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Insertion of Foreign DNA into an Established Mammalian Genome Can Alter the Methylation of Cellular DNA Sequences†

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The insertion of adenovirus type 12 (Ad12) DNA into the hamster genome and the transformation of these cells by Ad12 can lead to marked alterations in the levels of DNA methylation in several cellular genes and DNA segments. Since such alterations in DNA methylation patterns are likely to affect the transcription patterns of cellular genes, it is conceivable that these changes have played a role in the generation or the maintenance of the Ad12-transformed phenotype. We have now isolated clonal BHK21 hamster cell lines that carry in their genomes bacteriophage λ and plasmid pSV2neo DNAs in an integrated state. Most of these cell lines contain one or multiple copies of integrated λ DNA, which often colocalize with the pSV2neo DNA, usually in a single chromosomal site as determined by the fluorescent in situ hybridization technique. In different cell lines, the loci of foreign DNA insertion are different. The inserted bacteriophage λ DNA frequently becomes de novo methylated. In some of the thus-generated hamster cell lines, the levels of DNA methylation in the retrotransposon genomes of the endogenous intracisternal A particles (IAP) are increased in comparison to those in the non-λ-DNA-transgenic BHK21 cell lines. These changes in the methylation patterns of the IAP subclone I (IAPI) segment have been documented by restriction analyses with methylation-sensitive restriction endonucleases followed by Southern transfer hybridization and phosphorimager quantitation. The results of genomic sequencing experiments using the bisulfite protocol yielded additional evidence for alterations in the patterns of DNA methylation in selected segments of the IAPI sequences. In these experiments, the nucleotide sequences in >330 PCR-generated cloned DNA molecules were determined. Upon prolonged cultivation of cell lines with altered cellular methylation patterns, these differences became less apparent, perhaps due to counterselection of the transgenic cells. The possibility existed that the hamster BHK21 cell genomes represent mosaics with respect to DNA methylation in the IAPI segment. Hence, some of the cells with the patterns observed after λ DNA integration might have existed prior to λ DNA integration and been selected by chance. A total of 66 individual BHK21 cell clones from the BHK21 cell stock have been recloned up to three times, and the DNAs of these cell populations have been analyzed for differences in IAPI methylation patterns. None have been found. These patterns are identical among the individual BHK21 cell clones and identical to the patterns of the originally used BHK21 cell line. Similar results have been obtained with nine clones isolated from BHK21 cells mock transfected by the Ca^{2+}-phosphate precipitation procedure with DNA omitted from the transfection mixture. In four clonal sublines of nontransgenic control BHK21 cells, genomic sequencing of 335 PCR-generated clones by the bisulfite protocol revealed 5′-CG-3′ methylation levels in the IAPI segment that were comparable to those in the uncloned BHK21 cell line. We conclude that the observed changes in the DNA methylation patterns in BHK21 cells with integrated λ DNA are unlikely to preexist or to be caused by the transfection procedure. Our data support the interpretation that the insertion of foreign DNA into a preexisting mammalian genome can alter the cellular patterns of DNA methylation, perhaps via changes in chromatin structure. The cellular sites affected by and the extent of these changes could depend on the site and size of foreign DNA insertion.

Established mammalian and other eukaryotic genomes apparently allow the insertion of foreign DNA sequences both naturally, e.g., upon virus infections (5, 33) or via the gastrointestinal tract (34, 35), and under experimental conditions exemplified by the genomic fixation of foreign DNA after the application of various transfection or microinjection protocols. While investigating the oncogenic transformation of hamster cells after infection with human adenovirus type 12 (Ad12), we have studied in considerable detail the insertion of Ad12 DNA into the hamster cell genome and some of its consequences (for recent reviews, see references 6 and 7). Among the sequelae of foreign DNA insertion, we have concentrated on the de novo methylation of the integrated foreign DNA (27, 41, 42) and on alterations in the patterns of methylation in several cellular genes and DNA segments (15). The integration of foreign (Ad12) DNA into the hamster cell genome is not nucleotide sequence or chromosomal site specific (5, 16, 18). Ad12 DNA, λ DNA, and probably any other foreign DNA can be inserted in multiple copies at many different sites, sometimes partly fragmented, and often at a single chromosomal location.

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† This report is dedicated to R. Walter Schlesinger on the occasion of his 85th birthday.

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The described alterations in cellular DNA methylation patterns upon foreign DNA insertion are conceivably a reflection of more general changes in chromatin structure in the affected cells. Implications of DNA methylation for chromatin structure and vice versa have been investigated in several laboratories (17, 22, 30, 44). We have frequently observed that up to >50 genome equivalents of Ad12 DNA, i.e., an integrate of foreign DNA measuring 1 to 2 megabase pairs in total length, have been inserted into the established hamster cell genome. The addition of such a large segment of foreign DNA could well lead to structural rearrangements in the cellular genome. The extent and nature of such structural chromatin changes are unknown, but analyses of alterations of DNA methylation patterns might be a reliable indicator and motivate more refined analyses.

We now report that the insertion of bacteriophage λ DNA, as a paradigm foreign DNA, into an established hamster genome can alter methylation patterns in certain segments of cellular DNA. It is conceivable that the endogenous retroviral intracisternal A particle (IAP) subclone I (IAPI) sequences are particularly susceptible to undergo methylation changes upon eliciting structural perturbances in a mammalian genome. We also demonstrate by Southern blot hybridization and by genomic sequencing experiments that the BHK21 cell population used in all of these experiments shows uniform, and not mosaic, methylation patterns in the IAPI DNA sequences.

MATERIALS AND METHODS

Clonal lines of BHK21 cells with integrated bacteriophage λ and plasmid pSV2neo DNAs. The BHK21 hamster cell line (ATCC CCL 10) was kept in continuous monolayer culture in Dulbecco medium enriched with 10% fetal calf serum. These cells were transfected with a mixture of 5 μg each of bacteriophage λ DNA and of plasmid pSV2neo DNA (38); the latter carries the gene for neomycin phosphotransferase under the control of the simian virus 40 early promoter. The Ca^{2+}-phosphate precipitation technique (12) was used for transfection experiments. Neomycin-resistant cell clones were isolated by G418 (Gibco) selection by adding 1 mg of G418 per ml of medium 2 days after transfection. Neomycin-resistant single-cell clones isolated 2 weeks after the start of the G418 selection were recloned twice as single cells on microtiter plates and screened for the presence of integrated λ DNA by conventional restriction endonuclease and Southern blot hybridization analyses (19, 37). During all subsequent passages, G418 was omitted from the culture medium. In mock-transfection experiments, DNA was omitted from the otherwise complete transfection mixture. In other control experiments, BHK21 cells were recloned up to three times on microtiter plates, and IAPI methylation patterns were determined for 66 of these BHK21 cell clones as well as for 9 BHK21 cell clones previously subjected to a mock-transfection protocol. In further control experiments, the IAPI segment in nontransgenic, subcloned BHK21 cells was analyzed by the bisulfite protocol of the genomic sequencing method (11).

Cell line T637 was derived by transforming BHK21 cells by infection with human Ad12 (40).

Analyses of the BHK21 cell lines carrying integrated λ DNA. The presence of λ-specific sequences in the cellular DNA or RNA was assessed by analyzing cellular DNA or total cellular RNA by conventional Southern or Northern blotting experiments, respectively, using ^{32}P-labeled λ DNA or pSV2neo DNA as the hybridization probe. The cellular DNA or RNA (2) was extracted by standard protocols. The DNA was cleaved with *Eco*RI or *Pst*I prior to Southern blot hybridization experiments. DNA probes were ^{32}P labeled by the oligonucleotide labeling procedure (10).

Interphase nuclei or metaphase chromosomal spreads of the λ DNA-carrying cell lines were prepared and screened for the presence of integrated λ or pSV2neo DNA by the fluorescent in situ hybridization (FISH) procedure according to previously published methods (15, 21, 33). In some of the experiments, λ DNA and pSV2neo DNA were jointly used as hybridization probes; in other experiments, λ DNA was used alone. DNA probes used for hybridizations were biotinylated by nick translation (31). Hybridized biotinylated DNA probes were visualized in a sandwich procedure by subsequently reacting chromosome spreads with fluroescein isothiocyanate-tagged avidin. After extensive washing, chromosome preparations were counterstained with propidium iodide and examined and photographed in an Olympus BH2 microscope.

Determination of methylation patterns in cellular IAPI sequences and in integrated λ DNA in the λ DNA-containing BHK21 cell clones. (i) Restriction enzyme and Southern blot hybridization analyses and quantitation by phosphorimager evaluation. Cellular DNA was cleaved with *Hpa*II, *Msp*I, or *Hha*I, and the DNA fragments were separated by electrophoresis on 0.8 to 1% agarose gels, Southern blotted onto Qiagen Nylon-Plus membranes, and hybridized to ^{32}P-labeled λ DNA or pSV2neo DNA or to cloned IAPI DNA (15).

Autoradiograms of *Hpa*II, *Hha*I, or *Msp*I cleavage patterns of the cellular IAPI segments (see Fig. 5) were evaluated, and band intensities were quantitated by using a Fuji X BAS 1000 phosphorimager. For each fragment band in individual lanes, relative intensities in photostimulated luminescence units were calculated. Details of these analyses are described in Table 1, footnote *a*.

(ii) Genomic sequencing method. The genomic sequencing technique based on the bisulfite protocol (11) was applied to determine exactly which 5′-CG-3′ dinucleotides in the cellular IAPI segments in the genomes of the λ DNA-containing BHK21 clones or the T637 cell line were methylated. Similar experiments were performed with the DNAs from four clonal sublines of nontransgenic BHK21 cells. Details of the bisulfite protocol of the genomic sequencing procedure as used in our laboratory were described previously (24, 36, 45). Briefly, the genomic DNA was alkali denatured in 0.3 M NaOH for 15 min at 37°C and for 3 min at 95°C. The DNA was then treated with sodium bisulfite. The bisulfite solution was prepared by dissolving 8.1 g of sodium bisulfite (Sigma) in 15 ml of degassed water by gently inverting the tube; 1 ml of 40 mM hydrochinone was subsequently added. The solution was adjusted to pH 5 by adding 0.6 ml of freshly prepared 10 M NaOH. The denatured DNA solution (110 μl) was mixed with 1 ml of the bisulfite solution, overlaid with mineral oil, and incubated at 55°C for 16 h in a water bath in the dark. Subsequently, the DNA was purified by using glassmilk (Gene Clean II Kit; Bio 101 Inc.). A selected segment in the IAPI sequence was amplified by PCR with appropriate oligodeoxyribonucleotide primers (see Fig. 6). Reaction products were then cloned into the pGEM-T vector (Promega) and transfected into *Escherichia coli* XL1BlueMRF′ by standard methods (14). A number of clones were isolated, and the nucleotide sequences were determined with an Applied Biosystems 377 DNA sequencer by standard methods. The bisulfite reaction converted all C residues into U residues and then, after PCR amplification, into T residues, whereas the 5-methyldeoxycytidine (5-mC) residues were refractory to this chemical conversion reaction. Thus, a C residue in the eventually determined nucleotide sequence proved the presence of a 5-mC residue in this position in the original genomic nucleotide sequence. All bona fide C residues scored as Ts.

RESULTS

Rationale and design of the study. In investigations on integrated Ad12 genomes in Ad12-transformed hamster cell lines and Ad12-induced hamster tumor cells, we found extensive changes in the methylation patterns of cellular genes and cellular DNA segments, notably in the endogenous IAP retrotransposons and in the major histocompatibility complex region (15). Infection of BHK21 cells with Ad12 virions did not elicit such changes. Since in Ad12-infected and Ad12-transformed hamster cells similar patterns of early Ad12 gene transcription were observed (28), it was unlikely that the presence of early Ad12 gene products was responsible for the increases in cellular DNA methylation.

While a contribution of the transformed or oncogenic phenotype to the changes in DNA methylation of cellular DNA segments in Ad12-transformed cells is very likely, we have now investigated whether the integration of foreign DNA into the established BHK21 hamster cell genome by itself might have similar consequences. Therefore, a set of BHK21 hamster cell clones which contained one or multiple copies of bacteriophage λ DNA as integrates on different chromosomes was generated. Due to cotransfection and selection, the cell lines also carried integrated pSV2neo plasmid DNA, usually in the same chromosomal location as λ DNA (see Fig. 2). The prokaryotic viral DNA was not known to be expressed or capable of inducing a transformed phenotype in mammalian cells. Hence, if changes in cellular DNA methylation patterns were observed in λ DNA-carrying BHK21 cells, these changes would most likely be due to the insertion of foreign DNA. The possibility that the BHK21 cell genomes could be mosaics with respect to the patterns of methylation in the retrotransposon sequences of IAPI DNA even prior to foreign DNA insertion had to be investigated. The IAPI sequences are located on many different chromosomes in very specific patterns of distribution (15, 20, 23).

Clonal BHK21 cell lines carrying integrated λ genomes. The clonal BHK21 cell lines that had been selected upon cotrans-

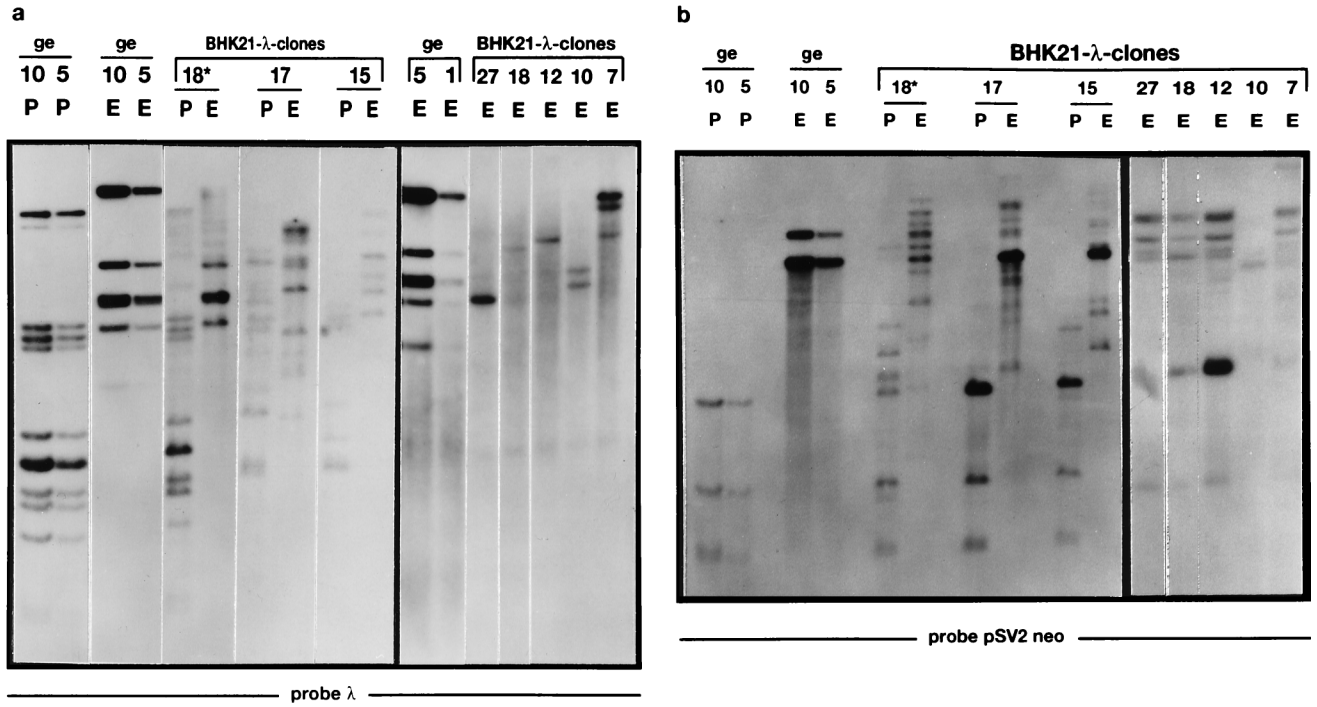

FIG. 1. Clonal cell lines of BHK21 cells that carry integrated λ DNA and pSV2neo DNA. (a) Integration patterns of λ DNA in some of the clonal, exemplarily selected λ DNA-transgenic BHK21 cell lines. The DNA extracted from BHK21-λ clones as indicated was cleaved with *Eco*RI (E) or *Pst*I (P), and the fragments were separated by electrophoresis on a 0.6 or 0.8% agarose gel. The DNA was then transferred by Southern blotting to a Qiagen Nylon-Plus membrane, and the λ DNA-specific fragments were visualized by hybridization to ^{32}P-labeled λ DNA followed by autoradiography. As size and quantity markers, λ DNA cut with *Eco*RI or *Pst*I was coelectrophoresed. Amounts of 1, 5, or 10 genome equivalents (ge) of λ DNA were used. (b) Integration patterns of the pSV2neo plasmid used in cotransfection experiments. Experimental conditions were identical to those described for panel a except that ^{32}P-labeled pSV2neo DNA was used as the hybridization probe. The same Qiagen Nylon-Plus filter as shown in panel a was used in these experiments, after removing the λ DNA probe by boiling in 0.1% sodium dodecyl sulfate–0.1 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The BHK21-λ18 and -λ18* clones are two distinct λ DNA-carrying BHK21 cell clones.

fection with pSV2neo DNA and λ DNA were analyzed by Southern blot hybridization for the presence and arrangement of the bacteriophage λ and pSV2neo plasmid DNAs and by the FISH method for the chromosomal locations of these foreign DNA molecules. A large number of λ DNA-containing clonal BHK21 cell lines were generated. The analytical Southern hybridization data from some of these cell lines are presented in Fig. 1a. Comparisons with fragment intensities in the marker lanes of *Eco*RI- or *Pst*I-cleaved authentic bacteriophage λ DNA revealed that the generated λ DNA-containing cell lines had less than 1 copy to more than 50 copies of the foreign DNA chromosomally integrated. The cleavage patterns of the cellular DNAs with *Eco*RI or *Pst*I demonstrated the presence of off-size DNA fragments with homologies to λ DNA. These off-size fragments were most likely due to junction fragments representing cellular DNA linked to the integrates or to partly rearranged λ DNA, as previously documented with many transformed cell lines and tumors for integrated Ad12 DNA (16, 18, 27, 39).

In some of the experiments, the integration patterns of the cotransfected pSV2neo DNA were also determined (Fig. 1b). The results documented the presence of multiple copies of the cotransfected plasmid DNA. Even after the removal of the selective drug G418, the plasmid DNA persisted in most of the analyzed cell lines.

In all aspects investigated, the integration patterns of bacteriophage λ DNA inserted into the hamster cell genome by transfection resembled those of Ad12 DNA integrated upon the infection and transformation of hamster cells with Ad12 virions. It was not the aim of the present study to investigate the integration patterns of λ DNA in more detail. We intended, rather, to concentrate on the analyses of methylation patterns in cellular DNA and their alterations in the λ DNA-transgenic cell lines.

We also determined the chromosomal locations of the integrated λ DNA molecules on the hamster chromosomes in metaphase spreads of the appropriately colchicin-pretreated, clonal λ DNA-transgenic hamster cell lines. The integrates were visualized by applying the FISH technique with biotinylated λ DNA as the hybridization probe and fluorescein-tagged avidin for the detection of integrates under UV optics. The FISH data were important for the interpretation of our results and hence are shown for several of the clonal cell lines investigated (Fig. 2). The integrated λ DNA was located on one chromosome, with the exception of cell line BHK21-λ48 (data not shown), which carried foreign DNA in two different chromosomal locations. Each of the cell lines studied carried the integrated λ DNA molecules on a different chromosome (Fig. 2b to f). In that respect, the data obtained with the clonal λ DNA-carrying hamster cell lines again resembled those derived from hamster tumor or transformed cell lines containing Ad12 DNA (16, 18).

For the cell line BHK21-λ15 (Fig. 2e and f), we also determined the chromsomal location of the cotransfected pSV2neo plasmid DNA in the λ DNA-carrying hamster cell line by using either a mixture of biotinylated λ and pSV2neo DNAs (Fig. 2e) or biotinylated λ DNA by itself (Fig. 2f) as the hybridization probe. The data in Fig. 2e and f documented that the pSV2neo DNA colocalized with λ DNA on one chromosome. Similar results (not shown) were obtained for other cell lines carrying λ and pSV2neo DNAs.

We conclude that the foreign DNA, either Ad12 DNA inte-

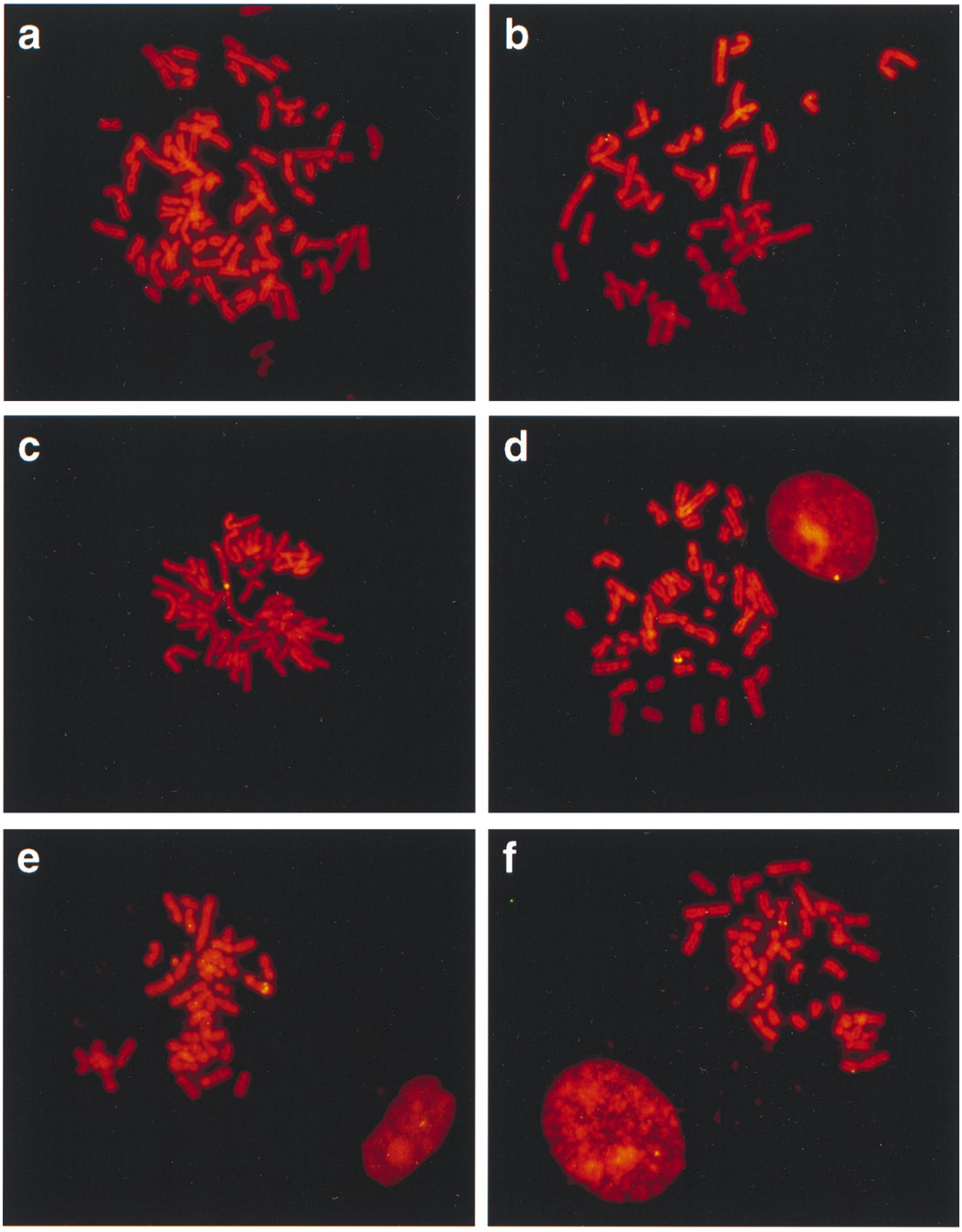

FIG. 2. FISH analyses of the chromosomal locations of the integrated λ genomes and the integrated pSV2neo plasmids in a series of BHK21 cell lines rendered transgenic for λ DNA and pSV2neo DNA. Experimental details are described in the text. (a) Control BHK21 cell devoid of foreign DNA; (b) BHK21-λ7; (c) BHK21-λ17; (d) BHK21-λ18*; (e and f) BHK21-λ15. (a and f) Biotinylated λ DNA alone was used as hybridization probe; (b to e) a mixture of biotinylated λ and pSV2neo DNAs was used for hybridization. The finding of a single signal demonstrated that both transgene DNAs were located at one chromosomal site, which was different for each cell line.

grated upon virion infection and tumor induction (16) or λ DNA after cotransfection with pSV2neo DNA and G418 selection, can be integrated at many different chromosomal locations but, with only one exception, at only one site in a given transgenic cell line. There is no evidence that the methods of transfer of foreign DNA into mammalian cells, i.e, virus infection or DNA transfection, would affect the mode of foreign DNA integration. Only in rare cases can the foreign DNA be integrated at more than one chromosomal site in one clonal cell line. The cotransfected plasmid DNA frequently colocalizes with the λ DNA that has been used as an experimental transgene. The morphologies of the clonal BHK21 cell lines transgenic for integrated λ DNA are indistinguishable from that of the original BHK21 cells used in this study (data not shown). Morphological transformation of the BHK21 cells by the integration of λ DNA and pSV2neo DNA has not been observed.

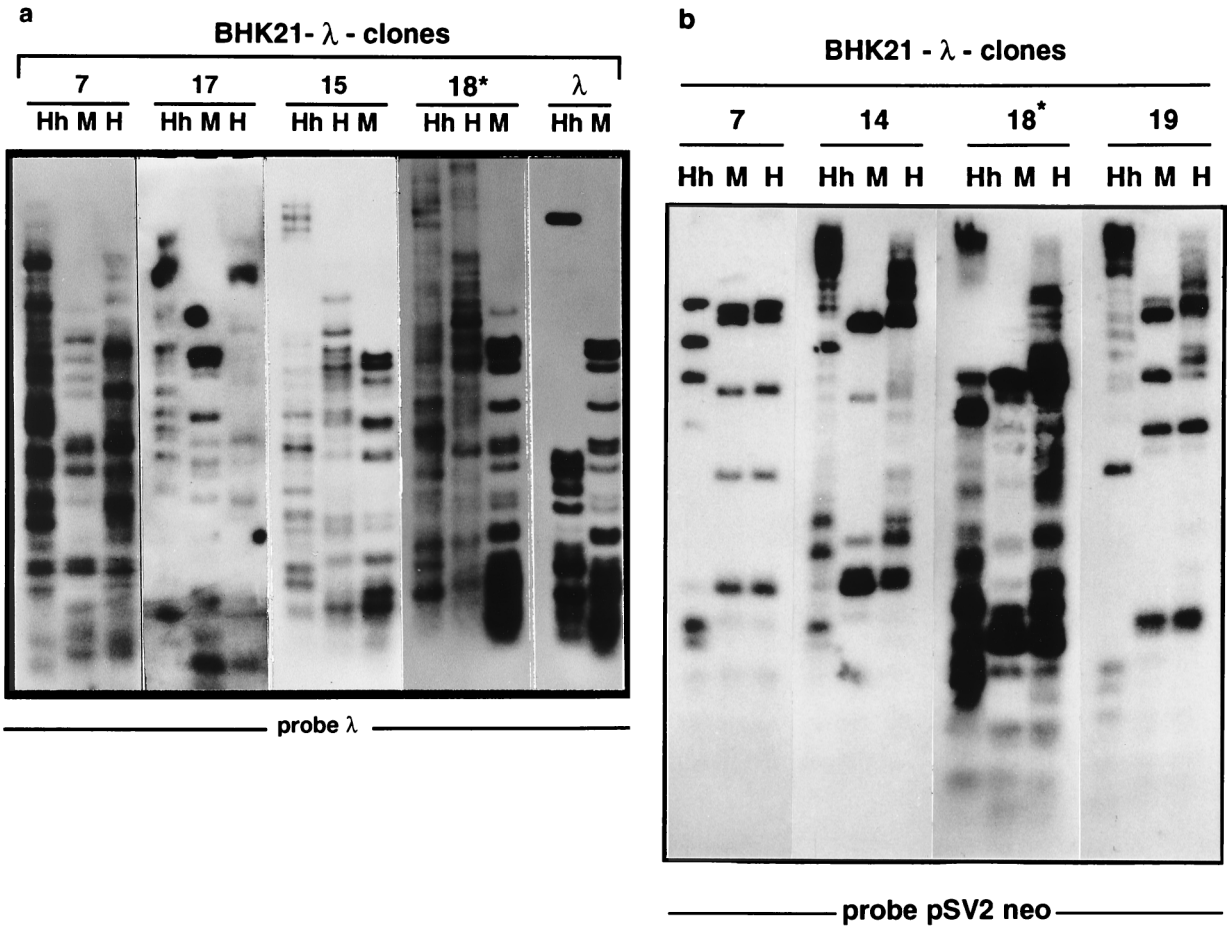

FIG. 3. De novo methylation of the integrated λ DNA (a) or pSV2neo DNA (b) in several clonal BHK21 cell lines transgenic for λ and pSV2neo DNAs. The DNAs from the λ and pSV2neo DNA-transgenic cell lines were isolated and cleaved with *Hpa*II (H), *Hha*I (Hh), or *Msp*I (M). Subsequently, the DNA fragments were separated by electrophoresis in 1.0% agarose gels and analyzed as described in the legend to Fig. 1. ^{32}P-labeled λ DNA (a) or ^{32}P-labeled pSV2neo DNA (b) was used as the hybridization probe.

De novo methylation of integrated λ DNA. Foreign DNA, e.g., Ad12 DNA, covalently inserted into an established mammalian genome became readily de novo methylated (18, 26, 27, 41, 42). Integrated λ DNA in the clonal λ DNA-transgenic BHK21 cell lines was frequently, but not always, extensively de novo methylated (Fig. 3a). Similarly, the cotransfected and integrated pSV2neo DNA could also be de novo methylated (Fig. 3b). The DNA extracted from the λ DNA-carrying cell lines was cleaved with *Hpa*II, *Msp*I, or *Hha*I, and the fragments were separated by electrophoresis on 1% agarose gels, blotted by the Southern procedure, and hybridized to ^{32}P-labeled λ DNA. Routinely, 10 U of restriction endonuclease per μg of DNA was used. Control experiments using 5 or 30 U per μg of DNA yielded identical results. The autoradiograms for the DNAs from several of the λ DNA-transgenic BHK21 cell lines demonstrated marked de novo methylation of the transgenic λ and pSV2neo DNAs (Fig. 3). With continuous passage of the cells, the extent of transgene methylation increased, probably due to the spreading of de novo methylation (27).

It is concluded that the integrated λ DNA and pSV2neo DNA can become heavily de novo methylated in many of the generated λ DNA-transgenic BHK21 cell lines. The exact site of the initiation of de novo methylation has not been determined. Apparently, most of the multiple integrated copies of λ DNA are congruently methylated in similar patterns. The de novo methylation has been shown both for the 5′-CCGG-3′ (*Hpa*II) and the 5′-GCGC-3′ (*Hha*I) sites in the transgenic λ and pSV2neo DNAs which were, of course, unmethylated at these sites prior to transfection.

The integrated λ and pSV2neo DNAs in the clonal BHK21 cell lines are not detectably transcribed. Total RNA was extracted from several of the λ DNA-transgenic clonal BHK21 cell lines by published methods (2) or by using extraction kits (Qiagen), electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, and blotted onto Qiagen Nylon-Plus membranes. Subsequently, the RNA was analyzed for the presence of λ DNA-specific sequences by hybridization to ^{32}P-labeled λ DNA. In the RNA preparations from 20 different BHK21 cell lines (8 are shown in Fig. 4) carrying integrated λ DNA, λ DNA-specific transcripts were not detectable (Fig. 4a). Similarly, pSV2neo-specific signals could not be detected on Northern blots (data not shown). The same RNA blots yielded RNA signals when the single-copy cellular gene for serine proteinase (Fig. 4b) or the multi-copy IAPI segment was used as the ^{32}P-labeled hybridization probe. We therefore conclude that the integrated λ and pSV2neo DNA molecules in the different hamster cell lines are not transcribed as detectable by RNA blot analyses. Transcription of a single-copy cellular gene, however, is readily detected by Northern blotting.

Altered methylation patterns in the cellular IAPI segments of several λ DNA-transgenic clonal BHK21 hamster cell lines. (i) Restriction analyses with methylation-sensitive endonucle-

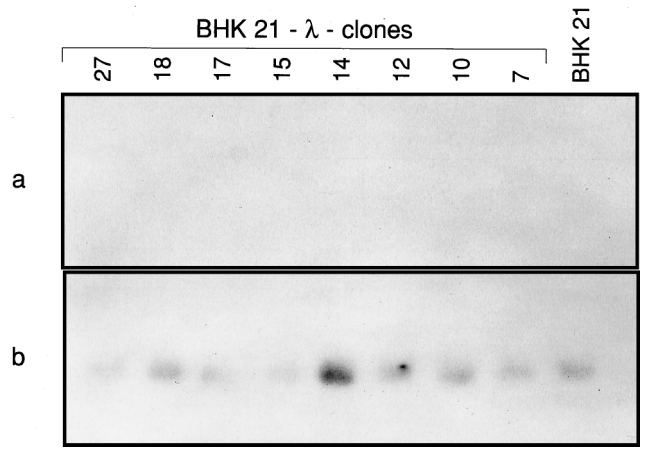

FIG. 4. BHK21 cells transgenic for bacteriophage λ DNA do not detectably transcribe this DNA. An autoradiogram of an RNA (Northern) blotting experiment in which 30 μg of total RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde is shown. Upon transfer of the RNA to a Qiagen Nylon-Plus membrane, the RNA was hybridized to ^{32}P-labeled λ DNA (a) or to the ^{32}P-labeled pBluescript-cloned serine proteinase gene from Syrian hamster (b). In this autoradiogram, RNA samples from only eight of the BHK21 cell lines transgenic for λ DNA were analyzed. When ^{32}P-labeled pSV2neo DNA was used as the hybridization probe, results similar to those in panel a were obtained (not shown).

ases and phosphorimager quantitations. The cellular DNAs from several of the λ DNA-transgenic BHK21 cell lines were isolated from early passages (passages 6 to 8) and were cleaved with the methylation-sensitive restriction endonuclease *Hpa*II or *Hha*I or, as a control, with *Msp*I. Routinely, 10 U of restriction endonuclease per μg of DNA was used. Control experiments using 5 or 30 U per μg yielded identical results. The fragments were separated by electrophoreses on 0.8 to 1% agarose gels, and the DNA fragments were transferred by Southern blotting to Qiagen Nylon-Plus membranes and hybridized to ^{32}P-labeled IAPI DNA cloned in plasmid pBR322. The origin of the IAPI subclone (25) was described elsewhere (15). Some of the autoradiograms of these Southern blot hybridization experiments are shown in Fig. 5. The data demonstrated that the levels of methylation of the IAPI segments in the DNAs from the cell lines BHK21-λ7, -λ10, -λ12, -λ18, and -λ27 were markedly increased in the 5′-GCGC-3′ (*Hha*I) and also in the 5′-CCGG-3′ (*Hpa*II) sequences in comparison to the same DNA segments and sequences in the original, nontransgenic BHK21 cells. In a total of 13 λ DNA-transgenic cloned BHK21 cell lines, similar changes in the methylation of the IAPI segment were observed. In 64 additional λ DNA-transgenic BHK21 cell lines tested, similar alterations in the methylation of the IAPI DNA segments were not apparent. Thus, methylation changes in the IAPI segment could be documented in about 17% of the cell lines investigated. Of course, in the clonal lines without changes in the IAPI segment, different parts of the hamster genome might be affected.

The alterations in the cleavage patterns on the autoradiograms shown in Fig. 5 were analyzed in more detail by using a phosphorimager. The intensities of corresponding fragment bands in the original BHK21 cell line and several of the cell lines transgenic for λ DNA (BHK21-λ7, -λ10, -λ12, -λ18, and -λ27) were compared. The relative intensities of each of the fragment bands were calculated for each of the cell lines, with the total amounting to 100%. Next, for each of the fragment bands quantitatively analyzed, the band intensity measured in BHK21 DNA was arbitrarily set to the value 1.00. The intensities of corresponding fragment bands in the λ DNA-transgenic BHK21 cell lines mentioned above were normalized relative to these 1.0 values for DNAs from the nontransgenic BHK21 cell lines. Thus, changes in band intensities were readily apparent. To various degrees in different λ DNA-transgenic cell lines, the intensities in the lower-molecular-mass bands had decreased and those in the higher-molecular-mass fragment bands had correspondingly increased (Table 1). Similar changes in fragment band intensities were recorded for the *Hpa*II and the *Hha*I cleavage patterns, although the changes were more pronounced in the *Hha*I patterns, as already apparent from a visual inspection of the autoradiogram in Fig. 5. The *Msp*I patterns were also analyzed as negative controls and showed minor changes, if any, in some of the fragments. These analytical data quantitated the increases in DNA methylation in the IAPI segments in the clonal BHK21 cell lines carrying integrated λ DNA. Several of the 5′-CCGG-3′ (*Hpa*II) and 5′-GCGC-3′ (*Hha*I) sequences in the cellular IAPI DNA segments had become increasingly de novo methylated in the λ-transgenic cell lines. It was reported that hamster cells carried up to 900 copies of IAP DNA per haploid genome (20). Surprisingly, the multiple copies of IAPI DNA seemed to be congruently de novo methylated, at least to some extent, although they were located on many different chromosomes. We had found previously that the changes in IAP DNA methylation were similar in the IAP subsegments I to IV (15). In similar experiments, we also investigated several different cloned hamster DNA segments used in an earlier study (15) as hybridization probes with the same blots with DNA from the same λ DNA-transgenic cell lines, but we did not find alterations in their methylation patterns.

In BHK21 cells the integration of foreign DNA into the cellular genome has led to increases in the levels of DNA methylation in certain segments of the endogenous retrotransposon IAPI DNA in the cellular genomes. These transgenic

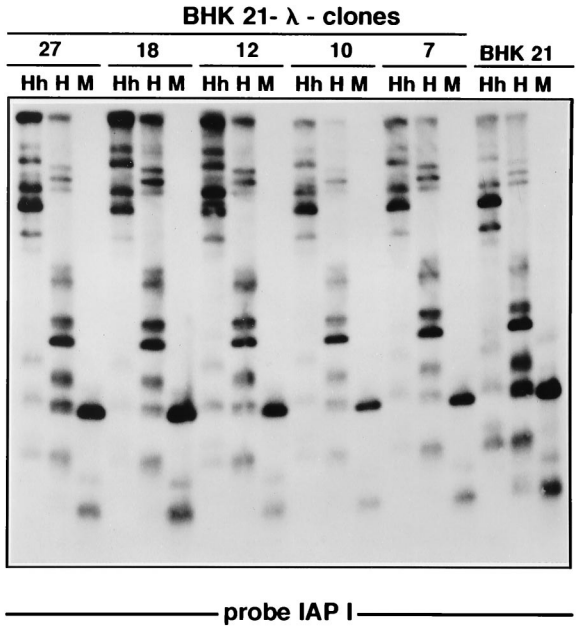

FIG. 5. Increases in DNA methylation in the 5′-CCGG-3′ (*Hpa*II [H]) and 5′-GCGC-3′ (*Hha*I [Hh]) sequences in the IAPI segments of five cloned BHK21 cell lines with integrated λ DNA in comparison to DNA from the non-λ-DNA-transgenic BHK21 cell lines. Experimental procedures were similar to those described in the legend to Fig. 3, except that ^{32}P-labeled IAPI DNA was used as the hybridization probe. The data for the BHK21-λ7 clone have been shown previously (15). The results of phosphorimager analyses are presented in Table 1.

TABLE 1. Phosphorimager analyses of data on DNA methylation[a]

Restriction enzyme	Fragment	Relative intensity in cell line:					
		BHK21	BHK21-λ7	BHK21-λ10	BHK21-λ12	BHK21-λ18	BHK21-λ27
*Hpa*II	1	1.00	1.36	1.35	1.78	1.39	0.92
	2	1.00	2.57	2.16	2.88	2.56	1.68
	3	1.00	3.07	1.80	3.21	2.65	1.71
	4	1.00	1.78	1.62	1.99	1.41	1.41
	5	1.00	2.08	1.61	1.77	1.55	1.30
	6	1.00	1.82	1.80	1.52	1.21	1.19
	7	1.00	1.57	1.38	0.81	1.26	1.17
	8	1.00	1.43	1.55	1.03	1.25	1.16
	9	1.00	0.63	0.69	0.89	0.93	1.08
	10	1.00	0.29	0.49	0.54	0.47	0.75
	11	1.00	0.54	0.58	0.88	0.85	0.91
	12	1.00	0.23	0.27	0.35	0.37	0.39
*Hha*I	1	1.00	1.86	2.19	1.97	2.28	1.64
	2	1.00	2.95	2.48	2.88	4.19	1.79
	3	1.00	2.86	2.95	2.78	3.35	2.92
	4	1.00	2.25	2.64	2.73	2.99	2.29
	5	1.00	2.12	1.89	2.46	2.38	2.31
	6	1.00	1.27	1.12	0.77	0.83	1.25
	7	1.00	0.93	0.88	0.78	0.60	0.72
	8	1.00	0.65	0.60	0.48	0.30	0.44
	9	1.00	0.64	0.61	0.60	0.42	0.50
	10	1.00	0.82	0.57	0.60	0.60	0.51
	11	1.00	0.83	0.62	0.52	0.55	0.46
	12	1.00	0.70	0.88	0.86	0.87	0.94
	13	1.00	0.50	0.64	1.00	1.07	0.78
	14	1.00	0.36	0.36	0.58	0.57	0.44
	15	1.00	0.59	0.54	1.01	0.79	1.10
*Msp*I	1	1.00	1.04	1.14	1.23	1.11	1.16
	2	1.00	1.05	0.87	0.97	0.80	0.78
	3	1.00	0.91	0.76	0.56	0.85	0.74

[a] Analyses of the cleavage patterns in the IAPI segments of five different clonal BHK21 cell lines carrying integrated λ DNA are shown. All fragment bands in the autoradiograms of Fig. 5 were numbered from 1 to 12 (*Hpa*II), from 1 to 15 (*Hha*I), or from 1 to 3 (*Msp*I), from top to bottom, i.e., from high to low molecular mass. The data presented in Fig. 5 were analyzed by quantitating the intensities in individual fragment bands with the aid of a phosphorimager. In comparing fragment band intensities for each individual band in the total cleavage patterns between BHK21 DNA, the internal standard for comparisons, and the DNAs of any of the BHK21-λ clones, the intensity values for the fragments in BHK21 DNA were arbitrarily set to 1.00. The light intensities for the corresponding fragments on the autoradiograms for all of the BHK21-λ clones were then calculated relative to those of the internal BHK21 standard. Thus, intensity comparisons are feasible only by reading the values in Table 1 across. Decreases of values in the lower-molecular-mass bands and increases in the higher-molecular-mass bands were due to increases in DNA methylation. These analyses were performed for the *Hpa*II, *Hha*I, and *Msp*I cleavage patterns. Since *Msp*I was not sensitive to DNA methylation in the 3′-cytidine residue in the sequence 5′-CCGG-3′, marked changes in relative intensities in the λ DNA-transgenic cell lines were not expected and not observed. The *Msp*I data represented an internal control.

BHK21 cells do not express the λ genome and are not morphologically transformed. It is conceivable that the extent and locations of changes in cellular DNA methylation patterns are dependent on the site of foreign DNA insertion. Upon freezing, thawing, and continuous culture of the λ DNA-transgenic cell lines to passage 40 and higher, differences in IAPI segment methylation patterns are no longer apparent in comparison to the patterns in the DNA of BHK21 cells. Perhaps, under the conditions of continuous culture, the cells with altered methylation patterns have selective disadvantages for propagation.

(ii) Analyses of changes in DNA methylation by using the bisulfite protocol of the genomic sequencing technique. Since the restriction and Southern blot hybridization data on increases in IAPI segment methylation revealed rather congruent changes in many of the IAPI genome copies, it appeared to be feasible to document these changes even more precisely by applying the genomic sequencing procedure (11). By this method, each 5-mC residue in a sequence can be determined independently of its location at a specific restriction endonuclease site. Experimental details are described in Materials and Methods. The map in Fig. 6 designates the locations of the 28 5′-CG-3′ dinucleotides in the p3–p4 segment of the IAPI region (25). The map also indicates the primers used in the PCR amplification step prior to sequencing of the bisulfite reaction products. The primers were selected in a segment of the IAPI region that had shown the most obvious changes in DNA methylation as determined by *Hpa*II or *Hha*I cleavage and Southern blot hybridization (Fig. 5; Table 1). The 5′-CG-3′ dinucleotides were investigated for the presence of 5-mC residues in DNA from the cell lines BHK21, T637, BHK21-λ7, and BHK21-λ10 in 123, 79, 58, and 73 cloned PCR products, respectively. In Tables 2 and 3 the percentages of 5-mC residues in the 5′-CG-3′ positions 1 to 34 were calculated for the DNAs from individual cell lines. These values were the averages of >330 independently sequenced cloned, PCR-generated molecules. Values can be compared only between different DNA sources for a given 5′-CG-3′ position; hence, lines in Tables 2 and 3 have to be read across. Table 2 presents the percentages of cloned PCR products that are methylated at the individual 5′-CG-3′ clones for each of the four cell lines (BHK21, T637, BHK21-λ7, and BHK21-λ10) investigated. For more immediate comparisons, the values for each 5′-CG-3′ site were normalized relative to the level of methylation at the same site in the reference cell line BHK21, for which the values were arbitrarily set to 1.000 (Table 3).

In the p3–p4 segment, three *Hha*I sites and two *Hpa*II sites are present (Fig. 6; Tables 2 and 3). The most marked overall increases in 5-mC contents in the 28 5′-CG-3′ sites in the p3–p4 IAPI subsegments are apparent in the Ad12-transformed cell line T637 (Tables 2 and 3). For the two λ DNA-transgenic cell lines, BHK21-λ7 and BHK21-λ10, the most significant increases in methylation at 5′-CG-3′ sites are highlighted. In both λ DNA-transgenic cell lines, the 5′-CG-3′ sites 2, 3, 6 to 8, 20, 21, 33, and 34 have methylation values well above those in the BHK21 reference cell line and even approach methylation levels found in the Ad12-transformed hamster cell line T637 (positions 2, 8, 20, 21, 33, and 34) (Tables 2 and 3; Fig. 7). We have shown previously that the increases in DNA methylation in the IAPI segment in cell line T637 can be readily documented by *Hpa*II and *Hha*I restriction followed by Southern blot hybridization (see Fig. 1 of reference 15). Apparently, the alterations of DNA methylation i.e., increases at some sites and decreases at other sites, in the λ DNA-transgenic cell lines have to be evaluated site by site and can be different at individual sites in each λ DNA-transgenic cell line (Tables 2 and 3; Fig. 7). The data reflect the expected polymorphisms in 5′-CG-3′ sequences in the IAPI segment with a haploid copy number of 900 (20, 25) in the hamster genome. In many of the cloned molecules from the IAPI segment p3–p4, 5′-CG-3′ dinucleotides 23 to 27 (Fig. 6) are deleted or altered to non-5′-CG-3′ dinucleotides (dinucleotides 19 and 35) and have therefore been omitted from the quantitation in Tables 2 and 3 and Fig. 7.

For each of the Ad12 or λ DNA-transgenic cell lines investigated as well as for the BHK21 reference cell line, a large number of PCR-generated clones were sequenced after bisulfite treatment of the genomic DNA. The data in Tables 2 and 3 are based on >330 individually cloned DNA molecules in which 5-mC residues were determined. These results are also presented graphically in Fig. 7. The percentage of methylated 5′-CG-3′ dinucleotides in each of the 28 dinucleotide positions in the p3–p4 segment of the IAPI region for the reference cell

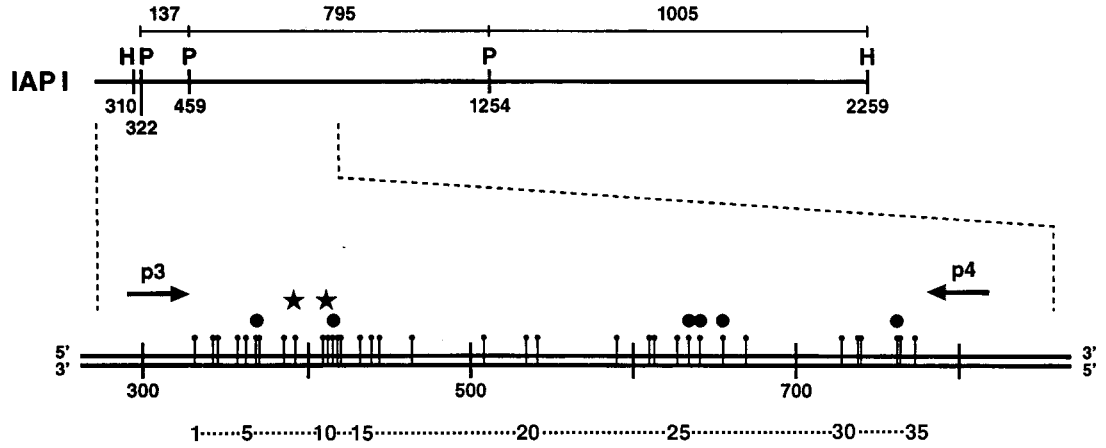

FIG. 6. Methylation analyses of the IAPI segments in the BHK21 and T637 cell lines and in the λ DNA-transgenic clonal BHK21 cell lines BHK21-λ7 and BHK21-λ10 by using the bisulfite protocol of the genomic sequencing procedure. A map of subclone I in the IAP retrotransposon in hamster cells (25) is shown. The locations of the primers used in the amplification step of the genomic sequencing procedure following the bisulfite reaction with clonal DNAs are designated with horizontal arrows. Several segments were genomically sequenced. The data shown in Tables 2 and 3 were derived from the primer p3–p4-flanked subsegment of the IAPI region, which contains 28 5′-CG-3′ dinucleotide sequences (vertical lines), as published previously (25). For experimental details see the text and the footnotes to Tables 2, 3, and 4. *Hpa*II (stars) and *Hha*I (circles) sites are indicated. Numbers refer to the published nucleotide sequence (25).

line BHK21 was compared with that in the same positions in cell lines T637, BHK21-λ7, and BHK21-λ10. The most extensive increases in DNA methylation were apparent in the Ad12 DNA-transgenic and transformed cell line T637. Alterations—often increases—were also seen for the two λ DNA-transgenic cell lines. A clustering of alterations in methylation sites in the IAPI segments was not apparent in any of the clonal lines investigated (see also Tables 2 and 3).

(iii) Genomic sequencing control experiments with the IAPI p3–p4 segments from four nontransgenic, subclonal BHK21 cell lines. Since methylation at 5′-CG-3′ sites in the 900 haploid IAP copies in hamster cells is expected to be polymorphic, a control analysis was performed. The levels of 5′-CG-3′ methylation at the 28 sites of the IAPI p3–p4 segments in the BHK21 reference cell line and four subclones of this cell line, sublines BHK21-1, BHK21-2, BHK21-3, and BHK21-4 (the latter mock transfected), were compared to assess the naturally occurring polymorphic fluctuation of methylation in this segment. The data in Table 4 were obtained by the same experimental methods of genomic sequencing with the bisulfite protocol and of normalization as described for Table 3 (also compare Fig. 7). However, the sequence determinations for PCR products cloned upon bisulfite treatment of DNAs from the BHK21 reference cell line, from the Ad12-transformed hamster cell line T637, and from the four different subclonal cell lines BHK21-1, BHK21-2, BHK21-3, and BHK21-4 were completely independent of the experiments described for Tables 2 and 3 and Fig. 7. Totals of 68, 43, 50, 60, 54, and 60 (Σ = 335) clones, respectively, were sequenced. As expected, the four BHK21 subclones showed some polymorphism in the levels of 5′-CG-3′ methylation in the IAPI p3–p4 segment, but practically all values were similar to the equivalent values in the BHK21 nontransgenic reference cell line (compare Tables 3 and 4). In contrast, the values for cell line T637 were again decisively higher than those for the BHK21 reference cell line (Table 4).

The results of these control genomic sequencing experiments validate the changes in 5′-CG-3′ methylation in the IAPI p3–p4 segment documented for the Ad12 DNA-transgenic cell line T637 and for the λ DNA-transgenic cell lines BHK21-λ7 and BHK21-λ10 (Tables 2, 3, and 4; Fig. 7).

TABLE 2. DNA methylation of individual 5′-CG-3′ dinucleotides in the p3–p4 segment of the IAPI DNA region[a]

5′-CG-3′ position	% Methylation in cell line:			
	BHK21	T637	BHK21-λ7	BHK21-λ10
1	39.8	77.3	42.6	47.2
2	51.8	82.4	72.0	70.1
3	54.8	81.6	65.3	65.7
4	46.6	70.3	51.0	66.7
5	79.6	88.9	86.5	83.3
6[b]	65.2	90.4	76.9	76.9
7	73.0	90.0	79.6	83.8
8	65.4	86.1	78.8	77.5
9[c]	67.9	85.1	67.3	85.5
10	67.9	86.8	64.3	64.3
11[c]	87.5	93.3	84.9	87.9
12[b]	48.6	67.1	39.2	56.5
13	66.0	83.1	62.5	78.1
14	72.1	94.7	64.3	82.4
15	67.6	83.3	57.7	77.9
16	68.4	76.7	57.7	77.5
17	51.0	75.0	52.0	52.3
18	48.6	58.0	35.8	47.1
20	42.0	79.5	66.0	62.0
21	27.8	61.5	41.1	53.4
22	37.8	70.1	43.6	37.3
28	44.1	69.2	43.9	35.8
29	35.0	54.5	35.2	34.3
30	60.0	92.9	60.7	52.5
31	50.9	77.8	77.4	45.5
32	73.5	83.6	80.8	73.5
33[b]	58.9	77.6	82.1	72.6
34	65.2	86.3	82.1	76.5

[a] For details, see the legend to Fig. 7 and Results. The values are the averages of methylated 5′-CG-3′ dinucleotides in the DNAs from 123 DNA clones of BHK21 cells (reference cell line), 79 DNA clones of T637 cells, 58 DNA clones of BHK21-λ7 cells, and 73 DNA clones of BHK-λ10 cells. For all of these clones, the DNA sequence was determined after the genomic DNA had been treated with bisulfite, the p3–p4 segment had been amplified by PCR, and individual PCR products had been molecularly cloned in the pGEM-T vector (Promega). In our BHK21 cells, 5′-CG-3′ dinucleotide positions 23 to 27 were frequently deleted compared to the published sequence (25), and positions 19 and 35 were altered to non-5′-CG-3′ (TG) dinucleotides. Those sequences were therefore not included in the analyses.

[b] *Hha*I (5′-GCGC-3′) sites.

[c] *Hpa*II (5′-CCGG-3′) sites.

TABLE 3. Normalized levels of DNA methylation at individual 5′-CG-3′ dinucleotides[a]

5′-CG-3′ position	DNA methylation level in cell line:			
	BHK21	T637	BHK21-λ7	BHK21-λ10
1	1.000	1.942	1.070	**1.186**
2	1.000	1.591	**1.390**	**1.353**
3	1.000	1.489	**1.192**	**1.199**
4	1.000	1.509	1.094	**1.431**
5	1.000	1.117	1.087	1.046
6[b]	1.000	1.387	**1.179**	**1.179**
7	1.000	1.233	1.090	**1.148**
8	1.000	1.317	**1.205**	**1.185**
9[c]	1.000	1.253	0.991	**1.259**
10	1.000	1.278	0.947	0.947
11[c]	1.000	1.066	0.970	1.005
12[b]	1.000	1.381	0.807	**1.163**
13	1.000	1.259	0.947	**1.183**
14	1.000	1.313	0.892	**1.143**
15	1.000	1.232	0.854	**1.152**
16	1.000	1.121	0.844	**1.133**
17	1.000	1.471	1.020	1.025
18	1.000	1.193	0.737	0.969
20	1.000	1.893	**1.571**	**1.476**
21	1.000	2.212	**1.478**	**1.921**
22	1.000	1.855	**1.153**	0.987
28	1.000	1.569	0.989	0.812
29	1.000	1.557	1.006	0.980
30	1.000	1.548	1.012	0.875
31	1.000	1.528	**1.521**	0.894
32	1.000	1.137	1.099	1.000
33[b]	1.000	1.317	**1.394**	**1.233**
34	1.000	1.324	**1.259**	**1.173**

[a] See Table 2, footnote *a*. The values in Table 2 were normalized relative to the values for the reference cell line BHK21, which were set to 1.000. Boldface indicates the most significant increases in methylation at 5′-CG-3′ sites in the two λ DNA-transgenic cell lines.
[b] *Hha*I (5′-GCGC-3′) sites.
[c] *Hpa*II (5′-CCGG-3′) sites.

The two different analytical methods used to demonstrate changes in the DNA methylation patterns in the IAPI region document alterations in the levels of DNA methylation in several clonal BHK21 cell lines carrying integrated λ DNA as foreign DNA compared to nontransgenic BHK21 cells. The methylation increases in the Ad12-transformed T637 cell line reported earlier (15) have again been documented in two independent experiments (Tables 2, 3, and 4) by using a highly sensitive technique. Remarkably, these changes in DNA methylation involve many of the 900 copies of the IAP retrotransposon sequences in a similar way; otherwise, we would not have been able to detect these changes in DNA methylation patterns in the IAP segment by the two independent methods applied. We have also isolated many λ DNA-transgenic BHK21 cell clones (83%) which do not show any changes in DNA methylation in the IAPI segment analyzed (data not shown).

We conclude that the insertion of foreign (Ad12 or λ) DNA into the established BHK21 hamster cell genome can elicit changes in the methylation in at least some of the 5′-CG-3′ sequences in the IAPI retrotransposon genomes. These changes are more pronounced in the Ad12-transformed BHK21 cell line T637 and have also been documented for the λ DNA-transgenic cell lines.

The changes in cellular DNA methylation patterns are not due to the Ca^{2+}-phosphate precipitation protocol used for BHK21 cell transfection. The standard Ca^{2+}-phosphate transfection procedure is toxic for mammalian cells in culture. We therefore had to ascertain that the application of this technique was not responsible for the observed alterations in DNA methylation patterns in the IAPI segments of the BHK21 cells. BHK21 cells were mock transfected by the Ca^{2+}-phosphate precipitation protocol, i.e., DNA was not added to the transfection mixture. The cellular DNA was then isolated either from the total cell population or from BHK21 cell clones after three rounds of single-cell cloning. Subsequently, the cellular DNA was cleaved with *Hpa*II, *Msp*I, or *Hha*I, and the fragments were separated by electrophoresis on 0.8% agarose gels, transferred to Qiagen Nylon-Plus membranes, and hybridized to the ^{32}P-labeled IAPI DNA clone. The autoradiograms from DNA samples derived from nine different pretreated BHK21 cell clones and from three different uncloned BHK21 cultures provided no evidence for differences in DNA methylation patterns in the IAPI segments of BHK21 cells compared to the DNA samples from untreated control cultures. (The data are not shown but were similar to those in Fig. 8). Apparently, the Ca^{2+}-phosphate transfection protocol by itself, including biochemical selection and single-cell cloning, does not cause changes in the methylation patterns of the cellular IAPI segment.

The IAPI segments in the DNAs from 66 isolated and propagated nontransgenic BHK21 cell clones exhibited the same methylation patterns as that from untreated BHK21 cells. BHK21 cells were recloned up to three times on microtiter plates by seeding the cells at <1 cell per 10 wells. Clones derived from single cells were propagated to the level of one 75-cm^2 monolayer culture, and the DNA was extracted and investigated for changes in IAPI segment methylation patterns by cleavage with the methylation-sensitive restriction endonucleases as described above. The results from 66 different single-cell clones showed identical methylation patterns in the IAPI segments and were identical to those from the bulk culture of BHK21 cells. Representative data of *Hpa*II cleavage patterns of DNAs from 16 different clones are shown (Fig. 8). Similarly, the DNAs from all 66 different BHK21 clones showed identical *Hha*I cleavage patterns (data not shown).

In the 66 BHK21 cell clones tested, the IAPI segment exhibits methylation patterns identical to those in the bulk culture of BHK21 cells and among individual BHK21 cell clones. Although the presence of minor variations among cells obviously cannot be completely ruled out, the control data presented provide no evidence for significant mosaics in the IAPI methylation patterns of BHK21 cells. We therefore consider it unlikely that the alterations in DNA methylation in the IAPI segments of the BHK21 cell clones that are transgenic for integrated λ DNA or have been transformed by Ad12 can be attributed to variations in these patterns among different cells in the BHK21 cell population and to the serendipitous selection of cell clones with such aberrant methylation patterns. The data in Table 4, which are based on a much more sensitive technique, have led to the same conclusion.

DISCUSSION

Alterations in cellular DNA methylation patterns. This study has been undertaken to determine whether changes in DNA methylation patterns of cellular genes and DNA segments observed in Ad12-transformed hamster cells (15) are due to the transformed state of the cells, to the insertion of foreign DNA into an established mammalian genome, to a combination of both events, or to preexisting mosaics in the methylation patters in the cellular IAPI segments. The data presented in this report support the interpretation that the in-

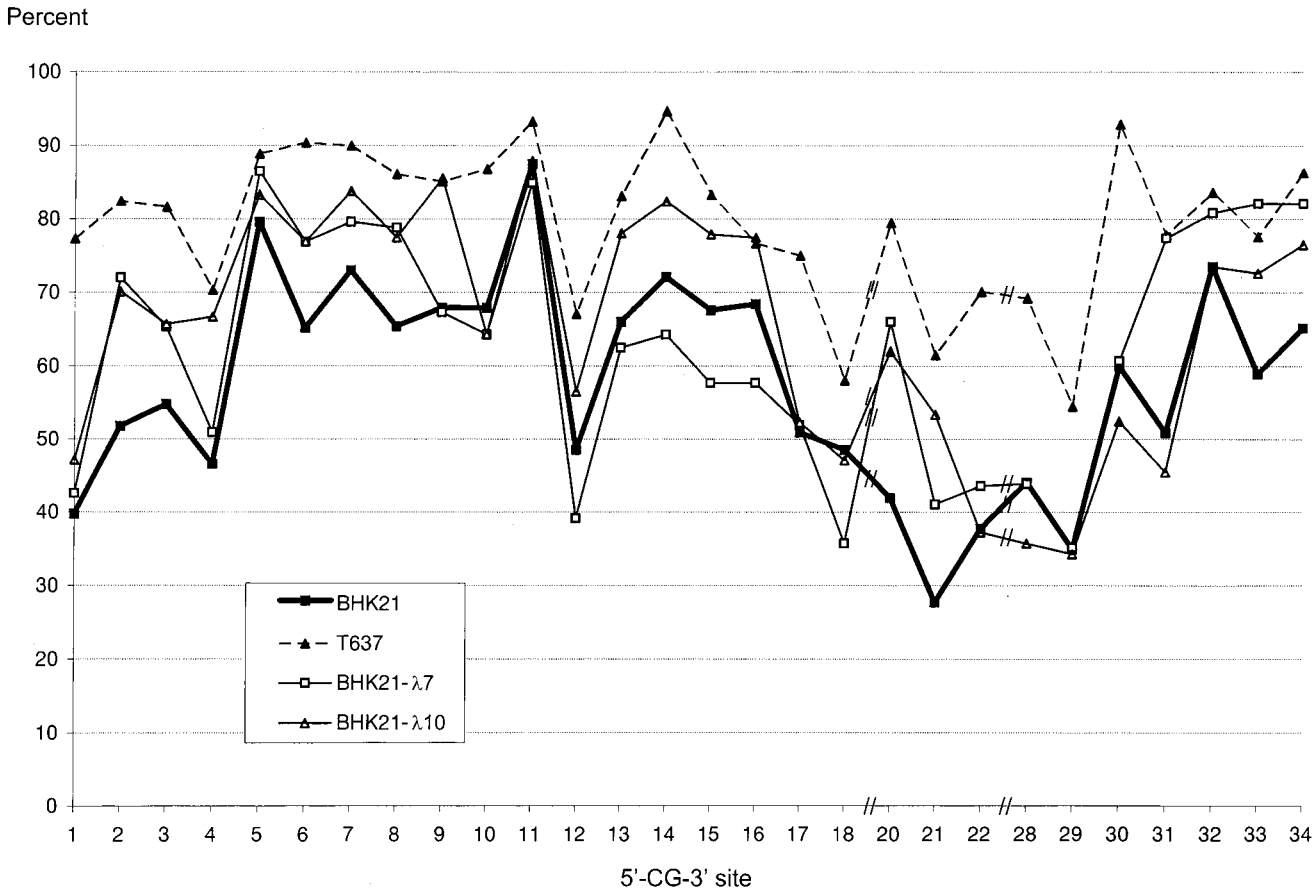

FIG. 7. Alterations in DNA methylation at 28 5′-CG-3′ sites in the p3–p4 segment (Fig. 6) of the IAPI retrotransposon sequence in the Ad12-transformed hamster cell line T637 and in two λ DNA-transgenic BHK21 hamster cell lines, BHK21-λ7 and -λ10. Experimental details are described in the text. The numbering of the 5′-CG-3′ dinucleotides corresponds to that in Fig. 6. During the genomic sequencing experiments, 123 DNA clones from the reference BHK21 cell line, 79 DNA clones from the Ad12-transformed T637 cell line, 58 DNA clones from the BHK21-λ7 cell line, and 73 DNA clones from the BHK21-λ10 cell line were sequenced. The percentage values represent the average of methylated 5′-CG-3′ dinucleotides at each site for DNA from each of the cell lines. In many of the clones, 5′-CG-3′ positions 23 to 27 were in a deleted segment as part of a naturally occurring polymorphism in this region compared to the published nucleotide sequence (25). Furthermore, 5′-CG-3′ positions 19 and 35 were altered to 5′-TG-3′ in many clones and were therefore omitted from this analysis.

tegration of nontranscribed bacteriophage λ DNA in the hamster genome is associated with such changes in some of the clonal transgenic BHK21 cell lines, notably in the IAPI retrotransposon sequences. It is conceivable that these repetitive sequences, which were integrated into the hamster genome probably several million years ago (43), may be particularly susceptible to alterations in DNA methylation patterns subsequent to the insertion of foreign DNA. Moreover, the sites and the extent of changes in methylation patterns of cellular DNA may be dependent on the sizes and locations of the foreign DNA integrates.

Alterations in DNA methylation can be most convincingly documented with a method that permits the analysis of each individual 5′-CG-3′ dinucleotide in a sequence. In several studies performed in this laboratory (24, 36, 45), we found the bisulfite protocol of the genomic sequencing technique (11) to be most reliable and to yield reproducible results. The data in Tables 2, 3, and 4 have been adduced from genomic sequencing studies extending over a DNA stretch of 522 (p3–p4)(Fig. 6 and 7) nucleotide pairs and encompassing 28 5′-CG-3′ dinucleotides in the IAPI DNA region. The p3–p4 segment carries deletions in many of the analyzed molecules, isolated by cloning upon PCR amplification, compared to the published sequence (25). These data are based on the genomic sequencing of over 330 individual DNA molecules and demonstrate that alterations in DNA methylation patterns in cellular DNA segments upon the integration of foreign DNA can best be documented by applying the genomic sequencing technique. This result has been strengthened by the genomic sequencing data adduced from four clonal sublines of nontransgenic BHK21 cell lines. The levels of DNA methylation in the IAPI p3–p4 segments in these sublines varied to some extent but were close to those in the original BHK21 cell line (Table 4).

As described in detail (Fig. 1 and 2), at the sites of foreign DNA integration in the BHK21-λ cell lines, λ DNA colocalizes with pSV2neo DNA, usually at one chromosomal insertion site. Since these cell lines have been selected for resistance against G418, the pSV2neo DNA has to be transcribed in these cells only during the selection period, whereas λ DNA transcription has never been detected in the transgenic cell lines tested. When considering possible mechanisms by which foreign DNA integration may lead to alterations of cellular DNA methylation patterns and possibly chromatin structure, the transcriptional activity in the pSV2neo part of the transgenic DNA might have to be debated. However, by the time that cellular methylation patterns were determined, G418 selection had been discontinued. Moreover, pSV2neo-specific signals could not be detected by RNA blot experiments.

TABLE 4. Normalized levels of DNA methylation at individual 5′-CG-3′ dinucleotides in the IAPI (p3–p4) segment of the BHK21 cell line (reference), in cell line T637, and in four different nontransgenic BHK21 subclonal cell lines[a]

5′-CG-3′ position	DNA methylation levels in cell line:					
	BHK21 (68)[b]	T637 (43)	BHK21-1 (50)	BHK21-2 (60)	BHK21-3 (54)	BHK21-4 (60)
1	1.000	1.445	0.850	0.602	1.062	0.837
2	1.000	1.255	0.931	0.840	1.148	1.091
3	1.000	1.154	0.874	0.831	0.822	0.822
4	1.000	1.276	1.050	0.841	0.855	0.855
5	1.000	1.050	1.014	1.002	1.027	1.027
6[c]	1.000	1.149	0.996	1.010	1.010	1.010
7	1.000	1.094	0.899	0.987	0.946	0.938
8	1.000	0.999	0.900	0.823	0.910	0.901
9[d]	1.000	1.062	0.894	0.847	0.892	1.013
10	1.000	1.086	0.894	0.996	1.107	0.880
11[d]	1.000	1.058	1.076	1.014	1.120	1.125
12[c]	1.000	0.977	0.843	0.628	0.870	0.915
13	1.000	1.146	1.106	1.018	1.123	1.039
14	1.000	1.231	1.061	0.963	0.994	0.930
15	1.000	1.048	0.917	0.998	0.940	0.962
16	1.000	1.083	0.785	0.787	0.920	0.983
17	1.000	1.279	0.777	0.569	0.678	0.976
18	1.000	1.728	1.395	0.966	1.111	1.277
20	1.000	1.123	0.788	0.915	0.946	0.899
21	1.000	1.534	0.810	0.704	1.230	1.112
22	1.000	1.182	0.912	0.523	1.043	0.818
28	1.000	1.085	0.986	0.775	0.890	0.901
29	1.000	0.842	0.738	0.611	0.861	0.927
30	1.000	1.247	1.107	1.089	1.063	1.116
31	1.000	1.337	0.906	0.825	1.047	0.986
32	1.000	1.050	0.909	1.006	0.948	0.945
33[c]	1.000	1.194	0.891	0.992	0.982	1.049
34	1.000	1.213	0.933	0.999	1.090	1.035

[a] Experimental details and methods of calculation are described in the footnotes to Tables 2 and 3 and the legend to Fig. 7. However, the data here are derived from completely independent sequence determinations of cloned PCR products in the IAPI p3–p4 segment (Fig. 6). The subclonal cell line BHK21-4 had been mock transfected, with λ DNA omitted from the transfection mixture.
[b] The number of clones individually sequenced are given in parentheses.
[c] *Hha*I (5′-GCGC-3′) sites.
[d] *Hpa*II (5′-CCGG-3′) sites.

We have shown previously that the infection of BHK21 cells with Ad12 and the concomitant transcription of early Ad12 genes do not elicit detectable changes in cellular DNA methylation patterns (15). Minor changes in a subpopulation of the cells cannot be ruled out. In this system, there is a complete block of Ad12 DNA replication (6, 7, 33). Arrays of newly synthesized Ad12 DNA thus cannot be formed. Moreover, it is unlikely that the transfection protocol by itself is capable of altering cellular DNA methylation patterns. It is impossible to rule out categorically the existence of mosaics in IAP methylation patterns in BHK21 hamster cells. However, in a total of 75 isolated subclonal cell lines that we have investigated, uniform and completely stable methylation patterns have been observed in the IAPI segments, whereas in the λ DNA-transgenic cell lines about 13 of 77 exhibited changes in the methylation of the IAPI p3–p4 segment. Genomic sequencing in the IAPI p3–p4 segment has not revealed major differences in DNA methylation in four BHK21 subclones (Table 4).

We therefore favor the interpretation that the alterations observed in the methylation patterns of the IAPI segments upon integration of bacteriophage λ DNA have been induced by the integration of foreign DNA. In the Ad12-transformed hamster cell lines, notably in the cell line T637, which has been directly derived from BHK21 cells by Ad12 transformation (40), the increases in DNA methylation have been more pronounced, probably because of the transformed phenotype of these cells. These increases in DNA methylation are stable in the TR3 revertant of the Ad12-transformed cell line T637, which has lost all of the multiple copies of integrated Ad12 DNA, as detected by Southern blotting (15). There is evidence from several lines of studies (see, e.g., references 1, 9, 13, and 15) that the transformed or oncogenic phenotype of mammalian cells is frequently associated with alterations in cellular DNA methylation patterns. Upon freezing, thawing, and long-term cultivation of the λ DNA-transgenic BHK21 cell lines, the alterations in IAPI DNA methylation patterns are no longer apparent. The cells with altered patterns might have selective growth disadvantages and thus disappear from the cell population.

The integration of λ DNA into the hamster cell genome is not known to transform cells to the oncogenic phenotype. However, it is conceivable that specific integration events with the concomitant alterations in cellular DNA methylation and expression patterns are, however rarely, capable of eliciting the oncogenic transformation events.

Foreign DNA integration and cellular chromatin structure: possible mechanisms. We have set out to investigate whether foreign DNA insertion into an established hamster cell genome can be related to detectable structural changes in cellular chromatin. Given these premises, certain cellular DNA segments might be exposed more directly to the cellular DNA methyltransferase systems from which they might have been protected prior to the insertion of foreign DNA. Since both after viral infection and after the transfection of foreign DNA,

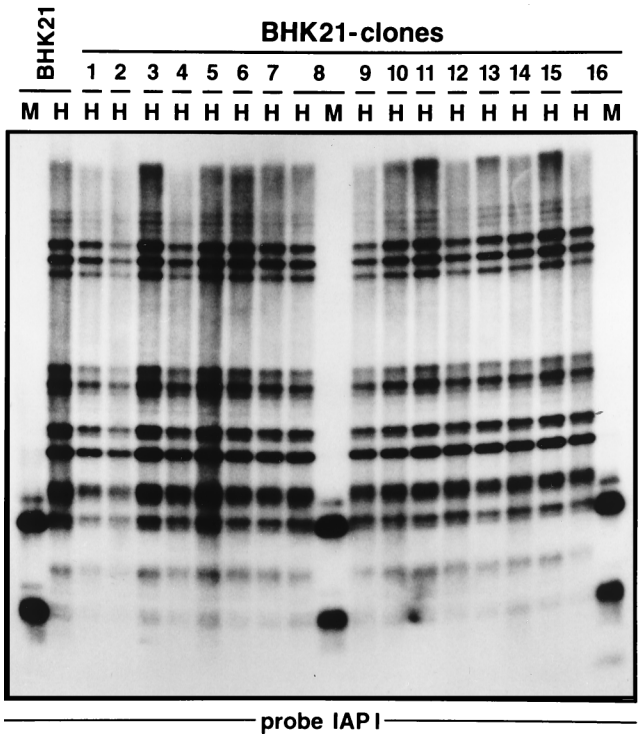

FIG. 8. The IAPI segment methylation patterns obtained upon *Hpa*II (5′-CCGG-3′) cleavage were identical in 66 nontransfected and 9 mock-transfected individual BHK21 cell clones and did not differ from those in DNA preparations from unselected, uncloned BHK21 cells. As examples, the IAPI DNA hybridization results with *Hpa*II (H)-cleaved DNAs from unselected BHK21 cells and from 16 individual BHK21 cell clones are shown. M, *Msp*I control pattern.

multiple copies of the foreign DNA can become inserted into the recipient genome, major structural perturbations due to the addition of large DNA blocks may ensue. Preliminary data (not shown) are consistent with this interpretation but need further refinement.

We also pursue the possibility that, depending on the sites of foreign DNA insertion, different genomic segments can be subject to changes in methylation patterns, because in the nucleus of a living cell different segments of individual chromosomes have unique and highly specific spatial interrelationships. Thus, by integrating foreign DNA arrays at a given site, specific genome neighborhoods of that site would be primarily affected. Insertions at a different site would affect other regions of the genome. It will also be interesting to investigate to what extent signal transmissions via the nuclear matrix upon insertion of foreign DNA will be able to affect the methyltransferase systems of the cell.

General implications. In many experimental procedures, the insertion of foreign DNA into an established mammalian or plant genome has become everyday practice, usually with the goal of expressing a foreign gene or of eliminating or restoring the function of an endogenous gene. The possibly farther-reaching sequelae of such manipulations have frequently not been contemplated. Changes in cellular DNA methylation patterns as a consequence of foreign DNA insertion would call for a precise, case-by-case analysis of alterations in cellular DNA methylation and transcription patterns with the potential for functional consequences. It has been known for almost two decades that DNA methylation and transcription patterns are functionally related (3, 4, 8, 29, 41). In Ad-transformed cells or in Ad12-induced tumor cells, altered transcription patterns in 5 of 40 investigated (in part randomly selected) cellular DNA segments and genes have been described (32).

The experimental findings reported here can be considered in a wider general context when interpreting experiments in viral oncogenesis or with transgenic organisms.

(i) Studies on the mechanism of viral oncogenic transformation. The process of viral oncogenic transformation might be causally related to changes in cellular DNA methylation and transcription patterns.

(ii) Studies on transgenic organisms. The most obvious interpretations of data gleaned from such studies on transgenic organisms may not always coincide with reality. The insertion of foreign DNA in these experiments could influence the activity of many more genes than the ones directly affected by the knock-in or the knockout procedure.

(iii) Schemes developed for human somatic gene therapy. The stable insertion of foreign DNA into the human genome could have far-reaching consequences with undesirable side effects.

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