In vitro methylation of total and foldback DNAs in normal and virus-transformed cells

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The levels of the in vitro methylation of total and palindromic DNAs in nuclei isolated from normal and virus-transformed cells are compared. The methylation rate of total DNA in normal rat kidney cells is much higher than that detected in normal mouse fibroblasts. However, for both cell species, while the maximal rate of DNA methylation is observed in the mid-logarithmic phase of the cell culture growth, palindromes are always found to be more heavily methylated than total DNA. The 5-methylcytosine content of DNA, especially of palindromes, is higher in virus-transformed cells than in untransformed cells.

DNA methylation *Palindrome* *Cell growth cycle* *Viral transformation*

1. INTRODUCTION

Several years ago we showed that enzymatic modification of eukaryotic DNA depends on its genetic organization. In HeLa cells, in fact, the hybrids of sheared methylated DNA with pre-processed messenger RNAs (purified from nuclei) contained a large amount of 5-methylcytosines, while the hybrids of the same DNA with processed messenger RNAs (purified from polysomes) contained, if any, 5-methylcytosines [1]. In agreement with knowledge of the transcriptional unit and splicing [2,3], such results unequivocally suggested that methylation does not significantly involve the informative DNA, i.e., the structural gene sequences, but preferentially involves the non-informative DNA, i.e., the intervening, intron sequences complementary to those parts of messenger RNA which are removed during processing [1,4]. Then, as expected, the kinetics of reassociation showed that the hypomethylated DNA is mainly made up of unique sequences, while the hypermethylated DNA is made up of repetitive and palindromic sequences [5]. The palindromic sequences reach a specific methylation, during the S-phase, which is much higher than that of the repetitive sequences [5]. However, a decrease of transcription corresponds, in the S-phase, to the hypermethylation of this DNA class and of total DNA [5–7].

This investigation shows increased methylation of total and palindromic DNAs of transformed cells relative to untransformed cells.

2. MATERIALS AND METHODS

Normal rat kidney cells (NRK) and normal mouse fibroblasts (3T3), Rous sarcoma virus (RSV)-transformed NRK cells and Simian virus 40 (SV40)-transformed 3T3 cells were grown in monolayer [8], while nuclei were isolated from them as in [9] and methylated in vitro [6]. These were suspended in 0.003 M Tris–HCl (pH 7.4) and incubated with *S*-[^{3}H]adenosyl-L-methionine ([^{3}H]SAM) for 30 min at 37°C under magnetic

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stirring. DNA was prepared from isolated nuclei as in [9,10].

Each portion of DNA, purified from nuclei of normal and virus-transformed cells, was brought to 2 × SSC and sparged with nitrogen for 10 min before shearing by sonication. After dialysis and deionization on Sephadex G-25, the DNA fragments obtained were estimated to be about 600 nucleotides long by sedimentation in an alkaline sucrose gradient, using DNA length standards [5]. The double helix state of these fragments was checked through T_m analysis. The sheared DNA was thus denatured at 100°C for 15 min, reannealed to a C_0t value of 0.1 in 0.12 M sodium phosphate buffer (pH 6.8), and percolated through a first hydroxyapatite column [11]. The yield of the material retained in 0.12 M buffer was approx. 15%. This partially purified DNA fraction was denatured again for 10 min at 100°C and immediately re-chromatographed on hydroxyapatite. In this case, the C_0t value was estimated to be below 0.001, while rechromatography reduced the yield of DNA to about one third, i.e., to about 5% of the total genome [5,12]. Unlike this fraction, which contained inverted repeats of the type ABC...CBA ($c/c_0 = e^{-kt}$), sedimentation in a CsCl gradient at neutral pH (9–13) suggested that the fraction which did not reassociate in the second step represented ordinary repetitive sequences, including satellites, of the type ABC...ABC...ABC ($c/c_0 = 1/1 + kC_0t$). Digestion of C_0t 0.001 reannealed DNA with *Aspergillus oryzae* nuclease S1 [1,14], subsequent denaturation and reannealing to C_0t 0.001 abolished the ability of this DNA to reassociate at the rate of unimolecular reactions. hence, 5 units enzyme/μg DNA in 10 μl of 0.01 M NaCl/0.3 M sodium acetate/0.003 M $ZnSO_4$ (pH 4.6) were incubated at 37°C for 45 min. The reaction was stopped by addition of 0.4 M sodium phosphate buffer at pH 6.8. After the addition of 50 μg carrier DNA, the sample was precipitated at 0°C with 1.5 M $HClO_4$, run at 10000 × g for 20 min, washed twice with ice-cold 0.5 M $HClO_4$, dissolved in Insta-gel, and counted in a Packard Tri-Carb 460 CD scintillation radiospectrometer.

The total DNAs and the palindromic DNA fractions were hydrolysed to bases and chromatographed to determine their 5-methylcytosine content [6].

Hydroxyapatite was prepared by a minor modification of the method in [15]. Sephadex G-25 was obtained from Pharmacia (Uppsala). RNase (Worthington, Bedford, MA), type II nuclease S1 (Sigma, München) and pronase (Merck, Darmstadt) were of high purity grade. Calf thymus DNA was purchased from Calbiochem-Behring (Lucerne). DNA length standards were obtained from Boehringer (Mannheim). [^{3}H]SAM (80.8 Ci/mmol) was furnished by New England Nuclear (Boston, MA). The SSC solution consisted of 0.15 M sodium citrate at pH 7.0.

3. RESULTS

3.1. *Culture growth cycle of normal and virus-transformed cells*

The NRK cell cultures grow more slowly than do the 3T3 cell cultures (fig.1a,b). The former are characterized by a 'lag' phase which is much longer than that of the latter. The mid-point of the logarithmic phase of growth of the NRK culture is thus retarded for about 10 h compared with that of the 3T3 culture.

After transformation, the lag phase is shortened to about 20 h both for RSV-NRK and SV40-3T3 cultures (fig.1a,b). However, the mid-point of the logarithmic phase of growth of the RSV-NRK cell culture remains retarded when compared with that of the SV40-3T3 cell culture, as it does in the case of NRK cells compared with 3T3 cells.

3.2. *Differential methylation of total DNA in nuclei isolated from normal and virus-transformed cells*

Nuclei from NRK and 3T3 cells and those from the same cells transformed with RSV and SV40, respectively, were methylated at short intervals throughout the culture growth cycle. In agreement with the fact that DNA methylation takes place almost exclusively during the S-phase [4–7], the maximal rate of formation of 5-methylcytosines on total DNA is detected in relation to the mid-point of the logarithmic phase of growth of the cell culture (fig.1c,d) when the mitotic index is the highest [16]. Obviously, the highest number of S-phases corresponds to the highest value of the mitotic index [16]. DNA becomes methylated, meanwhile, only during these S-phases [6]. The timing of methylation thus does not depend upon the cell species or the transforming viral type. Con-

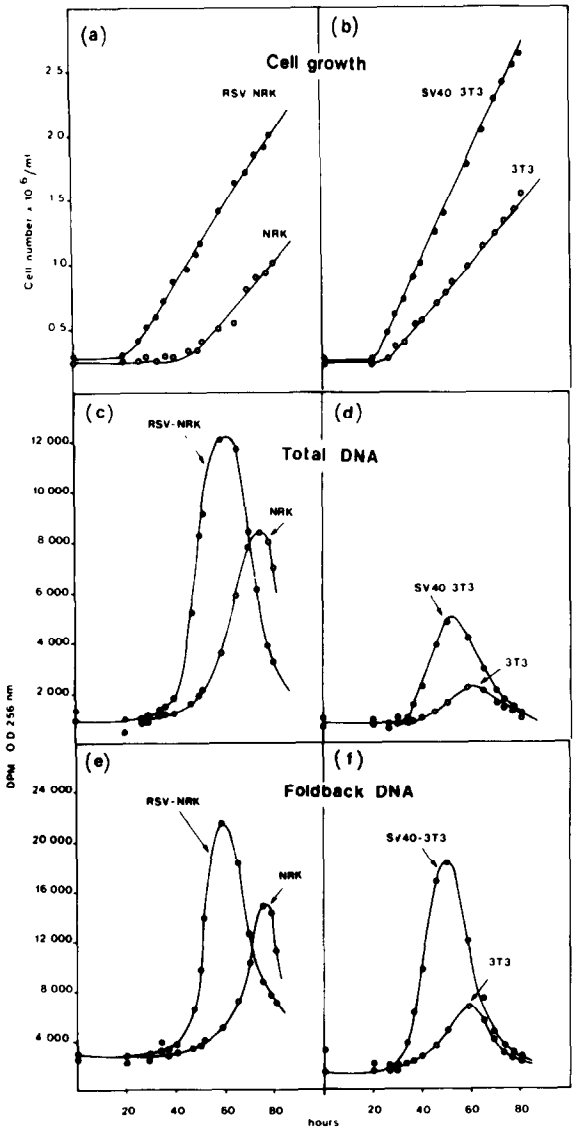

Fig.1. In vitro methylation rate of total and foldback DNAs from normal and virus-transformed cells. These were cultivated in monolayer at 37°C under a continuous flow of 5% CO_2 in air (a,b). At the indicated times during the culture growth cycle, 1×10^8 nuclei from both normal (○—○) and virus-transformed (●—●) cells were suspended in 1 ml Tris buffer and treated for 30 min with 10 μCi [^{3}H]SAM. The purified total (c,d) or foldback (e,f) DNAs were hydrolysed to bases and chromatographed to measure the radioactivity of the 5-methylcytosine spot. The points represent the mean of 3 experiments.

forming with the mid-point of the logarithmic phase of growth of normal and virus-transformed cells (fig.1a,b), there occur large shifts of the timing of the maximal rates of DNA methylation when the cell cultures are compared before and after transformation (fig.1c,d). Starting from the time of the culture inoculum, the maximal rate of DNA methylation coincides with the 75th hour for NRK cells, 60th hour for RSV-NRK cells, 60th hour for 3T3 cells, and 50th hour for SV40-3T3 cells.

There is a striking quantitative difference between DNA methylation in normal NRK and 3T3 cell nuclei. In NRK nuclei (fig.1c), the specific methylation rate of DNA is roughly twice as high as that observed in 3T3 nuclei (fig.1d). However, the methylation rate of total DNA is lower in normal and higher in virus-transformed nuclei. Throughout the cell culture growth cycle, there is at least a 100% increase of this rate for both RSV-transformed NRK (fig.1c) and SV40-transformed 3T3 (fig.1d) cells when compared with the corresponding normal cells. At the 75th hour from the culture inoculum the NRK nuclei show a maximal rate of DNA methylation which corresponds to 8500 dpm/*A*, while at the 60th hour those transformed with RSV show a value of about 12000 dpm/*A* (fig.1c). At the 60th hour from the culture inoculum the 3T3 nuclei show a maximal rate of DNA methylation corresponding to 2250 dpm/*A*, whereas at the 50th hour those transformed with SV40 show a value of 5000 dpm/*A* (fig.1d). Thus, viral transformation leads to a significant increase in the concentration of 5-methylcytosine on host cell DNA. Because the coding DNA is hypomethylated in comparison with the uncoding DNA [1,4], this increase should involve further specific sequences of a regulatory nature. Alternatively, the integrated DNA was shown to be poorly methylated [4].

3.3. *Hypermethylation of palindromes in nuclei isolated from growing normal and virus-transformed cells*

We have shown elsewhere for HeLa cells that palindromes are methylated during the S-phase in parallel with methylation of total DNA, while their methylation pattern is much higher than that of total DNA [5]. This is strikingly confirmed by the present study which involved other cell species.

Always in correspondence to the mid-point of the logarithmic phase of cell culture growth (fig.1a,b), both NRK and 3T3 cell nuclei showed that the maximal rates of methylation of their palindromic fractions (fig.1e,f) are roughly twice as high as those established for the corresponding total DNAs (fig.1c,d). At these cell culture growth stages, while the specific methylation of total DNA in NRK and 3T3 nuclei is 8500 and 2250 dpm/A, respectively (fig.1c,d), that of palindromes in these nuclear species is 15000 and 7000 dpm/A, respectively (fig.1e,f).

In addition, the specific methylation of palindromes, purified from RSV-NRK and SV40-3T3 nuclei, is at least twice as high as that of palindromes purified from normal NRK and 3T3 nuclei. The maximal rates of methylation for NRK and 3T3 nuclei correspond to 15000 and 7000 dpm/A, respectively, while those for RSV-NRK and SV40-3T3 nuclei are 21500 and 18200 dpm/A, respectively (fig.1e,f). These differences are even more significant considering the fact that palindromes show in general a specific methylation pattern which is higher than that of the total DNA [5]. In other words, palindromes of virus-transformed cells are hypermethylated to a greater extent with respect to the total DNA fraction while the rate of formation of 5-methylcytosines on DNA definitely appears to be a function of the culture growth cycle and the cell species. An important fact is that the observed increase of methylation of total DNA and supermethylation of the corresponding palindromic fraction are induced in the same fashion either by RNA (RSV) or DNA (SV40) oncoviruses, although integration occurs via inverse transcription in the first case and directly in the second.

4. DISCUSSION

The first result of this research was expected. Since DNA methylation occurs during the S-phase of the cell cycle [6], we supposed that its highest rate occurs during the logarithmic phase of the culture growth cycle, when the cells proliferate fastest [16]. This was shown to be true for both normal and virus-transformed cells.

The second result was not to be foreseen, since one could not predict whether the DNA of normal cells, with respect to DNA of transformed cells, would be methylated much more or much less. The finding of a higher rate of the host cell DNA methylation following the virus transformation represents a new fact to be elucidated. First of all, this phenomenon could be due to an increased effect of methylases or to a rearrangement of chromatin induced by transformation. Alternatively, in virus-transformed cells, hypermethylation of DNA could be due to a higher metabolic activity in general. Lastly, the main cause of the increased methylation rate of DNA in transformed cells might simply be the increased culture growth rate which characterizes these cells.

Foldback DNA in P 815 mastocytoma cells as well as that of lymphocytes of cow suffering from chronic lympholeucosis are hypermethylated as compared with respective foldback DNA from normal cells [17–19]. In agreement with this, this paper also shows that concomitant hypermethylation of palindromes corresponds to the increase of total DNA methylation following transformation. However, as already mentioned, during the S-phase palindromes were found to be more heavily methylated than total DNA [5]. Therefore, the fact that this DNA class in virus-transformed cells is even more methylated than in untransformed cells suggests that the increment of 5-methylcytosine on transformed DNA should not be exclusively explained as a result of an increased rate of cell growth. More likely, the additional methylation of palindromes might also be caused by the products expressed by the integrated oncogenes to change regulation of gene expression in transformed cells. In relation to this, it was suggested that palindromes might represent acceptor sites which could interact with regulatory proteins [20]. In fact, most of the binding sites for the regulatory proteins and restriction enzymes show a two-fold axis of rotational asymmetry, which is also a feature of palindromes [21,22]. From this point of view, hypermethylated palindromes might interfere with the binding of RNA-polymerase either at the level of the same promoter or at the level of the intron sequences.

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