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THE USE OF HYBRID SOMATIC CELLS AS AN APPROACH TO MITOCHONDRIAL GENETICS IN ANIMALS[1]

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ABSTRACT

We have studied the fate of parental mitochondrial DNA (mtDNA) in hybrid somatic cells derived by Sendai virus-induced fusion of human cells and mouse or rat cells. Many hybrid cell strains were obtained which contained sequences from both human and rodent mtDNA after 40 to 60 population doublings. Some strains were subcloned and cultured further for up to 150 doublings; a large fraction of these strains contained both parental mtDNA sequences at that time.

The relation between human and rodent mtDNA sequences was tested in some of the hybrid cell strains. In a high fraction of strains tested the human and rodent mtDNA sequences were linked to each other by what are most likely covalent bonds. This linkage may be described as "recombination" of mtDNA sequences from two different animals.

MITOCHONDRIA from all organisms studied contain DNA; this DNA is required for the formation of functional mitochrondria (see BORST 1972). Therefore, we may regard the mitochondrial DNA (mtDNA) as the carrier of a mitochondrial genome. The study of the function, regulation, and transmission of this genome is currently an active field. The experience with bacteria and bacteriophages clearly showed that studies of this type are greatly aided by the availability of genetic tools which, when combined with biochemical analysis, will lead to the elucidation of the mechanisms involved.

Genetic tools are available for the study of mitochondrial biogenesis in yeast (DUJON, SLONIMSKI and WEILL 1973; SAGER 1972; WILKIE 1972; BOLOTIN *et al.* 1971), and to a lesser extent in Neurospora (see SAGER 1972) and Paramecium (BEISSON and BEALE 1973). In multicellular animals the choice of genetics tools for this purpose is extremely limited. Attempts are being made at present to improve this situation in several ways: One aspect of these studies is the search in cultured cells for mutants (or variants) for mitochondrial functions. The most interesting mutants would be those which showed a cytoplasmic mode of inheritance. The search for cell strains resistant to certain antibiotics is a promising approach since cytoplasmically-inherited mutants resistant to chloramphenicol and similar drugs have been found in yeast (THOMAS and WILKIE 1968; LIN-

[1] Abbreviations: mtDNA: mitochondrial DNA; mt-rRNA: mitochondrial ribosomal RNA; cRNA: complementary RNA, referring to RNA transcribed *in vitro* with *Escherichia coli* RNA polymerase; mt-cRNA: cRNA transcribed from mtDNA as template; nDNA: nuclear DNA.

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NANE *et al* 1968). A preliminary report of success in this search has appeared (SPOLSKY and EISENSTADT 1972). However, it will be difficult to determine whether such variants are due to a mutation in the nuclear or the cytoplasmic (mitochondrial) genome. In yeast it was possible to assign certain drug-resistant mutants to the mitochondrial genome by showing their relation to the "petite" mutation (COEN *et al.* 1970) which is known to be a mutation of mtDNA (MOUNOLOU, JACOB and SLONIMSKI 1966). In cultured animal cells a different method will be required. The studies discussed below may prove to be a basis for such experiments.

An approach complementary to the search for mutants is the development of methods by which the reassortment of genetic elements from nuclei and mitochondria may be studied. One method, already successful but limited in scope, is the study of interspecific hybrids obtained by conventional breeding. If any cellular component is analyzably different in two animal species and a hybrid can be bred between these species it is possible to test which parent determines the structure of this particular component. In this way it can be decided whether that component is inherited in a Mendelian fashion or not. This approach has been used to show that mtDNA is inherited maternally and cytoplasmically in frogs (DAWID and BLACKLER 1972). The experiment was based on the fact that the mtDNA's of two frog species which can be crossed are sufficiently different to be distinguishable. Whether this approach can be extended to other molecules depends on finding dissimilarities in other mitochondrial components. If, for example, the cytochrome oxidase polypeptides of the two frog species were distinguishable one could determine whether the sequence of these polypeptides is inherited in a Mendelian (i.e., chromosomal) or non-Mendelian fashion (presumably *via* genes on mtDNA). If these polypeptides proved to be very similar in the two species the experiment is not feasible. Thus, the method is limited by the fact that only closely related animals can be crossed.

The production of hybrid cells by fusion of somatic cells is an alternate approach. It allows the hybridization of cells from very distant animals like man and mouse. Thus, very different genetic elements can be brought together in the same cell, making it more likely that phenotypic differences would be found which could be correlated with the genetic make-up of the hybrid cell. This approach has serious limitations, however. In general, it is not possible to make a hybrid cell "to order"—suitable cells are fused and grown under various conditions in the hope that the desired combination of genetic elements will arise. Manipulation is possible to a certain extent only. This limitation is exemplified by two articles which investigated the fate of parental mtDNA in hybrid cells (CLAYTON *et al.* 1971; ATTARDI and ATTARDI 1972). These authors studied mouse–human hybrid cells which contained predominantly mouse chromosomes and found only mouse mtDNA. In contrast, ELICEIRI (1973) reported the synthesis of both parental types of mt-rRNA in mouse-hamster hybrids, suggesting the presence of both types of mtDNA in the cell population.

Before hybrid cells can become a useful tool for the study of mitochondrial biogenesis it must be demonstrated that cells can be produced which contain both

parental mtDNA molecules or parts of these molecules in the same cell. We have obtained such hybrid cells and report some of their properties in this paper.

Production and properties of hybrid cells: Hybrid cells were produced with the aid of Sendai virus from cells of freshly dissociated embryonic tissues of the rat or mouse and the human cell line VA-2. Hybrid colonies were grown in HAT medium as described previously by COON and WEISS (1969) and COON, HORAK and DAWID (1973). Individual colonies consisting of 500 to 1000 cells were isolated and transferred to another culture plate. After growth corresponding to about 25 population doublings the chromosomes of 50 to 100 metaphases of each cell strain were inspected and the hybrid nature of the cells were established. After additional 15 to 35 doublings, samples were taken for karyological and biochemical analysis. Some of the cell strains were cultured further and from some strains subclones were derived at this time. Hybrid cell strains derived from freshly dissociated, primary rodent cells (either embryonic or adult) and the human cell line, VA-2, show the heretofore uncommon property of segregating either human or rodent chromosomes. Thus, cell strains were obtained which had about equal numbers of human and rodent chromosomes, as well as strains where one or the other type of chromosome predominated. This conclusion is supported by both karyotype and isoenzyme studies that will be presented elsewhere (COON and MINNA, to be published; MINNA and COON, to be published; COON, HORAK and DAWID 1973) and by the analyses presented below.

Distinguishing mtDNA's by molecular hybridization: Our method for distinguishing mtDNA's from different animals is based on the large differences in nucleotide sequence between these DNA's. We prepared cRNA transcribed by *E. coli* RNA polymerase from pure mtDNA and labeled one cRNA with ^{3}H (e.g., human ^{3}H-mt-cRNA), the other with ^{32}P (e.g., rat ^{32}P-mt-cRNA). These cRNA's were combined and hybridized to a set of filters containing mixtures of rat and human mtDNA in known proportions. After the filters were washed the $^{3}H/^{32}P$ ratio of RNA bound to each filter was plotted against the percentage of rat mtDNA in the mixture of DNA on the filter. In this way a standard curve was obtained (Figure 1). When filters containing DNA of unknown rat–to–human ratio were hybridized in the same vial the isotope ratio of RNA bound to these filters gave a quantitative measure of the proportion of rat mtDNA in that DNA sample. The proportion of nuclear DNA (nDNA) of two species in a hybrid cell sample could be estimated by the same method, using cRNA transcribed from nDNA. While the latter assay is less accurate, we found that its results correlated quite well with chromosome composition of hybrid cells. Further details and technical considerations of these assays are presented elsewhere (COON, HORAK and DAWID 1973).

Rat–human hybrid cells: We have investigated 33 rat–human hybrid cell strains. Of these, 19 contained both rat and human nDNA's and mtDNA's when first tested at a time about 40 to 60 population doublings after fusion. The filled circles in Figure 2 present these data by plotting a point at the position corresponding to the percentage rat mtDNA and rat nDNA of each hybrid cell strain. The percentage of human DNA is the difference between percent rat DNA and

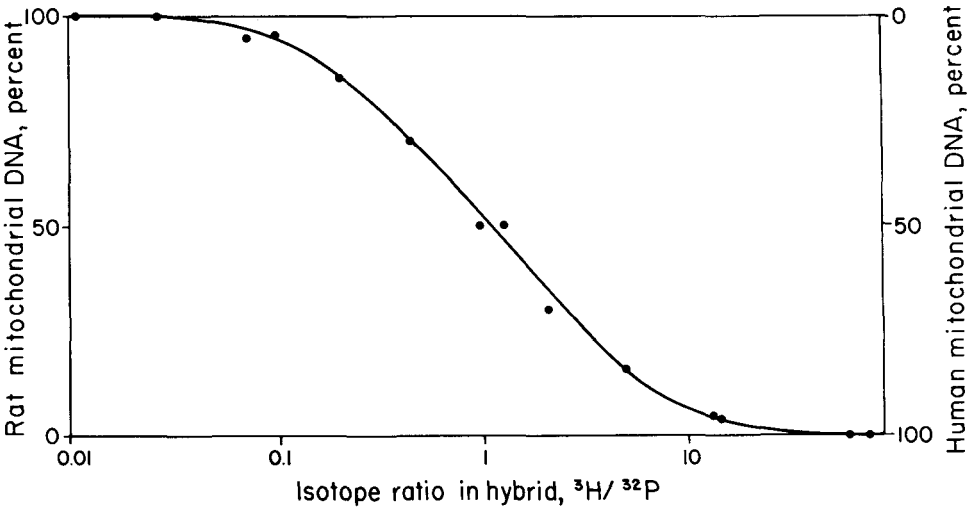

FIGURE 1.—Calibration curve for the assay of the proportion of rat and human mtDNA. Membrane filters containing mixtures of pure rate and human mtDNA in variable known proportions were hybridized in the same vial with a mixture of ^{3}H-cRNA transcribed from human mtDNA and ^{32}P-cRNA transcribed from rat mtDNA. The isotope ratio of the RNA bound to each filter is plotted against the percentage of either mtDNA on the same filter.

100. (The open symbols in the figure refer to studies on recombination described below.) There is a loose correlation between the retention of human chromosomes and mtDNA in the hybrid cells. In general, the proportion of chromosomes (nDNA) is more balanced while the proportion of mtDNA is more extensively segregated. This suggests that mtDNA tends more strongly than the chromosomes to segregate into pure types. Not shown in Figure 2 are the remaining 14 strains which contained only human nDNA and mtDNA at this point; these cell strains were segregants rather than parentals, since they had been found to be hybrid with respect to the morphology of their chromosomes at an earlier point in their history. No pure rat segregants were found at this time. After additional periods of growth some of the cell strains lost all human mtDNA, but no strains were observed which had lost all human chromosomes.

To test whether both rat and human mtDNA's were present in the same cell in hybrid cell strains we derived sets of subclones from several strains. These subclones were initiated 40 to 60 doublings after fusion. After the subclones had grown up (about 25 doublings) they were tested for their mtDNA and nDNA composition. Both rat and human mtDNA's, as well as both nDNA's, were found in most members of five sets of subclones (35 to 40 clones). Only 5 out of 40 subclones had lost all human mtDNA but even in these subclones human chromosomes (nuclear DNA) were still present. These experiments show that individual cells in hybrid strains at a time 40 to 60 doublings after fusion contained both rat and human mtDNA sequences.

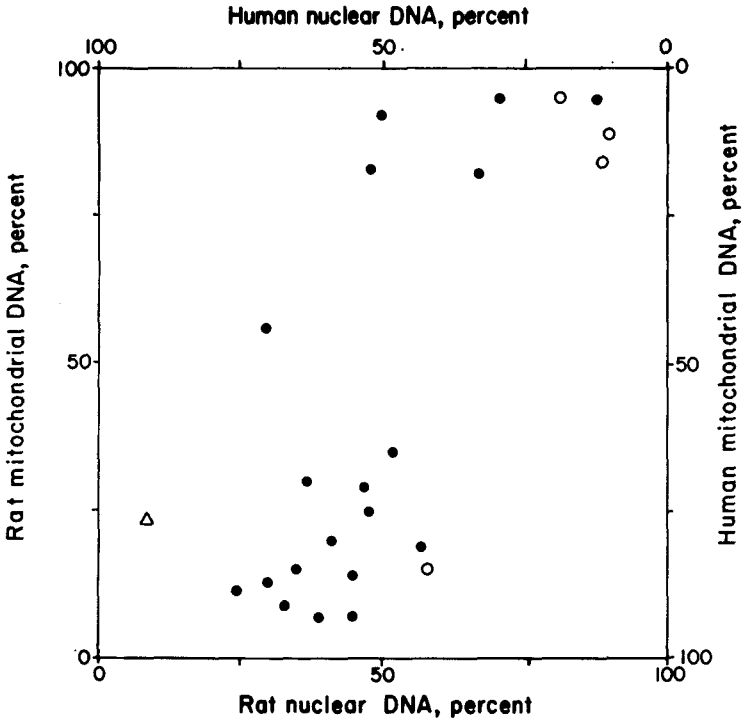

FIGURE 2.—Summary of DNA composition of rat–human hybrid cell strains. The solid points show the composition of 19 hybrid strains when first tested, 40 to 60 doublings after fusion. For each strain a point was plotted at the position corresponding to its percentage of rat mtDNA and rat nDNA (or human DNA's on reverse scales). The open circles refer to strains which were tested for the presence of recombinant molecules of mtDNA (see text, below). Open circles: strains containing recombinant mtDNA molecules; triangle: strain containing no detectable recombinant molecules.

Mouse–human hybrid cells: Our results with these cells are summarized in Figure 3. Arrows connect measurements made at one time to measurements on the same cell strain about 25 doublings later. Open symbols refer to studies in recombination of mtDNA which will be described below. Several facts emerge. First, all cell strains which contain more than 50% mouse nDNA (i.e., mouse chromosomes) contain pure mouse mtDNA. This may be a general property of mouse–human hybrid cells and could explain the results of CLAYTON *et al.* (1971) and of ATTARDI and ATTARDI (1972); these authors found exclusively mouse mtDNA in hybrid cells containing predominantly mouse chromosomes. Second, in spite of this apparent tendency of the mouse mtDNA to be retained preferentially we have found that cells which have lost all mouse chromosomes lose all mouse mtDNA as well. This loss may be slow, however: we observed several strains which contained no measurable mouse nDNA (which means less than 5%) but did contain large amounts of mouse mtDNA (Figure 3). These strains lost their mouse mtDNA during the next 25 generations of growth (Figure 3).

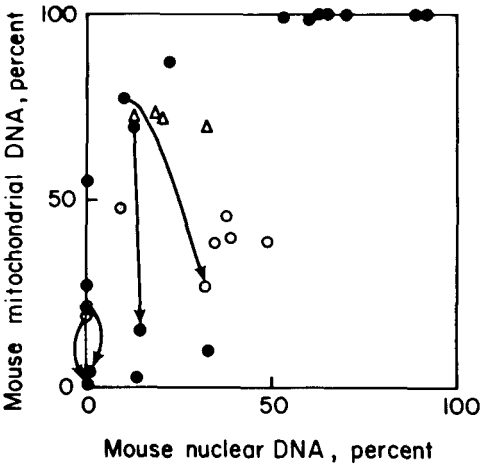

FIGURE 3.—Summary of DNA composition of mouse–human hybrid cell strains. Solid points refer to strains after 40 to 60 doublings, except that arrows lead from several points to DNA values measured in the same strains after an additional 25 doublings. Open circles: strains containing recombinant mtDNA molecules; open triangles: strains which did not contain such molecules.

Third, cell strains containing a low to moderate proportion of mouse chromosomes are relatively stable in retaining both mouse and human mtDNA's. Subclones were also derived from some mouse–human hybrid strains. Several were found which contained both types of mtDNA.

Apparent recombination of rodent and human mtDNA in hybrid cells: In a cell containing sequences of mtDNA from two different species these sequences could occur in different arrangements. Separate mitochondria from each species could coexist independently in the same cell. In this case the different mtDNA's would not come into direct contact. Since mitochondria appear to fuse *in vivo* (see LEHNINGER 1965) this possibility is not very probable. Second, intact molecules of human and of rodent mtDNA could coexist in the same organelles. Third, the different mtDNA's could interact and be linked to each other in various ways. To distinguish between the last two possibilities we carried out experiments based on the technique illustrated in Figure 4. Human and mouse DNA's differ in density by about 8 mg/cm^3. A mixture of human and mouse mtDNAs (actually whole-cell DNA's of which the mtDNA forms a small fraction) was banded in a CsCl gradient, the gradient was fractionated, and each fraction adsorbed to a membrane filter. The filter set was hybridized with a mixture of differentially labeled cRNA's, as described above for the quantitative assay of human and rodent mtDNA's. Each mtDNA binds its own cRNA and there is little cross-hybridization (Figure 4), but there is some overlap between the two bands, due to the small density difference. The large amount of nuclear DNA in the gradient does not interfere since this DNA does not hybridize with cRNA transcribed from mtDNA and does not inhibit the hybridization of mtDNA with its cRNA (see also DAWID and BLACKLER 1972; COON, HORAK and DAWID 1973).

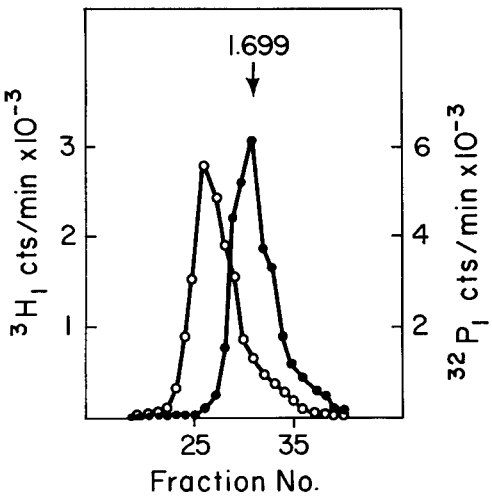

FIGURE 4.—Separation of mouse and human mtDNA. A mixture of mouse and human DNA's was banded in a CsCl gradient in a no. 65 angle rotor of the Beckman centrifuge. Fractions were collected, separately adsorbed to membrane filters and hybridized together to a mixture of ^{3}H-human mt-cRNA (open circles) and ^{32}P-mouse mt-cRNA (closed circles).

Figure 5 shows a gradient which analyzed DNA from a hybrid cell strain in the same way. The pattern of hybridization is quite different from the control in Figure 4. The DNA which contains human mtDNA sequences and which hybridizes with ^{3}H-cRNA bands as a broad peak, and the mouse mtDNA sequences (^{32}P-label) largely overlap with this ^{3}H peak. The thin solid and broken lines are marker DNA bands which allow the estimation of densities in the gradient. The major peak of ^{3}H-label occurs at about the density of human mtDNA (1.707 g/cm^3), and a large proportion of ^{32}P-label is bound at the same position. We interpret this fact to mean that molecules occur which consist of mostly human mtDNA sequences linked to a smaller proportion of mouse mtDNA sequences. This situation is schematically illustrated in Figure 6, A1 or B2. The interpretative drawings in Figures 6 and 7 are made on the assumption that the linkage between mouse and human mtDNA sequences is a covalent tandem linkage of parts of the two mtDNA's, best described as recombination. Other possible interpretation will be discussed below. The gradient of Figure 5 also contains molecules which band at a lower density, close to or slightly higher than that of mouse mtDNA (1.699 g/cm^3). These molecules are best interpreted as recombinants with a high proportion of mouse and a lower proportion of human mtDNA sequences, as illustrated in Figure 6, A2.

Due to the relatively small density difference between human and mouse mtDNA's these gradients have limited resolution and do not allow the determination of the exact ratio of sequences in the molecules which are present. The gradient of Figure 5 could be explained by two types of recombinant molecules as symbolized in Figure 6, A1 and A2. But it is equally possible that the situation obtains which is shown in Figure 6B: pure human and pure mouse mtDNA's and

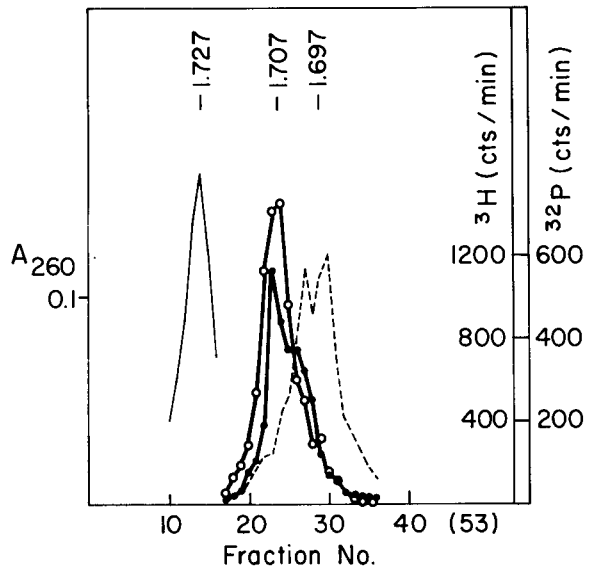

FIGURE 5.—Analysis of the DNA from a mouse–human hybrid strain in a CsCl gradient. Open and closed circles refer to hybridization with human and mouse mt-cRNA's as described in the legend to Figure 4. The thin solid line is a band of DNA from *Pseudomonas aeruginosa* (1.727 g/cm^3); the thin broken line refers to the band of human nDNA (1.697 g/cm^3). These two marker bands were used to estimate the position of density 1.707 in the gradient.

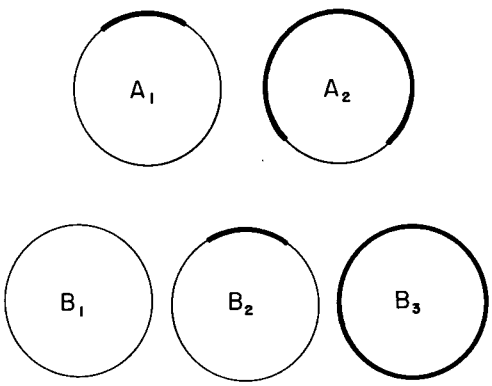

FIGURE 6.—Hypothetical models of recombinant mtDNA molecules. Thin line refers to human, thick line to mouse mtDNA sequences. For explanations see text.

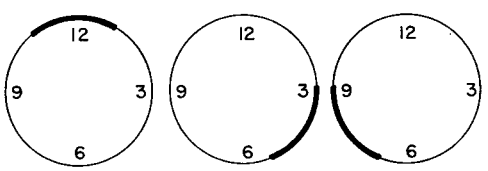

FIGURE 7.—Hypothetical models for recombinant mtDNA molecules with a constant proportion but different actual sequences of mouse and human sequences. Numbers are arbitrary being used to distinguish different parts of the molecules.

only one type of recombinant molecule (Figure 6, B2) could explain the observed pattern. Many combinations of these types of molecules are possible. Figure 6 shows the *minimum* heterogeneity in mtDNA molecules which is necessary to explain the gradient pattern. Much larger heterogeneity may exist: this cell strain may contain pure human and pure mouse mtDNA's and a large series of recombinant molecules spanning the range between these two extremes. We cannot decide between these possibilities at present. However, even the minimum heterogeneity necessary to explain the observations is remarkable in view of the fact that mtDNA in the cells of any one animal species is a homogenous population of molecules (see BORST 1972).

Once recombination of the type illustrated in Figure 6 is assumed to be the explanation of the observed pattern an additional question of possible heterogeneity arises. Even if the proportions of human and mouse mtDNA sequences in the recombinant molecules were known, we would not know whether a subpopulation of molecules with a certain percentage of human sequences is homogenous. This point is illustrated in Figure 7: if we knew that the only recombinant type in a certain cell strain contained 20% mouse sequences this could still include many different molecules if each contained a different 20% of the mouse mitochondrial genome. Another complication arises from the fact that the recombined molecules may not be standard-size mtDNA circles (i.e., 5 μm in circumference). In Figures 6 and 7 the implicit assumption has been made that the molecules would be standard circles, but this is a speculative assumption at present.

We must consider alternate explanations for the gradient pattern (Figure 5) which do not involve covalent linkage of parts of the two mtDNA's. One possibility might be that of interspecific catenanes, i.e., molecules connected like links of a chain (see HUDSON and VINOGRAD 1967). This interpretation is not very likely since the linked molecules which we observed are not equal combinations of the two parental mtDNA's. To produce by catenation a complex containing about 80% human and 20% mouse mtDNA one would have to involve a standard-size human molecule and a small mouse molecule that might have arisen by deletion. This mechanism would involve two steps, deletion and catenation. Also, we have observed that in mtDNA from a hybrid cell strain which had been sheared to a molecular weight between 1 and 1.5×10^6 the human and mouse components remained linked to a considerable extent (HORAK, DAWID and COON, unpublished). These facts make catenation a less likely mechanism.

Another possibility is that the mouse mtDNA's could suffer deletions to yield fragments which would, fortuitously, have densities similar to the density of human mtDNA, and *vice versa*. This appears not very likely since one might expect to obtain fragments of other densities as well (which is not the case), and also because there is some evidence that mammalian mtDNA's have very little intramolecular base compositional heterogeneity (WOLSTENHOLME, KIRSCHNER and GROSS 1972).

Rat–human hybrid cell DNA was analyzed by the same gradient centrifugation method. The density difference between rat and human mtDNA is only

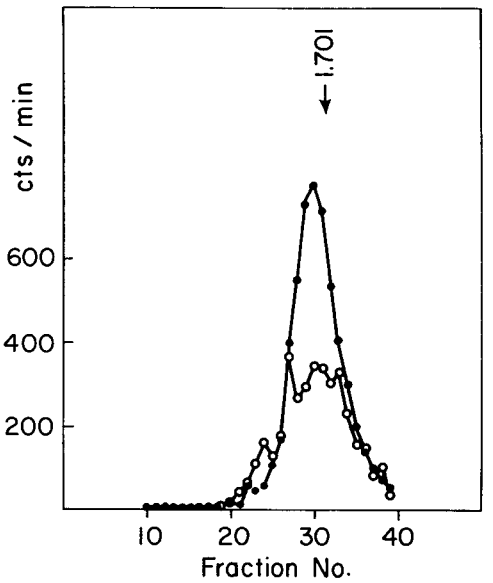

FIGURE 8.—Analysis of DNA from a rat–human hybrid strain in a CsCl gradient. Open and closed circles refer to hybridization with human and rat cRNA, respectively, as described in the legend to Figures 4 and 5. The position estimated to have a density of 1.701 is shown; the marker bands have not been plotted in this gradient.

about 6 mg/cm^3, reducing resolution somewhat. Figure 8 shows an example of hybrid cell DNA containing 84% rat mtDNA sequences. The human mtDNA sequences banded in CsCl at the same position as the rat sequences, both at a density about 1 mg/cm^3 higher than the density of pure rat mtDNA. This fact suggests that all the human mtDNA in the sample was linked to rat mtDNA and that rat sequences accounted for most of the complex molecules. Whether all rat sequences are linked to human ones or whether pure rat mtDNA also occurs in this sample cannot be decided (see also above).

In addition to examples of apparent recombination in hybrid cell strain DNA we also observed cases in which the two parental mtDNA's appeared to be present without interaction. However, due to the significant overlap of the bands (see Figure 5) a small amount of recombination would not be detectable; thus, the strains we classified "nonrecombinant" may actually contain some recombined DNA molecules.

With this reservation in mind, we classified all strains which were analyzed by gradient centrifugation. The results were incorporated into Figures 2 and 3 (open symbols). In both figures the open circles refer to strains which contained recombinant mtDNA molecules and the open triangles refer to strains where the mtDNA molecules appeared to be unlinked (nonrecombinant). Thus, 4 rat and 7 mouse–human hybrid strains contained recombinant molecules, while 1 rat and 4 mouse–human hybrid strains did not. We conclude that the appearance of recombinant mtDNA molecules in hybrid cells is not a rare phenomenon. Furthermore, this phenomenon arises both in "high-human" and "high-rodent" cells.

It appears possible that the exposure of these cells to Sendai virus may in some way be related to mitochondrial fusion. We have no information on this possibility.

The frequent occurrence of recombinant mtDNA molecules in hybrid cells suggests that such events may also take place in normal animal cells. Provided that recombination is reciprocal, it would not be possible to detect this event by biochemical means in such cells. It could be detected by genetic techniques if markers on mtDNA of animals were available.

Recombination of markers on yeast mtDNA has been observed some time ago (COEN *et al.* 1970). It has been shown more recently by molecular techniques that different yeast mtDNA molecules become physically linked to each other concurrently with the recombination of genetic markers (MICHAELIS, PETROCHILO and SLONIMSKI 1973). The latter finding encourages us to think that the molecular recombination observed in our experiments may have genetic significance which could be revealed when suitable markers become available.

The potential use of hybrid somatic cells for mitochondrial genetics: MtDNA's from different animals can replicate in the same cell for extended periods, and these mtDNA molecules interact in a way which most likely corresponds to recombination. These observations should be the basis for several types of genetic experiments on mitochondrial biogenesis. Hybrid cells could be used to correlate the presence of mtDNA of one species with the presence of certain enzymes or other proteins corresponding to the same species. If the suitable combination of genetic elements can be found, such experiments could establish which proteins (if any) are coded for by animal mtDNA.

Another future course of study would attempt to determine which chromosomes of one species are required for the maintenance of mtDNA from the same species. If specific chromosomes can be implicated, we might conclude that these chromosomes carry genes for certain mitochondrial components which have to interact with the products of mtDNA in a species specific way.

If mutants or variants in animal cells are found which lead to modifications of certain mitochondria-related properties (e.g., chloramphenicol resistance, oligomycin resistance, etc.), it might be possible to determine the nuclear or mitochondrial localization of the mutated genes with the aid of hybrid somatic cells. Indeed, hybrid cells appear to be the only way in which such a localization could be achieved in cultured cells. It is not adequate to show that, for example, a chloramphenicol-resistant variant has resistant mitochondrial ribosomes. This would only prove that resistance is not due to permeability, but it would not decide whether the nuclear or mitochondrial genome carried the mutation. Since mitochondrial ribosomal proteins are synthesized in the cytoplasm (KÜNTZEL 1969; LIZARDI and LUCK 1972) and very probably are coded for by nuclear DNA, it is quite possible that a nuclear mutation could lead to the drug resistance of mitochondrial ribosomes.

Hybrid cells may also offer the opportunity to investigate the rules according to which mtDNA's recombine. This would concern the question of whether certain specific sites are involved or whether recombination can take place at any

point along the DNA; and whether large regions of nucleotide homology are required. This is particularly interesting since it offers the opportunity to study some molecular aspects of recombination in animal cells, which is otherwise difficult.

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