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Abstract

For the purpose to clarify the distribution of DNA in mouse ascites sarcoma cells (SR-C3H) induced by Rous sarcoma virus (Schmidt-Ruppin strain), quantitative assays were carried out by SCHMIDT-THANNHAUSERSCHNEIDER'S method using subcellular fractions isolated from SR-C3H cells and C3H mouse liver as a control tissue, and simultaneously electron microscopic observations were conducted with the rotary shadowed preparations of the SDS-phenol extracted nucleic acids by the protein monolayer technique. The results are briefly summarized as follows. 1. The RNA/DNA ratios in SR-C3H cells and liver cells were 2.3 and 3.7, while those in nuclear fraction of SR-C3H cells and liver cells were 0.34 and 0.56, respectively. The electron micrographs of nuclear nucleic acids revealed a DNA-RNA complex-like structure. 2. DNA and RNA contents of SR-C3H mitochondria were found to be 3.1 and 24 fl-g per mg of protein, respectively, which proved to be greater than those of liver mitochondria. The mean values of the contour length of circular DNA molecules in highest frequency group observed in the electron micrographs were 4.88 μ in SR-C3H mitochondria and 5.08 μ in mouse liver mitochondria. There could be observed circular molecules of duplicated-length in both mitochondrial DNA's and small circular molecules in SR-C3H mitochondrial DNA. 3. In the microsomal and supernatant fractions of SR-C3H cells and mouse liver cells, the ratios of DNA to RNA gave several percent by chemical analysis and this percentage was particularly high in the supernatant of SR-C3H cells. On the other hand, in the electron micrographs, the fibrous structure was significantly recovered in the supernatant nucleic acids of SR-C3H cells, but with difficulty in the other three fractions. This fibrous structure measured 1.13 μ in the mean value of the length and was considered to be DNA as it readily disappeared after the treatment with DNase.

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**STUDIES ON NUCLEIC ACIDS IN ROUS SARCOMA VIRUS-
INDUCED MOUSE ASCITES SARCOMA CELLS
DISTRIBUTION AND ELECTRON MICROSCOPY OF NUCLEIC
ACIDS IN SUBCELLULAR FRACTIONS AND CIRCULAR
DNA IN MITOCHONDRIAL FRACTION**

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Mouse ascites sarcoma cells, induced by Schmidt-Ruppin strain of Rous sarcoma virus, are the cell line (SR-C3H/He) established by YAMAMOTO *et al.* (1, 2). This line contains no directly demonstrable infectious virus, but the virus can be recovered when the cells are inoculated back into the wing web of young chicken (1, 2) or cultured with chicken embryo fibroblasts by the addition of UV-irradiated HVJ (3). This indicates that the viral genome persists in the mouse ascites sarcoma cells in masked form. Rous sarcoma virus (RSV), on entering into normal chicken cells, rapidly converts the latter to malignant form. BADER (4) has reported that DNA function of the host cell is required continuously for virus growth, and in the same manner TEMIN (5, 6) has proposed an hypothesis in the conversion by RSV of a normal chicken embryo fibroblast to a cancer cell, that the viral RNA in the infected cell directs the synthesis of DNA homologous to itself and that this DNA provirus then acts as a template for the production of further viral RNA. According to recent study by Temin it is said that the synthesis of nonchromosomal DNA is required for the formation of the provirus (5, 7). As the viral genome of the mouse ascites sarcoma (SR-C3H) cells is in masked form, it is of a prime interest to study the nucleic acids from virus-induced cancer cells with respect to its state and location in order to clarify the role played by a virus in carcinogenesis.

Many investigators (8—15) place a great emphasis on mitochondrial DNA as a cytoplasmic gene. Therefore, with the object to reconsider the behaviors of genetic materials in virus-induced cancer cells, we studied the distribution of nucleic acid in the subcellular fractions of SR-C3H cells and mouse liver, separated by the differential centrifugation, and examined by electron microscopy nucleic acids extracted from each subcel-

lular fraction, with special reference to circular molecules of DNA in mitochondrial fractions.

As a result it was found that the mitochondrial circular DNA molecules in SR-C3H cells were slightly shorter in the contour length than those in mouse liver, and circular molecules of varying size were observed in the both mitochondrial DNA's and small circular molecules in the SR-C3H mitochondria. On the other hand, the fibrous linear structures, probably DNA molecules, were readily observable by electron microscopy in the supernatant fraction of SR-C3H cells fractionated under identical conditions as the mouse liver cell supernatant where such structures were hardly detectable.

MATERIALS AND METHODS

Preparation of SR-C3H cells

SR-C3H cells, kindly supplied by Prof. T. YAMAMOTO and Dr. M. TAKEUCHI (Institute of Medical Science, University of Tokyo), were maintained by intra-peritoneal transplantation into C3H/He mouse every 8th or 10th day. Each of the experimental animal was inoculated intraperitoneally under aseptic conditions with 0.1 ml of the ascites. On the 6th day, the mice were decapitated, ascites was collected, and it was immediately stored in ice water bath. The ascites was washed with sucrose solution (containing 0.25 M sucrose, 0.01 M tris-chloride buffer, pH 7.6, and 0.1 mM EDTA) and then centrifuged at 0—4° for 5 seconds at 800 xg to remove erythrocytes. The washing and centrifugation repeated 4 times usually can remove erythrocytes completely.

Subfractionation of SR-C3H cells and mouse liver cells

The SR-C3H cells suspended in the sucrose solution were homogenized by teflon homogenizer, and filtered through the 2 layers of tetoron cloth. The fractionations were carried out by a modified method of HOGEBOM (16) at 0—4°. The homogenate so obtained was centrifuged at 600xg for 10 min. The sediment was suspended in the sucrose solution supplemented with 0.003 M magnesium chloride and centrifuged. The washing was repeated once more, and the sediment was taken as the nuclear fraction. The first supernatant was layered on 0.34 M sucrose, centrifuged at 1,000xg for 10 min, of which the upper layer was carefully collected, centrifuged at 8,000xg for 10 min., washed in the sucrose solution thrice to remove the fluffy layer, and the residue was taken as mitochondrial fraction. The supernatant removed of mitochondria was centrifuged at 10,000xg for 30 min., and after removing the residue, the supernatant was ultracentrifuged at 105,000xg for 60 min., and the sediment was taken as the microsomal fraction. By adding 2-fold volume of ethanol to the final supernatant, the precipitates was taken as the supernatant fraction.

C3H mouse liver was similarly homogenized by glass and teflon homogenizers in the sucrose solution, and filtered through the 2 layers of tetoron cloth. The

filtrate was taken as a starting material. The liver homogenate was subfractionated by the differential centrifugation method in an identical manner as with SR-C3H cell subfractionation.

Estimation of nucleic acid

Separation of nucleic acid from the subcellular fractions was carried out by the method of SCHMIDT-THANNHAUSER (17) and SCHNEIDER (18) as described by VOLKIN and CHON (19). Perchloric acid was used to remove of acid soluble compounds. Solubilized RNA and DNA were measured by the orcinol (20) and the indole method (21) respectively and the values calculated were referred to those obtained with standard solution of commercial yeast RNA (Sigma) and calf thymus DNA (Sigma), respectively, with the aid of ultraviolet absorbancy at 260 m μ using a Hitachi recording spectrophotometer (EPS-3T).

Preparation of nucleic acid

Nucleic acids were isolated from the various subcellular fractions by SDS-phenol extraction (22). Material was suspended in the buffered saline (0.14 M NaCl, 0.01 M tris-Cl, pH 8.1, and 0.001 M EDTA) and treated with polyvinyl sulfate (23) (10 μ g/ml) or bentonite (24) (0.5 % in final) and sodium lauryl sulfate (SLS, 0.5 % in final). Following the addition of an equal volume of buffer saturated phenol, the preparation was shaken for 10 min. at room temperature by hand and centrifuged. All the operations except the shaking was carried out at approximately 4°. Phenol extraction of aqueous phase was further repeated 2 times. Nucleic acid was precipitated by adding 2 volumes of chilled ethanol to aqueous phase and storing in a freezer at -20°.

Preparation of mitochondrial circular DNA

DNA was extracted from mitochondrial fraction by a modified method of MARMUR (25). Mitochondrial fraction was washed in EDTA-saline (0.1 M EDTA, 0.15 M NaCl, pH 8.0) and suspended in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4). Following the addition of SLS to 2 % in final, the suspension was heated at 60° for 10 min., and cooled in ice bath. After the addition of 5N sodium perchlorate to 1N, the preparation was mixed with an equal volume of chloroform-isoamylalcohol (24 : 1), shaken for 20 min. and centrifuged. The nucleic acid from the aqueous phase was precipitated by adding 2 volumes of chilled ethanol and stored over night at -20°. The precipitate was collected by centrifugation, drained, and dissolved in SSC, which was followed by the hydrolysis with RNase (Type 1A from Sigma, 50 μ g/ml) at 37° for 30 min. (25), and then the chloroform extraction was repeated. DNA was precipitated by adding 2 volumes of chilled ethanol, and stored at -20°.

Estimation of protein

The amount of protein was measured by the Biuret's method (26) while comparing it with that of bovine serum albumin (Fraction V, Armor) as standard.

Electron microscope observations

The protein monolayer spreading technique for electron microscope visualization described by FREIFELDER and KLEINSCHMIDT (27) was employed with nucleic acid of varying concentration in the range of 0.1 to 1.0 of optical density

at 260 m μ . The monolayered nucleic acid was picked on the collodion-carbon-coated grid, rinsed with ethanol, and dried. The rotary shadowing was carried out on the grid with platinum-paradium (80 : 20, 30 mg, the product of Oken Shoji Co.) at an angle of 6° from the distance of 5 to 8 cm using a Hitachi vacuum evaporator (HUS-3B). Photographs were taken in a Hitachi electron microscope (11-D), magnification at 10,000.

RESULTS

Distribution of nucleic acid in subcellular fraction of SR-C3H cells and mouse liver cells

The contents of nucleic acids, DNA and RNA, of each subcellular fraction of SR-C3H cells and mouse liver tissue are illustrated in Tables 1 and 2. The mouse liver tissue was used just as a rough reference, because

TABLE 1. DISTRIBUTION OF NUCLEIC ACIDS IN SUBCELLULAR FRACTIONS OF SR-C3H MOUSE ASCITES SARCOMA CELLS

Fractions	Protein %	DNA $\mu\text{g}/\text{mg prot.}$	Total %	RNA $\mu\text{g}/\text{mg prot.}$	Total %
SR-C3H cells	100	27.7	100	64.1	100
Nuclear	24.3	70.6	62.0	24.3	9.2
Mitochondrial	3.8	1.6	0.7	31.8	1.9
Microsomal	5.1	6.7	1.2	157.4	12.6
Supernatant	29.7	2.4	2.6	44.0	20.5

TABLE 2. DISTRIBUTION OF NUCLEIC ACIDS IN SUBCELLULAR FRACTIONS OF C3H MOUSE LIVER

Fractions	Protein %	DNA $\mu\text{g}/\text{mg prot.}$	Total %	RNA $\mu\text{g}/\text{mg prot.}$	Total %
Liver homogenate	100	12.8	100	47.4	100
Nuclear	12.1	55.6	52.7	30.9	7.9
Mitochondrial	11.1	1.1	1.0	21.7	5.1
Microsomal	5.3	7.2	3.0	209.0	23.4
Supernatant	22.0	0.8	1.3	24.3	11.3

the normal control cells for SR-C3H ascites cells were difficult to obtain. The yield of protein in nuclear fraction of SR-C3H was more than that of the liver but the protein recovery in mitochondrial fraction of SR-C3H was less than that of the liver. However, the recovery of total protein by the method described in the text is not satisfactory because of washing of each fraction. In SR-C3H cells there can be seen a decrease in the ratio of RNA and an increase in the ratio of the concentration of DNA to protein as compared with the case of liver homogenate. The RNA/DNA ratio in SR-C3H was 2.3 and that in liver homogenate was 3.7, while in nuclear

fractions it was 0.34 and 0.56, respectively. On the other hand, the amount of DNA to protein in the mitochondrial fraction of SR-C3H was greater than that in the liver isolated by the same method, but the DNA contents in both mitochondria seemed to be greater than those in more purified mitochondria.

The content of DNA per mg protein in microsomal fractions was higher than that in mitochondrial fractions, and DNA was detected in supernatant fractions by the present method. But DNA's in these fractions were calculated up to 7 per cent of respective RNA content, particularly high was the DNA content in the supernatant fraction of SR-C3H cells.

Contents of DNA and RNA in mitochondrial fraction

In the liver mitochondrial fraction it appears to contain practically no contamination, but in the mitochondrial fraction from SR-C3H cells fractionated by the identical methods it can be anticipated that such fraction would contain some nuclear and microsomal materials. Therefore, we separated mitochondria from SR-C3H cells into light and heavy fractions by centrifugation at 6,000 xg for 10 min. In the assays it was found that the heavy fraction contained a greater amount of DNA than the light fraction, but no difference in the RNA contents between the two fractions. These findings seem to suggest that the mitochondrial fraction is prone to a greater contamination of nuclear fragments but after repeated washing it contains less microsomal material. On the other hand, the mitochondrial fraction of SR-C3H cells separated by the centrifugation method contain 3.1 μ g DNA and 24 μ g RNA per mg of protein in the average of six preparations. Moreover, when these preparations are treated with DNase (5 μ g/ml) overnight at 4° the content of DNA is reduced by one-third. Even then this reduced DNA content seems to be still higher than that in the normal liver mitochondria.

Possible distribution of DNA in the microsomal and supernatant fractions

In order to clarify more precisely the DNA materials in the microsomal and supernatant fractions of SR-C3H cells and mouse liver cells, demonstrable as acid-insoluble substances by the indole method, we extracted the nucleic acids by the phenol method. As shown in Table 3, the nucleic acids so extracted from microsomal and supernatant fractions both of the SR-C3H cells and liver contain DNA in the amount 3 to 6 per cent to that of RNA. The indole method is tolerably sensitive for the analysis of DNA in RNA, but it is not so completely a reliable method to detect DNA in the presence of a large amount of RNA. Therefore, in order to ascertain whether or not the indole-positive substances would diminish after DNase

TABLE 3. EFFECT OF DNase TREATMENT ON NUCEIC ACIDS EXTRACTED FROM SUPERNATANT AND MICROSOMAL FRACTIONS OF SR-C3H MOUSE ASCITES SARCOMA CELLS AND C3H MOUSE LIVER. TREATMENT WITH DNase I WAS CARRIED OUT IN ICE BATH FOR 30 MIN. THE CONTENTS ARE EXPRESSED AS THE RELATIVE CONCENTRATION TO THE ORIGINAL RNA CONTENT. PER CENT OF RECOVERY (% REC.) OF DNA WAS CALCULATED BY THE PRECIPITATION BY ETHANOL AFTER THE TREATMENT.

	Before treatment		After treatment	
	RNA	DNA	RNA	DNA (% Rec.)
SR-C3H ascites sarcoma				
Supernatant fraction	100	5.4	95.6	4.2 (74.1)
Microsomal fraction	100	3.0	90.0	2.0 (66.7)
C3H mouse liver				
Supernatant fraction	100	3.7	93.3	2.0 (54.0)
Microsomal fraction	100	3.6	92.4	2.4 (66.6)

treatment, the nucleic acids were placed in the medium containing the buffered saline, 0.005 M magnesium chloride and 10 μ g DNase per ml (Type I, Sigma), which was dipped in ice bath for 30 min and nucleic acids were recovered by ethanol precipitation. After the treatment, the ratio of DNA to RNA in the recovered nucleic acids of each fraction tended to be similar, each decreasing to two-thirds of the original (Table 3). On the other hand, the nucleic acids extracted from the supernatant of SR-C3H and liver contained a considerable amount of RNA of high-molecular weight. Therefore, the high molar saline fractionation (28, 29) was carried out. Namely, we examined RNA of high-molecular weight precipitated in 1.0 M saline at 4° for over night. As shown in Table 4,

TABLE 4. SALT FRACTIONATION OF NUCLEIC ACIDS EXTRACTED FROM SUPERNATANT FRACTIONS OF SR-C3H MOUSE ASCITES SARCOMA CELLS AND C3H MOUSE LIVER

Nucleic acids from supernatant	Per cent of total RNA	DNA/RNA (%)
SR-C3H mouse ascites sarcoma		
Salt-soluble	31	15.0
Salt-insoluble	69	3.5
C3H mouse liver		
Salt-soluble	29	8.0
Salt-insoluble	71	4.3

this operation resulted in the recovery of saline-soluble fractions of about 30 % of the total RNA from the supernatant of either SR-C3H or liver, and the ratio of DNA to RNA was increased in the both salt-soluble fractions, particularly so in SR-C3H preparation. This result may indicate that the presence of DNA cannot be definitive in the high concentration of RNA as mentioned in the foregoing, none the less, the content of DNA in the supernatant of SR-C3H seemed to be higher than the other three fractions.

Electron microscope observation of the extracted nucleic acids

It is observed in electron micrograph that the double stranded nucleic acid is seen as a fibrous form and the single stranded one as an aggregated particle-like or globular form by the rotary shadowing method with the protein monolayer technique.

In the nucleic acid isolated from the nuclear fraction of SR-C3H both the fibrous and globular shapes are seen individually as shown in Fig. 1, and a globular structure on the fibrous structure seems to appear in a complex form in some cases as shown in Fig. 1B. Circular molecules are not observable in these fractions.

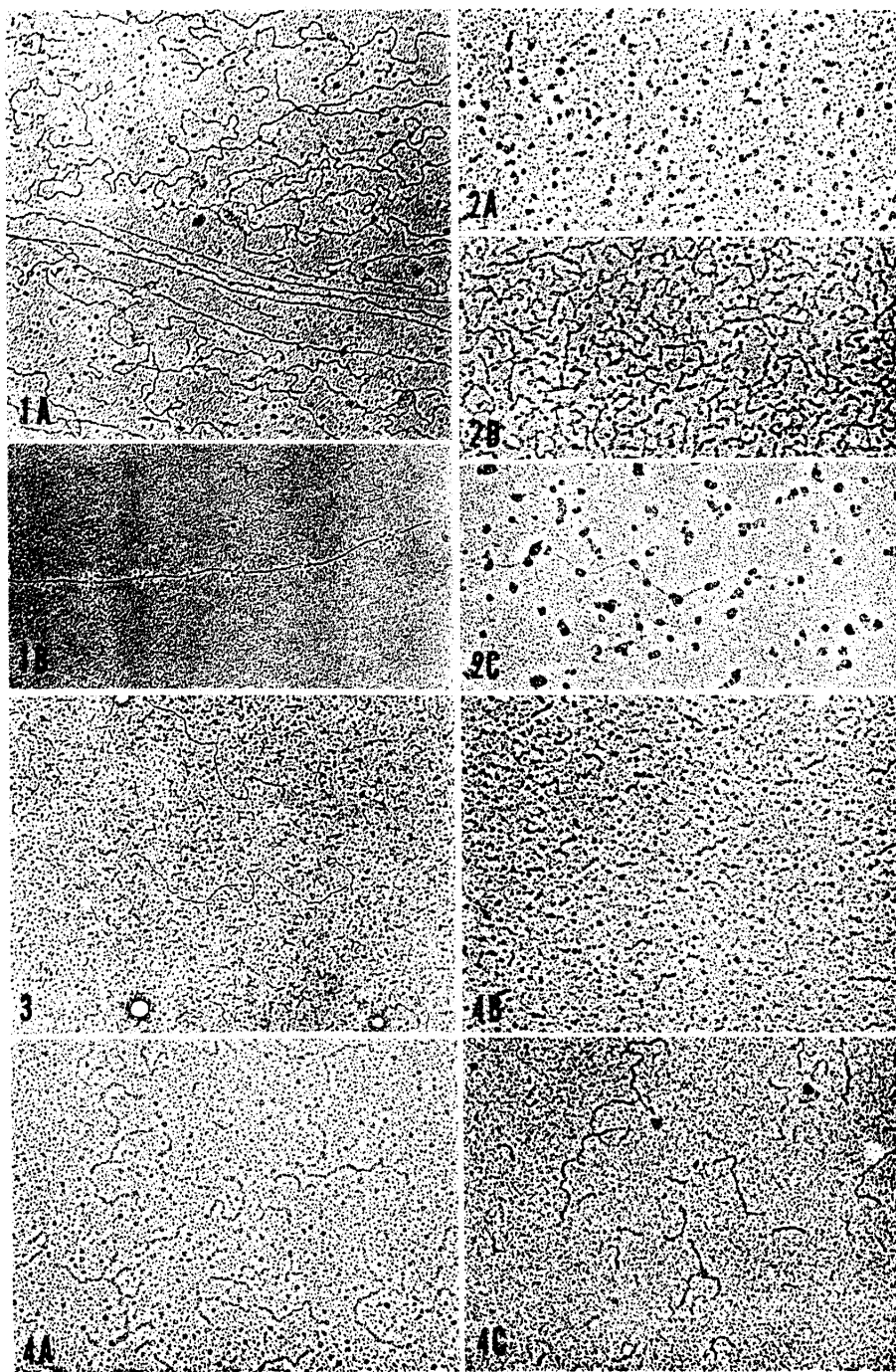
In the electron micrograph of the nucleic acid extracted from microsomal fraction there can be seen hardly any clear-cut fibrous structure, but aggregates of varying sizes. This seems to have resulted from different concentrations of the nucleic acid used to prepare the monolayer (Fig. 2 A & B), but sometimes there appears a chain-like structure as shown in Fig. 2 C.

In the nucleic acid extracted from the supernatant of SR-C3H cells, the fibrous linear structures can be seen without difficulty intermingled with small globular particles (Fig. 3). However, the fibrous one is observable only with difficulty in the case of liver supernatant nucleic acid.

The saline-soluble nucleic acid of SR-C3H supernatant was chromatographed on the methylated serum albumin Kieselguhr column, to be described elsewhere (30), and the fraction was obtained in the DNA eluting region. This fraction contained the DNA and RNA in equal two halves. This was collected by the ethanol precipitation through the dialysis. The electron micrograph (Fig. 4 A) reveals fibrous and globular structures. The linear fibers are more prone to be digested by DNase in 30 min (Fig. 4 B), and the globular one has almost disappeared by the treatment with RNase for 30 min (Fig. 4 C). The contour length distribution of the linear fibers is summarized in Fig. 5, and their length is less than 5 μ , the mean value being $1.13 \pm 0.70 \mu$.

Circular molecules of mitochondrial DNA

The DNA extracted from the mitochondrial fraction of SR-C3H showed open, twisted circular, and linear molecules by rotary shadowing electron microscopic observation, and the DNA from liver mitochondria was nearly of circular form. Thus, it appears that the cancer mitochondrial DNA fraction may allow the contamination of the nuclear DNA fragments. The contour lengths of circular DNA molecules of SR-C3H mitochondria are summarized in Fig. 6, where the mean value of the



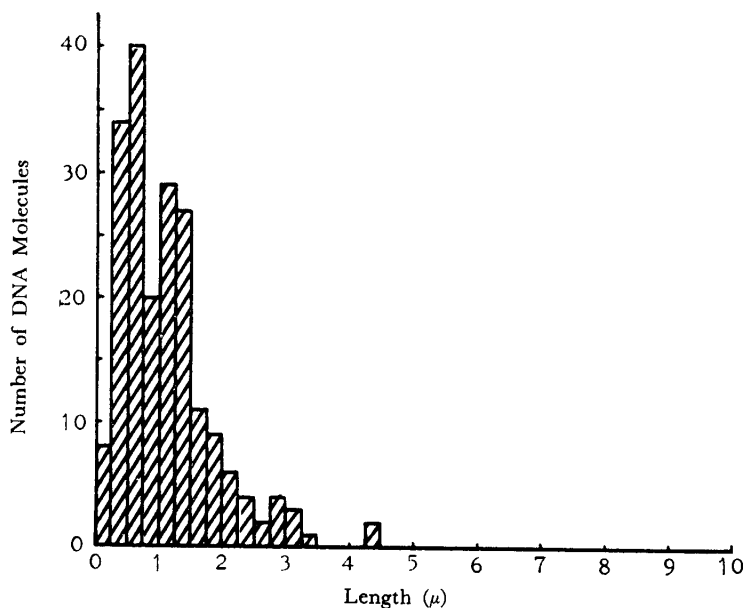


Fig. 5 Contour length distribution of DNA molecules isolated from the supernatant fraction of SR-C3H cells.

Total number of DNA molecules: 200

Mean value of the length: $1.13 \pm 0.70 \mu$

highest frequency group is $4.88 \pm 0.29 \mu$. The small (approximately 1μ in length) and large molecules (approximately double or triple in length) are also observable (Fig. 8). On the other hand, the contour lengths of liver mitochondrial DNA molecules are illustrated in Fig. 7, where the mean value of the highest frequency group is $5.08 \pm 0.34 \mu$, which is slightly longer than that of SR-C3H mitochondrial DNA molecules. The large

Fig. 1 Electron micrographs of nucleic acids extracted from nuclear fraction of SR-C3H cells. Magnification at 30,000. (A) Fibrous and globular shapes are seen individually. (B) Globular structure attached on the fibrous structure.

Fig. 2 Electron micrographs of nucleic acid extracted from microsomal fraction of SR-C3H cells. Magnification at 30,000. (A) Globular particles, (B) aggregates of globular particles in varying size, and (C) a chain-like structure, in which globular particles are connected by a fibrous structure.

Fig. 3 Electron micrograph of nucleic acid extracted from supernatant fraction of SR-C3H cells. Linear fibers are seen intermingled with small globular particles. Magnification at 30,000.

Fig. 4 Electron micrographs of nucleic acid in DNA eluting region on MAK-column chromatography of the saline-soluble nucleic acid extracted from supernatant fraction of SR-C3H cells. (A) Both fibrous structure and globular shape particles are seen. (B) After the DNase treatment fibrous structure has disappeared and (C) after the RNase treatment globular particles disappeared. Magnification at 30,000.

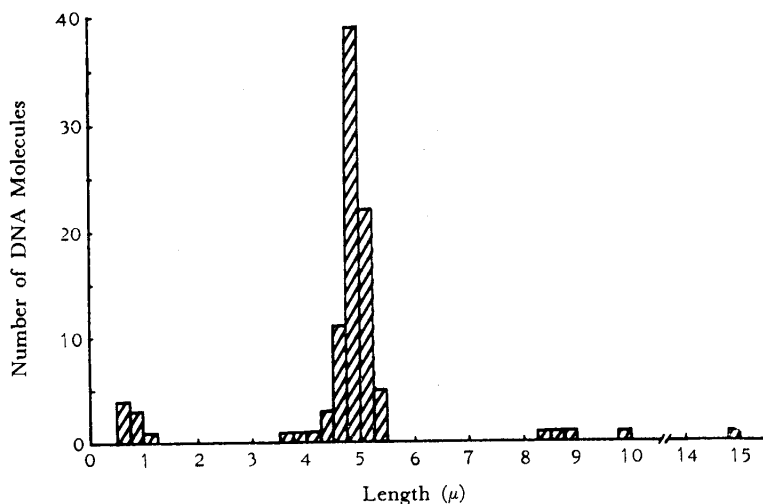


Fig. 6 Contour length distribution of mitochondrial circular DNA molecules isolated from SR-C3H cells.

Total number of DNA molecules: 96

Mean value of the contour length of the highest frequency group:
 $4.88 \pm 0.29 \mu$

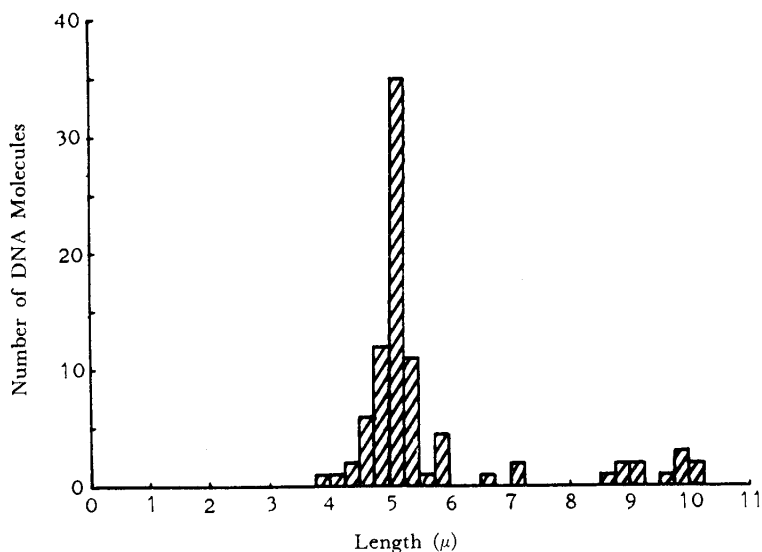


Fig. 7 Contour length distribution of mitochondrial circular DNA molecules isolated from C3H mouse liver.

Total number of DNA molecules: 97

Mean values of the contour length of the highest frequency group and secondary group: $5.08 \pm 0.84 \mu$ and $9.65 \pm 0.54 \mu$, respectively

circular molecules are also found in liver mitochondrial DNA (Fig. 9), calculated to be $9.65 \pm 0.54 \mu$ in the mean value of contour length that may imply a duplicate form, but the small circular molecules are not observable.

Ultraviolet absorption characteristics of extracted DNA's

The ultraviolet absorption characteristics (31) of DNA's extracted from individual subcellular fractions of SR-C3H cells were determined with the samples solubilized by 0.5N perchloric acid at 90° for 20 min after removing alkaline hydrolizate in acid precipitable nucleic acid. Alkaline hydrolysis was carried out in 0.3N KOH at 37° for 16 hours. The ratios illustrated in Table 5 are summed up to varying values of individual DNA's indicating some differences in the nature of the DNA's.

TABLE 5. ULTRAVIOLET ABSORPTION CHARACTERISTICS OF DNA'S EXTRACTED FROM SUBCELLULAR FRACTIONS OF SR-C3H MOUSE ASCITES SARCOMA CELLS. THE ABSORPTION CHARACTERISTICS OF DNA'S WERE DETERMINED ON THE SAMPLE HEATED IN 0.5N PERCHLORIC ACID AT 90° FOR 20 MIN.

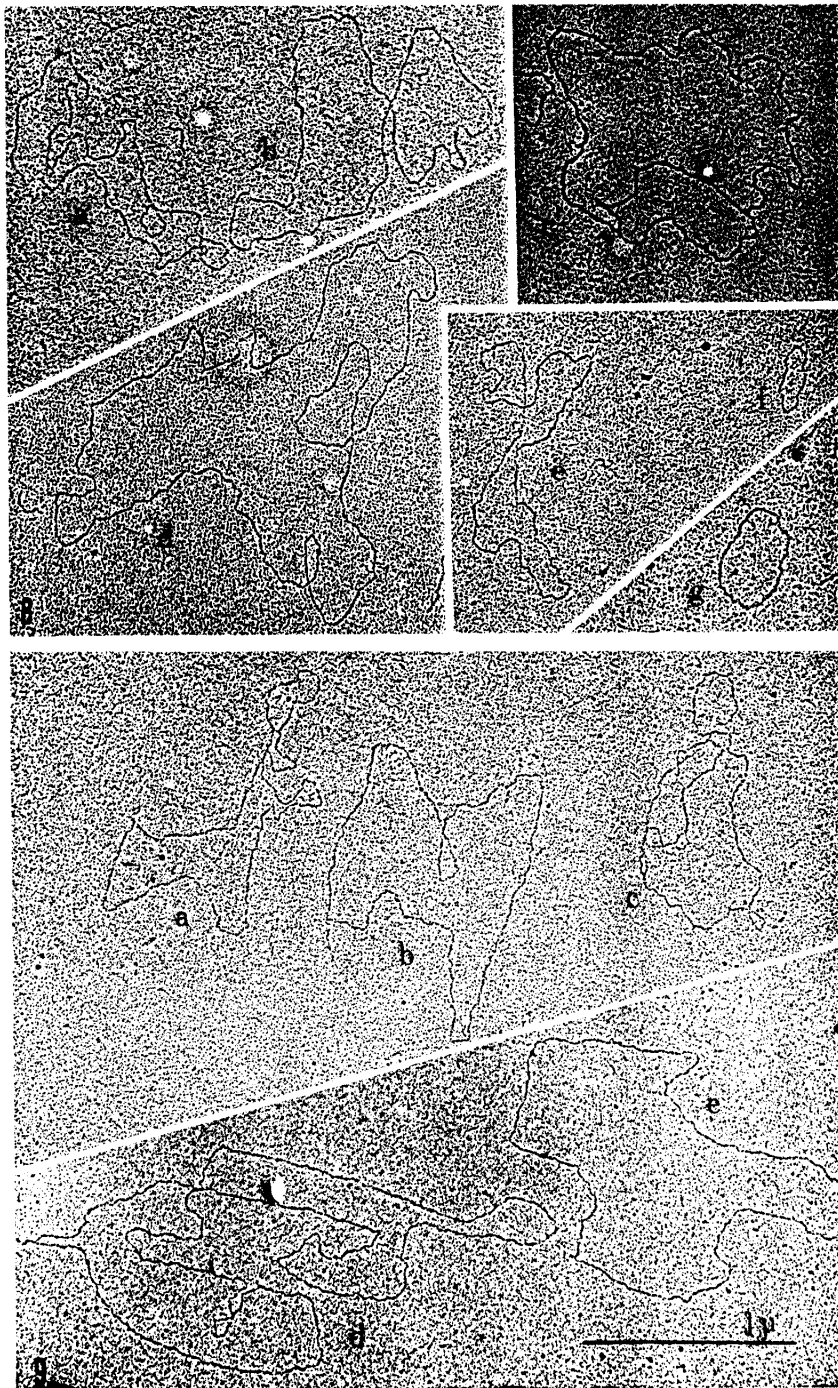
Ratio	DNA's			
	Nuclear	Mitochondrial	Microsomal	Supernatant
290 m μ : 260 m μ	0.40	0.51	0.53	0.54
280 m μ : 260 m μ	0.79	0.86	0.87	0.91
(290 m μ + 280 m μ) : 260 m μ	1.19	1.37	1.40	1.45

DISCUSSIONS

Normally all tumor cells are characterized by a decrease in the amount of RNA in proportion to DNA (32, 33) and a relative increase in the concentration of DNA over protein (32). Such a tendency can also be observed in the SR-C3H cells as compared with the mouse liver cells. The DNA in the acid-precipitable substance are distributed in each subcellular fraction separated by the routine differential centrifugation method.

The ratio of DNA to RNA is higher in the nuclear fraction of the SR-C3H than that in the liver. By electron microscopy, a DNA-RNA complex-like structure is observed in the nucleic acid extracted from the nuclear fraction of SR-C3H cells, and this structure is similar to that seen in the micrograph of HeLa cells, reported by BRUNFAUT *et al.* (34).

The DNA of mitochondria has recently aroused an interest as the cytoplasmic gene. The contents of DNA (12) and RNA (35) of cancer mitochondria are about several-fold higher than those of normal one (36, 37). The content of DNA of SR-C3H mitochondria seems to be higher than that of the mouse liver mitochondria. However, the DNA extracted from SR-C3H mitochondria reveals both circular and linear molecules,



while the DNA from the liver mitochondria almost invariably shows circular molecules in electron micrographs. These evidence suggest that a linear molecule reflects contamination of the nuclear DNA or denatured mitochondrial DNA in the SR-C3H mitochondrial fraction, whose isolation is difficult without nuclear material, but isolated SR-C3H mitochondria are intact on the oxidative phosphorylation as measured by oxygraphic method (38). Animal mitochondrial DNA's comprise circular molecules of approximately 5μ in contour length (8—15). The circular molecules (4.88μ) of SR-C3H mitochondria are slightly shorter than mouse liver mitochondria (5.08μ) in the mean value of the highest frequency group under the identical preparatory procedures, and small and large circular molecules are observable in the SR-C3H mitochondrial DNA. These findings resemble well those reported of rat ascites hepatoma (12) and human hepatoma mitochondria (11). Circular molecules of duplicated length are also observed in the mouse liver mitochondrial DNA, but none of small ones. It has been reported that the mitochondrial DNA of mouse tissue measures 4.74μ in L-cell (14), 4.96μ (13) and 5.10μ (15) in liver cells in the mean value of contour length, but none of small and large circular molecules. Small circular DNA in animal tissue has been demonstrated in HeLa cell (39, 40), rat ascites hepatoma (12), human hepatoma (11) and pig sperm (41), but the relation to the nature of cancer is obscure. Sometimes, a replicating form (9) is observable in the SR-C3H mitochondrial circular DNA (Fig. 8).

DNA's can be detected by the chemical method, in acid-insoluble materials in the microsomal and the supernatant fractions of SR-C3H cells and liver cells, especially so in the supernatant of SR-C3H cells. The ultraviolet absorbancy observations on the characteristic properties of the DNA's have suggested individual differences in the base component of subcellular fraction DNA's. However, electron microscope observations of nucleic acids reveal readily a fibrous structure in the SR-C3H supernatant fraction extracted by SDS-phenol method, but difficult to observe it in

Fig. 8 Electron micrographs of mitochondrial circular DNA of SR-C3H cells. Contour lengths of the DNA molecules in (a), (b) and (c) are 4.61μ , 5.0μ and 4.78μ , respectively, and the form in (c) is seem to be replicating one. Contour length of the open large circular molecules in (d) is 8.63μ and of small molecules in (e), (f) and (g) are 4.03μ , 0.70μ and 1.19μ , respectively. Magnification at 28,500.

Fig. 9 Electron micrographs of circular DNA isolated from C3H mouse liver mitochondria. Contour lengths of the DNA molecules in (a), (b), (c), (d) and (e) are 4.93μ , 5.09μ , 5.13μ , 10.0μ and 5.09μ , respectively, and the molecule (10.0μ) in (d) is duplicated form, in which two circular molecules measuring 4.96μ and 5.04μ in contour length are connected by a short bridge. Magnification at 28,500.

the other fractions. Since this fibrous structure of nucleic acid from the supernatant of SR-C3H cells is digested by DNase, it seems to represent DNA. These results indicate that there is a possibility of the presence of DNA in the supernatant of fraction SR-C3H cells. However, as it is generally considered that in the cell fractions prepared by the aqueous isclation procedures a considerable amount of nuclear components is transferred to the supernatant fraction (42), it remains still uncertain whether the fibrous structure is originally present in the supernatant fraction or derived from the soluble nuclear materials. GREENBERG and UHR (43) have reported that some mouse tumor cell DNA's contain a greater amount of satellite DNA than the DNA's from normal mouse tissue, and SCHIDKRAUT and MAIO (44) contend that all the satellite DNA's in the intact cell of mouse are derived from the nucleoli. Regarding this problem analysis of base compositions of the DNA in the subcellular fractions of the mouse ascites sarcoma cells is being made.

SUMMARY

For the purpose to clarify the distribution of DNA in mouse ascites sarcoma cells (SR-C3H) induced by Rous sarcoma virus (Schmidt-Ruppin strain), quantitative assays were carried out by SCHMIDT-THANNHAUSER-SCHNEIDER's method using subcellular fractions isolated from SR-C3H cells and C3H mouse liver as a control tissue, and simultaneously electron microscopic observations were conducted with the rotary shadowed preparations of the SDS-phenol extracted nucleic acids by the protein monolayer technique. The results are briefly summarized as follows.

1. The RNA/DNA ratios in SR-C3H cells and liver cells were 2.3 and 3.7, while those in nuclear fraction of SR-C3H cells and liver cells were 0.34 and 0.56, respectively. The electron micrographs of nuclear nucleic acids revealed a DNA-RNA complex-like structure.

2. DNA and RNA contents of SR-C3H mitochondria were found to be 3.1 and 24 μ g per mg of protein, respectively, which proved to be greater than those of liver mitochondria. The mean values of the contour length of circular DNA molecules in highest frequency group observed in the electron micrographs were 4.88 μ in SR-C3H mitochondria and 5.08 μ in mouse liver mitochondria. There could be observed circular molecules of duplicated-length in both mitochondrial DNA's and small circular molecules in SR-C3H mitochondrial DNA.

3. In the microsomal and supernatant fractions of SR-C3H cells and mouse liver cells, the ratios of DNA to RNA gave several percent by chemi-

cal analysis and this percentage was particularly high in the supernatant of SR-C3H cells. On the other hand, in the electron micrographs, the fibrous structure was significantly recovered in the supernatant nucleic acids of SR-C3H cells, but with difficulty in the other three fractions. This fibrous structure measured 1.13μ in the mean value of the length and was considered to be DNA as it readily disappeared after the treatment with DNase.

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REFERENCES

1. YAMAMOTO, T. and TAKEUCHI, M.: *Japan J. Exp. Med.* **37**, 43, 1967
2. TAKEUCHI, M., HINO, S. and YAMAMOTO, T.: *Japan J. Exp. Med.* **37**, 107, 1967
3. YAMAGUCHI, N., TAKEUCHI, M. and YAMAMOTO, T.: *Japan J. Exp. Med.* **37**, 83, 1967
4. BADER, J. P.: in "*The Molecular Biology of Viruses*" (ed. COLTER, J. S. and PARANCHYCH, W.), Academic Press, New York & London, p. 679, 1967
5. TEMIN, H. M.: in "*The Molecular Biology of Viruses*" (ed. COLTER, J. S. and PARANCHYCH, W.), Academic Press, New York & London, p. 709, 1967
6. TEMIN, H. M.: *Cancer Res.* **26**, 212, 1966
7. TEMIN, H. M.: *J. Cell Physiol.* **69**, 1, 1967
8. VAN, BRUGGEN, E. F. J., RUNNER, C. M., BORST, P., RUTTENBERG, G. J. C. M., KROON, A. M. and STEKHOVEN, F. M. A. H. S.: *Biochim. Biophys. Acta* **161**, 402, 1968
9. KIRSHNER, R. H., WALSTENHOLME, D. R. and GROSS, N. J.: *Proc. Natl. Acad. Sci. U. S.* **60**, 1466, 1968
10. HUDSON, B. and VINOGRAD, J.: *Nature* **216**, 647, 1967
11. ODA, T.: *Proc. Jap. Cancer Assoc. 26th Ann. Meet.* p. 11, 1967; *Saishin-Igaku* **23**, 464, 1968 (in Japanese); *Jap. J. Clin. Med.* **27**, Supple. 67, 1969
12. INABA, K.: *Acta Med. Okayama* **21**, 6, 1967
13. SINCLAIR, J. H. and STEVENS, B. J.: *Proc. Natl. Acad. Sci. U. S.* **56**, 508, 1966
14. NASS, M. M. K.: *Proc. Natl. Acad. Sci. U. S.* **56**, 1215, 1966
15. KROON, A. M., BORST, P., VAN BRUGGEN, E. F. J. and RUTTENBERG, G. J. C. M.: *Proc. Natl. Acad. Sci. U. S.* **56**, 1836, 1966
16. HOGEBOOM, G. H.: "*Method in Enzymology*" (ed. COLOWICK, S. P. and KAPLAN, N. O.), I, Academic Press, New York, p. 16, 1955
17. SCHMIDT, C. and THANNHAUSER, S. J.: *J. Biol. Chem.* **161**, 83, 1945
18. SCHNEIDER, W. C.: *J. Biol. Chem.* **164**, 747, 1946
19. VOLKIN, E. and COHN, W. E.: "*Methods of Biochemical Analysis*" (ed. GLICK, D.), I, Interscience, New York, p. 287, 1954
20. ZAMENHOF, S.: "*Method in Enzymology*" (ed. COWICK, S. P. and KAPLAO, N. O.), III, Academic Press, New York, p. 696, 1957
21. CERIOTTI, G.: *J. Biol. Chem.* **214**, 59, 1955

22. YOSHIKAWA-FUKUDA, M., FUKUDA, T. and KAWADA, Y.: *Biochim. Biophys. Acta* **103**, 383, 1965
23. FELLING, J. and WILEY, C. E.: *Arch. Biochem. Biophys.* **85**, 313, 1959
24. FRANKEL-CONRAT, H., SINGER, B. and TSUGITA, A.: *Virology* **14**, 54, 1961
25. MARMUR, J.: in "Method in Enzymology" (ed. COLOWICK, S. P. and KAPLAN, N. O.), Academic Press, London, VI, p726, 1963
26. GORNALL, A. G. and BARDAWIKK, C. J.: *J. Biol. Chem.* **177**, 751, 1949
27. FREIFELDER, D. and KLEINSHMIDT, A. K.: *J. Mol. Biol.* **14**, 271, 1965
28. KAWADA, Y.: *Ann. Rept. Inst. Virus Res. Kyoto Univ.* **2**, 219, 1959
29. GREEMAN, D. L., KENNY, F. T. and WICKS, W. D.: *Biochem. Biophys. Res. Commun.* **17**, 449, 1964
30. YAMAMOTO, G.: in preparation
31. KALF, G. F. and GRÉCE, M. A.: in "Method in Enzymology" (ed. GROSSMAN, L. and MOLDAVE, K.), Academic Press, New York & London, XII, p. 533, 1967
32. LESLIE, I.: in "The Nucleic Acid" (ed. CHARGAFF, E. and DAVIDSON, J. N.), Academic Press, New York, II, p. 1, 1955
33. IWANAMI, Y. and ISOYAMA, S.: *Proc. Jap. Cancer Assoc. 22nd Gen. Meet.* p. 108, 1963
34. BRUNFAUT, M., WINCKELMANS, D. S., FAURÈS, A. M. and ERRERA, M.: in "The Cell Nucleus", Proc. of the Internat. Symp., Taylor & Francis Ltd., London, p. 100, 1966
35. FREEMAN, K. B.: *Biochem. J.* **94**, 494, 1965
36. NASS, S., NASS, M. M. K. and HENNIX, U.: *Biochim. Biophys. Acta* **95**, 426, 1965
37. O'BRIEN, T. W. and KALF, G. F.: *J. Biol. Chem.* **242**, 2172, 1967
38. YAMAMOTO, G.: unpublished data
39. RADLOFF, R., BAUER, W. and VINOGRAD, J.: *Proc. Natl. Acad. Sci. U. S.* **57**, 1514, 1967
40. OMURA, S., ODA, T. and YAMAMOTO, S.: *Proc. Jap. Assoc. 27th Ann. Meet.* p. 33, 1968
41. HOTTA, Y. and BASSEL, A.: *Proc. Natl. Acad. Sci. U. S.* **53**, 356, 1965
42. ALLFREY, V.: in "The Cell" (ed. BRACHET, J. and MIRSKY, A. E.), Academic Press, New York, I, p. 193, 1959
43. GREENBERG, L. J. and UHR, J. W.: *Biochem. Biophys. Res. Commun.* **27**, 523, 1967
44. SCHILDKRAUT, C. L. and MAIO, J. J.: *Biochim. Biophys. Acta* **161**, 76, 1968