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Methylation patterns in the gene for the alpha subunit of chorionic gonadotropin are inherited with variable fidelity in clonal lineages of human fibroblasts

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ABSTRACT

Cytosine methylation in DNA of an endogenous single-copy gene locus, the α-subunit of human chorionic gonadotropin (α-hCG), was assessed in a mass culture and individual clonal lineages of human diploid fibroblasts. Progressive hypomethylation at -CCGG- sites in this gene occurred during the replicative lifespan of the mass culture and was shown to arise randomly during clonal expansion. Thus, some clones and subclones lost -CCGG- methylation in the α-hCG gene region while others maintained essentially complete methylation. These data indicate significant interclonal variability in the fidelity with which DNA methylation is transmitted in an endogenous gene.

INTRODUCTION

Cytosine methylation in the dinucleotide CpG is the only base modification known to occur post-synthetically in vertebrate DNA (1), and considerable evidence now supports a correlation between the loss of CpG methylations in the vicinity of a gene and its initial expression in specific tissues during development (2-5). Occasional exceptions to this correlation (6,7) and the lack of remethylation in genes subsequently repressed (2-4,8) suggest that DNA hypomethylation may be an effect rather than a cause of gene expression. However, studies with specific genes that were methylated in vitro and then transfected into animal cells (9,10) show an inverse correlation between the degree of methylation, particularly in the 5' control region (11) and gene expression. Taken together with the striking derepression effects of 5-azacytidine (2-5), a specific inhibitor of eukaryotic cytosine methylases, these studies argue strongly that losses of methylation serve as primary switches for the initiation of gene expression during development.

Analyses of cloned DNA sequences transfected into permanent mouse cell lines have indicated that most -CCGG- sites lose 30-85% of initial methylations over 25 cell generations (9,10) but often stabilize thereafter

(12). Although the relation between the transfected DNA and endogenous chromosomal DNA is unknown, these studies, in concert with recent comparisons of specific gene methylation patterns in active and inactive human X-chromosomes (13,14), indicate that DNA methylation can be faithfully inherited in eukaryotic cells. However, the long-term stability of endogenous gene methylation patterns during repeated replication of normal mortal cells has been less well explored.

In previous studies, we examined CpG methylation extent and patterns in mass cultures and eight clones of human diploid fibroblasts at -CCGG- sites of the gene regions for the α-subunit of human chorionic gonadotropin (α-hCG), ß-globin, α-globins, and ß- and γ-actin genes (15,16). We observed apparent drift, occasionally to increased methylation but more frequently to hypomethylation, and similar results were obtained by Wolf and Migeon for the γ-globin gene and a sequence of unknown function on the X-chromosome (17). However, because of the brief replicative lifespan of these diploid human fibroblasts, we could not obtain subclones to confirm unequivocally that the observed methylation changes arose during propagation of individual cell lineages in vitro rather than during in vivo development. In the latter case, most of our data might be accounted for by the heterogeneity already extant between individual fibroblast clones, allowing clonal succession among the various cell types comprising the mass culture (18,19). We have studied the α-hCG gene locus, a single copy gene (20) expressed in a wide variety of normal and neoplastic cells (21), and we now present evidence that hypomethylation develops progressively at CpG sites within the α-hCG gene region in a fraction of clones and subclones from a long-lived strain of human fibroblasts.

MATERIALS AND METHODS

Cell Culture

Human fibroblast strain HSC172, derived from lung of a third trimester female fetus (22), was grown in Eagle's medium supplemented with 15% fetal calf serum. Subculturing was carried out at a 1:8 split, counting three mean population doublings (MPD) each time the cells grew to confluence (15,18). As currently grown in our laboratory, these cells cease dividing at 75-80 MPD.

Cloning and Subcloning of Fibroblasts

To derive clones from uncloned (mass) cultures at early passage (~20 MPD) cells were plated at 100 cells/100 mm petri dish. After two weeks,

individual, well-demarcated colonies were harvested (18), and individual isolates expanded as required. For subcloning, individual clonal populations were again plated followed by isolation and expansion of individual colonies. Sub-subcloning was carried out as above on selected subclones. All mass cultures, clones, subclones and sub-subclones were propagated routinely until they became replicatively senescent (15,18,23).

DNA Isolation

DNA was prepared from fibroblast mass cultures, clones, subclones and sub-subclones with over 90% yield as described (23).

Restriction Enzyme Digestion and DNA Electrophoresis

Aliquots of DNA were digested for 2 h at 37°C with MspI or HpaII restriction endonucleases at ratios of 6-12 U/µg DNA and electrophoresed in 0.8-1.5% agarose slab gels at 2V/cm to resolve high molecular weight DNA (15). Gels were stained with ethidium bromide, photographed under ultraviolet light through a red filter, transferred by the method of Southern (24) to nitrocellulose paper (Schleicher & Schull, BA-85) and hybridized for 18 h at 42°C with denatured ^{32}P-DNA probe labeled by nick translation (25) or by hexanucleotide priming (26), yielding specific activities of 0.5-5 x 10^9 CPM/µg. Filters were rinsed at 55°C in 0.1 x SSC with 0.2% SDS, covered in Saran wrap and exposed at -70°C for 2-14 days to Kodak XAR film in Dupont Lightning Plus enhancement screen cassettes. α-hCG cDNA probe, a 440 bp fragment encompassing ~70% of the 5' coding portion of this gene (20), was used as before (16). Dehybridization prior to mitochondrial DNA (mtDNA) probing (27) was carried out by heating filters at 90°C for 20 minutes in a low-salt buffer (28), repeating this step, then reprobing as above.

RESULTS

Replication-Dependent Hypomethylation of CpG in the α-hCG Gene Region

Cells frozen at 10 MPD were reconstituted and propagated an additional 66 MPD, and DNA samples were prepared from mass (uncloned) cultures at early (30 MPD) and late (76 MPD) passage. Purified DNA was then digested with the restriction enzymes MspI or HpaII in separate aliquots, and the resultant DNA fragments analyzed on Southern blots probed with ^{32}P-labeled α-hCG DNA. As seen in lanes a and b of Figure 1A, the same pattern of hybridizing bands was observed at early and late passage for DNA digested with MspI. Identical DNA aliquots were also digested with HpaII, which recognizes the same -CCGG- site as MspI, but which cleaves only if neither

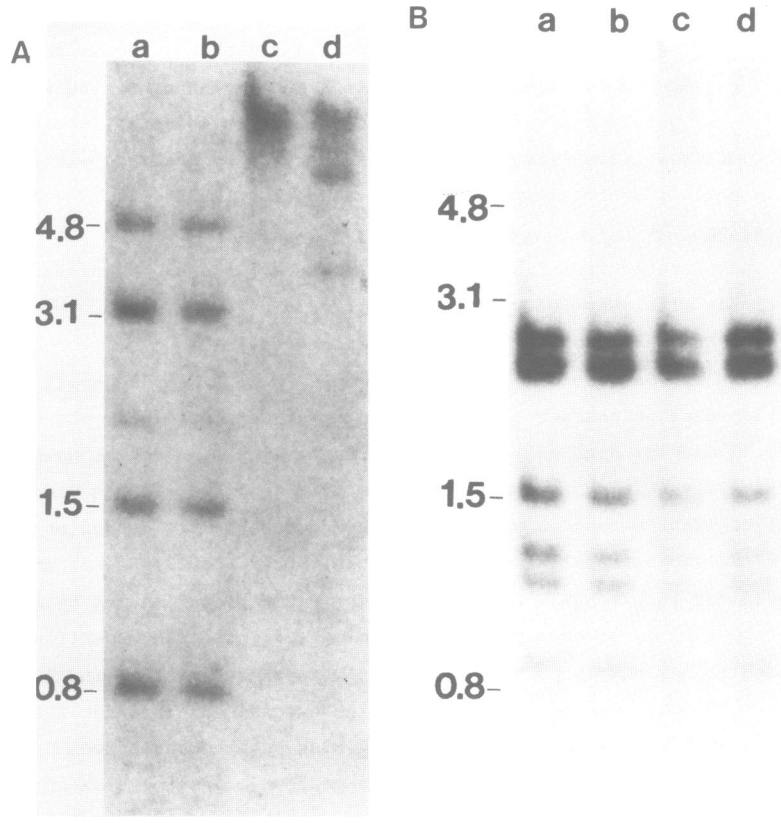

Figure 1 DNA methylation patterns during the replicative lifespan of human fibroblast mass cultures. (A.) Autoradiograph of DNA fragments from the α-hCG gene region hybridizing to an α-hCG ^{32}P-DNA probe following digestion with restriction endonucleases and Southern transfer. DNA samples were obtained at early passage (30 MPD) lanes a and c, or at late passage (76 MPD) lanes b and d, and 3 μg of each digested with MspI (lanes a and b) or HpaII (lanes c and d). Fragment sizes in kilobase pairs (Kbp) were determined from PM2/HindIII markers. (B.) Uniform banding patterns of endogenous fibroblast mtDNA. The filter used in A was dehybridized and re-probed with ^{32}P-mtDNA. Patterns are identical in all lanes, indicating that -CCGG- sites in mtDNA are unmethylated and that DNA was cleaved completely with HpaII.

DNA strand is methylated at the internal cytosine of this site (26,27). DNA samples from fibroblasts at early passage, following complete digestion with HpaII (Fig. 1A, lane c) showed only high molecular weight DNA hybridizing to the α-hCG probe, indicating extensive methylation of CpG dinucleotides within -CCGG- sites of this gene region. At late passage,

however, new HpaII bands appeared at lower molecular weights (lane d) although still not at sizes corresponding to the MspI-generated fragments, indicating hypomethylation of some -CCGG- sites in or adjacent to the α-hCG coding sequences. Although this loss of methylations was most pronounced at the latest passage examined, it was already apparent well before these cells reached the end of their replicative lifespan. To ensure that differences between MspI and HpaII cleavage patterns were specifically due to hypomethylation, three sets of controls were routinely carried out. First, DNA was treated with both a 6- and 12-fold excess of MspI and HpaII enzymes and revealed identical hybridization patterns, strongly suggesting that digestion had gone to completion. Second, digestions with a variety of restriction enzymes which do not contain CpG in their cleavage recognition sites, indicated no passage-dependent changes in digestion (data not shown). Third and most important, the mitochondrial genome, present in about 3,000 copies per diploid fibroblast, has been shown to be essentially unmethylated in these cells (27), and thus served as a highly sensitive and reliable internal control for the completeness of HpaII digestion. Upon probing of the same filters (following dehybridization of the α-hCG probe) with ^{32}P-mtDNA, complete cleavage was observed in each case with HpaII enzyme (Fig. 1B), as evidenced by restriction patterns identical to those generated using MspI, with essentially complete absence of partial digestion products (discrete fragments >3 Kbp). In total, these results clearly indicate that altered banding patterns following HpaII digestion must be due to loss of methylations rather than to other factors.

Loss of CpG Methylations During Clonal Replications

Clones were isolated from the mass culture at early passage, and following additional proliferation of 30-45 MPD, DNA was prepared from each clone at ~50-65 MPD. Analysis of HpaII-digested DNA samples by Southern blot hybridization with the α-hCG probe revealed a spectrum of clonal differences in the extent of passage-dependent hypomethylation (Fig. 2). At mid-passage (~50 MPD), clone A retained essentially full methylation of α-hCG DNA (lane a), clones B and C appeared slightly hypomethylated (lanes b and c), while clones D and E (lanes d and e) had prominent bands of hybridization appearing below the high molecular weight, essentially undigested, material seen in highly methylated clone DNA (cf. lane a). Moreover, at later passage (65 MPD), while DNA from clone A remained undigested by HpaII and hence presumably highly methylated in the α-hCG

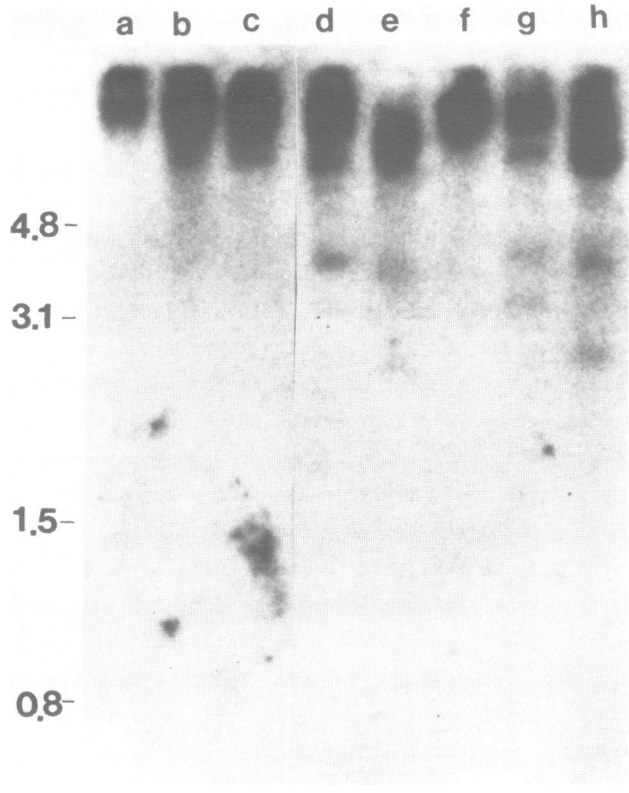

Figure 2 Interclonal variation in methylation pattern of the α-hCG gene region. Autoradiograph of DNA fragments hybridizing to the α-hCG ^{32}P-cDNA probe. DNA samples (3 μg per lane) were obtained from individual fibroblast clones at ~50 MPD (a-e) and where possible at ~65 MPD (f-h) and digested with HpaII: clone A (lanes a and f), clone B (lane b), clone C (lane c), clone D (lanes d and g) and clone E (lanes e and h). Sizes indicated at left, in Kbp, represent positions of MspI-generated fragments run in parallel. Lanes a-c and d-h were spliced from an autoradiogram of a single filter which ensures equality of handling including probe hybridization, rinsing and autoradiographic exposure.

region (lane f), hybridization to DNA of clones D and E (lanes g and h) was further shifted toward lower molecular weight DNA fragments, indicative of decreasing methylation. As noted previously (15,16), the lower-molecular-weight bands are present in sub-stoichiometric amounts, indicating that only a fraction of the cells have lost methyl groups from specific -CCGG- sites during continued proliferation of these clones.

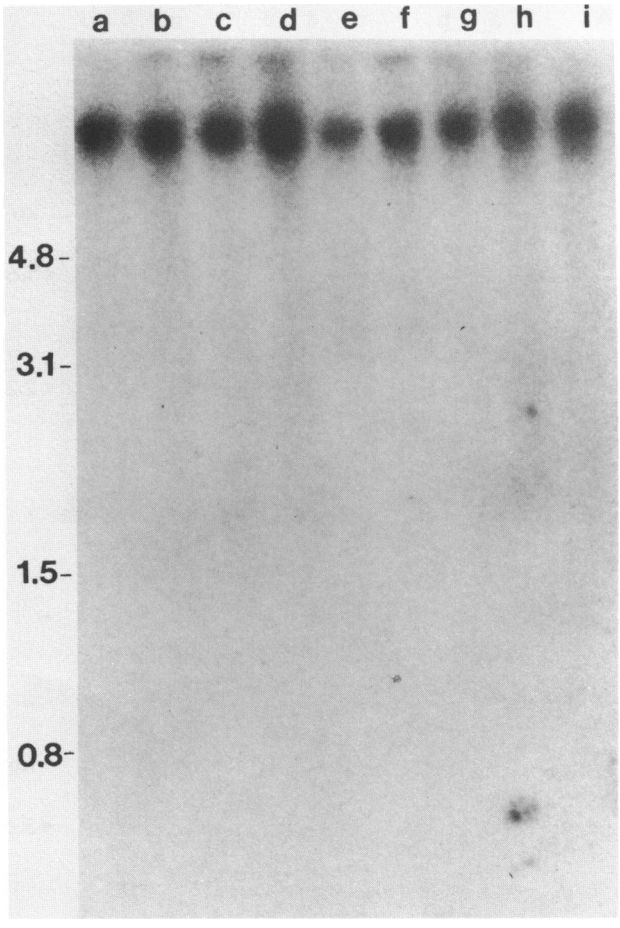

Figure 3 Maintenance of methylation patterns in the α-hCG gene region of clone A, its subclones and sub-subclones. DNA samples were analyzed as in legends to Figures 1 and 2, following digestion with HpaII: clone A at middle passage (~50 MPD, lane a) and late passage (~65 MPD, lane b); subclones A_1, A_2 and A_3 of clone A, at 50 MPD (lanes c-e); A_3 subclone at 65 MPD (lane f) and three A_3 sub-subclones at 65 MPD (lanes g-i).

A Variety of CpG Hypomethylations Arises in Subclones, but Individual Lineages Differ in Methylation Stability: Two clones with the longest replicative capacity, clone A which appeared to maintain α-hCG methylation at a high level, and clone E which underwent extensive cytosine hypomethylation, were utilized for further subcloning. In Fig. 3, α-hCG hybridization is shown for HpaII-digested DNA from clone A at middle and late passage (lanes a and b), from three subclones of clone A at middle

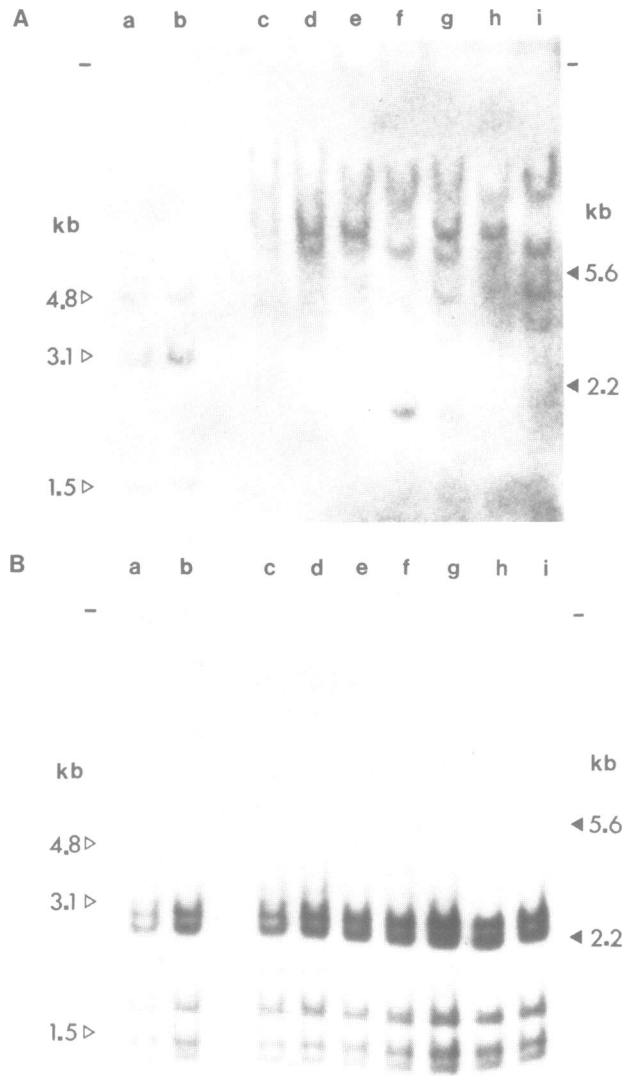

Figure 4 DNA methylation patterns in subclones of clone E. (A.) α-hCG gene region. DNA samples (3 µg) of clone E and five of its subclones were digested with MspI (lanes a and b) or HpaII (lanes c-i) at 12 units/µg, and autoradiographs prepared following hybridization to the α-hCG probe. Lane a: clone E at ~50 MPD: lane b: subclone E7 at ~60 MPD; lane c: clone E at ~50 MPD; lane d: clone E at ~65 MPD; lane e: subclone E3 at ~60 MPD; lane f: subclone E7 at ~60 MPD; lane g: subclone E13 at ~60 MPD; lane h: subclone E17 at ~60 MPD; lane i: subclone E20 at ~60 MPD. Sizes in right margin correspond to PM2/HindIII markers. (B.) Endogenous fibroblast mtDNA. The filter in A was dehybridized and reprobed with human ^{32}P-mtDNA as in Fig. 1B. The faint bands in lane b (>5.6 Kb) represent ≤5% of mtDNA molecules incompletely digested with MspI.

passage (lanes c-e), one of these subclones at late passage (lane f), and three of its sub-subclones (lanes g-i) at late passage. It is apparent that the high level of α-hCG-region DNA methylation noted for clone A is preserved throughout this branching lineage. In contrast, five subclones of clone E, which had undergone passage-dependent hypomethylation (Fig. 2, lanes e and h), all demonstrated varying degrees and loci of hypomethylation (Fig. 4A) as evidenced by the distinctive banding patterns obtained upon α-hCG probing of DNA samples digested to completion with HpaII. As before, complete HpaII digestion was confirmed by reprobing filters with mtDNA (Fig. 4B).

DISCUSSION

CpG dinucleotides are under-represented at least fourfold in the vertebrate genome comprising less than 1% of all dinucleotides (29). The symmetric cytosines of these loci are approximately 50-70% methylated in a variety of mammalian tissues (30) and in human diploid fibroblasts (15), while hemimethylation is thought to occur only transiently at DNA replication forks (7). Indeed, hemimethylated CpG sites are the optimal substrate for eukaryotic DNA methylases (2-4), indicating a simple mechanism for the somatic inheritance of methylation patterns.

If loss of methylations is one of several prerequisites for developmental gene expression, occasional cell lineages which became hypomethylated at normally "silent" gene loci, might only rarely become transcriptionally active and would translate relatively few of the resultant transcripts into ectopic protein products. Nevertheless, surveys of human fibroblasts indicate that up to 41% of strains may produce radioimmunoassayable hCG (31,32), suggesting that the α- and/or ß gene locus may be exceptionally vulnerable to derepression in these cells. Preliminary results for the HSC172 strain have shown no detectable α-hCG protein, although other strains were positive by radioimmunoassay; consistent with this, Northern blots showed no α-hCG mRNA although initial- and intermediate-length RNA transcripts appeared to be present (S. Goldstein et al., in preparation), indicating that regulation is occurring at post-transcriptional levels.

In conclusion, we have documented the accumulation of hypomethylated loci in the α-hCG gene region, as human diploid fibroblasts traverse their limited replicative lifespan. Individual clones isolated from the mass culture clearly differed from each other in their ability to maintain an

initially high level of α-hCG DNA methylation, through the course of repeated replications. Because different patterns were seen in individual subclones, this hypomethylation must have arisen during *in vitro* propagation of each clone. These data suggest that clones or cell types within the initial mass culture vary considerably in their levels of cytosine methylases (33), in putative methyl cytosine demethylases (34), or in chromatin structure near the α-hCG gene locus, such that certain clones are consequently vulnerable to sporadic hypomethylation in this region, as a stochastic process. Although we have utilized genetically stable human diploid fibroblasts which enable precise clonal analysis of gene methylation, we emphasize that cultured cells may differ in methylation controls compared to their *in vivo* counterparts. However, if the lability of methylation patterns we observe *in vitro* also obtains *in vivo*, there could be important consequences in somatic cells. Thus, hypomethylation events leading to transcription might not impair functioning of a tissue or an organism if they occurred sporadically. However, the cumulative effect of many clonal gene derepressions could lead to a progressive deterioration of adaptive function and predisposition to malignant and degenerative disease, as may be seen in foci of excessive or prolonged cell turnover.

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