EFFECT OF 5-AZACYTIDINE ON RIBOSOMAL GENE EXPRESSION IN AFRICAN GREEN MONKEY RAMT CELL LINE

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UDC 612.6.05:575.117.2].014.46.08

KEY WORDS: chromosomes; African green monkey cells; rDNA; nucleolar organizing regions; Ag-staining; 5-azacytidine

In the last decade the role of methylation in the regulation of gene expression and in cell differentiation has been actively studied at various levels (molecular, biochemical, cytologic) [5]. Investigations have demonstrated the existence of a mechanism of gene repression through DNA methylation [4, 12]. However, data contradictory to this idea have also been obtained. It has been shown that both transcriptionally active and inactivated genes may have methylated bases in their composition [11, 15].

Ribosomal genes are a very convenient system with which to study the role of methylation in the mechanism of gene repression. First, they are represented in the genome by many copies, clustered in nucleolar-organizing regions (NOR) of particular chromosomes (five pairs of acrocentric chromosomes in man). Second, there exists a method of specific staining of regions of localization of ribosomal genes (Ag-staining), by the use of which it is possible to distinguish transcriptionally active clusters from silent ones at the cytologic level [3]. Finally, it is possible to induce expression of silent genes on the basis of the action of the cytidine analog, 5-azacytidine (5-AC), on DNA; incorporated into DNA in the process of synthesis, this compound cannot be methylated, for it contains a nitrogen atom instead of the carbon atom in position 5 of the pyrimidine ring [9, 10].

The aim of this investigation was to study correlation between incorporation of 5-AC and activation of previously inactivated ribosomal genes, using cytogenic methods.

EXPERIMENTAL METHOD

Experiments were carried out on a transformed African green monkey cell line, RAMT, isolated by V. I. Stobetskii et al. [2]. This line is interesting because it contains two heteromorphic nucleolar-organizing chromosomes, differing from those in the initial cells in the structure and morphology of their NOR [2]. Cells were cultured on Eagle's medium with 10% bovine serum. Immediately after seeding of the cells or at the 24th hour of culture, a freshly prepared solution of 5-AC ("Serva," West Germany) was added to the cultures in a concentration of 2 to 16 μM for the next 17-34 h of culture. The 5-AC either was left until fixation of the cells, or 17 h before fixation the medium was changed for one not containing 5-AC. Cells cultured in the absence of 5-AC constituted the control. The preparations were obtained by the standard method. For analysis, the preparations were stained first with azure-eosin, after which the same preparations were stained with silver nitrate [8]. From 50 to 100 metaphases from each variant of the experiment were analyzed.

EXPERIMENTAL RESULTS

During analysis of the chromosomes, in preparations obtained from the control cultures and those stained with azure-eosin, an extensive secondary constriction was found in one chromosome (structurally reorganized) in 100% of cells, in the middle of the arm. In less than 40% of cells, a poorly defined secondary constriction was found in another chromosome in under 40% of cells (Fig. 1a). This chromosome, in the original cells, was identified as one carrying an actively functioning NOR. On Ag-staining, strong binding of silver was found in

Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bochkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 11, pp. 606-608, November, 1989. Original article submitted April 20, 1988.

0007-4888/89/0011-1631$12.50

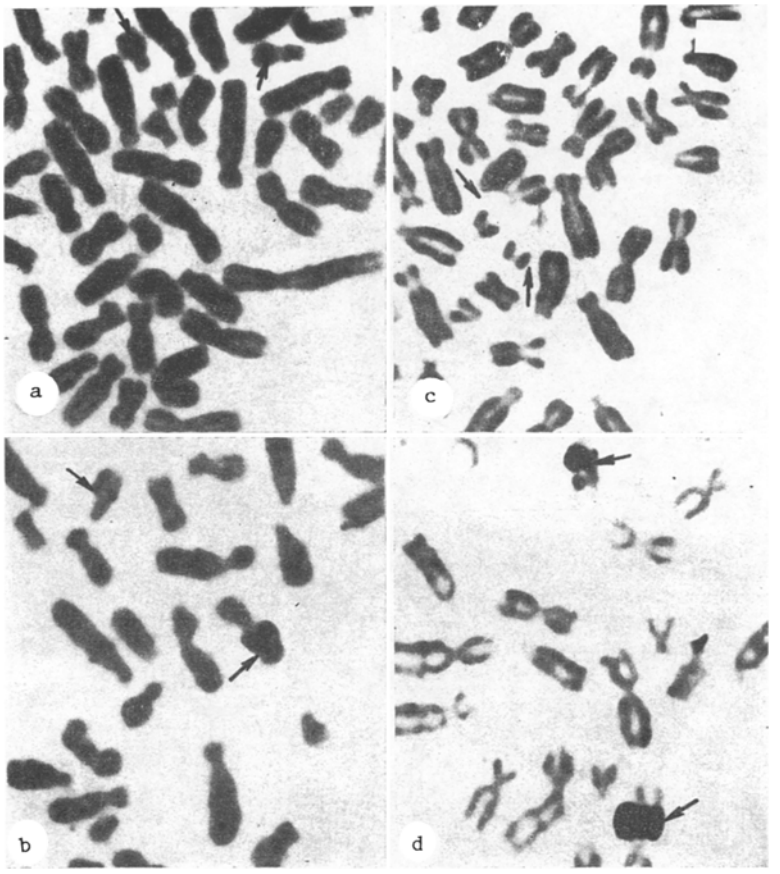

Fig. 1. Fragments of metaphase plates from cultures of African green monkey RAMT cells. a, c) Stained with azure-eosin; b, d) stained with silver nitrate. Arrows indicate nucleolar-organizing chromosomes: bold arrow – structurally modified (M-NOC), thin arrows – structurally normal (N-NOC); a, b) control cultures; c, d) cultures treated with 5-AC.

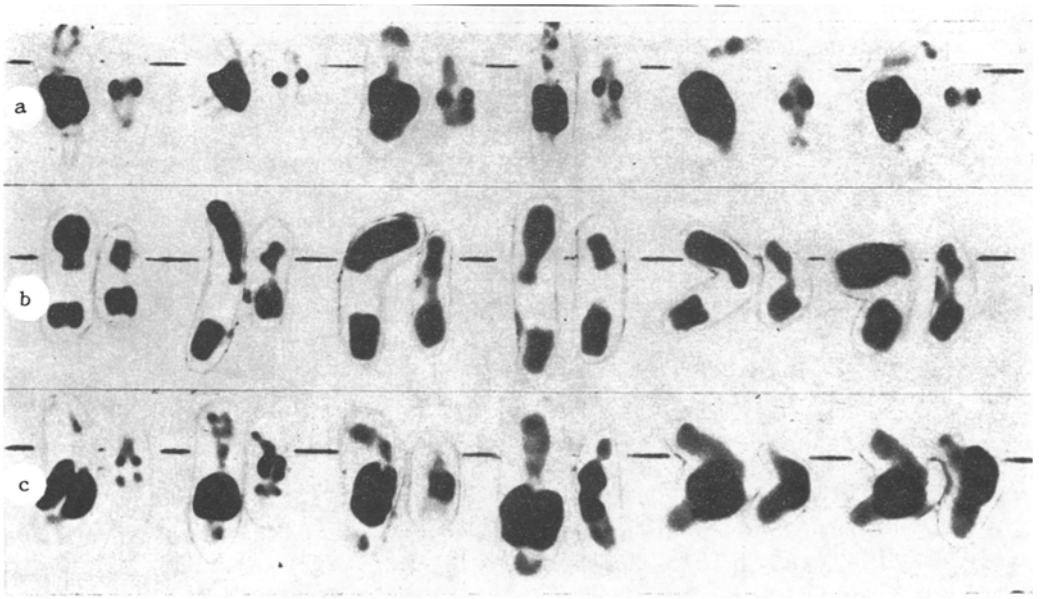

Fig. 2. Analysis of nucleolar-organizing chromosomes from African green monkey RAMT cells. a) Control; b, c) cells cultured in the presence of 5-AC; a-c) Ag-staining, b) staining of chromosomes with azure-eosin.

the region of the extended secondary constriction of the modified nucleolar-organizing chromosome (M-NOC) in all the cells analyzed, and weak binding (only one grain of silver to each chromatid) in the other structurally normal nucleolar-forming chromosome (N-NOC) in fewer than 50% of cells (Figs. 1 and 2). Analysis of preparations obtained from cells cultured in the presence of 5-AC gave the following results. With a concentration of 5-AC of 16 μM, two NO chromosomes could be detected in all cells stained with azure-eosin, easily in all experiments, because of the presence of a secondary constriction in them; in N-NOC in 20-30% of cells, moreover, the extent of the region of secondary constriction was equal to the length of the secondary constriction in M-NOC (Fig. 2b, c). In about 30% of cells, in preparations from a series of experiments in which the medium was changed, interchromatid asymmetry was discovered in the manifestation of the secondary constriction in N-NOC: in one chromatid the constriction was very clearly defined, in the other it was much weaker. A dose-dependent effect of 5-AC on NOR morphology also was discovered. With 5-AC in a concentration of 8 μM the number of cells in which the constriction in N-NOC was well defined reached 50%, with 5-AC in a concentration of 10 μM it reached 80%, and 16 μM – 100%. Ag-staining revealed clear correlation between the extent of the secondary constriction in the chromosomes and the intensity of silver impregnation in these regions (Fig. 2b, c).

Ag-staining was observed in all cells analyzed in both chromosomes; in N-NOC it was always more intense than in the control.

These results are interesting with respect to answering the question of the role of methylation in the regulation of gene expression. Direct correlation was found between incorporation of 5-AC into DNA and intensification of NOR staining in the chromosomes by silver nitrate. On incorporation of 5-AC, N-NOC became Ag-positive in 100% of cells (compared with 50% in the control), and under these circumstances the intensity of Ag-staining increased considerably.

The data on derepression of genes with the aid of 5-AC were obtained previously for viral DNA [4] and genes of eukaryotes, including man [11, 13]. In the investigations cited the effect was assessed biochemically and with the aid of molecular methods. Two other investigations are known in which 5-AC was used to derepress ribosomal genes, but the effect was evaluated at the cytologic level [6, 7]. However, the results obtained in these studies in our view are insufficiently convincing for one very important reason: the authors did not draw up criteria for saturation of NOR staining when using the Ag-method. According to tables presented in [6], in the experimental cultures five or six chromosomes per set were stained with Ag in most cells, and cells with two and eight Ag-stained acrocentric chromosomes were observed also. In investigations conducted in our laboratory on a large number of individuals, and with the intensity of Ag-staining assessed in points (from 0 to 3), the minimal number of Ag-stained chromosomes per cell was found to be seven, and in this case the NOR of these chromosomes from one cell to another had the same intensity of silver staining characteristic of each of the chromosomes [1].

As a result of this investigation data on derepression of ribosomal genes were obtained at the cytologic level during incorporation of 5-AC into DNA. Meanwhile the question of the relative number of copies of genes of rRNA in NOR of N-NOC remains unanswered. We know that 5-AC, incorporated into DNA, leads to despiralization of regions of chromosomes rich in 5-methylcytosine [14]. The answer to the question of the possible correlation between the extent of the secondary constriction induced by 5-AC in NOR and the relative number of copies of rDNA may be obtained by carrying out hybridization in situ, using rRNA probes. Such an investigation is currently in progress. The interchromatid asymmetry in the length of the secondary constriction in N-NOC in some cells which was observed in our experiments can be explained by differences in the quantity of 5-AC incorporated into each chromatid, as a result of which some cells went through two replication cycles in the presence of 5-AC.

The authors are grateful to V. I. Stobetskii for providing the cultures of RAMT cells for the investigation.

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c-src LOCUS DETERMINES INCREASED RATE OF Na^+,K^+-COTRANSPORT AND INCREASED CALCIUM CONTENT IN (SHR × WKY)F_2 HYBRID ERYTHROCYTES

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UDC 612.111.015.31:546.41].08

KEY WORDS: c-src locus; calcium; Na^+, K^+-cotransport

Spontaneously hypertensive Okamoto-Aoki rats, (SHR, Wistar-Kyoto strain) are nowadays used as an experimental model of essential hypertension in man, for which they are the closest prototype [7]. In both forms of pathology characteristic disturbances have been found in the cation-transport function of the plasma membrane in various types of cells (the so-called membrane defect), and the key role of these disturbances in the pathogenesis of these forms of primary hypertension has been demonstrated [5]. Further development of these investigations has shown that a high proportion of these membrane disturbances can be reproduced by activating intracellular protein kinases, and it has been suggested that products of cell oncogenes may participate in the genesis of the membrane disturbances in these forms of hypertension [4, 6]. Recently, by the method of restriction analysis of DNA of SHR rats and of WKY controls to them (normotensive Wistar–Kyoto rats) interlinear polymorphism of the c-src locus was found, characterized by SHR (src^S) and WKY (src^W, HindIII: 3.4 kbp, src^S, 4.1 kbp, src^W; PstI: 4 kbp, src^S, 4.6 kbp, src^W) alleles [1].

In the investigation described below linking of c-src alleles with Na,K-cotransport and the calcium content in erythrocytes (in the presence of the Ca-ATPase inhibitor, Na_3VO_4), was studied for the first time in (SHR × WKY)F_2 hybrids. Previously work wiith such hybrids demonstrated positive correlation of the above-mentioned parameters with the blood pressure [2].

EXPERIMENTAL METHOD

Male second generation hybrids between SHR and WKY, aged 8 weeks, were used. They were obtained by crossing F_1 sibs, progenies of SHR♂ × WKY♀ and SHR♀ × WKY♂ pairs. SHR and WKY rats were obtained in 1973 from the Montreal Institute of Clinical Research, and both lines of rats were maintained under strict genetic supervision in the animal house of the laboratory.

Central Research Laboratory, Ministry of Health of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 11, pp. 608-609, November, 1989. Original article submitted March 6, 1989.

 0007-4888/89/0011-1634$12.50