low Ca^{++} spot roughly covers the area of microvesicles aggregated into the 'Spitzenkörper', which can look bright yellow by light reflection in the centre of the alizarin-stained hyphal tips (figure 2, a).

We have attempted to check the apico-basal correlation of purple alizarin-Ca^{++} concentration along the gradient in vegetative hyphae of the same fungi using a more specific reagent, the metallochrome indicator Arsenazo III (Merck), designed to complex Ca^{++} ions into a blue-violet complex absorbing maximally at 685 nm[17,20]. As with alizarin, increasing gradients of Arsenazo III staining, initiated by a pinkish reaction in the apices, were obtained in all cases (figure 3). Rounded mitochondria were most often more densely stained than their surrounding hyaloplasm, developing only a pinkish-violet hue. This last observation is in line with our previous assumptions of a homeostatic control of calcium concentrations along polarly elongating hyphae[16]. Subapical sequestration of Ca^{++} by respiratorily-active mitochondria could therefore lead to the continuous captation, in the subapical and distal zones of the hyphae, of the Ca^{++} ions pumped from their tips, maintaining the Ca^{++} in such tips at the low concentration compatible with a hyaloplasmic gelation able to prevent the entrance of mitochondria.

1 The support of the 'Fonds National Suisse de la Recherche Scientifique' is acknowledged and we thank Miss E. Herz for her technical assistance.
2 G. Turian, Experientia *34*, 1277 (1978).
3 R. Emerson, Lloydia *4*, 77 (1941).
4 E.C. Cantino, The Fungi II, chapt. 10. Academic Press, New York 1966.
5 G. Turian, Experientia *32*, 989 (1976).
6 E. Racker and R. Wu, Regulation of cell metabolism. Ciba Found. Symp. Churchill, London 1959.
7 G. Charlot and D. Bézier, Méthodes modernes d'analyse quantitative minérale. Masson, Paris 1945.
8 H.J. Vogel, Am. Nat. *98*, 435 (1964).
9 G.M. Roomans and A. Boekestein, Protoplasma *95*, 385 (1978).
10 M. Zalokar, Am. J. Bot. *46*, 555 (1959).
11 M. Girbardt, Protoplasma *67*, 413 (1969).
12 S.N. Grove and C.E. Bracker, J. Bact. *104*, 989 (1970).
13 G. Turian, J. gen. Microbiol. *79*, 347 (1973).
14 N. Howell, C.A. Zuiches and K.D. Munkres, J. Cell Biol. *50*, 721 (1971).
15 J.S. Condeelis and D. Lansing Taylor, J. Cell Biol. *74*, 901 (1977).
16 G. Turian, Archs Sci., Genève *31*, 239 (1978).
17 T.J. Lea, Nature *269*, 108 (1977).
18 C.F. Robinow, J. Cell Biol. *17*, 123 (1963).
19 U.P. Roos and G. Turian, Protoplasma *93*, 231 (1977).
20 E.J. Harris, Nature *274*, 820 (1978).

Heterogeneity of DNA methylation in murine L5178Y lymphoblasts[1]

J. Sawecka, L. Kornacka and J. Malec

Department of Biochemistry, Institute of Haematology, Chocimska 5, PL-00-957 Warsaw (Poland), 27 October 1978

Summary. Comparison of the extent of methylation in mouse DNA fragments rendered $MgCl_2$ soluble after mild DNase II digestion of nuclei, with different reassociation rate and nucleoli-bound, revealed the existence of 3 regions of the genome particularly 5-methylcytosine-rich: the sequences considered to be related to the transcriptionally active chromatin with the highest content of this base and fast reassociating, as well as nucleolar DNA with somewhat lower proportion of the methylated cytosines.

5-methylcytosine (5MC) is known to be the only minor base in eukaryotic DNA. Some observations indicate that its intragenomic distribution is not random but there are regions more and less 5MC-rich[2-4]. The significance of such heterogeneity is, however, not understood. Moreover, the mutual quantitative ratio of the proportion of 5MC in the particular fragments of the genome is difficult to evaluate, as the data reported by various authors were obtained from various kinds of cell, in different fragments of DNA and by different methods of 5MC estimation. To get some further information on the nature of the intragenomic heterogeneity of DNA methylation in the present work, we compared the proportion of 5MC formation in several DNA fragments of mouse leukemic lymphoblasts cultured in vitro. Bearing in mind the presumed role of DNA methylation in the regulation of transcription[5], we concentrated first of all on DNA fractions considered to vary in their function in genetic transcription, i.e. transcriprionally active and inactive chromatin (prepared by the digestion of nuclei by DNase II), sequences differing in the reassociation rate and nucleolar DNA.

Materials and methods. L5178Y murine leukemia cells were grown in Eagle's medium supplemented with L-asparagine, folic acid and 10% calf serum[6]. For fractionation of chromatin by DNase II digestion, the procedure of Gottesfeld et al.[7,8] was followed. DNA was isolated by the procedure described by Butterworth[9]. For reassociation experiments, the resulting DNA was additionally purified by centrifugation for 30 min at 10,000 × g in the presence of acid-washed Norite and the final preparation ($A_{260}/A_{280} > 2.0$) sheared by sonication to the average length of molecules of about 500 nucleotides as determined by agarose gel electrophoresis. The solution of fragmented DNA was dialyzed against 0.01 M sodium phosphate buffer, heat denatured at 100 °C for 10 min, cooled in dry-ice acetone and brought to a suitable phosphate concentration by adding 1.0 M sodium phosphate buffer; reassociation was performed at 65 °C in 0.05 M (fraction reassociated to Cot = 0.01) and 0.12 M phosphate buffer; double stranded DNA fragments were separated from single stranded molecules on hydroxyapatite columns at 65 °C by elution with 0.4 and 0.12 M phosphate buffer, respectively. Following the separation of fraction reassociated to Cot 0.01, the subsequent fractions of decreasing repetitiveness were isolated by subjecting the previous fractions of non-reassociated DNA to another schedule of dialysis, heat denaturation and reassociation, as described by Church[10]. For the isolation of nucleoli, the procedure was based on the method of Muramatsu et al.[11] with some modifications described for the isolation of nucleoli from human leukemic lymphocytes[12]. The proportion of 5MC in DNA was determined on 12 N $HClO_4$ hydrolysates after separation of bases by 2-dimensional paper chromatography on Whatman no 1 paper with solvent systems propan-2-ol-12 M HCl-water (85:22:18) and methanol-12 M HCl-water (91:26:13)[13].

Results and discussion. The experimental design was to submit DNA of L5178Y cells prelabelled with deoxy-[U-C^{14}]-cytidine to 3 fractionation procedures: 1. Limited digestion of chromatin with DNase II. 2. Preparation of fractions with different reassociation rate. 3. Isolation of

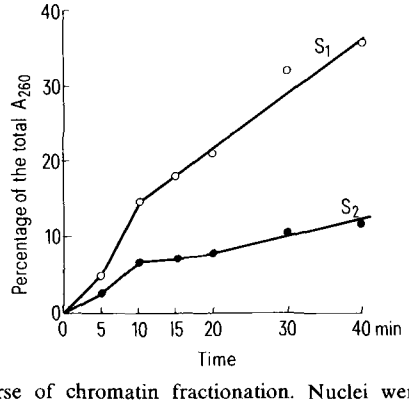
Proportion of 5-methylcytosine in various DNA fragments of L5178Y cells, measured by the extent of deoxy-[U-C^{14}]-cytidine incorporation

Fraction of DNA	% of total DNA	$\frac{5MC}{C+5MC} \times 100$	Relative proportion of 5MC
Whole	-	5.40	1.0
1. Fractions from DNase II digestion			
Mg^{++}-soluble digest (S_2) 15 min		13.56	2.51
Mg^{++}-soluble digest (S_2) 40 min		11.95	2.21
Mg^{++}-insoluble digest 15 min		5.45	1.01
Undigested residue 15 min		4.74	0.87
2. Reassociation fractions			
Cot 0.01 (rechr.)	12.7	9.78	1.81
Cot 0.01-20	10.25	6.15	1.14
Cot > 20	69.10	4.53	0.84
3. Nucleolar DNA	3.10	7.20	1.33

DNA was prelabelled with deoxy-[U-C^{14}]-cytidine by growing the cells in the presence of this precursor for about 2 generations. 1. Gently lyzed nuclei were treated with DNase II and various fractions recovered as described in the figure. 2. Reassociation rate of DNA fragments was assayed by chromatography on hydroxyapatite and expressed as Cot = $\frac{1}{2} \times A_{260} \times$ reassociation time (h). The reproducibility of fractionation procedure as regards the nature of reassociation fractions was monitored by their thermal denaturation profiles, which revealed characteristic features in particular fractions. 3. The purity of the nucleolar preparations was monitored by toluidine-blue staining and RNA/DNA ratio which was about 1.0. All results are mean values from 2 experiments.

nucleoli-bound DNA and comparison of the level of 5MC in those fractions with that in the total DNA of the same cells.

The figure shows the time course of chromatin fractionation by limited digestion of nuclei with DNase II. In this method, the fraction considered to be transcriptionally inactive is separated by centrifugation and precipitation with 2mM $MgCl_2$. The soluble active chromatin fraction has been shown to consist of tissue-specific subsets of middle and non-repetitive DNA sequences[7]. As is shown in the table, DNA fragments so obtained have the highest proportion of 5MC among all DNA fragments separated by us. The 2nd 5MC-rich region of the genome comprises the fast-reassociating sequences and contains twice the level of this base that occurs in slow-reassociating and unique sequences. Some concentration of the methylated cytosines occurs also in the nucleolar DNA. This effect may be due, however, at least partly, to the 3-4-fold enrichment of mouse nucleolar DNA in fast-reassociating sequences when compared with DNA from whole nuclei[14]. Such a pattern of intragenomic distribution of 5MC-rich regions may be similar in other species, as Adams et al.[3] observed the relatively higher content of this base in the first 10% of material rendered acid-soluble by treatment of CHO nuclei with DNase I, and some other authors[2,4] reported the highest content of methylated cytosines in fast-reassociating sequences when compared with moderately and slow-reassociating as well as with unique sequences. The functional significance of such intragenomic distribution of methylated cytosines is difficult to explain, especially because the 5MC-richest regions of the genome seem to comprise both the unique and moderately repeated DNA sequences of active chromatin, postulated to represent transcribed and control regions of the genome[15], and highly repetitious DNA whose function in transcription is still debatable. It seems, however, that the comparison of the intragenomic pattern of DNA methylation in different tissues of the same organism, and different developmental stages of the same kind of cell, may shed some light on this question.



Time course of chromatin fractionation. Nuclei were lyzed by suspension in 0.2 mM EDTA, pH 7, and sodium acetate was added to a final concentration of 25 mM (pH 6.6). After repeated incubation with DNase II (at 5 units/A_{260} unit) in 24 °C, the pH of the samples was raised to 7.5 with 0.1 M Tris-HCl (pH 10). Chromatin was separated into a 1st supernatant (S_1) and pellet by centrifugation. The S_1 was further fractionated by the addition of $MgCl_2$ to 2 mM and the final supernatant of Mg^{++}-soluble chromatin (S_2) was recovered by centrifugation. Aliquots of each fraction were taken for estimation of absorption at 260 nm in 0.1 N NaOH.

1 Acknowledgments. This research was supported by the Polish National Cancer Program PR-6, grant No. 1317/13.

2 G.J. Kirianov, G.A. Romanov and B.F. Vanyushin, Dokl. Akad. Nauk SSSR *219*, 1007 (1974).
3 R.L.P. Adams, E.L.McKay, J.T. Douglas and R.H. Burdon, Nucl. Acid Res. *4*, 3097 (1977).
4 M.J. Browne, A.C.B. Cato and R.H. Burdon, FEBS Letters *91*, 69 (1978).
5 R.L.P. Adams, J. Thurnbull, E. Smillie and R.H. Burdon, in: Postsynthetic modifications of macromolecules, p.39, Akademiai Kaido, Budapest 1975.
6 J. Malec, L. Kornacka and M. Wojnarowska, Arch. Immunol. Ther. Exp. *23*, 693 (1975).
7 J.M. Gottesfeld, G. Bagi, B. Berg and J. Bonner, Biochemistry *15*, 2472 (1976).
8 J.M. Gottesfeld and P.J.G. Butler, Nucl. Acid Res. *4*, 3155 (1977).
9 P.H.W. Butterworth, in: Subnuclear Components, p.295. Ed. G.D. Birnie. Butterworths, London 1976.
10 R.B. Church, in: Molecular Techniques and Approaches in Developmental Biology, p.223. Ed. M.J. Chrispeels. J. Wiley, New York 1973.
11 M. Muramatsu, K. Smetana and H. Busch, Cancer Res. *23*, 510 (1963).
12 J. Malec, M. Wojnarowska and L. Kornacka, Haematologia *5*, 25 (1971).
13 G.R. Wyatt, Biochem. J. *48*, 584 (1951).
14 C.L. Schildkraut and J.J. Maio, Biochim. biophys. Acta *161*, 76 (1968).
15 E.H. Davidson and R.J. Britten, Q. Rev. Biol. *48*, 565 (1973).