Mitochondrial DNA and nuclear DNA from normal rat liver have a common sequence

(Southern blot/mammalian genome)

HERBERT I. HADLER, BOGOMIR DIMITRIJEVIC, AND RAVI MAHALINGAM

Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, IL 62901

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ABSTRACT Although Pst I does not cut the circular mitochondrial genome of the rat, BamHI generates from this genome two unequal fragments of DNA. Each of these fragments was cloned in pBR322. Nuclear DNA was digested from rat liver singly or doubly with Pst I and BamHI, and it was demonstrated that nuclear DNA shared a common sequence with the larger mitochondrial DNA BamHI fragment. The cloned larger mitochondrial DNA fragment was further subdivided with HindIII into four pieces that were labeled and then used to probe the double-digested nuclear DNA. The hybridization data showed that the common sequence is less than 3 kilobase pairs long and lies within the part of the mitochondrial genome containing the D-loop and a portion of the rRNA genes. It therefore appears that, as in lower eukaryotes, there are shared sequences between the nuclear and mitochondrial genomes in mammals.

In current editorial reviews (1, 2) the first evidence of the movement of DNA between intracellular genomes of eukaryotes during evolution was collected. Thus an ATPase gene had migrated from the mitochondria to the nucleus during the evolution of *Saccharomyces cerevisiae* and *Neurospora crassa* (3). Mitochondria and chloroplasts of maize possess a closely homologous 12-kilobase-pair (kb) segment of DNA (4). Mitochondria and nuclei of *S. cerevisiae* have multiple common fragments of DNA (5). Mitochondria and nuclei of the sea urchin (*Strongylocentrotus purpuratus*) have common pieces of DNA (6). This is also true for the locust *Locusta migratoria* (7). Wright and Cummings (8) ascribe the senescence of a fungus, *Podospora anserina*, to the active mobilization of genetic elements from the mitochondrion to the nucleus. It is cogent to note that the observation by Chilton et al. (9) dealing with transformation is analogous. These workers reported that when plant cells are transformed by infecting bacteria, DNA from the bacterial plasmid becomes integrated into the nuclear genome of the host.

We also have been examining the putative movement of mtDNA into the nuclear genome of eukaryotes because of a hypothesis that we presented some twelve years ago (10). This hypothesis stated that as a result of pressure on the mitochondria by carcinogens and their metabolites mitochondrial genetic material may be integrated into nuclear DNA (nDNA) just like the genetic material of an oncogenic virus. Since 1971 many other examples of such pressure have been observed (11-20). Indeed such pressure now has added significance because of the recent report that human oncogenes express a mitochondrial and bacterial ATPase (21).

We chose to test our hypothesis by using probes of normal mtDNA to compare nDNA derived from normal tissue with nDNA derived from tumor tissue. During the course of this investigation we observed that the mammalian mtDNA and nDNA from normal rat liver have a common sequence. Irrespective of the validity of our hypothesis this experimental finding with normal mammalian intracellular genomes is significant in its own right and is the subject of this report.

MATERIALS AND METHODS

Isolation of mtDNA. Mitochondria were isolated from Sprague-Dawley rats (purchased from Holtzman, Madison, WI). The twice washed mitochondrial pellet was obtained as described (22) except 250 mM sucrose/100 mM Tris-EDTA, pH 7.5 replaced 250 mM sucrose. Once-banded mtDNA was isolated by a modification of the method of Bogenhagen and Clayton (23). The mitochondria were not subjected to hypotonic treatment. The lysis buffer was 100 mM Tris-EDTA/10 mM NaCl, pH 7.5. An additional step to remove protein and colored substances was included. After adding CsCl to the lysate and centrifuging at 20,000 × g for 30 min at 4°C we adjusted the cleared lysate (corresponding to 13.5 g of liver) to a density of 1.3 g/ml of CsCl and a volume of 3.0 ml and layered it in a 5-ml polyallomer tube over 1 ml of CsCl density 1.6 g/ml and 1 ml of CsCl density 1.7 g/ml. After spinning in a Beckman SW 50.1 rotor for 20 hr at 170,000 × g at 20°C, the nucleic acids were pelleted. The bottom of the tube was pierced with a 18-gauge needle directed from the top, the lower viscous 1.5 ml was collected and subjected to CsCl/ethidium bromide (1.6 g/ml and 150 μg/ml, respectively) isopycnic centrifugation (160,000 × g, 48 hr, Beckman SW 50.1 rotor, 20°C). The lower band was collected with an 18-gauge needle, the dye was completely removed by four extractions with isooamyl alcohol and dialyzed overnight with 4 changes of 10 mM Tris-HCl/1 mM Na2EDTA, pH 7.5 (TE buffer). The DNA was precipitated by the addition of 1/10 vol of 3 M NaOAc (pH 7.0) and 2 vol of ethanol and then washed by centrifugation with 70% ethanol and, after brief drying in vacuo at room temperature, was dissolved in TE buffer and stored at 5°C.

Cloning of mtDNA. The once-banded mtDNA was digested with BamHI to yield a mixture of linear fragments 10.8 and 5.2 kb. Transformation of *Escherichia coli* strain HB101 was carried out as described (24). The clones were selected by growing the ampicillin-resistant colonies on tetracycline plates (25). After the plasmid DNA was digested with BamHI, electrophoresis showed that only colonies with the 5.2-kb piece of mtDNA inserted into pBR322 had been isolated. To obtain a recombinant of pBR322 with the 10.8-kb piece of mtDNA as the insert, the mixture of mtDNA cut with BamHI was sized by gel electrophoresis and the 10.8-kb fragment was recovered from the gel (26). In addition the pBR322 that had been linearized with BamHI was dephosphorylated with alkaline phosphatase.

Abbreviations: nDNA, nuclear DNA; kb, kilobase pairs; NaCl/Cit, standard saline citrate.
(27). Two colonies selected as before had the 10.8-kbp piece of mtDNA inserted into pBR322.

Growth, Isolation, and Characterization of Plasmid DNA. Plasmid pBR322 or recombinant plasmids with the 10.8 kbp or 5.2 kbp of mtDNA inserted into the BamHI site were grown in E. coli strain HB101 in L-broth fortified with ampicillin. Chloramphenicol was added to increase the yield of plasmid DNA. Lysis at pH 8.0 used Tris-HCl, sucrose, lysozyme, Na2EDTA, and 2% Triton X-100. The lysate, which was cleared at 40,000 rpm for 1 hr at 10°C in a Beckman SW 41Ti rotor, was extracted with phenol and then with isomyl alcohol. The nucleic acid was precipitated with NaOAc and ethanol, washed with 70% ethanol, dried, and dissolved in TE buffer. After treatment with RNase and extraction in succession with phenol and isomyl alcohol the nucleic acids were precipitated again as described above. The nucleic acid was now banded in TE buffer with CsCl (1.56 g/ml)/ethidium bromide (150 μg/ml) in a vertical head (Sorvall TV-850) at 40,000 rpm at 15°C for 24 hr. The lower band was collected, processed, and stored as described for the preparation of mtDNA.

Plasmid pBR322 and the two recombinant plasmids were characterized by digestion with BamHI followed by electrophoresis with a submerged horizontal, 6-mm thick 1% agarose gel (28) after 16 hr in 40 mM Tris base/20 mM NaOAc/1 mM Na2EDTA adjusted to pH 7.9 with HOAc (E buffer) at a voltage gradient of 1.3 V/cm. The gel was stained in E buffer with ethidium bromide at 0.5 μg/ml for 10 min and photographed with a transilluminator using a UV light, 254 nm, a UV filter for the camera, Polaroid film and a Polaroid MP-7 camera.

Isolation of DNA. A homogenate of rat liver in 250 mM sucrose/1 mM MgCl2 was spun at 900 x g for 15 min at 4°C. The pellet from 1.5 g of liver was suspended (homogenizer) in 10 ml of 2.2 M sucrose/30 mM MgCl2 and layered over 22 ml of the same medium and spun at 112,500 x g for 1 hr at 4°C in a Beckman SW 28 rotor. The clear gelatinous pellet of nuclei (29) was processed as the mitochondrial pellet described above to yield nDNA in TE buffer. There was only a single broad band after isopycnic centrifugation.

Labeling of Probes. Probes were labeled by the method of Rigby et al. (30) and were purified by extraction with phenol and then by chromatography on Sephadex 100.

Hybridization of Labeled Probes to Southern Blots of Sized Restriction Fragments of nDNA Digested with Restriction Enzymes. The nDNA (500 μg) in 1 ml was incubated at 37°C for 16 hr with 420 units (5-fold excess) of Pst I (Bethesda Research Laboratories) in a buffer recommended by the manufacturer. Completeness of digestion was confirmed by incubating in parallel 1 μg of pBR322 added to a 50-μl aliquot of the main digest and running a gel. After completeness of digestion was confirmed, the reaction mixture, which had been stored at 4°C, was extracted first with phenol and then with isomyl alcohol, and the DNA was precipitated in the cold (−70°C for 2 hr) by the addition of NaOAc and ethanol. The precipitate was collected by centrifugation (16,000 x g for 10 min at 0°C), washed with 70% ethanol, dried briefly in vacuo at room temperature, and redissolved in TE buffer to give a solution of once-digested nDNA. To obtain twice-digested nDNA, the above digestion with 2.5-fold excess of enzyme and monitoring for completeness of digestion was carried out with 250 μg of once-digested nDNA. The twice-digested nDNA was also extracted, precipitated, washed, dried, and dissolved in TE buffer.

Electrophoresis was carried out in a horizontal 6-mm thick slab of 1% agarose submerged 1 cm in E buffer at room temperature for 112 hr. The slots were 7.5 x 2 x 5 mm. Each slot was loaded with 40 μl of a solution that contained the designated amount of nDNA and 2% Ficoll, 2 mM Na2EDTA, and orange G. The voltage gradient was 0.7 V/cm. A glass plate was carefully placed on the gel after the DNA had been driven into the gel. The buffer was not circulated.

The method of Southern (31) as described by Jeffreys and Flavell (32) was used to transfer the cut DNA to nitrocellulose, for hybridization (65°C for 2 days in a box), for washing the strips (18 x 4 cm), and for autoradiography. During hybridization, 50 ml of the solution in the box contained 2.5 μg of probe of specific activity 1 x 106 cpm/μg of DNA. The following wash solutions, modelled after Denhardt (33), were used at 65°C to treat the nitrocellulose before and after hybridization. Before hybridization the strips of nitrocellulose were washed for 30 min in 3X standard saline citrate (NaCl/Cit/1 X NaCl/Cit is 150 mM NaCl/15 mM trisodium citrate) then treated for 3 hr with 10X Denhardt’s solution (0.2% Ficoll/0.2% polyvinylpyrrolidine/0.2% bovine serum albumin in 3X NaCl/Cit). This solution was replaced by a similar solution supplemented with denatured and sheared salmon testes DNA at 50 μg/ml (Sigma), poly A at 10 μg/ml (Sigma), and 0.1% NaDodSO4 and incubated for 1 hr. After hybridization the strips were successively washed at 65°C as follows: 3 washes for 5 min then 2 washes for 30 min with 3X NaCl/Cit/1X Denhardt’s solution/0.1% NaDodSO4 and 2 more washes for 45 min each with 0.1X NaCl/Cit/0.1% NaDodSO4.

The molecular weight markers were various restriction fragments of pBR322 and recombinant DNA.

Other portions of nDNA were processed as described above except Pst I was replaced by BamHI or a combination of BamHI with Pst I.

Autoradiography was for either 3 days or 7 days at −70°C. The x-ray film was a Kodak XAR-5 without preflash. Dupont Cronex Lightning Plus intensifying screens were used.

To obtain sharp reproducible marker bands of unfragmented mtDNA it was necessary to digest once-banded mtDNA with Pst I, presumably to digest away accompanying nDNA.

Preparation of pBR322, p4.0, p2.1, and p1.7. The large recombinant was digested with BamHI and the desired fragments of DNA were separated in E buffer on 1% agarose by electrophoresis and electroelution (34). The aqueous solution was successively extracted with phenol and then isomyl alcohol, and the DNA was precipitated as described above with NaOAc and ethanol. The precipitate was dissolved in low-salt buffer (200 mM NaCl/20 mM Tris-HCl/1 mM Na2EDTA, pH 7.4) and passed through an ELUTIP-d column (according to the directions of the manufacturer, see ref. 35). The DNA adhered to the column. The DNA was eluted with high-salt buffer (1.0 M NaCl/20 mM Tris-HCl/1 mM Na2EDTA, pH 7.4) precipitated with NaOAc and ethanol as described above and stored in TE buffer. Labeling with 32P was as described above.

RESULTS

As recorded by others (36) and noted in Fig. 1 circular mtDNA from rat contains 16 kbp. Digestion of mtDNA with BamHI yields two linear pieces (36–39)—namely, 10.8 and 5.2 kbp. Pst I does not cut rat liver mtDNA (36). HindIII cuts the 10.8-kbp fragment into four fragments (36–39), which are 3.0, 4.0, 2.1, and 1.7 kbp. These enzymes do not discriminate between the two polymorphic forms of rat mtDNA (36–39).

Each fragment of mtDNA obtained by digestion with BamHI was cloned into the BamHI site of pBR322. Data confirming this are shown in Fig. 2. Each recombinant of DNA was labeled with 32P by nick-translation (30) to yield one probe with the 10.8-kbp insert (p) and another probe with the 5.2-kbp insert (sp). These probes of cloned mtDNA were of course free of any contaminating nDNA. In addition, five other probes of
DNA were used—pBR322 alone and the four fragments (3.0, 4.0, 2.1, and 1.7 kbp) derived from the cloned 10.8-kbp piece by digestion with HindIII. The four probes are designated p3.0, p4.0, p2.1, and p1.7, respectively.

The results of hybridizing blots of Pet I digests of nDNA with lp are shown in Fig. 3. Similar hybridization was carried out with sp but the results are not shown. Bands 1 and 3 in lanes f, g, and i, which hybridized to both lp and sp and are in the same position of marker mtDNA (lane b), were presumed to be unfragmented nonintegrated mtDNA contaminating the digest of nDNA. Because it migrated faster than band 1, band 3 was presumed to be form 1 of mtDNA and band 1 was presumed to be either form 2 or form 3 of mtDNA or a mixture of the two forms. Band 5, which was about 10.8 kbp and thus decidedly smaller than unfragmented mtDNA, was hybridized to lp and not hybridized to sp. This band could contain a portion of mtDNA integrated into the nuclear genome.

In this experiment and in those shown in Figs. 4 and 5, there are two pairs of lanes with nDNA digests. One pair of lanes (f and g) was loaded with 10 μg of nDNA digested once and twice with excess of the same enzyme. The other pair of lanes (h and i) was loaded with 30 μg of nDNA digested once and twice with excess of the same enzyme. Thus artificial bands due to incomplete digestion should fade away after the successive digestions.

The result of hybridizing blots of BamHI digests of nDNA to lp are shown in Fig. 4. Similar hybridization was carried out with sp but the results are not shown. Preliminary experiments indicated that the bands present either at 10.8 kbp by hybridization to lp or at 5.2 kbp by hybridization to sp would be so intense that they could obscure neighboring bands. Thus, to circumvent this difficulty the locating lanes (a, b, c) were first exposed to x-ray film and the section of the blot for lanes d, e, f, g, and i that correspond to the intense band was cut out and relocated at the top of the blot before exposing lanes d, e, f, g, h, and i to fresh x-ray film. Band 11 (about 14 kbp) was not due to incomplete digestion. It was larger than 10.8 kbp and very faintly hybridized to sp. DNA in this band could contain a portion of mtDNA covalently integrated into the nuclear genome. Band 13, hybridized only to lp, was a piece of fragmented nonintegrated mtDNA contaminating the digest of nDNA.

The results of hybridizing lp to blots of nDNA digested once and twice by the combination of Pet I with BamHI are shown in Fig. 5. Similar hybridization was carried out with sp but the results are not shown. Band 17 likely is the same as band 11 because it was much less intense with sp. Although this band could contain a portion of mtDNA integrated into the nuclear genome, the result is equivocal because of the fading of the band after extra digestion (lanes g and i). Band 19, which hybridized only to lp, was nonintegrated fragmented mtDNA of 10.8 kbp. Band 21 (8.6 kbp) hybridized to lp and not to sp. This band survived extra digestion and was absent when the enzymes were singly Pet I or BamHI. Band 21 accordingly is a
portion of mtDNA covalently integrated into nDNA with one end a Pst I site and the other end a BamHI site.

To further narrow down the region of homology lp was re-

FIG. 5. Hybridization of lp to Southern blot of sized restriction fragments of nDNA digested simultaneously with Pst I and BamHI. These data are from a single blot and the alignment of all lanes is identical to that on the original single piece of nitrocellulose. The procedure is similar to that described for Fig. 4, except the nDNA was digested with BamHI and the strip of nitrocellulose at the 10.8-kbp mark was cut out and relocated on top before exposure to the second x-ray film. Lanes a and e contain size markers in kbp, indicated by numbers next to lane a, O, origin. The numbers 11 and 13 identify certain bands discussed in the text. Lane b, 10 μg of once-digested nDNA. Lane c, 113 pg of once-banded mtDNA that was digested with BamHI. Lane d, 10 μg of once-digested nDNA and 113 pg of once-banded mtDNA that was digested with Pst I. Lanes f and g, 10 μg each of nDNA that was digested once and twice, respectively. Lanes h and i, 30 μg each of nDNA that was digested once and twice, respectively.

FIG. 6. Hybridization of p3.0, p4.0, p2.1, and p1.7 to Southern blots of sized restriction fragments of nDNA digested simultaneously with Pst I and BamHI. The procedure is described in the legend to Fig. 3. Four different blots are shown. The probes used with each blot, from left to right, were p1.7, p2.1, p3.0, and p4.0. The left lane of each blot contains size markers in kbp, indicated by numbers on left. O, origin. The right lane for each blot had 30 μg of twice-digested nDNA. The arrow indicates the 8.6-kbp band.

placed by its HindIII fragments p3.0, p4.0, p2.1, and p1.7. As shown in Fig. 6 the nDNA was again digested successively with the combination of Pst I and BamHI. Each of the four blots has an intense band at about 10.8 kbp because of nonintegrated fragmented mtDNA. This is a positive control, which showed that each probe was effective. Only p3.0 hybridized to the 8.6-kbp band. The intensity of the band at 10.8 kbp was so diminished, that it was no longer necessary to cut out and relocate any bands before the final exposure of each blot to x-ray film.

In the 8.6-kbp piece of DNA there are at least five kbp of nDNA. It is important to note that these results were obtained under conditions of high stringency compatible with perfectly matched hybrids (32).

Additional blots with 30 μg of twice-digested nDNA corresponding to the enzymes used in Figs. 3, 4, and 5 were hybridized to a probe derived from pBR322. No bands were detected (data not shown). The results represented by Figs. 3, 4, 5, and 6 were obtained with nDNA prepared from at least two different groups of rats. Only the bands seen in Fig. 6 were present even after exposing the four blots to x-ray film for 4 months.

The results do not eliminate the possibility that there are other portions of mtDNA covalently integrated into the nuclear genome.

DISCUSSION

The results show that nDNA derived from rat liver yield, after double digestion with Pst I and BamHI, an 8.6-kbp piece of DNA that contains a mtDNA sequence found within the 3.0-kbp segment of the mitochondrial genome (Fig. 1). This piece of nDNA has a Pst I site at one end and a BamHI site at the other end and is not homologous with mtDNA, which is either clockwise or counterclockwise to the 3-kbp mitochondrial segment.

Further investigation could describe with greater precision the sequence of covalently integrated mtDNA and its possible function in evolution, development, transformation, and aging.

Our experimental approach, which is applicable to isolable nDNA contaminated with nonintegrated mtDNA, also can be used to investigate pathological conditions because, unlike other strategies (5), it is not restricted to mutant organisms deficient in mitochondria. In such mutants the isolable nDNA is not contaminated with nonintegrated mtDNA. Is the observation true for humans, other mammals, and other higher eukaryotes?
the homology due to a portion of the 16S rRNA gene, the 12S rRNA gene, the D-loop region (see Fig. 1), or a tRNA gene?

The results thus far do not favor either tRNA or rRNA genes. Because tRNA genes are scattered around the whole mitochondrial genome (38) possibly more than one of the probes would be expected to show homology with the nuclear genome. Such was not the case. The same argument holds for rRNA genes as p4.0, which contains much of the mitochondrial 16S rRNA gene, did not show homology with the 8.6-kbp piece of nDNA. In addition, Rothblum et al. (40) isolated and characterized clones of rat rRNA nuclear genes. The largest piece in the cloned DNA, with one end a BamHI site and the other end a Psi I site, is 4.0 kbp (12.7–8.7). Thus the 8.6-kbp piece of nDNA cannot be present in the DNA that was cloned.

The remaining section of mtDNA, which could be homologous with the 8.6-kbp piece of the nuclear genome, includes the D-loop region. The D-loop region in mammalian mtDNA has attracted considerable attention (41–45). It contains the origin of replication for the H strand. The region has been completely sequenced for man (41, 42), cow (44), and mouse (45) and partially sequenced for rat (43). This region in rat (43) has 2-fold rotational symmetry, palindrome structures, a G cluster, A+T-rich regions, possible single-stranded hairpin structures, and is largely noncoding. Because this region of mtDNA is so species specific, Clayton (46) suggested that the D-loop region is a candidate for an interactive role with the nuclear genome. Our findings support this view.

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