Genomic sequencing reveals absence of DNA methylation in the major late promoter of adenovirus type 2 DNA in the virion and in productively infected cells

Christina Kämmer, Walter Doerfler*

Institut für Genetik, Universität zu Köln, Weyertal 121, D-50931 Köln, Germany

Received 7 February 1995

Abstract By using methylation-sensitive restriction endonucleases, we have previously provided evidence that adenovirus type 2 (Ad2) virion DNA or free intranuclear Ad2 DNA in productively infected hamster or human cells is not methylated. We have now chosen a different experimental approach and have investigated the major late promoter (MLP) sequence of Ad2 DNA for the presence of 5-methyldeoxycytidine (5-mC) residues with the genomic sequencing technique. This study has been prompted by the finding that the MLP of Ad2 DNA can be inactivated by sequence-specific methylation in experiments in which a MLP–chloramphenicol acetyltransferase construct has been transcribed in a cell-free system from HeLa cell nuclear extracts. Virion Ad2 DNA and Ad2 DNA isolated from productively infected human or hamster cells between 1 and 48 h post-infection (p.i.) have now been analyzed. There is no evidence for the presence of 5-mC in the cytidine positions in the MLP of any of these Ad2 preparations. We conclude that DNA methylation does not seem to play a role in the early–late control of this viral promoter. The sensitivity of the genomic sequencing technique does not permit us to exclude the unlikely presence of 5-mC in a few Ad2 DNA molecules.

Key words: Adenovirus–human cell system; Absence of promoter methylation; Late viral transcription; Promoter control

1. Introduction

The fifth nucleotide in DNA, 5-methyldeoxycytidine (5-mC) serves as a long-term genetic signal for permanent promoter inactivation in eukaryotic cells [1,2]. The inter-relationship between long-standing gene inactivation and specific patterns of DNA methylation has been recognized in integrated adenovirus genomes in mammalian cells [3–5]. Early in our investigations on the state of DNA methylation in integrated adenovirus genomes, the question arose as to whether specific parts of the free, non-integrated viral genome could be methylated in the virion DNA and could subsequently become demethylated in the course of the infection cycle. Could the switch in transcriptional activity between early and late viral genes thus be effected? Could methylation also play a role in the inactivation of the late genes of the adenovirus type 12 (Ad12) genome in abortively infected hamster cells? By extensive analyses with the aid of methylation-sensitive restriction endonucleases on non-integrated adenovirus genomes in the nuclei of productively or abortively infected cells, we have not obtained any evidence for the presence of 5-mC residues in free adenoviral genomes in abortively or productively infected cells [6,7].

In cell-free transcription experiments, we have demonstrated that the in vitro methylation of specific 5′-CCGG-3′ and 5′-GCGC-3′ sequences in the major late promoter (MLP) of adenovirus type 2 (Ad2) DNA can lead to its transcriptional inactivation [8]. It was, therefore, conceivable that, in Ad2 virion DNA and/or in intracellular Ad2 DNA early in productive infection, one or a few 5-mC residues in sites not recognized by restriction enzymes in the MLP sequence of Ad2 DNA might contribute to the inactivity of this promoter early in infection. We have investigated the MLP sequence of Ad2 DNA in the virion both early (starting 1 h p.i.) or late (up to 48 h p.i.) in productively Ad2-infected human KB or hamster BHK21 cells for the presence of 5-mC by the genomic sequencing method. This technique permits the analysis of all deoxycytidine (C) residues in a sequence for the presence of 5-mC [9,10]. In this study, 5-mC residues have not been found at any time in the MLP sequence of Ad2 DNA. We conclude that DNA methylation is not found in non-integrated adenoviral genomes after the productive infection of cells and hence most likely does not play a role in the regulation of early vs. late promoter activity in Ad2 genomes.

2. Materials and methods

Human KB or hamster BHK21 cells growing in monolayer cultures were inoculated with twice CsCl-purified Ad2 at multiplicities of infection of about 10 or 100 plaque forming units (PFU) per cell, respectively. At different time intervals post-infection (addition of inoculum = 0 h), i.e. at 1, 2, 3, 5, 7, 8, 10, 20 h p.i. of KB cells or 6, 10, 30, 48 h p.i. of BHK21 cells, the total intranuclear DNA was isolated by standard procedures [11]. As control DNA, the DNA from purified Ad2 virions or from uninfected KB or BHK21 cells was also prepared. DNA preparations were precleaved with *Hin*dIII at 37°C overnight; the DNA was re-extracted with phenol/chloroform and subsequently ethanol precipitated. The DNA was then treated with 100% hydrazine at 20°C for 10 min for the cytidine (C) reaction. Nucleotide-specific cleavage was completed by incubation in 1 M piperidine at 87°C for 30 min [12]. The DNA was then lyophilized and vacuum-dried three times from aqueous solution. DNA fragments were resolved by electrophoresis at 1200 V, 26 mA, overnight on a sequencing gel (80 × 20 × 0.5 cm) in 6% polyacrylamide (acrylamide:bisacrylamide = 39:1), 8.4% urea, 0.5 × TEB (TEB is 89 mM Tris, 89 mM borate, 2 mM EDTA) and then electrotransferred (15 V for 30 min; 30 V for 30 min) to a nitrocellulose membrane (Gene-Screen from Dupont). The DNA was crosslinked to the matrix by UV irradiation at a lamp distance of 20 cm for 3 min.

*Corresponding author. Fax: (49) (221) 470 5163.

SSDI 0014-5793(95)00248-0

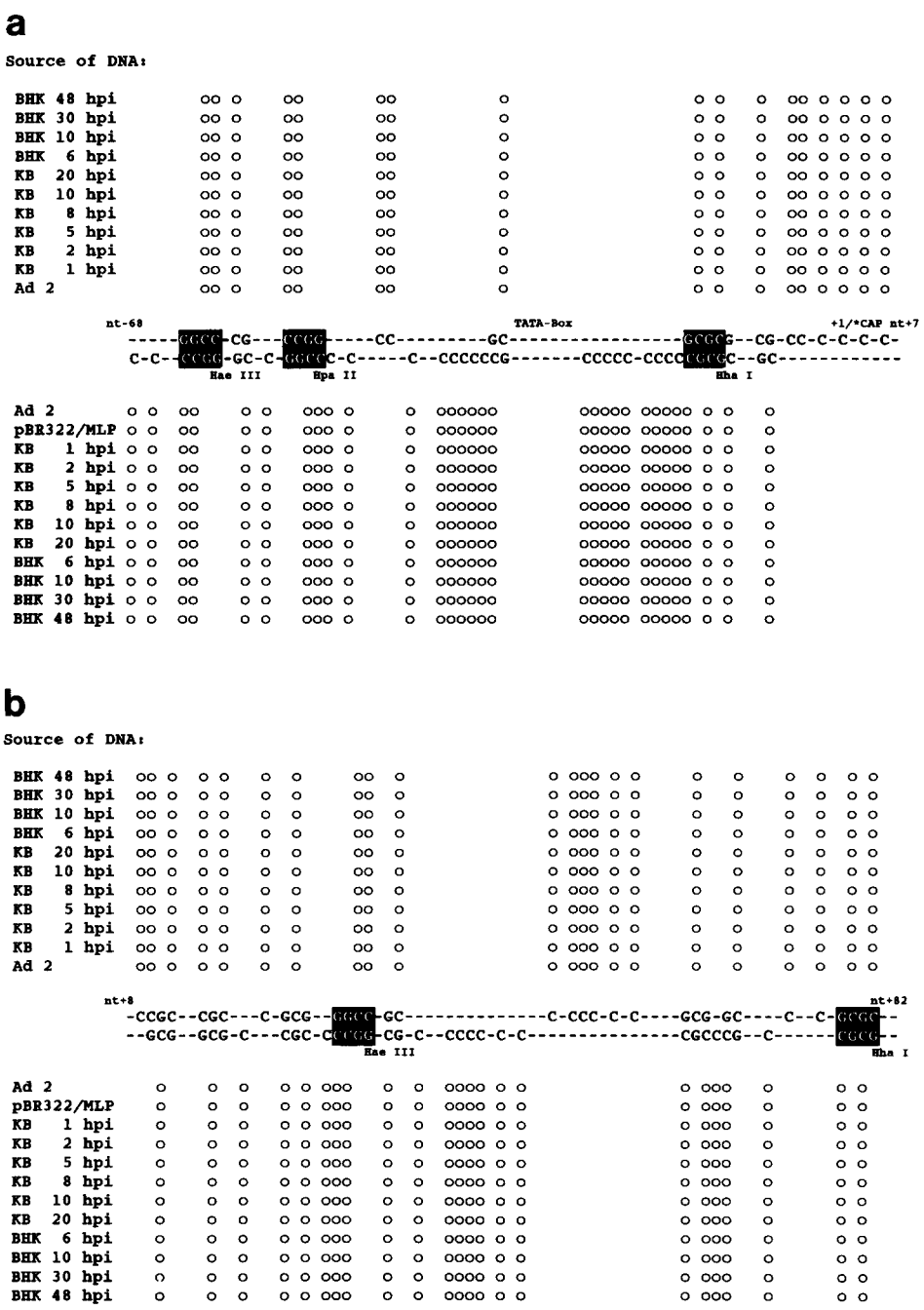
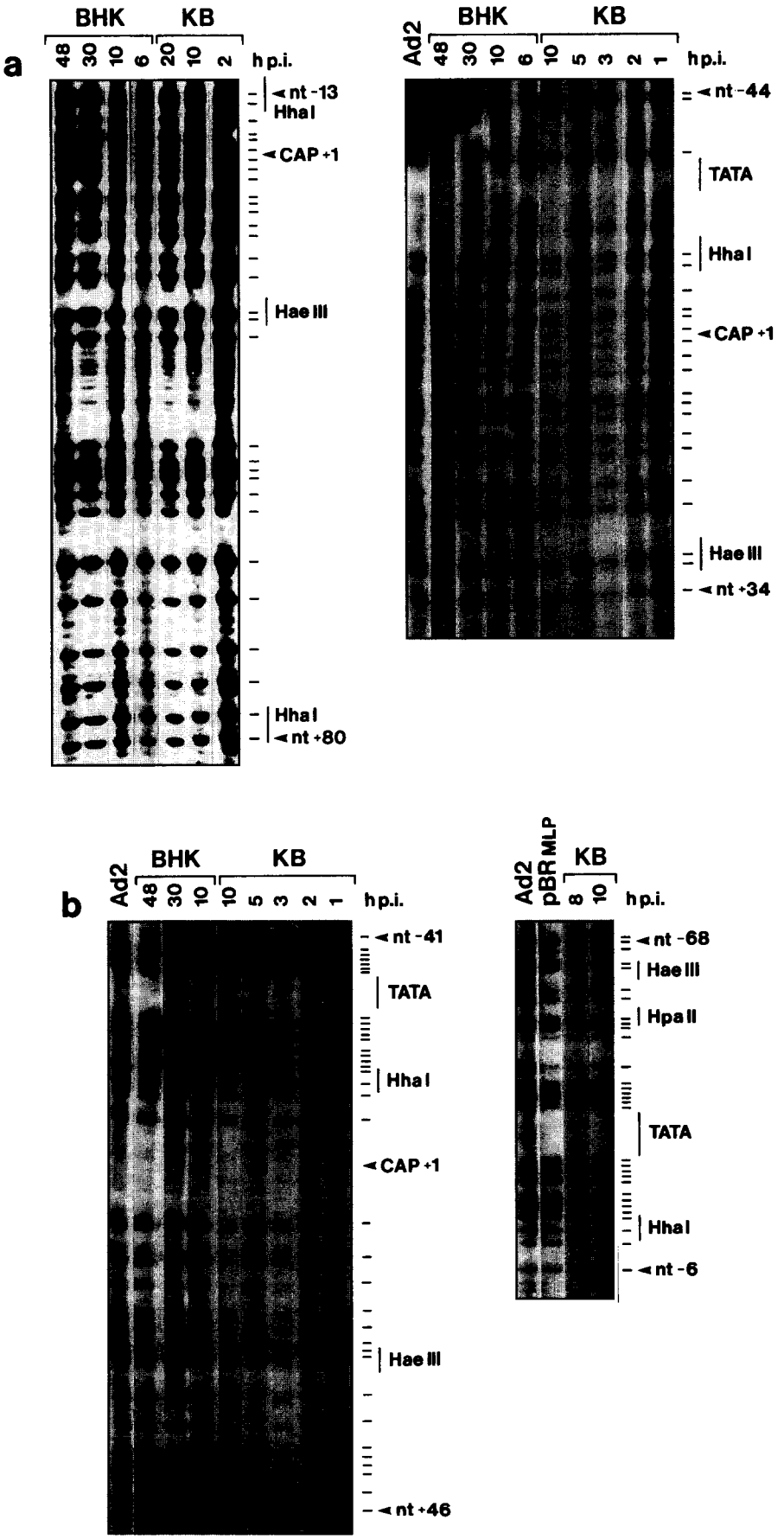


Fig. 1. The −68 to +82 MLP promoter sequence of Ad2 DNA is not methylated in virion DNA or in viral DNA isolated early or late from productively infected KB or BHK21 cells. Details of the schematic presentation have been described in the text. The sequences in parts (a) and (b) are left-to-right continuous.

To prepare the hybridization probe, the 5780–6230 MLP nucleotide sequence of Ad2 DNA [13] was first cloned into the M13mp18 or the M13mp19 DNA vector. Single-stranded probes were then synthesized by annealing 20 nucleotide primers to the M13 DNA sequence and extending the chain by using Klenow DNA polymerase [14] and deoxyribonucleoside triphosphates including ^{32}P-labeled C (500 μCi; 6000 Ci/mM). The synthesized double strands were cleaved by *Dde*I (M13mp18) or *Hin*dIII (M13mp19) to generate fragments of about 100

→

Fig. 2. Autoradiograms of genomic sequencing gels following hydrazine reactions of Ad2 DNA samples as indicated. These are exemplary samples of numerous experiments performed. The positions of C residues, as marked by horizontal lines, are known from the published nucleotide sequence of Ad2 DNA [13], and sequence landmarks (CAP site, TATA box, *Hpa*II, *Hha*I, and *Hae*III sites) are indicated as well as the nucleotide numbers in the upstream (−) or downstream (+) part of the promoter. This numbering system is identical to the one used in Fig. 1. The Ad2 MLP promoter cloned in the vector M13mp18 (a) or the same promoter cloned in the vector M13mp19 (b) was used as the hybridization probe. Thus, promoter sequences in the rightward-transcribed (a) or the leftward-transcribed DNA complement (b), respectively, are visualized on the autoradiogram of the genomic sequencing gel.


a
BHK
KB
48
30
10
6
20
10
2
h.p.i.
◄nt -13
HhaI
◄CAP +1
HaeIII
HhaI
◄nt +80
Ad2
BHK
KB
48
30
10
6
10
5
3
2
1
h.p.i.
◄nt -44
TATA
HhaI
◄CAP +1
HaeIII
◄nt +34
b
Ad2
BHK
KB
48
30
10
10
5
3
2
1
h.p.i.
◄nt -41
TATA
HhaI
◄CAP +1
HaeIII
◄nt +46
Ad2
pBR MLP
KB
8
10
h.p.i.
◄nt -68
HaeIII
HpaII
TATA
HhaI
◄nt -6

nucleotide pairs which were purified by electrophoresis on a denaturing polyacrylamide gel. The single-stranded, labeled probes were eluted and used for DNA–DNA hybridization.

Filters were prehybridized for 1 h at 68°C and hybridized with the ^{32}P-labeled probe in a rotating glass tube overnight at 68°C in 10 ml of 0.25 M Na_2HPO_4, 1 mM EDTA, 0.25 NaCl, 7% SDS, and 10% polyethylene glycol (PEG 8000). Subsequently, the filters were washed at 68°C once for 5–10 min in 500 ml of 100 mM Na_2HPO_4, 5% SDS, 5 mM EDTA, and six times at 68°C for 5–10 min in 500 ml each of 50 mM Na_2HPO_4, 1% SDS, 5 mM EDTA. The filters were dried and autoradiographed at −80°C on Kodak XAR film.

3. Results and discussion

The schemes in Fig. 1a,b show the nucleotide sequence of the MLP of Ad2 DNA between nucleotide positions −68 and +82, relative to the nucleotide of transcriptional initiation at +1, the CAP site. Only the C and some of the G residues are indicated, the A and T residues are replaced by dashes (−) for improved graphical presentation. In addition to the CAP site, the TATA (Goldberg–Hogness) box and several restriction endonuclease sites are also shown, including the *Hha*I and *Hpa*II sites the in vitro methylation of which leads to promoter inactivation [8]. *Hae*III sites are also designated.

In genomic sequencing gels, the presence of a C-band after reacted the DNA had with hydrazine signifies that the DNA is not methylated in this nucleotide. Replacement of a C by a 5-mC residue would lead to the loss of the C-band in the autoradiogram, since 5-mC residues do not react with hydrazine [15]. The scheme in Fig. 1 summarizes the results of some 15–20 independent genomic sequencing experiments performed for each of the DNA types listed. An open circle stands for absence of methylation, i.e. for the presence of a C-band in the corresponding sequence position on autoradiograms of genomic sequencing gels (see, Fig. 2a,b). In addition to Ad2 virion DNA and Ad2 DNA extracted from Ad2-infected KB cells between 1 and 20 h p.i. or from Ad2-infected BHK21 cells between 6 and 48 h p.i., the pBR322 DNA-cloned 5780–6230 nucleotide fragment of Ad2 DNA propagated in an *E. coli* host has also been analyzed as a positive control devoid of 5′-CG-3′ methylation. In all experiments and with all DNA and control samples, C residues in both Ad2 complements have been visualized by hybridization to the M13mp18 or the M13mp19 probe.

In the promoter sequence analyzed, all expected C residues, known from the total nucleotide sequence of Ad2 DNA [13], are represented as bands on the autoradiograms of genomic sequencing gels (○). Thus, none of the C residues in any dinucleotide combination, in particular not in the 5′-CG-3′ dinucleotides, the main known target for the mammalian host cell's DNA methyltransferase system, is methylated in any of the DNA preparations investigated. We can not rule out the possibility that a small proportion of Ad2 DNA molecules carry an occasional 5-mC since a full C band and one that is slightly weaker cannot be distinguished by this method with certainty.

Exemplary autoradiograms of sequencing gels visualizing the C residues on the rightward-transcribed (Fig. 2a) or on the leftward-transcribed strand (Fig. 2b) are also presented. Based on the published sequence of Ad2 DNA in the MLP [13], all C residues yield signals (designated by a horizontal line) on this autoradiogram and in at least 15–20 similar experiments in which this Ad2 DNA segment was investigated for the presence of 5-mC residues by the genomic sequencing method. The presence of all expected C bands in the virion DNA and in the intracellular Ad2 DNA from human or hamster cells at any time after infection demonstrates that the MLP sequence of Ad2 DNA is not methylated in any of the DNA preparations investigated.

We, therefore, conclude that the MLP of Ad2 DNA is devoid of 5-mC both in virion DNA and in the intracellular free Ad2 DNA, both early and late after the productive infection of human KB or of hamster BHK21 cells with Ad2. DNA methylation thus does not play a role in the regulation of early vs. late gene expression of Ad2 DNA. The sensitivity of the method does not allow us to rule out the unlikely possibility that a subset of the molecules might carry isolated 5-mC residues.

We have tried to assess the capacity of the genomic sequencing and the restriction-Southern blot hybridization methods to detect small amounts of 5-mC. In reconstitution experiments, unmethylated preparations of the pBR322 plasmid containing the cloned 5780–6230 nucleotide fragment of Ad2 DNA have been mixed with increasing amounts of the same construct in the 5′-CG-3′ DNA methyltransferase-methylated form or vice versa. These mixtures were analyzed by the genomic sequencing method or by restriction enzyme analyses (employing *Msp*I and *Hpa*II), respectively. The results (not shown) demonstrate that with the genomic sequencing technique about 10% of unmethylated sequences can be recognized with some difficulty in a background of 90% methylated sequences. On Southern blots upon restriction and hybridization to a ^{32}P-labeled probe, 5% of methylated DNA are still clearly visible in a background of 95% unmethylated DNA. Thus, both methods have high but nevertheless limited levels of resolution and detectability for methylated sequences.

We conclude that restriction analyses with methylation-sensitive enzymes followed by Southern blot hybridization is the more sensitive method to trace small amounts of methylated DNA molecules. The genomic sequencing method, though somewhat less sensitive, detects, however, 5-mC in nucleotide sequence positions which are not recognized by any of the methylation- sensitive enzymes. Although it is unlikely that a small percentage of the intracellular Ad2 DNA molecules are methylated, this possibility can not be rigourously ruled out with any of these even very sensitive methods.

Acknowledgements: C.K. is indebted to Stefan Kochanek for introducing her to the technique of genomic sequencing. We thank Petra Böhm for expert editorial work. This research was supported by the Deutsche Forschungsgemeinschaft through SFB274-A1 and by a grant through Schwerpunkt 'Virulenzfaktoren und Wirtstropismus animaler Viren'.

References

[1] Doerfler, W. (1983) Annu. Rev. Biochem. 52, 93–124.
[2] Doerfler, W. (1993) Prog. Nucleic Acid Res. Mol. Biol. 46, 1–36.
[3] Doerfler, W. (1989) Nucleic Acids Mol. Biol. 3, 92–119.
[4] Sutter, S. and Doerfler, W. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, 565–568.
[5] Sutter, D. and Doerfler, W. (1980) Proc. Natl. Acad. Sci. USA 77, 253–256.

[6] Vardimon, L., Neumann, R., Kuhlmann, I., Sutter, D. and Doerfler, W. (1980) Nucleic Acids Res. 8, 2461–2473.
[7] Wienhues, M. and Doerfler, W. (1985) J. Virol. 56, 320–324.
[8] Dobrzanski, P., Hoeveler, A. and Doerfler, W. (1988) J. Virol. 62, 3941–3946.
[9] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991–1995.
[10] Saluz, H.P. and Jost, J.P. (1987) A Laboratory Guide to Genomic Sequencing, Birkhäuser, Basel.
[11] Stabel, S., Doerfler, W. and Friis, R.R. (1980) J. Virol. 36, 22–40.
[12] Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560–564.
[13] Roberts, R.J., Akusjärvi, G., Aleström, P., Gelinas, R.E., Gingeras, T.R., Sciaky, D. and Pettersson, U. (1986) Dev. Mol. Virol. 8, 1–51.
[14] Klenow, H., Overgaard-Hansen, K. and Patkar, S.A. (1971) Eur. J. Biochem. 22, 371–381.
[15] Ohmori, H., Tomizawa, J.I. and Maxam, A.M. (1978) Nucleic Acids Res. 5, 1479–1485.