Distribution of repetitious sequences in chick nuclear DNA

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ABSTRACT

By an improved method of hydroxylapatite chromatography, the reassociated sequences of chick nuclear DNA were isolated, and their base composition analysed. By increasing the amount of reassociation, the G + C content of the renatured sequences decreased progressively to reach a mean value corresponding to that of the total DNA. In order to study the distribution of the families, or group of families having different amount of reassociation, DNA was fractionated by CsCl density gradient centrifugation. Fractions having different G + C content were obtained, and their reassociation rates analysed. At high C_{ot} value of renaturation ($C_{ot}=50$) the amount of reassociated sequences included in the high or in the low buoyant density DNA fractions was approximately the same, but their G + C content was as expected different. At lower C_{ot} values of renaturation (between C_{ot} of 0.2 and the C_{ot} of 10), the results indicated an heterogeneity of the repeated sequences in the A + T rich DNA fractions, as compared to the G + C rich ones.

INTRODUCTION

Part of the Eukaryote genome is composed of repetitious DNA sequences which are characterized by their rate of reassociation.^{1,2} Exemples of such sequences are provided by the distinct nuclear satellite DNA's which have been isolated from the DNA of several species.^{3,4,5} In species with no distinct satellite DNA a discrete class of repetitious DNA was found burried within the main band DNA.⁶ Recently it was demonstrated that in the human genome, the most rapidly renaturing sequences constitutes 10% of the genome and appear closely spaced, while the slowly reassociating sequences are dispersed.⁷ In our experiments using a modified technique of hydroxylapatite chromatography and direct base composition analysis of nuclear DNA from chick fibroblast cells, we are investigating the distribution of repetitious DNA with different frequencies of repetition among DNA fractions having different G + C content. Finally we are reporting the isolation of single stranded DNA from native DNA.

MATERIALS AND METHODS

Cell culture : Secondary chick fibroblast were grown and labeled for approximately three cell generation in Roux bottles, with Eagle's medium minus inorganic phosphate, 10% calf serum prealably dialysed against the phosphate free medium and 50 μ Ci/ml carrier free 32 P (Saclay).

Isolation of DNA.

Nuclei were isolated as described by Fernandez and Pogo.⁸ They were suspended in 0.01 M Tris-HCl pH 8.3, EDTA 0.01 M and lysed by incubating for 30 min. at 37°C in the presence of 0.1% sodium lauryl sulfate (Merck) and 100 μ g/ml of pronase (Sankyo). The concentration of sodium lauryl sulfate was increased to 1% and the nuclei reincubated for 15 min. at 37°C. The DNA extracted according to the technique of Marmur⁹ was dissolved in 0.1xSSC, and incubated for 30 min. at 37°C with 20 μ g/ml of pancreatic ribonuclease (RNAase A Worthington), and 10 units/ml of RNAase T_I (Sankyo). Reextracted with chloroform-isoamyl alcohol, precipitated with ethanol and redissolved in and dialysed against NaCl 0.014 M, Tris 0.01 M (pH 8.3), EDTA 0.002 M. The specific activity determined by counting aliquots in a liquid scintillation spectrometer was about 1.5x10⁵ cpm/ μ g DNA.

Preparative density gradient centrifugation.

Nuclear DNA (200 μ g) was sheared with a calibrated needle BD 26 using 10 strokes with a 5 ml syringe, and dissolved in CsCl in 0.01 M Tris (pH 8.5), plus 2 mM EDTA to make the density approx. 1.7 g/ml. Placed in tubes suitable for the 50 Ti rotor of the model L ultracentrifuge, overlayed with paraffin oil, and centrifuged at 33000 rev./min. at 18°C for 72 hr. The tubes were pierced at the bottom with a needle and fractions were collected. For isolation of the fractions differing in G + C content, aliquots were taken and their buoyant densities measured by the analytical density gradient centrifugation as previously described.¹⁰ The fractions with the same buoyant density were pooled, dialysed against NaCl 0.014 M, Tris 0.01 M (pH 7.8), EDTA 0.002 M, and their final buoyant density determined by the analytical density gradient centrifugation.

Improved method of hydroxylapatite fractionation.

Previous investigators^{11,12,13} found that the double stranded DNA elutes at 0.25 M phosphate buffer pH 6.9, whereas the single stranded DNA elutes at 0.15 M. In the present study elution was carried on at pH 7.85 where the separation of the single and the double stranded DNA

is greatly increased. Instead of column chromatography, a procedure which facilitates the processing of relatively large number of samples was used. Aliquots of the labeled DNA to be analysed, were diluted to 4 ml with NaCl 0.10 M, EDTA 0.001 M, and mixed with 0.5 g. of hydroxylapatite (Biorad laboratories). Adsorption was achieved by gently shaking at room temperature for 5 min. The hydroxylapatite was pelleted by low speed centrifugation, and the supernate collected. Hydroxylapatite was then washed twice by the same procedure, with 0.01 M phosphate buffer pH 7.85. Step-wise elution of the different secondary structure of the DNA was performed by resuspending the hydroxylapatite pellet in 4 ml phosphate buffer of increasing molarities. The tubes were incubated in a water bath at 56°C for 8 min. with intermittent agitation. Hydroxylapatite was again pelleted by low speed centrifugation at room temperature, and the supernate collected. The process was repeated twice for each given concentration of the phosphate buffer.

Carrier DNA was added to aliquots of each step-eluted fraction, and cold trichloroacetic acid (TCA) was added to bring the final concentration to 5%. After incubation of 10 min. in an ice bath, the acid precipitable material was collected by filtering through whatman glass fibre paper GF/B. Each tube was washed twice with 4 ml of TCA 5%, which was poured into the filters. The filters were dried and the radioactivity determined in a liquid scintillation spectrometer.

The results were normalized by adding the values of the radioactivity recovered in the two successive supernates at each elution step, and expressing the sum as per cent of the total radioactivity recovered from the elutions. Provided the pH of the phosphate buffer was carefully controlled, reproducible results were obtained in all assays, and about 95% of the radioactivity was recovered.

Renaturation of the DNA.

DNA was sheared by passage of 2 ml DNA solution (100 µg/ml) in a 5 ml syringe through a 26 gauge syringe needle with a maximum pressure applied 10 times by hand.

Denaturation of DNA: Alkaline denaturation was used. To 8 parts of DNA solution at room temperature, was added one part of 1.0 M NaOH. After 15 min. incubation at room temperature, the solution was cooled to 0°C and one part of cold 2.0 M NaH_2PO_4 was added to neutralize the solution. The denatured DNA solution was used within one hour.

Renaturation of DNA: The rate of renaturation of DNA, was followed by

the elution from the hydroxylapatite as described above, after incubation at 66°C in 0.3 M NaCl during different time periods of a constant DNA concentration.

Determination of DNA base composition.

A portion of ^{32}P labeled DNA obtained as described in the different figures was incubated in 0.04 ml with pancreatic deoxyribonuclease (100 $\mu\text{g}/\text{ml}$) in 0.01 M Tris (pH 7.0) plus 0.01 M MgCl_2 for 30 min. at 37°C. After heating for 5 min. at 100°C, the pH was adjusted to 8.8 with 1.0 M glycine buffer (pH 9.2), and 0.01 unit (1 unit = 1 μmole of bis-(p-nitrophenyl) phosphate hydrolysed per min.) of snake venom phosphodiesterase (Sigma) was added. After 30 min. at 37°C, 0.01 μmole of each of the four unlabeled 5'-deoxyribomononucleotides were added. The pH was adjusted to approx. 3.5 with HCl. The solution was applied to Whatman N° 3 paper and high voltage electrophoresis was carried out in 0.05 M sodium citrate buffer (pH 3.5) at 2000 V for 150 min. The paper was cut after determining the U.V. spots corresponding to the migration of carrier nucleotides, and counted in a liquid scintillation spectrometer.

RESULTS

Fractionation of DNA by an improved method of hydroxylapatite chromatography.

The secondary structure of native or alkali denatured nuclear DNA was analysed by using a modified technique of hydroxylapatite chromatography. Native DNA isolated as described in materials and methods, and analysed by the hydroxylapatite chromatography, contained two eluted fractions; a major one with a main peak of elution at 0.55 M phosphate buffer pH 7.85, and a small one, corresponding to about 2% of the total which was constantly eluted with a main peak of elution at 0.15 M as the denatured DNA (Fig. I). Evidence for this DNA being single stranded comes also from its susceptibility to S_1 nuclease. This enzyme, (kindly provided by Dr. J. Hurwitz) preferentially degrades single stranded DNA.²⁹ Thus by incubating this isolated fraction for 30 min. at 37°C in acetate buffer pH 4.5 in the presence of S_1 nuclease and rechromatographing on hydroxylapatite, all the fraction was degraded and therefore did not adsorb to hydroxylapatite. This fraction was also degraded after incubation for 30 min. at 37°C in the presence of DNAase, whereas it was not degraded when incubated in the presence of RNAase.

Fig. 1. Hydroxylapatite chromatography of native and denatured nuclear DNA. Native (top), or alkali denatured (bottom) ^{32}p labeled nuclear DNA from chick fibroblastic cells, were loaded on hydroxylapatite. The fractions were eluted at different phosphate buffer (pH 7.85) molarities and processed for TCA precipitation as described in Materials and Methods.

Fig. 2. Hydroxylapatite chromatography of nuclear DNA denatured and renatured at different C_0t values. Isolated ^{32}P labeled nuclear DNA was sheared, denatured, renatured at the indicated C_0t values and analysed by hydroxylapatite chromatography as described in Materials and Methods. The fractions eluted at different molarities of phosphate buffer, were processed for TCA precipitation, and their radioactivity measured in a liquid scintillation spectrometer.

Fig. 3. Fractionation of ^{32}P labeled nuclear DNA by $CsCl$ density gradient centrifugation. Chick fibroblast nuclear DNA was sheared, and centrifuged in $CsCl$ density gradient as described in Materials and Methods. Fractions were collected and the TCA precipitable radioactivity measured. The buoyant density of DNA from different regions of the gradient was analysed by analytical density gradient centrifugation. Fractions having approximately the same buoyant density were pooled and dialysed against $NaCl$ 0.014 M, Tris 0.01 M (pH 7.8), EDTA 0.002 M. The respective buoyant densities (indicated in the figure) of the pooled dialysed DNA fractions were finally determined.

As a control the fraction corresponding to double stranded DNA was incubated in the presence of the S_1 nuclease and rechromatographed on hydroxylapatite. All the radioactive material was adsorbed and eluted upon rechromatography at the same phosphate buffer molarities. Furthermore, the base composition of this single stranded DNA fraction was determined, and it was found that its G + C content (40.2%) was slightly different from that of double stranded DNA (see table 2). Finally, the hydroxylapatite elution profiles (Fig. 4) of the separated DNA fractions obtained by density gradient centrifugation, showed that only the high buoyant density DNA contain a fraction which was eluted as single stranded DNA. These results, confirm our previous data obtained by other procedures with DNA from other animal cells.²³

Reassociation of chick nuclear DNA.

The nuclear DNA used in these studies was sheared as described in materials and methods. The alkaline sucrose gradients showed that the sedimentation coefficient of the bulk of the DNA was comprised between 15 and 18 S, indicating a molecular weight of $1.3 - 2.1 \times 10^6$ Daltons which corresponds to a single strand chain length of 4000 to 6000 nucleotides, approximately.¹⁴

Reassociation analysis was accomplished by allowing reassociation to occur for various periods of time and determining the amount reassociated by fractionation on hydroxylapatite (Fig. 2). It was considered as reassociated DNA only the fractions eluted at phosphate buffer molarities comprised between 0.50 M and 0.70 M, and as unreassociated DNA the fractions eluted at 0.15 - 0.20 M. Upon rechromatography these pooled fractions were reeluted as sharp peaks, corresponding to those of native double stranded DNA and denatured DNA respectively. Intermediate fractions (eluted between 0.25 and 0.45 M) were not taken into account since they may corresponded to partially reassociated or mismatched sequences.

The time course of reassociation for chick DNA showed that between the C_{ot} of 2 and the C_{ot} of 50, the reassociated amount increased from about 10 to 35% respectively. Furthermore, the base composition analysis of the DNA reassociated at different C_{ot} values (table I) shows that the G + C content of the reassociated sequences decreases as the amount of reassociation increases, to reach at C_{ot} 50 a G + C content identical to that of the total DNA.

Table I. Base composition of chick DNA after renaturation at different C_{ot} values and hydroxylapatite fractionation.

C_{ot} of renaturation	phosphate buffer moles/l	Moles %				
		C	A	G	T	G+C
2	0.15-0.20	19.1	29.3	19.4	32.2	38.5
	0.50-0.60	22.3	25.9	24.2	27.6	46.5
10	0.15-0.20	18.9	30.0	19.6	31.5	38.5
	0.50-0.60	21.5	26.4	23.1	29.0	44.6
50	0.15-0.20	18.6	30.2	19.6	31.5	38.2
	0.50-0.60	21.1	28.1	21.7	29.1	42.8

Nuclear DNA renatured at different C_{ot} values was analysed by hydroxylapatite chromatography as shown in Fig. 2. Fractions eluted at 0.15-0.20 M and 0.50-0.60 M phosphate buffer, which correspond to unrenatured and renatured DNA sequences respectively, were pooled and the base composition was determined on aliquots. After enzymatic hydrolysis to 5'-deoxyribomononucleotides and high voltage electrophoresis, over 97% of the radioactivity present in the hydrolysate, migrated with the four 5'-deoxyribomononucleotides added as ultraviolet markers.

It appears then that the repetitious DNA fractions consist of several families of repeated sequences with different G + C content and with different frequencies of repetition which probably range from hundreds of copies to thousands of copies per genome.

Fractionation of nuclear DNA by the CsCl density gradient centrifugation.

In order to study the distribution of these different families of repeated sequences, the DNA was fractionated by centrifugation to equilibrium in CsCl (Fig. 3). DNA fractions having similar buoyant densities were pooled and dialysed as described in materials and methods. Aliquots were tested for susceptibility to alkaline hydrolysis for 20 hr at 37°C. No significant amount of radioactivity was rendered trichloroacetic acid soluble, indicating no contamination by RNA. The buoyant densities of the different DNA fractions were determined by analytical density gradient centrifugation. The base composition of the DNA fractions (table 2) were determined by degradation to 5'-mono nucleotides by the combined action of pancreatic deoxyribonuclease and snake venom phosphodiesterase followed by high voltage paper electrophoresis. The G + C contents of the different fractions was linearly related to their density satisfying the equation previously described.¹⁵

Table 2. Base composition of native unfractionated and fractionated chick fibroblast nuclear DNA.

Density of DNA fractions g./ml	phosphate buffer moles/l	Moles %				
		C	A	G	T	G+C
Unfractionated	0.15-0.20	19.7	30.6	20.5	29.2	40.2
	0.50-0.60	20.2	28.7	22.4	28.7	42.6
I.708	0.50-0.60	23.8	26.0	25.2	25.0	49.0
I.700	0.50-0.60	19.3	28.7	20.9	31.1	40.2
I.696	0.50-0.60	17.7	31.1	19.2	32.0	36.9

Nuclear DNA unfractionated and fractionated by CsCl density gradient centrifugation as shown in Fig. 3, was analysed by the hydroxylapatite chromatography as shown in Fig. 1 and 4. Fractions eluted at 0.15 - 0.20 M and 0.50 - 0.60 M phosphate buffer which correspond to single and double stranded DNA respectively, were pooled and the base composition determined on aliquots as described in materials and methods.

Distribution of reassociated sequences of DNA fractions having different G + C content.

In considering models of the organization of the genome, it is important to know the arrangement of the repeated sequences in DNA. The approach used here involves the fractionation of the total genome in CsCl density gradients (Fig. 3). Three main fractions were obtained having a G + C content of 49.0, 40.2, and 36.9% respectively (table 2). The reassociation analysis, and the fractionation of the reassociated sequences were accomplished as described in materials and methods. The amount of reassociated sequences was plotted as shown in fig. 5.

The amount of reassociation of the G + C rich fraction did not increase from the C_{0t} of 0.2 to the C_{0t} of 10, and was at a relatively high ratio (20% of the fraction). The base composition analysis of the reassociated sequences (table 3) showed a G + C content higher than that of the original fraction. Furthermore, no variations of the G + C content was observed between the reassociated sequences obtained at these C_{0t} values.

Between the C_{0t} of 10 and the C_{0t} of 50, the amount of reassociation increased by 17% and the G + C content of the reassociated sequences obtained at the C_{0t} of 50 decreased to reach approximately the same

value as the original fraction. This indicates that the high buoyant density DNA fractions, consists of probably one or several families of highly repeated sequences with the same G + C content and with the same frequency of repetition. Another family of less repeated sequences with probably less G + C content appears to be also included in this DNA fraction.

Density of DNA fractions g./ml	C ₀ t of renaturation	Moles %				
		C	A	G	T	G+C
I.708	2	25.8	22.7	28.3	23.2	54.1
	10	25.3	22.7	27.9	24.1	53.2
	50	24.4	23.9	26.4	25.3	50.8
I.700	2	19.8	28.5	21.5	30.2	41.3
	10	20.4	28.6	21.4	29.6	41.8
	50	18.0	28.5	21.7	31.8	39.7
I.696	2	19.5	29.8	20.5	30.2	40.0
	10	18.4	29.9	20.7	31.0	39.1
	50	17.6	29.9	19.9	32.5	37.5

Table 3. Base composition of DNA fractions having different G + C content, after renaturation and fractionation by the hydroxylapatite chromatography.

DNA fractions having different buoyant densities (Fig. 3) were renatured at different C₀t and chromatographed on hydroxylapatite. Fractions eluted at the phosphate buffer molarities corresponding to the renatured sequences as plotted in fig. 5, were pooled and the base composition determined on aliquots as described in materials and methods.

The amounts of reassociation of the low buoyant density fraction (having a G + C content of 36.9%) increased from 5 to 32% between the C₀t of 0.2 to the C₀t of 10 respectively, and a very slight increase was observed between the C₀t of 10 and the C₀t of 50. The G + C content of the sequences reassociated at the different C₀t values, varied from 40.0 to 37.5% (table 3). This indicates that this fraction unlike the G + C rich one, consists of several families of repeated sequences, having different G + C content.

The amount of reassociation of the main band DNA fraction (having a G + C content of 40.2%), showed the lowest ratio of redundant sequences (8% at C₀t of 2) but the repeated sequences represented as much as 25% of the fraction at a C₀t of 50. The G + C content of the reassociated

Fig. 4. Hydroxylapatite chromatography of the DNA fractions obtained by the CsCl density gradient centrifugation. DNA fractions having different buoyant densities (shown in Fig. 3.) were analysed by the hydroxylapatite chromatography as described in Materials and Methods. The fractions eluted at different molarities of phosphate buffer were processed for TCA precipitation.

Fig. 5. Renaturation at different C_0t values of nuclear DNA fractions having different buoyant densities. DNA fractions having different buoyant densities, (Fig. 3.) were denatured and renatured at different C_0t values. They were analysed by the hydroxylapatite chromatography as described in Materials and Methods. Aliquots of the fractions eluted at different molarity of phosphate buffer were processed for TCA precipitation and their radioactivity measured. The fractions eluted from 0.50 M to 0.70 M phosphate buffer, were summed and considered as the "renatured fractions". Fraction H. (●—●), Fraction M. (○—○), Fraction L. (▲—▲).

sequences obtained at any C_{ot} values, was the same and corresponded to that of the original fraction. It appears then that this fraction, representing the major part of the DNA, contained several families of repeated sequences having low frequency of repetition, and with probably the same G + C content.

DISCUSSION

The presence of families of repeated sequences has been established through the study of renaturation kinetics of denatured DNA.^{1,16} In the mouse, the satellite DNA is more or less a pure fraction in terms of base sequence, but it does not represent the entire population of the repeated sequences. The satellite DNA comprises approx. 10% of the total mouse genome^{17,18} whereas repetitious DNA of the mouse comprises approx. 35% in the total genome.¹

Hydroxylapatite chromatography, separates the DNA on the basis of the particular conformations present at the time of elution.¹⁹ In our experiments this technic was modified, and reassociated sequences were separated from the unreassociated ones. The base composition of the reassociated sequences of chick nuclear DNA, indicates that the G + C content of these sequences is inversely proportional to the C_{ot} of renaturation. When nuclear DNA from other species, e.g. chinese hamster is examined with this method, the amount of reassociated sequences obtained, corresponded to the amount obtained with chick DNA, but the G + C content of the reassociated sequences did not vary and corresponded to that of native DNA.²⁰ Thus it is suggested that heterogeneous repeated sequences constitute important part of the animal cell DNA. Because of this heterogeneity, nuclear DNA was fractionated in fractions having different G + C content, and families of repeated sequences within each fraction were analysed. The fact that both fast and slower reassociating DNA sequences were isolated from the G + C to the A + T rich DNA fractions, indicates that the repeated DNA sequences occurs in the entire spectrum of the chick genome. But the amount of the reassociated sequences as well as the G + C content of these sequences varied according to the DNA fraction analysed. Previous workers^{21,22} showed that in the mouse and in *Xenopus*, unique sequences transcribe in vivo. However it is still debatable whether repetitious DNA ever functions as template for RNA synthesis in vivo. They may be also important for maintaining the physical integrity of the chromosome.

In the present investigations we are also reporting evidences for a single stranded DNA within the chick genome. The relative amount of this single stranded DNA appears to be related to the buoyant density of the DNA. Thus confirming our previous results in other animal cell.²³ The origin of this single stranded DNA is so far unknown, its existence has been postulated in bacteria²⁴ and in mammalian cells²⁵ It was related to the newly synthesized DNA, but its amount do not increase when chick cells were synchronized and the DNA pulse labeled.²⁶ A model was proposed for the recognition sites needed for control purposes of higher organisms²⁷ where it was suggested that those sites might represent unpaired single stranded DNA stretches of double stranded DNA. Recently it was reported that nucleolar DNA contain a G + C rich DNA fraction in a single stranded form.²⁸ In the present work, and according to the G + C content of the single stranded DNA we obtained, it is suggested that DNAs other than nucleolar DNA can be found in the single stranded form.

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