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Foreign DNA Integration

GENOME-WIDE PERTURBATIONS OF METHYLATION AND TRANSCRIPTION IN THE RECIPIENT GENOMES*

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In hamster cells transgenic for the DNA of adenovirus type 12 (Ad12) or for the DNA of bacteriophage λ, the patterns of DNA methylation in specific cellular genes or DNA segments remote from the site of transgene insertion were altered. In the present report, a wide scope of cellular DNA segments and genes was analyzed. The technique of methylation-sensitive representational difference analysis (MS-RDA) was based on a subtractive hybridization protocol after selecting against DNA segments that were heavily methylated and hence rarely cleaved by the methylation-sensitive endonuclease *Hpa*II. The MS-RDA protocol led to the isolation of several cellular DNA segments that were indeed more heavily methylated in λ DNA-transgenic hamster cell lines. By applying the suppressive subtractive hybridization technique to cDNA preparations from nontransgenic and Ad12-transformed or λ DNA-transgenic hamster cells, several cellular genes with altered transcription patterns were cloned from Ad12-transformed or λ DNA-transgenic hamster cells. Many of the DNA segments with altered methylation, which were isolated by a newly developed methylation-sensitive amplicon subtraction protocol, and cDNA fragments derived from genes with altered transcription patterns were identified by their nucleotide sequences. In control experiments, no differences in gene expression or DNA methylation patterns were detectable among individual nontransgenic BHK21 cell clones. In one mouse line transgenic for the DNA of bacteriophage λ, hypermethylation was observed in the imprinted *Igf2r* gene in DNA from heart muscle. Two mouse lines transgenic for an adenovirus promoter-indicator gene construct showed hypomethylation in the *interleukin 10* and *Igf2r* loci. We conclude that the insertion of foreign DNA into an established mammalian genome can lead to alterations in cellular DNA methylation and transcription patterns. It is conceivable that the genes and DNA segments affected by these alterations depend on the site(s) of foreign DNA insertion.

In various natural and experimental scenarios, mammalian genomes become the targets for foreign DNA insertions. Many DNA- and RNA-containing viruses are capable of integrating their genomes into the genomes of their host cells. Numerous experiments designed for the artificial transfer of genes into mammalian cells aim at the permanent fixation of these genes in established eukaryotic genomes. In transgenic organisms that have been propagated from successfully transformed embryonal cells, all cells carry integrated foreign DNA. We have started to investigate the structural and functional consequences of the insertion of foreign DNA into established mammalian genomes. The *de novo* methylation of the integrated DNA and alterations in the patterns of DNA methylation in the recipient genomes at the site of insertion and remote from it have been of particular interest (for review, see Ref. 1). By using different techniques including the bisulfite protocol of the genomic sequencing technique (2, 3), we have documented extensive changes in the patterns of DNA methylation at several cellular sites remote from the loci of insertion of the DNA of adenovirus type 12 (Ad12)[1] and lesser changes in cells transgenic for the DNA of bacteriophage λ (4, 5). Because λ DNA is not transcribed in transgenic mammalian cells, alterations of methylation patterns subsequent to foreign DNA insertion are not dependent on foreign gene transcription. It has been shown earlier that cellular DNA sequences immediately abutting the foreign DNA integrates also exhibit changes in DNA methylation (6, 7). It is presently unknown by what mechanisms the insertion of foreign DNA affects the organization and function of the recipient genome. Does the site of foreign gene integration determine where the remote effects occur, and is a critical size of integrated DNA required? We surmise that the acquisition of many kilobases or even a megabase of inserted DNA alters the chromatin topology and can thus influence the function of specific parts of the genome. At present, these interdependencies are essentially unknown. We have therefore started a step-by-step analysis of these alterations by studying changes in transcription and DNA methylation patterns.

In this report, we have utilized a differential hybridization method with cDNAs prepared from λ DNA-transgenic cells as compared with nontransgenic cells. We have used the method of methylation-sensitive representational difference analysis (MS-RDA) and the novel method of methylation-sensitive amplicon subtraction (MS-AS) to detect cellular DNA segments with altered methylation patterns in transgenic cells. These genome-wide scanning methods have led to the isolation of several cellular genes and DNA segments with changes in DNA methylation or transcription. Differentially methylated DNA segments obtained with the MS-RDA method usually are highly repetitive and show no homology to known sequences. The newly developed MS-AS method on the other hand has facilitated the cloning of DNA segments with high C+G con-

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[1] The abbreviations used are: Ad12, adenovirus type 12; MS-RDA, methylation-sensitive representational difference analysis; MS-AS, methylation-sensitive amplicon subtraction; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; IAP, intracisternal A particle; IL-10, interleukin 10; PSL, photostimulated luminescence.

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tents, which have been characterized by their nucleotide sequences; and their functions have been derived from GenBank™ data collections.

EXPERIMENTAL PROCEDURES

Transgenic Mice—The origin of mouse lines transgenic for a construct carrying the chloramphenicol acetyltransferase gene under the control of the E2A late promoter of adenovirus type 2 (pAd2E2AL-CAT) has been described (8, 9). Microinjection of the M-*Hpa*II (5′-CCGG-3′) premethylated construct generated the founder animal of mouse line 8-1, and microinjection of unmethylated DNA generated that of line 7-1.

Cell Lines—The Ad12-transformed hamster cell line T637 (with multiple copies of integrated Ad12 DNA (10)) was described elsewhere. The clonal BHK21 cell lines transgenic for the DNA of bacteriophage λ and plasmid pSV2neo (11) were presented earlier (5). After selection, these cell lines were cultivated without the addition of G418. For control experiments, nontransgenic BHK21 cells were recloned three to four times, and the thus established clonal lines were used. All cell lines were cultivated in Dulbecco's medium with 10% fetal calf serum.

Standard Techniques of Molecular Biology—DNA extraction, including RNase treatment, Southern transfer hybridization (12, 13), restriction analyses with the methylation-sensitive endonucleases *Hpa*II, *Hha*I, or the control enzyme *Msp*I, and electrophoresis in 0.8–1.0% agarose gels were all detailed earlier (*e.g.* Ref. 14). Autoradiograms were evaluated quantitatively by using a Fuji X-BAS 1000 phosphorimager.

Identification and Isolation of Differentially Methylated DNA Segments by MS-RDA (15)—After *Hpa*II cleavage of tester or driver DNAs and ligation of the fragments to Rhpa adaptors, amplicons were prepared by 25–35 PCR cycles using the Rhpa24 oligonucleotides as primers under the conditions described elsewhere (16). In these experiments, DNA from the cell line T637 or from the λ DNA-transgenic BHK21 cell lines was the "driver" and the DNA from the nontransgenic BHK21 cells the "tester" DNA. Amplification products were cut with *Msp*I and purified by gel filtration. Only the tester amplicon preparation (1 μg) was subsequently ligated to 500 pmol of the Jhpa adaptor (designations and sequences of adaptors available on request). All adaptors carried a CG overlap, which was ligated to the GC overlap of the *Hpa*II and *Msp*I cleavage products. When selective hybridization was performed, only self-reannealed tester molecules with adaptors at both ends became amplified efficiently in the following PCR. Adaptor-ligated tester molecules only self-reannealed in the absence of homologous partners in the driver amplicon pool. Amounts of 40–100 ng of the ligation product were mixed with 30–40 μg of driver amplicon DNA. The mixture was phenol-extracted, ethanol-precipitated, and dissolved in 3 mM EDTA, 3 mM HEPES, pH 8.0. The subtractive hybridization was performed by denaturing the tester-driver DNA mixture at 96 °C for 10 min followed by reannealing at 67 °C for 18 h in the presence of 1 M NaCl. After adding 45 μl of a prewarmed dilution buffer (1 M NaCl, 8 mM Tris-HCl, pH 7.4, 0.8 mM EDTA, pH 8.0), one-tenth of the product was amplified with the Jhpa24 primer for 13–15 cycles. The PCR solution was then heated to 72 °C for 10 min, and the linearly amplified fragments were cleaved with 100 units of mung bean nuclease in the presence of 1 mM $ZnSO_4$. Double-stranded DNA was further amplified by PCR for 20–35 cycles using the Jhpa24 primer.

In a second cycle of competitive hybridization, 10–40 ng of the PCR product was ligated to the Nhpa adaptor and again mixed with 40 μg of driver amplicon DNA. The product was cloned into the pGEM-T vector (Promega) and transfected into XL1BlueMRF′ bacteria. The insert from positive clones was PCR-amplified using the SP6 and T7 primers and restricted with *Msp*I. To test for the effectiveness of the MS-RDA protocol, linearized and *in vitro Hpa*II-premethylated pGL2 control vector (Promega) or the pN3 plasmid containing human proto-RET cDNA p51 (17) was added in 1 or 2 genome equivalents to the driver DNA prior to *Hpa*II cleavage. The same amount of unmethylated plasmid was added to the tester DNA. Southern blot analyses of amplicons, first- and second-round difference products with the pGL2-control or the pN3 probe, revealed the levels of enrichment of the exogenously added DNAs in the course of the MS-RDA procedure. The nucleotide sequences of all primers and adaptors referred to in this section as well as the annealing temperatures chosen in individual experiments were not reproduced here but will be available on request.

Screening and Analysis of Second-round Difference Products—PCR-amplified plasmid inserts were denatured and gridded identically on two GeneScreen Plus membranes. These membranes were then hybridized against ^{32}P-labeled amplicon DNA from either the tester or the driver. Putative difference products as determined by their differences in signal intensities between tester and driver were selected and used for Southern blot analyses with cellular DNA. The nucleotide sequences of differentially methylated clones were determined with an Applied Biosystems 377 DNA sequencer by standard methods (18), and a homology search was performed at a GenBank™ Web site.

Isolation of Differentially Expressed Genes: Subtraction of cDNA Libraries—Differences in gene expression between transformed or transgenic cells *versus* nontransgenic BHK21 cells were determined by the cDNA subtraction method (19). Poly(A$^+$) mRNA was isolated using an mRNA isolation kit (Roche Molecular Biochemicals). From both the tester and the driver cell lines, cDNA libraries were constructed by oligo(dT) priming with 2 μg of poly(A$^+$) mRNA. The protocol specified by CLONTECH was followed for the subtraction of the cDNA libraries. For PCR reactions, the Advantage 2 polymerase mix (CLONTECH) was used. Difference products were cloned into the pGEM-T vector (Promega) and transfected into competent XL1BlueMRF′ cells. Insert-positive colonies were cultivated in 96-well plates, 3 μl of each culture was used for PCR with the SP6 and T7 primers to amplify the plasmid inserts. The PCR products were denaturated in 0.8 M NaOH, 50 mM EDTA and subsequently arrayed on GeneScreen Plus membranes. The two identical membranes were hybridized against the ^{32}P-labeled cDNA libraries from either tester or driver. Positive clones were selected and further analyzed by Northern blot experiments using either cytoplasmatic RNA isolated as described (20) or poly(A$^+$) mRNA.

MS-AS: A Novel Strategy for Identifying Differentially Methylated Sequences in Complex Genomes—This new protocol (21) facilitates comparisons between the representations of DNA fragments that are present in one amplicon sample but not in another. Under the conditions chosen, only fragments of up to 2.5 kilobase pairs were effectively PCR-amplified. Larger fragments derived from methylated and therefore *Hpa*II cleavage-resistant DNA segments were not or were inefficiently amplified. Differential methylation in the tester and driver DNAs will therefore result in different representations. Conventional RDA required several rounds of hybridization, more material, and favored the amplification of repetitive sequences. To overcome vast differences in abundance, the newly developed subtraction protocol included two separate initial subtractive hybridizations with representations (amplicons) of genomic DNA in combination with suppressive PCR (19, 22). Genomic tester or driver DNAs (5 μg) were cleaved with excess amounts of *Hpa*II. After ligating 200 ng of the *Hpa*II fragments to the Rhpa adaptor, amplicons were prepared by PCR using the Rhpa24 primer as described (16). The PCR products were cut with *Rsa*I to create blunt ends. The tester DNA was subdivided into two 500-ng portions, which were ligated to either adaptor L or R. Two rounds of hybridization followed. In the first, 2 μl of *Rsa*I-cleaved driver amplicon (1–2 μg) was added to 1 μl of tester DNA (50 ng) and to 1 μl of hybridization buffer containing 200 mM HEPES, pH 8.3, 2 M NaCl, 0.08 mM EDTA, pH 8.0, 40% (w/v) polyethylene glycol (PEG 8000). The solution was overlaid with mineral oil, heat-denatured (98 °C, 2.5 min), and annealed for 8 h at 68 °C. For the second hybridization, the two primary hybridization samples were mixed without denaturation. Denatured driver (500 ng in 1 μl) was added and hybridized for an additional 14 h at 68 °C and then diluted with 200 μl of preheated dilution buffer (20 mM HEPES, pH 8.3, 50 mM NaCl, 0.2 mM EDTA). The hybridization products were subsequently amplified by primary and nested PCRs. After ligating the PCR products into the pGEM-T vector, difference products were screened and analyzed as described above.

RESULTS

Differential Expression of Cellular Genes in BHK21 Hamster Cells Transgenic for Ad12 or λ DNA—The cDNA subtraction method was applied to reverse transcripts of mRNAs isolated from the Ad12-transformed BHK21 hamster cell line T637 or from BHK21 cells transgenic for the DNA of bacteriophage λ, *e.g.* cell lines L10 and L18. After one cycle of subtractive hybridization to a cDNA preparation from nontransgenic BHK21 cells, the PCR products were cloned into the pGEM-T vector and arrayed on GeneScreen Plus membranes. The DNA was then hybridized to ^{32}P-labeled cDNA from nontransgenic BHK21 cells, from T637 cells (Fig. 1*A*), or from the λ DNA-transgenic cell line L10 (Fig. 1*C*). The clones with array numbers as indicated in Fig. 1, *B* and *D*, showed marked differences, and these cDNAs were used for RNA transfer hybridization (Northern blot) experiments with RNAs from BHK21 cells (*B*), T637 cells (*T*), or the L10 cell line. All differ-

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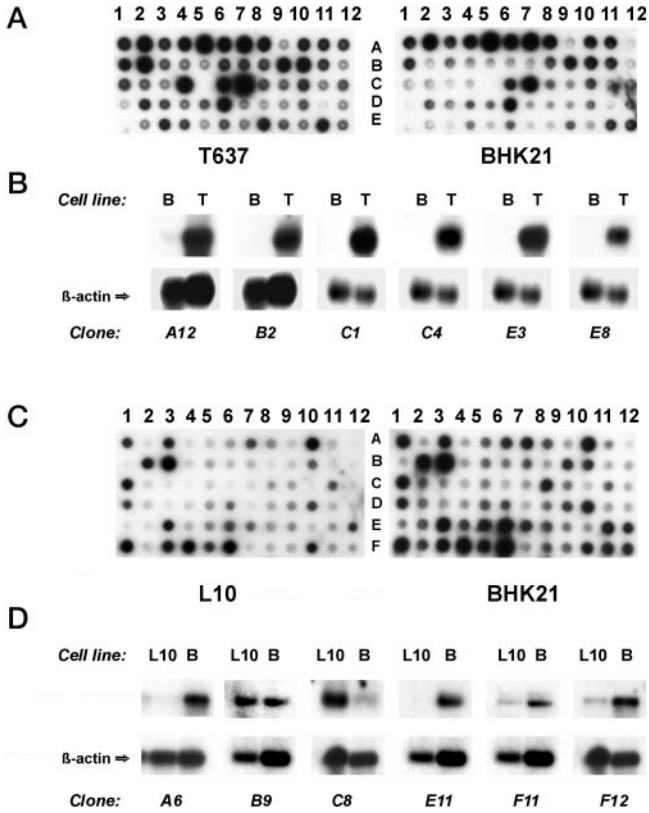

FIG. 1. **Differential transcription of cellular genes in BHK21 hamster cells transgenic for Ad12 or λ DNA.** Individual PCR-amplified difference products were gridded on GeneScreen Plus membranes as dot blot arrays of difference products after one round of cDNA subtraction. Membranes were hybridized against ^{32}P-labeled cDNA preparations from nontransgenic BHK21 or T637 cells (*A*) or from the λ DNA-transgenic cell line 10 (*L10*) (*C*). Clones with marked differences in signal intensities between BHK21 (*lanes B*) and T637 (*lanes T*) or L10 were analyzed further in Northern blot experiments using either 30 μg of cytoplasmic RNA (*B*) or 2 μg of poly(A$^+$) mRNA (*D*). Samples were analyzed by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde. Upon transfer to GeneScreen Plus membranes, the RNA was hybridized to ^{32}P-labeled difference clones with array numbers as indicated in *B* and *D* or to β-actin (*open arrows*) as shown in the *lower panels* of *B* and *D*. Results from phosphorimager analyses of signals obtained in *D* are described in the text.

ential cDNA clones were strongly expressed in T637 but not in BHK21 cells.

For the two λ DNA-transgenic cell lines L10 and L18, 198 cDNA clones each were analyzed by these methods. Phosphorimager analyses of signal intensities on Northern blot experiments revealed differences between different mRNA levels of up to 6-fold. Transcription of the E11 clone was not detectable in the λ DNA-transgenic cell line L10 (Fig. 1*D*). Several of the differentially expressed clones from cell line L10 were identified by their nucleotide sequences, and the most convincing data base matches for the nucleotide sequences of some of these clones were included in Table I. The differential cDNA arrays presented in Fig. 2 revealed seven cDNA segments, clones 80, 4, 10, 29, 31, 32, 58, which were expressed in cell line L18 but not, or to a very limited extent, in BHK21 cells. The numbers 80, 4, 10, 29, 31, 32, 35, 58 designated individual cDNA clones that were selected for DNA array analyses. The transcription of known genes, murine DNA methyltransferase (*Dnmt1*), *ADPRT*, *β-actin*, *IAPI*, or *pSV2neo*, showed no differences between the L18 and the BHK21 parental cell line (Fig. 2). At the time the L18 line was analyzed, G418 selection had long been discontinued, and hence *pSV2neo* expression was not required. In several λ DNA-transgenic cell lines without *pSV2neo* expression, differences in DNA methylation or transcription were observed. In contrast, some of the cell lines still expressing the resistance marker did not exhibit these effects. In the λ DNA-transgenic cell lines, λ DNA transcripts were never found. Moreover, we observed no differences in methylation in the cDNA clones analyzed between the transgenic and nontransgenic cells. Of course, this experimental protocol based on cDNA comparisons precluded promoter analyses that would have been necessary for functionally meaningful investigations of correlations between promoter methylation and activity.

Differentially Methylated Cellular DNA Fragments in Hamster Cell Lines Transgenic for Ad12 (T637) or Bacteriophage λ DNA (L12, L18)—Methylation patterns in a wide selection of cellular DNA sequences were compared between nontransgenic BHK21 cells and Ad12 DNA- or λ DNA-transgenic BHK21 cells. The methods of MS-AS or MS-RDA compare representations (amplicons) of DNA fragments and have not been applied previously in analyses with transgenic cell lines. Amplicons were produced by cleavage of genomic DNA with *Hpa*II followed by PCR amplification of these fragments using a universal adaptor that was ligated to the *Hpa*II fragments as primer annealing site. The conditions chosen for PCR favored the amplification of small fragments derived from hypomethylated and therefore *Hpa*II-restricted genomic areas. In contrast, large *Hpa*II fragments resulting from highly methylated DNA were not enriched by PCR. Differences in DNA methylation between the two cell lines resulted in different fragment representations. The goal of each subtractive hybridization method was to enrich and clone DNA fragments present in only one amplicon. After amplicon preparation, the universal adaptors were removed, and only the tester amplicon fragments were ligated to new adaptors.

During the subtractive hybridization of adaptor-ligated tester fragments to excess amounts of driver amplicon, only tester fragments without a homologous partner in the driver pool self-reannealed and hence carried adaptor sequences on both ends of the DNA molecule. Fragments common to tester and driver formed heterohybrids with adaptor sequences on only one terminus. The PCR following the subtractive hybridization step led to the exponential amplification of self-reannealed tester fragments. MS-RDA required several cycles of subtractive hybridization and PCR to enrich differentially methylated DNA fragments from the tester cell lines, as the method did not take into account the large differences in relative abundance of individual DNA sequences.

MS-AS used a normalization step and a special form of PCR to adjust for the sequence abundance in the tester amplicon. A detailed protocol for the MS-AS method has been described elsewhere (21). All differentially methylated DNA fragments derived from both low abundance and repetitive genomic sequences were equally enriched by MS-AS. In addition, the number of false-positive fragments of repetitive DNA fragments was reduced by the internal normalization step of the MS-AS protocol.

Individual DNA clones, which were identified by DNA array hybridization (data not shown), were isolated and used as ^{32}P-labeled hybridization probes with DNA from the nontransgenic BHK21 cells, the Ad12-transformed BHK21 cell line T637, or the λ DNA-transgenic BHK21 cell lines L12 or L18. These DNAs had all been cleaved with *Msp*I (*M*), *Hpa*II (*Hp*), or *Hha*I (*Hh*) prior to electrophoresis and Southern transfer. The data presented in Fig. 3, *A* (T637) and *B* (L12, L18), demonstrate the differences in cleavage patterns for the methylation-sensitive restriction endonucleases *Hpa*II and *Hha*I between the DNAs from the nontransgenic BHK21 cells and the Ad12 DNA (T637, Fig. 3*A*)- or λ DNA-transgenic cell lines (L12, L18,

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TABLE I

Database homologies of differentially expressed DNA-segments

Details of these analyses were described in the text.

Cell line	Clone	Data base homologies	Nucleotide identities	Transcription compared with BHK21[a]	Changes in levels of transcription normalized to level of β-actin expression[b]
			(%)		
T637	A12	Mouse *endo B cytokeratin* mRNA	390/431 (91)	+	>10
	B2	mRNA for placental calcium-binding protein	318/355 (90)	+	No expression in BHK21
	C1	Human *Thy-1 glycoprotein* gene	112/132 (85)	+	No expression in BHK21
	C4	Mouse mRNA for pEL98 protein	408/478 (85)	+	No expression in BHK21
	E3	*Rattus norvegicus* mRNA for keratin 18	283/308 (92)	+	No expression in BHK21
	E8	None	None	+	No expression in BHK21
λ 10	A6	*C. griseus* thrombospondin protein	107/110 (97)	−	3.9
	B9	None	None	+	3.9
	C8	Mouse *cofilin isoform* mRNA	217/250 (87)	+	2.5
	E11	None	None	−	No expression in L10
	F11	Human chromosome 19, BAC CIT-B-470f8 (BC330812)	141/157 (90)	+	0.6
	F12	Mouse *thrombospondin* gene	347/396 (88)	−	6.3
λ 18	80-I	Mouse *superoxide dismutase* 2 mRNA	424/458 (93)	+	8.7
	32-II	Rat ribosomal protein S12 mRNA	223/240 (93)	+	7.5

[a] +, indicates higher levels of expression of the cloned DNA segment in the transgenic cell line compared with the non-transgenic BHK21 cell line; −, the underexpression in the transgenic cell line.

[b] These data were derived from phosphorimager analyses.

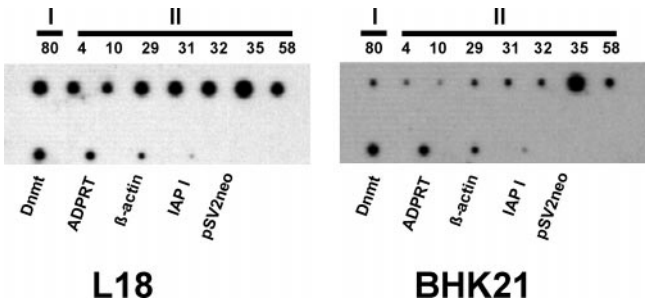

FIG. 2. **Differential gene transcription in the λ DNA-transgenic BHK21 cell line L18.** Shown are reverse Northern dot blot arrays of preselected difference products after one round of cDNA subtraction with the λ DNA-transgenic cell line L18 as tester and the nontransgenic BHK21 cell line as driver. Difference clones were gridded on GeneScreen Plus membranes together with known genes, murine *DNA methyltransferase* (*Dnmt1*), *ADPRT*, *β-actin*, *IAPI*, or *pSV2neo*. The DNAs were hybridized to ^{32}P-labeled cDNA libraries from L18 or BHK21 cells.

Fig. 3*B*). The probe numbers refer to individual clones isolated by MS-AS (Fig. 3*A*, probes *t3, t4, t7, R-1, R-3, R-4*) or MS-RDA (Fig. 3*B*, probes *18, 79, 80, 32, 44*). The data base homologies and gene assignments identified by the nucleotide sequences of the probes characterized in Fig. 3*A* are listed in Table II. Probe 44 used in the L18 experiment of Fig. 3*B* corresponds to intracisternal A particle (*IAP*) DNA that had been analyzed previously in a different set of λ DNA-transgenic cell lines using different methods (4, 5). All of the differentially methylated clones identified by the MS-RDA technique and the *IAP* sequences (24) selected by the MS-AS method represented repetitive sequences in the hamster genome. Most of the repetitive sequences with altered methylation patterns are most likely not linked to the insertion site of the transgene, but are located *in trans* on different chromosomes. When the DNAs from non-transgenic single cell-cloned BHK21 isolates were analyzed using the same probes and methods, no differences in *Hpa*II or *Hha*I cleavage patterns were observed (data not shown).

We conclude that the selection methods used to identify differentially methylated DNA sequences in Ad12 DNA- or λ DNA-transgenic BHK21 cell lines yield a spectrum of DNA sequences in which the methylation patterns have been altered as a consequence of foreign DNA insertion into the hamster cell genome.

Alterations in Levels of DNA Methylation in the DNA from Transgenic Mice—The B6D2$_{F1}$ mouse strains 7-1 and 8-1 carried a transgene construct that contained the adenovirus type 2 E2A late promoter controlling the chloramphenicol acetyl transerase gene (pAd2E2AL-CAT) (8). Other mouse strains (a gift of Klaus Schughart, Strasbourg, France) were transgenic for the DNA of bacteriophage λ DNA. We investigated about 10 different known mouse genes as ^{32}P-labeled hybridization probes with DNA from nontransgenic or transgenic mice that had been cut with *Hpa*II or *Msp*I. For *IL-10* and *Igf2r* (insulin-like growth factor 2 receptor, an imprinted gene), differences in the cleavage patterns of *Hpa*II were observed (Fig. 4, *A–C*).

The *IL-10* and *Igf2r* loci mapping to different chromosomes were hypomethylated in liver DNA from the transgenic animals 8-1 (Fig. 4*A*) and 7-1 (Fig. 4, *B* and *C*). For the transgenic mouse line 7-1, the *Bam*HI/*Hpa*II fragment patterns in the *IL-10* (Fig. 4*B*) and *Igf2r* (Fig. 4*C*) genes were determined. Both genes exhibited partial methylation phenotypes (methylation mosaicisms). The relative signal intensities in each lane reflected the degree of methylation at a given site. The results of phosphorimager analyses of the decrease in DNA methylation at the *IL-10* and *Igf2r* loci in the transgenic mouse line 7-1 documented the loss of methylation to a range between 11 and 25% (Tables III and IV). The photostimulated luminescence (PSL) of each fragment band was measured (Fig. 4, *B* and *C*) and the percentage relative to the sum of total intensity/lane (relative signal intensity, %PSL) was calculated for the *Bam*HI/*Hpa*II cleavage pat-

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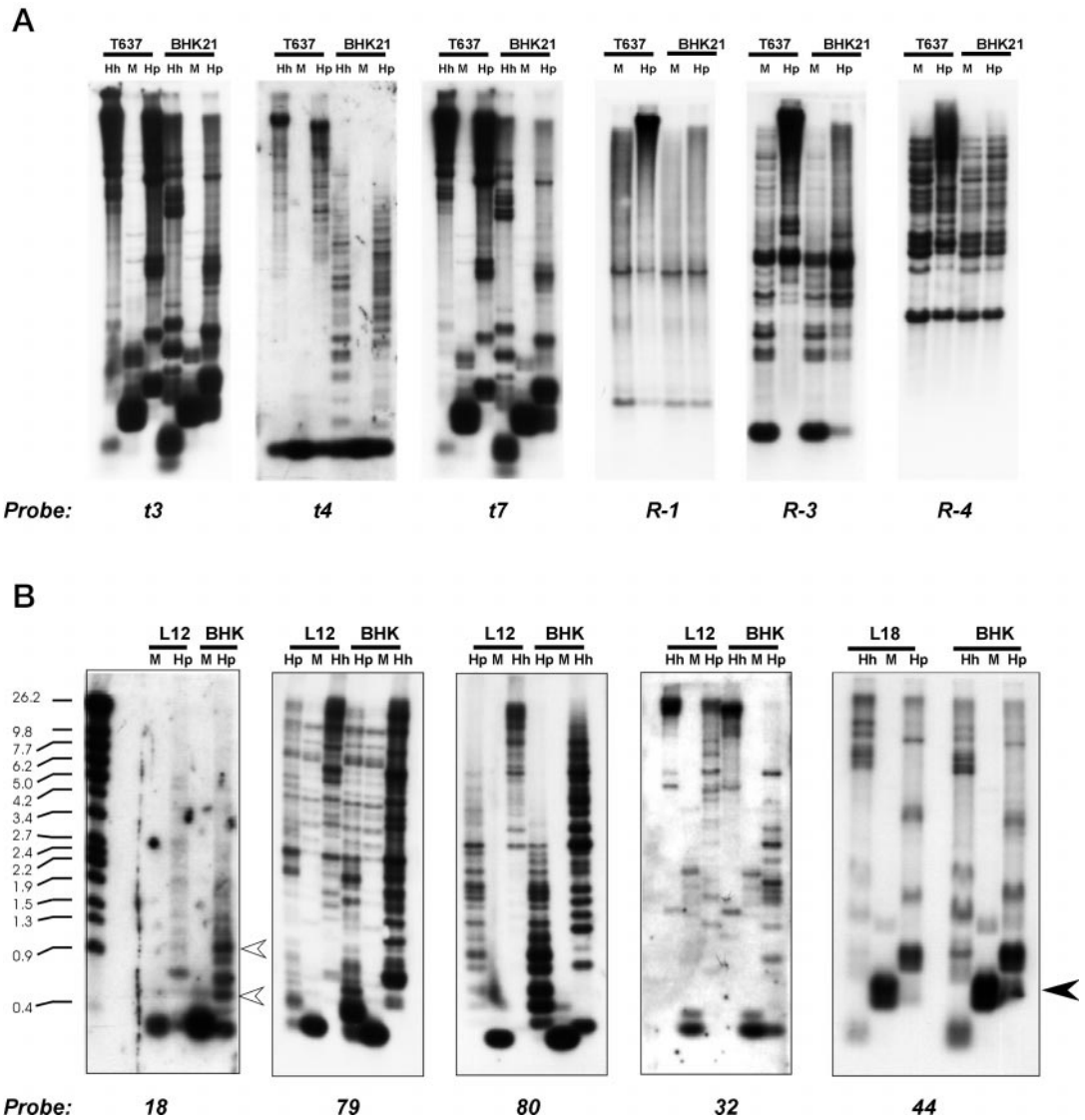

FIG. 3. *A*, detection of *de novo* methylated cellular genes and DNA segments in the Ad12-transgenic BHK21 cell line T637 by applying the method of MS-AS is shown. The DNA (5 μg) extracted from BHK21 or T637 cells was cleaved with *Hpa*II (*Hp*), *Hha*I (*Hh*), or *Msp*I (*M*), and the fragments were separated by electrophoresis on a 1% agarose gel. The DNA was then transferred by Southern blotting to a GeneScreen Plus membrane and hybridized to difference products isolated after one round of MS-AS (for details, see "Experimental Procedures"). Most of the six differentially methylated clones (*t3, t4, t7, R-1, R-3, R-4*) were identified by their nucleotide sequence homologies to known genes (see Table II). *B*, an increase in DNA methylation of cellular DNA segments in the λ DNA-transgenic BHK21 cell line L12 or L18 is shown. By applying the methylation-sensitive representational analysis to *Hpa*II cleaved genomic DNA from the nontransgenic BHK21 cell line compared with the λ DNA-transgenic BHK21 cell line L12 or L18, several differentially methylated DNA segments were isolated. Experimental procedures for the Southern blot experiments were similar to those described in *A*. The *open arrowheads* indicate differences in the *Hpa*II cleavage patterns between the DNAs from cell lines BHK21 and L12 when clone18 DNA was used as a hybridization probe. The *closed arrowheads* show the differences in the *Hpa*II cleavage patterns between cell lines BHK21 and L18 using the hybridization probe 44.

TABLE II
Database homologies of DNA segments with altered methylation patterns

Cell line	Clone	Database homologies	Nucleotide identities
			(%)
T637	t3	*IAP*	455/462 (98)
	t4	Mouse 45S pre rRNA gene	123/125 (98)
	t7	*IAP*	268/274 (98)
	t9	Rat *thymosin* β-4 mRNA	196/207 (95)
	R-1	None	
	R-3	*Mesocricetus auratus* clone e23 retroviral-like pol protein mRNA	99/107 (93)
	R-4	Human hamster hybrid cosmid clone 38a13	475/542 (88)
λ 12	a6	IAP	214/222 (88)
	a73	None	
	a83	Rat *thymosin* β-4 mRNA	172/181 (95)
	a91	None	

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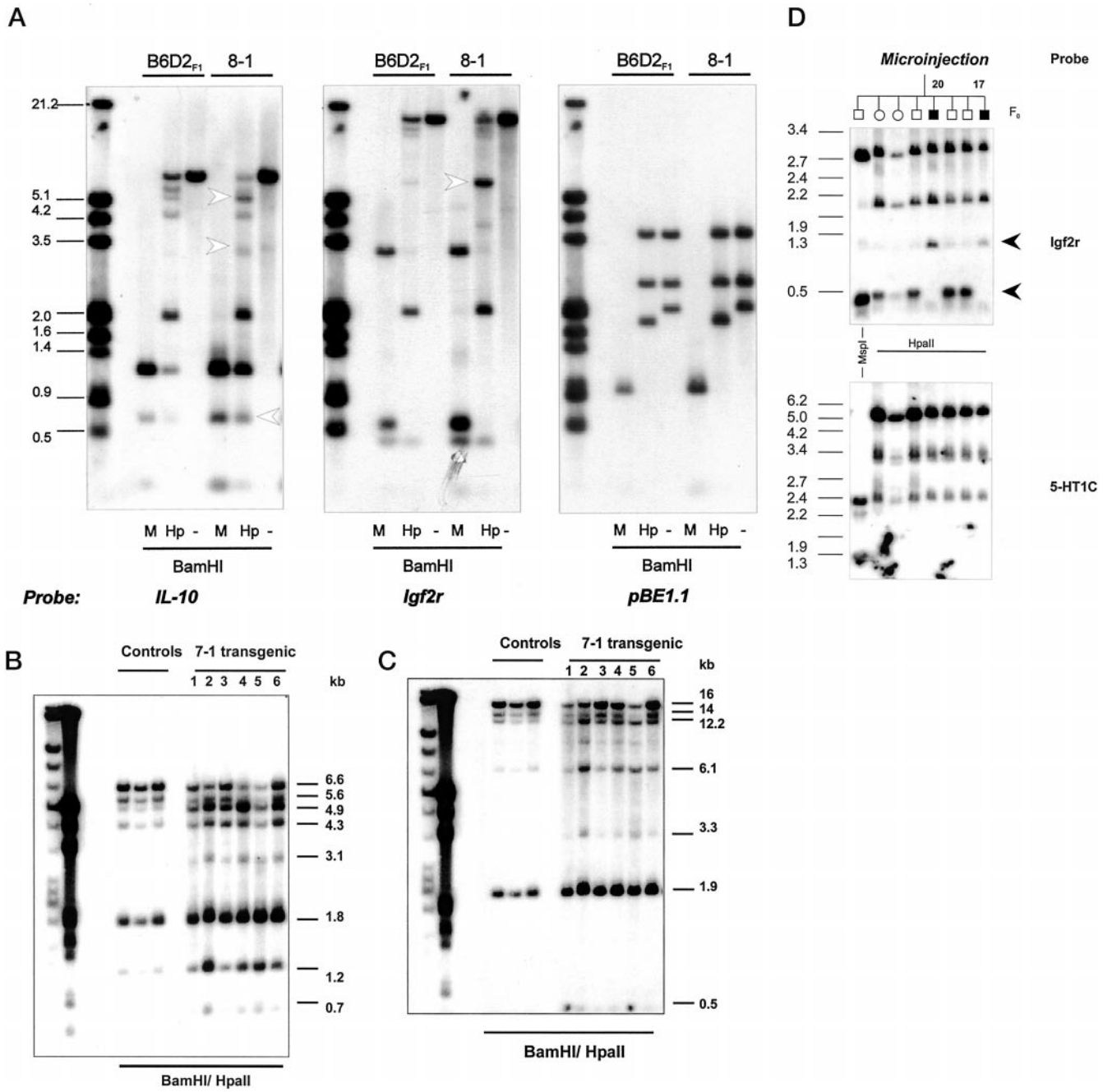

FIG. 4. **Changes in DNA methylation patterns in transgenic mouse strains.** *A*, loss of methylation in the *IL-10* and *Igf2r* genes in pAd2E2AL-CAT-transgenic mouse line 8-1. Genomic liver DNA (20 μg) extracted from male animals of the transgenic mouse line 8-1 or from male nontransgenic $B6D2_{F1}$ (hybrid C57BL/6 × DBA/2) animals with the same genetic background was cleaved with *Bam*HI only (−) or in combination with *Hpa*II *BamHI*/*Hp*) or *Msp*I (*BamHI*/*M*). Experimental procedures for the Southern blot experiments were carried out as described in the legend to Fig. 3*A*. *The open arrowheads* indicate increases in relative signal intensities in the *Bam*HI/*Hpa*II cleavage patterns of *IL-10* and *Igf2r* in the DNA of the transgenic mouse line 8-1 compared with the nontransgenic $B6D2_{F1}$ control animals. Increases in signal intensities of smaller fragments are due to the loss of methylation at these loci in transgenic animals. After transfer of the genomic DNA fragments, the DNA was hybridized to 12 different known gene probes. The results of three of these hybridization experiments with the same membrane were shown as examples. *B* and *C*, loss of methylation in the *IL-10* (*B*) and *Igf2r* (*C*) loci in transgenic animals of the pAd2E2AL-CAT transgenic 7-1 mouse line. Liver DNA (20 μg) from male transgenic 7-1 mice and from nontransgenic control animals with the same genetic background ($B6D2_{F1}$) was cleaved with *Bam*HI and *Hpa*II. Further experimental procedures for the Southern blot experiments (12) were similar to those described in *A*. Loss of methylation in the *IL-10* and *Igf2r* loci as evaluated by phosphorimager analyses of the fragment patterns ranged from 12 to more than 25%. The observed changes in DNA methylation were tissue-specific for liver DNA. *D*, hypermethylation in the *Igf2r* locus of λ DNA-transgenic mice. DNA extracted from heart muscle of λ DNA-transgenic CD-1 animals or from nontransgenic CD-1 animals of the same litter was cleaved with *Hpa*II or *Msp*I. As hybridization probe, a 3.0-kilobase pair fragment of the imprinted *Igf2r* locus (23) or of several other cellular genes (here the *5-HT1C* gene probe was shown as an example) were used. Both male founder animals (*filled squares*) showed hypermethylation specifically in the *Igf2r* locus of heart muscle DNA as compared with nontransgenic litter mates. No differences were apparent with the *5-HT1C* hybridization probe. The *arrowheads* indicate differences in *Hpa*II cleavage patterns between transgenic and nontransgenic litter mates.

terns of *IL-10* (Table III) and *Igf2r* (Table IV). The marker in Fig. 4*B* indicates the positions of fragment bands included in the phosphorimager analyses. The standard deviations of signal intensities for *Bam*HI/*Hpa*II DNA fragments from nontransgenic control mice were always below 1% (Tables III, IV). Numerous control hybridization probes, *e.g. pBE1*, a glycosylase gene (Fig.

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TABLE III

Phosphorimager analyses of the IL-10 BamHI/HpaII cleavage patterns

—, measured PSL yielded background values; %PSL, percentage of signal intensity of a specific fragment band as compared with the total signal intensity in each lane; kb, kilobase pair.

	Control animals			Average controls	7–1 Transgenic animals					
	1	2	3		1	2	3	4	5	6
kb	*%PSL*	*%PSL*	*%PSL*	*%PSL*	*%PSL*					
6.6	36.8	36.6	36.9	36.8 (±0.15)	22.6	10.8	25.5	11.5	11.2	20.23
5.6	17.1	16.8	15.9	16.6 (±0.62)	13.4	11.8	14.5	10.1	8.1	15.63
4.9	8.5	9.8	8.9	9.1 (±0.67)	18.5	20.7	20.5	32	16.3	21.63
4.3	9.8	9.5	8	9.1 (±0.96)	9.3	9.7	10.2	11.9	8.9	12.1
3.1	0.5	0.1	0.2	0.3 (±0.2)	0.9	2.5	1.1	2.7	3.6	2.1
1.8	22.9	23.1	24	23.3 (±0.58)	32.1	27.5	25.3	26.3	37	25.1
1.2	2.5	2.5	2.9	2.6 (±0.23)	5.4	15.7	3.9	6.6	13.5	3.9
0.7	2	1.8	1.5	1.8 (±0.25)	—	1.3	—	—	1.39	—

TABLE IV

Phosphorimager analyses of the Igf2r BamHI/HpaII cleavage patterns

%PSL, percentage of signal intensity of a specific fragment band as compared with the total signal intensity in each lane; kb, kilobase pair.

	Control animals			Average controls	7–1 Transgenic animals					
	1	2	3		1	2	3	4	5	6
kb	*%PSL*	*%PSL*	*%PSL*	*%PSL*	*%PSL*					
16	38.7	38	38.2	38.3 (±0.36)	13.5	13	29.7	22.7	13.2	29.7
14	15.3	15.4	15.7	15.5 (±0.21)	11.2	10.2	14.5	12.5	5.6	14.6
12.2	10.7	10	9.6	10 (±0.56)	11.8	13	11.5	12.5	12.1	10.3
6.1	2.5	3.1	2.7	2.8 (±0.31)	5.2	5.8	3.8	4.8	2.2	3.2
3.3	5.1	4.9	5.3	5.1 (±0.2)	6.7	10.9	5.6	8.9	11	5.6
1.9	23.6	25.3	25.4	24.8 (±0.83)	39.24	43.21	32.15	36.5	45.9	34.5
0.5	2.5	1.7	2	2.1 (±0.40)	11.53	1.1	1.6	0.9	7.9	1.4

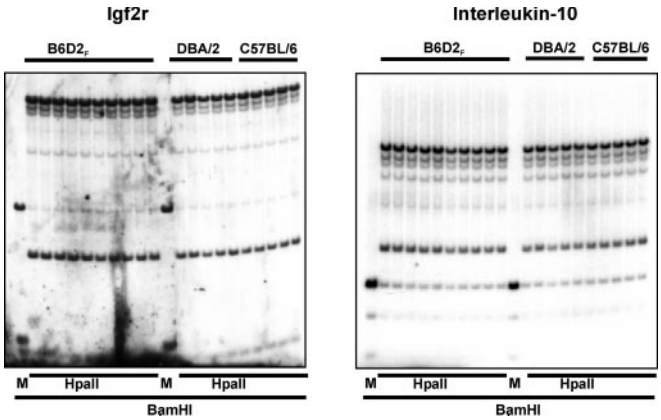

FIG. 5. **Methylation status of the *IL-10* and *Igf2r* loci in nontransgenic control animals.** Liver DNA (20 μg) from nontransgenic control animals of the DBA/2, C57BL/6, or hybrid $B6D2_{F1}$ mouse strains was cleaved with *Bam*HI and *Hpa*II. All experimental procedures for the Southern blot experiments and the abbreviations used were similar to those described in the legend to Fig. 4.

4*A*), revealed no changes in DNA methylation at these sites on the same membranes.

When *Bam*HI/*Hpa*II-cut DNA from nontransgenic control mice from the B6D2F1 hybrid mouse strain or from the parental C57BL/6 or DBA/2 strain was probed with ^{32}P-labeled *IL-10* or *Igf2r* DNA, identical cleavage patterns were observed (Fig. 5). These data demonstrate that the methylation patterns in the *IL-10* and *Igf2r* genes do not vary when comparisons are made between different control animals or between any of the three mouse strains. The significant changes in DNA methylation at the *IL-10* and *Igf2r* loci seem to be specific for the transgenic animals of the 8-1 and 7-1 lines. Other transgenic mouse lines, *e.g.* lines 6-2 and 5-8, which carried the same transgene construct, albeit at different loci (8), showed no alterations in the *IL-10* and *Igf2r* methylation patterns (data not shown). Because *IL-10* and *Igf2r* are located on different mouse chromosomes, we reason that at least one of the genes with altered DNA methylation is located *in trans* to the transgene insertion site.

In two additional mouse strains transgenic for the DNA of bacteriophage λ, strains 17 and 20, hypermethylation of the *Igf2r* gene was observed in the DNA from heart muscle (Fig. 4*D*). The founder animals (*filled squares* in Fig. 4*D*) of the λ DNA-transgenic mouse lines showed increased methylation at the *Igf2r* site as compared with the same site in their nontransgenic litter mates (*open squares* in Fig. 4*D*). Differences in *Hpa*II cleavage patterns are indicated by *black arrows*. Numerous other gene probes, *e.g. 5-HT1C*, revealed no changes in DNA methylation at these sites (Fig. 4*D, lower panel*). The MS-RDA analysis of λ DNA-transgenic founder animals compared with nontransgenic animals did not lead to the isolation of aberrantly methylated genomic DNA fragments. Instead, DNA segments with restriction fragment length polymorphisms in *Hpa*II sites were isolated irrespective of the organs from which the DNA was prepared.

DISCUSSION

The integration of foreign DNA into established mammalian genomes can be considered a frequent event. Virus infection, microinjection, or transfection of foreign DNA can lead to the permanent insertion of foreign DNA into the recipient genome. It is unknown how frequently and by what exact mechanism this insertional recombination proceeds. It is not unusual for multiple copies of the foreign DNA to be inserted at a single site. Hence, arrays of foreign DNA comprising a total sequence of up to 1 megabase pair and more can be added to mammalian genomes. At present, information is lacking as to what extent this megabase acquisition of foreign genetic material can lead to perturbations of the chromatin structure at or remote from the sites of insertion. There is evidence that in the nucleus of the cell individual chromosomes are neighboring one another in a unique spatial relationship (25–27). Thus, structural alterations at one site on one chromosome might be transmitted to and affect the structure and function of loci on adjacent chromosomes, *i.e. in*

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trans, hence over considerable genetic distances.

Can these structural and functional consequences of foreign DNA insertion be assessed by current technology? In the present communication and in previous work from our laboratory (4, 5), we have analyzed hamster cells transgenic for and transformed by Ad12 DNA or transgenic for the DNA of bacteriophage λ DNA as well as mice transgenic for the latter DNA. By applying several independent subtractive hybridization protocols previously not used in the analyses of methylation and transcription patterns in transgenic cell lines or animals, we were able to expand this analysis to a genome-wide survey and demonstrate marked changes in transcription and methylation patterns in cells that carried integrated Ad12 or λ DNA. Several of the clones with aberrant methylation patterns in the transgenic cell lines were derived from repetitive DNA sequences in the genome irrespective of the techniques applied, either MS-RDA or MS-AS. This finding either reflects a bias of these PCR-based techniques for highly abundant DNA templates or indicates that repetitive sequences are particularly prone to undergoing changes in DNA methylation when foreign DNA is inserted into an established genome. In several independent experiments using either the MS-RDA or the MS-AS method, a specific CpG-rich region from the endogenous *IAP* genomes was cloned and found to be hypermethylated in transgenic cells.

Control experiments, in which patterns of methylation have been assessed by the same set of methods in a large number of subcloned lines of nontransgenic BHK21 cells have never shown differences in methylation or transcription patterns among individual BHK21 cell clones for the probes tested (see also Ref. 5). Hence, we have not pursued the possibility that the BHK21 cell population could be mosaic with respect to methylation or transcription patterns. Similar control experiments performed with the DNA from three different mouse strains showed no differences in the *Hpa*II cleavage patterns with the *IL-10* and *Igf2r* probes (Fig. 5).

As the integrated λ DNA is not detectably transcribed in the transgenic BHK21 cells, the possibility that products of the integrated foreign DNA might be involved in the observed alterations is unlikely. The *pSV2neo* gene, usually present as a cointegrate with λ DNA, has usually been silenced in the λ DNA-transgenic cell lines, since G418 selection had been discontinued. Several of the λ DNA- and *pSV2neo*-transgenic cells, which still expressed *pSV2neo*, did not show alterations in transcription and methylation patterns. In contrast, in the Ad12-transformed hamster cell line T637 and Ad12 genes are transcribed, and their products may contribute to the induction of changes in transcription and methylation patterns (4). However, revertants of the T637 cell line devoid of Ad12 genomes, *e.g.* TR3 or Ad12 DNA-transgenic cell lines (*e.g.* H-Ad12neo2/5), which do not express the integrated Ad12 DNA, still exhibit the marked alterations in methylation patterns (4). On the other hand, analyses of a different Ad12-transformed hamster cell line, A2497-3, or of Ad12-infected BHK21 cells, both of which express parts of the Ad12 genome, show no altered methylation (4). Thus, factors other than transgene transcription and/or viral gene products must be decisive in eliciting alterations in cellular DNA methylation and transcription patterns.

It is conceivable that the selection of combinations of genes and DNA segments with altered methylation and transcription patterns depends on the sites of foreign DNA insertion and on the loci adjacent to these sites. This selection may also be subject to possible structural and functional effects. In this context, it will be important to investigate whether the insertion of foreign DNA *per se* can contribute to the mechanism of oncogenesis in virus-induced and/or "naturally occurring malignancies."

Of course, the observation that the insertion of foreign DNA into the mammalian (hamster, mouse) genome can have structural and functional consequences for the target cell will be of interest for the interpretation of experiments in which regimens have been applied that lead to foreign DNA insertion, such as in gene transfer studies, transgenic animals (knock-out, knock-in), and human (somatic) gene therapy. The interpretation of the results adduced with these methods may be complicated.

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