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The Initiation of De Novo Methylation of Foreign DNA Integrated into a Mammalian Genome Is Not Exclusively Targeted by Nucleotide Sequence

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The de novo methylation of foreign DNA integrated into the mammalian genome is a fundamental process whose mechanism has not yet been elucidated. We have studied de novo methylation in adenovirus type 12 (Ad12) genomes inserted into the genomes of Ad12-induced hamster tumor cells. De novo methylation of Ad12 DNA, which is not methylated in the virion, is initiated in two paracentrally located regions and spreads from there across the integrated Ad12 genomes. (i) After extensive cultivation of cloned Ad12-induced hamster tumor cell lines, the same segments in integrated Ad12 DNA in different cell lines become methylated or remain unmethylated, depending on their positions in the viral genome. (ii) When Ad12 DNA or Ad12 DNA fragments are transfected into hamster cells and permanent cell lines are established by selection for the cotransfected neomycin phosphotransferase gene, patterns of de novo methylation in terminally or internally located segments of Ad12 DNA are different from those in Ad12-induced tumor cell lines. (iii) A detailed study on the topology of the integrated viral genomes in the Ad12-transformed hamster cell lines T637 and A2497-3 and in the Ad12-induced hamster tumors T191, T1111(1), and T181 has been performed. Some of the integrated viral genomes are inserted into the cellular genome in an orientation colinear with the virion genome; others have been rearranged. An originally internally located Ad12 DNA segment has become transposed to the left-terminal sequences of the viral genome in several cell lines and tumors. In the complete Ad12 genomes, the internally located *Pst*I-D fragment becomes extensively methylated at the 5′-CCGG-3′ and 5′-GCGC-3′ sequences. When this DNA segment has been juxtaposed to the left-terminal, hypomethylated fragment of Ad12 DNA in rearranged genomes, the *Pst*I-D fragment remains unmethylated. We therefore reason that the initiation of de novo methylation in integrated Ad12 DNA cannot be directed exclusively by the nucleotide sequence. Other parameters, such as site of integration, conformation of integrates, mode of cell selection, or chromatin structure related to transcriptional activity, may play decisive roles.

Foreign DNA integrated into eukaryotic genomes is frequently de novo methylated in specific patterns (16, 21, 24, 39, 40). During early embryonic development in mammals, DNA sequences become unmethylated and subsequently become de novo methylated in the same specific patterns that existed before demethylation (32). The inactivation of major parts of the X chromosome has also been related to de novo methylation (7). The differential imprinting of specific genes in the human or the mouse genome on the paternally or the maternally inherited chromosome has been related to differing patterns of methylation of allelic sequences in the two different chromosomes (17, 18, 27, 29, 38, 41). Furthermore, foreign genes fixed in mammalian cells in experiments aimed at gene therapy approaches are often inactivated after transient states of activity. The de novo methylation of the foreign DNA is probably responsible for this unintended shutoff.

Adenovirus type 12 (Ad12) DNA integration and de novo methylation have been studied extensively (for reviews, see references 3 and 4), numerous cell lines carrying integrated Ad12 genomes are available, and the entire Ad12 nucleotide sequence of 34,125 bp has recently been determined (35). Moreover, the Ad12 genome or selected fragments of Ad12 DNA can be introduced into the hamster cell genome by cotransfection of Ad12 DNA and the gene for neomycin phosphotransferase (34).

The mechanism of insertional recombination between Ad12 (foreign) DNA and mammalian DNA seems to be characterized by considerable pliability. Analyses of nucleotide sequences across a number of sites of linkage between viral and cellular DNAs have failed to provide evidence for sequence-specific insertion of adenovirus DNA (3, 4, 13, 19). On the other hand, we have observed five different Ad12-induced hamster tumor cell lines with strikingly similar, if not identical, sites of Ad12 DNA integration (25). There seems to be a preference for viral DNA insertion into transcriptionally active cellular DNA sequences (31).

The topology of multiple copies of integrated Ad12 genomes has been analyzed. Some genomes appear to be integrated in an orientation colinear with Ad12 DNA, and some of them are flanked on one or both termini not by cellular DNA but by Ad12 DNA segments derived from internal parts of the viral genome.

In this paper we provide several lines of evidence that for integrated foreign (Ad12) genomes in hamster cells, the nucleotide sequence cannot be the sole determinant in targeting the initiation of de novo methylation.

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TABLE 1. Characteristics of Ad12-induced tumors, Ad12-transformed cell lines, and tumor-derived cell lines

Tumor	Cell line	Mode of transformation	Reference	No. of Ad12 genomes/cell[c]	State of integrated viral DNA
T181		Tumor induction by Ad12 in newborn hamsters	24	5	Intact, colinear
T191	H191	Tumor induction by Ad12 in newborn hamsters	24	3	Intact, colinear, rearranged *Pst*I-D[a]
T201/1		Tumor induction by Ad12 in newborn hamsters	24	5–10	
T201/2	H201/2	Tumor induction by Ad12 in newborn hamsters	24	10	Intact, colinear
T201/3	H201/3a	Tumor induction by Ad12 in newborn hamsters	24	3	Intact, colinear
T211	H211	Tumor induction by Ad12 in newborn hamsters	24	5	Intact, colinear
T271	H271	Tumor induction by Ad12 in newborn hamsters	24	3–5	Intact, colinear
T281	H281	Tumor induction by Ad12 in newborn hamsters	24	5	Intact, colinear
T1111(1)		Tumor induction by Ad12 in newborn hamsters	16	10–11	Intact, colinear, rearranged *Pst*I-D[a]
T1112(2)		Tumor induction by Ad12 in newborn hamsters	16	8–9	Intact, colinear
T5	HT5	Two animal passages of H313 cells	24	10	Intact, colinear
	T637	Ad12-transformed BHK cells	37	20–25	Intact and fragment; rearranged *Pst*I-D[a]
	TR12	Revertant of T637	9	1	Intact and fragment
	A2497-3	Hamster embryo cells transformed by Ad12	1	17	Intact and fragment; rearranged *Pst*I-D[a]
	HA12/7	Hamster embryo cells transformed by Ad12	49	2–3	Intact and fragment (13)
	HEK-12	Human embryonic kidney cells transfected with the *Eco*RI-C fragment of Ad12 DNA	46	<5	ND[b]
	H-Ad12neo5	BHK21 cells transfected with Ad12 DNA	This report	<5	Colinear, terminal deletions
	H-Ad12neo2	BHK21 cells transfected with Ad12 DNA	This report	<5	ND
	EcoRI-C3	BHK21 cells transfected with the *Eco*RI-C fragment of Ad12 DNA	This report	<5	ND
	EcoRI-C7	BHK21 cells transfected with the *Eco*RI-C fragment of Ad12 DNA	This report	<5	ND
	PstI D13	BHK21 cells transfected with the *Pst*I-D fragment of Ad12 DNA	This report	<5	ND
	PstI-D16	BHK21 cells transfected with the *Pst*I-D fragment of Ad12 DNA	This report	<5	ND

[a] See Fig. 5 to 7.
[b] ND, not determined.
[c] Estimates.

MATERIALS AND METHODS

The techniques used in this study have been described earlier (15, 24–26, 30).

Ad12-transformed cell lines, Ad12-induced tumor cell lines, and cell lines obtained by transfection of Ad12 DNA or of Ad12 DNA fragments. The characteristics of the cell lines and their derivations (previously described) are summarized in Table 1. All cells were propagated in culture with Dulbecco medium supplemented with 10% fetal calf serum.

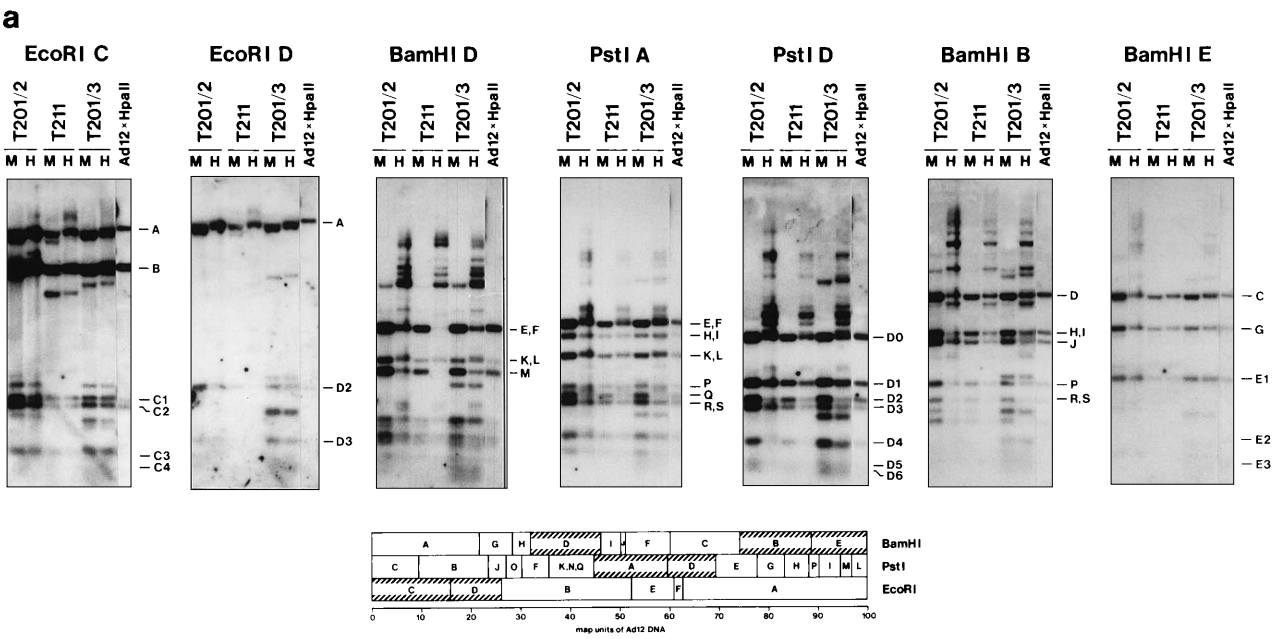
Fixation of Ad12 DNA or of Ad12 DNA fragments in BHK21 cells by cotransfection with the pSV2neo construct. Four micrograms of Ad12 DNA or of the *Eco*RI-C (nucleotide 1 to 5574) or *Pst*I-D (nucleotide 20.885 to 24.053) fragment of Ad12 DNA was cotransfected into BHK21 cells by the $CaCl_2$ method (8) with 4 μg of the pSV2neo construct, which carries the neomycin phosphotransferase gene under the control of the early simian virus 40 promoter (34). Prior to transfection, the Ad12 DNA fragments were excised from their vectors and gel purified twice. At 48 h after transfection, transformed cells were selected for neomycin resistance for 2 weeks in Dulbecco medium containing 10% fetal calf serum and 1 mg of G418 (Pharmacia) per ml. Single-cell clones were recloned twice under selective conditions, which were maintained until passage 4 of the cells. At various passage levels, nuclei were isolated by the Nonidet P-40 method, and DNA was extracted by standard procedures. Cell lines generated in this way were designated *Eco*RI-C3 or -C7, which carried the *Eco*RI-C fragment of Ad12 DNA, and *Pst*I-D13 or -D16, which carried the *Pst*I-D fragment of Ad12 DNA (see Fig. 10). Cell lines obtained after cotransfection of intact Ad12 DNA were termed H-Ad12neo2 or H-Ad12neo5 (see Fig. 9).

Extraction of nuclear DNA, restriction analyses, and Southern blot hybridization. Methods for extraction of nuclear DNA, restriction analyses, and Southern blot hybridization were as outlined in detail elsewhere (15, 24, 25).

Molecular cloning of off-size fragments. About 100 μg of genomic DNA from T637 or T191 cells was restricted with 10 U of *Bam*HI per μg. The fragments were separated by velocity sedimentation over a sucrose gradient (10 to 40% in 1 M NaCl–20 mM Tris [pH 8.0]–10 mM EDTA [pH 8.0]). The gradients were fractionated, and individual fractions (500 ng) were electrophoresed in a 0.6% agarose gel. *Bam*HI-restricted genomic DNA (20 μg) as a control was coelectrophoresed on the same gel. The fractions containing the off-size fragments were identified by hybridization against the ^{32}P-labeled terminal Ad12 DNA fragments. The DNA from the off-size region was ligated into the *Xba*I (Boehringer)- and *Bam*HI (New England Biolabs)-double-cleaved vector λ Gem12 (Promega). Equimolar amounts of vector and insert DNAs were used for ligation (T4 ligase; New England Biolabs) at 15°C overnight. The complete ligation reaction mixture, without pretreatment, was incubated with Gigapack gold packaging extracts II (Stratagene) as described by the manufacturer. About 40,000 PFU per plate were screened. The λ phages were amplified on plate stocks. About 40,000 plaques per 13-cm-diameter dish were plated. The plaques were transferred to Hybond N+ nylon membranes, and the plaques containing off-size fragments were identified by hybridization to the ^{32}P-labeled terminal fragments of Ad12 DNA. DNA was isolated with the Quiagen Lambda DNA preparation kit as described by the manufacturer. The sizes of the λ DNA inserts were compared with those of the genomic off-size fragments by agarose gel electrophoresis and Southern blot hybridization (33).

Analysis of rearranged Ad12 DNA sequences by PCR. The PCR mixture contained 500 ng of genomic template DNA, 100 ng of each primer, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates, and 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.) in a total volume of 50 μl. The Ad12-specific primers were 20- to 23-mers, each with a CG content of between 58 and 68%. A total of 30 cycles, consisting of denaturation for 1 min at 92°C followed by a 1-min annealing step at 68°C and a 1-min polymerization step at 72°C, were performed. One-fifth of the reaction volume was loaded on a 1% agarose gel; the products were visualized by ethidium bromide staining and were alkali transferred to Hybond N+ nylon membranes (Amersham). The authenticities of the PCR products were confirmed by hybridization to the Ad12 *Pst*I-D fragment.

Isolation of cytoplasmic RNA, transfer to Hybond nylon membranes, and hybridization to ^{32}P-labeled fragments of Ad12 DNA. Standard methods were employed as described elsewhere (15, 23, 26, 30). Some of the hybridization experiments used single-stranded RNA probes which were transcribed in the presence of the four ribonucleoside triphosphates, with one or two of them ^{32}P labeled, with T7 or T3 DNA-dependent RNA polymerase from individual pBluescript-cloned *Hin*dIII or *Pst*I fragments of Ad12 DNA (35).



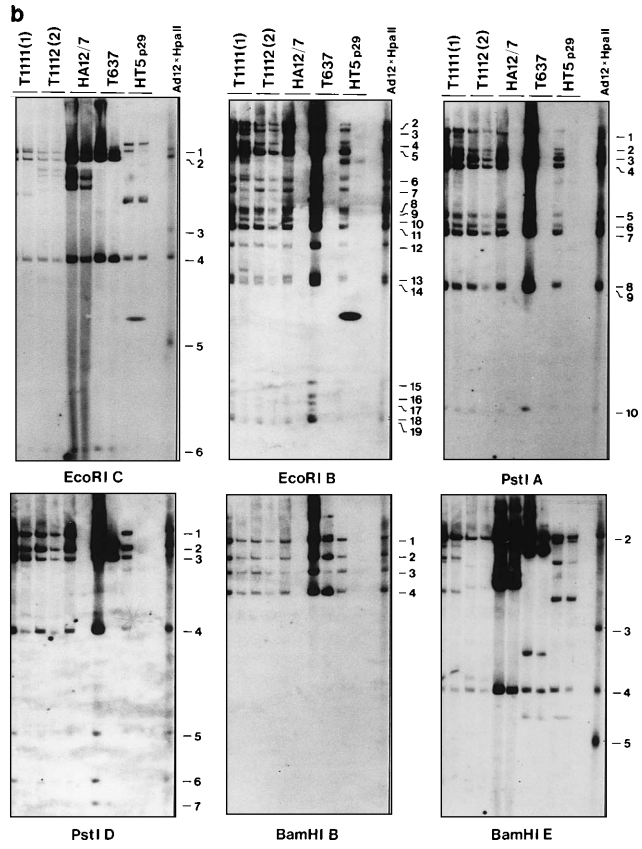


FIG. 1. Initiation of de novo methylation in integrated Ad12 genomes in the paracentrally located regions *Pst*I-D, *Bam*HI-D, and *Bam*HI-B in the DNA of Ad12-induced tumors (a) and in tumors and cell lines (b). (a) The DNA from tumor T201/2, T211, or T201/3 or from Ad12 was cleaved with *Hpa*II (H) or *Msp*I (M), and the fragments were separated by electrophoresis on 0.8% agarose gels and transferred to Hybond N+ nylon membranes. (b) The DNA from tumor T1111(1) or T1112(2) or from cell line HA12/7, T637, or HT5 (passage 29 [p29]) was cleaved with *Msp*I (left lanes) or *Hpa*II (right lanes). The fragments were separated by electrophoresis on a 5% denaturing polyacrylamide gel and transferred to Hybond N+ nylon membranes. The shift of DNA fragments to the higher-molecular-weight range in the *Hpa*II lanes demonstrated a high degree of methylation of 5′-CCGG-3′ sites in the corresponding Ad12 DNA segment. In different hybridization experiments, the membrane-bound DNA was hybridized to the ^{32}P-labeled Ad12 DNA fragments as indicated at the top (a) or bottom (b) of each autoradiogram. The map locations of the probes were apparent from the map at the bottom of panel a. The Ad12 DNA fragments are indicated at the right of each blot. In panel a, D0 to D6, C1 to C4, and E1 to E3 refer to the *Hpa*II fragments inside the *Pst*I-D (or *Eco*RI-D), the *Eco*RI-C, and the *Bam*HI-E fragments, respectively. Fragment designations are explained in the maps in Fig. 2 and its legend. The restriction map of Ad12 DNA was taken from reference 35 with minor modifications. In panel b, the *Hpa*II fragments are numbered (1 to 19) according to decreasing size. The tumor and cell line designations for the upper autoradiograms also apply to the lower ones.

'Determination of nucleotide sequences. All DNA nucleotide sequences were determined by using the dideoxy chain termination method (28). Appropriate 19- to 24-nucleotide primers were utilized for PCR. The nucleotide sequences were computer analyzed in an Applied Biosystems 373A DNA sequencer as previously reported (35). Oligodeoxyribonucleotide primers used for sequence analyses or for PCRs were synthesized in an Applied Biosystems 381A DNA synthesizer.

Nucleotide sequence of Ad12 DNA and restriction maps. The nucleotide sequence of Ad12 DNA was presented earlier (35), and the map in Fig. 2c was drawn on the basis of published data (35). The nucleotide sequence of Ad12 DNA was previously made generally available under EMBO Data Library accession number X73487.

RESULTS

Experimental approach. Approximately 17% of all the 5′-CG-3′ sequences in the Ad12 genome could be screened for methylation by the restriction endonucleases *Hpa*II (5′-CCGG-3′) and *Hha*I (5′-GCGC-3′) (35), which were used to cleave the DNAs of the different Ad12-transformed hamster cell lines or of the Ad12-induced hamster tumor cell lines. The fragments were separated by electrophoresis on 0.8% agarose gels resolving fragments down to 500 bp or on 5% denaturing polyacrylamide gels resolving fragments of between 1,000 and 50 bp. Of the 76 possible *Hpa*II fragments and the 182 possible *Hha*I fragments in Ad12 DNA (35), 61 fragments (representing 4% of all the 5′-CG-3′ sites) and about 100 fragments (representing 6.7% of all 5′-CG-3′ sites), respectively, were investigated in this study. Appropriate fragments of Ad12

DNA were ^{32}P labeled and used as hybridization probes in Southern blot experiments (15, 33).

In some of the previously studied Ad12-transformed hamster cell lines and Ad12-induced tumor cell lines (Table 1), fragments of the Ad12 genome, in addition to the intact or nearly intact genomes, were integrated and possibly rearranged to different locations. Thus, Ad12 DNA segments with the same nucleotide sequence, e.g., the *Pst*I-D fragment, could be investigated for methylation in different environments of intact or rearranged viral DNA sequences.

The de novo methylation of integrated Ad12 DNA is initiated in two paracentrally located regions of the viral genome. Although multiple copies of Ad12 DNA were integrated in the genomes of the cell lines investigated (24, 25), distinct patterns of DNA methylation could be recognized in the integrated Ad12 sequences by Southern blot hybridization. The data in Fig. 1a demonstrate that de novo methylation in the Ad12-induced tumors T201/2, T211, and T201/3 was initiated in the paracentrally located regions of the restriction fragments *Bam*HI-D, *Pst*I-D, and *Bam*HI-B. Some of the 5′-CCGG-3′ sequences in these Ad12 genome segments were not cut by *Hpa*II, as shown by the analyses of the larger and the smaller DNA fragments in agarose gels (Fig. 1a) or in polyacrylamide gels (Fig. 1b). The disappearance of fragments after *Hpa*II restriction (right lanes in Fig. 1b) demonstrated a high degree of methylation in the *Eco*RI-B, *Pst*I-A, *Pst*I-D, and *Bam*HI-B fragments of Ad12 DNA in the cell lines HA12/7 and HT5 and in fragments *Eco*RI-B and *Pst*I-A in cell line T637.

These results and additional data are summarized in Fig. 2. The paracentrally located *Pst*I-D fragment of Ad12 DNA (map in Fig. 2c) was already partly methylated in the tumor cells and became progressively further methylated soon after explantation and the establishment of continuously cultivated cell lines (Fig. 2b). The left-terminal 15% and right-terminal 10% of the integrated Ad12 DNA and the flanking cellular sequences remained hypomethylated or unmethylated in the 5′-CCGG-3′ and 5′-GCGC-3′ sequences even upon prolonged passage of cell lines (Fig. 1 and 2a and d). In Ad12-transformed or tumorigenic cells, hypomethylation and expression of the left-terminal Ad12 DNA sequences were possibly selected for. The scheme in Fig. 2d illustrates the gradual spreading of de novo methylation over extensive parts of the integrated Ad12 genomes.

Analysis of initiation and progression of de novo methylation in the *Pst*I-D fragment of Ad12 DNA. By using *Hpa*II subfragments in the *Pst*I-D fragment of Ad12 DNA as ^{32}P-labeled hybridization probes (*Msp*I map; Fig. 2c), the initiation of de novo DNA methylation in the DNAs from several Ad12-induced tumors and from established cell lines was assessed. Restriction patterns with *Hpa*II (*Msp*I) were determined for cellular DNAs from the Ad12-induced tumors T181, T191, T201/2, and T211 (24), T1111(1) and T1112(2) (16), and T5 (16, 25) with *Hpa*II fragments D0, D4, D2, D1, and D3 as hybridization probes (Fig. 3a and 4). The lack of ability to be cleaved by *Hpa*II (Fig. 3a, right lane for each tumor) was most notable for fragments D0, D4, D2, and D1, whereas fragment D3 was still cut by *Hpa*II.

Similar analyses were performed with the DNAs from the Ad12-transformed cell lines A2497-3, T637, and HA12/7 (36, 40) and from the Ad12-induced hamster tumor cell lines H281 (clone IID3, passage 26) and H191 (clone E7, passage 18) (Fig. 3b). The region between *Hpa*II fragments D0 and D3 (map in Fig. 4) was de novo methylated in all 5′-CCGG-3′ sequences. However, not all of the Ad12 genomes were equally completely methylated, as demonstrated by the appearance of *Hpa*II fragments smaller than 3,169 bp. The 3,169-bp *Pst*I-D fragment comprised the subfragments D0 to D7 (Fig. 4). In the DNA from the tumor T5, some of the Ad12 molecules were still left unmethylated at the 5′-CCGG-3′ sites between D4 and D5 and between D7 and D3. In contrast, all Ad12 genomes were methylated at the *Hpa*II sites located between D2 and D1 and between D0 and D4 (Fig. 4d to f).

The schematic drawings in Fig. 4 illustrate a detailed size class analysis for the *Hpa*II fragments and designate the locations of all the 5′-CCGG-3′ (*Hpa*II) sequences and the lengths of the generated fragments in the *Pst*I-D segment of Ad12 DNA. The temporal course of disappearance of specific *Hpa*II fragments suggested that de novo methylation of integrated Ad12 DNA was initiated between *Hpa*II fragments D2 and D1 (Fig. 4a to c). During tumor development and cell cultivation, the gradient of de novo methylation in the integrated Ad12 DNA extended predominantly to the left of the region between D2 and D1, one of the centers of initiation of de novo methylation (Fig. 4). It was remarkable that the de novo patterns of methylation and their progression were very similar and nonrandom in independently induced Ad12 hamster tumors.

Rearrangements of internal segments of the Ad12 genome in chromosomally integrated viral genomes. Sequences close to the termini of colinearly integrated Ad12 genomes in Ad12-transformed hamster cells can be markedly rearranged (5, 13, 16, 36). After determination of the nucleotide sequence of Ad12 DNA (35), more detailed analyses of the structures of these rearranged Ad12 DNA sequences have become feasible. The methods employed in these investigations were (i) Southern blotting of DNA fragments and identification of the locations of specific Ad12 DNA segments by using small Ad12 DNA fragments as hybridization probes, (ii) molecular cloning of off-size fragments of Ad12 DNA and determination of their nucleotide sequences, and (iii) characterization by PCR.

In the experiment documented in Fig. 5a, DNA from the Ad12-transformed cell line T637 (36, 37, 40) was cleaved with *Hin*dIII, the fragments were electrophoretically separated on a 0.8% agarose gel, and the DNA on the same strip of nylon filter was then hybridized to Ad12 DNA or to the cloned *Eco*RI, *Pst*I, or *Bam*HI fragment indicated in the figure. All hybridization probes were ^{32}P labeled (6). Prior to rehybridization, the filter-bound probe was removed by being boiled in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 30 min. The *Hin*dIII fragments (A to P) of Ad12 DNA were coelectrophoresed as internal size markers. All off-size fragments are indicated by arrowheads and were designated, according to decreasing size, OS1 to OS8. Locations of individual Ad12 fragment probes are shown on the Ad12 DNA map (Fig. 2c). There were numerous off-size bands, not only with homologies to the terminal virion DNA fragments *Eco*RI-C and *Bam*HI-E, as expected, but also with homologies to the internal Ad12 DNA segments *Pst*I-D, -E, -G, and -H. Similar data were obtained upon cleaving T637 DNA with *Eco*RI, *Pst*I, *Bam*HI, *Hpa*II, or *Hha*I (autoradiograms not shown; Fig. 5b). The estimated sizes of these off-size fragments and their homologies on the Ad12 genome (see map in Fig. 2c) are summarized in Table 2. It was concluded that in cell line T637 the terminal Ad12 DNA *Eco*RI-C fragments were linked to cellular DNA or to rearranged Ad12 DNA segments which were derived from the *Pst*I-D, -E, -G, and -H block of internally located segments in authentic Ad12 DNA. The data demonstrated that *Pst*I fragments D, E, G, and H of Ad12 DNA were also represented in the authentic virion size class positions. Hence, a fraction of the integrated Ad12 DNA in cell line T637 was not rearranged.

Similar analyses on the nature of off-size fragments of Ad12 DNA were performed with the DNAs from the Ad12-induced

a

|---------- EcoRI C* ----------

C5	C2 = R/S	C6	C4	B	C1 = Q	A	MspI / HpaII
143	504	83	226	2462	555	4251	bp
[ ]	0	0	0	0	0	0	T181
[ ]	0	0	0	0	0	0	T191
[ ]	0	0	0	0	0	0	T201/2
[ ]	0	0	0	0	0	0	T211
[ ]	0	0	0	0	0	0	T201/3
[ ]	0	0	0	0	0	0	T1111(1)
[ ]	0	0	0	0	0	0	T1112(2)
[ ]	[ ]*	0	0	1	1	1	T5
[ ]	0		0	1	1	1	H191
[ ]	[ ]*	0	0	2	2	2	H201/2
[ ]	0	0	0	0	0	1	H211
[ ]	1	0	0	0	1	2	HA12/7
[ ]	0	0	0	1	0	1	T637
[ ]	0	0	0	0	0	[ ]	HEK-12
[ ]	0		0	0	1	1	Eco RI C7
[ ]	1		1	1	2	1	Eco RI C3
[ ]	[ ]*		[ ]*	2	2	2	H-Ad12neo5

b

---------- PstI A *----------|

|----------PstI D *----------|

A4	A8	E/F = A-1	H/I = D0/A0	D4	D5	D6	Q = D2	N/O = D1	D7	R/S = D3	MspI / HpaII
482	196	1245	1089	255	142	111	515	639	99	460	bp
1	0	0	1	1	1	1	1	1	1	0	T181
0	0	0	1	1	1	1	1	1	1	0	T191
1	1	1	1	1	1	1	1	1	1	0	T201/2
1	0	1	1	1	1	1	1	1	1	0	T211
1	1	1	1	1			1	1		1	T201/3
1	1	0	1	1	1	1	1	1	1	0	T1111(1)
0	1	1	1	1	0	1	1	1		0	T1112(2)
2	2	2	2	2	2	2	2	2	2	2	T5
2	2		2	2			2	2		2	H191C
2	1	2	2	2	2	2	2	2	2	2	H201/2
1	2	1	1	1	2	1	1	1	2	1	H211C
2	2	2	2	2			2	2	2	2	HA12/7
2	2	2	2	2	2	2	2	2	0	0	T637
			[ ]		0		0	[ ]*		0	PstI D13
			[ ]	1			1	1		1	PstI D16
			2	2			2	2		2	H-Ad12neo2
			2	2			2	2		2	H-Ad12neo5

FIG. 2. Schematic presentation of the initiation and progression of de novo methylation in integrated Ad12 genomes in Ad12-induced tumors (T), in a number of Ad12-induced tumor cell lines (H), in Ad12-transformed cell lines (HA12/7, T637, HEK-12), or in cell lines generated by transfection with Ad12 DNA or Ad12 DNA fragments. Data similar to those from the autoradiograms in Fig. 1 are graphically summarized. (a) Status of 5′-CCGG-3′ methylation in the left-terminal *Eco*RI-C fragment of Ad12 DNA in the indicated tumors and cell lines. The *Hpa*II fragments, their sizes, and the nomenclature used are explained in the text. (See also the *Msp*I map in panel c.) 0, absence of methylation in both *Hpa*II sites flanking a fragment; 1 and 2, methylation of one or both of the flanking sites in some (1) or all (2) of the integrated copies of Ad12 DNA. Brackets indicate that the *Msp*I fragment was missing because some of these fragments were covalently linked to cellular DNA. (b) Status of 5′-CCGG-3′ methylation in the paracentrally located sequences of the *Pst*I-A and *Pst*I-D fragments. The organization and symbols are as for panel a. (c) Restriction maps of Ad12 DNA (35). Letters indicate fragment designations and numbers above the map indicate base pairs. (d) Summary of the initiation (light shading) and progression (heavy shading) of de novo methylation in integrated Ad12 genomes. On the bottom, the transcriptional map of Ad12 DNA (35) is shown. The cellular DNA sequences flanking the integrated Ad12 DNA are indicated by open bars. Unfilled areas in the Ad12 map designate the absence of methylation at 5′-CCGG-3′ and 5′-GCGC-3′ sites. MLP, major late promoter.

hamster tumors T1111(1) (16) and T181 (24) upon cleavage with the restriction endonucleases listed in Table 2. The results of Ad12 DNA fragment hybridizations with *Hin*dIII-cleaved T1111(1) tumor DNA yielded consistent results. Approximate sizes and map derivations of the off-size fragments generated upon cleavage of T1111(1) DNA with *Hin*dIII, *Eco*RI, *Bam*HI, or *Msp*I are summarized in Table 3, and the equivalent data for the Ad12-induced tumor T181 (autoradiograms not shown) are presented in Table 4. Again, the data demonstrated that both Ad12 termini, i.e., the *Eco*RI-C (Table 3) or the *Hin*dIII-G (Table 4) fragment from the left end of Ad12 virion DNA and the *Bam*HI-E (Table 3) or the *Pst*I-L (Table 4)

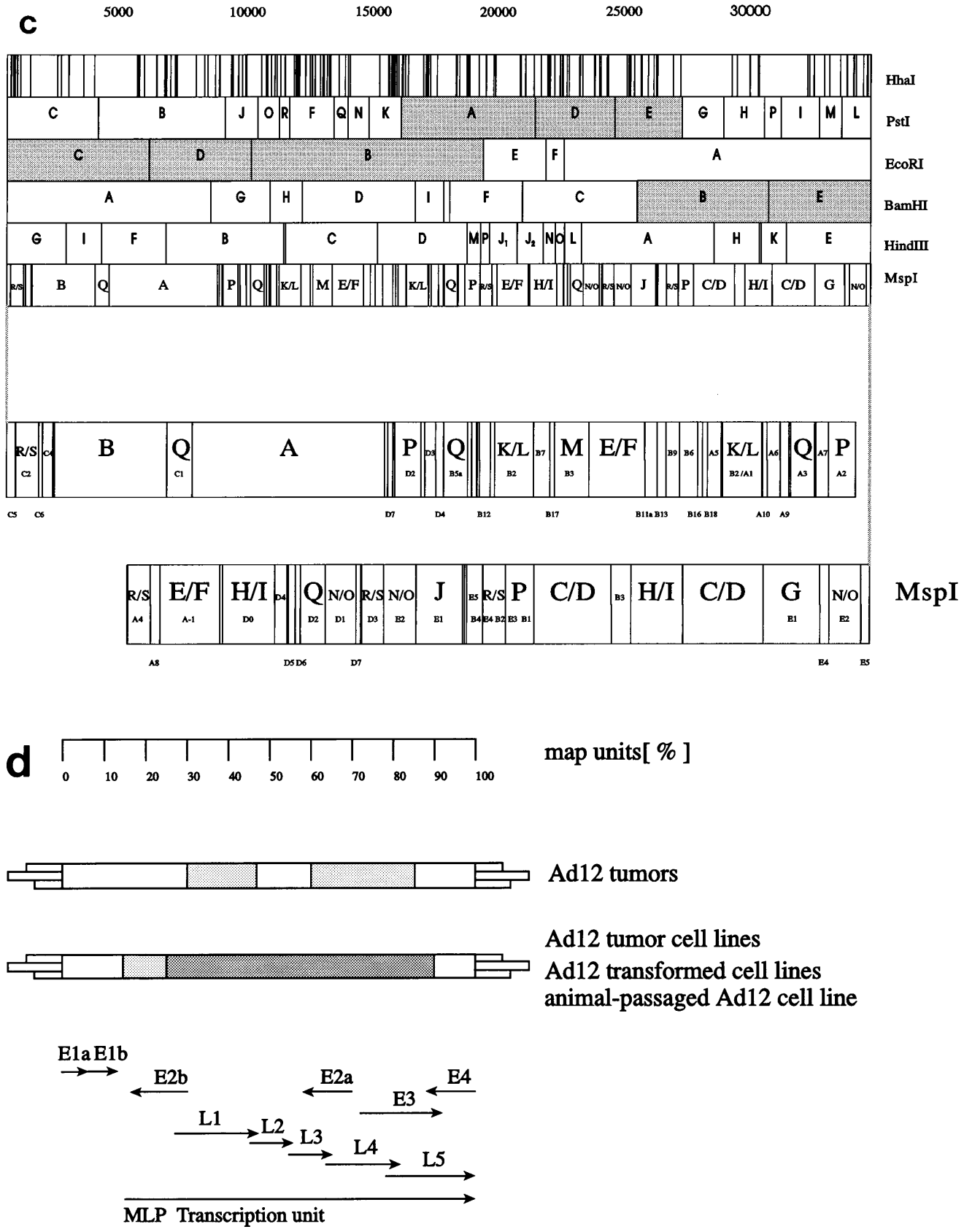


FIG. 2—*Continued.*

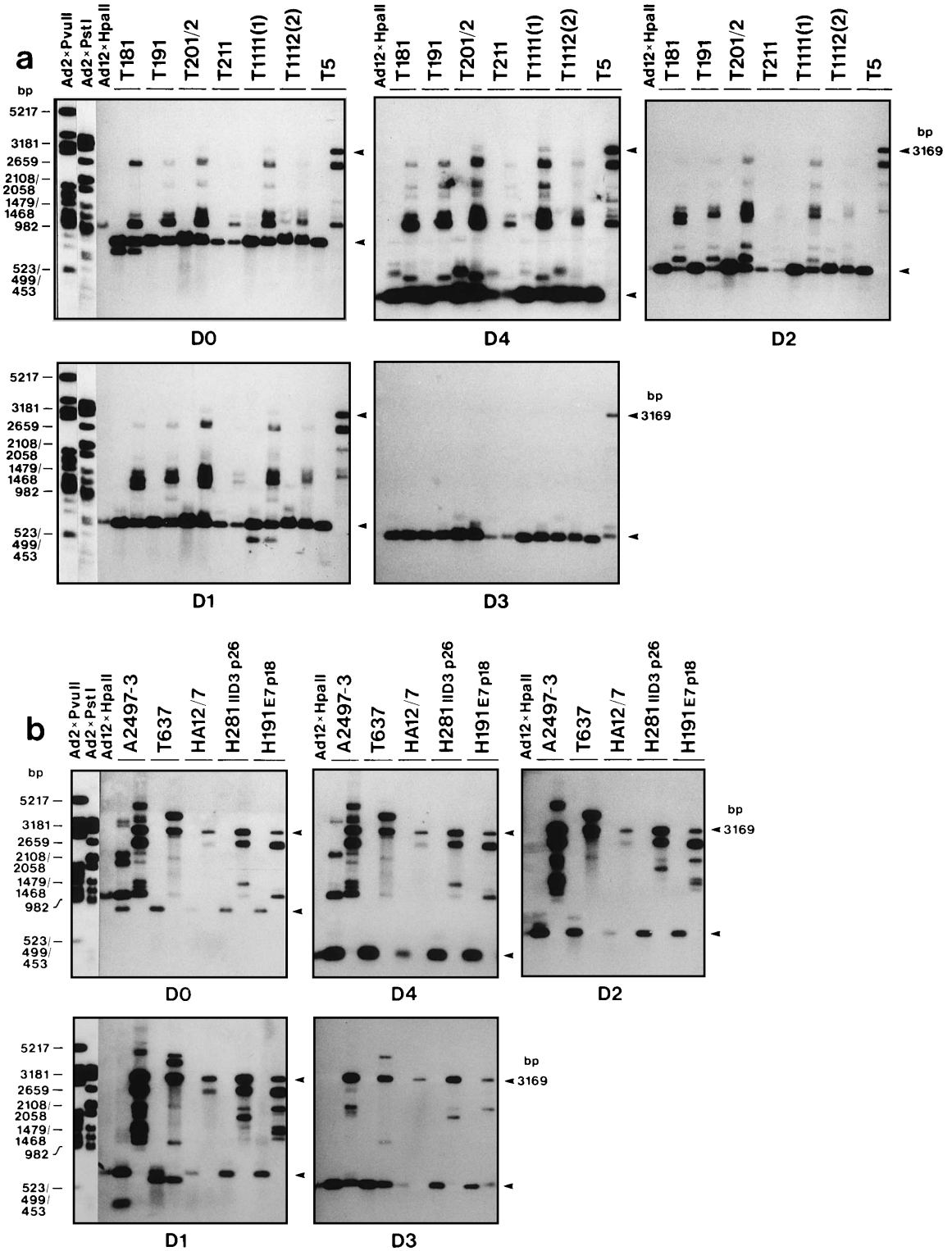


FIG. 3. Analysis of de novo methylation in the *Pst*I-D fragment of integrated Ad12 DNA in tumors and cell lines. DNA from tumors T181, T191, T201/2, T211, T1111(1), T1111(2), or T5 (a) or from cell line A2497-3, T637, HA12/7, subclone IID3 of H281 in passage 26 (p26), or subclone E7 of H191 in passage 18 (b) was cleaved first with *Msp*I (left lane in each pair) or *Hpa*II (right lane) and then with *Pst*I. The fragments were alkali transferred to Hybond N+ nylon membranes and hybridized to the isolated ^{32}P-labeled *Hpa*II subfragments from the *Pst*I-D segment of Ad12 DNA as indicated. Coelectrophoresed Ad2 or Ad12 DNA fragments served as size markers. Fragment lengths are indicated on the left. Arrowheads designate the 3,169-bp *Pst*I-D fragment derived from integrated Ad12 DNA molecules that were completely methylated in all 10 *Hpa*II sites in this fragment. Arrowheads also designate the *Msp*I fragments D0, D1, D2, D3, and D4. The locations of the hybridization probes were apparent from the *Msp*I-*Hpa*II maps of the Ad12 *Pst*I-D fragment in Fig. 2b and c and Fig. 4.

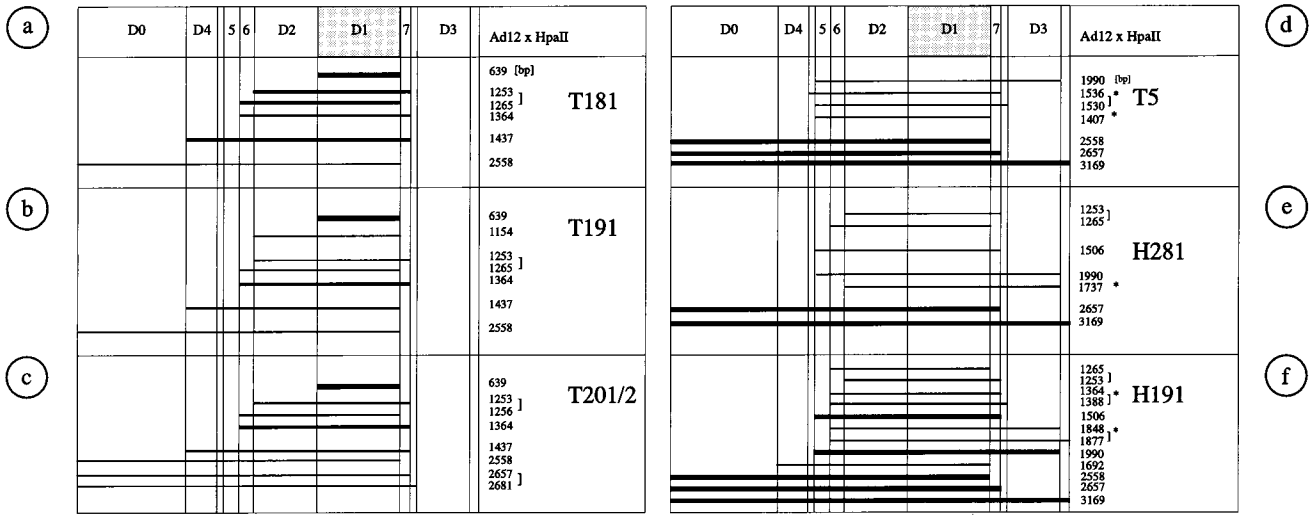


FIG. 4. Initiation and progression of de novo methylation in the region D0 to D3 of 5′-CCGG-3′ sequences in the *Pst*I-D fragment of integrated Ad12 DNA in Ad12-induced tumors (a to d) and in cell lines derived from them (e and f). A schematic summary of the data presented in Fig. 3 is shown. The *Hpa*II map of the *Pst*I-D fragment is described in the legends to Fig. 1 and 2b and c. The horizontal bars in the graph reflect fragment lengths as indicated in the rightmost columns. Bracketed fragments could not be distinguished. Fragments marked with an asterisk were cell line specific. The thickness of the bars relates to the preponderance of the designated fragment on the Southern blots in Fig. 3. Similar analyses were performed after hybridization with the probes D0, D4, D2, and D3 (not shown).

fragment from the right end of Ad12 virion DNA, were displaced to off-size positions because of linkage to cellular or rearranged viral DNA.

The data presented so far documented that in Ad12-transformed hamster cells and in Ad12-induced hamster tumors, a considerable proportion of the integrated Ad12 genomes was inserted intact and organized colinearly with the genome orientation in the virion. Strikingly, in the DNAs from some cell lines and tumors, segments from the internally localized *Pst*I-D segment extending into the *Pst*I-A, -E, -G, and -H segments were rearranged, and some of them were found in conjunction with the left [T637, T1111(1), and T181 (Tables 2 to 4)] or the right [T1111(1) and T181 (Tables 3 and 4)] terminus of integrated Ad12 DNA. This internal viral DNA segment seemed to be predisposed to independent recombination events.

Analyses of rearranged viral genome regions by PCR. The schemes in Fig. 6a to c present the arrangement of the left-terminal *Pst*I-C fragment of Ad12 DNA relative to the internal *Pst*I-D fragment in Ad12 virion DNA (Fig. 6a) and arrangements in integrated reorganized Ad12 genomes in the cell line T637 (Fig. 6b) or in the Ad12-induced tumor T191 and the cell line derived from it, H191 (24) (Fig. 6c). The drawings also indicate the map locations of oligodeoxyribonucleotide primers used in PCRs and the sizes of PCR products obtained with specific primer combinations. Obviously, in Ad12 virion DNA (Fig. 6a) the left-terminal *Pst*I-C fragment and the internal *Pst*I-D fragment were located almost 20 kbp apart, and PCR with the indicated primers did not yield any products (data not shown). Such products could arise only if these two Ad12 DNA segments had been juxtaposed because of rearrangements upon integration of Ad12 DNA, as in cell line T637 (Fig. 6b) and in tumor T191 or in cell line H191 (Fig. 6c). PCRs with primer combinations as indicated in Fig. 6c were performed with hamster liver DNA and Ad12 virion DNA as controls, with T191 DNA, and with DNA from cell line H191 in passage 3, 17, or 29 of subclone E7 of cell line H191 (Fig. 6d). PCR products were visualized in ethidium bromide-stained gels, and their authenticities were confirmed by Southern blotting of the analytical agarose gel and hybridization to the ^{32}P-labeled *Pst*I-D fragment of Ad12 DNA. Ad12 virion DNA fragments generated by cleavage with *Bam*HI or with *Pst*I served as size markers. The actual PCR product sizes obtained (Fig. 6d) supported the model shown in Fig. 6c for the juxtaposition of the internal *Pst*I-D fragment to the vicinity of the left viral DNA terminus, and their identities were confirmed by nucleotide sequence analyses of clone 23 from T191 DNA (Fig. 5b; see Fig. 7c). The PCR data obtained with DNA from cell line T637 are not shown.

*Msp*I-*Pst*I- or *Hpa*II-*Pst*I-cleaved DNAs from tumors T181 and T1111(1) or from cell lines A2497-3 and T637 were hybridized to *Msp*I subfragment D0 or D1 (Fig. 6e) or D4, D2, or D3 (not shown) of the Ad12 *Pst*I-D segment in order to determine which parts of the *Pst*I-D segment were rearranged. Rearranged DNA of the *Pst*I-D segment appeared in off-size fragments comprising the sequences of *Pst*I-D0 in T181 or the sequences of *Pst*I-D1 in A2497-3, T1111(1), and T637. Additional *Msp*I restriction length polymorphisms could not be determined in the Ad12 segments *Eco*RI-D, *Eco*RI-B, *Pst*I-A, *Pst*I-E, and *Bam*HI-B for tumors T1111(1) and T181 and in *Eco*RI-D, *Eco*RI-B, *Pst*I-A, and *Pst*I-E for T637. These data demonstrated that sequences around *Pst*I-D0 and *Pst*I-D1 became preferentially rearranged.

Nucleotide sequence arrangements in reoriented integrated Ad12 genomes determined in molecularly cloned off-size fragments of Ad12 DNA. In Fig. 5, the cleavage patterns of Ad12-specific DNA fragments in cell line T637 (Fig. 5) or in tumor T191 (Fig. 5b) upon cleavage with *Eco*RI, *Hin*dIII, or *Bam*HI (Fig. 5a) or with *Eco*RI or *Bam*HI (Fig. 5b) were schematically presented. In particular, the off-size Ad12 DNA fragments, which hybridized to left-terminal (L), right-terminal (R), or internal (I) segments of the Ad12 genome, were identified. Most of these off-size fragments were molecularly cloned in λ DNA as the vector, and their nucleotide sequences were determined (14a). The nucleotide sequence data for some of the off-size fragments, i.e., *Bam*HI-OS4 (Fig. 7b) and *Bam*HI-OS5 (Fig. 7a) from T637 DNA (Fig. 7a; Table 2) and OS1 (Fig. 7c) from T191 DNA are summarized in Fig. 7. The nucleotide sequence data and the restriction fragment maps derived from them confirmed the extensive rearrangements of viral DNA segments originally located inside the authentic Ad12 virion

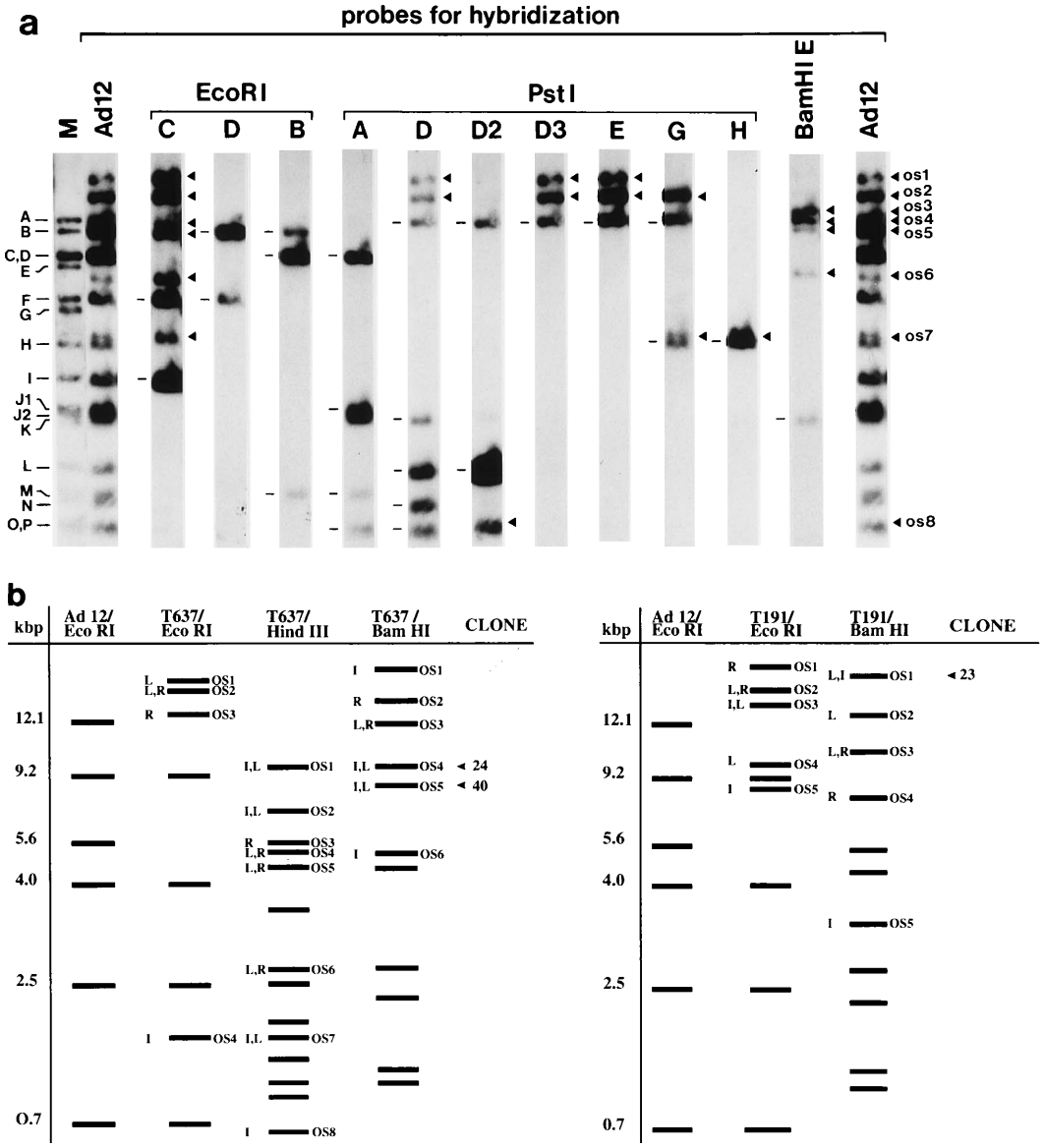


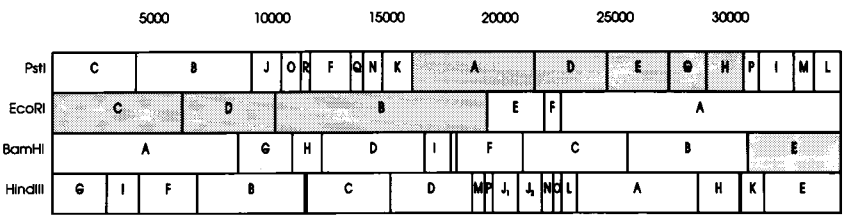
FIG. 5. (a) Localization of internal Ad12 DNA segments in off-size (OS1 to OS8) viral DNA fragments in *Hin*dIII-cleaved T637 DNA. Experimental details and the organization of the autoradiograms shown are explained in the text. The ^{32}P-labeled hybridization probes were total Ad12 DNA (Ad12) and the indicated *Eco*RI or *Pst*I fragments or the *Bam*HI-E fragment of Ad12 DNA. For size markers (M), *Hin*dIII-cut Ad12 DNA was coelectrophoresed. After transfer to Hybond N+ membranes (Amersham), the DNA fragments were hybridized to ^{32}P-labeled Ad12 DNA. In these experiments, 20 μg of cellular DNA was restricted. (b) Schematic representation of Ad12-specific DNA fragments generated by cleavage of the DNA from cell line T637 (left panel) or from tumor T191 (right panel) with *Bam*HI, *Eco*RI, or *Hin*dIII and analysis by Southern blot hybridization. The schemes present a summary of primary hybridization data similar to those shown in Fig. 1 and 3. The gel positions and sizes of the authentic *Eco*RI fragments of Ad12 virion DNA are shown in the left lanes. The off-size fragments of integrated Ad12 DNA (OS1 to OS8), which do not comigrate with any of the authentic virion DNA fragments, are designated, and their homologies to the left (L) Ad12 DNA terminus (fragment *Hin*dIII-G) or to the right (R) Ad12 DNA terminus (fragment *Bam*HI-E) are graphically indicated. I, hybridization to the internal fragment *Pst*I-D. Clones 24 and 40 correspond to *Bam*HI-OS4 and -OS5, respectively, from T637 DNA (Table 1), and clone 23 corresponds to *Bam*HI-OS1 from tumor T191. Details of the cloning procedure into λ Gem 12 DNA are described in Materials and Methods.

genome and their translocation to one of the termini of integrated Ad12 DNA. Details of the schemes presented are described in the legends to Fig. 5b and 7. The Ad12-specific off-size bands BamHI-OS2 from cell line T637 and BamHI-OS1 from tumor T191 were linked to cellular DNA (14a).

Hypomethylation of rearranged Ad12 DNA sequences in cell lines T637 and H191 and in tumors T181 and T1111(1). Restriction enzyme analyses, PCR experiments, and nucleotide sequence determinations revealed rearrangements of sequences in the *Pst*I-D fragment in at least five independent Ad12-induced tumors or Ad12-transformed hamster cells, i.e., T181, T1111(1), T-H191, A2497-3, and T637. These rearrangements juxtaposed *Pst*I-D sequences into the vicinity of the left or right terminus of Ad12 DNA. Nucleotide sequence analyses revealed no cellular DNA at these sites. Surprisingly, the *Hpa*II sites in these rearranged sequences were hypomethylated in the DNA from the Ad12-induced tumors T181 (hybridization to D0) and T1111(1) (hybridization to D1) (Fig. 3a) and from cell line T637 (555-bp fragment in Fig. 8). In the DNAs from cell line T637 (Fig. 1b and 8) and from tumor T1111(1) (data

TABLE 2. Survey of Ad12 DNA segments identified in off-size fragments in integrated Ad12 DNA from the Ad12-transformed hamster cell line T637 by Southern blot hybridization to cloned Ad12 DNA fragments[a]

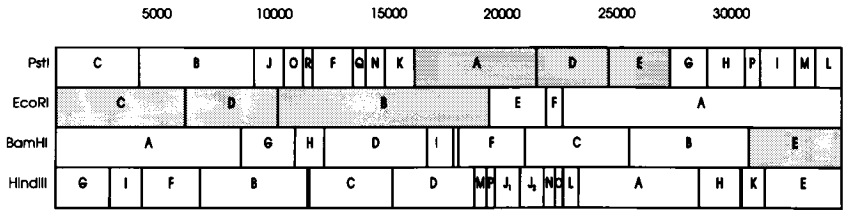
	[Kbp]	EcoRI C	EcoRI D	EcoRI B	PstI A	PstI D	PstIE	G	H	BamHI E
T637 x HindIII										
OS1	10	+	-	-	-	+	+	-	-	-
OS2	7	+	-	-	-	+	+	+	-	-
OS3	5.6	-	-	-	-	-	-	-	-	+
OS4	5.3	+	-	-	-	-	-	-	-	+
OS5	4.5	+	-	-	-	-	-	-	-	+
OS6	2.9	+	-	-	-	-	-	-	-	+
OS7	1.7	+	-	-	-	-	-	+	+	-
OS8	0.5	-	-	-	-	+	-	-	-	-
T637 x PstI										
OS1	5.2	+	-	-	-	-	-	-	-	+
OS2	4.7	+	-	-	-	+	-	-	+	-
OS3	3.9	+	-	-	-	+	-	-	-	-
OS4	3,4	-	-	-	-	-	+	-	-	-
OS5	2,9	-	-	-	-	-	-	-	-	+
OS5a	2.2	-	-	-	-	+	-	-	-	-
OS6	1.8	-	-	-	-	-	-	-	-	+
T637 x BamHI										
OS1	>20	-		-		-				-
OS2	17	-		-		-				+
OS3	12	+		-		-				+
OS4	10	+				+				-
OS5	9	+				+				-
OS6	5.2	-				+				-
T637 x HpaII										
OS1	0.55	+			-	+	-			-
OS2	1.1	-			-	+	-			-
OS3	4.0	+			-	+	-			-
OS4	4.2	+			-	+	-			-
T637 x HhaI										
OS	0.25	-				+				-



[a] These data summarize the hybridization results shown in Fig. 1a (*Hin*dIII) and results (not shown) obtained in a similar way by cleaving T637 DNA with *Pst*I, *Bam*HI, *Hpa*II, or *Hha*I. The cloned hybridization probes with which the off-size fragments OS1 to OS8 have been identified are indicated in the top row and are hatched in the restriction map of Ad12 DNA shown at the bottom. +, positive hybridization in an OS band with the designated cloned restriction fragment; −, absence of a hybridization signal.

TABLE 3. Survey of Ad12 DNA segments identified in off-size fragments in integrated Ad12 DNA from the Ad12-induced tumor T1111(1)[a]

	[Kbp]	EcoRI C	EcoRI D / EcoRI B	PstI A	PstI D	PstI E		BamHI E
T1111(1) x HindIII								
OS1	7	+	-	-	+	+		-
OS2	6.5	+	-	-	-	-		-
OS3	6	-	-	-	+	+		+
OS4	5.5	+	-	-	-	-		+
OS5	5.3	+	-	-	-	-		+
OS6	3.6	+	-	-	+	-		-
OS7	2	+	-	-	-	-		-
OS8	0.7	-	-	-	+	-		-
T1111(1) x EcoRI								
OS1	17	+			+			
OS2	16	-			+			
OS3	15	-			+			
T1111(1) x BamHI								
OS1	7.0	-			+			-
OS2	9.0	+			+			-
T1111(1) x MspI								
OS	0.6	-			+			+



[a] See text and Table 2, footnote *a*.

not shown), some of the *Hpa*II sites located on either side of the junction between the left-terminal sequences of Ad12 DNA and its rearranged *Pst*I-D fragment were also unmethylated.

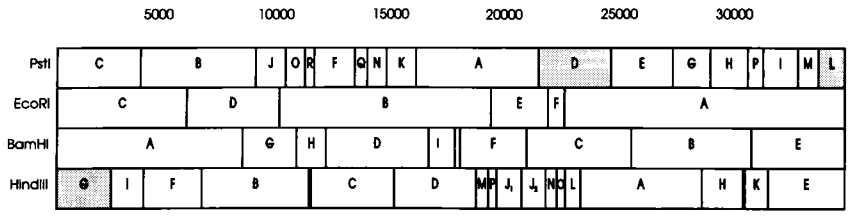
These interpretations were further documented by the purification of some of the rearranged Ad12 sequences by velocity sedimentation on a sucrose gradient (fraction T637B2 in Fig. 8) followed by cleavage with *Msp*I or *Hpa*II and subsequently with *Pst*I, Southern blotting, and hybridization to subfragment D1 or D3 of the *Pst*I-D segment or to the left-terminal *Hin*dIII-G fragment of Ad12 DNA (Fig. 8). The results revealed that the 5′-CCGG-3′ sequences at the junction of the left-terminal fragment and the rearranged internal *Pst*I-D fragment of Ad12 DNA were unmethylated. Similarly, sequences in the relocated left-terminal *Eco*RI-C2 and ΔB fragments, as well as in the *Pst*I-D3 segment at nucleotides 23565 and 24025, showed no methylation. The data indicated that in cell line T637, the rearranged parts of the integrated Ad12 genomes were unmethylated at the 5′-CCGG-3′ sequences over a range of about 9 kbp of Ad12 DNA.

Selection for viral gene expression and/or site of insertion might influence the patterns of de novo methylation in integrated Ad12 DNA. It was conceivable that the differential patterns of Ad12 DNA methylation observed in Ad12-transformed cells or in Ad12-induced tumor cell lines (24) (Fig. 2d) were a consequence of the selection for Ad12-transformed or tumor cells that constitutively expressed the terminal parts of the Ad12 genome (26, 30) (see Table 5). It was thought that functions encoded in these viral DNA segments were involved in Ad12-induced oncogenesis (for a review, see reference 45). Would a different mode of selection give rise to altered patterns of DNA methylation in the integrated viral genomes?

Ad12 DNA was therefore fixed in the hamster genome by cotransfection into BHK21 hamster cells with the pSV2neo plasmid (34). Cells resistant to G418 were subsequently selected and cloned. Two cell lines thus generated, H-Ad12neo5 between passages 15 and 58 and H-Ad12neo2 in passage 41 (Fig. 9), were further investigated. These cell lines exhibited the morphology of BHK21 cells and not that of Ad12-transformed cells. Cleavage of the DNA from these cells with *Msp*I (Fig. 9, left lane for each passage) or *Hpa*II (right lanes) and subsequent hybridization to the ^{32}P-labeled *Eco*RI-C (Fig. 9, left panel) or *Pst*I-D1 (right panel) fragment of Ad12 DNA revealed that either part of the Ad12 genome could become extensively methylated at the 5′-CCGG-3′ sequences. Cell line H-Ad12neo2 did not contain the left-terminal *Eco*RI-C fragment of Ad12 DNA (Fig. 9), and cell line H-Ad12neo5 lacked the left terminal *Msp*I *Eco*RI-C2 and -C4 fragments. These results demonstrated that even the integrated left-terminal sequences in the *Eco*RI-C fragment of Ad12 DNA could become de novo methylated in hamster cells. Ad12 DNA was probably integrated at sites different from those in the Ad12-induced

TABLE 4. Survey of Ad12 DNA segments identified in off-size fragments in integrated Ad12 DNA from the Ad12-induced tumor T181[a]

	[Kbp]	G		EcoRI D	EcoRI B	PstI A	PstI D	PstIE	G	H		L
T181 x HindIII												
OS1	5.2	+					-					+
OS2	4.6	+					-					-
OS3	2.2	-					+					-
OS4	0.8	-					+					-
T181 x PstI												
OS1	5.2	+					-					-
OS2	5.3	+					+					+
OS3	4.6	+					-					+
OS4	2.7	-					+					-
T181 x EcoRI												
OS1	17.7	+					+					+
OS2	10	+					-					-
OS3	4.6	-					+					-
OS4	0.8	-					+					-
T181 x BamHI												
OS1	16	+					-					-
OS2	12	+					-					+
OS3	3.6	+					+					-
T181 x MspI												
OS	0.6	-					+					-



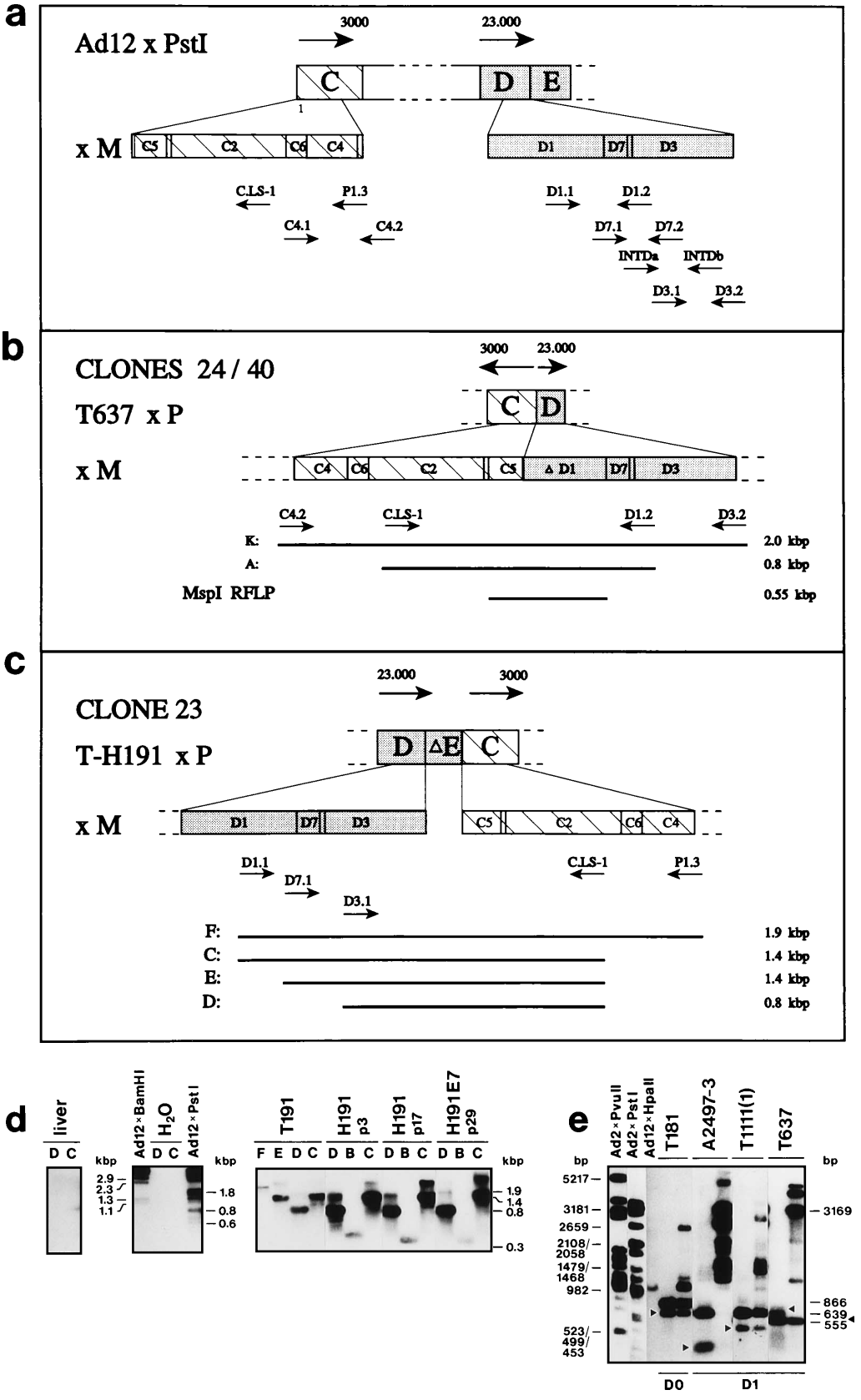
[a] See text and Table 2, footnote *a*.

tumor cell lines or Ad12-transformed cell lines described above. These data argued against the notion that the left-terminal segment of Ad12 DNA contained sequences that by themselves prevented their de novo methylation.

In other cotransfection experiments with pSV2neo, the left-terminal *Eco*RI-C fragment or the paracentrally located *Pst*I-D fragment of Ad12 DNA (maps in Fig. 1a and 2c) was covalently fixed in the BHK21 genome. Upon cotransfection of the left-terminal *Eco*RI-C fragment (nucleotides 1 to 5574) with the pSV2neo construct into BHK21 cells and subsequent G418 selection and cloning, cell lines *Eco*RI-C3 (Fig. 10a) and -C7 (Fig. 10b) arose, in which most copies of the *Eco*RI-C fragment resisted cleavage or were cleaved by *Hpa*II, respectively. Upon cotransfection of the *Pst*I-D fragment with pSV2neo, cell lines PstI-D16 and -D13 were generated. In the *Pst*I-D-transformed cell line PstI-D13 (Fig. 10d), the integrated *Pst*I-D fragment was hypomethylated; i.e., it was cleavable by *Hpa*II even at higher passage levels (passage 55) (Fig. 2b and 10d). In another cell line, PstI-D16, the *Pst*I-D fragment became partly methylated at 5′-CCGG-3′ sites; i.e., it was not cut completely by *Hpa*II at higher passages (passage 55) (Figs. 2b and 10c).

The results from these transfection experiments and their comparison with results gleaned from Ad12-transformed hamster cell lines or Ad12-induced hamster tumor cell lines again demonstrated that a nucleotide sequence by itself was not sufficient to determine patterns of de novo methylation in integrated Ad12 genomes in hamster cells.

Transcriptional activities in segments of the integrated Ad12 genomes. In Ad12-transformed hamster cells, early viral DNA segments were transcribed (26, 30). The major late promoter of Ad12 DNA was silenced in hamster cells by a mitigator element (47, 48). We examined the cytoplasmic RNAs from a number of Ad12-transformed hamster cell lines and from Ad12-induced hamster tumor cells for the presence of specific transcripts from the Ad12 genome. RNA was resolved by electrophoresis on 2.2 M formaldehyde-containing agarose gels and hybridized to specific fragments of Ad12 DNA or their RNA transcripts, which were ^{32}P labeled. The data in Table 5 demonstrate that in the cell lines investigated, the E1 and E4


a
Ad12 x PstI
3000
23.000
C
D
E
x M
C5
C2
C6
C4
D1
D7
D3
C.LS-1
P1.3
D1.1
D1.2
C4.1
C4.2
D7.1
D7.2
INTDa
INTDb
D3.1
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b
CLONES 24 / 40
T637 x P
x M
C4
C6
C2
C5
Δ D1
D7
D3
C4.2
C.LS-1
D1.2
D3.2
K:
2.0 kbp
A:
0.8 kbp
MspI RFLP
0.55 kbp
c
CLONE 23
T-H191 x P
x M
D
ΔE
C
D1
D7
D3
C5
C2
C6
C4
D1.1
D7.1
D3.1
C.LS-1
P1.3
F:
1.9 kbp
C:
1.4 kbp
E:
1.4 kbp
D:
0.8 kbp
d
liver
D C
kbp
2.9
2.3
1.3
1.1
Ad12×BamHI
H2O
Ad12×PstI
D C
1.8
0.8
0.6
T191
H191 p3
H191 p17
H191E7 p29
F E D C
D B C
D B C
D B C
1.9
1.4
0.8
0.3
e
Ad2×PvuII
Ad2×PstI
Ad12×HpaII
T181
A2497-3
T1111(1)
T637
bp
5217
3181
2659
2108/
2058
1479/
1468
982
523/
499/
453
3169
866
639
555
D0
D1

FIG. 6. Identification of rearranged *Pst*I-D segments of Ad12 DNA close to the left terminus of integrated Ad12 DNA in cell lines T637 and H191 and in tumor T191. The models in panels a, b, and c and the PCR data in panel d are described in the text. The subfragments inside the *Pst*I-C and -D fragments were obtained by *Msp*I (M) cleavage (Fig. 2c). In the PCRs (d), 0.5 μg of T191 or H191 DNA from different cell passages was used. The capital letters in panel d relate to the PCR primer combinations explained in panel c. In analogy to the PCR experiments presented in panel d, the rearranged Ad12 integrates of clones 23 and 40 in T637 DNA gave rise to identical PCR products of 2.0 and 0.8 kbp with primer sets K and A, respectively (b) (data not shown). In addition, rearranged Ad12 *Pst*I-D sequences in clones 23 and 40 were apparent as an *Msp*I fragment of 555 bp (b). (e) Analysis of methylation in the 5′-CCGG-3′ sequences or rearranged *Pst*I-D segments in tumors and cell lines. DNA [20 μg from tumors T181 and T1111(1) or from cell lines A2497-3 and T637] was cleaved with *Msp*I and *Pst*I (left lanes) or *Hpa*II and *Pst*I (right lanes). The fragments were alkali transferred to Hybond N+ nylon membranes (Amersham) and hybridized to the isolated ^{32}P-labeled HpaII subfragment D0 or D1 from the *Pst*I-D segment of Ad12 DNA. The locations of the hybridization probes were apparent from the *Msp*I-*Hpa*II maps of the Ad12 genome in Fig. 2c.

regions were transcribed. These regions corresponded to the left- and right-terminal segments of Ad12 DNA, respectively, which were hypomethylated in the cell lines studied. Thus, transcriptional activity was again associated with hypomethylation in this system (39).

DISCUSSION

Modes of Ad12 DNA integration. In hamster cell genomes, Ad12 DNA molecules are integrated predominantly intact and in an orientation colinear with that in virion DNA. Many lines of evidence from analyses of integrated adenovirus DNA sequences argue for the insertion at many different, and not at specific, cellular genomic sites in different tumors and cell lines. It is likely that transcriptionally active regions have an enhanced propensity for insertional recombination with foreign (Ad12) DNA (31). We have also reported (25) that under certain, so far undefined, cell culture conditions, Ad12-induced tumor cell lines with sites selective for integration of Ad12 DNA have arisen. In several instances, e.g., cell lines HA12/7 (13) and T637 (this report) and tumor T1111(1) (19), rearrangements of viral DNA at both termini of the integrated Ad12 DNA have been observed.

We have now documented in detail the following new aspects of integration of foreign Ad12 DNA. (i) In at least five independently Ad12-induced hamster tumors and Ad12-transformed hamster cell lines, i.e., T181, T191, T1111(1), T637, and A2497-3, internal Ad12 DNA sequences comprising the *Pst*I-D, -E, G, and -H segments of Ad12 DNA are rearranged. (ii) Some of the rearranged *Pst*I-D sequences are flanked by left- or right-terminal Ad12 DNA sequences. It is conceivable that patchy sequence homologies between the *Pst*I-D sequence and the inverted *Pst*I-C sequence of Ad12 DNA (data not shown) may have played a role in generating these recombination events leading to rearrangements of integrated Ad12 DNA.

Rearrangements in parts of the integrated Ad12 DNA sequences. It is striking that in several independently isolated Ad12-transformed hamster cell lines and in several independently Ad12-induced hamster tumors, very similar rearrangements of integrated Ad12 DNA segments have been observed. Apparently, nucleotide sequences in and around the *Pst*I-D fragment of Ad12 DNA, i.e., the viral DNA segment between nucleotides 20885 and 24053, have a propensity to recombine with other cellular or viral DNA sequences, although they are located inside the intact Ad12 DNA molecule and cannot recombine via free ends. The same viral DNA segment has been reported to recombine with hamster cellular preinsertion sequences more frequently than other parts of the Ad12 genome in a cell-free recombination system employing fractionated nuclear extracts of BHK21 hamster cells (12). This increased recombination frequency in vitro is not due to direct sequence homologies between the *Pst*I-D fragment of Ad12 DNA and the preinsertion hamster DNA sequences. It will have to be determined what nucleotide sequence, structural, or signal features of this viral DNA fragment could be responsible for its behavior in recombination experiments.

The search for signals involved in the initiation of de novo methylation of integrated foreign DNA. It has been considered that a specific nucleotide sequence may be related to the ini-

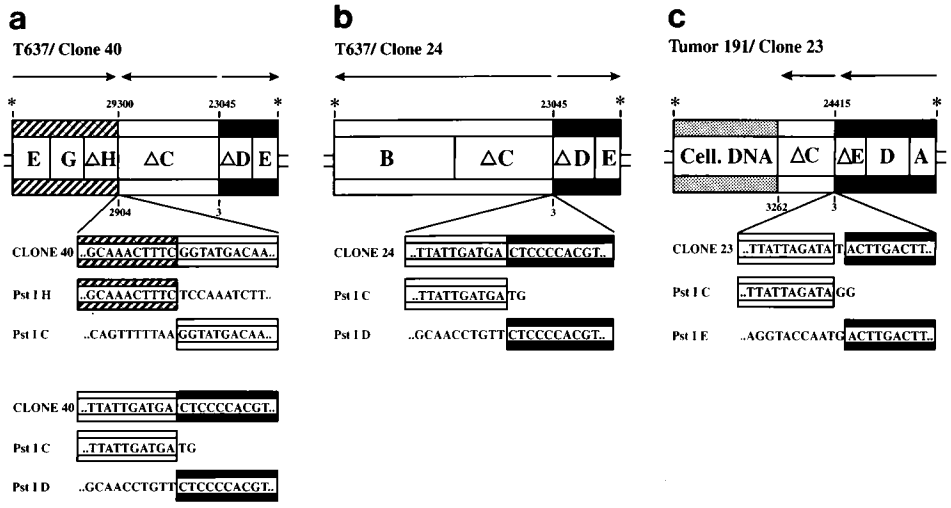

FIG. 7. Arrangements of viral DNA fragments and nucleotide sequences at sites of junction in the off-size fragments *Bam*HI-OS5 (clone 40) (a) and OS4 (clone 24) (b) from cell line T637 (Fig. 5b) and in *Bam*HI-OS1 (clone 23) (c) from tumor T191 (Fig. 5b). The asterisks indicate the *Bam*HI sites with which the off-size fragments have been cloned into the λ DNA vector. Nucleotide numbers are those of the authentic Ad12 DNA sequence (35). The nucleotide sequences at various transition sites were determined and shown graphically; e.g., *Pst*I-H shows the sequence of Ad12 DNA in the block designated ΔH (hatched bar) up to the junction site and then continues with the authentic sequence of the *Pst*I-H fragment (no bar) that has been deleted in the junction site sequence.

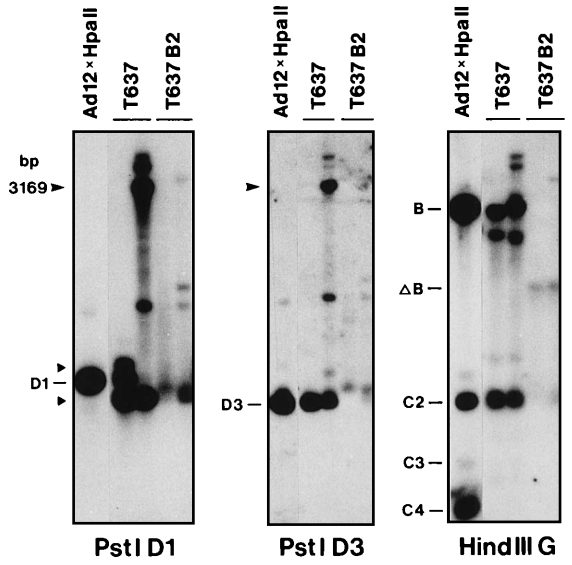

FIG. 8. Some of the integrated *Pst*I-D segments of Ad12 DNA in cell line T637 were rearranged and remained unmethylated. The DNA in a sucrose gradient fraction from *Hin*dIII-cut T637 DNA (T637B2) was cleaved first with *Msp*I (left lanes) or *Hpa*II (right lanes) and subsequently with *Pst*I. *Hpa*II-cleaved Ad12 DNA served as a size marker. The fragments were blotted and hybridized to the indicated Ad12 DNA fragments, which were used as ^{32}P-labeled probes. The *Msp*I-B fragment of Ad12 DNA was cut by *Hin*dIII, generating a shorter (ΔB) fragment in the sucrose gradient fraction of T637B2 DNA. The arrowheads pointing to the 700- and 555-bp fragments indicate an *Msp*I polymorphism in T637 DNA, due to the rearranged *Pst*I-D fragments.

tiation of de novo methylation (10, 22, 42). The results with integrated Ad12 DNA sequences presented here suggest that the mechanism of de novo DNA methylation cannot be dependent exclusively on specific DNA sequences. Other parameters, like sites of foreign DNA insertion and differences in the sequence environment, as well as the chromatin structure (14) and gene expression in different parts of the cellular or viral genome, can also determine the de novo patterns of DNA methylation.

Why not sequence alone? The following observations are not compatible with the notion that a certain nucleotide sequence by itself is sufficient to elicit the de novo methylation reaction.

(i) In the Ad12-induced hamster tumors and even in long-term-cultured cell lines, some of the integrated Ad12 molecules are still unmethylated at the 5′-CCGG-3′ sites, whereas other Ad12 genomes are disproportionately methylated to a high degree (Fig. 1a and 3a).

(ii) In cell lines established from Ad12-induced hamster tumor cells, de novo methylation reproducibly commences in two paracentrally located internal segments of the integrated Ad12 DNA molecule (Fig. 2d). In the long-term-cultured Ad12-transformed hamster cell lines T637, A2497-3, and HA12/7 and in the Ad12-induced hamster tumor cell line HT5, the *Pst*I-D segment of Ad12 DNA is heavily methylated in 5′-CCGG-3′ sequences in the continuous and intact Ad12 genomes. This viral DNA segment is thus defined as one of the methylation centers in integrated Ad12 DNA. However, sequences from the same *Pst*I-D segment can be integrated as separate fragments outside the intact Ad12 genome, e.g., repositioned to the vicinity of the hypomethylated left or right termini of the intact Ad12 genomes. These rearranged *Pst*I-D fragments are hypomethylated or unmethylated at 5′-CC GG-3′ sites. The locations of their integration sites and/or the proximity to the hypomethylated terminal Ad12 segments might limit accessibility of the cell's de novo methylation machinery.

(iii) When the intact Ad12 DNA molecule or its *Eco*RI-C or *Pst*I-D fragment is transfected into BHK21 cells and genomically fixed by G418 selection for expression of the cotransfected neomycin phosphotransferase gene, methylation patterns in the integrated Ad12 DNA segments arise that are different from those observed in Ad12-transformed or Ad12-induced tumor cells. In the intact Ad12 genomes fixed by transfection, the left-terminal *Eco*RI-C fragment can become heavily methylated. In different cloned cell lines carrying the integrated *Eco*RI-C fragment of Ad12 DNA, the viral 5′-

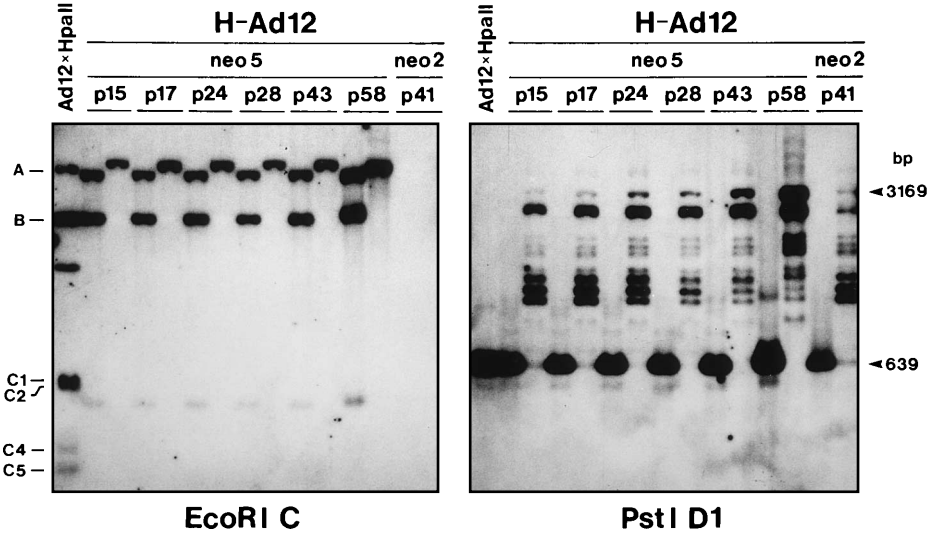

FIG. 9. Upon transfection of Ad12 DNA and selection for neomycin resistance, even the integrated left-terminal *Eco*RI-C fragment of Ad12 DNA became de novo methylated at 5′-CCGG-3′ sequences. Experimental details are described in the text. DNA from cell line H-Ad12neo2 or H-Ad12neo5 in the indicated passages (p) after cloning was cleaved with *Msp*I (left lanes) or with *Hpa*II (right lanes), transferred to Hybond N+ membranes, and hybridized to the ^{32}P-labeled *Eco*RI-C (left panel) or *Pst*I-D1 (right panel) fragment. The *Hpa*II fragments of Ad12 DNA were coelectrophoresed as markers.

TABLE 5. Transcription of the E1 and E4 regions of the integrated Ad12 DNA in Ad12-induced tumors, tumor cell lines, and Ad12-transformed cell lines[a]

Region	Ad12 tumors			Ad12-induced tumor cell lines			Ad12-transformed cell lines		
	Methylation in:		Transcription	Methylation in:		Transcription	Methylation in:		Transcription[b]
	*Hha*I sites	*Hpa*II sites		*Hha*I sites	*Hpa*II sites		*Hha*I sites	*Hpa*II sites	
E1	0	0	ND[c]	0	0	+	0	0	+
E4	0	0	ND	0	0	+[d]	0	0	+
Major late promoter[e]	0	0	ND	1	1	ND	2	1/2	−

[a] Experimental details are described in the text. The symbols 0, 1, and 2 are the same as in Fig. 2. Ad12 tumors are T181, T191, T201/1, T201/2, T1111(1), and T1111(2); Ad12-induced tumor cell lines are H191, H211, HT5, and H281 IID3; Ad12-transformed cell lines are HA12/7, A2497-3, and T637.
[b] Partly based on previously published work (26, 30).
[c] ND, not done.
[d] Transcription of the E4 region in the tumor line HT5 was very weak.
[e] The major late promoter of Ad12 DNA was silenced by a mitigator element (47, 48).

CCGG-3′ sequences can become heavily methylated or remain unmethylated in different cell lines, perhaps depending on the site of integration into the host cell genome. Similar observations have been made for the *Pst*I-D fragment of Ad12 DNA in different cloned cell lines. The 5′-CCGG-3′ sequences in the *Pst*I-D fragment can become significantly de novo methylated or remain hypomethylated.

(iv) The *Pst*I-D sequences of Ad12 DNA have been found to be de novo methylated in a cell line-specific pattern (Fig. 3b and 6e and data not shown). In cell lines kept in culture for several months (e.g., H191, H281, H-Ad12neo2, and H-Ad12neo5) or even years (e.g., A2497-3, HA12/7, and HT5), only a portion of the integrated Ad12 genomes is completely methylated at 5′-CCGG-3′ (Fig. 3b and 4d to f) and 5′-GCGC-3′ (data not shown) sites in the *Pst*I-D fragment.

Previously published results on the state of methylation in integrated Ad2 genomes in hamster cells also argue against the notion that nucleotide sequences predominantly direct de novo methylation. The late E2A promoter in integrated Ad2 genomes is methylated at all 5′-CG-3′ sequences in the Ad2-transformed hamster cell line HE1 but is unmethylated at all of these sequences in the HE2 cell line (43, 44). Similarly, after the fixation of the late E2A promoter of Ad2 DNA by microinjection into mouse oocytes, the 5′-CCGG-3′ sequences in this construct have been found to be methylated in some transgenic mouse strains but not in others (17).

In the symmetric recombinant SYREC between the left terminus of Ad12 DNA and human cellular DNA (2), the cellular DNA sequences are not methylated at 5′-CCGG-3′ sites, whereas the same cellular sequences as part of the human genome in KB cells are extensively methylated (2). Nuclear location, but not nucleotide sequence by itself, can also affect DNA methylation.

In the DNA from the Ad12-induced hamster tumor T1112(2), the left-terminal Ad12 sequences are flanked by unmethylated cellular DNA of about 1 kbp in length, whereas

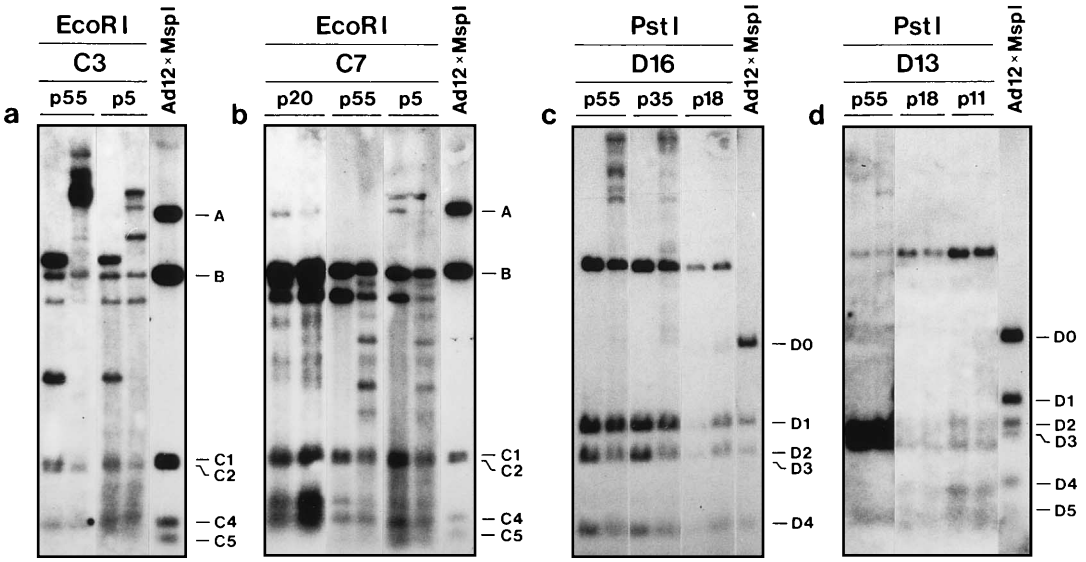

FIG. 10. The de novo methylation of Ad12 DNA fragments inserted by transfection into the hamster genome differed from that in intact Ad12 genomes in tumors, tumor cell lines, and Ad12-transformed hamster cell lines. Cell lines EcoRI-C3 (a) and -C7 (b) were generated by cotransfecting BHK21 hamster cells with the isolated *Eco*RI-C fragment of Ad12 DNA and the pSV2neo construct. Similarly, cell lines PstI-D16 (c) and -D13 (d) were obtained by cotransfection of the isolated *Pst*I-D fragment of Ad12 DNA and the pSV2neo construct (see Materials and Methods). At the indicated passage levels (p), the DNA was isolated and then cleaved with *Msp*I (left lanes in each passage set) or *Hpa*II (right lanes), blotted, and hybridized to the ^{32}P-labeled *Eco*RI-C fragment (a and b) or *Pst*I-D fragment (c and d) of Ad12 DNA. The DNA isolated from cell line EcoRI-C7 (p20) and cleaved with *Msp*I or *Hpa*II was hybridized to ^{32}P-labeled Ad12 DNA. *Msp*I-cut Ad12 DNA was coelectrophoresed as a size marker.

the same sequences on the unoccupied chromosome and at the preinsertion site in nontransformed hamster cells are unmethylated (20). Changes in DNA methylation in cellular DNA sequences in the vicinity of a retroviral integration site have also been described (11).

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