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Mitochondrial DNA of Yoshida Ascites Tumour Cells. Physicochemical Properties and Biological Function

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Mitochondrial DNA from Yoshida A.H. 130 cells, has been characterized by determination of the buoyant density by CsCl equilibrium density gradient centrifugation and the thermal denaturation and renaturation behaviour. These studies have been carried out parallelly on nuclear DNA from the same cells in order to search for possible differences between both DNAs.

Mitochondrial DNA of Yoshida cells presents an equlibrium in CsCl of 1.7154 g/cm^3 and a sharp melting with a T_m of 92° C.

Nuclear DNA presents an equilibrium of 1.7030 g/cm^3 and a T_m of 88° C.

The guanine plus cytosine content in both DNAs has been calculated from tumour results and compared with the content in normal rat liver cells. M-DNA of tumour cells presents a higher guanine plus cytosine content than N-DNA, whereas in normal liver cells is higher in N-DNA. N-DNAs of both normal and tumour cells have the same guanine plus cytosine content, whereas M-DNA from tumour cells presents a significant increase (about 35 %) with regard to this from normal liver cells.

The presence of unusual forms like catenated or circular oligomers in M-DNA has been first described in tumour cell mitochondria (5, 6, 7). These results gave rise to the idea that unusual forms could be characteristic of tumour cells M-DNA, A few years later, was also demonstrated the presence of these unusual forms in normal cells M-DNA (1, 2).

Having excluded this morphological property in order to characterize tumour cells M-DNA, we have studied from a biochemical point of view M-DNA and N-DNA from Yoshida hepatoma A.H. 130 tumour cells, in order to search for possible differences at a biochemical level

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between tumoral and normal liver cell DNAs.

In this paper, values for the guanine plus cytosine (G-C) content in nuclear and mitochondrial DNA from Yoshida tumour cells are determinated and compared to the G-C content in nuclear and mitochondrial DNA from rat liver cells (3). We have employed the buoyant density and thermal denaturation studies as analytical procedures, in order to calculate the G-C content of these nuclear and mitochondrial DNAs from tumour cells.

Materials and Methods

Preparation of mitochondria. Mitochondria from Yoshida hepatoma cells were isolated in semisterile conditions as described in a previous paper (9).

Preparation of nuclei. Wistar rats bearing tumours were sacrificed and collection of ascitic fluid, washing of the cells and homogenization were carried out under the same conditions as for the preparation of mitochondria. After homogenization the tumour cell homogenate was centrifuged at 600×*g* for 20 minutes and supernatant was discarded. The precipitate was resuspended in solution S (0.25 M sucrose, 1 mM Na EDTA) containing 0.1 M citric acid (400:1 v/v) and centrifuged for 20 minutes at 200 × *g*. The supernatant was collected and again centrifuged at 800 × *g* for 10 minutes. The precipitate was washed once in solution S under the same conditions. The pellet obtained in the previous washing was resuspended in 1.62 M sucrose in TMK (50 mM Tris buffer, (pH 7.4), 5 mM $MgCl_2$, 2.5 M KCl) and centrifuged at 95,000 × *g* for 90 minutes at 4° C. Supernatants were discarded an the precipitates resuspended in solution S after previously rinsing the tubes containing pellets with solution S.

Extraction of M-DNA. Mitochondrial DNA was isolated by a slight modification of the BORST procedure (4).

The mitochondrial suspension was mixed with an equal volume of cold 0.3 M NaCl, 0.2 M Na EDTA (pH 8) followed by a lytic treatment with 0.1 times the previous total volume of 22 % SDS (sodium dodecyl sulfate). No previous treatment with DNase as described by BORST (4) was carried out. The mixture was carefully mixed by magnetic stirring until the mitochondrial suspension became clear broth. The mixture could additionally be swirled briefly in a 30° C water bath and after the suspension became clear (within 30 seconds) it was cooled again in an ice bath to 0° C. 5 M $NaClO_4$ was added to a final concentration of 1 M followed by about half a volume of chloroform-isoamyl alcohol (24:1 v/v). The mixture was carefully shaken, periodically, 10 times every 3 minutes, in MSE Mistral centrifuge 250 ml glass stoppered flasks and afterwards centrifuged in a MSE Mistral centrifuge at 1,300 × *g* for 15 minutes at 4° C. The top aqueous layer obtained after centrifugation was carefully collected by means of a Pasteur pipette. DNA was obtained from this fraction by precipitation overnight with two volumes of 95-100 % cold ethanol at —20° C. The filamentous precipitate was then collected by centrifugation at 1,300 × *g* for 15 minutes at —20° C.

Preparation of nuclear DNA. Nuclear DNA was prepared from purified nuclei by the same procedures as described for M-DNA.

DNA purification. The pellet, containing DNA, was resuspended in an equivalent amount of the primitive mitochondrial volume of 0.1×SSC (0.015 M NaCl, 0.0015 M Na citrate final pH 7.2) and then dialyzed for at least 3 × 8 hours against at least 3 × 2 liters of 0.1 × SSC. The dialisate was incubated for 30 minutes with 50 μg pancreatic ribonuclease (E.C. 2.7.7.16) (Worthington) per ml at 30° C and deproteinized once more with chloroform as was described before for DNA

isolation. Aqueous layer fraction containing DNA was further purified in one layer MAK (methylated albumin kieselguhr, Serva and BDH respectively) column prepared according to SUEOKA (12) and exhaustively washed with 0.44 M NaCl, 0.05 M sodium phosphate buffer (final pH 6.8). The DNA in 0.1 × SSC was layered on the top of the MAK column (3.0 cm ∅, 4 cm height). The MAK column previously covered by aluminium foil (to avoid the action of the light), was eluted with 1.2 M NaCl, 0.05 M sodium phosphate buffer (final pH 6.8) at an elution velocity about 3 ml per minute. Fractions containing 2-3 ml were collected in a LKB ultrorac fraction collector. All chromatographycal procedures were carried out in a cold chamber at 4° C. Diluted DNA aliquots were concentrated by centrifuging at high speed (120,000 × *g*) for 15 hours and the top two thirds of the fluid removed by sucking with a Pasteur pipette. Optical density was controlled, and if no high content in DNA was present in these fractions, discarded. The occasional pellet can then be resuspended in the lower fraction of the supernatant. DNA quantitative determination was achieved by optical density determination at 260 nm. Protein quantitative determination was carried out by the WADDELL procedure (13) modified by MURPHY (10).

CsCl equilibrium density gradient centrifugation. The DNA purified as previously described was dialyzed against 1 mM Na EDTA (pH 7.4) for 16 hours. To 3.5 ml fractions containing 35-40 μg DNA per ml, 0.5 ml of 0.5 M sodium phosphate buffer (pH 7.4) and 5.35 g CsCl (Merck) was added. The pH of the solution was controlled and carried if necessary to neutral values. The (refractive index) of the solution was measured by means of a thermostated Abbe refractometer at 25° C, and adjusted if necessary to a $\eta_{D\ 25^\circ}$ of 1.4000 by adding CsCl or sodium phosphate buffer. The mixture was centrifuged in a polyallomer tube of the S.W. 56 Ti Spinco rotor at 298,000 × *g* for 35 hours at 4° C in a Spinco L 50 B centrifuge. Following the run, the tube was punctured and 20 drop fractions were collected. The fraction's density was determinated by means of refractometer and the optical density at 260 nm after previous dilution with 0.6 ml of 25 mM sodium phosphate buffer, 1 mM Na EDTA (final pH 7.4), was read in 1 cm light path cuvettes.

Thermal denaturation measurement. Melting properties of DNAs in solution were monitored out at 260 nm by heating 1.0 ml samples in 1 ml stoppered glass cuvettes, to avoid solvent evaporation, in a spectrophotometer equipped with a thermoregulated cuvette holder. Correction factors for the difference in temperature inside the cuvettes were obtained as well as for variations in specific volume for different temperatures. The DNA solutions were equilibrated for 10 minutes at each temperature. The DNA content in these solutions was about 5-10 μg per ml in 1 × SSC. Corrections for water specific volume values were achieved.

Results

Mitochondrial and nuclear DNA prepared by the above described procedure, presented a typical nucleic acid spectrum. The profiles of the equilibrium density in CsCl for both mitochondrial and nuclear DNA from tumour cells, are presented in figures 1 and 2. The refractive index at 25° C has been determinated in the maximal containing DNA fractions, in order to calculate the buoyant density values according to SCHILDKRAUT (11). The values obtained for mitochondrial and nuclear DNA are 1.7154 and 1.7030 g/cm³ respectively (table I).

Mitochondrial and nuclear DNA in 1 × SSC presented the thermal denatur-

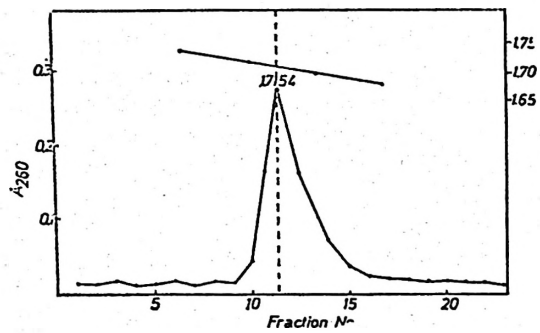

Fig. 1. *Buoyant density of mitochondrial DNA in CsCl (in neutral sodium phosphate buffer).* Experimental conditions as described in Materials and Methods.

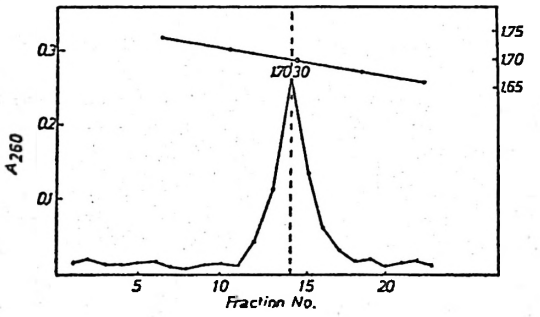

Fig. 2. *Buoyant density of nuclear DNA in CsCl (in neutral sodium phosphate buffer).*

Table I. *Buoyant density and thermal denaturation of the mitochondrial and nuclear DNAs from rat liver and Yoshida cells.*

	ρ in CsCl (g/cm³)		Tm in SSC (°C)	
	M-DNA	N-DNA	M-DNA	N-DNA
Rat liver	1.701	1.703	85.6	87
Yoshida hepatoma A.H. 130	1.715	1.703	92	88

ation behaviour shown in figures 3 and 4 respectively. The Tm values obtained (table I), are 92 and 88° C respectively; on slow cooling M-DNA regained its original hypochromicity, indicating complete renaturation (8). The cooling profile of N-DNA in contrast, was that observed for a complex polynucleotide in which the original double helical structure is not reached again.

Base composition of mitochondrial and nuclear DNA. A significant reproducible difference in base composition between

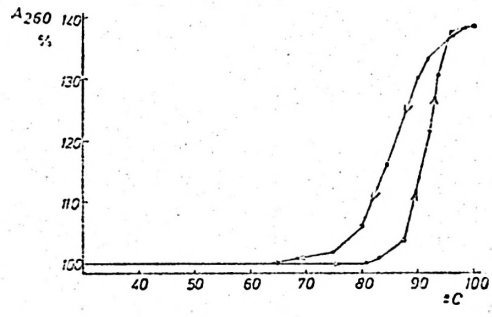

Fig. 3. *Thermal denaturation in SSC of mitochondrial DNA.*
Arrows pointing to the right represent variation in optical density at 260 nm during heating, arrows pointing to the left, variations in optical density during slow cooling. Experimental conditions as described in Materials and Methods.

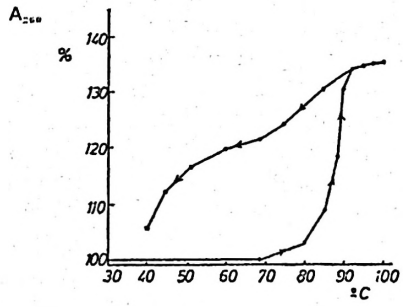

Fig. 4. *Thermal denaturation in SSC of nuclear DNA.*
Symbols are as is figure 3.

Table II. *Guanine + cytosine content (%).*

	From buoyant density values		From Tm values	
	M-DNA	N-DNA	M-DNA	N-DNA
Rat liver	42	44	40	43
Yoshida hepatoma A.H. 130	57	44	55	46

mitochondrial and nuclear DNA was observed as result of the melting temperature and buoyant density values. The buoyant density of mitochondrial and nuclear DNAs corresponded to a calculated G-C content of 57 and 44 % respectively. The melting temperature of mitochondrial and nuclear DNA corresponded to a G-C content similar to those above calculated from the bouyant density values, that is 55 % for M-DNA and 46 % for N-DNA.

Discussion

As it can be concluded from the above described results mitochondrial and nuclear DNAs from Yoshida cells show concordant values in G-C content in both techniques. The studied M-DNA from Yoshida cells seems to be quite different from normal rat liver M-DNA in base composition, showing a greater content in G-C for Yoshida M-DNA of about 35 % in relation to normal rat liver M-DNA (table II), whereas N-DNA from normal and Yoshida tumour cells show similar values in G-C content. It is interesting to point out that M-DNA shows a rapid and complete renaturation curve (fig. 3). This renaturation may be either due, as well known, to homogeneity in base sequence or to an inability of the strands to separate under denaturing conditions (8). Separation may be prevented by cross links (8), or because the DNA consists of intact circular molecules in which both strands are covalently continuous.

These results gave rise to a conclusion of a visible change in physical properties and therefore in nucleotides content between normal and tumour cells only regarding the M-DNA, without any changes in physical properties between both N-DNAs. It is also interesting to point out that the G-C content in normal rat liver cells is 5.75 % lower in N-DNA than in M-DNA. Whereas in tumour cells, M-DNA shown a significant increase in the G-C content.

Resumen

El DNA mitocondrial de células de Yoshida A.H. 130 ha sido caracterizado por determinación de su *buoyant density* por centrifugación en gradiente de equilibrio en CsCl y su comportamiento en la desnaturalización térmica y renaturalización. Estos estudios han sido realizados paralelamente con DNA nuclear de las mismas células a fin de conocer las posibles diferencias entre ambos.

El M-DNA de células de Yoshida presenta una ρ de 1,7154 g/cm^3 y una rápida desnaturalización con un Tm de 92° C. El N-DNA presenta una ρ de 1,7030 g/cm^3 y un Tm de 88° C.

A partir de estos resultados ha sido calculado el contenido en guanina y citosina en ambos DNAs comparándose posteriormente con el contenido en los DNAs de células normales de hígado de rata. El M-DNA de células tumorales presenta un contenido en guanina y citosina más alto que el N-DNA; por el contrario, en células normales es más alto en el N-DNA. Los N-DNAs de células normales y tumorales presentan igual contenido en guanina y citosina; por el contrario, el M-DNA de células tumorales presenta un notable aumento ($\sim$ 35 %) respecto al contenido en el M-DNA de células normales.

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References

1. ASHWELL, M. and WORK, T. S.: *Ann. Rev. Biochem.*, 39, 251-290, 1970.
2. BORST, P.: *Ann. Rev. Biochem.*, 41, 333-376, 1972.
3. BORST, P. and KROON, A. M.: *Int. Rev. Cytol.*, 26, 107-190, 1969.
4. BORST, P., RUTTENBERG, G. J. C. M. and KROON, A. M.: *Biochim. Biophys. Acta*, 149, 140-155, 1967.
5. CLAYTON, D. A., DAVIS, R. W. and VINOGRAD, J.: *J. Mol. Biol.*, 47, 137-153, 1970.
6. CLAYTON, D. A., SMITH, C. E., JORDAN, J. M., TEPLITZ, M. and VINOGRAD, J.: *Nature*, 220, 976-979, 1968.
7. HUDSON, B., CLAYTON, D. A. and VINO-

GRAD, J.: *Cold Spring Harbour Symp. Quant Biol.*, 33, 435-442, 1968.
8. MARMUR, J., ROUND, R., SCHILDKRAUT, C. I.: *Progr. Nucl. Acid. Res.*, 1, 231-300, 1963.
9. MONTALVO, A., PICCOLELLA, E., FRIGOLA, A. and GUERRITORE, D.: *Boll. Soc. it. Biol. Sper.*, 48, 233-236, 1972.
10. MURPHY, J. B. and KIES, M. W.: *Biochim. Biophys. Acta*, 45, 382, 1960.
11. SCHILDKRAUT, C. L., MARMUR, J. and DOTY, P.: *J. Mol. Biol.*, 4, 430, 1962.
12. SUEOKA, N. and CHENG, T. Y.: *J. Mol. Biol.*, 4, 161, 1962.
13. WADDELL, W. J.: *J. Lab. Clin. Med.*, 48, 311, 1956.