig. 2). The nature of the concentrically arranged pattern seen in the cytoplasm is unknown. It was observed in only a few cells. × 35,000.

RESULTS

Analysis of F2 Cells

COLONY-FORMING EFFICIENCY OF DIFFERENT CELL LINES IN THE PRESENCE OF EB: Fig. 1 demonstrates the cloning efficiencies of various cell lines as a function of EB concentration. Human HeLa cells were most sensitive. A concentration of 1.0 μg EB/ml over a period of 12 days was completely lethal and no colonies could be scored. The other two cell lines Nil-2E and the SV40-transformed HK15-SV, which are of Syrian hamster origin, both proved to be highly sensitive to EB. The F5-1 cell, also of Syrian hamster origin, was found from the very first exposure to be less sensitive to EB than other sensitive cell lines under these test conditions. Nonetheless, the action of EB resulted not only in a significant drop of colony formation, but in a striking reduction in colony size. The F2 cell, however, which had been maintained in the presence of 2 μg EB/ml for several months at the time of the experiment, shows nearly the same cloning efficiency and colony size in the presence of 2 μg EB/ml as in normal medium. Although at higher concentrations of the drug a considerable decrease in the cloning efficiency of the F2 cell was observed, it will be seen later that cell lines growing in the presence of 4, 8, and 16 μg EB/ml could be derived from the F2 cell population, but not from the F5-1 parental cells.

ELECTRON MICROSCOPY: Fig. 2 illustrates the ultrastructure of representative mitochondria of the parental cell F5-1, showing round and filamentous mitochondrial profiles with a relatively dense matrix and numerous cristae. F5-1 cells treated with 1 μg of EB/ml for 4 days show enlarged mitochondrial profiles, a relatively electron-transparent matrix, and fewer cristae than the untreated cells (Fig. 3). Some cristae are arranged in whorl-like patterns, similar to results with EB-sensitive L cells (19). In contrast, the F2 cell grown for 16 mo in the presence of 2 μg EB/ml has an essentially normal ultrastructure (Fig. 4).

DETERMINATION OF CYTOCHROMES: Fig. 5 illustrates the reduced-oxidized spectra of total cytochromes in control, EB-treated F5-1 cells, and EB-resistant F2 cells, obtained at 77°K by the trapped steady-state method (34). The α region of the spectrum (*A*) reveals the characteristic bands of cytochromes $a + a_3$, b, and c at 599, 558, and 547 nm, respectively. In spectrum *B*, in which the F5-1 cells were treated for 11 days with EB, the absorption bands of cytochromes $a + a_3$ and c disappear and only the absorption of b cytochromes is demonstrated with an α maximum at 557 nm and a shoulder at 552 nm. In spectrum *C*, in

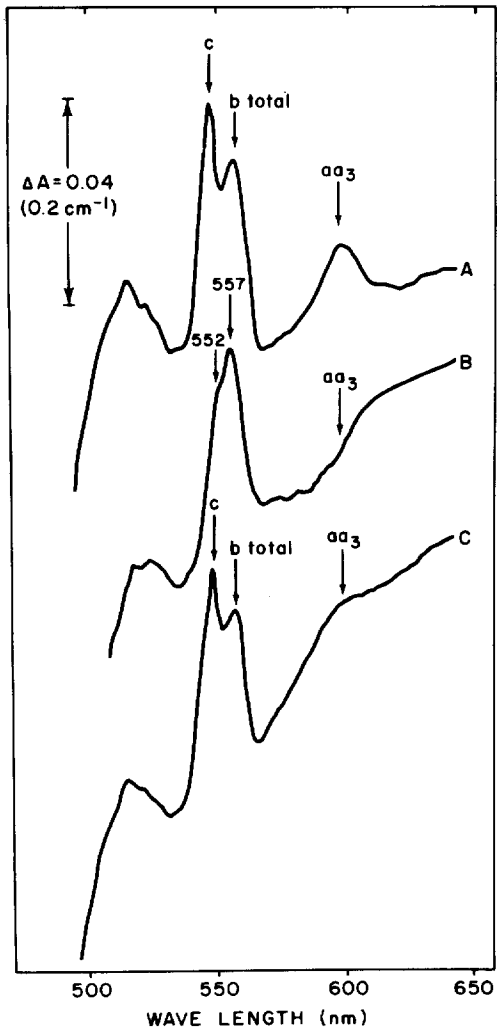


FIGURE 5 The oxidized-minus-reduced absorption spectra of the cytochromes of untreated control F-51 cells (spectrum *A*), F5-1 cells treated with 2 μg EB/ml for 11 days (spectrum *B*), and EB-resistant F2 cells treated for 8 mo with 2 μg EB/ml (spectrum *C*). The cells were suspended in PBS at 57.0, 15.0, and 56.0 mg protein/ml, respectively. Each reference sample was supplemented with 5 μM rotenone to oxidize the cytochromes in the respiratory chain, withdrawn 1 min later, and injected into a spectrophotometer cuvette which had been precooled to the liquid nitrogen temperature (33). Each measuring sample was treated with sodium dithionite, withdrawn 2 min later, and treated in the same way as the reference sample. Light path, 0.2 cm; temperature 77°K.

which the EB-resistant F2 cells are characterized, an α absorption of cytochrome $a + a_3$ is detectable, but is reduced as compared with the control F5-1 cells; however, cytochromes *b* and *c* appear to be almost the same.

When the reduced-oxidized spectra of cytochromes of the mitochondrial respiratory chain that are sensitive to cyanide were determined, the α region of the spectra revealed the bands of cytochromes *a*, *b*, c_1, and *c* at 599, 559, 553, and 547 nm, respectively (not shown). In F5-1 cells treated with EB for 11 days, all the respiratory cytochromes have disappeared and only the *b* cytochrome has remained or increased in amount. This *b* cytochrome with an α maximum at 559 nm seems to be different from the *b* cytochrome shown in Fig. 5 (*B*), which has an α maximum at 557 nm with a shoulder at 552 nm. This difference has not been further characterized. From these spectra the contents of the cytochromes were calculated (Table I). The results in Table I also show the ability of the F2 cell to recover within 4 days in the presence of normal medium to yield the same amounts of cytochromes as the parental F5-1 cell.

Characterization of F4, F8, and F16 Cell Lines

ISOLATION OF EB-RESISTANT CELLS: 1 yr after the isolation of F2 and subcultivation in EB-containing medium for more than 50 passages, the cells were exposed to medium containing 4, 8, and 16 μg EB/ml. The experiment was performed in roller bottles containing approximately 1×10^8 cells. All tissue cultures were refed in weekly intervals with new medium, containing the respective dye concentration. The cells exposed to 16 μg EB/ml all died during the second week, so did F5-1 control cells exposed to all EB concentrations tested. In the bottles containing 4 or 8 μg EB/ml a small number of the F cells survived and continued replicating. After passaging the cells for more than a month, an obviously resistant population had grown out in each bottle, which replicated without any noticeable impairment in the presence of the dye. At this time F8 cells were again exposed to 16 μg EB/ml with the intention of obtaining cells resistant to this dye concentration. The bottle was refed in weekly intervals for several weeks and retained some surviving cells which attached to the glass and grew out. After

TABLE I

Cytochrome Content of F5-1 Cells and EB-Induced F2 Cells

Cells	Concentration (nmol/g protein)				Relative amounts (%)*			
	c	$a + a_3$	*b*	*b* total‡	*c*	$a + a_3$	*b*	*b* total
F5-1 (3)§	32.2 ± 0.7	8.7 ± 0.7	11.8 ± 1.9	22.1 ± 1.1	100	27	38	69
F5-1 (2 μg EB/ml)‖ (1)	n.d.	n.d.	n.d.	87.0	—	—	—	—
F2 (2 μg EB/ml)¶ (3)	28.0 ± 2.8	5.9 ± 1.1	9.1 ± 3.0	25.0 ± 5.5	100	21	32	89
F2 (0 μg)** (3)	33.6 ± 1.4	9.5 ± 0.6	11.5 ± 2.8	21.0 ± 9.0	100	28	34	63

* Cytochrome *c* content is taken as 100%.

‡ Total of cytochromes determined by the dithionite-reduced minus the oxidized, difference spectrum.

§ Numbers of experiments are given in parentheses.

‖ EB treatment for 11 days.

¶ EB treatment for 8 mo.

** EB treatment for 8 mo, then drug was omitted from medium for 4 days.

n.d. = not detectable.

more than 1 mo we obtained a cell population replicating in the presence of 16 μg EB/ml. All three cell lines F4, F8, and F16 have been passaged since then over very high dilutions in 5-day intervals in the presence of EB in their respective concentrations. The growth rate of the F16 cell is approximately 80% that of the parental F5-1 cell.

ELECTRON MICROSCOPY: Figs. 6 and 7 show the parental F5-1 cells treated with 8 and 16 μg EB/ml for 4 days. Abnormal, irregular-shaped mitochondrial profiles are apparent. There is a heterogeneous population of mitochondria, especially in F5-1 cells exposed to 16 μg EB/ml, ranging from irregular small profiles with a condensed matrix to swollen profiles with few or poorly defined cristae. The latter types of mitochondria are barely distinguishable from the background (Fig. 7). In contrast to the parental cells, the EB-resistant F8 and F16 cells have relatively intact-appearing mitochondria, although some distinctive features are apparent. The F8 mitochondria of most cells are filamentous and packed with cristae which are in both the orthodox and condensed configuration, although the difference may not be easily apparent because of a much denser matrix in these than in normal F5-1 mitochondria (Fig. 8). In F16 cells most mitochondria display a highly condensed cristae conformation (Fig. 9).

MITOCHONDRIAL DNA: The sensitivity of the parental cells F5-1 to EB is also reflected in the mitochondrial DNA profiles obtained after exposure of the cells for 4 days to 0, 2, 8, and 16 μg of EB/ml (Fig. 10). It is apparent that in EB-treated cells no [^{3}H]thymidine was incorporated into closed circular mitochondrial DNA of region I, and this region contained almost no circular DNA molecules as detected by electron microscopy. The incorporation of [^{3}H]thymidine into DNA II (consisting here mostly of nuclear DNA) was not inhibited. In contrast, in EB-resistant F2, F4, F8, and F16 cells, [^{3}H]thymidine was clearly incorporated into closed circular mitochondrial DNA, and the DNA was located in the normal density region of the gradient (Fig. 11). A shoulder banding in the lower density region of the DNA I peaks may indicate that a small proportion of circular DNA molecules had an increased degree of supercoiling (39, 21). The presence of this shoulder also implies that at least some EB penetrated into the mitochondria.

Fig. 12 shows electron micrographs confirming the presence of closed circular mitochondrial DNA in F2 and F8 cells. Both DNA monomers and catenated dimers were present, the former in larger number. In F5-1 and F2 cells, there were approximately 10% dimers and oligomers in the fractions of covalently closed mitochondrial DNA.

Growth of F Cells in Normal Medium and Reexposure to EB

When F16 cells were grown for 5 days in medium lacking EB, all mitochondrial profiles seen still appeared in the condensed conformation, similar to the appearance of F8 and F16 mito-

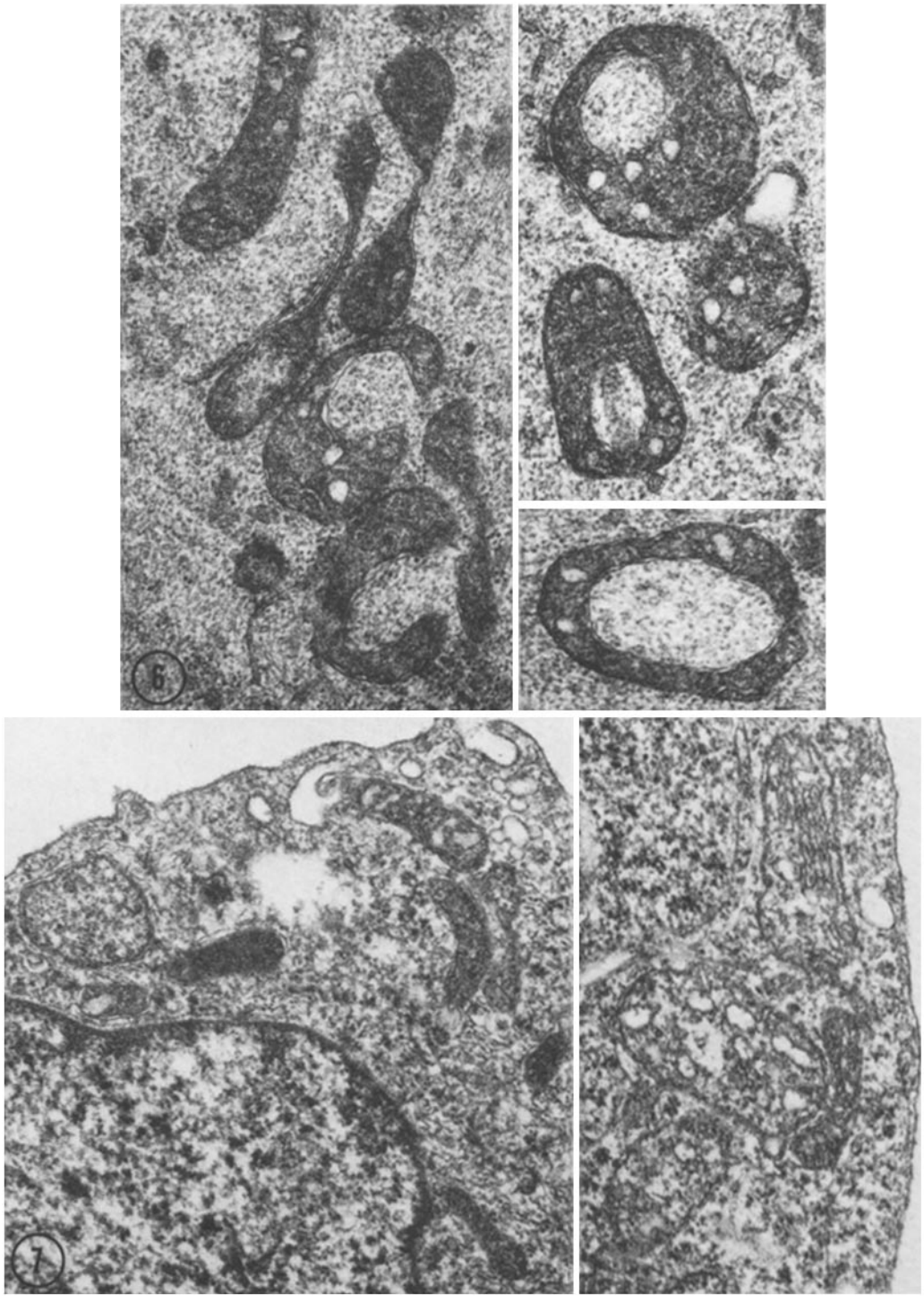


FIGURE 6 Electron micrographs of parental F5-1 cells treated with 8 μg EB/ml for 4 days. Mitochondrial profiles appear in irregular shapes; the matrix is condensed and shows rounded electron-transparent areas. × 35,000.

FIGURE 7 Parental F5-1 cell treated with 16 μg EB/ml for 4 days. Mitochondrial ultrastructure appears similar to that shown in Fig. 6, but in addition there are many swollen profiles with a low matrix density and lacking cristae. Many small dense profiles are present with a poorly defined ultrastructure. × 35,000.

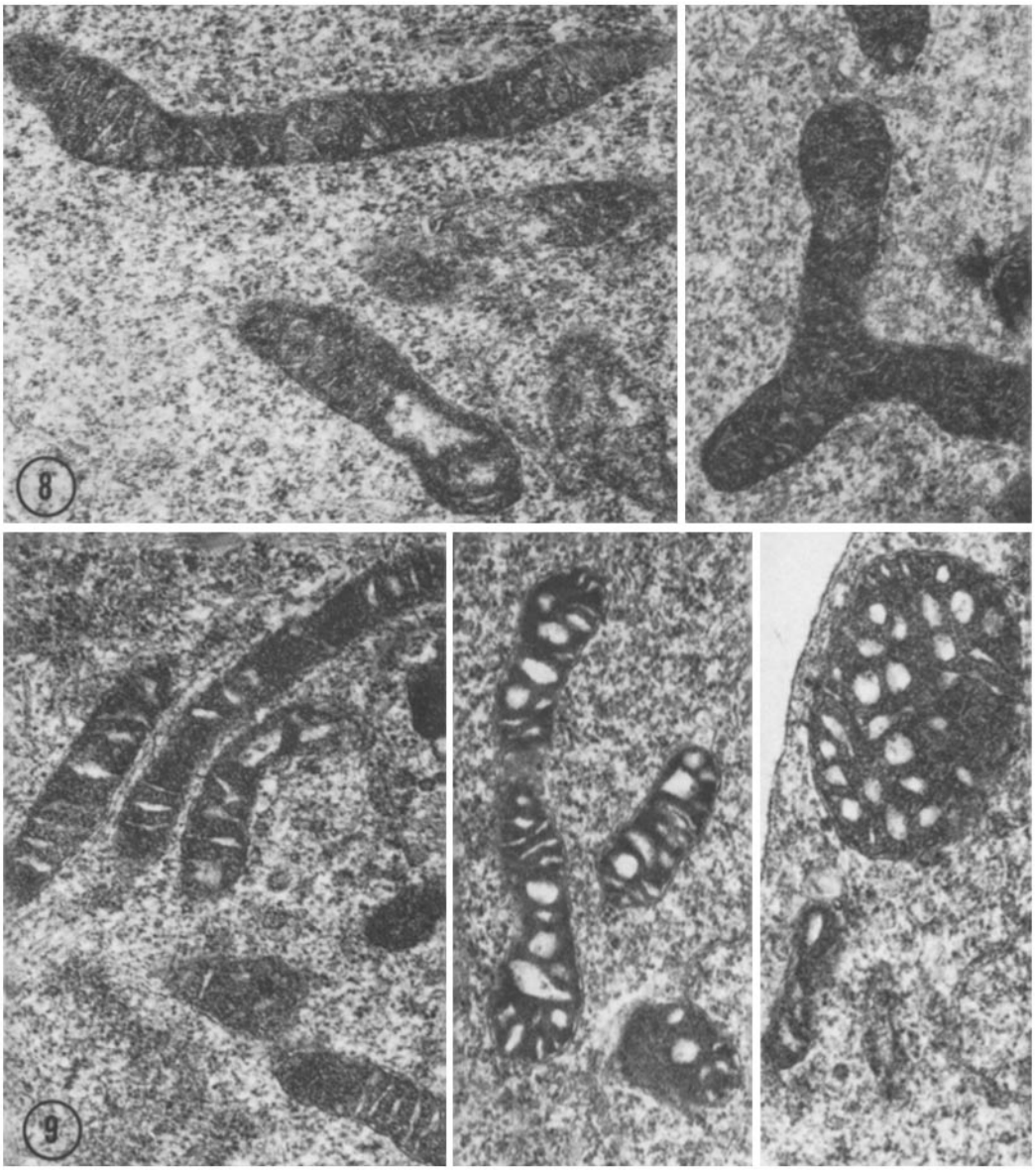


FIGURE 8 Electron micrographs of EB-resistant F8 cells maintained in 8 μg EB/ml for 4 mo. Mitochondria appear relatively normal; most are in the condensed configuration. × 35,000.

FIGURE 9 EB-resistant F16 cells grown in the presence of 16 μg EB/ml for 4 mo. Mitochondrial conformation appears highly condensed. × 35,000.

chondria grown in the presence of EB (Figs. 8, 9). However, there was a reversal to normal cytochrome content (Table I) and normal banding of mitochondrial DNA in CsCl-EB gradients (Fig. 13) when F2 and F4 cells were grown in medium lacking EB. To determine whether the newly acquired resistance to EB is possibly lost during growth in normal medium or is a stable characteristic of the cells, F4, F8, and F16 cells were passaged in normal medium for more than 1 mo. Then the cells were reexposed to media containing EB in their respective concentrations. Reexposure of F16 to EB (16 μg/ml) had no noticeable influence on the behavior and propagation of these cells in

tissue culture. The resistance to EB was thus maintained.

DISCUSSION

The results reported here present the first available evidence that mammalian cells resistant to EB can be obtained which grow under conditions that are normally lethal to other obligate aerobic eucaryotic cells. In the latter, the mitochondria appear to be a prime target for the action of EB, as described in the Introduction. The EB-resistant cells are derived from surviving clones of the SV40-transformed hamster cell line F5-1 after treatment with a sublethal concentration of EB, followed by treatment of the derivatives of these clones with increasingly higher concentrations of the drug. The new EB-resistant cell lines F2, F4, F8, and F16 reveal traits that distinguish them from the parental cell line F5-1.

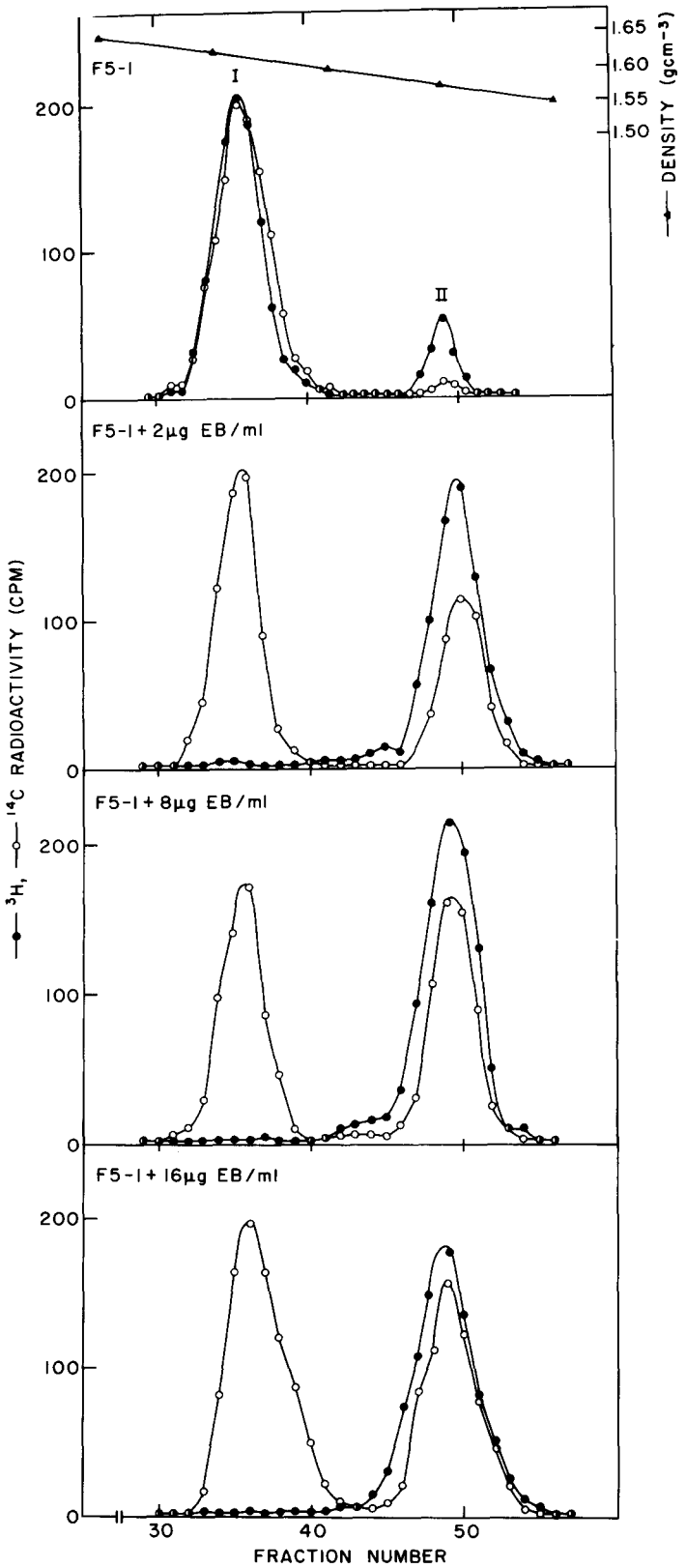
First, in the F cell there are relatively small yet reproducible phenotypic differences with respect to (*a*) mitochondrial cytochrome content, (*b*) morphology, and (*c*) structure of closed circular DNA.

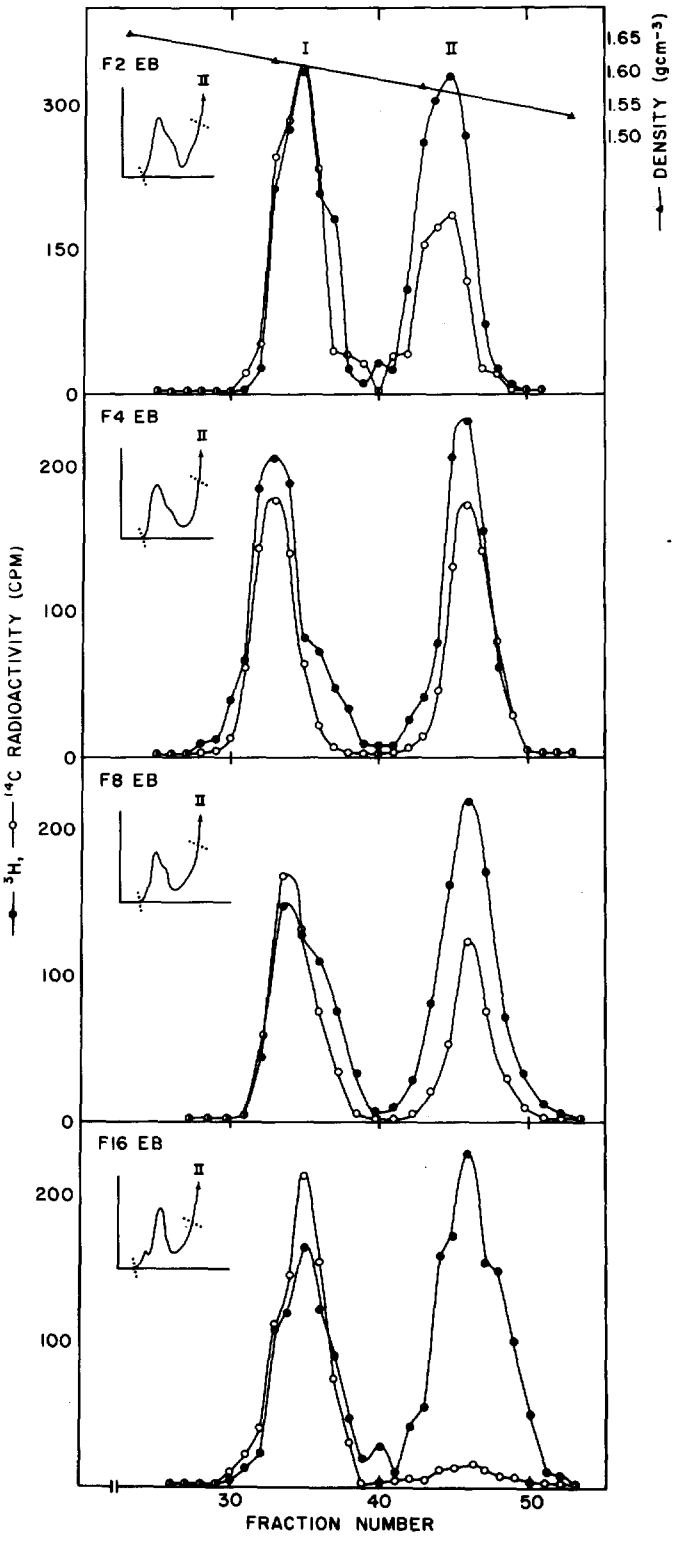
(*a*) In the F2 cell, which is the precursor of the other F cell lines grown in higher concentrations of EB, the amounts of cytochromes $a + a_3$ appear considerably decreased (Fig. 5 and Table I) as compared to amounts in untreated F5-1 cells. This decrease is not as great as the decline of cytochromes $a + a_3$ to undetectable amounts of EB-sensitive F5-1 cells treated with 2 μg EB/ml. The cytochrome analysis of the newer F4, F8, and F16 cell lines has not yet been performed. An interesting fact is that a major consequence of EB action is expressed preferentially at the level of cytochromes $a + a_3$. These cytochromes, according to present knowledge, are at least partially gene products of mitochondrial DNA and are also preferentially affected in respiratory petite mutants of yeast (11, 12) and in many tumors (e.g., 35).

(*b*) The most pronounced change in ultrastructure was an increased mitochondrial matrix density of F8 and F16 cells. Especially in F16 cells, the ultrastructural organization of the mitochondria resembles the condensed or unorthodox conformation that has been related to states of mitochondrial energy transfer and oxidative phosphorylation in other tissues (40, 41). The condensation of the matrix also resembles that found in mitochondria treated with the mitochondrial protein synthesis inhibitor chloramphenicol (42) or in mitochondria of glutamine-deprived cells (43). The morphological appearance of F cell mitochondria may be related to changes in energy production. A direct comparison in all these cases cannot be made, however, since metabolic studies of the F cells have not yet been carried out. In contrast, the mitochondria of the EB-sensitive parental cells F5-1, when grown in the presence of EB, show as expected greatly abnormal and degenerative ultrastructural changes comparable to those observed in other EB-sensitive cells (19–21). It is possible that the few small dense mitochondria observed especially in some F5-1 cells treated with 16 μg EB/ml (Fig. 7) represent residual resistant mitochondria, similar to those seen in L cells after long-term treatment with EB (19).

(*c*) The closed circular mitochondrial DNA I was was well detectable in F cells. The mitochondrial DNA I of F4 and F8 cells (Fig. 11) showed a small shoulder in CsCl-EB gradients, which may indicate a small change in superhelix density of a portion of the DNA (39, 21). This shoulder was not apparent in F16 cells, except for a small DNA peak of doubtful significance located in the region between DNA I and II. Whether these effects reflect the amounts of EB that penetrate into the mitochondria can only be tested when radioactive EB becomes available. In contrast to the EB-resistant F cell, the parental F5-1 cell when exposed to EB showed no trace of covalently closed mito-

FIGURE 10 Mitochondrial DNA of parental F5-1 cells centrifuged to equilibrium in CsCl-EB gradients. Control cells (upper frame) and cells treated with 4, 8, and 16 μg EB/ml for 4 days are shown. [^{3}H]Thymidine (1.5 μCi/ml) was added during the last 2 days of the 4-day treatment with EB. Region I represents closed circular DNA (DNA I), region II nicked circular and linear mostly nuclear DNA (DNA II). Mitochondria were isolated from 4×10^8 cells in each case. DNA was first purified in a CsCl-EB gradient (300 μg EB/ml), then region I and part of II were centrifuged in a second CsCl-EB gradient (200 μg EB/ml) in the presence of ^{14}C-labeled marker DNA (SV40 DNA I and II) as shown above. No labeled mitochondrial DNA I is apparent in EB-treated cells.


F5-1
I
II
1.65
1.60
1.55
1.50
DENSITY (gcm⁻³)
F5-1 + 2µg EB/ml
F5-1 + 8µg EB/ml
F5-1 + 16µg EB/ml
200
100
0
—● ³H, —○ ¹⁴C RADIOACTIVITY (CPM)
30
40
50
FRACTION NUMBER


F2 EB
F4 EB
F8 EB
F16 EB
I
II
1.65
1.60
1.55
1.50
DENSITY (gcm⁻³)
300
150
0
200
100
0
200
100
0
200
100
³H, ¹⁴C RADIOACTIVITY (CPM)
30
40
50
FRACTION NUMBER

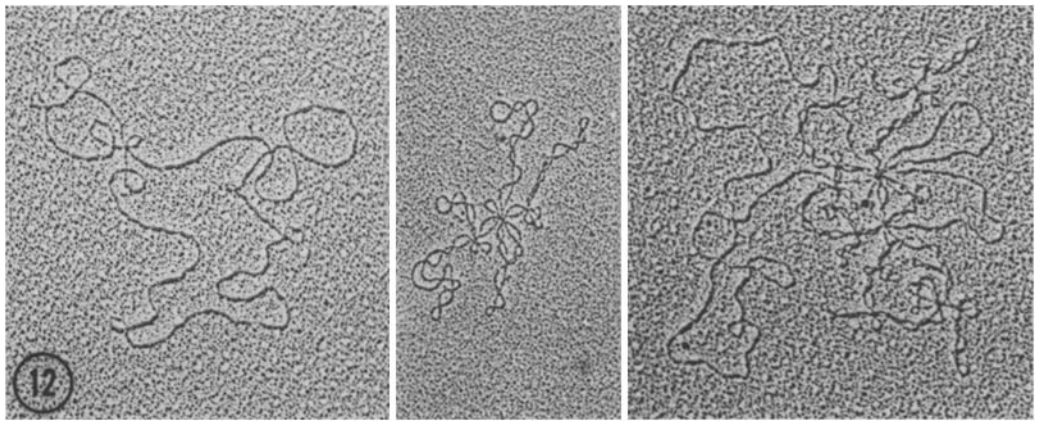


FIGURE 12 Electron micrography of circular DNA molecules of F2 mitochondria isolated from region I of a parallel gradient to that shown in Fig. 11, with no SV40 marker DNA added. A relaxed monomer of F2, a supercoiled monomer of F8, and a dimer of F2 (presumably catenated, consisting of a supercoiled and a relaxed monomer) are shown. × 40,000.

chondrial DNA in the normal position of cesium chloride gradients, in agreement with results with other EB-sensitive cells (20, 21).

Most importantly, there is strong indication for a genetic change in the F cell, given by the fact that the resistance of this cell has been hereditarily transmitted through hundreds of cell generations and was retained even after a period of growth in EB-free medium (during which time some phenotypic differences temporarily returned to normal). It is unlikely that there is a correlation between ease of developing resistance to EB and a species specificity of Syrian golden hamster or SV40 transformation since the cloning experiments with SV40-transformed HK15/SV cells and hamster-derived Nil-2E cells proved these cells to be sensitive to EB. Also, studies with HeLa cells have shown that, even in these highly EB-sensitive cells, EB-resistant clones can be obtained which, after passages for several months, show altered physical characteristics of the mitochondria (44).

Future genetic experiments will be needed to clarify whether the resistance to EB is the result of a gene mutation, either chromosomal or non-chromosomal, spontaneous or induced. A possible explanation for the resistance of the F cell to EB could be a change in the composition of the cellular and/or mitochondrial membranes causing a decrease in the amount of dye that penetrates into the cell and/or mitochondria. Such change(s) could conceivably be either the result or cause of a mutation in DNA (nuclear or mitochondrial). An affinity of EB for increased binding to the mitochondrial membrane has been reported which causes an energy-linked transition in the membrane (45). This could result in a structural change of the inner mitochondrial membrane that may also alter enzymes and/or the attachment site of mitochondrial DNA. It has been suggested that petite mutants of yeast can be obtained efficiently as a result of a hereditarily or transiently induced membrane abnormality (46, 47). A membrane change in the F cell is suggested by our preliminary experiments in which the rate of L-

FIGURE 11 Mitochondrial DNA of EB-resistant F2, F4, F8, and F16 cells, maintained in the presence of 2, 4, 8, and 16 μg EB/ml, respectively. The DNA profiles are obtained from the second CsCl-EB centrifugation, as described in Fig. 9. Representative profiles from several experiments with each cell are shown. [^{3}H]Thymidine was added to the cultures as follows: to F2, 2 μCi/ml for 48 h; F4 and F8, 2.5 μCi/ml for 96 h; F16, 2 μCi/ml for 48 h. The DNA profiles correspond to material isolated from 4×10^8 cells. ^{14}C-labeled marker DNAs: F5-1 DNA I for F2 DNA; SV40 DNA I plus DNA II for F4, F8 DNA; SV40 DNA I for F16 DNA. Mitochondrial DNA I, with a slight right-hand shoulder, is apparent.

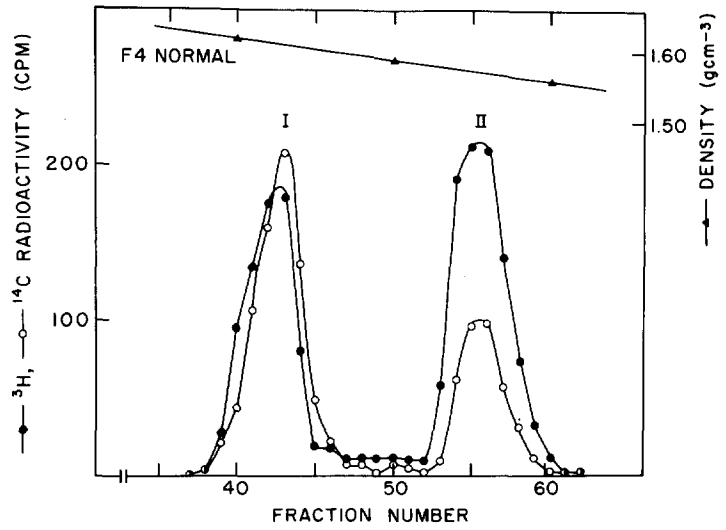


FIGURE 13 Mitochondrial DNA of F4 cells grown in normal, EB-free medium for 3 wk. Other conditions as described for F4 cells in Fig. 10.

fucose and glucosamine incorporation into F cells differed from that in F5-1 cells, although the pattern was not analogous to that described for actinomycin D-resistant cells (4). To shed light on the molecular basis for resistance to EB (48), experiments are planned using radioactive EB (soon available to us) to study the fate of EB, as well as isolated F and F5-1 mitochondria and surface membranes to characterize their composition and enzyme patterns. It may also be possible to isolate cells resistant to even higher concentrations of EB than used thus far. Another object of future studies is the possible genetic localization of factor(s) responsible for EB resistance.

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