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Author(s)	HORI, Samuel H.
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On the Distribution of Nucleic Acids in Tumor Cells of the MTK-Sarcoma III[1)]

By
Samuel H. Hori

(Zoological Institute, Hokkaido University)

Most of the studies heretofore published on the investigation of nucleic acids of malignant cells depend on smear material. So far as the author is aware, Bayreuther (1952) seems to be the first to use sectioned material of the ascites tumor for the study of chromosomes. It is usual that many experiments regarding the ascites tumor have been carried out with smear preparations, with the one exception of the investigation by electron microscopy to which the sectioned material is well-adapted. So far as the nucleic acid study is concerned, the smear preparations of tumor cells are unsatisfactory for accurate and detailed research of cell structure. While working for the improvement of method in the field of cytochemistry, the present author has found a simple technique available for the cytochemical study of ascites-tumor cells. The present paper describes the method with some results obtained herewith.

Material and methods

The ascites tumor used as material in the present study was the MTK-sarcoma III which malignantly propagates in the peritoneal cavities of albino rats. Wistar albinos weighing about 60 g were supplied as hosts of the tumor propagation. The paraffin sections of ascites tumor cells were prepared according to the following procedure :

1) Fix by carefully dropping tumor-ascites in alcohol-chloroform-acetic acid mixture with a pipette. The drops immediately coagulate in the mixture into a spherical form. Alcohol-acetic acid or Bouin's solution is also available for this purpose, but the best results were obtained with alcohol-chloroform-acetic acid. Leave the fixed material in the solution for 1 hour.

2) Rinse the material with several changes of 95% alcohol, dehydrate with absolute alcohol, make clear with xylol and embed in paraffin as usual.

3) Section the paraffin material 2–3 μ thick, mount the sections on slides and deparaffinize as usual. Jung's sliding microtome is more conveniently used for

1) Contribution No.352 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan

making thin sections than Minot's rotary microtome.

The methods of staining for demonstration of desoxyribonucleic acid follow Stowell's modification of Feulgen-reaction (1945), according to the following procedure:

1) Prepare three slides, and treat one of them with trichloracetic acid (TCA) at 90°C for 15 minutes.
2) After the TCA-treatment, hydrate the slide with 1N-hydrochloric acid for 8 minutes, together with one of the untreated slides.
3) Rinse the slides in distilled water and transfer to the Feulgen-stain, together with an unhydrated slide. Leave the slides in the stain for 90 minutes in the case of the hydrated ones, and for 50 minutes in the case of unhydrated one.
4) Wash the slides in tap water, dehydrate, clear and mount in balsam.

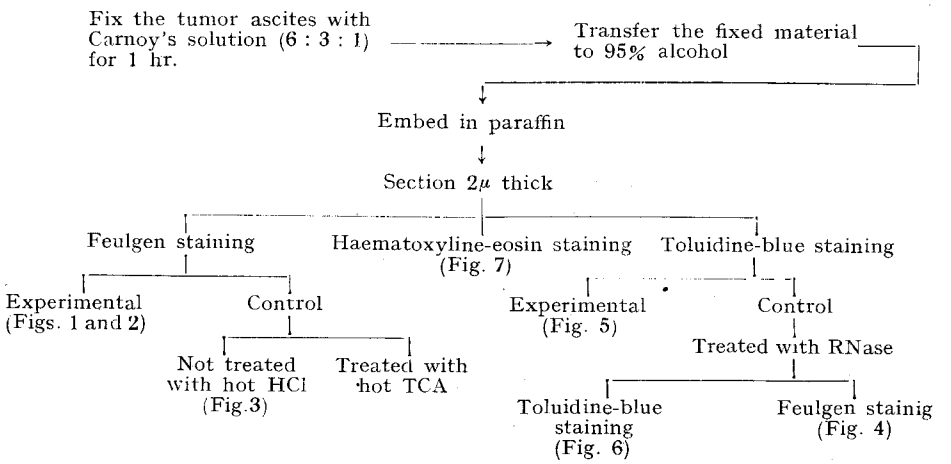
Basic fuchsin used was parafuchsin (pararosaniline, Merck). It is of the high quality required for cytochemical experimental uses. Feulgen reagent was prepared following the indication by Stowell. It was preserved in refrigerator and used within 1 month. Bleaching solution was made every time wanted.

Staining method for the test of ribonucleic acid is as follows:

For the demonstration of RNA localities in cells, toluidine blue in a buffered solution was applied to sections according to Lison's method.

1) Prepare three slides.
2) Cover two of them with several drops of the 0.02% ribonuclease solution and the other one with distilled water. Place the three slides in a container, on the bottom of which a wet filter paper has been placed for protection of ribonuclease solutions from evaporating. Keep this container in incubator for 90 minutes at 65°C.

Table 1



3) Treatment of the slides with enzymes or distilled water is followed by rinsing in distilled water.
4) Place the two slides, one digested with ribonuclease and the other not digested, simultaneously in the staining solution consisting of one part of 0.1% toluidine blue solution and one part of phosphate buffer at pH 4.6.

After staining, slides were submerged in phosphate buffer solution at pH 4.6, in order to remove the dye adhering to the cells.
6) Immerse slides into 4% ammonium molybdate solution.
7) Apply Feulgen reaction to the slide which has not been stained with toluidine blue after treatment with ribonuclease.

In addition, the double staining method with haematoxylin and eosin was also used for comparison.

The procedure above mentioned is summarized in Table 1.

Experimental results

The Feulgen technique having been applied as described above, the distribution of desoxyribonucleic acids in tumor cells was investigated ; the results obtained are as shown in the following.

The Feulgen stainability was more intensive in the peripheral regions of both nucleus and nucleolus than in their inner parts. The tumor cells were classified into two groups regarding the distribution of the Feulgen positive material in the nucleus. The one type, referred to as A-type, is characterized by the presence of prominent Feulgen positive elements distributed within the nucleus with a reticulum-like constitution. There is present a body which lies in the central part of the nucleus and is Feulgen negative. In all probability it is a true nucleolus in view of its staining reaction. The boundary of this body is diffusedly outlined showing the Feulgen positive reaction. This evidence sufficiently suggests that the nucleolus of this type consists of two parts, a main body that is Feulgen negative and a peripheral zone made of Feulgen positive material. The A-type cells could be divided again into two groups; the cells in which the nuclei contain Feulgen positive material in relatively plenty with a compact distribution are referred to as A1-type, and those designated A2-type are represented by the nuclei which show the Feulgen positive masses scattered along or partially adhering to the nuclear membrane. In the A-type cells (Fig. 1), the Feulgen positive material surrounding the nucleolus often showed amoeboid configuration characterized by irregular processes stretching bridge-like between the nucleolus and the nucleus borders. They seem to be common in the tumor-preparations obtained at any time, particularly abundant in those taken from the middle part of a transplant generation. The evidence seems to indicate that the A-type cells are in physiologically high activity, and further that they must be very active in DNA synthesis. More probably the A2-type cells can undergo division in view of their nuclear structures.

Further observations revealed that there is another type of cells, designated herein as B-type cells; they are characterized by a less content of the Feulgen positive material in the nucleus. The Feulgen colorability of these cells is weaker than that of the A-type cells. The nucleolus shows also the peripheral covering of the Feulgen positive material in these cells, though it is rather sharply outlined. The B-type cells are very abundantly observable in the tumor-preparations taken from the later days of a transplant generation, though A– and B– type tumor cells were found with variable frequency in samples through a whole transplant generation. Tumor cells of a transitory type were also observable. Considered from their nuclear structure, the cells of B-type possess low mitotic activity.

Next, the distribution pattern of the Feulgen positive material was compared between the tumor cells and the ordinary cells. For the ordinary material, liver cells were employed. After observation it was found that the liver of adult rat showed only one type of cells with regard to the Feulgen positive material; they are the B-type cells, since the nucleoli show a well-defined peripheral zone of Feulgen positive material. That material in the nucleus shows a reticulum-like distribution. In this respect they simulate the tumor cells. Treatment of the sample with TCA at 90°C for 15 minutes resulted in inhibition of coloration with Feulgen reagent and also loss of stainability by the Feulgen method in the non-hydrated material (Fig. 3).

Brachet used pyronine for identifying ribonucleic acid in the cells stained with the Unna-Pappenheim solution. The phosphate buffer solution of toluidine blue at pH 4.6 to 4.8 was also used to identify RNA by Lison (1936). By immersing the cells in Lison's solution, RNA is selectively colored, and its specificity to RNA is insured by impairment of stainability after treatment with RNase. The application of Lison's method with RNase treatment has furnished the following results in the tumor material:

Blue coloration was obtained in both the cytoplasm and nucleolus, and violet coloration was attained both in the nuclei and chromosomes. The most intensive coloration occurred in the regions bordering the nucleolus and the nucleus, and also near the nucleus and the cytoplasm. Remarkable is the fact that the reticular substances of the nucleus and the chromatin associated with the nucleolus were colored with stains (Fig. 5). It is probable that the nuclear substances thus colored may be the chromatin elements which have already been identified as the DNA-containing substance by the application of the Feulgen technique. There is a difficulty in dividing the material stained with toluidine blue into two as could be done in the Feulgen positive material. A similar situation was obtained after examination of the liver material stained with toluidine blue: most positive reactions appeared in the juxtanuclear cytoplasm and also in the body consisting of both the nucleolus and nucleolus-associated chromatin. The reticular chromatic elements in the nucleus were also colored by the stain

though their coloration was not so deep as in the other parts. Treatments with distilled water at 65°C for 90 minutes resulted in the dissolution of RNA to a certain extent.

Discussion

It has been a well-accepted fact that the nucleolus is surrounded by heterochromatin. But the results of the present observations have raised a question whether or not the nucleoli contain DNA, because the chromatin of the A-type cells is closely associated with nucleoli of red coloration in case of application of the Feulgen technique.

Ogata (1883), working with the distinction between a true nucleolus and a chromosome-nucleolus through the Feulgen reaction, made it clear that the former was Feulgen positive and the latter was Feulgen negative. Wermel (1927) found that the nucleolus did not contain Feulgen-positive material. Kaufmann et al. (1951) investigated the distribution of nucleic acids in fixed cells using the method of enzymatic hydrolysis ; they confirmed that the nucleolus contained no DNA. Bauer (1933) reported in the nucleus of insect oocytes two different examples of Feulgen-positive nucleoli. Davidson (1947) found in the nucleolus of mammalian liver cell that there is an outer zone of Feulgen-positive material. The results of the present study with the rat liver cell employing the Feulgen reaction provide basis for the same conclusion as that of Davidson : the liver cell shows an ourter zone of Feulgen-positive material very distinctly with a well-defined Feulgen-negative mass lying inside (Fig. 8 and 9). Caspersson (1950) has commented upon the definition of the nucleolus as follows ; "the definition of the word nucleolus is not precise in cytological literature. The term, as used in the literature, often covers nucleolus-associated chromatin and other endonuclear structures. In the older literature, large heterochromatin parts of the chromatin are also included. For cytochemical work an exact definition is badly needed. The following definition seems suitable ; Nucleoli are dense, round, as a rule optically homogeneous, endonuclear bodies consisting of proteins in high concentrations, rich in diamino acids and associated with the cytoplasmic protein formation. Often they contain some ribose nucleotides (rarely small granular inclusions which probably are of chromatic character may occur)." Caspersson has also described the nucleolus-associated chromatin as the material inside of which the nucleolus appears when a cell starts growth.

In addition to the above evidence, the present observations have revealed that there was a strongly positive Feulgen reaction markedly present in the peripheral zone of the nucleolus. Thus the present author is quite in harmony with Caspersson's statement that the nucleolus is not Feulgen-positive, but it is surrounded by the nucleolus-associated chromatin. Further, results of the present experiments have suggested that the morphological relationship between the nucleolus and the nucleolus-associated chromatin is influenced by the physiological condition of the cell. The occurrence of the two types of tumor cells may probably represent this situation.

Considered from the fact that the location of the RNA in cells is similar to that of DNA, it seems probable that the so-called chromatin substances in the nucleus may contain

mixed nucleic acids composed of both DNA and RNA. Caspersson (1950) has reported that RNA appears around the nucleolar membrane and diffuses out into the cytoplasm at a time when cell growth starts. Recent advances in electron microscopy have, however, revealed that the nucleolar membrane does not actually exist (Borysko and Bang, 1951). If the nucleolar membrane does exist, the portion around the nucleolar membrane should be occupied by the nucleolus-associated chromatin. Caspersson's report that RNA appears around the nucleolar membrane implies, therefore, that the nucleolus-associated chromatin may contain RNA. This statement is supported by the results of the present experiments. Furthermore, Caspersson (1950) and Stowell (1948) reported that juxtanuclear cytoplasm contains a large amount of RNA.

The results of the present experiments have indicated a possibility that much RNA may be also contained in a juxtacytoplasmic nuclear region. This possibility seems to the author to be a probability. Future study is needed for further clarification of this point with improved technique.

Summary

The distribution of desoxyribonucleic acids was investigated in tumor cells of the MTK-sarcoma III by the application of the Feulgen technique. It was found that the tumor cells could be classified into two types with regard to the distribution of the Feulgen-positive material in the nucleus, and further that the two types of tumor cells may represent the morphological relationship between the nucleolus and the nucleolus-associated chromatin which is influenced by the physiologial condition of the cell.

By using ribonuclease in conjunction with toluidine blue solution in a buffered condition, a possibility was suggested that RNA may exist in the nuclei both of tumor cells and of normal liver cells. Furthermore, Feulgen reaction after treatment with ribonuclease furnished evidence that both RNA and DNA were intimately associated within the chromatins, and that they are densely accumulated in the peripheral zone of both the nucleus and the nucleolus.

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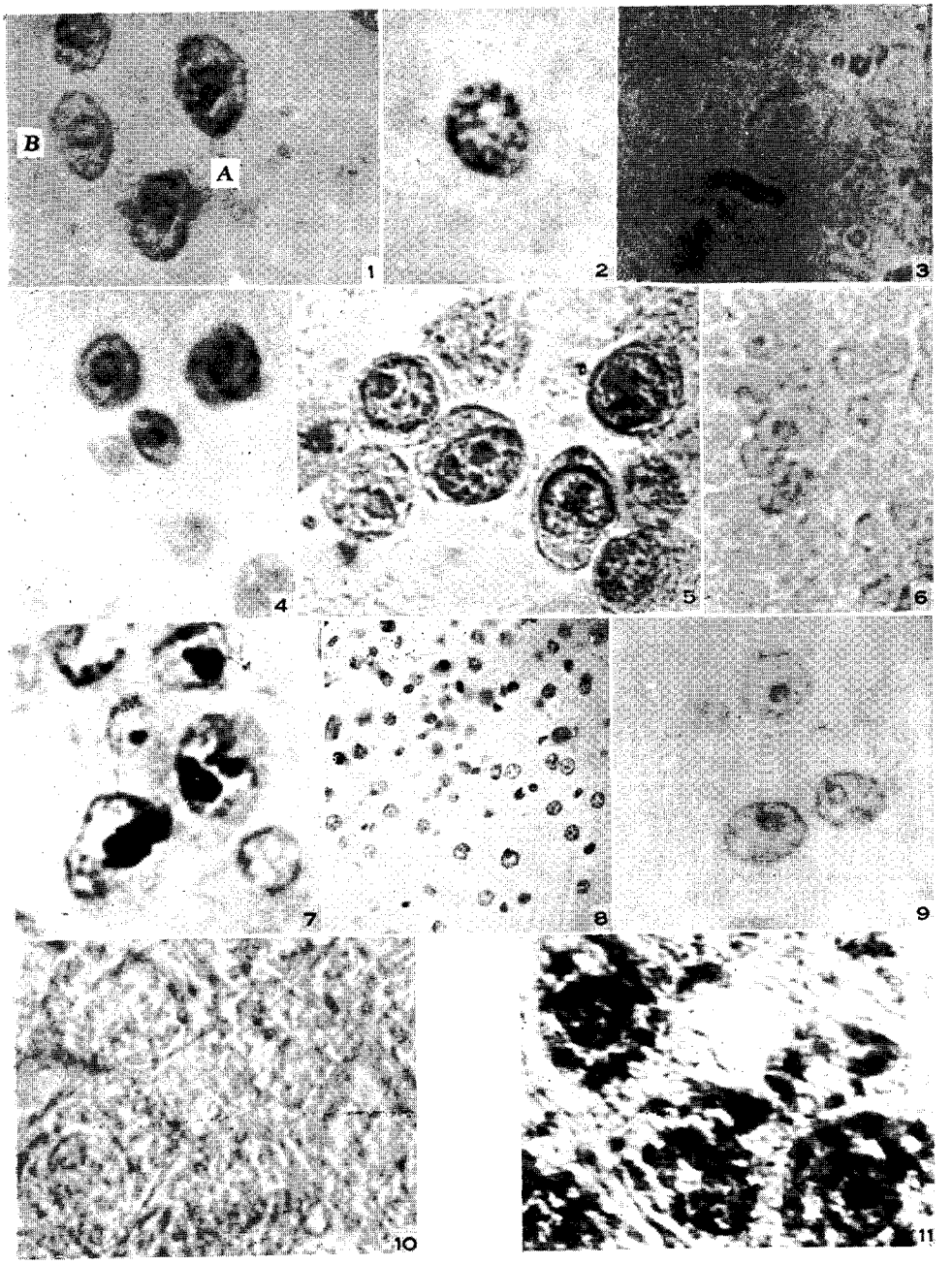
Explanation of Plate XIX

Figs. 1–7. Tumor cells of the MTK-sarcoma III.
Fig. 1. Feulgen stain of tumor cells. A-type cells and B-type cells. ×1600.
Fig. 2. Feulgen stain of tumor cells. The cell which starts to divide. ×1600.
Fig. 3. Section treated with Feulgen technique without hydrolysing with hot HCl. ×1600.
Fig. 4. Section treated with RNase, and then stained with Feulgen reagent. ×1600.
Fig. 5. Toluidine-blue stain. ×1600.
Fig. 6. Section treated with RNase, and then stained with toluidine blue. ×650.
Fig. 7. Haematoxyline-eosin stain. ×1600.
Figs. 8–11. Liver cells of rat.
Fig. 8. Feulgen stain of liver cells. ×350.
Fig. 9. Feulgen stain of liver cells. ×1600.
Fig. 10. Toluidine-blue stain of liver cells. ×1600.
Fig. 11. Section treated with RNase, and then stained with toluidine blue. ×1600.



S. H. Hori: *Distribntion of Nucleic Acids in Tumor Cells*