IN SITU HYBRIDIZATION OF CLONED REPEATING DNA SEQUENCES AND DIFFERENTIAL STAINING OF HUMAN CHROMOSOMES

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The study of the structural and functional organization of human chromosomes is aimed at discovering the distribution of genetic elements and different types of DNA sequences in chromosomes, their interconnection with known cytologic differences between chromosomal regions and, ultimately, the creation of complete cytogenetic maps of the human genome [2]. To determine the chromosomal localization of genes several methods are used: analysis of family trees, somatic cell hybridization, fractionation of isolated metaphase chromosomes, and so on [1-3]. However, the only direct approach by which the chromosomal localization of genetic elements can be established is the method of *in situ* hybridization of nucleic acids on chromosomes [10]. By means of this method data have now been obtained on the distribution of different types of repeating DNA sequences in human chromosomes and, in particular, of satellite DNAs I-IV in regions of the juxta-centrometric heterochromatin of chromosomes 1, 9, 13-15, 16, 21, 22, and Y [11], and of 5S ribosomal genes in the distal region of the long arm of the first chromosome [16], 18 and 28S ribosomal genes in regions of secondary constriction bands in the short arms of chromosomes 13-15 and 21-22 [8], and of histone genes in the distal region of the long arm of chromosome 7 [6]. During the last few years the method of *in situ* hybridization has been successfully used to localize unique DNA sequences in chromosomes, such as the insulin gene in the short arm of chromosome 11 [12], the α-globin gene in chromosome 16 [9], and the β-globin gene in the short arm of chromosome 11 [14].

Meanwhile, the use of this approach is made more difficult by a number of as yet unsolved technical problems which hinder its wide use in chromosome mapping studies. The first of these problems is the relatively low efficiency of hybridization of radioactive samples with fixed metaphase chromosomes, due to several factors: loss of up to 80% of DNA in the course of obtaining chromosome preparations for hybridization, incomplete denaturation of chromosomal DNA, incomplete hybridization, insufficiently high specific radioactivity of the DNA samples studied, etc. [1]. Analysis of the end results of hybridization also depends on reliable identification of all chromosomes, which is possible only by simultaneous investigation of the character of distribution of the radioactive label in differentially stained chromosomes.

The object of this investigation was optimization of experimental conditions of hybridization of DNA sequences, cloned in bacterial plasmids, on chromosomes *in situ*, followed by differential staining of the chromosomes.

EXPERIMENTAL METHOD

To obtain chromosome preparations, peripheral blood lymphocytes cultured by the standard method were used. To obtain whole and metaphase chromosomes, 5-fluoro-2-deoxyuridine and uridine in final concentrations of 0.1 and 2.5 μg/ml respectively were added to the culture medium after 48 or 72 h. Thymidine was then added 18 h later to the culture flasks in a final concentration of 2.5 μg/ml to remove the pre-existing block to DNA synthesis. Hypotonic treatment and fixation of the cells were carried out 5-6 h later by the usual methods. Preparations of metaphase chromosomes were kept at 37°C and used in the experiments in the course of 7-10 days. To work out the conditions of hybridization, a total preparation of human

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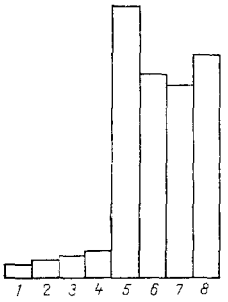

Fig. 1. Efficiency of DNA hybridization on chromosomes *in situ* as a function of experimental conditions. Abscissa, No. of experimental variant; ordinate, mean number of grains of silver (in percent) per metaphase plate. Hybridization efficiency of variant No. 5 conventionally taken to be 100%.

genome DNA, isolated by the method in [4], and individual sequences of human alphoid DNA (clones pHS 05 and pHS 53), cloned in the writers' laboratory [5], were used as radioactive samples. Incorporation of radioactive label ([^{3}H]thymidine triphosphate) into the DNA samples was carried out by means of the DNA-polymerase substitution reaction [15]. Hybridization of the radioactive DNA samples with metaphase chromosomes was carried out in the following experimental versions:

1) by the method described in [10] — chromosome preparations were treated with 0.2 M HCl for 30 min and with 100 μg/ml of RNase in 2SSC (0.3 M NaCl and 0.03 M sodium citrate) for 1 h at 37°C. Denaturation was then carried out in 0.07 M NaOH for 2 min, followed by washing off in alcohols; hybridization was carried out in 2SSC at 65°C for 17 h (the hybridization mixture, in a volume of 25 μl, contained 0.01 μg DNA with specific radioactivity of 15×10^6 cpm/μg), the preparations were washed in two changes of 2SSC at 65°C, after which they were washed again in 2 changes of 2SSC at room temperature and in alcohols;

2) the same as in case 1, but leaving out treatment with HCl and RNase;

3) the same as in case 2, but with reducing the denaturation time in NaOH to 30 sec;

4) by the method described in [7] with certain modifications: Treatment with HCl and RNase was omitted, chromosome denaturation was carried out in 70% formamide, 2SSC at 70°C for 2 min; the preparations were then washed while the temperature was raised to 39°C in two changes of 50% formamide and 2SSC in the course of 30 min, and in two changes of 2SSC at room temperature in the course of 30 min, and finally in alcohols;

5) the same as case 4, but with an increase in the concentration of the DNA sample in the hybridization mixture to 0.1 μg;

6) the same as case 4, but with addition of dextran sulfate-500 to the hybridization mixture in a final concentration of 10%;

7) the same as case 6, but denaturation was carried out in 70% dimethylsulfoxide in 1SSC at 70°C for 2 min;

8) the same as case 6, but denaturation was carried out in 0.07 N NaCl in the course of 30 sec.

The preparations were covered with type M emulsion (Moscow Photographic Plate Factory) and exposed at 4°C for 3-7 days. After development of the autoradiographs and staining of the chromosomes, the number of grains of silver was counted above the metaphase plates (10 cells were analyzed in each version). To detect G- and C-types of segmentation of metaphase chromosomes, the autoradiographs were stained with a 3% solution of Romanovsky—Giemsa stain in 0.02 M phosphate buffer, pH 6.8.

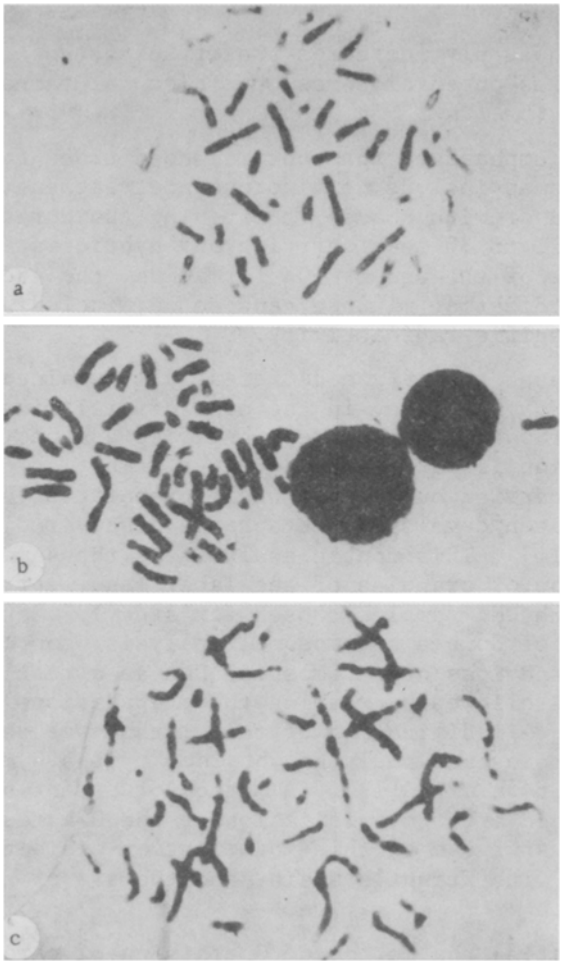

Fig. 2. Metaphase plates (from preparations of cultures of human peripheral blood lymphocytes) with differentially stained chromosomes (C–G segmentation) after hybridization procedure of clone pHS 53 (a, b) and clone pHS 05 (c) *in situ*.

EXPERIMENTAL RESULTS

The main results of analysis of dependence of hybridization efficiency on the experimental conditions are shown in Fig. 1. In this case a total preparation of genome DNA was used as the radioactive sample. Data obtained in version No. 5, which differed from all others by the fact that the DNA content in the radioactive sample was increased by one order of magnitude, were conventionally taken to represent 100% efficiency of hybridization. It will be understood that the practical use of this version was associated with increased utilization of of the test DNA and radioactive precursors. It is more useful to use versions Nos. 6-8 in which, as follows from the graph, the hybridization efficiency approached that in version No. 5, but with a substantially lower DNA concentration in the sample and, on the other hand, it is much higher than the hybridization efficiency in control versions Nos. 1-4, due to the use of the dextran sulfate-500 "amplifier." In addition, the use of formamide, dimethyl sulfoxide, and weak solutions of alkalies (not more than 30 sec) in the principal versions as denaturing agents contributes to better preservation of the morphology of fixed metaphase chromosomes, which is very important for subsequent use of differential staining methods.

It was next decided to study to what degree addition of dextran sulfate-500 to the hybridization mixture as "amplifier" affects the specificity of the hybridization process. For this purpose experiments were carried out using a cloned fragment of the genome (clone pHS 53), characterized by a highly specific location in the juxta-centrometric region of human chromosome 11, as the radioactive sample. Both in the control version and when dextran sulfate-500 was used, the same distribution of label was observed in chromosome 11 (Fig. 2a, b). The

use of dextran sulfate thus not only increases the efficiency of recording of the results of hybridization of DNA samples on chromosomes, but also maintains the specificity of the hybridization process itself *in situ*.

On the whole it must be emphasized that optimal conditions for location of cloned repeating DNA sequences in chromosomes include no vigorous pretreatment of the chromosome preparations (for example, with acid or with RNase), shortening the duration of denaturing treatment of the chromosomes with alkali to 30 sec, carrying out hybridization at a relatively low temperature (to 37°C) on account of the use of 50% formamide, the addition of up to 10% of dextran sulfate-500 to the hybridization mixture, and so on, including thorough washing of the preparations to remove nonspecific radioactivity.

Several approaches are used to analyze differentially stained chromosomes in *in situ* hybridization experiments. For instance, in the case of preliminary staining of chromosomes by the Q method, suitable metaphase plates are identified and photographed on the luminescence microscope before hybridization is carried out. After selection and photographic recording of metaphases hybridization is carried out on the whole preparation and autoradiographed and prepared. After these have been developed, metaphases discovered previously are photographed again in transmitted light [11]. This double analysis of the same metaphase plates ensures reliable regional localization of granules of the label (and, correspondingly, regions of hybridization of granules of the DNA samples chosen for study). Besides the difficulty and laboriousness of this method of double chromosomal analysis, another defect of the method just described is the increased loss of chromosomal DNA as a result of photodegradation, during ultraviolet irradiation, followed by washing the preparations before hybridization of the radioactive DNA samples. A modification of this method was described not long ago, whereby differentially stained chromosomes could be obtained by the Q method after the hybridization procedure had been carried out *in situ* [13]. A method of detection of G-segmentation of chromosomes also has been described after hybridization of the DNA test samples [6, 7]. The essential conditions for successful use of this modification are denaturation of the chromosomes in formamide, staining with Wright's stain, and the use of preparations with long prometaphase chromosomes.

In the present investigation the method of G staining of chromosomes through the photographic emulsion after development of the autoradiographs, by Romanovsky–Giemsa stain, was used. Important conditions when this method is used are use of the chromosome preparations not later than 7 days after they are made, shortening the denaturation time of the chromosomes in 0.07 M alkali to 30 sec, and staining with 3% dye solution for 30-40 min (Fig. 2). The use of formamide and, in particular, of dimethylsulfoxide instead of alkali (as denaturing agents), while preserving the other conditions mentioned above, enables C segmentation of the chromosomes to be revealed also.

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SENSITIVITY OF BALB/c AND WR MICE TO THE IMMUNOSUPPRESSANT ACTION OF CYCLOPHOSPHAMIDE AND THIOPHOSPHAMIDE

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Previously [1] the writers found significant interlinear differences in sensitivity of mice to the immunosuppressant action of cyclophosphamide (CP) and thiophosphamide (thiotepa) *in vivo*. It was shown that the degree of sensitivity of mice of different lines to the immunosuppressant action of the compound is maintained whatever immunosuppressant is used.

The aim of this investigation was to determine how universal this phenomenon really is. The basis for the investigation consisted of previous findings indicating that WR/Y mice are highly sensitive to the mutagenic action of thiotepa [3] but relatively resistant to the mutagenic action of CP [2]. It was interesting to discover whether this rule also applies to the immunosuppressant action of these compounds.

EXPERIMENTAL METHOD

Male BALB/cIYsto and WR/Y mice (black mice) weighing 22-25 g were used. The immunosuppressants used were CP (as the Soviet preparation cyclophosphan) and thiotepa (synthesized at the S. Ordzhonikidze All-Union Pharmaceutical Chemical Institute). Sheep red blood cells (SRBC) were used as antigen. The mice were immunized intravenously with SRBC in a dose of 5×10^8 cells, after which the immunosuppressant was injected intraperitoneally. The interval between injection of antigen and compound was 24 h in the case of CP, but thiotepa was injected immediately after immunization. The number of antibody-forming cells (AFC) in the mouse spleen was determined by Jerne's method [6] 4 days after injection of the antigen. Animals not receiving immunosuppressant treatment served as the control. The results were expressed in percentages of the control and were subjected to statistical analysis by Student's test. Differences were considered to be significant at the $P \leq 0.05$ level.

EXPERIMENTAL RESULTS

Values obtained for relative immunosuppression of WR mice by the use of CP (Table 1) and thiotepa (Table 2) were compared with those obtained for BALB/c mice which, as previous investigations [1] showed, were highly resistant to the immunosuppressant action of these compounds.

It will be clear from Table 1 that the sensitivity of WR and BALB/c mice to the immunosuppressant action of CP was virtually equal, whereas WR mice were significantly more sensitive to the immunosuppressant action of thiotepa than BALB/c mice (Table 2). Interlinear differences were discovered when maximal and intermediate doses of the compound were used.

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