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Fate of Mitochondrial DNA in Human–Mouse Somatic Cell Hybrids

(density gradient centrifugation/ethidium bromide/karyotype)

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ABSTRACT **Several hybrid lines between human and mouse somatic cells, containing one or two complements of mouse chromosomes and a reduced complement of human chromosomes, have been examined for the presence of mouse and human mitochondrial DNAs. For this analysis, advantage was taken of the fact that these two types of mitochondrial DNA have a buoyant density difference in CsCl gradients of 0.008 g/cm³. In all the hybrid clones analyzed, which retained an average number of human chromosomes estimated conservatively to vary from 5 to 23, only mitochondrial DNA of mouse character was detected. It seems likely that either repression of relevant human genes by the mouse genome or loss of human chromosomes is responsible for these results. If the latter explanation is true, since chromosome loss under the conditions used here was substantially a random process, one would have to assume that the activity of nuclear genes distributed in many chromosomes is required for the survival of mitochondrial DNA.**

A considerable amount of evidence has accumulated in recent years indicating that, although mitochondrial DNA (mit-DNA) has a crucial role in the biogenesis of functionally active mitochondria, the nucleus provides a major part of the information required for the synthesis of mitochondrial constituents (see, for reviews, refs. 1 and 2). The existence of nuclear mutations in yeast that produce a "petite" phenotype (3, 4) is an example of evidence of such nuclear control. In animal cells, limitation of genetic tools has thus far hampered the analysis of the genetic control of mitochondrial structure and function. Recently, however, the development of the techniques of somatic cell genetics, and, in particular, of somatic cell hybridization, has opened the way to this type of analysis. Particularly promising in this respect appears to be the use of some interspecific somatic cell hybrids, which exhibit a preferential loss of chromosomes of one parental source. Thus, somatic cell hybrids between human and mouse cells, either from permanent cell lines or from freshly explanted diploid cultures, undergo a rapid and extensive loss of human chromosomes, while retaining all or almost all of the parental mouse chromosomes (5, 6). In the case of hybrids containing more than one complement of mouse chromosomes, the loss of human chromosomes is slower and less massive (7, 8). Recently, the isolation of an exceptional human–mouse hybrid clone exhibiting an apparently full complement of human chromosomes, but only a partial complement of mouse chromosomes, was reported (8).

Abbreviations: mit-DNA, mitochondrial DNA; BrdU, 5-bromodeoxyuridine.

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In the present work, the fate of parental mit-DNA was investigated in several human–mouse hybrid cell lines. For this analysis, advantage was taken of the possibility of resolving mouse and human mit-DNAs on the basis of their difference in buoyant density in CsCl gradients. Only mouse-type mit-DNA was detected in all hybrid clones examined, even in hybrids estimated conservatively to contain an average of at least 23 residual human chromosomes per cell.

MATERIALS AND METHODS

Cells and Media. All cell lines were grown attached to glass or plastic tissue-culture bottles in the Dulbecco-Vogt modification of Eagle's medium [or in Ham's F12 medium (9) in the case of 3T3-4E] containing 10% calf serum, and further supplemented as specified below:

(*a*) Human cell line: Clone VA2-B, deficient in hypoxanthine:guanine phosphoribosyl transferase activity [derived from the line W1 18-VA2 transformed by Simian Virus 40 (10)]: it was grown in the presence of 3 μg/ml of 8-azaguanine.

(*b*) Mouse cell lines: LM (TK^-) Cl 1D, a subline of L cells deficient in thymidine kinase (11): it was grown in the presence or absence of 30 μg/ml of bromodeoxyuridine (BrdU); another L cell derivative, A9, deficient in hypoxanthine:guanine phosphoribosyl transferase (12): it was grown in the presence of 3 μg/ml of 8-azaguanine; 3T3-4 E, a permanent fibroblast line lacking thymidine kinase (7).

(*c*) Hybrid cell lines:

(i) Clones F and F6 from a cross between human diploid line FH10 and 3T3-4 E (8).

(ii) Clones E31 and F23 from a cross between VA2-B and Cl 1D (8): they were grown in medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT) (13) until 2–3 days before radioactive labeling, when aminopterin was eliminated. Clone F51 from the same cross, which grew very slowly in the presence of aminopterin, was maintained in medium containing hypoxanthine and thymidine alone. For all three clones, at the time of addition of labeled thymidine, the medium was changed to one supplemented with hypoxanthine only.

All hybrid cell lines used were isolated and analyzed by Jami *et al.* (8) in this laboratory.

Labeling Conditions. All isotopes were obtained from CEA, France. [Methyl-³H]thymidine (7 or 26 Ci/mmol; 0.33–0.83 μCi/ml) was used to label DNA of A9 (for 96 hr) and

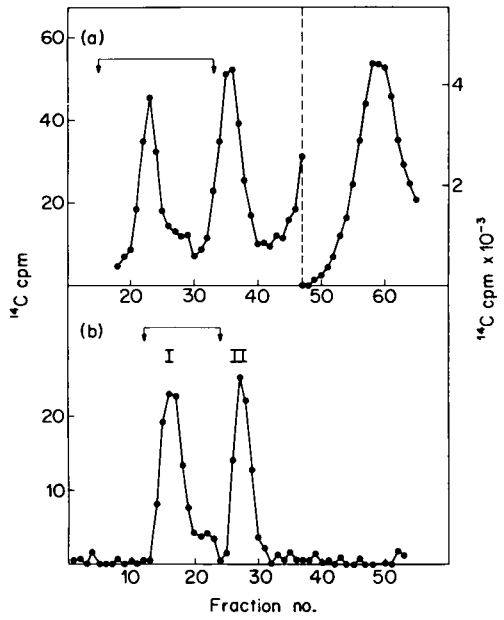

FIG. 1. Isolation of closed-circular mit-DNA from VA2-B cells labeled with [methyl-^{14}C]thymidine. (*a*) Velocity sedimentation of total mit-DNA through a CsCl–ethidium bromide solution. (*b*) The fractions indicated by *arrows* in (*a*) were pooled and run to density equilibrium through a CsCl–ethidium bromide gradient.

of the hybrid cell lines (for 48 or 72 hr). [Methyl-^{14}C]-thymidine (55.3 Ci/mol; 0.020–0.070 μCi/ml) was used to label the DNA of A9 and VA2-B cells (for 72 hr). DNA of Cl 1D cells was labeled with [5-^{3}H]deoxycytidine (18 Ci/mmol; 0.33–0.41 μCi/ml), and that of 3T3-4 E cells, with [2-^{3}H]deoxyadenosine (10 Ci/mmol; 0.41 μCi/ml), both for 72 hr.

Isolation of mit-DNA. Cells were detached from the bottles by trypsinization at 37°C for 5–10 min, washed twice with 0.01 M phosphate buffer (pH 7.0)–0.16 M NaCl (14), and swollen in hypotonic buffer for 45–60 min. Homogenization and differential centrifugation to isolate the mitochondrial fraction were described (15). The mitochondrial pellet was resuspended in 0.25 M sucrose–0.01 M Tris buffer (pH 6.7) (2.5 ml per 0.5–1.0 ml of the original volume of packed cells). Total mit-DNA was extracted from the pellet by a modification of the procedure of Smith, Jordan, and Vinograd (16). Briefly, after addition of $MgCl_2$ to 1.5 mM, NaCl to 0.01 M, and RNase and DNase, each to 100 μg/ml, the suspension was incubated at 2°C for 45 min, adjusted to 3 mM EDTA, and centrifuged at 10,000 rpm for 10 min in the Servall SS-34 rotor. The final mitochondrial pellet was resuspended in 2.4 ml of lysing buffer [0.01 M Tris buffer (pH 7.4)–0.01 M EDTA–1.2% sodium dodecyl sulfate]. When lysis was complete, Pronase was added to 75 μg/ml: after 30 min incubation at 37°C, the lysate was adjusted to 1 M CsCl, left at 2°C for about 60 min, and centrifuged at 15,000 rpm for 15 min in the Servall SS-34 rotor. The supernatant was centrifuged in the Spinco 50 rotor at 35,000 rpm for 16–17 hr. The pelleted nucleic acids were dissolved in 1.0 ml of 0.01 M Tris buffer (pH 7.4)–0.01 M EDTA, and total mit-DNA was separated from degraded nuclear DNA and RNA by centrifugation of this solution through a 2-step gradient (1 ml CsCl, ρ = 1.76, below 3.2 ml CsCl, ρ = 1.40, in 0.01 M Tris buffer (pH 7.4)–0.01 M EDTA–100 μg/ml of ethidium bromide) in the SW 50L Spinco rotor at 38,000 rpm for 5 hr. Fig. 1*a* shows a typical pattern obtained from VA2-B cells labeled with [^{14}C]thymidine: the lower band represents closed-circular mit-DNA, the intermediate, open-circular mit-DNA, and the upper, degraded nuclear DNA. The fractions corresponding to the lower band were pooled and rerun to equilibrium in a CsCl–ethidium bromide density gradient (17) in the SW 50L rotor at 33,000 rpm for 48 hr. Fractionation and analysis of radioactivity led to a pattern like that shown in Fig. 1*b*. The lower band represents closed-circular mit-DNA, and the upper band, mostly open-circular mit-DNA arising from nicking of closed molecules. Only the lower band was used for further analysis.

The ethidium bromide was removed either by dialysis of the DNA samples against standard saline citrate [SSC: 0.15 M NaCl–0.015 M Na citrate (pH 7.0)] containing 1 mM EDTA for 3 days (18), or by passage through a Dowex 50W-X8 column (17).

Analysis of Density of mit-DNA in CsCl Gradients. Suitable aliquots of purified closed-circular mit-DNA in SSC containing 1 mM EDTA or in 0.01 M Tris buffer (pH 7.4)–0.01 M EDTA, from two differently labeled sources were mixed and brought to ρ = 1.70 with CsCl, usually in a total volume of 3 ml. Centrifugation was in the SW 50L Spinco rotor at 20°C for 72 hr at 33,000 rpm. 3-Drop fractions were collected from the bottom of the tubes directly on 25-mm paper filters, which were washed with trichloroacetic acid, ethanol, and ether, dried, and counted in a scintillation counter. (Samples in the peak regions were counted for 60 min or up to at least 500 counts). Several fractions throughout the gradient were collected in glass tubes for determination of their refractive index. The density (ρ) values reported in the figures have been determined by use of the equation:

$$\rho^{25°C} = 10.860/n_D^{25°C} - 13.497 \quad (19),$$

but have not been corrected for the small contribution to the density made by the buffer components.

RESULTS

Characteristics of hybrid clones

The origin and general behavior of the hybrid clones used in this work have been described (8). Table 1 shows the karyological characteristics of the parental and hybrid strains, based on examination of 20 metaphases for each, as they appeared within 1–2 days of the time when the cells were collected for the isolation of mit-DNA.

Clones F and F6, resulting from a cross of FH10 (carrying no selective marker) and 3T3-4E, and isolated in HAT selective medium, showed no evidence of the persistence of any human parental cells by karyological examination. Both these hybrids appeared to consist of cells with two sets(2 S) of mouse chromosomes, and only a part of the original human chromosome complement (Fig. 2). On the basis of the excess of the total number of chromosomes over that expected for two sets of mouse chromosomes, one could estimate an average of 11 residual human chromosomes per cell in the case of the F line, and of 31 human chromosomes in the case of the F6 line. By use of the more stringent criterion of the

number of metacentric chromosomes (thus not taking into account the possible residual human acrocentric chromosomes), the minimum estimate of human chromosomes would be an average of 8 for the F line and 17 for the F6 line.

The other three clones analyzed were obtained from a cross between VA2-B and Cl 1D, and were isolated in HAT selective medium. Two of these clones, which were maintained in HAT medium, contained full complements of mouse chromosomes (one set for F23 and two sets for E31) and were deficient in human chromosomes, having retained, on the average, six and 22–24 human chromosomes, respectively, as estimated on the basis of total chromosome number,

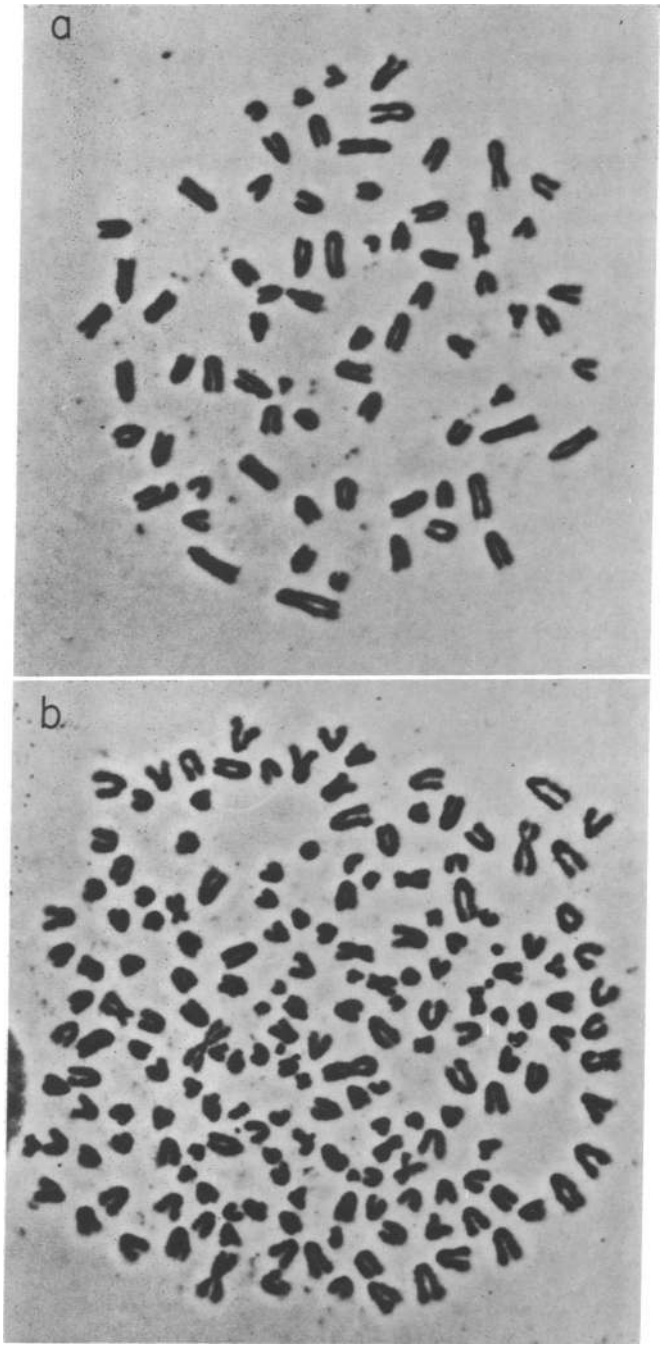

FIG. 2. (*a*) Mitotic figure of a 3T3–4 E parental cell containing 71 chromosomes. (*b*) Mitotic figure of an F6 hybrid cell containing in total 178 chromosomes, of which 16 are metacentric.

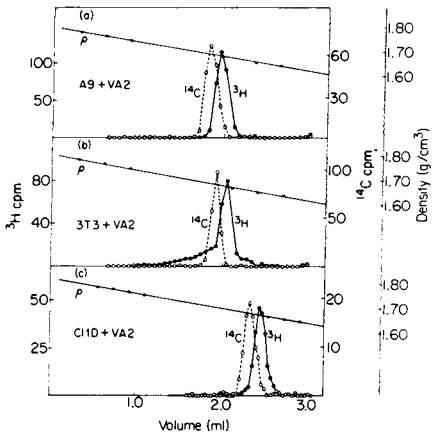

FIG. 3. Buoyant densities in CsCl of mouse and human mit-DNAs. Mixtures of [methyl-^{14}C]thymidine-labeled VA2-B mit-DNA with [2-^{3}H]deoxyadenosine-labeled 3T3–4 E mit-DNA (*a*), [methyl-^{3}H]thymidine-labeled A9 mit-DNA (*b*), or [5-^{3}H]deoxycytidine-labeled Cl 1D mit-DNA (*c*).

or at least five and 19–23 human chromosomes, as estimated on the basis of the excess of metacentric chromosomes. The third clone, F51, at the start of growth for the preparation of mit-DNA, was unique in that it contained an apparently full complement of human chromosomes, while having only 6–30 mouse chromosomes. Since this clone grew very poorly in selective medium, aminopterin had to be removed. At the time of final karyological examination, clone F51 resembled closely the parental VA2-B cells, but appeared to contain, on the average, about five more chromosomes per cell.

Resolution of human and mouse mit-DNAs by CsCl density equilibrium analysis

Previous reports (for summary, see ref. 20) had given an indication that human and mouse mit-DNAs differ enough in buoyant density so as to be separable in CsCl density gradients. CsCl density gradient centrifugation of a mixture of ^{3}H-labeled mit-DNA from mouse cells (3T3-4 E or A9) and ^{14}C-labeled mit-DNA of human source VA2-B, showed the mouse DNA to be reproducibly less dense by 0.008 g/cm^3 (Fig. 3*a* and *b*, respectively). Attempts to isolate closed-circular mit-DNA from Cl 1D cells (resistant to BrdU and grown routinely in the presence of 30 μg/ml of this drug to prevent reappearance of cells with thymidine kinase activity), with the use of widely different conditions of labeling, cell detachment and breakage, and of the isolation procedure for mit-DNA, were all unsuccessful. A small amount of apparently linear mit-DNA was obtained from Cl 1D under the conditions described in *Methods*, but this material gave a broad band in a CsCl gradient in the density region from $\rho = 1.70$ to $\rho = 1.73$, indicating that BrdU had been incorporated into mit-DNA. It was only upon the elimination of BrdU from the growth medium of Cl 1D that closed-circular mit-DNA could be isolated from this source; this DNA showed the same density difference with respect to the VA2-B mit-DNA marker (0.008 g/cm^3) (Fig. 3*c*), as did the other mouse cell lines. A9 mit-DNA was chosen to serve as a marker in the analysis of mit-DNA of the human–mouse hybrid cells (see below), since it could be conveniently labeled with [methyl-^{14}C]thymidine.

TABLE 1. *Characteristics of the human-mouse hybrid and parental cell lines*

Cell line	Total number of chromosomes (*a*)	Total number of mouse chromosomes expected (*b*)	Estimate of number of human chromosomes (*a–b*)	Total number of metacentric chromosomes (*c*)	Number of metacentric chromosomes expected from mouse parent (*d*)	Estimate of number of human metacentric chromosomes (*c–d*)
Parental						
VA2-B	72.7 (67–80)	—	—	—	—	—
LM (TK$^-$) Cl 1D	52.1 (47–54)	—	—	8.8 (7–11)	—	—
3T3-4 E	72.0 (67–82)	—	—	—	—	—
Hybrid						
(*A*) FH10 x 3T3-4 E						
F	155.1 (147–162)	144.0 (2 S)	11.1	8.4 (6–12)	—	8.4
F6	174.6 (157–195)	144.0 (2 S)	30.6	16.8 (10–25)	—	16.8
(*B*) VA2-B x Cl 1 D						
E31$_I$*	128.3 (114–144)	104.2 (2 S)	24.1	36.5 (26–51)	17.6 (2S)	18.9
E31$_{II}$*	126.1 (105–144)	104.2 (2 S)	21.9	40.6 (33–48)	17.6 (2S)	23.0
F23	58.2 (52–65)	52.1 (1 S)	6.1	13.5 (9–17)	8.8 (1S)	4.7

* Cultures independently grown from the same original stock for 24 (I) and 40 (II) days.

Analysis of mit-DNA from hybrid strains

Using ^{14}C-labeled mit-DNA from A9 cells as marker, we examined the hybrid cell lines listed in Table 1 for the presence of mouse and human mit-DNAs. Fig. 4 shows the type of mit-DNA present in the two hybrid cell lines resulting from the FH10 x 3T3-4E cross. In both cases, the radioactivity of the hybrid mit-DNA coincides almost perfectly with that of the mouse marker, despite the fact that the hybrid cells contain a substantial number of residual human chromosomes (in particular F6, with an average of at least 17 human chromosomes per cell). A similar result was obtained with clones E31 and F23 derived from the VA2-B x Cl 1D cross (Fig. 5*a* and *b*). Reconstruction experiments showed that the presence in the hybrid cells of 10 percent human mit-DNA could have been detected. Two cultures grown independently from clone E31 and the culture grown from clone F23, had, on the average, at least 19, 23, and 5 human chromosomes per cell, respectively. Clone F51, which at the time of labeling showed a karyotype similar to the parental VA2-B, with, however, an average of five chromosomes in excess over the parental number, contained only human-type mit-DNA (not shown); however, it is difficult to say, on the basis of the available data, whether the VA 2-B-similar cells were derived from hybrid cells having lost almost all the mouse chromosomes, or contaminating parental VA2-B cells, which took over the culture after the removal of aminopterin from the medium.

DISCUSSION

The main result reported here is that in human–mouse somatic cell hybrids still retaining a substantial number (up to an average of at least 23) of the original human chromosomes, only mouse-type mit-DNA was detectable. Two main explanations can be entertained for this result. The first is that the human nuclear and/or mitochondrial genes necessary for survival of human mit-DNA are repressed in the hybrid cell, independently of any chromosome loss, while the corresponding mouse genes are expressed normally. The second possibility is that the disappearance of human mit-DNA

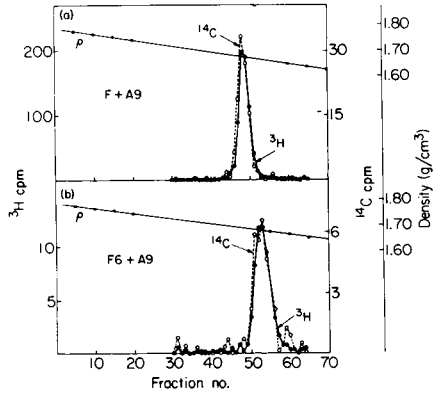

FIG. 4. Buoyant density in CsCl of mit-DNA from human–mouse hybrid cell lines, F and F6, derived from a cross between FH10 and 3T3-4 E. Mixtures of [methyl-^{14}C]thymidine-labeled A9 mit-DNA with [methyl-^{3}H]thymidine-labeled F (*a*) or F6 (*b*) mit-DNA.

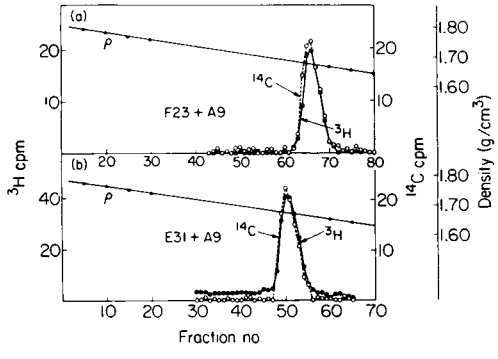

FIG. 5. Buoyant density in CsCl of mit-DNA from human–mouse hybrid cell lines, F23 and E31, derived from a cross between VA2-B and Cl 1D. Mixtures of [methyl-^{14}C]thymidine-labeled A9 mit-DNA with [methyl-^{3}H]thymidine-labeled F23 (*a*) or E31 (*b*) mit-DNA.

is a consequence of the loss of a part of the human chromosomes. Since cell hybrids at the initial stages after cell fusion, when little or no human chromosome loss has occurred, cannot be analyzed for technical reasons, it is not possible to distinguish between these two alternatives. This, however, should be feasible to test by the use of interspecific cell hybrids exhibiting little or no chromosome loss.

Since no obvious selective pressure existed for the retention of particular human chromosomes under the conditions used here [apart from the chromosome bearing the thymidine kinase gene (5, 21, 22)], it is likely that the loss of human chromosomes was substantially random. Therefore, if the disappearance of human mit-DNA is a consequence of the loss of part of the human chromosomes, to account for the present results one has to assume that the nuclear genes whose activity is essential for the survival of human mit-DNA are distributed in many chromosomes. This would not be a surprising result, since it is likely that replication of mit-DNA is coordinated with the growth and division of mitochondria, and since the greater part of the information for the synthesis of mitochondrial protein and lipid constituents is of nuclear origin. One would have to conclude, however, that mouse nuclear gene products cannot substitute, or substitute only in part, for the corresponding human nuclear gene products, and that, if any hybrid mitochondria are formed, they are selected against in the mitochondrial population.

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