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# Tumor-associated mutations of rat mitochondrial transfer RNA genes

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## ABSTRACT

Mitochondrial DNA is a sensitive target of chemical carcinogens (Backer and Weinstein (1980) Science 209, 297-299), suggesting that mutations of the mitochondrial genome occur in tumor cells. We examined this point by comparing mitochondrial DNA sequences in four rat tumors with those of normal rat liver. Some novel mutations found in the tRNA genes of tumor mitochondria were as follows: nucleotides deletions in the amino-acyl acceptor stem of the $tRNA^{Tyr}$ gene or in the anticodon stem of the $tRNA^{Trp}$ gene and insertions in the "TψC" loop of the $tRNA^{Cys}$ gene. These structures are extraordinary compared with those of the tRNA genes of other mammals, indicating that these mutations are each associated with a corresponding tumor.

## INTRODUCTION

The mammalian mitochondrial genome has circular DNA of about 16 kilobases (kb) length arranged in an extremely economic fashion, genes for rRNAs, tRNAs and poly A-containing mRNAs being encoded with only a few spacer nucleotides. Recently, the complete nucleotide sequences of human (1), mouse (2) and bovine (3) mitochondrial DNAs (mtDNAs) were determined and in each case all corresponding genes were found to be organized in the same order. The protein coding genes that have been identified so far encode the components necessary for electron transport and oxidative phosphorylation.

The mitochondrial genome is thought to be a target for some chemical cacinogens and to be altered in tumor cells, because their metabolites are covalently linked to more mtDNA than nuclear DNA (4) and inhibit DNA synthesis (5) or protein synthesis (6) in mitochondria. If mutations of mtDNA occur in tumor cells, the following questions arise: (i) What kind of mutations occur in mitochondrial genes? (ii) Is a certain mutation characteristic of each tumor? (iii) How are mutations in the mitochondrial genome involved in carcinogenesis?

To answer these questions, we tried to detect genomic mutations in mtDNAs of existing rat tumor cell lines, that is, Morris hepatoma (#5123D), Yoshida sarcoma (YS), ascites hepatomas AH-130 and AH-7974. In a series of experiments, we found insertions, deletions or substitutions of nucleotides in the genes coding for 16S rRNA, tRNAs and/or poly A-containing mRNAs of YS, AH-130 and AH-7974 mitochondria. The present paper reports studies with particular attention to variations in the tRNA genes found in YS, AH-130 and AH-7974, because the structural features of standard tRNA molecules have been well characterized and it is relatively easy to assess interspecific differences other than polymorphic changes. Other mutations observed in the protein coding region, as well as in the 16S rRNA gene, will be reported elsewhere.

## MATERIALS AND METHODS

### Tumor cell lines

Morris hepatoma (#5123D) cells were obtained a solid tumor from Buffalo rats. Yoshida sarcoma (YS) and ascites hepatoma AH-130 were harvested from the peritoneal cavitiy of Donryu rats, and ascites hepatoma AH-7974 cells from that of Buffalo rats.

### Gene cloning

MtDNAs were prepared from rat tumor cells and normal rat liver of the Donryu strain and digested with restriction endonuclease BamHI (7). The resulting BamHI DNA fragments, BamA (11 kb) and BamB (5.2 kb), were cloned using pBR322 as a vector.

### DNA sequencing

5'-End labeling of the DNA fragment, strand separation and sequence analysis by a chemical method were performed as described by Maxam and Gilbert (8).

### Gel electrophoresis and autoradiography

Agarose gel electrophoresis was carried out on horizontal slab gel in 40 mM Tris-HCl (pH 7.8), 20 mM sodium acetate, 2 mM EDTA, 0.2 μg/ml ethidium bromide at 60-70 V. Polyacrylamide gel electrophoresis was carried out on vertical slab gel (1.0 mm x 14 cm x 33 cm) in 45 mM Tris-borate (pH 8.2), 2 mM EDTA at 400-500 V. Gels were stained with 2 μg/ml ethidium bromide and photographed using Polaroid film. Sequencing gels of 8 or 15 % polyacrylamide (0.3 or 0.5 mm x 35 cm) containing 7 M urea were prepared and subjected to electrophoresis at 1000-1800 V. Autoradiography was performed with Kodak X-Omat XRP-1 film using an intensifying screen.

RESULTS

Comparison of fine restriction maps and electrophoretic mobilities of mitochondrial DNA fragments of normal rat liver and tumor cells

To detect changes of nucleotide sequences in BamA fragments of 11 kb of mtDNAs from cells of normal liver and the four different tumor cell lines, we compared the restriction maps and electrophoretic mobilities of the restriction fragments on 0.5-2.0 % agarose gel and 5 % polyacrylamide gel, respectively. The restriction maps of BamA fragments were made with various restriction endonucleases, that is, HindIII, EcoRI, HhaI, HaeIII, HinfI, Sau3A, TaqI and MboII in combination. The results clearly showed that the mtDNAs of ascites hepatoma cells AH-130 and AH-7974 both differed from that of normal rats in terms of their restriction enzyme cleavage site (newly created or disappearing) and the electrophoretic mobilities of the restriction fragments. Only a few fragments of Yoshida sarcoma (YS) differed in electrophoretic mobility. Some of the changes located in the region of the HindB/EcoC fragment of BamA are shown in Fig.1, where five tRNA genes are clustered around the origin of light-strand replication ($O_L$). In AH-130 and AH-7974, a new HaeIII site was created in the $tRNA^{Tyr}$ gene and one of the TaqI or HinfI sites was lost in the URF2 (unidentified reading frame 2) gene and the $tRNA^{Cys}$ gene, respectively (Fig.1a). On electrophoretic separation of the fragments obtained by HinfI digestion of the HindB/EcoC fragment, it was observed that the 0.14 kb-fragment from YS had higher mobility than that of the fragment from normal liver (Fig.1b). This 0.14 kb-fragment contains the genes for the N-terminal region of cytochrome c oxidase subunit I (COI), $tRNA^{Tyr}$ and the 5'-half of $tRNA^{Cys}$, as shown in Fig.1a. It was also observed that the mobility of the 0.42 kb-fragment from AH-130 or AH-7974 was less than that of the fragment from normal liver (Fig.1b), in which the genes for $tRNA^{Ala}$, $tRNA^{Trp}$ and the C-terminal region of URF2 are located (Fig.1a). On the other hand, hepatoma #5123D was identical to normal liver in its restriction map and the electrophoretic mobilities of the various restriction fragments of BamA so far examined. Divergences observed in the HindB/EcoC fragment reflected the mutation of the tRNA gene in YS, AH-130 or AH-7974 mitochondria.

Comparison of nucleotide sequences of tRNA genes from normal liver and tumor cell mitochondria

To determine what kinds of mutation occur in the tumor cells, we compared the regions containing the five tRNA genes in mitochondria of normal liver and tumor cells. The nucleotide sequences of 480 base pairs (bp) from

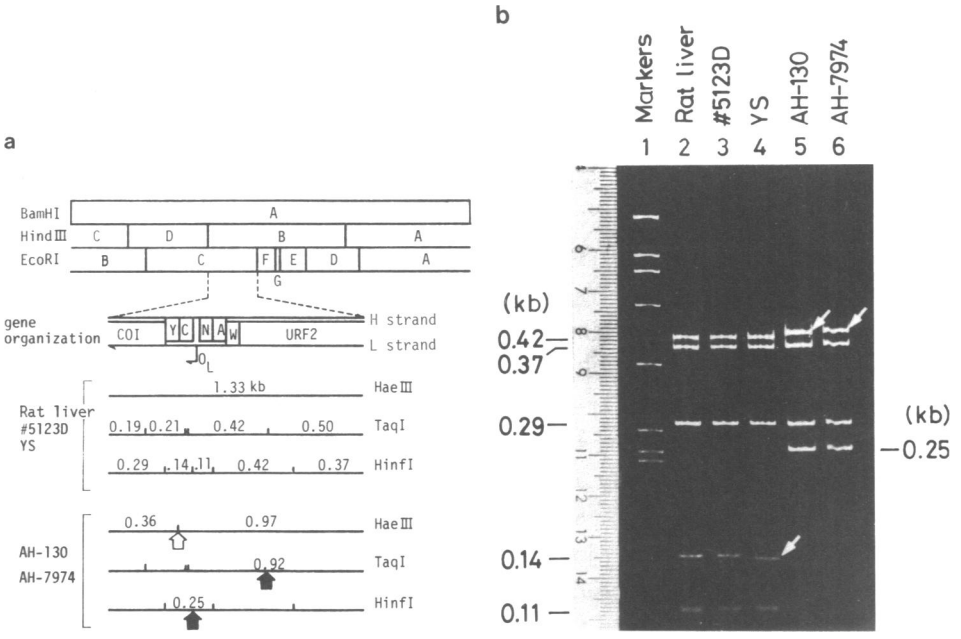


Fig. 1 Restriction maps and electrophoretic patterns of the HindB/EcoC fragment of mitochondrial DNA from rat liver and tumor cell lines. (a) Top: Restriction maps of the BamA fragments of rat liver mtDNA obtained with HindIII and EcoRI. Center: Gene organization of the HindB/EcoC fragment, where the tRNA genes are indicated by the one-letter amino acid code. Genes for $tRNA^{Tyr}$(Y), $tRNA^{Cys}$(C), $tRNA^{Asn}$(N) and $tRNA^{Ala}$(A) are located in the H strand, while genes for $tRNA^{Trp}$(W), cytochrome c oxidase subunit I (COI) and unidentified reading frame 2 (URF2) are in the L strand. The presumptive replication origin of the L strand ($O_L$) is located in between the $tRNA^{Cys}$ and $tRNA^{Asn}$ genes. Bottom: Restriction maps of the HindB/EcoC fragments of mtDNAs from rat liver and tumor cell lines for HaeIII, TaqI and HinfI. Cleavage sites were identical in the rat liver, Morris hepatoma (#5123D) and Yoshida sarcoma (YS), while, in AH-130 and AH-7974, a new HaeIII site appeared(⌂) and each one of the TaqI and HinfI sites disappeared (⬟). (b) Electrophoretic patterns of the HindB/EcoC fragments of mtDNAs from rat liver and tumor cell lines digested with HinfI. The HindB/EcoC fragment (1.33 kb) digested with HinfI was separated by 5 % polyacrylamide gel electrophoresis, and stained with ethidium bromide. The AluI fragments of pBR322 DNA were used as size markers (lane 1). Samples in lanes 2, 3, 4, 5 and 6 are of rat liver, #5123D, YS, AH-130 and AH-7974, respectively. The numerical figures on the left of the photograph are sizes of DNA fragments of rat liver DNA, while that on the right is the size of a new DNA band of AH-130 and AH-7974. These values were estimated by 2 % agarose gel electrophoresis. Arrows in the photograph show DNA bands with different mobilities from those of rat liver DNA.

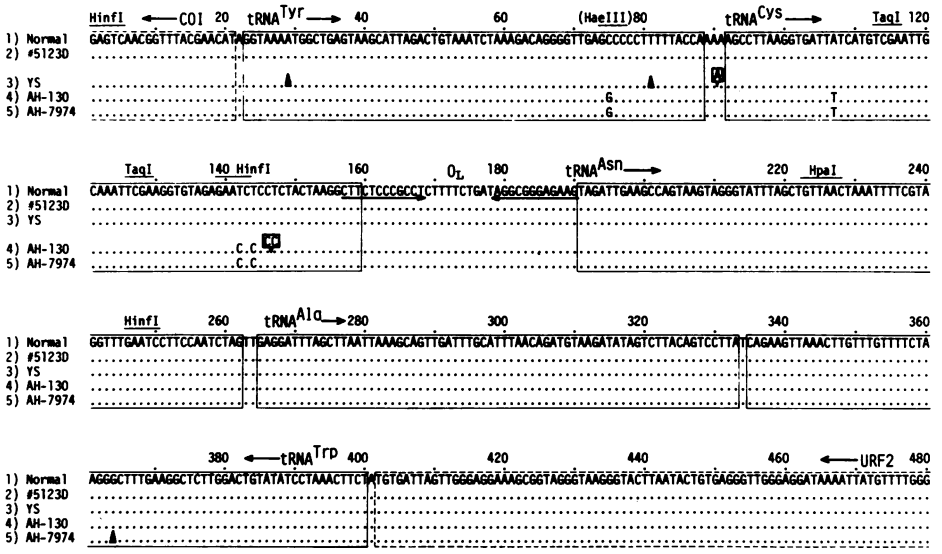


Fig. 2 Nucleotide sequences of the DNA region containing a cluster of five different tRNA genes.
The first sequence is the H-strand sequence of the rat liver DNA. The tRNA genes, identified by looking for a clover-leaf structure, are shown in boxes with solid lines. The 5'-end and 3'-end of COI and URF2 genes, respectively, are indicated by broken lines. The polarities of genes are indicated by arrows. Sequences 2, 3, 4 and 5 correspond to those of #5123D, YS, AH-130 and AH-7974, respectively. Identical nucleotides are shown by dots. Black triangles indicate deletions. ▽ is an inserted nucleotide sequence.

the HinfI site within the 5'-end region of the COI gene were compared and are shown in Fig.2. The nucleotide sequence of #5123D was identical with that of the normal rat, which is consistent with the data from restriction mapping and electrophoretic analysis. In contrast, significant changes of nucleotide sequences were detected in the genes for $tRNA^{Tyr}$, $tRNA^{Cys}$ and $tRNA^{Trp}$ of the other tumor mitochondria. In ascites hepatoma cells AH-130 and AH-7974, four nucleotide replacements were observed at positions 75, 107, 142 and 144. The C⟶G transversion at position 75 created a new HeaIII site in the $tRNA^{Tyr}$ gene and the T⟶C transition at position 142 eliminated one of the HinfI sites in the $tRNA^{Cys}$ gene. In AH-130, di-nucleotide CC was inserted at a position next to 146th nucleotide in the $tRNA^{Cys}$ gene. On the other hand, the 364th nucleotide G of the $tRNA^{Trp}$ gene was deleted in AH-7974. In the YS sequence, two nucleotides A and T were deleted at positions 29 and 81, respectively, of the $tRNA^{Tyr}$ gene, while

one nucleotide was inserted into the spacer region next to the 90th nucleotide, resulting in a change in mobility of the 0.14 kb-HinfI fragment on polyacrylamide gel electrophoresis (Fig.1b). In addition, the trinucleotide TGG inserted into the URF2 gene region of the 0.42 kb-fragment of AH-130 and AH-7974 (data not shown), resulting in reduced mobility of this fragment (Fig.1b).

Structural feature of mitochondrial tRNA genes from rat tumor cells and mammals

To examine the difference of mitochondrial tRNA genes of rat tumor cells from those of normal liver, we compared the secondary structures of the genes for $tRNA^{Tyr}$, $tRNA^{Trp}$ and $tRNA^{Cys}$, deduced from the nucleotide sequences of these four rat tumor cells, and those of rat liver, mouse, bovine and human cells. As shown in Fig.3a, the aminoacyl acceptor stem of the $tRNA^{Tyr}$ (boxed region ) is completely conserved in the four mammalian species, but one of seven base pairs in the stem structure is deleted in the YS $tRNA^{Tyr}$ gene, that is two bases A and T at positions 29 and 81, respectively (Fig.2). As a consequence, the product of this gene would have a shorter aminoacyl acceptor stem consisting of six base pairs (Fig.3a,YS(i)). If an A-A pair is formed, seven base pairs may be constructed using a spacer nucleotide adjacent to the 5'- and 3'- ends of the gene (Fig.3a, YS(ii)).

Although the anticodon stem of $tRNA^{Trp}$ in the four different mammalian species is well conserved, one nucleotide G in the right hand side of the stem in the AH-7974 gene was deleted (Fig.3b). This deletion might result in formation of two alternative stems of either abnormal (Fig.3b, AH-7974 (i)), or different structure, where one nucleotide from the extra-loop region could compensate for this deletion in the anticodon stem (Fig.3b, AH-7974 (ii)). In the latter case, the extra loop would consist of three nucleotides, a size that has been reported in only three tRNAs (3,9).

The size and sequence of the "TΨC" loop of mitochondrial tRNA gene were comparatively variable in the four different mammalian species. The "TΨC" loop of the $tRNA^{Cys}$ gene consisted of eight nucleotides in rats and six nucleotides in other species (Fig.3c). In AH-130, however, this loop was ten nucleotides long because of a dinucleotide CC insertion. This is extraordinary in comparison with this tRNA gene in other mammals and even with other tRNA genes of mammalian mitochondria which have a loop of nine nucleotides at most (1-3,9-12).

As mentioned above, nucleotide deletion or insertion in tRNA genes of tumor mitochondria resulted in various changes of the secondary structure,

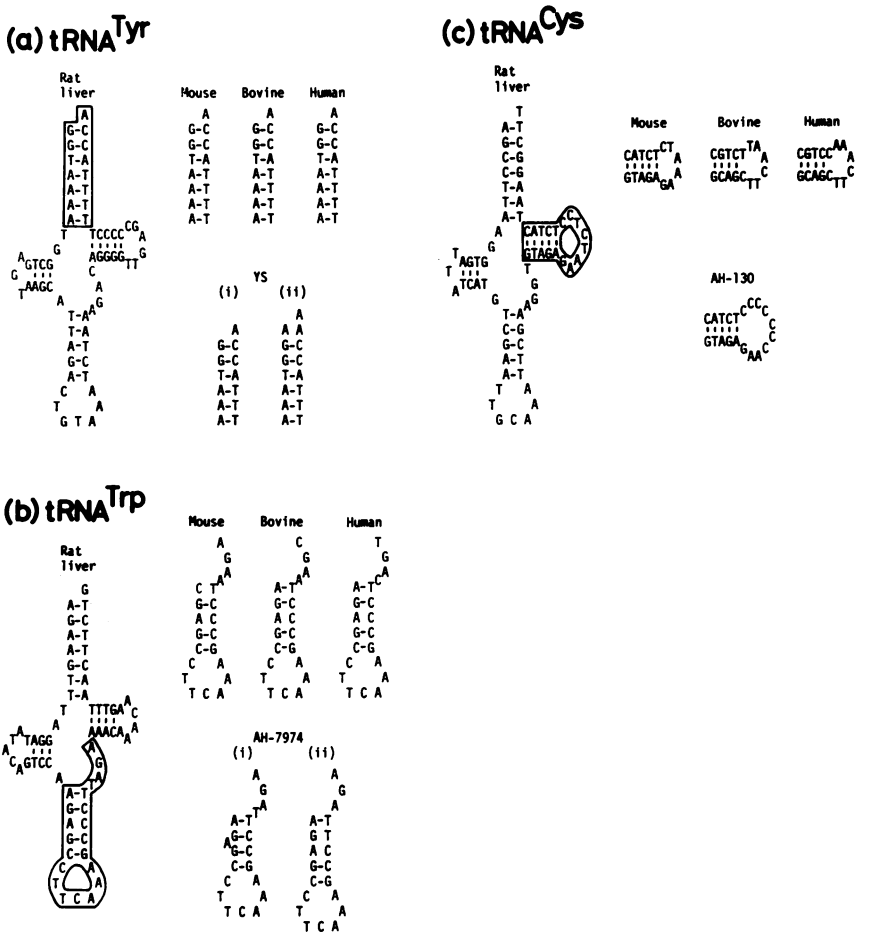


Fig. 3 Comparison of secondary structures of mitochondrial tRNA genes from rat liver, tumor cells and mammals.
The divergent region in the tRNA gene from the tumor is shown in the secondary structure of the normal gene as a boxed-in region and is also compared with its counterparts in other mammalian species, mouse (2), bovine (3) and human (1). (a) Comparison of aminoacyl acceptor stems of $tRNA^{Tyr}$ genes. In the case of YS, either (i) or (ii) would be possible. (b) Comparison of regions consisting of anticodon stems and extra loops in $tRNA^{Trp}$ genes. In the case of AH-7974, either (i) or (ii) would be possible. (c) Comparison of "TΨC" loops of $tRNA^{Cys}$ genes.

which have not been found either in mitochondrial tRNAs or in other tRNAs (13). Therefore, these variations of mitochondrial tRNA genes seem to be specifically associated with the respective tumor cells. Other changes of

nucleotide sequence by base replacement in the tRNA genes from AH-130 and AH-7974 were only observed in the loop regions such as "TΨC" loop of the $tRNA^{Tyr}$ gene, and the "D" and "TΨC" loops of the $tRNA^{Cys}$ gene (Fig.2). This is discussed in detail in the DISCUSSION.

## DISCUSSION

In the present work, several mutations of the mitochondrial tRNA genes were demonstrated in rat tumor cell lines, such as Yoshida sarcoma (YS), ascites hepatomas AH-130 and AH-7974, but not in Morris hepatoma so far as examined. Nucleotide deletions occurred in sequences of the YS $tRNA^{Tyr}$ and AH-7974 $tRNA^{Trp}$ genes, which were, in turn, well conserved in rat, mouse, bovine and human mitochondria (Fig. 3a and b). In addition, the insertion of two nucleotides resulted in the formation of a "TΨC" loop of the AH-130 $tRNA^{Cys}$ gene containing ten nucleotides, which was outside the range of size of "TΨC" loops in either mitochondrial or cytoplasmic tRNAs (1-3,9-12). Therefore, these variations seem to be mutations characteristic of the respective tumor cell lines. However, the products of these mutated genes are apparently functional for the following reasons: (i) If the products had no function, protein synthesis would be inhibited, because the mitochondrial genome has a single gene for each tRNA molecule. (ii) Mitochondrial tRNAs or tRNA genes are more variable than cytoplasmic or procaryotic ones and do not satisfy several criteria for the secondary structure of standard tRNA (e.g., mitochondrial $tRNA^{Ser}_{AGY}$ has no "D" loop or "D" stem), indicating that tRNA molecules with this extent of variation are still fully functional in mitochondria (1-3). However, it is not known whether these mutated tRNAs in tumor cells are completely functional. If not (low fidelity and/or low velocity of protein synthesis), mutation could cause an abnormality of cell function in the tumor cells.

As described above, the sequence of about 1.7 kb of #5123D mtDNA, that has been sequenced so far (partly shown in Fig. 2), was completely identical with that of normal liver. Thus mutation does not readily occur during passage of tumor cells. That is, the mutations found in YS, AH-130 and AH-7974 mitochondria must have occurred during the period of chemical carcinogen(s) administration rather than during cell passages. As AH-130 and AH-7974 cells originated from hepatomas induced by DAB (dimethylamino azobenzene) in different rats, it seems probable that these two tumors have certain variations in common. In fact, the nucleotide replacements were observed in the "D" and "TΨC" loop regions of tRNA genes from both AH-130 and AH-7974 in the same

fashion (Fig. 2). As there is less conservation of these loop regions in rat, mouse, bovine and human (1-3), it is, however, not clear yet whether the variation in the "D" and "TΨC" loop regions were associated with specific properties of the tumor cells or polymorphic changes in the rats, from which the tumor cells were derived. Our preliminary experiments showed that the restriction patterns of mtDNAs from the liver of rats treated with DAB showed a similar pattern to those of mtDNAs from AH-130 and AH-7974 cells. We, therefore, suspect that the base replacement found in AH-130 and AH-7974 mtDNAs is induced by DAB administraion.

In this paper, we observed "tumor-associated mutations" in mitochondrial tRNA genes from YS, AH-130 and AH-7974 cells, but the direct correlation between such mutations and carcinogenesis remains to be investegated.

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