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Interclonal variation in methylation patterns for expressed and non-expressed genes

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ABSTRACT

A mass culture of human diploid fibroblasts, and eight clones isolated from that mass culture, were examined for methylation patterns in several regions of DNA. Plasmid-inserted cDNA sequences were used as probes for α-hCG, β-globin, A_γ- and G_γ-globin, and β- and γ-actin gene regions. Each probe revealed a different clone-specific pattern of DNA methylation, indicating a striking degree of inter-clonal heterogeneity, for those gene regions which are not normally expressed in diploid fibroblasts (α-hCG, γ-globin and β-globin). Intra-clonal variation was also evident in many instances, implying that heterogeneity could arise de novo in pure cell clones during serial passage. Thus methylation patterns, in particular for repressed genes, appear to be unstably inherited in these cells, and this instability may lead to random derepression in some cell lineages during mitotic growth.

INTRODUCTION

It has been proposed that specific cytosine methylations at CpG loci in DNA might be important heritable determinants of gene repression during cell differentiation (1-4). An inverse relationship between gene expression and cytosine methylation has been corroborated by many studies (1-10), but a number of exceptions have also been documented. For example, human placenta (3) and certain permanent cell lines of human (3) and rabbit (5) derivation have very low levels of methylation at CpG loci in the β-globin gene clusters despite their nonerythropoietic origins. Moreover, exogenous DNA transfected into permanent mouse cell lines can lose prior methylations (11,12) can, on rare occasions, gain methylations in vivo (11), or can remain unmethylated yet unexpressed in their new environment (13). Taken overall, the evidence indicates that hypomethylation may be a necessary but insufficient condition for gene expression (4,13). Nevertheless, the fidelity with which specific patterns of DNA methylation are transmitted in endogenous gene loci of diploid cells remains to be determined. Such experi-

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ments cannot readily be performed with animal tissues because of their polyclonal developmental history, but can be achieved with a homogeneous population of cultured human diploid fibroblasts.

We have therefore examined methylation patterns of human fibroblasts, both in mass culture and in clonal isolates after extended propagation *in vitro*. We find evidence of marked clonal heterogeneity in methylation of human γ-globin genes and α-hCG genes, whereas methylation of the β-globin gene and the β-actin gene family is substantially less variable between fibroblast clones. These observations suggest that gene methylation patterns are not always rigidly maintained over many cell generations, may be more variable in some gene regions than in others, and could be responsible for the leaky expression of "silent" genes which has previously been reported in several normal cell strains (14).

MATERIALS AND METHODS

DNA Isolation from Mass Culture and Clones of Human Diploid Fibroblasts

Fibroblast strain A2 was established as previously described from a forearm biopsy of a normal 11 year old male donor, and grown in Eagle's medium with 15 percent fetal calf serum (15). Clones were isolated by low density plating and subsequent identification of attached single cells, followed by propagation of each clone. DNA was prepared from mass-culture cells and from each clone with >90 percent yield, as previously described (16).

Preparation of DNA Probes and Hybridization to Nitrocellulose Filter Transfers of Electrophoresed DNA Fragments

Plasmid DNAs were prepared by standard procedures (17) from transfected *E. coli*. Plasmid pHγG1 and pHβG1 (17), containing human $^{G}\gamma$-globin and β-globin cDNA sequences, respectively, were the kind gift of P.F.R. Little and R. Williamson. Plasmid pCGα, containing the cDNA sequence of human chorionic gonadotropin α subunit (αhCG), was the kind gift of I. Boime (18). Plasmid pA1, containing a chicken β-actin cDNA sequence, was the kind gift of D. Cleveland (19). Globin cDNA inserts were cleaved out with HpaII, recovered by pressure elution from frozen agarose gels followed by adsorption to DEAE-cellulose (Whatman DE52), elution in 1 M NaCl/6.5 M urea, and ethanol precipitation. Insert and plasmid DNAs were labeled with ^{32}P-dATP and/or dCTP by nick-translation (20) to a specific activity of 10^8-10^9 cpm/μg.

Total human fibroblast DNA (6 μg in 50 μl) was digested 2 h at 37°C with

MspI or HpaII enzyme (6 units / μg DNA), brought to 1 x Eco-RI buffer, and digested 2 h at 37°C with 5 units Eco-RI/μg DNA. Samples were ethanol precipitated, resuspended, and electrophoresed on 1.5 percent agarose gels. DNA was transferred to nitrocellulose paper (Schleicher and Schuell BA85) and hybridized 18 h at 42°C with denatured probe (1.2×10^7 cpm in 20-25 ml) as described by Wahl et al. (21) with the addition of 20 μg/ml each poly(rA) and poly(rC). Filters were then rinsed 20 min at 68°C in 5 x SSC, and 4 x 20 min at 55°C in 0.1 x SSC (1.0 x SSC in the case of the β-actin probe), covered in Saran wrap and exposed at -70°C for 3-14 days to Kodak XAR film in enhancement screen cassettes. Prior to subsequent reutilization, filters were rinsed 20 min at 80-90°C in the "dehybridization buffer" of Thomas (22).

RESULTS

Human fibroblast DNA, from the A2 mass culture and from eight clones isolated from the parent culture, was digested to completion with 6-fold excess of either MspI, which cleaves -CCGG- and -C$\overset{*}{C}$GG- (23,24), but apparently not -$\overset{*}{C}$CGG- (3), or HpaII, which cuts -CCGG- and -$\overset{*}{C}$CGG-, but not -C$\overset{*}{C}$GG- (25). ($\overset{*}{C}$ indicates 5-methylcytosine.) Each sample was then digested with excess Eco-RI in order to introduce scissions which would not depend upon methylation state (26,27). These DNA samples were then electrophoresed in 1.5 percent agarose and transferred to nitrocellulose filters (21,28). Similar digestions with twice the enzyme : DNA excess gave nearly identical results, and served as replicates in subsequent hybridizations.

In the first hybridizations, filters were incubated with ^{32}P-DNA probe containing an insert sequence of chicken β-actin cDNA. Cross hybridization occurs with human DNA sequences (19), producing a pattern of at least a dozen bands after MspI plus Eco-RI digestion (Figure 1). The pattern after HpaII plus Eco-RI cleavage was shifted to higher molecular weight bands, but the multiplicity of bands precluded a complete analysis of the autoradiographic data (Figure 1). Two of these bands (at 1.2 kb and 1.5 kb after MspI + Eco-RI) were more prominent than the rest, and survived high stringency rinses relatively unattenuated (not shown), implying greater homology to the probe. These are presumably fragments containing the β-actin sequence (Figure 5 in reference 19), while the more weakly hybridizing bands are believed to include fragments coding for multiple γ-actin genes (19). For either pair of restriction digestions, the hybridization pattern did not appear to vary greatly from clone to clone, although extensive methylation

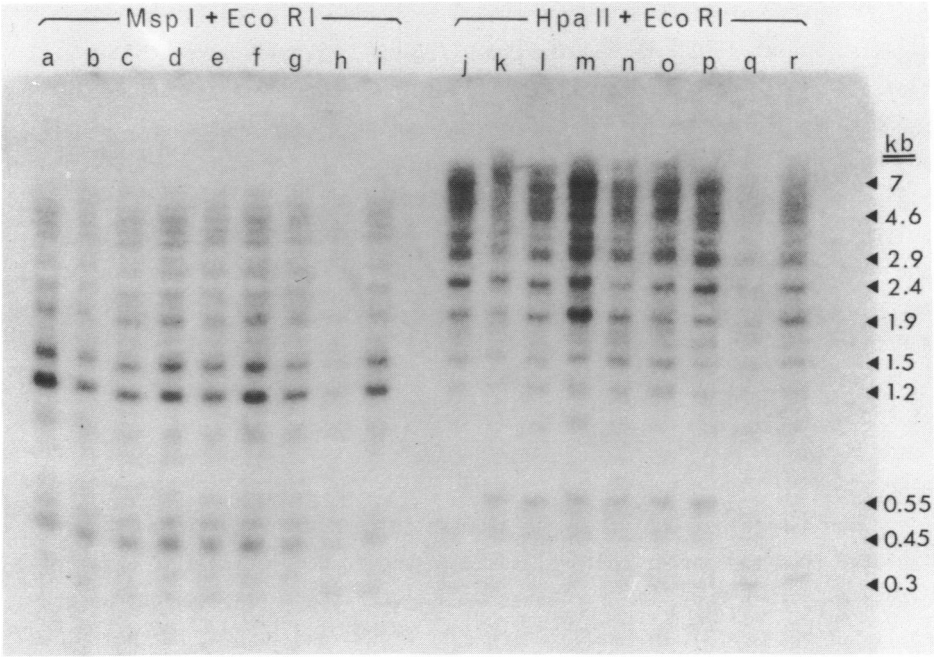

Figure 1. Interclonal variation of methylation patterns in the β- and γ-actin gene regions. Autoradiograph of Southern-transferred DNA fragments hybridizing to a β-actin ^{32}P-cDNA probe. DNA samples were obtained from A2 diploid human fibroblasts at 28 mean population doublings (a,j) and eight clones isolated from that strain: clone 1 (b/k), clone 2 (c/l), clone 3 (d/m), clone 4 (e/n), clone 5 (f/o), clone 6 (g/p), clone 7 (h/q), and clone 8 (i/r). All DNA loads were 6 μg, except clone 7 which was 3 μg. DNA samples were digested with MspI and Eco-RI (a-i) or HpaII and Eco-RI (j-r). Fragment sizes in kilobase pairs (kb) were determined from PM2/Hind III and lambda/Hind III markers.

of -CCGG- 3' cytosines in these gene regions was evident from a comparison of HpaII vs. MspI fragment sizes. Only the HpaII+Eco-RI band at 0.55 kb appeared to differ between DNA samples, being absent in the mass culture (Figure 1, lane j) and two clones (lanes q and r).

The ^{32}P-DNA probe was dissociated from the filters by "melting" at 80-90°C (22) after which autoradiography revealed very little residual ^{32}P. The filters were then rehybridized with a ^{32}P- DNA probe for the α-subunit of human chorionic gonadotropin (α-hCG). The resulting autoradiograph (Figure 2) showed quite uniform hybridization to DNA samples from the mass culture (lane a) and from all eight clones (b-i) digested with MspI plus Eco-RI. Four principal bands of hybridization were seen for each sample, at

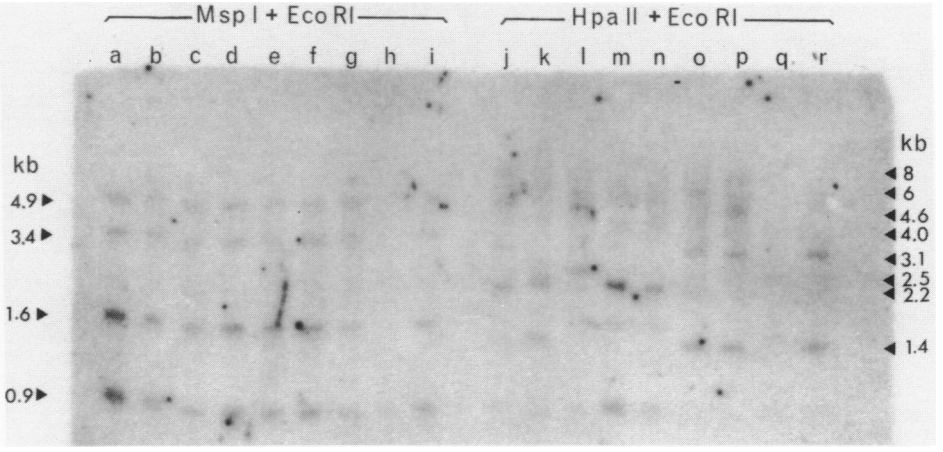

Figure 2. Interclonal variation of methylation patterns in the α-hCG gene region. This is the same filter as shown in Figure 1, following dehybridization and reassociation with ^{32}P-α-hCG DNA probe. Lanes and markers correspond to the legend for Figure 1.

0.9 kb, 1.6 kb, 3.4 kb, and 4.9 kb. In contrast, following HpaII plus Eco-RI digestion (Figure 2, j-r), some or all of these bands were shifted to higher molecular weight, producing a distinctive pattern of bands for each clone (presumably reflecting their locus-specific patterns of -CCGG- methylation). New fragment sizes appearing in these digests included 1.4 kb (lanes j,k,o, p,r), 2.2 kb (j-n), 2.5 kb (l,q), 3.1 kb (j,l,o,p,r), 4 kb (m,n), 4.6 kb (j,l,p), and several higher molecular weight species. The variability between clones in HpaII cleavage sites (not seen after MspI and Eco-RI) indicates a striking heterogeneity of methylation patterns among these fibroblast clones, in the α-hCG gene region (Figure 2) yet not in the β- and γ-actin region (Figure 1).

Replicate filters were next hybridized to a ^{32}P-DNA probe containing a $^{G}\gamma$-globin cDNA sequence, subjected to stringent washes, and autoradiographed (Figure 3). In this case, hybridization to DNA samples digested with MspI plus Eco-RI was not as uniform as found for the first two probes, but instead the relative proportions of $^{A}\gamma$-globin bands at 2.6 kb, 1.5 kb, 1.05 kb and 0.6 kb, and of $^{G}\gamma$ bands at 4.7 kb, 1.6 kb, and 0.95 kb, varied distinctively between clones (Figure 3, a-i), indicating variability in methylation of -CCGG- outer (5') cytosines. Relative band intensities were still more variable between clones (and between the mass culture and the clones) after HpaII plus Eco-RI cleavage, indicative of variable methyla-

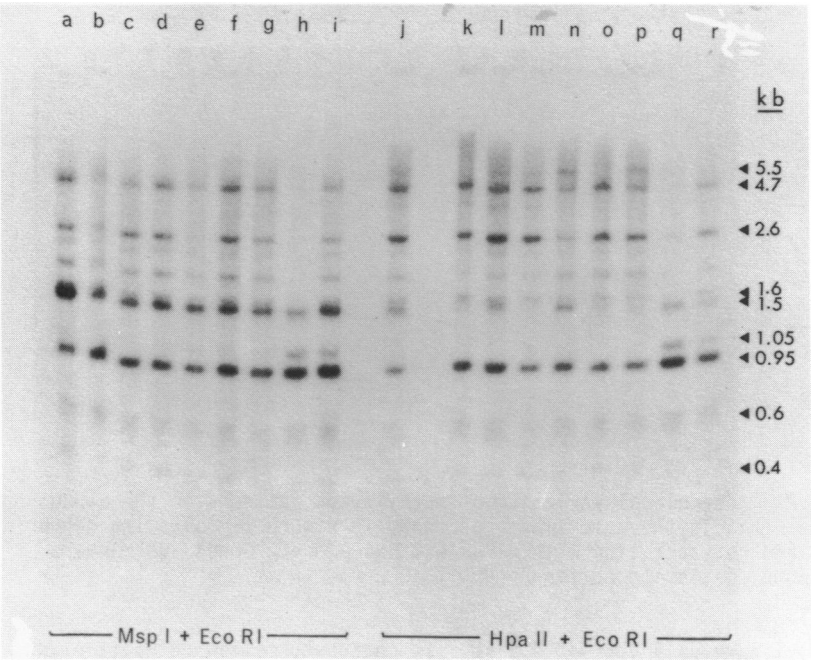

Figure 3. Interclonal variation of methylation patterns in the γ-globin gene region. Lanes and markers correspond to the legend for Figure 1.

tion at internal (3') cytosines of -CCGG- loci. In particular, the relatively complete disappearance of the 1.5 kb band should be noted in Figure 3, lanes k, m, o, p and r; and of the 2.6 kb band in lanes b, e, h, n, and q. The appearance of a 1.05 kb band is limited to lanes h,i, q, and r; and the band at 5.5 kb is most prominent in lanes n and p, although also apparent in lanes j, l and m. Very similar results were reported recently (29); they are included here for comparison with the other data. These hybridization results thus reveal far more extensive inter-clonal heterogeneity than had been observed with the β-actin probe (Figure 1), and a more complex distribution of DNA between bands than was obtained for the α-hCG gene (Figure 2).

After dissociation of the γ-globin probe DNA, the same filters were rehybridized with a cloned β-globin cDNA probe (Figure 4). In this instance, after MspI plus Eco-RI cleavage, two β-globin-specific bands of hybridization were observed at 5.1 kb and 0.8 kb, plus a band at 2.3 kb due to cross-hybridization with the 5' δ-globin DNA fragment (3). Although these bands

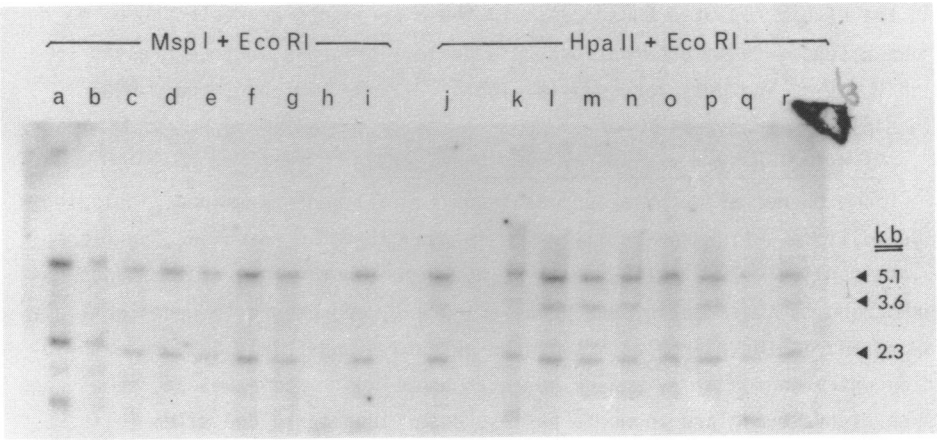

Figure 4. Interclonal variation of methylation patterns in the β-globin gene region. This is the same filter as shown in Figure 3, following dehybridization and reassociation with ^{32}P-β-globin cDNA probe. Lanes and markers correspond to the legend for Figure 1.

varied in intensity lane by lane, within each lane they were present in roughly the same proportions for all DNA samples (Figure 4, a-i). Following HpaII plus Eco-RI digestion, a new β-globin band could be seen at 3.6 kb which was especially pronounced in five of the clones (Figure 4, l,m,n, p,r). Appearance of this band was correlated with disappearance of a faint 3' β-globin band at 0.8 kb (not shown), and was believed to result from methylation at the inner (3') cytosine of a -CCGG- site flanking the β-globin gene. While the variety of methylation patterns was not as great as that observed for α-hCG and γ-globin gene regions, clonal heterogeneity was again clearly evident for the single -CCGG- site assessed in the β-globin gene region.

DISCUSSION

We have recently found that the extent of methylation in total DNA drifts in an apparently random fashion during serial culture of human diploid fibroblasts, whereas the methylation of three highly repeated DNA families remains relatively constant at 70-80 percent (29). This instability of total DNA methylation and contrasting stability of repetitive fractions of DNA was also supported by comparisons of clones isolated from the mass culture fibroblasts which showed substantial heterogeneity both in total DNA methylation and in the pattern of γ-globin-linked DNA methylation, yet not

in the highly repeated DNAs (29). In the experiments presented here, we have extended these observations to a variety of genes, both expressed (β- and γ-actin) and not expressed (β-globin, $^{A}\gamma$ and $^{G}\gamma$-globin, and α-hCG) in diploid fibroblasts.

Methylation as a Measure of Gene Repression

Some degree of methylation was found for all genes examined, including the multiple actin gene family which is thought to be essential for fibroblast survival (19). Although it is not known whether all of the bands hybridizing to the β-actin cDNA probe (Figure 1) correspond to genes actively expressed in the fibroblast (19), the extensive and fairly uniform methylation which we found may appear somewhat anomalous. Nevertheless, some genes such as ovalbumin are found to possess both "invariant" CpG sites (either methylated in all tissues or unmethylated in all tissues) and "variable" sites (tissue specific, correlated with gene expression) (1). Since the MspI/HpaII assay measures only those CpG methylations occurring within the -CCGG- recognition site, many other -CpG- sites will be missed in this assay, possibly including those correlated with expression. de Crombrugghe (30) has recently reported a similar absence of correlation between expression and assayable methylations, for the collagen α2(I) gene in several chicken tissues.

Interclonal Heterogeneity

Marked differences appeared between clones in their gene-specific banding patterns following HpaII plus Eco-RI digestions, reflecting variable extents of methylation at inner (3') cytosines of 5'-CCGG-3' sites (Figures 1-4).

Interclonal heterogeneity was particularly apparent in the α-hCG hybridizations, yielding distinctive patterns of prominent HpaII plus Eco-RI bands for each clone (Figure 2, j-r). The mass culture showed some degree of hybridization at many of the band positions represented in the clones. In the β-globin hybridizations, methylation differences betwen clones were limited to variations in the relative intensity of the 3.6 kb band (Figure 4, lanes j-r). Again, the mass culture represented a rough average of the eight clones. The relatively simple β-globin banding pattern may reflect a paucity of -CCGG- sites in this region of DNA, since digestion with Eco-RI alone yields β-globin bands at 5.1 and 3.6 kb (data not shown). In marked contrast, the β-actin probe revealed a great many -CCGG- sites, predominantly methylated at 3' cytosines (thereby producing a profound difference in fragment sizes following HpaII vs. MspI digestion) and yet the great

majority of these sites did not vary between clones (Figure 1).

The γ-globin gene region produced a rather complex pattern (Figure 3) wherein most bands present in HpaII digestions were also present at a different intensity after MspI, and vice versa. This is interpreted as evidence of partial methylation at both the inner and outer cytosines of -CCGG- sites, generating similar high-molecular-weight products in each instance. The unusually high level of outer cytosine methylation in -CCGG- sites of the human γ-globin gene region has been reported previously (3).

Assignments of bands to specific γ-globin genes could be made in several instances due to mapping information obtained in our laboratory (29) and elsewhere (3, and references therein). The 2.6 kb band was attributed to methylation-dependent extension of the 1.5 kb $^{A}\gamma$ fragment. The 1.05 kb band appeared in only two clones (lanes h,i,g,r) and was believed to be a truncated form of the 1.5 kb $^{A}\gamma$ DNA fragment, due to cleavage at a -CCGG- site which was normally doubly methylated. A somatic mutation in clones h/g and i/r, or in their putative common ancestor, could also account for this band, but this was deemed unlikely in view of its appearance in two out of eight independently isolated clones. Moreover, a faint band at this position could sometimes be discerned in the mass-culture DNA (Figure 3, lane a). Finally a band of 5.5 kb (seen principally in lanes n and p, but also detectable in lanes j and r) appeared to result from two or more inner cytosine methylations extending a 0.95 kb $^{G}\gamma$ DNA fragment.

The multiplicity of bands observed in the α-hCG and γ-globin gene regions (in excess of the fragment number expected for a homogeneous population of cells) clearly implies heterogeneity arising de novo within many clones. Thus the patterns of methylation cannot be stably inherited in these instances, over the span of ~ 25 MPD required for expansion of clones. Evidence of inter- and intra-clonal heterogeneity for X-chromosome and γ-globin region methylation in human fibroblasts has been reported recently (31), entirely consistent with the findings presented here for specific gene regions.

Relevance to cellular senescence

The replicative lifespan of diploid human fibroblasts is limited (32), and in particular, cells of the A2 strain cease to divide approximately 62 mean population doublings (MPD) after establishment in culture. Since we have found that progressive drift occurs during the replicative lifespan, in the level of total DNA methylation (29), it is possible that the observed clonal heterogeneity of gene methylation reflects inter-clone differences in

their remaining growth potential (15,33). Maximal MPD levels, determined for four of these clones, were tightly clustered between 54-57 MPD. Since clones were harvested at approximately 45 MPD while still growing actively, they were essentially equidistant from the ends of their lifespans. Thus the differences in DNA methylation patterns observed were unlikely to result from terminal deterioration of senescent fibroblast cultures. It is possible, of course, that random drift of DNA methylation contributes to cellular senescence, since it would be expected to alter the normal pattern of gene expression (4).

Does methylation stability depend on gene expression ?

Taken together, these data support a surprisingly high degree of cell-to-cell variation in gene methylation patterns for diploid human fibroblasts. It may be significant that the greatest interclonal heterogeneity was apparent in genes (αhCG, γ-globin and, to a lesser extent, β-globin) which are normally repressed in fibroblasts, while nearly all of the -CCGG- site methylations in β- and γ-actin gene regions (expressed and potentially expressed, respectively, in fibroblasts) were essentially constant in all clones examined. Further studies with additional probes will clearly be necessary in order to ascertain whether methylation patterns are always less well maintained in "silent" loci than in expressed genes.

Preferential instability of repressed genes, if substantiated, cannot be a simple consequence of methylations being lost more readily than gained (11,12) since β-actin-homologous regions of DNA are highly methylated and yet relatively conserved in methylation pattern between clones (Figure 1). It is possible that the more closed chromatin conformation associated with repressed genes (34,35) impedes methylase access to hemimethylated sites, or alternatively that selection in culture acts against methylation changes for genes which are functionally important.

Low levels of globin gene transcription have been reported in non-erythropoietic cultured cells (36), which could be accounted for by a small fraction of derepressed cells in the culture rather than uniformly low "leakiness" of transcriptional control in all cells. Sporadic clonal derepression of genes, perhaps originating from instability of methylation inheritance, could also explain the low but significant levels of polypeptide hormones produced by three out of 18 normal human fibroblast strains (14).

In summary, clones isolated from a mass culture appear strikingly heterogeneous in their gene specific methylation patterns. Moreover, this heter-

ogeneity is more complex than differences in the level of total DNA methylation (29) would indicate. Because different gene regions appear to vary independently in different clones, simple clonal differences in methylase activity cannot be responsible. Initial evidence suggests that inconstancy of methylation patterns may be greatest in repressed gene regions. If gene methylation is important in maintaining the repressed state (4), then demethylation leading to leaky expression of silent genes may occur quite commonly in individual cell lineages even though the extent of leakiness for any given gene appears low when averaged over a cell population.

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