MOLECULAR-CYTOGENETIC APPROACH TO MAPPING METHYLATED POLYMORPHIC RESTRICTION FRAGMENTS OF HUMAN RIBOSOMAL RNA GENES

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It has been shown that the nontranscribed spacer (NTS) of human ribosomal RNA (rRNA) genes is characterized by interindividual polymorphism of the lengths of the DNA fragments formed through the action of restriction endonuclease BamH I [1, 6, 8], and that the inheritance of fragments of each infrequently found variant is linked, i.e., the fragments are located in only some and not all of the 10 human nucleolar organizers (NO) [2]. Technical difficulties in the way of obtaining DNA from individual acrocentric chromosomes carrying NO have impeded mapping of concrete classes of polymorphic fragments from concrete chromosomes. The writers have suggested that to map polymorphic fragments of genes in a state of repression, a new approach can be developed, based on the use of methylation of the cytosine bases of DNA. The central part of the heterogeneous region of NTS contain a recognition site of restriction endonuclease Sal I, which does not cleave methylated DNA. It may be expected that if a significant proportion of copies of rRNA genes in individual NO is methylated, combined attacks by restriction endonucleases BamH I and Sal I will give a different restriction picture from that found in DNA samples from individuals with functionally active ribosomal genes in all NO.

In a human population rare individuals are found with an excess of rRNA genes in one of their acrocentric chromosomes; methods of identification of these chromosomes and of demonstration of an excess of rRNA genes and of their repression by methylation are known in cytogenetics [4, 9].

We considered that a combined study of such cases by the use of the above-mentioned molecular and cytogenetic methods would result in accurate chromosome mapping of the methylated class of polymorphic restriction fragments of rRNA genes. The investigation described below was undertaken for this purpose.

EXPERIMENTAL METHOD

Blood from normal persons and from an individual with an acrocentric chromosome with morphology suggesting an excess of rRNA genes was used as the test object. Chromosomes were identified as described previously [3]. NO of metaphase chromosomes were revealed by the method of Ag staining [5]. Hybridization *in situ* was carried out by the method in [6]. The basic stages of the method included denaturation of the preparations in 7% formamide at 70°C for 3 min, hybridization for 18 h at 37°C, exposure under type M emulsion (Photographic Chemical Research Institute) for 5 weeks. The hybridization mixture contained: 50% formamide, 10% dextran sulfate, and calf thymus DNA as the carrier DNA. The probe was ^{3}H-28S ribosomal DNA (rDNA). A subfragment of the 28S rDNA gene, cloned from the rat gene library and generously provided by V. V. Nosikov, was used as the probe for *in situ* hybridization.

Human DNA was isolated from whole blood leukocytes [1]. Deproteinization was carried out by the phenol-chloroform method [1]. Restriction hydrolysis of DNA, electrophoresis,

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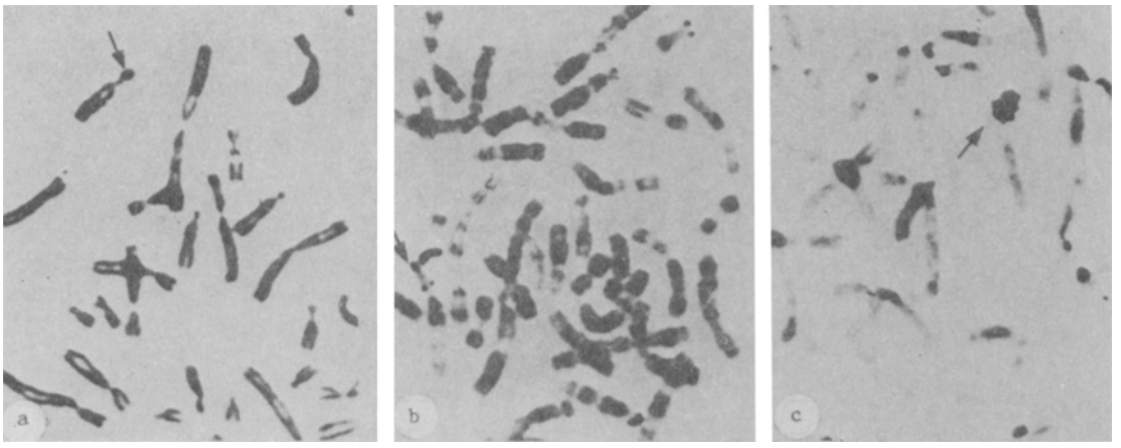

Fig. 1. Cytogenetic characteristics of chromosome 13 with enlarged short arm (13p+): a) uniform staining with azure-eosin; b) stained with silver nitrate; c) hybridizatin in situ with ^{3}H-28S rRNA probe. Arrow indicates chromosome 13p+.

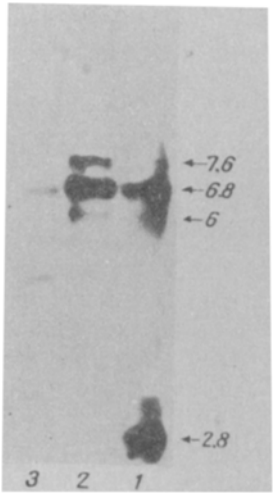

Fig. 2. Molecular characteristics of NTS of rRNA genes of an individual with a 13p+ chromosome. Length of fragments (in kilobase pairs). 1, 2, and 3) Hydrolysis of total DNA by restriction endonucleases BamH I and Sal I, BamH I, and BamH I + Hpa II respectively, followed by hybridization with subfragment of 28S rRNA gene. Arrows indicate position of polymorphic fragments.

and blot hybridization were described previously [1]. DNA was labeled by the nick-translation method [1]. The phosphorus-labeled precursor ^{32}P-dTTP (8.1×10^{7} MBq/mmole) was used for hybridization on nitrocellulose filters. For hybridization in situ, three tritium-labeled nucleotides with specific radioactivity of 1.85×10^{6} and 9.99×10^{5} MBq/mmole were used in the reaction mixture for hybridization in situ. The ^{32}P-DNA and ^{3}H-DNA were purified from nucleotides by gel-filtration on a column with Sephadex 50 (fine). Specific radioactivity of the ^{32}P-DNA thus obtained was $(1\text{-}5) \times 10^{8}$ cpm/μg, and that of the ^{3}H-DNA was $(1\text{-}5) \times 10^{7}$ cpm/μg.

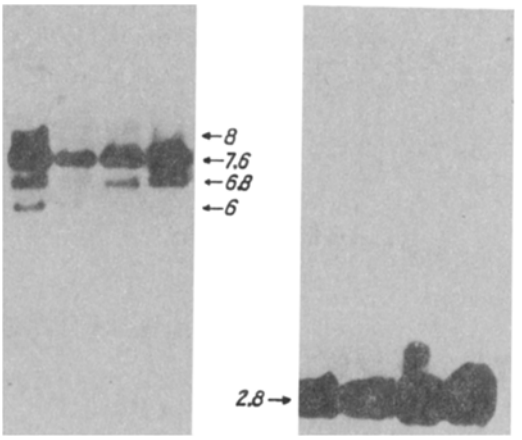

Fig. 3. Molecular characteristic of heterogeneous region of NTS of rRNA genes of persons with 2NO stained with silver nitrate. DNA of four individuals hydrolyzed by BamH I (a) and BamH I and Sal I (b), and hybridized with ^{32}P-probe of subfragments of 285 rRNA genes. Triangles indicate polymorphic fragments whose length are given in kilobase pairs.

EXPERIMENTAL RESULTS

Besides the very considerable size of the short arm of chromosome 13 (Fig. 1a) of the individuals studied, two small regions, intensely stained regions were found by Ag staining methods at both ends of the arm (Fig. 1b). We know that such regions contain functioning rRNA genes [7]. In size and intensity of staining, the total quantity of Ag-positive material of chromosome 13p+ corresponded approximately to its contents in any other acrocentric chromosome of the set (Fig. 1b).

On hybridization in situ with the ^{3}H-28S rDNA probe several times more label was contained in the short arm of chromosome 13p+ than in the most strongly labeled acrocentric chromosome of the same individual (Fig. 1c). Comparison of the results of Ag staining and hybridization in situ leads to the conclusion that the enlarged arm of marker chromosome 13 carries a large number of copies of rRNA genes, most of which are functionally inactive.

The molecular characteristics of the heterogeneous region of NTS of the rRNA genes of the individual with a 13p+ chromosome are illustrated in Fig. 2. Equal quantities of DNA, hydrolyzed by restriction endonucleases and hybridized with a fragment of the 28S rRNA gene were applied to three lanes. DNA on the 2nd lane was hydrolyzed by restriction endonuclease BamH I, which revealed the most varied heterogeneity of structure of the NTS, adjacent to the 28S rRNA sequence. Heterogeneity in this DNA is represented by fragments 6, 6.8, and 7.6 kilobase pairs (kb) long. On the first lane DNA from the same individual was hydrolyzed by a combination of restriction endonucleases BamH I and Sal I. The recognition site of restriction endonuclease Sal I is located approximately in the central part of the polymorphic BamH I restriction fragments. After double restriction with BamH I and Sal I fragments all nonmethylated polymorphic fragments ought to have been hydrolyzed and to appear in the region with lower molecular weights; the length of the newly formed fragments, moreover, was about 2.8 kb and the methylated fragment ought to have remained unchanged. In fact, after hydrolysis of the DNA by two restriction endonucleases the intensity of the 6 and 7.6 kb fragments was much reduced, whereas the 6.8 kb fragment was virtually completely preserved. These results were confirmed by the use of another restriction endonuclease (Hpa II), which also does not hydrolyze methylated DNA. The 3rd lane shows the results of hydrolysis of DNA by a mixture of restriction endonucleases BamH I and Hpa II. This combination of restriction endonucleases leads to disappearance of the 6 and 7.6 kb fragments, and a reduction in the intensity of the 6.8 kb fragment. The difference between tracks 1 and 3 are due to the fact that restriction endonuclease Hpa II has a sequence of four nucleotides in its recognition

site, and it therefore hydrolyzes DNA by a greater degree than Sal I. Patterns of DNA from four control individuals hydrolyzed by restriction endonuclease BamH I (a) and by a combination of endonucleases BamH I and Sal I (b), followed by hybridization with a subfragment of the 28S rRNA gene, are shown in Fig. 3. In these subjects, NO of all 10 acrocentric chromosomes were revealed by Ag staining at the ordinary intensity. Total DNA, hydrolyzed by restriction endonuclease BamH I reveals a definite set of polymorphic restriction fragments of NTS of rRNA genes (Fig. 3a). After double restriction by BamH I and Sal I (Fig. 3b) all polymorphic structural variants are hydrolyzed and the hydrolysis products revealed in newly formed fragments with lower molecular weights. Consequently, DNA of control individuals possessing rRNA genes on all acrocentrics in the usual numbers, do not contain clusters of methylated structural variants of NTS.

This investigation thus fully confirmed the hypothesis that restriction fragments of DNA or rRNA genes, polymorphic in length, can be mapped on the individual chromosome. The condition for success is methylation of the given fragment and its presence as a cluster of copies on the individual chromosome. The problem of what is the minimal number of repressed structural variants which can be mapped by the new method has not yet been settled. In our case, we deliberately chose NO with an unusually high content of them. However, according to our preliminary data, the DNA framgent contained in a repressed NO of usual size can be mapped. If this is confirmed, there will be a real possibility of studying the state of heterogeneity of NTS of rRNA genes in a state of repression, in any of the 10 human NO.

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