Interspecific Recombination of Mitochondrial DNA Molecules in Hybrid Somatic Cells

(CsCl gradient centrifugation/RNA·DNA hybridization/biogenesis of mitochondria/cytoplasmic genetics)

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ABSTRACT The mitochondrial DNA (mtDNA) in rodent-human hybrid somatic cells was studied in strains that contain nucleotide sequences from both parental mtDNAs. A test for linkage of rodent to human mtDNA was devised on the basis of the density and sequence differences between these DNAs. When a mixture of rodent and human mtDNAs was banded in a CsCl gradient and each fraction hybridized with a mixture of complementary [³H]RNA transcribed from human mtDNA and complementary [³²P]RNA transcribed from rodent mtDNA, each DNA was detected as a distinct and separate band at its expected density by specific hybridization with its complementary RNA. In some of the hybrid cell strains, the mtDNA sequences derived from the two species did not separate in the CsCl gradients. This result is interpreted as evidence for linkage between sequences from the two parental mtDNAs. While the exact nature of the linkage and the structure of the molecules containing both types of sequences are not known, the evidence supports the conclusion that the linkage is realized by a covalent bond. An event leading to the covalent bonding of these different sequences may be described as recombination. Among 18 hybrid cell strains examined, 13 contained large proportions of recombinant molecules. These molecules were found in both mouse-human and rat-human strains, and in strains containing more human or more rodent mtDNA sequences.

We have reported on rat-human and mouse-human hybrid cell strains that contained mitochondrial DNA (mtDNA) sequences from both parental cells after many generations of growth and after one or two steps of subcloning (1). The existence of cells hybrid for mtDNA posed the question of the relation of the different mtDNA sequences to each other, since fusion of the different mitochondria in the hybrid cell appeared likely. We report here experiments that show that in many such cell strains the two different mtDNAs interact in a way that most likely represents recombination between these molecules.

MATERIALS AND METHODS

The conditions of cell culture and Sendai virus-induced cell fusion have been described (1).

DNA was extracted from whole cells as described (1). In some experiments partially purified mtDNA was prepared.

Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; cRNA, complementary RNA, referring to RNA transcribed *in vitro* with RNA polymerase.

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For this purpose cells were broken by homogenization in 10 mM Tris·HCl, pH 8.0, 50 mM NaCl, 1.5 mM MgCl₂, nuclei and debris were pelleted at 1500 rpm (600 × *g*), and mitochondria were collected by centrifugation at 10,000 rpm (12,000 × *g*) for 15 min. The mitochondria were washed twice in 0.25 M sucrose, 30 mM Tris·HCl, pH 7.4, 1 mM EDTA, and the DNA was extracted and purified as described (1). In such preparations, mtDNA is enriched but not pure since no isopycnic banding nor DNase treatment of the mitochondria was done. These treatments were omitted to increase the yield of DNA from relatively small samples of cells.

Purified mtDNA from rat or mouse liver and from human placenta was prepared and transcribed with *Escherichia coli* RNA polymerase (EC 2.7.7.6) as described (1). RNA·DNA hybridization experiments were done as described (1). In all experiments, [³H]cRNA transcribed from human mtDNA and [³²P]cRNA transcribed from rodent mtDNA was used. Each hybridization reaction contained from 2 to 10 × 10⁵ cpm of each complementary RNA (cRNA) per ml. Specific activities of the cRNAs and input ratios of ³H- and ³²P-labeled cRNA varied. Therefore, isotope ratios of RNA bound to DNA do not reflect directly the proportion of rodent and human mtDNAs, but are converted into such proportions with the aid of a calibration curve (see ref. 1).

DNA was fractionated in CsCl gradients (2) in a no. 40 or 65 fixed-angle rotor of a Beckman centrifuge. Fractions were collected through a hole punctured in the bottom of the tube. All densities are referred to *E. coli* DNA as a standard with a density of 1.710 g/cm³ (3). For estimation of densities in preparative CsCl gradients, we included DNA from *Pseudomonas aeruginosa* (density 1.727 g/cm³). In some gradients the peak of nuclear DNA (nDNA) was localized by hybridization (see Fig. 1). Mouse or rat nDNA band at 1.699 and human nDNA at 1.697 g/cm³. Densities between two marker bands were estimated, taking into account the nonlinearity of such gradients (4). These density estimations are accurate within 1–2 mg/cm³.

RESULTS

Propagation of mtDNA Sequences from Both Parental Cells in Hybrid Somatic Cells. Our previously reported results (1) and some additional experiments are summarized as follows. Hybrid cell strains were obtained by fusion of freshly dissociated cells from rat or mouse embryos with the human cell lines VA-2 or D 98/AH-2. In these hybrid cells, preferential segregation of either the human or the rodent chromosomes has been observed. At 40 to 60 population doublings after

FIG. 1. Separation of human and mouse mtDNAs in a CsCl gradient. A mixture of 30 μ g of human and 25 μ g of mouse whole-cell DNA and 22 μ g of *P. aeruginosa* DNA was centrifuged to equilibrium and fractionated. The peak of *P. aeruginosa* DNA was located by its absorbance. Fractions lighter than this peak were adsorbed to filters and hybridized with [3 H]cRNA transcribed from human mtDNA and [32 P]cRNA transcribed from mouse mtDNA. After processing and counting, the filters were washed with chloroform and the RNA was removed by heating to 50° in 50% formamide. The filters were then rehybridized with [3 H]cRNA transcribed from human nDNA. The fraction number in parentheses is the last fraction of the gradient (4.5 ml). (O) [3 H]cRNA hybridization: human mtDNA sequences; (●) [32 P]cRNA hybridization: mouse mtDNA sequences; (thin solid line) A_{260} : *P. aeruginosa*; (thin dashed line) rehybridization with [3 H]cRNA: human nDNA sequences.

fusion, 19 of 34 independently derived rat-human hybrid strains contained both rat and human mtDNA sequences as well as chromosomes from both species. Nine mouse-human hybrid cell strains were found that contained mtDNA sequences from both species at the same time after fusion. Subclone sets from five different rat-human and five mouse-human hybrid strains still contained mtDNA sequences of both the human and rodent type after 25 to 150 additional doublings. These data show that the mtDNAs (or parts thereof) from two different animal species had replicated for an extended period in the same cell.

Molecular Test for Linkage (Recombination) Between Rodent and Human mtDNA Sequences. The test is based on (i) the density difference between human and either mouse or rat mtDNA (see ref. 5) and (ii) the specificity of hybridization of cRNA with its template DNA (1). We have found that under stringent conditions of hybridization followed by treatment of the hybrid with ribonuclease, cross-hybridization between human mt-cRNA and either rat or mouse mtDNA is only 1–2% of the homologous hybridization level.

The separation of human and mouse mtDNAs is shown in Fig. 1. A mixture of equal amounts of whole-cell DNA from the two species was used in this experiment. The use of whole-cell DNA is possible because cRNA transcribed from pure mtDNA does not hybridize with nDNA (1). In this way it is not necessary to purify mtDNA from small samples of hybrid cells, which is often difficult. About 20 μ g of whole-cell DNA containing about 20–100 ng of mtDNA is required for each gradient. After equilibrium was established, the gradient was fractionated and the absorbance

FIG. 2. Separation of human and rat mtDNA in a CsCl gradient. A mixture of 30 μ g of human and 23 μ g of rat whole-cell DNA was banded and hybridized as described in *Methods* and the legend of Fig. 1, using human [3 H]cRNA and rat [32 P]cRNA. (O) [3 H]cRNA: human mtDNA sequences; (●) [32 P]cRNA: rat mtDNA sequences.

of each fraction was measured starting at the bottom and reading through the peak of *P. aeruginosa* DNA. DNA in the fractions to the light side of the *P. aeruginosa* peak were adsorbed individually to membrane filters and annealed with a solution containing a mixture of [3 H]cRNA transcribed from human mtDNA and [32 P]cRNA transcribed from mouse mtDNA. After the filters were processed and counted, they were rehybridized with cRNA transcribed from human nDNA (Fig. 1). This latter hybridization provided a second density marker. Human mtDNA (Fig. 1: open circles) was clearly separated from mouse mtDNA (closed circles). The densities estimated for the two mtDNA peaks using the two DNA markers as reference agree quite well with the densities determined in the analytical ultracentrifuge (1.700 and 1.707 as compared to 1.699 and 1.707). Fig. 2 shows a similar control gradient for a mixture of rat and human whole-cell DNA. The separation is less complete due to the smaller density difference (1.707 and 1.701).

To demonstrate, under the present conditions of analysis, that cross-hybridization of rodent and human mtDNA sequences is low and no significant level of radioactive cRNA is bound nonspecifically to nDNA, we banded whole-cell DNA from each species separately and hybridized the DNA-containing fractions to a mixture of homologous and heterologous cRNA. An example with rat DNA is shown in Fig. 3.

FIG. 3. Analysis in a CsCl gradient of pure rat whole-cell DNA. The fractions were hybridized with cRNAs as described in the legend to Fig. 2. The symbols have the same meaning as in Fig. 2.

FIG. 4. Analysis in CsCl of DNA from the mouse-human hybrid strain VMPS 10D. The symbols have the same meaning as in Fig. 1.

While cRNA transcribed from rat mtDNA hybridized well with the rat mtDNA present in the sample, the human cRNA was bound at a very low level. Similar results were also obtained with mouse and with human whole-cell DNAs.

Hybrid Cells Containing Recombinant mtDNA Molecules. The analysis of the DNA of a mouse-human hybrid cell strain is shown in Fig. 4. The marker peak of human nDNA is not shown, but its density (1.697) is indicated. This strain contained 39% mouse mtDNA sequences (and 61% human mtDNA). In contrast to the control (Fig. 1), the bands of human and mouse mtDNA sequences have not separated from each other in the gradient. The major peak of human mtDNA sequences banded at a density close to that or slightly lower than that of pure human mtDNA. DNA in the same region also hybridized extensively with mouse cRNA. Both peaks of hybridization were quite broad and extend into the density region where pure mouse mtDNA bands. However, the ratio of human-to-mouse sequences decreased from higher to lower density. This experiment, and similar experiments described below, show that molecules in which human and mouse mtDNA sequences are linked to each other occur in these cells. Since the preparation was treated with Pronase, sodium dodecyl sulfate, and phenol during purification, it is unlikely that DNA fragments are held together by "protein linkers" or by nonspecific aggregation. In order to explain the gradient pattern of Fig. 4, we must assume the presence of at least two types of linked molecules: some containing a high human-to-mouse ratio, and others containing a high mouse-to-human ratio of mtDNA sequences. In addition, "pure" molecules of human and of mouse mtDNA may have been present. Because of the low density difference between

FIG. 5. Analysis of DNA from the mouse-human hybrid strain VMSC 10F. The symbols have the same meaning as in Fig. 1.

FIG. 6. Analysis in a CsCl gradient of sheared DNA from the hybrid strain VMSC 10F (see Fig. 5). The DNA was sheared by sonication to obtain an average molecular weight of 1.2×10^6 , as measured by band centrifugation in an analytical ultracentrifuge. The DNA was then banded in a CsCl gradient and analyzed by hybridization. The symbols have the same meaning as in Fig. 1.

the two mtDNAs the resolution of these gradients is not very high. Thus, we cannot decide whether material banding between 1.708 and 1.705 g/cm³ consists of only molecules with a human-to-mouse ratio of, for example, 8:2, or whether there is a mixture of pure human molecules and recombinant molecules with a similar or slightly lower human-to-mouse ratio. The same type of argument (substituting mouse for human) holds for the molecules banding at about 1.700 g/cm³.

The mtDNA from another cell strain with a low proportion of mouse mtDNA is shown in Fig. 5. Again, a large fraction of the mouse mtDNA sequences band at a position coincident with the human sequences, and at a density close to that of human mtDNA. Since this cell strain contained 93% human mtDNA sequences (Table 1) one might expect that most or all recombinant molecules would be "high-human" and band at human density. A portion of the DNA molecules that contain mouse mtDNA sequences bands at lower densities. There is also a shoulder of human sequences in these low-density regions, but the significance of this shoulder is not certain. Thus, the gradient of Fig. 5 could be explained by assuming that it contains high-human recombinants and possibly pure human mtDNA; and, either pure mouse mtDNA or high-mouse recombinants, or both. These interpretations attempt to explain the observations with a minimum of heterogeneity in the population of mtDNA molecules. It is also possible that the population is very heterogeneous, from pure human molecules through many kinds of intermediate re-

FIG. 7. Analysis in CsCl of DNA from the rat-human hybrid strain VRSC 21-5S. The symbols have the same meaning as in Fig. 2.

FIG. 8. Analysis in a CsCl gradient of DNA from the mouse-human hybrid strain VMPOC 15B. The symbols have the same meaning as in Fig. 1.

combinant molecules to pure mouse molecules. The hybrid cell DNAs analyzed in Figs. 4 and 5 are similar in that both contain at least one type of linked mouse-human mtDNA molecule with predominantly human sequences. In addition, strain VMPOC 10D (Fig. 4) contains at least a second type of linked molecule with a predominance of mouse sequences.

One possible interpretation of the gradient profiles of Figs. 4 and 5 postulates that human and mouse mtDNA sequences in hybrid cells are linked by catenation (6) rather than by covalent bonds. To test this possibility, we sheared the DNA to fragments about one-eighth the size of intact mtDNA before analysis. When a mixture of mouse and human DNAs was sheared to an average molecular weight of 1.2×10^6 and subjected to gradient analysis, the two mtDNAs separated clearly even though the bands had broadened (experiment similar to Fig. 1 with slightly more overlap; not shown). Next, DNA from the hybrid strain VMSC 10F was sheared to the same size and analyzed for linked mtDNA sequences (Fig. 6). A comparison of Figs. 5 and 6 shows that almost an equal proportion of mouse sequences were linked to human sequences in the sheared sample as in the intact sample. A similar result was obtained with the DNA from another mouse-human hybrid cell strain. These experiments exclude the possibility that catenanes could be the basis for the linkage observed.

Linked, apparently recombinant, molecules of rat and human mtDNA were also observed. Fig. 7 shows an example from a twice subcloned hybrid cell strain that contained 84% rat mtDNA sequences. In this gradient all the human mtDNA sequences banded together with the rat mtDNA sequences, at a density close to or slightly higher than the density of rat mtDNA. This fact demonstrates that essentially all the human mtDNA sequences were linked to rat mtDNA sequences. The density of the recombinant molecules indicates that they were mostly rat. Whether any pure rat mtDNA molecules occurred cannot be decided at the resolution of these experiments. Because all human mtDNA sequences in this gradient banded at the density where rat mtDNA and nDNA are also found, it was important to show that little cross-hybridization or nonspecific binding of human cRNA occurred (Fig. 3).

Hybrid Cells Not Containing Detectable Recombinant mtDNA. Not all hybrid cell strains that contained both rodent and human mtDNA sequences exhibited a high proportion of linked mtDNA molecules. One example each is shown for mouse-human (Fig. 8) and for rat-human (Fig. 9) hybrid cell DNA. The two types of mtDNA in the gradient of Fig. 8 are not as well separated as in the control (Fig. 1), but this

FIG. 9. Analysis in a CsCl gradient of DNA from the rat-human hybrid strain VRSC 33. The symbols have the same meaning as in Fig. 2.

may be due to the small size of the sample or other technical reasons. The gradient in Fig. 9 is essentially identical to the control gradient (Fig. 2). Because of the limits in the resolution of the technique, the absence of recombinant molecules cannot be ascertained with high sensitivity. However, it is clear that in the samples shown in Figs. 8 and 9, most mtDNA molecules are separate, and recombinant molecules are either rare or absent. The distinction between these cases and the hybrid strains with recombinant molecules (Figs. 4, 5, and 7) is quite pronounced.

Frequency of Occurrence of Hybrid Cell Strains with Recombinant mtDNA Molecules. All hybrid cell strains tested for the presence of recombinant mtDNA by the gradient centrifugation method are listed in Table 1. Strains with patterns as shown in Figs. 8 and 9 were classified as negative. The data of Table 1 together with earlier results (1) allow the following conclusions. (i) Hybrid cell strains that contain mtDNA sequences from two animal species frequently contain recombinant mtDNA molecules in high proportions. Thirteen of 18 tested hybrid cell strains contained unequivocally linked (recombinant) mtDNA molecules. This proportion is not a measure of the frequency of recombination since the final result also depends on selective processes in the cell population whose importance cannot be estimated. (ii) Recombined mtDNA molecules occur in hybrid cells with predominantly rodent or predominantly human mtDNA sequences. (iii) Hybrid cell strains grown directly from a colony derived from fusing cells and subclonal populations derived from such hybrid cell strains may contain recombinant mtDNA molecules, or either type of population may fail to contain a high proportion of such molecules. (iv) Recombinant mtDNA molecules were found in hybrid cell populations that had undergone between 40 and 150 doublings after fusion.

DISCUSSION

Nature of Recombinant mtDNA Molecules. The gradient patterns of Figs. 4, 5, and 7, together with the method of purification of the DNA, makes it highly probable that hybrid cells contain mtDNA molecules in which human and rodent sequences are linked together without mediation by a non-DNA component, like a "protein linker." The possibility that linkage is topological rather than chemical, involving catenanes (6), is unlikely because of the results with sheared hybrid cell DNA (Fig. 6). The simplest interpretation is, therefore, that the linked molecules are the result of recom-

TABLE 1. *Recombinant and nonrecombinant mtDNA molecules in human-rodent hybrid cell strains*

Cell strain*	Sub-cloned†	Percent of mouse‡		Recombinant mtDNA
		mtDNA	nDNA	
VMSC 10F	Yes	7	N.D.§	Yes
VMSG 16	No	20	1	Yes
VME 15	No	27	32	Yes
VMSC 10 G	Yes	28	N.D.	Yes
VMPOC 15F	Yes	39	49	Yes
VMPOC 10D	Yes	39	35	Yes
VMPE 18F	Yes	40	39	Yes
VMPOC 18E	Yes	46	38	Yes
VMPth 18	No	48	9	Yes
VMPOC 15L	Yes	69	32	No
VMPOC 15B	Yes	73	20	No
VMSC 10E	Yes	71	13	No
VMSC 10B	Yes	73	19	No
Percent of rat‡				
		mtDNA	nDNA	
VRE 36A	Yes	15	58	Yes
VRSC 21-5S	Yes (2×)	84	89	Yes
VRSC 21-5N	Yes (2×)	89	90	Yes
VRSC 21-8	Yes	95	81	Yes
VRSC 33	No	24	8	No

* Strain designations allow comparison to ref. 1 and to figure legends. All rodent cells were derived from 15- to 18-day embryos, and human cells were the line VA-2(WI-18 VA-2, SV40-transformed, HGPRT⁻, see ref. 1). V = VA-2, M = mouse, R = rat, SC = spinal cord, SG = spinal ganglion, E = neural retina, OC = optic cortex, th = thalamus. P designates a separate series.

† Cell strains not subjected to subcloning had undergone 40 to 60 doublings after fusion. Subcloned strains had undergone 60 to 150 doublings.

‡ These proportions were determined by hybridization with cRNA as described in ref. 1.

§ N.D., not determined.

bination and consist of parts of rodent and human mtDNA connected by phosphodiester bonds, i.e., that the sequences from the different species now form sections of the same continuous DNA molecule.

An interpretation of the CsCl gradient analyses not involving linkage between rodent and human mtDNA sequences must also be considered. This interpretation assumes the occurrence of large deletions in one or both parental types of mtDNA molecules leading, fortuitously, to molecules that resemble in density the other mtDNA molecule in the hybrid cell. It does not appear likely that deletion would always produce fragments with such a density. Furthermore, there is some evidence that mammalian mtDNA may have very little intramolecular base-composition heterogeneity (7). Unless such heterogeneity is present, fragments will have the same density as parental molecules.

Therefore, we consider covalent linkage of parts of the parental molecules the most likely interpretation of these experiments. If this interpretation proves correct, there remain several unanswered questions regarding the structure of the recombinant molecules. Compositional heterogeneity is one open question. The gradient patterns require at least

the presence of one or two types of recombinant molecules and of one or the other parental molecule, depending on the exact nature of the particular experiment. Even this minimal heterogeneity is in marked contrast to the homogeneity of mtDNA in the cells of animals of any single species (8). However, it is possible that the heterogeneity is extensive in that molecules with variable proportions of human and rodent mtDNA sequences occur in such hybrid cell strains. Even if compositional heterogeneity is low, there could be large sequence heterogeneity; e.g., two molecules that are 80% mouse-20% human need not be identical since different human and mouse sequences may be involved in different recombinant molecules. A distinct question is the physical shape and size of recombinant mtDNA molecules, i.e., whether they are standard-size, 5- μ m circles.

Biological Implications of mtDNA Recombination. No genetic markers on the mitochondrial genome are available in animal cells. In contrast, several such markers are known in yeast, and recombination between these markers has been demonstrated (9). Recently, Michaelis *et al.* have correlated physical recombination of yeast mtDNA molecules with recombination of genetic markers (10). In this instance it is clear that the molecular recombination event is the basis for phenotypically recombinant cells. This observation encourages us to think that molecular recombination of mtDNA sequences in animal cells may be, at least in certain situations, correlated with the recombination of genetic markers. Although mtDNA recombination could be restricted to hybrid cells in which fusion was induced by Sendai virus, mtDNA recombination may occur frequently in nonhybrid animal cells. In such cells recombination could not be detected by molecular techniques, but could be detected by genetic methods if suitable mitochondrial markers became available. It is possible that the nucleotide sequences of one species inserted into the mitochondrial genome of another might themselves serve as genetic markers in further crosses. Other possible experimental applications of mtDNA recombination in hybrid cells have been discussed elsewhere (11).

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