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Repetitive Sequences in DNA of Mouse Tumor Cells

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INTRODUCTION

In recent years, experiments have shown that the genome of higher organisms contains a large amount of order which is manifested as repeated nucleotide sequences.(Britten and Kohne, 1968a, 1968b.) Non-repeated nucleotide sequences in the DNA can be distinguished from repetitive sequences by measuring the rate of reassociation of single stranded DNA. (Hoyer et al, 1964: Britten and Kohne, 1967)

Reassociation of single stranded DNA refers to the reforming of Watson-Crick base pairing of DNA strands under certain conditions.(Doty et al, 1960) The conditions for efficient reassociation of DNA are adequate salt concentration, adequate temperature of incubation, adequate size of DNA fragments examined and concentration of the DNA segments. (Britten and Kohne, 1968a)

Part of the eukaryotic DNA reassociates very slowly at a rate consistent with the genetic complexity of the organism, indicating that this fraction of DNA is composed of DNA segments repeated only once per genomic unit. Unexpectedly, however, part of the DNA reassociates at a much more rapid rate; this fraction of the DNA could be composed only of the same or nearly the same DNA units repeated many times. The number of repetitions would determine how much faster than the very slow reassociating non-repeated fraction of DNA this repeated fraction would reassociate.

McCarthy and Hoyer (1964) have examnied this repeated DNA to determine if there is any difference in repetition of composition of the repeated DNA from one cell type to another. Apparently, all the cells of the same organism have the same DNA composition and repetition, thus ruling out the hypothesis that repeated DNA is made locally for the purpose of assisting the process of differentiation of cell type.

Flamm et al (1969) found that the intermediate repetitive fraction appears to be transcribed into RNA. One interpretation of the sequence complexity suggested by the renaturation experiments is that genes coding for proteins comprise the unique fractions and that control signals of some nature constitute the intermediate sequences. The highly repetitive sequences are not transcribed and must play some structural role. (Walker et al, 1969: Levine, 1974)

Britten and Davidson(1971) propose a model for gene regulation of eukaryotic cell. According to the model, the repeated DNA in some way acts as a switching system for controlling the expression of the remainder of the genetic DNA. One of the most difficult issues for evolutionary theory is the appearance of new organs or of complex systems which carry out novel functions. They emphasized that at all times an internally consistent program of development for the organ must be present in

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the genome. The gene regulation systems which function during ontogeny are crucial and a million copies of a given set of sequences scattered throughout the genome would represent an exceptionally large number of possible new regulation relationships.

However, there is no evidence on the possible origin and function of repetitive sequences, but it would be interesting to determine if there are any changes of repetitive sequences between normal cells and tumor cells, because tumor cells are regarded as having a different gene regulation system. It is well known that chromosomal anomalies exist in many advanced tumors and it is often possible to find a variety of karyotypes within a given tumor. This finding has lead to the suggestion that deviation from the original diploid karyotype may represent manifestations of genetic evolution in which the dominating cell line represents those cells that are best adapted to their immediate enviroment. (Levan, 1967) This does not, of course, necessarily mean that such chromosomal anomalies are the cause of the tumorous state; they may simply be the result. (Braun, 1974)

The aim of this paper is to examine the complete extent of the reassociation kinetics of the DNA of mouse tumor cells, in order to determine differences of amounts and frequencies of repetitive sequences, especially intermediate fractions, between normal cell DNA and tumor cell DNA.

MATERIALS AND METHODS

The rodents used for the experiment were obtained from the laboratory of cancer pathology, Korea Atomic Energy Research Institute, by the courtesy of Dr. T.K. Yun. As inbred mice, C_3H/HeN and $C_{57}BL/6N$ were used for the transplantation of Yun's spontaneous tumors. (Yun and Sung, 1975a,b) N: GP (SW) as non-inbred mice were also used for the transplantation of Ehrlich ascites tumor and murine ascitic sarcoma cells (Sarcoma 180).

In ascitic tumor experiments, transplantation was performed by injecting 20 million cells in 0.2ml saline into the peritoneal cavity. In solid tumor experiments, the minced pieces were transplanted into the subcutaneous space of the back in the mouse by 12 gauge trocar needle. (Yun and Sung, 1975a,b)

The transplantable tumors used in the experiment were Ehrlich ascites tumor, sarcoma 180 Yun liposarcoma and Yun salivary adenocarcinoma. The results were analyzed by comparing the pooled normal liver cell DNA with that of tumor cells.

In ascitc tumor experiment, all the available fluid was collected with a syringe at 7~10 days after inoculation and then the packed tumor cell was obtained by spinning it down. It was homogenized by using two kinds of homogenizers alternatively; Potter-Elvejem type and Dounce type. Solid tumor-bearing mice were sacrificed by cervical fracture. When the size of the solid tumor increased to the diameter of 2~2.5cm, the tumor mass removed the necrotic debris and other connective tissue was thoroughly minced and homogenized with the same homogenizers as the above.

The DNA was isolated and purified by a modification of the Marmur method.(Paul and Gilmour, 1968) Other details of the procedure were identical to those in a previous report. (Lee, Choi and Seo, 1977)

Thermal denaturation (melting profiles) of DNA

The melting curves of DNA solutions of the tumor cells were studied by monitering the chan-

ge in a thermostatically controlled cell-block containg teflon stoppered quartz cell, using Pye Unicam SP 1750 spectrophotometer equipped with SP 876 temperature programme controller and its accessories. DNA was dissolved in 1 x SSC (0.15M NaCl-0.015M trisodium citrate) solution for the melting determination. The melting curve of all the native DNA samples was measured for the characterization of the purity. The Guanine-Cytosine (G-C) content of each tumor cell DNA was also obtained from the midpoint of melting temperature (Tm) of the melting curve.

Optical determination of the DNA reassociation

The DNA was sheared to about 400∼500 nucleotide pairs by using sonifier.(Model W185 Heat Systems-Ultrasonic Inc.) The DNA solution was sonified under ice cooling at maximum intensity for 1 min. 30 sec. with an interruption of 10 sec. between successive 20 sec. of the sonication. This sonicating condition was proved to be able to produce the fragment size of 400 ∼500 nucleotide pairs of DNA by the sedimentation velocity method using an analytical ultracentrifuge.(Lee, Choi and Seo, 1977)

Reassociation of the denatured DNA solution was also measured optically at 260 nm by hypochromicity as previous report. (Lee, Choi and Seo, 1977) The reassociation reaction of DNA was monitored. using the same spectrophotometer as the above.

For the short term reassociation, the cuvette containing the DNA sample was heated in a constant temperature water bath (Gallenkamp) at 100°C for 10 min. and immediately cooled to 60°C and then transferred to the cell-block in the spectrophotometer maintained at 60°C. The rate of reannealing was determined by the decrease of UV absorbance. The long term reassociation was carried out by incubating sealed aliquots of heat denatured DNA in a water bath at 60°C and extreme precaution was taken to prevent the evaporation of sample by varying devices. Periodically aliquots of sample were approximately diluted and then placed into the spectrophotometer, followed by absorbance measurement. Considering the dilution factor and the extent of reassociation, Cot* (the product of DNA concentration in mole and time in second) value was calculated from this measurement. The original DNA concentration was determined from the absorbance of the completely denatured sample. Each fraction was also corrected for collapse hypochromicity which occurs on cooling single-stranded DNA. Reassociation kinetics was thus followed optically as a function of Cot.

RESULTS

Melting curves of mouse tumor cell DNAs

All of the DNA preparations were first checked for the purity by the melting profile and the hypochromicity as well as the ratio of absorbance at 260 nm to that at 280nm. The melting curves of DNA of each tumors are shown in Fig. 1 and Fig. 2. The thermal denaturation of DNA is usually characterized by the temperature of the midpoint of transition, Tm, sometimes referred to as melting temperature which is known to depend on the Guanine-Cytosine (G-C) content of the DNA molecule except the high G-C content. The G-C contents of DNA of each tumor compared with that of the normal cells are summarized in Table I.

Cot 1 (mole x second/liter) results if DNA is incubated for 1 hour at a concentration of 83 μg/ml. which corresponds to an optical density of about 2.0 at 260 nanometers.

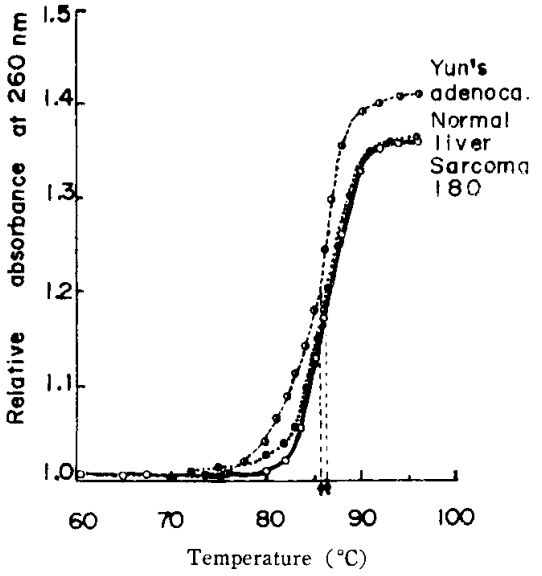

Fig. 1. Melting profiles of DNAs of normal liver cell, Sarcoma 180 and Yun adenocarcinoma in 1× SSC solution

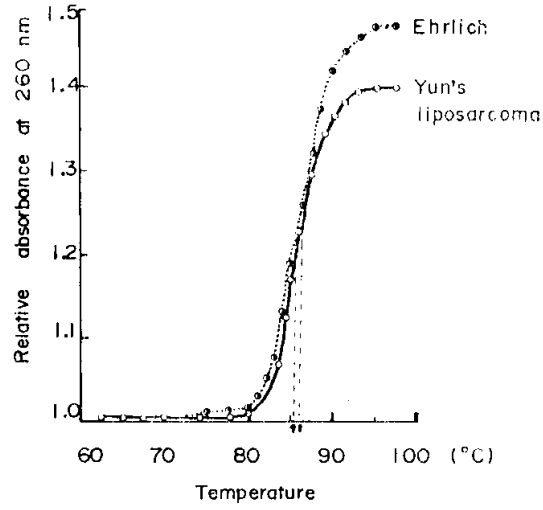

Fig. 2. Melting profiles of DNAs of Ehrlich ascites tumor and and Yun liposarcoma in 1×SSC solution.

Reassociation kinetics of tumor cell DNAs

The reassociation kinetics of normal liver cell DNA, Ehrlich ascites tumor DNA and Yun salivary adenocarcinoma DNA are shown in Fig. 3, Fig. 4 and Fig. 5. There are three

Table I. Tm value and Guanine-Cytosine contents of several mouse tumor cell DNAs

	Tm(°C)	G-C%*
Normal Liver Cell DNA	86.1	41.0
Ehrlich Ascites Tumor DNA	86.3	41.5
Sarcoma 180 DNA	86.1	41.0
Yun Salivary Adenocarcinoma DNA	85.8	39.5
Yun Liposarcoma DNA	85.7	40.0

*G-C content of DNA was estmated by following empirical formula according to Marmur and Doty (Lee, Choi and Seo, 1977)

$Tm = 69.3 + 0.41\ (GC)$ (GC): mole % Tm: °C

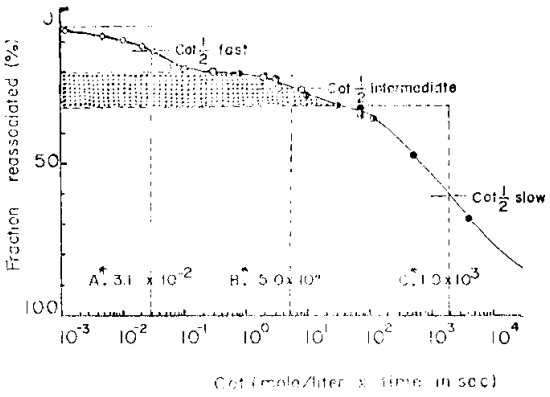

Fig. 3 The reassociation kinetics of normal liver cell DNA measured with spectrophotometer. The DNA sheared by sonication for 3 min. was incubated at 60°C in 0.12 M phosphate buffer.
open square (◇): 40 μg/ml (DNA concentration)
open circle (○): 83 μg/ml
half open circle (◐): 880 μg/ml
closed circle (●): 1100 μg/ml
*The dotted area indicates intermediate repetitive fraction.
**The values of A,B, and C in the figure are half Cot ($Cot\frac{1}{2}$) of each fraction.

fractions with different degrees of repetition; a highly repetitive, intermediate and a unique fraction. The Cot for half-reassociation of a fraction ($Cot\frac{1}{2}$) is a useful measure of the rate of reassociation of that fraction.

From the data of reassociation kinetics, the amount, repetition frequency and nucleotide length of repetition unit can be calculated. For instance, the slowest non-repeated DNA of

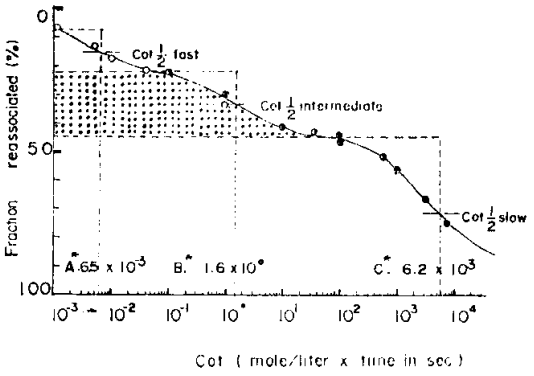

Fig. 4. The reassociation kinetics of Ehrlich ascites tumor cell DNA measured with spectrophotometer. The DNA sheared by sonication for 3 min. was incubated at 60°C in 0.12 M phosphate buffer.
open circle (○): 14μg/ml(DNA concentration)
half open circle (◐): 80μg/ml
closed circle (●): 1100 μg/ml
*The dotted area indicates intermediate repetitive fraction.
**The values of A.B. and C are half Cot (Cot $\frac{1}{2}$) of each fraction.

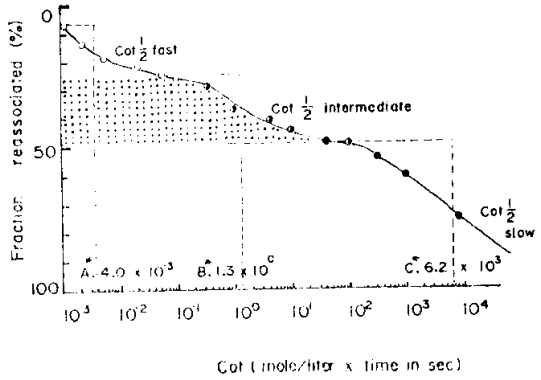

Fig. 5. The reassociation kinetics of the DNA of Yun salivary adenocarcinoma measured with spectrophotometer. The DNA solution was prepared by sonication for 3 min. and incubated at 60°C in 0.12 M phosphate buffer.
open circle (○): 28 μg/ml (DNA concentration)
half open circle (◐): 83 μg/ml
closed circle (●): 880 μg/ml
*The dotted Area indicates intermediate repetitive fraction.
**The values of A,B, and C are half Cot (Cot $\frac{1}{2}$) of each fraction.

normal liver cells is one-half reacted at a Cot of 1.9×10^{3}, whereas the fastest reassociating fraction is one-half reacted at a Cot of 3.1×10^{-2}, indicating that this fraction must have

Table II. The amount, repetition frequency and nucleotide length of repetition unit of highly repetitive sequence of tumor cell DNAs

	Amount(%) per total genome	Repetition frequency	Size (NTP*)
Normal Liver Cell DNA	14.0	610,000	3,300
Ehrlich Ascites Tumor DNA	16.0	950,000	9.400
Yun Salivary Adenocarcinoma DNA	19.0	1,600,000	6,900

*NTP: nucleotide pairs

Table III. The amount, repetition frequency and nucleotide length of repetition unit of intermediate repetitive sequence of tumor cell DNAs

	Amount(%) :per total genome	Repetitive frequency	Size (NTP*)
Normal Liver Cell DNA	11.5	380	520,000
Ehrlich Ascites Tumor DNA	23.0	3,900	330,000
Yun Salivary Adenocarcinoma DNA	24.0	4,800	270,000

*NTP: nucleotide pairs

Table IV. The amount of non-repeated DNA fraction in total genome of tumor cell

	Amount (%) :per total genome*
Normal Liver Cell DNA	68.5
Ehrlich Ascites Tumor DNA	55.0
Yun Salivary Adenocarcinoma DNA	51.0

*The word genome means haploid DNA content of a cell.

6.1×10^{5} copies compared to the non-repeated DNA.

Complete data of repetitive sequences of tumor cell DNAs and normal liver cell DNA are summarized in table II, III and IV.

DISCUSSION

The analysis of reassociation reactions of DNA shows that repetitive fractions of two tumor cell DNAs seem to be larger than that

of normal cell DNA in amount. The highly repetitive fractions of mouse tumor cell DNAs comprise about 16%～19% of the total DNA and vary from 950,000 to 1,600,000 copies, whereas the normal cell DNA contains 14% of highly repetitive fractions of the total DNA, averaging 610,000 copies. (Table. II) In the mouse, this DNA fraction corresponds to the mouse satellite DNA, (Hennig and Walker, 1970: Maio and Schildkraut, 1969) which has never conclusively been shown to be transcribed into RNA of any kind. (Santiago and Rake, 1972) Harel et al (1968) reported that this satellite did in fact transcribe rRNA, but later work of Flamm et al (1969) was not able to confirm this report.

The amount of intermediately repeated DNA fraction of tumor cells is 23～24% of the total DNA(genome) and the repetitive frequencies are 3,900～4,800 copies. Compaed with the data of normal cells, the tumor cell DNA contains on the average 10% more intermediate repetitive sequences. (Table III)

It is possible that the individual fractions of DNA are in fact composed of families of DNA, each family having a different repetition frequency, i.e. mixture, perhaps heterogeneous, of families. More thorough analysis of reassociation kinetics of mouse and hamster indicates that the highly repeated fraction is composed of a single family, while the intermediate fraction, is composed of a mixture of several small families repeated at different frequencies. Thus the repetition number of 380, etc., represents a weighted average of the various heterogeneous frequencies of that fraction, and the amount of DNA in that fraction represents the total of the various families. Within the limits, the amount of DNA and repetition number are useful indicators of that fraction, (Santiago and Rake, 1973)

On the basis of kinetic analysis, it has been reasoned that this DNA is transcribed into RNA and that the pattern of transcription of this DNA fraction varies from one tissue to another. This consideration, among others, led Britten and Davidson (1971) to suggest that repeated DNA is involved in the switching system for controlling the expression of the genetic DNA, in turn controlling differentiation. There is very good circumstancial evidence that this hypothesis is true, however, it is not easy to see why the tumor cell would need as much as 23%～24% and normal cell only 11.5% of its DNA in the form of intermediate repetitive sequence for such control of development and division.

Although the information presented here has not provide an explanation of the function of repetitive sequence, it might give some suggestion that the increase of amount in the intermediate fraction of tumor cell DNA would be related to some characteristics of tumor cell such as loss of autonomy and rapid cell proliferation.

SUMMARY AND CONCLUSION

The kinetics of reassociation of the DNA of mouse tumor cells has been examined to determine the amount and repetition frequency of various DNA fractions. Before the reassociation experiment the purity of DNA preparations was confirmed by its melting behavior(temperature denaturation). The Guanine-Cytosine content of DNA was also calculated from the melting curve. (Tm value)

The transplantable tumor used in the denaturation experiment were Ehrlich ascites tumor, Sarcoma 180, Yun salivary adenocarcinoma and Yun liposarcoma. Among 4 kinds of mouse tumors, Ehrlich ascites tumor and Yun salivary adenocarcinoma were used in the reassociation experiment. The results were analyzed by comparing the pooled DNA of normal liver cells

with that of tumor cells.

The following results were obtained;

1. The Guanine-Cytosine contents of each DNA are as follows.

Ehrlich ascites tumor	41.5%(Tm: 86.3°C)
Sarcoma 180	41.0%(Tm: 86.1°C)
Yun salivary adenocarcinoma	39.5%(Tm: 85.5°C)
Yun liposarcoma	40.0%(Tm: 85.7°C)
normal liver cell	41.0%(Tm: 86.1°C)

2. The amount of non-repetitive DNA of the tumor cells decreased by about 15 percent, compared with that of normal liver cell DNA.

3. There are great differences of amount and repetitive frequency between normal and tumor cell DNA, especially in the intermediate repetitive fraction. The amount of highly repetitive DNAs of tumor cells is increased 2∼5% over that of normal cell DNA. In the case of the intermediate fraction, its amount in the tumor cells is approximately twice that of normal cells. In addition to the increase in amount of repetitive sequences, the frequency of repetition is also increased in both fractions of each tumors. The average nucleotide repetition length in the intermediate fraction of the tumor cell DNA is approximately half that of the correspondng length in the normal cell DNA fraction

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≪국문초록≫

마우스 癌細胞 DNA의 반복염기서열 구조

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2종의 마우스 癌細胞(Fhrlich Ascites tumor, Yun Salivary Adenoca)에서 DNA을 추출하고, DNA 재결합 실험법을 이용하여 이를 반복염기 서열을 가진 분획(Repetitive sequence)과 비반복 염기서열분획의 두 부분으로 나누어 각각의 전체 DNA에서 차지하는 양을 계산하였고, 반복구조의 경우에는 비슷한 염기 서열의 반복횟수를 추정하였다. 이 결과는 정상 마우스 간세포 DNA의 재결합 실험결과와 비교·대조하였다.

비반복구조의 양을 Tumor cell DNA(암세포)와 정상의 그것과 비교해 보면, Ehrlich 복수암세포 DNA에서는 55%로 정상의 68.5%보다 13.5%가 작고 Yun Salivary Adenocarcinoma에서는 51%로 17.5%가 감소된 것으로 나타났다. 다시 말하면, 암세포 DNA에 있는 반복서열 구조가 그만큼 더 많이 존재한다는 시사가 된다.

반복구조분획은 재결합 속도가 가장 빠른 Highly repetitive sequence와 이보다는 느린 Intermediate Repetitive Sequence로 나누어 결과를 분석해 보면 2종의 암세포 DNA에서 모두 다 두 분획의 양적인 증가를 볼 수 있었으며, 특히 Intermediate repetitive fraction의 경우에 더욱 현저한 차이를 나타냈다. 반복횟수의 추정에서도 암세포 DNA의 반복 서열 구조의 횟수가 10∼12배 증가된 것으로 나타났다. 암세포의 세포조절기전의 상실과 Intermediate repetitive sequence의 양과 반복횟수의 증가를 연결시켜 보려고 시도하였다.

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