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Spreading of DNA Methylation across Integrated Foreign (Adenovirus Type 12) Genomes in Mammalian Cells

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The establishment of de novo-generated patterns of DNA methylation is characterized by the gradual spreading of DNA methylation (I. Kuhlmann and W. Doerfler, J. Virol. 47:631–636, 1983; M. Toth, U. Lichtenberg, and W. Doerfler, Proc. Natl. Acad. Sci. USA 86:3728–3732, 1989; M. Toth, U. Müller, and W. Doerfler, J. Mol. Biol. 214:673–683, 1990). We have used integrated adenovirus type 12 (Ad12) genomes in hamster tumor cells as a model system to study the mechanism of de novo DNA methylation. Ad12 induces tumors in neonate hamsters, and the viral DNA is integrated into the hamster genome, usually nearly intact and in an orientation that is colinear with that of the virion genome. The integrated Ad12 DNA in the tumor cells is weakly methylated at the 5′-CCGG-3′ sequences. These sequences appear to be a reliable indicator for the state of methylation in mammalian DNA. Upon explantation of the tumor cells into culture medium, DNA methylation at 5′-CCGG-3′ sequences gradually spreads across the integrated viral genomes with increasing passage numbers of cells in culture. Methylation is reproducibly initiated in the region between 30 and 75 map units on the integrated viral genome and progresses from there in either direction on the genome. Eventually, the genome is strongly methylated, except for the terminal 2 to 5% on either end, which remains hypomethylated. Similar observations have been made with tumor cell lines with different sites of Ad12 DNA integration. In contrast, the levels of DNA methylation do not seem to change after tumor cell explantation in several segments of hamster cell DNA of the unique or repetitive type. Restriction (*Hpa*II) and Southern blot experiments were performed with selected cloned hamster cellular DNA probes. The data suggest that in the integrated foreign DNA, there exist nucleotide sequences or structures or chromatin arrangements that can be preferentially recognized by the system responsible for de novo DNA methylation in mammalian cells.

Foreign genes introduced into cells by viral infection or by DNA transfection and integrated into the genomes of eukaryotic (mammalian) cells are often inactivated, presumably because of the de novo methylation of the inserted DNA. The mechanism of de novo methylation is not yet understood. It has sometimes been speculated that the cellular site of foreign DNA insertion has an influence on the rate and pattern of de novo methylation. Apparently, chromosomal association is one of the preconditions for foreign (adenovirus) DNA to be accessible to the DNA methyltransferase systems of the cell.

Adenovirus type 12 (Ad12) DNA, which is unmethylated in virions (9), is methylated de novo in distinct patterns at the 5′-CCGG-3′ sequences (25–27) upon integration into cellular DNA. These patterns do not seem to arise instantaneously upon viral DNA insertion but evolve in the course of many cell generations in culture (16). In one instance, cellular DNA sequences abutting a recently integrated Ad12 genome have become undermethylated in comparison with the allelic, nonoccupied sequence, which remained strongly methylated (18). In contrast, in the vicinity of an integrated retrovirus genome the adjacent cellular DNA has been found to be hypermethylated (10). Obviously, we do not yet understand the rules which govern the de novo methylation of integrated foreign DNA and changes in the status of methylation of the neighboring cellular DNA sequences.

Integrated adenovirus genomes facilitate investigations of the spreading of DNA methylation over an entire adenovirus genome of some 30 kbp. Methylation of the colinearly integrated Ad12 genome in hamster cells is initiated between 30 and 75 map units on the viral DNA and gradually spreads from there over the entire genome. The terminal viral and adjacent cellular sequences do not seem to be methylated. No concomitant change in methylation in a few selected hamster cellular DNA sequences has been observed.

MATERIALS AND METHODS

Induction of tumors in newborn hamsters by Ad12: tumor cell lines. Within 24 h of birth, hamsters (*Mesocricetus aureatus*) were injected with 1.5×10^7 PFU of Ad12 (1 U of optical density at 260 nm = 10^{10} PFU) which had been purified by three cycles of equilibrium sedimentation in CsCl density gradients. Tumors developed about 30 days after Ad12 injection. The animals were sacrificed 48 days after injection, and the tumor tissue was prepared. Part of the tumor tissue (about one-third) was rapidly frozen at −196°C and stored at −80°C for DNA preparation. From the bulk of the tumor mass (two-thirds), cell cultures were established either in Dulbecco medium containing 10% fetal calf serum or in conditioned medium containing 5% fresh serum. Conditioned medium consisted of 50% fresh Dulbecco medium and 50% medium in which BHK21 cells had been propagated to one-half or two-thirds confluency. The tumor cell lines were passaged for up to 5 months. Confluent monolayers were subdivided by briefly treating the cells with 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE) and dispersing them mechanically.

Preparation of DNA from tumor tissue or from cell lines. Tumor tissue was pulverized in a mortar in liquid N_2 at −196°C (1). The total DNA was purified by overnight

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TABLE 1. Induction of tumors in neonate hamsters by Ad12[a]

Hamster	Sex[b]	Injected material (amt)	No. of days from injection to:		Tumor diam (cm)	Tumor designation	Cell line designation	No. of Ad12 genome equivalents/cell
			Appearance of tumor	Extirpation				
1	F	Ad12 (1.5×10^7 PFU)	39	41	0.6	T201/2	H201/2	10
						T201/3	H201/3	3
2	F	Ad12 (1.5×10^7 PFU)	35	41	0.5	T211	H211	5
5	M	Ad12 (1.5×10^7 PFU)	32	40	1.0	T191	H191	3
6	M	Ad12 (1.5×10^7 PFU)	39	48	0.2	T271	H271	3–5
7	M	Ad12 (1.5×10^7 PFU)	41	48	0.1	T281	H281	5
8	M	T 24-2 Cl 2 cells (3.0×10^6)		63	2.4	T5	HT5	10

[a] Ad12 was injected subcutaneously into seven neonate Syrian hamsters. Five animals developed tumors at the site of injection. One tumor (T5) was induced by injection of an Ad12-induced hamster tumor cell line (15) which had been passaged once in a hamster.
[b] F, female; M, male.

incubation at 37°C in 10 mM Tris-HCl (pH 8.0)–25 mM EDTA–25 mM NaCl–0.5% sodium dodecyl sulfate (SDS)–1 mg of proteinase K per ml, several extractions in Tris-saturated phenol and in a 1:1 mixture of chloroform and phenol, and finally dialysis against TE.

Ad12-transformed hamster tumor cells growing in culture were frozen at −80°C in TE. The DNA was extracted from these cells by adjusting the solution to 1% SDS and 500 μg of proteinase K per ml and by incubation at 37°C for 4 h followed by phenol-chloroform treatment.

Restriction analysis, electrophoresis, and alkali transfer of cellular DNA. Cellular DNA was cut with restriction enzymes as indicated, and the fragments were separated by electrophoresis in agarose gels in 100 mM Tris-HCl (pH 8.3)–70 mM boric acid–25 mM EDTA. After electrophoresis, the DNA fragments were denatured for 2 to 4 h in 0.5 M NaOH–1.5 M NaCl and transferred in 0.25 M NaOH–1.5 M NaCl to nylon membranes.

Other methods. All other methods used in this study have been described previously. Ad12 DNA and its cloned fragments were prepared as described elsewhere (13). DNA was ^{32}P labeled by random priming using oligodeoxyribonucleotides (6). DNA-DNA hybridizations were performed in 0.3 M NaCl–0.03 M Na citrate–1% SDS–0.5% milk powder (Glücksklee, Hamburg, Germany)–10% dextran sulfate–300 μg of salmon sperm DNA per ml at 68°C for 16 to 20 h. The probe and carrier DNAs were denatured by boiling for 10 min. At the end of the hybridization reactions, the filters were washed for a total of 4 to 5 h at 68 to 70°C, successively in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na citrate), 2× SSC–0.1% SDS, and 0.1× SSC–1% SDS. The ^{32}P-labeled probe could be removed from the nylon (Hybond) membranes by boiling the membranes for 60 to 90 min in 2× SSC–0.1% SDS.

RESULTS

Design of experiments. Some of the characteristics of the Ad12-induced hamster tumors and the derived cell lines are reviewed in Table 1. The cell lines were termed H201/2, H201/3, H211, H191, H271, H281, and HT5; the estimated amounts of integrated Ad12 DNA that they carried are given in Table 1. The copy number of integrated Ad12 genomes that persisted was determined by the evaluation of band intensities on autoradiograms of Southern blots as described earlier (24). The total intracellular DNA was prepared from the tumor cells directly or from the established cell lines at different passage levels. The DNA was subsequently cleaved with *Hpa*II or *Msp*I (32) to assess the status of DNA methylation. In the Ad12-induced tumor cell lines studied in these experiments, patterns of DNA methylation were assessed by probing *Hpa*II- or *Msp*I-cleaved cellular DNAs with the ^{32}P-labeled Ad12 genome or with specific cloned segments of this viral genome. In this way, we were able to map the progression of DNA methylation across the entire integrated Ad12 genomes with increasing generation and passage numbers of the tumor cells in culture. It is conceivable that the increase in DNA methylation after the transition from the tumor in a living animal into cell culture was triggered by the dramatic change in growth conditions for the tumor cells.

Colinear integration of Ad12 DNA in hamster tumor cell lines. Analyses of integration patterns have been previously performed on many different adenovirus-transformed and Ad12-induced tumor cell lines (4, 11, 14–17, 24–27, 31). The DNA from Ad12-induced tumor T201/2, T201/3, T211, T191, T271, T281, or T5 was cleaved with restriction endonuclease *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I. Subsequently, the fragments were separated by electrophoresis on agarose gels and blotted (22), and the Ad12-specific DNA fragments were visualized by hybridization to the entire ^{32}P-labeled Ad12 DNA or the ^{32}P-labeled cloned terminal Ad12 DNA fragments and by autoradiography. From the intensity and the distribution patterns of Ad12-specific signals relative to precalibrated amounts of Ad12 reference DNA, which was cut with the same enzymes and coelectrophoresed, the copy number (Table 1) and the integration patterns of Ad12 DNA in the cells of individual cell lines could be deduced. As an example, the results of the *Hin*dIII cleavage of T191 and H191 DNAs are shown in Fig. 1. Not all of the data are presented here. In the tumors and cell lines analyzed, the viral DNA molecules were integrated in a mode colinear with that in Ad12 virion DNA. Upon restriction analyses and electrophoreses, the internal viral DNA fragments (cf. Fig. 1 and restriction maps of Ad12 DNA in Fig. 2) comigrated with the internal virion DNA fragments coelectrophoresed as references, whereas the terminal viral DNA fragments, *Hin*dIII-E and *Hin*dIII-G, were displaced to off-size positions because of their covalent linkage to cellular DNA (Fig. 1).

Gradual spreading of DNA methylation across integrated Ad12 DNA molecules. In Fig. 2, the autoradiograms shown in panels a document *Hpa*II-*Msp*I cleavage patterns of the DNA from Ad12-induced hamster tumors (T201/2 and T281) or from the corresponding cell lines (H201/2 and H281) and visualize all of the Ad12-specific DNA fragments. In these

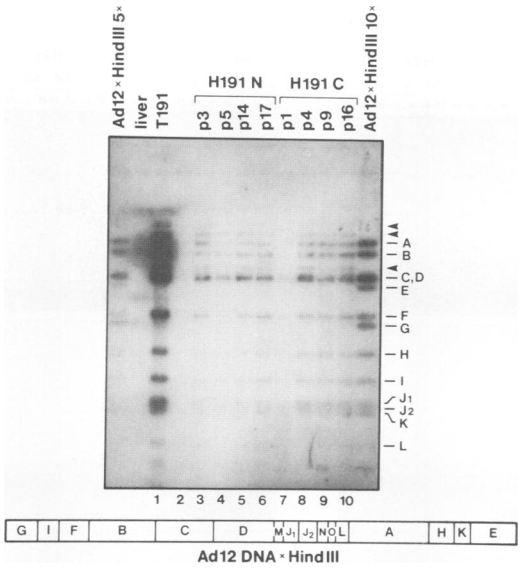

FIG. 1. Colinear pattern of Ad12 DNA integration in the Ad12-induced hamster tumor T191 and in the cell line, H191, derived from it. The cellular DNA (10 μg per lane; ~20 μg in lane T191) was cleaved with *Hin*dIII, and the DNA fragments were separated by electrophoresis on 0.8% agarose gels. Liver DNA from a non-Ad12-treated Syrian hamster was cut with *Eco*RI and used as carrier DNA (10 μg per lane of Ad12 virion DNA) or as a negative control. Carrier DNA did not contain Ad12 DNA sequences and was added to obtain the same DNA concentration in the lanes with marker DNA as in the lanes with DNA from Ad12-induced tumor cells. The DNA fragments were transferred by the alkali method (0.25 M NaOH, 1.5 M NaCl) to a Hybond N membrane and hybridized to [^{32}P]Ad12 DNA as a probe. Ad12 DNA in amounts of 5 or 10 genome equivalents (5× and 10×) per cell, corresponding to 10 μg of cellular DNA, was also cleaved with *Hin*dIII and coelectrophoresed with *Eco*RI-cut carrier DNA as a size and intensity marker. [^{32}P]Ad12 DNA was used as the hybridization probe. An autoradiogram of the agarose gel is shown. N and C, passage in normal Dulbecco medium (N) or in conditioned medium (C); p, passage; T, tumor; H, hamster tumor cell line. The arrowheads indicate the terminal viral DNA fragments, *Hin*dIII-E and *Hin*dIII-G, which are linked to cellular DNA and thus displaced to off-size positions.

Southern blot hybridization experiments, the entire Ad12 genome was used as the hybridization probe. The Ad12 genomes in the tumor cells extirpated from the animals exhibited a low level of DNA methylation at the 5′-CCGG-3′ sequences in that *Hpa*II and *Msp*I cleavage patterns were very similar (Fig. 2, part 2a). High-molecular-mass bands in the *Hpa*II patterns are presumably due to DNA methylation and to the lack of cleavage at some of the 5′-CCGG-3′ sequences. In the DNA of tumor T281 (Fig. 2, part 2), the *Hpa*II and *Msp*I cleavage patterns were apparently identical. Upon explantation of the cells from the tumor into culture and subsequent continuous passage in Dulbecco medium with 10% fetal calf serum (cell lines and normal medium) or in conditioned medium (see Materials and Methods), a striking increase in the levels of DNA methylation in the integrated Ad12 genomes was observed. *Hpa*II cleaved the DNA progressively less with increasing passage number, whereas *Msp*I cleavage was not interfered with. A clearly documented effect of normal versus conditioned medium on the rate of increase of methylation was not observed (Fig. 2).

Subsequently, cloned Ad12 DNA fragments were used as hybridization probes on the same blots to assess the mode of onset and increase in DNA methylation for different segments of the viral genome. The probes employed in panels a to f (Fig. 2) are described in the legend to Fig. 2. Map locations are shown in Fig. 2. The nylon membranes were washed off and rehybridized 11 to 12 times (see legend to Fig. 2). It is apparent from the autoradiograms in Fig. 2 that the onset and increase of DNA methylation vary in different parts of the viral genome.

Progressive disappearance of *Hpa*II fragments. The progression of DNA methylation in the integrated Ad12 genomes could be monitored by the gradual disappearance of *Hpa*II DNA fragments on Southern blots of the DNA from Ad12-induced tumors and the corresponding tumor cell lines (Fig. 3). The results corroborated the conclusions drawn in the previous section about the order in which individual segments of the integrated Ad12 genome became methylated. In the transition from tumor (designations beginning with T) to cell line (designations beginning with H) for T281 and H281, T271 and H271, T191 and H191, *Hpa*II E or F and M fragments were the first ones to disappear, indicating that DNA methylation commenced in these parts of the integrated viral genomes. Very small *Hpa*II fragments could not be resolved on these gels, and the corresponding regions of the Ad12 genome thus could not be analyzed. The block of fragments H to M was next to disappear, followed by the C or D fragment. At a later stage of passage, fragments A and B (in the left terminal 15 to 25 map units of the Ad12 genome) were displaced to the region of uncleaved fragments. It was striking that some of the *Hpa*II fragments, such as G, N or O, P or Q, and the off-size fragments (in cell line H281), did not disappear in some of the cell lines, even after a large number of passages. However, in cell line H191, the G fragment was one of the first to disappear. It is concluded that, with some exceptions in the details of the arising patterns, the modes of spreading of DNA methylation across integrated Ad12 genomes are similar in different Ad12-induced tumor cell lines, although some differences between cell lines do exist.

Comparison with DNA methylation patterns in previously analyzed Ad12-transformed cell lines. In the Ad12-transformed hamster cell lines T637, A2497/3, and HA12/7 (13, 26) and the Ad12-induced hamster tumor line H313 (16), some of the 5′-CCGG-3′ sites (in particular, the N or O fragment) in the integrated Ad12 genomes remained unmethylated even after many years of continuous passage of these cells in culture. The data in Table 2 provide evidence that in these cell lines, the *Hpa*II N or O fragments were again the ones whose 5′-CCGG-3′ flanks escaped methylation in the cell lines that had been cultivated for many years. Cell lines H281, H271, H191, H201/2, H201/3, and H211 were not kept in culture for comparable periods. Apparently, there were (unknown) signals in the Ad12 genome that prohibited DNA methylation at very selective sites.

Viral DNA-cellular DNA junction sites remain unmethylated. A detailed analysis was performed on the size distributions of the *Hpa*II and *Msp*I fragments in the left-terminal *Eco*RI-C fragment (Fig. 2, panels b) or in the right-terminal *Bam*HI-E fragment (panels h) of Ad12 DNA. The *Hpa*II fragments involving and close to the sites of junction remained *Hpa*II excisable even at later passages of the Ad12-induced hamster tumor lines. Thus, the 5′-CCGG-3′ sequences at or close to the sites of junction between cellular and viral DNA in the Ad12-induced tumors and in the cell lines established from them are not methylated. In tumor T1111/2 (17), the site of junction between viral and cellular DNAs is hypomethylated, whereas the same cellular prein-

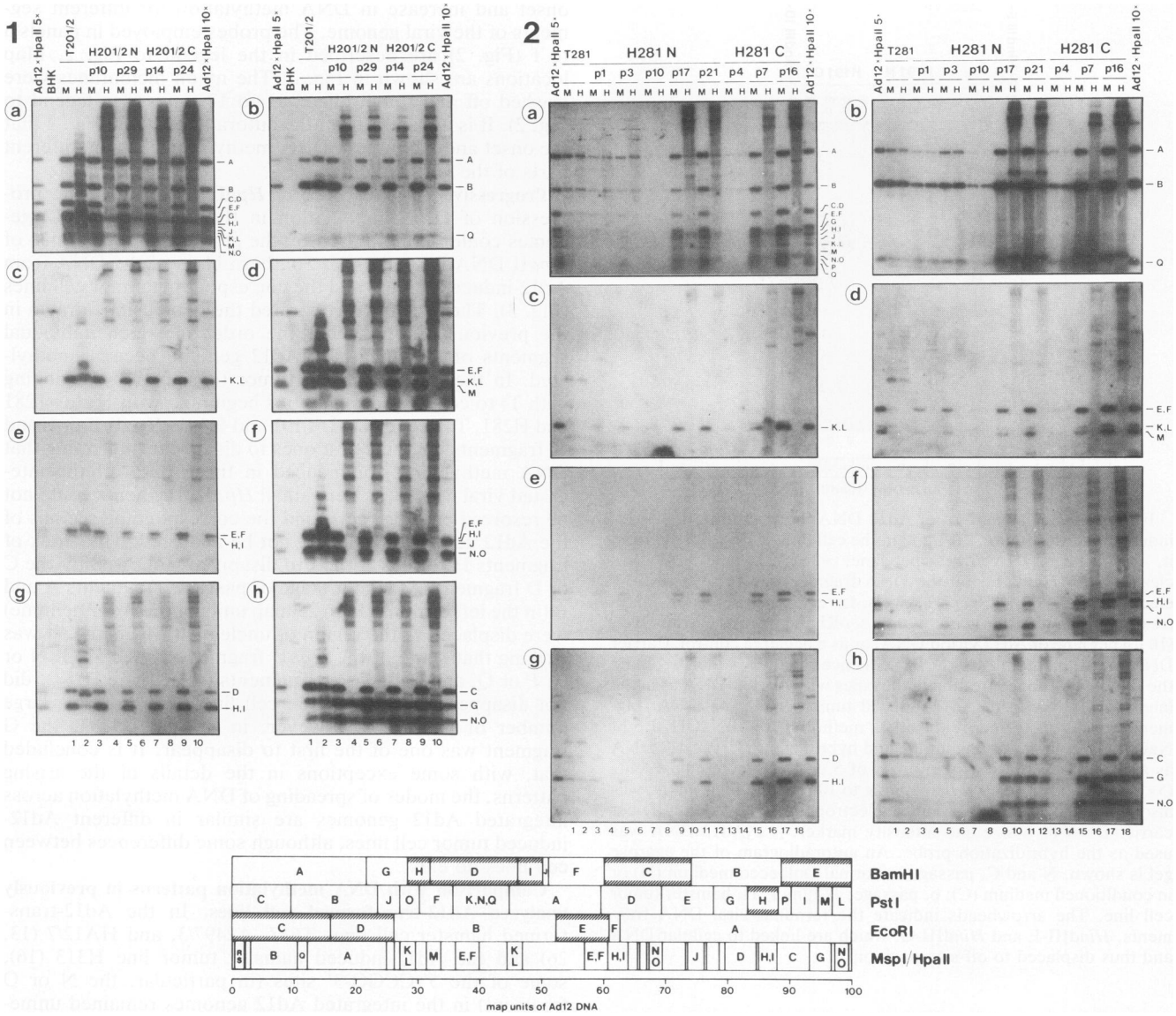

FIG. 2. Spreading of DNA methylation in integrated Ad12 genomes in Ad12-induced hamster tumor cell lines in cell culture. The experimental conditions were similar to those described in the legend to Fig. 1, except that the DNAs were cleaved with *Msp*I (M) or *Hpa*II (H). BHK21 cell DNA was used as a negative control or balance DNA (10 μg per lane) with Ad12 marker DNA. Tumor (T) DNA or DNA from tumor-derived hamster cell lines (H) was analyzed after different numbers of passages (p) in culture. The letters A to Q indicate the positions of individual *Msp*I-*Hpa*II Ad12 DNA fragments on a 0.8% agarose gel. The following ^{32}P-labeled hybridization probes were employed: Ad12 virion DNA (a) and the *Eco*RI-C fragment (b), the *Bam*HI-H fragment (c), the *Bam*HI-D fragment (d), the *Eco*RI-E fragment (e), the *Bam*HI-C fragment (f), the *Pst*I-H fragment (g), and the *Bam*HI-E fragment (h) of Ad12 DNA. The map locations of individual restriction fragments on the Ad12 genome are apparent from the map at the bottom. The DNAs from the following tumors and cell lines were analyzed: T201/2 and H201/2 (part 1) and T281 and H281 (part 2). The data for T271, H271, T191, and H191 are not shown. For autoradiograms a to h, the same blot was employed. After exposure with one probe, the nylon membranes were boiled for 60 to 90 min in 2× SSC–0.1% SDS to remove the previously hybridized probe DNA.

sertion site and the allelic site are completely methylated (18).

Mode of methylation spreading in the integrated Ad12 genomes. All of the hybridization results are summarized in Fig. 4. The distribution of sites of onset and gradual increase in the methylation of 5′-CCGG-3′ sequences followed a general pattern in different Ad12-induced tumors, in established tumor cell lines, and in Ad12-transformed cell lines kept in culture for many years (Table 2). Nevertheless, there was some variation in details (Fig. 4). In most instances, DNA methylation was initiated in the region between map units 30 and 75 of the integrated viral genomes early after the explantation of cells or even in the tumor itself. The right-terminal 80 to 95 map units assumed an intermediate position in this respect, whereas methylation progressed last to the left termini of the integrated Ad12 genomes. The right-terminal 2 to 5 map units and some 5′-CCGG-3′ sites on the left terminus never became completely methylated. In gen-

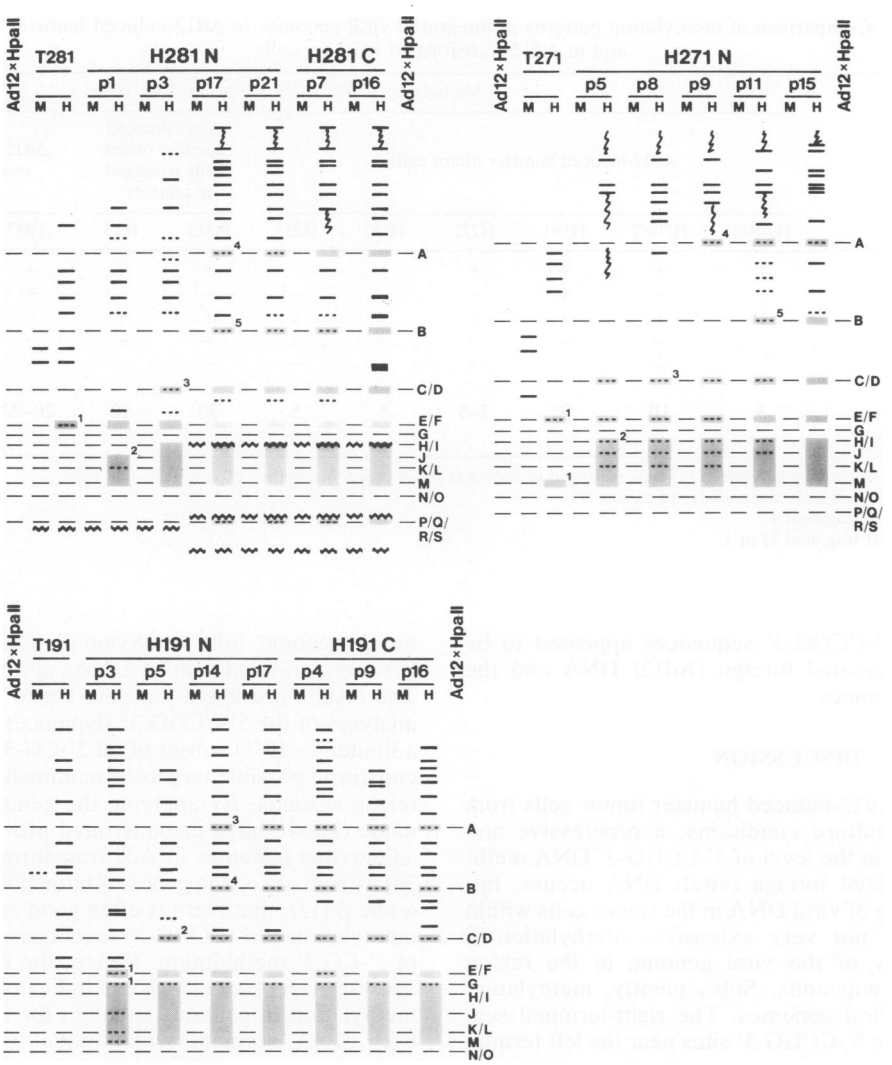

FIG. 3. Schematic presentation of the progression of DNA methylation in integrated Ad12 genomes in three Ad12-induced tumor cell lines. The fragment distribution of Ad12 DNA after cleavage with *Hpa*II (H) or *Msp*I (M) in Southern blots of three Ad12-induced tumor cell lines, H281, H271, and H191, is shown schematically. Primary autoradiographic data are presented in Fig. 1 and 2. The abbreviations are explained in the legends to Fig. 1 and 2. The numerals 1 to 5 refer to the sequence in which *Hpa*II fragments disappeared from the authentic Ad12 virion DNA positions on the autoradiograms. Dashed lines indicate partly methylated regions, and wavy lines indicate junction fragments. The shaded areas indicate fragment collections that completely disappeared.

eral, the hypomethylated state of the off-size fragments of Ad12 DNA generated by *Hpa*II or *Msp*I remained unaltered throughout the passage of the cell lines investigated (Fig. 2, panels b and h). The viral and cellular 5′-CCGG-3′ sequences close to and including the sites of junction between the endogenous cellular and the foreign viral DNA did not become methylated, even at late passages.

No change in methylation status of selected hamster cell sequences upon explantation of tumor cells. As an approximate measure of the extent of cellular DNA methylation, the DNAs from the tumors and from the established cell lines at low and high numbers of passages were cleaved with *Hpa*II and *Msp*I, and the distributions of the ethidium bromide-stained DNA fragments were compared. There was no evidence that the high degree of overall cellular DNA methylation in the 5′-CCGG-3′ sequences had changed as the number of passages increased. Moreover, we used the following randomly selected hamster cellular DNA segments as hybridization probes on tumor cell DNA or tumor cell line DNA after cleavage with *Hpa*II or *Msp*I: pBHK-3, a middle repetitive hamster sequence with a length of about 1.8 kbp; p7, a 1,768-bp hamster sequence, the preinsertion site from the Ad12-induced tumor line CLAC1 (23); p16, the 3.1-kbp preinsertion site from the Ad12-induced tumor T1111/2 (17); BH3-1, a subclone of p16 DNA devoid of the sequences from the intracisternal A particle; and pF19, the 5.5-kbp preinsertion clone from the Ad2-transformed hamster cell line HE5 (7). Some of the analyzed sequences were completely or strongly methylated at the 5′-CCGG-3′ sequences (pBHK-3 and p7); others were partly or weakly methylated (data not shown). A comparison of the methylation patterns in the DNA from the tumors with those in the DNA from the cell lines revealed no noticeable major shifts in the extent of DNA methylation for any of the probed cellular DNA segments. Of course, minor alterations in patterns of methylation could not be ruled out. Thus, major changes in DNA

TABLE 2. Comparison of methylation patterns in integrated viral genomes in Ad12-induced hamster tumor cells and in Ad12-transformed hamster cells

Ad12 *Hpa*II fragment(s)	Methylation at 5'-CCGG-3' sites in DNA from:										
	Ad12-induced hamster tumor cells						Ad12-induced hamster tumor cells passaged in animals[a]		Ad12-transformed hamster cells transformed in cell culture[b]		
	H201/3	H201/2	H191	H271	H281	H211	H313	HT5	T637	HA12/7	A2497-3
A–F	+	+	+	+	+		+	+	+	+	+
G	–	–	+	–	–	–	–	–	+	+	–
H–M	+	+	+	+	+	+	+	+	+[c]	+[d]	
N, O		–	–	–	–	–	–	–	–	–	–
Q–S		–		+	+	–	–	–	–	–	
No. of integrated Ad12 genome equivalents/cell	3	10	3	3–5	5	5	30	10	20–22	3–4	15–20

[a] The Ad12-induced hamster tumor cell line H313 was passaged in hamsters twice, resulting in the cell line HT5 (14–16).
[b] These Ad12-transformed cell lines were analyzed earlier (27).
[c] No methylation in *Hpa*II fragment J.
[d] No methylation in *Hpa*II fragment H or I.

methylation at the 5'-CCGG-3' sequences appeared to be restricted to the integrated foreign (Ad12) DNA and the flanking cellular sequences.

DISCUSSION

Upon transfer of Ad12-induced hamster tumor cells from the tumors to cell culture conditions, a progressive and site-specific increase in the level of 5'-CCGG-3' DNA methylation in the integrated foreign (viral) DNA occurs. Initially, the methylation of viral DNA in the tumor cells within the living animal is not very extensive. Methylation is initiated in the body of the viral genome in the region between 30 and 75 map units. Subsequently, methylation spreads across the viral genomes. The right-terminal segments and some of the 5'-CCGG-3' sites near the left termini never become fully methylated. The junction sequences between viral and cellular DNAs are not methylated either. The results presented here are based on and are limited to analyses of the 5'-CCGG-3' sequences which comprise only a limited (~10%) subset of all 5'-CG-3' dinucleotides which constitute possible targets of mammalian DNA methyltransferase systems. By applying the genomic sequencing technique (2), we have demonstrated previously, for integrated adenovirus genomes in Ad2-transformed hamster cells (29, 30) as well as for the genes of human tumor necrosis factors α and β (12), that there is often good agreement between the methylation at 5'-CCGG-3' sequences and the overall levels of 5'-CG-3' methylation. Hence, the results of *Hpa*II-*Msp*I analyses can serve as a useful indicator for patterns of DNA methylation in general, although for the analysis of details each DNA segment would have to be genomically se-

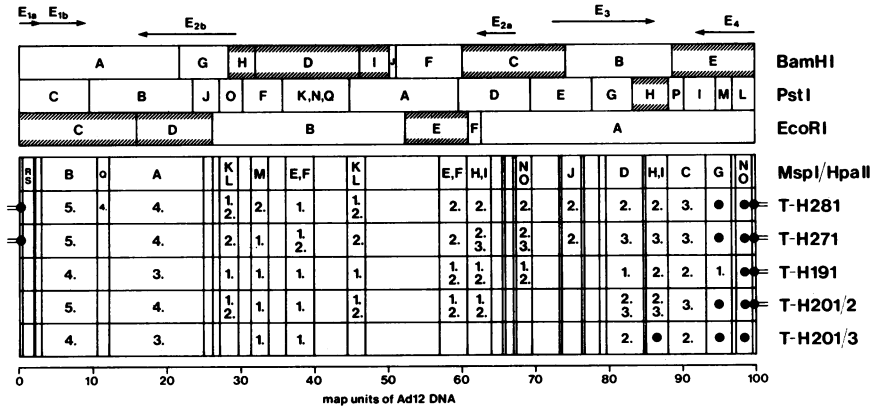

FIG. 4. Schematic summary of the mode of spreading of DNA methylation across the integrated Ad12 genomes. The data presented in Fig. 2 and 3 are summarized. On the restriction maps of Ad12 DNA, the hybridization probes used in these analyses are indicated (partly hatched). Above the maps, the locations of the early regions of the viral genome are indicated. The *Msp*I-*Hpa*II map with the designations of DNA fragments is also reproduced. This map is not complete, since the nucleotide sequence of Ad12 DNA has not yet been completely determined. In the restriction maps of the integrated Ad12 genomes for five independently generated sets of tumors and cell lines, the order of disappearance of individual fragments from their authentic gel positions is indicated by the numbers 1 to 5. This order reflected the progression of DNA methylation across the integrated viral genomes. The primary data for autoradiograms for the tumor-cell line assembly T201/3-H201/3 (T-H201/3) are not given in Fig. 2 or 3. Symbols: =, abutting hamster cell DNA sequences; ●, DNA fragments at or close to junction sites where the 5'-CCGG-3' sequences were not methylated.

quenced. Because of the technical difficulty and magnitude of this task, in most instances inherently limited restriction analysis will have to suffice. In some of the experiments, the methylation-sensitive restriction endonuclease *Hha*I (5'-GCGC-3') was also used for the analyses. In the tumors and cell lines T191 and H191, T201/2 and H201/2, T271 and H271, and T281 and H281, methylation of the integrated Ad12 sequences at 5'-GCGC-3' sequences paralleled that shown with *Hpa*II (data not presented).

Less definite statements can be made about changes in the levels of cellular DNA methylation following explantation of the Ad12-induced tumor cells into culture. The available evidence argues against massive changes in the patterns of DNA methylation in the endogenous hamster cellular genome. Alterations in segments not tested, of course, cannot be excluded.

In the inception of de novo patterns of DNA methylation, the gradual spreading of methylation, which starts at a few focal points, seems to be a general mechanism. We have previously documented this spreading at the nucleotide level by using the genomic sequencing method with hamster cells which had been transfected with a construct that contained the 5'-CCGG-3' in vitro-premethylated late E2A promoter of Ad2 DNA and carried this construct in the integrated form (19, 29, 30). In the study described here, the spreading of de novo methylation across an entire, recently integrated Ad12 genome of some 30 kbp was monitored. As a probably important difference from the experimental approach of our previous work (19, 29, 30), the integrated Ad12 genome was not premethylated before integration into the cellular genome. It is conceivable that the gradual inactivation of an entire chromosome, e.g., the X chromosomes in some mammals (8), is also related to the spreading of DNA methylation at the megabase pair level. Very detailed work on the methylation of the X-linked phosphoglycerate kinase 1 gene has recently been presented (20, 21) and supports this conclusion.

In the integrated Ad12 genomes, DNA methylation is not initiated at the termini of viral DNA. Thus, it is unlikely that spreading from a neighboring cellular focus of DNA methylation into the abutting viral DNA sequences is the only important determinant in the establishment of DNA methylation patterns. The notion that the site of foreign DNA integration determines the pattern and the speed of spreading of foreign DNA methylation does not receive support from these results. Methylation commences at locations between 30 and 75 map units on the integrated foreign Ad12 genome at sites which might be distinguished by a specific nucleotide sequence, structure, or chromatin assembly or a combination of these parameters. There is evidence, from work on the Thy-1 gene in embryonic stem cells, that certain nucleotide sequences might represent signals defining de novo methylation patterns (28). It is, of course, conceivable that the site of foreign DNA insertion afflicts these parameters via long-distance interactions. The adenovirus system might offer the opportunity to study these important problems. The phenomena reported in this article are documented by studies of six different and independently evolved Ad12-induced hamster tumor cell lines as well as by the results from several Ad12-transformed cell lines (Table 2). We therefore plan to investigate whether the integrated Ad12 genomes carry hitherto unknown signals that determine the site-specific inception and progression of DNA methylation.

The initiation of methylation spreading in the integrated Ad12 genomes is linked to the transfer of Ad12-induced tumor cells from a tumor-bearing animal into cell culture. Of course, the growth conditions in cell culture are very different from those in animals. Nevertheless, some of the 5'-CCGG-3' sites in the integrated Ad12 genomes in the tumors are already methylated. Is the shift in the culture milieu one of the decisive stimuli for extensive changes in the levels of DNA methylation? According to a limited set of data obtained with the Ad12-transformed cell line T637 (5) and the Ad2-transformed cell lines HE3/ATL1 and HE3/ATL2 (3), the reverse transfer of cells from culture to an animal does not seem to elicit changes in the levels of DNA methylation in the integrated Ad12 genomes. Similarly, the Ad12-induced tumor cell line H313 (15) has been passaged twice through a hamster, and the cells from the tumor generated in the animal (T5) have been reexplanted into culture (HT5) (Table 2). The patterns of DNA methylation in the integrated Ad12 genomes in the tumor cells before and after the hamster passage are indistinguishable (unpublished results). Our preliminary attempts to influence shifts in DNA methylation by choosing conditioned rather than fresh medium have not produced significant effects, except perhaps in cell line H281.

So far, we have met with considerable difficulties in the cloning of Ad12-induced tumor cells directly out of tumors. The results reported here are nevertheless not compromised by the nonclonal nature of the cell lines investigated. Initially, the tumor cells contained unmethylated or hypomethylated Ad12 genomes, and distinct methylation patterns developed gradually in six different examples with increasing times of cell culture.

It is, however, conceivable that in the course of cell cultivation those cells in which major parts of the integrated viral genome have been silenced by methylation have been selected. Similarly, the Ad12 genes encoded by the left and right termini of the viral genome may bestow growth advantages on hamster cells, and hence those cells could have preferentially survived, and the active viral genes could have remained unmethylated. However, we have shown that the late genes of Ad12 DNA cannot be expressed in hamster cells (33), probably because of the presence of a mitigator element in the Ad12 major late promoter (34). Thus, the interpretation arguing in favor of selective advantages for cells carrying silenced late viral genes appears less plausible. We therefore consider the discussed selection hypothesis less likely but cannot rule it out completely.

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