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DNA SEQUENCES HOMOLOGOUS TO MITOCHONDRIAL GENES IN NUCLEI FROM NORMAL RAT TISSUES AND FROM RAT HEPATOMA CELLS

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Using specific probes we show that sequences homologous to NADH dehydrogenase Subunit 6, and Cytochrome oxidase Subunits I, II, and III mitochondrial genes are present in nuclear DNA from various tissues. These mitochondrial-like sequences are also present in rat hepatoma nuclear DNA but with an abnormal organization and a higher copy number than in normal hepatocytes.

Several studies have recently shown that the nDNA of lower and higher eucaryotes contain sequences homologous to mitochondrial genes (1-7). In human and rat, mtDNA fragments corresponding to ND4 and ND5 genes, to some tRNA and to rRNA regions were found within the nuclear genome (8, 9). In this paper we extended this observation to COI, COII, COIII and ND6 genes, which suggests that every mitochondrial gene might have its nuclear counterpart. In a previous work we have described a cDNA clone isolated from an hepatoma cell line library, the pHT 13 clone, which corresponds to mRNAs present at a much higher level in rat hepatomas than in normal hepatocytes (10). This clone contains mitochondrial sequences with an abnormal organization, it includes parts of the ND6 and COI genes separated by 230 bases instead of 9 kb in normal mitochondrial genome (11). This observation strongly suggests that mtDNA organization is altered during chemical induced carcinogenesis.

The question then arises whether mitochondrial sequences present in nuclear genome are also modified during hepatocarcinogenesis. In this paper we show that COI, COII, COIII and ND6 sequences are present in the nuclear genome from hepatoma cells both at a higher copy number and with a modified organization as compared to the normal tissue.

MATERIALS AND METHODS

Cells and tissues. All normal and tumoral tissues were taken from Sprague-Dawley rats. Hepatocytes were obtained by collagenase perfusion of the rat livers (12). Purified hepatocytes or total liver always gave the same results. Liver carcinogenesis has been induced by giving

Abbreviations: nDNA, nuclear DNA; mtDNA, mitochondrial DNA; cDNA, complementary DNA; ND 1,4,5,6, NADH dehydrogenase subunits 1,4,5,6; COI,II,III, cytochrome oxidase subunits I,II,III; DENA, diethylnitrosamine.

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DENA to female Sprague-Dawley rats after a two-third hepatectomy ; malignant nodules and hepatocytes from the non-cancerous parts of the livers were prepared as already described (13).

DNA preparation and hybridization. Freshly isolated tissues, cells or tumors were homogenized in 10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Tris HCl, pH 7.5, 6 mM EDTA and 330 mM sucrose for isolation of nuclei and mitochondria as described (14 and submitted). Mitochondrial DNA was purified by CsCl gradient centrifugation according to Bogenhagen and Clayton (15). Nuclear DNAs were prepared according to Gross-Bellard *et al.* (16) to yield high molecular weight DNA.
DNA samples dissolved in TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA) were digested to completion by Hind III or Eco RI enzyme at 37°C for 4 to 8 h in the appropriate buffer. For Southern blot analysis, nDNA (10 µg) and mtDNA (0.25 µg) were submitted to 0.75% agarose gel electrophoresis in TBE buffer (40 mM Tris pH 8.3; 50 mM boric acid, 1 mM EDTA) and transferred to Gene Screen Plus filters. Hybridizations were performed for 48 h in 50% formamide as described (17).

Hybridization probes. The following probes have been used :

1. The complete 580 base pHT13 cDNA insert which contains at one end 144 bases of the N terminal region of COI gene and at the other end 210 bases of the N terminal region of ND6 gene (10).
2. The ND6 and COI single strand probes obtained by elongation using the Klenow enzyme of the recombinant Bluescript pHT13 clone.
3. A 14 kb Eco RI mouse mtDNA fragment containing the complete mouse genome except the ND1 gene and the 3' half of the 16S rRNA (18).
4. The 230 base fragment of the rat COII cDNA clone (19).
5. The 1 kb Eco RI-Pst I restriction fragment of the human CO III gene.
6. The rat pyruvate kinase cDNA.

All the DNA probes, except the single strand probes, have been labelled with the random multiprimer system (20).

RESULTS

Sequences homologous to mitochondrial ND6 andCOI genes are present in nuclear DNA from normal rat tissues

We have used two types of probes to determine whether sequences homologous to mitochondrial genes are present in nuclear DNA :

1. ND6 and COI sequences which are parts of the pHT13 cDNA clone (10).

2. A 14 kb Eco RI fragment of mouse mtDNA which contains the complete mitochondrial genome except the ND1 gene and the 3' half of the 16S rRNA gene (position 1750-4013) (18).

Figure 1 shows the hybridization pattern obtained after Eco RI digestion of nDNA from rat liver, brain, spleen and kidney and of mtDNA from rat liver : two fragments of 4.2 and 3.2 kb respectively were obtained with the mtDNA preparations, whereas with the nDNA samples two fragments of 5 and 3 kb respectively were observed. The differences in size between the nuclear and the mitochondrial restriction fragments were confirmed by hybridization with the 14 kb mouse probe : seven restriction fragments of 7, 4.2, 3.2, 2.7, 2, 1.4 and 0.8 kb respectively were obtained with Eco RI digested mtDNA and five fragments of 8, 5, 3, 2 and 0.2 kb respectively with Eco RI digested nDNA.

The extent of homology between the nuclear and the mitochondrial gene is very high, since the hybrids were resistant to 1 h of heating at 68°C in 0.1 SSC.

It has to be noticed that the same hybridization pattern was found with the nDNA from all the studied normal tissues.

The differences in restriction pattern observed between nuclear and mitochondrial DNAs demonstrate that the presence of mitochondrial sequences in nDNA cannot result from mitochondrial contamination.

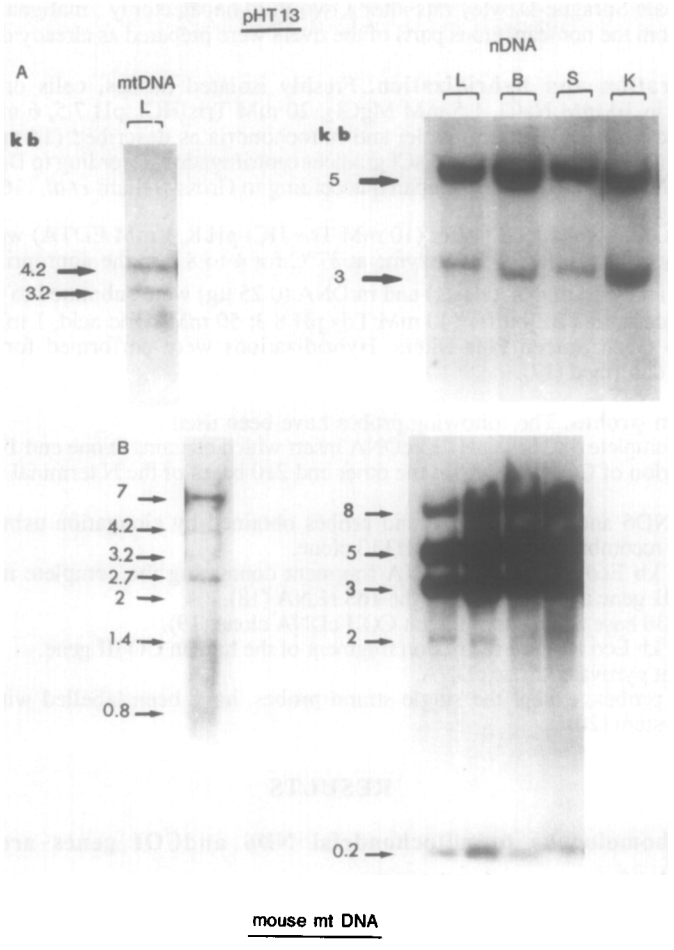

Figure 1. Mitochondrial sequences in nDNAs from normal rat tissues.
Southern blots were performed after complete Eco RI digestion in the appropriate buffer of 250 ng of mtDNA from normal liver (L) or 5 µg of nDNA from the following rat tissues : liver (L), brain (B), spleen (S) and kidney (K). The hybridizations were successively performed with the pHT 13 cDNA (A) and with the 14 kb Eco RI fragment of the mouse mtDNA containing the complete genome except the ND1 gene and the 3' half of the 16S rRNA gene (B). The probes were added to a 50% formamide solution and the hybridization was performed for 48 h at 42°C. The filters were exposed for 48 h with Kodak XAR 5 films, after washing in a 0.1 SSC solution at 68°C.

Sequences homologous to mitochondrial genes are present in nuclear DNA from hepatoma cells

Clone pHT 13 originated from an hepatoma cell cDNA library and shows an altered organization of the mtDNA genes ND6 and COI (10). In previous works (14 and submitted) using different mtDNA probes we have shown that various modifications of mtDNA have occurred during hepatocarcinogenesis. The question arises whether mitochondrial sequences in nDNA would have also been affected during hepatocarcinogenesis. Moreover modifications of the nuclear genome have been reported to be associated with the induction and the maintenance of the cancerous state *in vivo* (21, 22).

Nuclear DNA prepared from tumors induced *in vivo* by DENA and from hepatocytes from the non cancerous parts of the same livers, have been digested with Eco RI or with Hind III

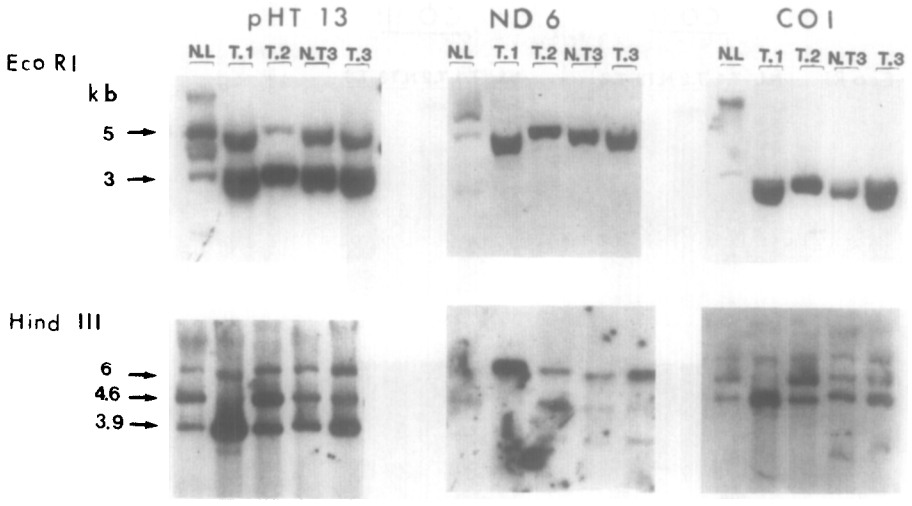

Figure 2. Mitochondrial ND6 and COI sequences in nDNAs from rat hepatomas.

Nuclear DNAs (5 µg) form normal liver (N.L), from liver tumors induced by DENA and isolated 15 months after the treatment (T.1, T.2, T.3) and from non cancerous parts of the liver (NT3) were digested to completion with either Eco RI or Hind III enzyme in the appropriate buffer and analyzed by Southern blots.
The complete pHT 13 cDNA insert and the two single-strand probes corresponding to ND6 and COI mitochondrial sequences were used for successive hybridizations.

restriction enzyme before Southern blot analysis; hybridization has been performed successively with the pHT13 cDNA probe or with the single strand probes corresponding to ND6 or COI sequence respectively (Figure 2).

The same two Eco RI fragments of 5 and 3 kb have been recognized by the pHT 13 probe in tumors (T) and in the hepatocytes from the non cancerous parts of the liver (N.T). However the signal was much higher in tumors and in non tumoral hepatocytes than in normal liver (N.L), although the same amounts of DNA have been loaded on the gels. The 5 and 3 kb bands can be assigned to ND6 and COI respectively. When normal liver nDNA has been digested with Hind III, three bands of 6, 4.6 and 3.9 kb respectively were detected with the pHT13 probe.The 6 kb band hybridized with the ND6 probe whereas the 4.6 and 3.9 kb bands were recognized by the COI probe. The relative intensities of the hybridization signal suggests that the same amounts of normal liver sequences recognized by ND6 and COI probes are present in nDNA. In tumors the intensity of the bands is higher than in normal liver, confirming the Eco RI pattern.

In order to determine whether other mitochondrial sequences are also present in nDNA, two other probes were used corresponding to parts of COII and CO III genes respectively. Figure 3 shows that both COII and COIII probes recognized nDNA sequences in normal liver and tumors, with different Eco RI and Hind III patterns. The two probes hybridized with a single 3 kb fragment after Eco RI digestion of the DNAs, the intensity of the signal being at least 10 times higher in tumors than in normal tissues. Hind III digestion of the DNAs revealed a more complex pattern with two fragments of 3.9 and 4.6 kb respectively, detected in nDNA from normal liver, whereas in nDNA from tumors at least two additional 2.2 and 2.9 kb fragments were generally found. The differences in the intensity of the signals cannot be attributed to higher amounts of DNA in the samples, as shown after hybridization of the filters with a pyruvate kinase cDNA probe (P.K).

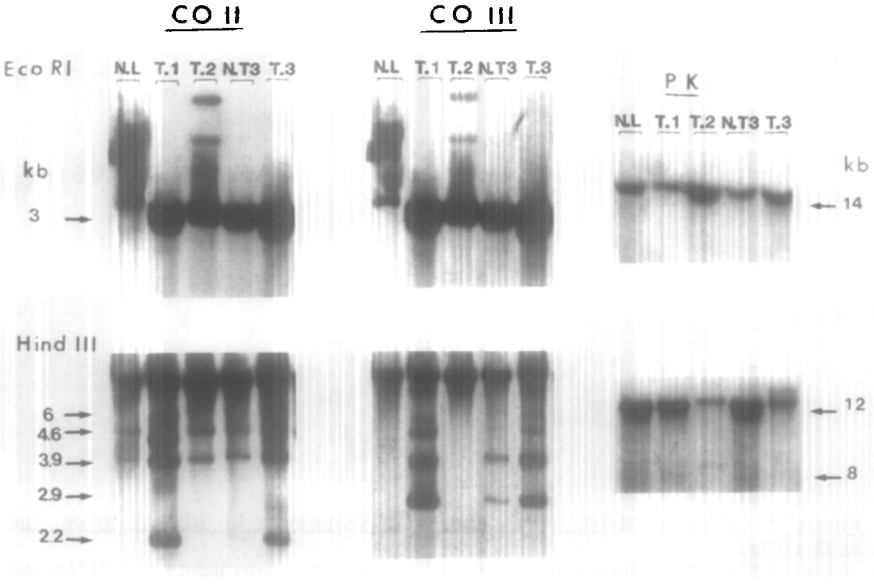

Figure 3. Mitochondrial COII and COIII sequences in nDNAs from rat hepatomas.
The filters used in the experiments shown in figure 2 were successively hybridized with a rat cDNA clone containing 230 bases from the COII gene, with a 1 kb Eco RI-Pst I fragment from the human COIII gene and with a rat pyruvate kinase cDNA clone (P.K).

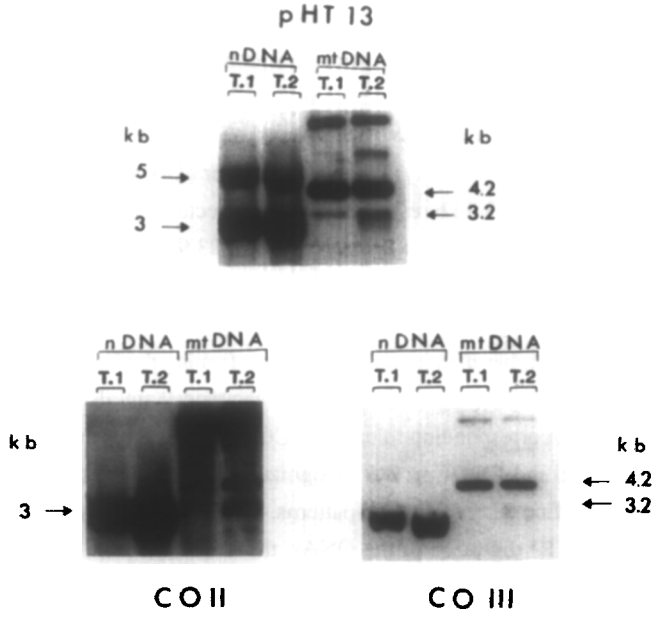

Figure 4. Comparison of the nuclear and the mitochondrial DNA fragments containing pHT 13, COII and COIII sequences.
Mitochondrial DNA and nuclear DNA from liver tumors (T.1 and T.2) were digested at completion with the Eco RI enzyme and submitted to Southern blot analysis. The hybridizations were successively performed with the pHT 13, COII and COIII sequences. After washing at 68°C in a 0.1SSC, 0.1% SDS solution during 45 min, the filters were exposed for 48 h.

Furthermore the hybridization signals observed in tumors cannot be attributed to contamination by mtDNA : Figure 4 shows that mtDNA from liver tumors digested with Eco RI (T1, T2) hybridized with two 4.2 and 3.2 kb fragments when the pHT 13 and the COII cDNAs were used as probes, a single 3.2 kb fragment was detected with the CO III probe.
It then appears that large portions of mtDNA are present in nuclear genome probably under a covalently integrated form.

DISCUSSION

Using both the pHT 13 clone, identified as containing rat mitochondrial sequences, and a DNA fragment containing mouse mitochondrial genes, we have shown that nDNAs from normal rat tissues and from cancerous hepatocytes contain ND6, COI, COII and COIII sequences. Because restriction fragments from mtDNA and from nDNA are different, contamination of nDNA by mitochondrial sequences can be excluded. Mitochondrial DNA fragments have been found in the nuclear DNAs from yeast, podospora, sea urchin, maize, rat and man (1-9, 23, 24). It has been shown that rat nuclear genome carries a DNA sequence homologous to the D-loop and the rRNA gene regions. We have extended this observation to several protein coding sequences. Moreover we show that the copy number and the organization of these mitochondrial-like nuclear sequences have been modified during chemically induced hepatocarcinogenesis. Uoch *et al.* have isolated from a human hepatoma cell library a cDNA clone which contains a mitochondrial ND4 sequence associated with the HBV nuclear sequence (Personal communication). Recently we have described in the same hepatomas, modifications of mtDNA, very early after carcinogen administration and maintained for at least 15 months. Indeed mtDNA has been shown to be the main target for most of the carcinogens (25, 26).
At least two hypotheses can be proposed to account for modification of the mtDNA-like nuclear sequences during cell transformation :
1. These sequences proceeded from those already present in normal cell nuclear genome. Cell transformation leads to their modifications.
2. The mitochondrial sequences were transferred into the nucleus as a consequence of the pressure on the mitochondria exerted by carcinogens or their metabolites.
Wilkie *et al.* (25) and Shay *et al.* (26) have postulated that modifications in mitochondrial DNA may represent an important step in carcinogenesis. Reid has suggested that insertion of mtDNA in nuclear DNA might activate oncogenes (27). Since the genetic code is different in mitochondria and in nuclei it is excluded that the mitochondrial-like sequences could code for a normal regulatory compound in nuclei.

In conclusion combination of mitochondrial and nuclear mutations might be critical for induction of carcinogenesis.

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