possibility of testing the general validity of this explanation.

Registry No. Lactate dehydrogenase, 9001-60-9.

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Rat Growth Hormone Gene Expression Is Correlated with an Unmethylated CGCG Sequence near the Transcription Initiation Site†

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ABSTRACT: The methylation status of the rat growth hormone (GH) gene was compared in DNA obtained from GH-producing and GH-nonproducing sources by digestion with three methylation-sensitive restriction enzymes. GH gene expression was correlated with an unmethylated *Tha*I site (CGCG) 144-bp upstream of the GH RNA transcription initiation site. This *Tha*I site was unmethylated in nine GH-producing subclones of the rat pituitary tissue culture cell line GH_3 and in greater than 50% of the DNA isolated from rat anterior pituitary, a gland containing GH-producing somatotroph cells as well as GH-nonproducing cells. In DNA prepared from GH-nonproducing tissues, e.g., rat spleen, kidney, liver, and brain, this *Tha*I site was entirely methylated. Furthermore, this site was entirely methylated in hybrid cells formed by the fusion of GH_3 cells with mouse fibroblasts in which GH production has been extinguished. DNA methylation at 10 other restriction sites located throughout the rat GH gene region failed to correlate with GH expression in GH-producing subclones of GH_3 cells as well as in GH-nonproducing $GH_3 \times LB82$ hybrid cells. We suggest that the conserved absence of methylation 144 bp 5′ of the RNA transcription initiation site of transcribed GH genes identifies a potential GH gene control region.

In eukaryotic DNA approximately 3-5% of the cytosines have undergone modification to 5-methylcytosine (Razin & Riggs, 1980). Although the function of DNA methylation in eukaryotes is not clear, the evidence is strong that specific cytosines are methylated in the 5′ flanking DNA of inactive genes. In many genes examined in eukaryotic tissues or cultured cells, DNA methylation occurs in a tissue-specific pattern. Methylcytosine is present 5′ of the nonexpressed genes while there is an unmodified cytosine in the expressed genes [for a review, see Yisraeli & Szyf (1984)]. Increases in gene transcriptional activity during development have also been correlated with decreases in gene methylation (Andrews et al., 1982; Bird et al., 1981; Colgan et al., 1982; Weintraub et al., 1981). More recently, DNA methylation changes 5′ of the albumin gene have been shown to accompany reversible gene activation and deactivation (extinction) in somatic cell hybrids (Ott et al., 1982, 1984).

In certain instances, experimentally induced alterations in gene methylation patterns have resulted in predictable changes in gene transcription. DNA methyltransferase activity is inhibited by 5-azacytidine (Taylor & Jones, 1982). Treatments with 5-azacytidine have resulted in DNA demethylation and activation of a number of genes including the rat GH[1] gene in GH_3 cells (Cherington & Tashjian, 1983; Lan, 1984). The chief limitation of this type of experiment is the inability to control the sites of demethylation. The influence of 5-methylcytosine within specific genes has been tested by in vitro methods to introduce 5-methylcytosine into cloned genes and gene transfer to study the effects of methylation on gene activity (Wigler et al., 1981; Stein et al., 1982; Vardimon et al., 1982). Methylation clearly depresses the activity of the globin

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[1] Abbreviations: GH, growth hormone; kbp, kilobase pairs; DMEM, Dulbecco's minimum essential medium; SSC, standard saline citrate, 0.14 M sodium chloride-0.015 M sodium citrate, pH 7.2; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; FCS, fetal calf serum; HS, horse serum; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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and adenovirus E2a gene promoters when measured this way (Busslinger et al., 1983a,b; Langner et al., 1984).

Whether methylation is a control signal or a result of gene inactivity may differ in individual genes. Deactivation of the albumin gene by somatic cell hybridization precedes gene methylation, suggesting that methylation is a consequence of albumin gene inactivity (Ott et al., 1984). Activation of chicken vitellogenin gene transcription by estrogen precedes demethylation of the 5′ flanking DNA at a site where the estrogen receptor can bind preferentially (Geiser et al., 1983; Jost et al., 1984). However, for other genes where 5-azacytidine activates expression (Compere & Palmiter, 1981; Groudine et al., 1981; Charache et al., 1983; Delers et al., 1984) or in vitro methylation inhibits it, DNA methylation may directly control gene expression in vivo. We show here that inactive rat GH genes contain 5-methylcytosine at a specific site in the 5′ flanking DNA and suggest this identifies an important GH gene control region.

Materials and Methods

Cell Culture. Unless otherwise noted, all cells were grown in Dulbecco's minimum essential medium (DMEM, NIH media unit) supplemented with 5% horse serum (Gibco, Inc.), 2% fetal calf serum (Biofluids, Inc.), and 2 mM glutamine. Monolayer cell cultures were maintained at 37 °C in a humidified 95% air–5% CO_2 atmosphere.

(A) GH_3. The GH_3 rat pituitary adenoma cells used in these experiments were obtained initially from Dr. Armen Tashjian, Jr. (Harvard University, Boston, MA) (Tashjian et al., 1968). The strain referred to in this paper as GH_3^m is this original cell population and was the rat parent in the hybrid cells described in this paper. We also selected for ouabain resistance and isolated from the GH_3^m population a clonal line of cells referred to in this paper as GH_3. After 1.5 years of continuous culture without ouabain present, GH_3 cells were seeded sparsely (10^2 cells/100 mm^2) in DMEM supplemented with 10% horse serum and 2.5% fetal calf serum. Eight random clones were isolated with the aid of cloning cylinders 3 weeks later. Only one colony per dish was picked to ensure that individual isolates were obtained.

(B) Cell Hybrids. The preparation and initial characterization of the GH_3 × LB82 (mouse transformed fibroblasts) somatic cell hybrid lines GL12, GL14, and GL16 have been described previously (Thompson et al., 1980). At the time DNA was prepared from these three hybrid clones for these studies (7 months postfusion), their modal chromosome numbers (determined from five to seven metaphase spreads) were 96, 90, and 107, respectively. GL12, GL14, and GL16 contained an average of 77, 77, and 98% of the parental rat chromosomes and 79, 75, and 82% of the parental mouse chromosomes, respectively. In all hybrid cell analyses, rat and mouse chromosomes were distinguished by fluorescent C-banding with Hoechst dye no. 33258 (Casperson & Zech, 1973). No GH RNA or protein production was detectable in GL12 (hybrid clone 1) or GL14 (hybrid clone 2) at this or any other time. GL16 (hybrid clone 3) expressed a low level of GH RNA and protein (Strobl et al., 1982). Subclones of the GL16 hybrid cell (denoted here 3-1 through 3-5) line were isolated after 2 years of continuous GL16 cell culturing. At the time DNA was prepared for these methylation studies, chromosome analyses performed on five or more metaphase spreads showed that some additional chromosome segregation had taken place. Subclones 3-1 through 3-5 contained respectively a modal chromosome number of 78, 72, 71, 85, and 91; in every clone, the proportions of rat and mouse chromosomes were 50:50. None of these clones produced GH as assayed by complement fixation (unpublished). Extinction of GH mRNA production in subclone 3-2, GL16 C19 in a previous publication (Strobl et al., 1984), was demonstrated by RNA dot blot analysis (Figure 7). Lane 5 shows the background hybridization of the GH cDNA probe to 2 μg of tRNA and lane 6 hybridization to 10 μg of total cell RNA prepared from this hybrid clone.

DNA Analysis. High molecular weight DNA (50–100 kbp) was isolated from tissue culture cells following the procedure of Steffen and Weinberg (1978). A crude nuclear pellet was obtained from rat, kidney, spleen, and liver by Dounce homogenization in 50 mM Tris, pH 7.3–50 mM EDTA–0.32 M sucrose and centrifugation (3000*g* for 15 min) before isolation of the DNA. Microscopic examination showed this pellet to be >95% nuclei with ≤5% contamination with intact cells. DNA was prepared from these nuclei by solubilization in 6 M guanidine hydrochloride and centrifugation through a pad of 5.7 M cesium chloride in the presence of guanidine hydrochloride (Kantor et al., 1980). The DNA band above the 5.7 M cesium chloride cushion was recovered and dialyzed against 10 mM Tris, pH 8–1 mM EDTA. A mixture of recrystallized phenol (Bethesda Research Labs, Inc.)–chloroform–isoamyl alcohol (25:24:1) was equilibrated with 10 mM Tris, pH 8–1 mM EDTA and kept at 4 °C in the dark. The DNA was then extracted twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) and ethanol-precipitated. The DNA was redissolved in 10 mM Tris, pH 8–1 mM EDTA at a concentration of 0.2 mg/mL before digestion with the various restriction enzymes. DNA was prepared from total brain tissue and anterior pituitary by this same method. DNA (20 μg/400 μL) was digested with 2–4-fold unit excess of enzyme (New England Biolabs, Inc., or Bethesda Research Labs, Inc.) at 37 °C for 18 h in the buffers recommended by the suppliers. The DNA digests were extracted once with an equal volume of the phenol mixture, precipitated with ethanol, and redissolved in 10 mM Tris, pH 8–1 mM EDTA at a concentration of 1 μg of DNA/μL. The completeness of all digestions was assessed in parallel aliquots of genomic DNA to which was added 2 μg of φX174 DNA to be sure the expected fragments were produced. Restriction enzyme digested DNA (15–20 μg/lane) was electrophoresed in 5 mm thick 0.7% agarose gels for 15 h at 30 V with 89 mM Tris–89 mM borate, pH 8.2–2 mM EDTA as the buffer. Seakem agarose (ME) was obtained from Microbiological Associates, Inc. The gels were stained in 1 μg/mL ethidium bromide (Sigma) in water for 10 min and photographed while transilluminated by 365-nm UV light. The DNA was nicked by exposing the gel to short-wave UV light (254 nm) for 5 min. The gels were next soaked in 0.2 M NaOH–0.5 M NaCl for 3 h and neutralized for 1.5 h in 0.4 M Tris, pH 6.5–3 M NaCl. DNA was transferred to nitrocellulose paper (Schleicher & Schuell) overnight in 20× SSC following the Southern method (Southern, 1973). Filters were baked at 80 °C in a vacuum oven for 2 h, and then prehybridized for 10–18 h (Wahl et al., 1979). A full-length rat GH cDNA isolated from a clone in pBR322 (Seeburg et al., 1977) was purified (Yang et al., 1980) and nick-translated with large-fragment DNA polymerase I (Bethesda Research Labs, Inc.) and DNase I (Worthington Biochemicals) to a specific activity of (2–8) × 10^8 cpm/μg of DNA (Rigby et al., 1977). The nitrocellulose filters were hybridized with 1 × 10^7 cpm of labeled GH cDNA/filter for 24 h at 42 °C, according to the dextran-sulfate hybridization procedure (Wahl et al., 1979). Filters were then washed 1.5 h at 65 °C with three changes of 3× SSC–0.1% SDS, washed 1 h at 55 °C with two changes of

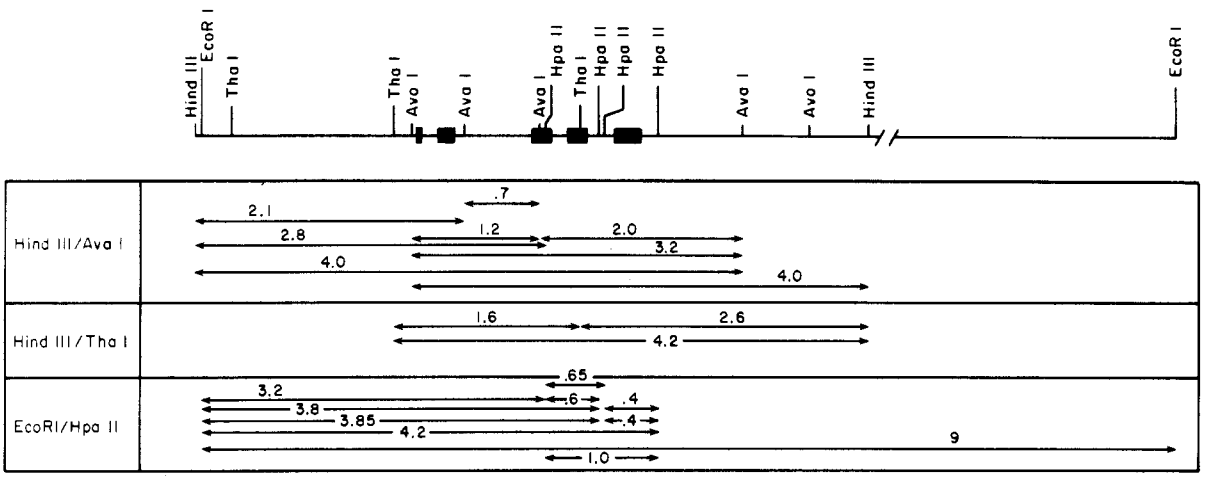

FIGURE 1: Genomic rat GH restriction endonuclease map. Restriction endonuclease recognition sites for *Hpa*II, *Ava*I, and *Tha*I were derived from the published rat GH DNA sequence (Barta et al., 1981) and by the use of standard plasmid mapping techniques. The sizes in kilobase pairs of the GH gene fragments observed after digestion of the genomic DNA preparations with *Hin*dIII plus *Ava*I, *Hin*dIII plus *Tha*I, and *Eco*RI plus *Hpa*II are indicated below the restriction map.

0.1× SSC–0.1% SDS, air-dried, and exposed to X-ray film (XAR-5, Kodak) at −70 °C. The X-ray film was preflashed and exposed for 2–10 days before development.

RNA Analysis. RNA was prepared from cultured cells and tissues by centrifugation through guanidine hydrochloride and 5.7 M cesium chloride (Kantor et al., 1980). The RNA pellets were redissolved in 0.5 sodium sarkosyl–2% β-mercaptoethanol–5 mM EDTA, extracted once with an equal volume of redistilled phenol–chloroform–isoamyl alcohol (25:24:1), and ethanol-precipitated. The purified RNA was then dissolved in water, and stock solutions were added to a final concentration of 10× SSC–3.6 M formaldehyde–2 μg of tRNA in 400 μL. These samples were heated at 60 °C for 15 min, quick-chilled on ice, and applied to nitrocellulose paper soaked in 10× SSC with a sampling manifold purchased from Schleicher & Schuell. Serial 1:1 dilutions were prepared in 10× SCC–3.6 M formaldehyde–2 μg of tRNA diluent and treated as described above. The nitrocellulose filter was air-dried overnight and then baked in vacuo for 2 h at 80 °C. Filter prehybridization and hybridization conditions were as described under DNA Analysis. The filters were washed for 1 h at room temperature in two changes of 2× SSC–0.1% SDS and 45 min in three changes of 0.1× SSC–0.1% SDS at 50 °C.

GH Production. GH is rapidly secreted by the GH_3 cells into the culture medium and is stable in this state (Bancroft et al., 1969). GH was measured in aliquots of the culture medium by complement fixation (Levine & Vunakis, 1967) with a baboon anti-rat GH antibody. To correct GH production for small differences in cell content per dish, all of the cells in each dish were harvested, washed 3 times by resuspension in PBS, sonicated by two 10-s bursts on a setting of 15 W of a Branson sonifier, and assayed for total cell protein following the method of Lowry et al. (1951). The data were expressed as μg of GH secreted (unit of time)$^{-1}$ (mg of total cell protein)$^{-1}$.

RESULTS

Distribution of Methylation-Sensitive Restriction Endonuclease Sites near the Rat GH Gene. The cleavage sites of the restriction endonucleases *Hpa*II, *Tha*I, and *Ava*I shown in Figure 1 were derived from DNA sequence data (Barta et al., 1981) and restriction endonuclease mapping of a genomic clone of the rat GH gene. The DNA fragment sizes indicated are those that were observed after hybridization of Southern blots of the digested DNA with a ^{32}P-labeled full-length GH cDNA. For clarity, fragment sizes that were not observed experimentally are not included in Figure 1.

Table I: Variability of GH Production by a Population of GH_3 Cells and Isolated GH_3 Subclones[a]

GH_3 subclone	clonal GH production relative to the GH_3 population
1 (n = 3)	2.7 ± 0.2
2 (n = 1)	0.6
3 (n = 4)	0.6 ± 0.2
4 (n = 1)	0.5
5 (n = 1)	1.3
6 (n = 3)	1.3 ± 0.9
7 (n = 4)	1.3 ± 0.3
8 (n = 4)	0.6 ± 0.1
$\bar{X}$	1.1

[a] Cells were plated at a density of 2 × 10^4 cells/cm^2. One to three days later, cells received fresh DMEM + 2% FCS + 5% HS. After 48 h, the medium was collected and stored frozen at −20 °C until assayed for GH by complement fixation. Cells present in the medium and attached to the dish were washed 3 times with PBS, pelleted by centrifugation (1500g, 5 min), and assayed for total cell protein. GH production by the GH_3 cell population and each clonal cell line was expressed as μg of GH secreted (24 h)$^{-1}$ (mg of total cell protein)$^{-1}$. All determinations were performed in triplicate. Basal GH production by the GH_3 cell population in these experients was 30.9 μg ± 11.0 per 24 h (n = 6). Relative GH production was calculated as the ratio of GH production by each subclone/GH production by the GH_3 population measured in parallel dishes in every experiment. Results presented represent one to four independent experiments (n). GH production by clones 2, 4, and 5 is the mean of duplicates in a single experiment. Replication of these measurements could not be performed due to a freezer failure, which resulted in the loss of these clones.

Clonal Variability in GH Expression and Methylation Patterns in GH_3 Cells. Comparisons of GH protein production by eight random GH_3 subclones were made. In Table I, the amount of GH produced by the uncloned GH_3 population is set equal to 1 and production of GH by the individual subclones is expressed as a ratio of this. The average GH production by all eight subclones ($\bar{X}$) is essentially that observed in the uncloned GH_3 cell population (1.1). In most instances, the relative production of GH by the uncloned GH_3 cell population and any one subclone varied by less than a factor of 0.5. An exception, subclone 1 produces 2.7 times more GH than the uncloned GH_3 cell population. A 2.5-fold difference in GH production is seen when GH_3 (uncloned) is compared with the ouabain-resistant GH_3^m subclone isolated previously

Table II: Comparison of GH Production by GH_3 and GH_3^m Strains of Rat Pituitary Adenoma Cells[a]

cell line	basal GH production [μg of GH (mg of protein) (24 h)$^{-1}$]
GH_3	19.8 ± 2
GH_3^m	7.9 ± 2

[a] Experimental details are described in Table I. Values represent the mean ± SD of triplicate determinations in three independent experiments.

(Table II). GH_3 cells produce 2–3 times more GH mRNA than GH_3^m when compared by RNA dot blot analysis (Figure 7).

There are four *Hpa*II sites in the rat GH gene region: one lies in the third exon, two lie in the fourth, and one lies in the 3′ flanking DNA. *Hpa*II cleaves the sequence CCGG and ^{m}CCGG but not C^{m}CGG (Sneider, 1980), and therefore, cleavage of genomic DNA by *Hpa*II is indicative of an unmethylated CpG dinucleotide at that site. Growth hormone gene methylation at the *Hpa*II restriction sites in GH_3 subclones is compared in Figure 2 after digestion of DNA with *Eco*RI and *Hpa*II. Five patterns of methylation were observed. GH_3 subclones 1–7 exhibit four different GH methylation patterns. The ouabain-resistant clone GH_3^m (lane 9) exhibits a fifth pattern of methylation. All DNAs were completely digested following treatment with *Eco*RI and *Hpa*II isoschizomer *Msp*I (data not shown). *Msp*I cleaves the sequence CCGG and C^{m}CGG (Busslinger et al., 1983). This indicates that the clonal heterogeneity in *Hpa*II digestion patterns results from methylation differences at the internal cytosine in the recognition sequence CCGG.

Extensive methylation of the GH gene in GH_3^m (lane 9) correlated with decreased GH protein and mRNA production as compared to the uncloned GH_3 population (lane 8). About 50% of the GH genes in GH_3^m are completely methylated at all four *Hpa*II sites, resulting in a 9-kbp digestion fragment. We do not know whether this is due to different subpopulations of GH_3^m cells that carry either methylated or unmethylated genes or whether each GH_3^m cell carries one methylated and one unmethylated gene. The 9-kbp fragment is weak in the uncloned GH_3 population (lane 8). The fragment pattern of the uncloned GH_3 population in contrast to that of GH_3^m indicates that the *Hpa*II site in the 3′ flanking DNA is almost entirely unmethylated. The *Hpa*II methylation patterns in GH_3 subclones 1–7 are more similar to the uncloned GH_3 population than the GH_3^m subclone. In all seven subclones, the *Hpa*II site in the 3′ flanking DNA is completely unmethylated. The four different patterns of GH methylation result from methylation differences at the three internal *Hpa*II sites. In general, the GH protein production levels are similar among the seven subclones. As the following analysis indicates, these methylation differences do not correlate with GH production levels. Subclone 1 produces 2.7 times more GH than the uncloned GH_3 population. The internal *Hpa*II methylation pattern in this subclone (lane 1) is not remarkably different from that of GH_3 (lane 8), suggesting a lack of correlation between internal *Hpa*II sites' methylation status and GH production levels. This finding is supported by two additional observations. Subclone 7 (lane 7) has the same internal *Hpa*II methylation pattern as subclone 1 yet does not produce as much GH protein as subclone 1. Subclone 6 (lane 6) is completely unmethylated at the internal *Hpa*II sites while the uncloned GH_3 population is partially methylated here, and both produce similar amounts of GH. We conclude that, among eight clonal lines of GH_3 cells, GH gene expression is correlated with an unmethylated *Hpa*II site in the 3′ flanking DNA. Variations in the degree of methylation at the three internal *Hpa*II sites occur frequently but do not correspond to variations in GH protein production levels.

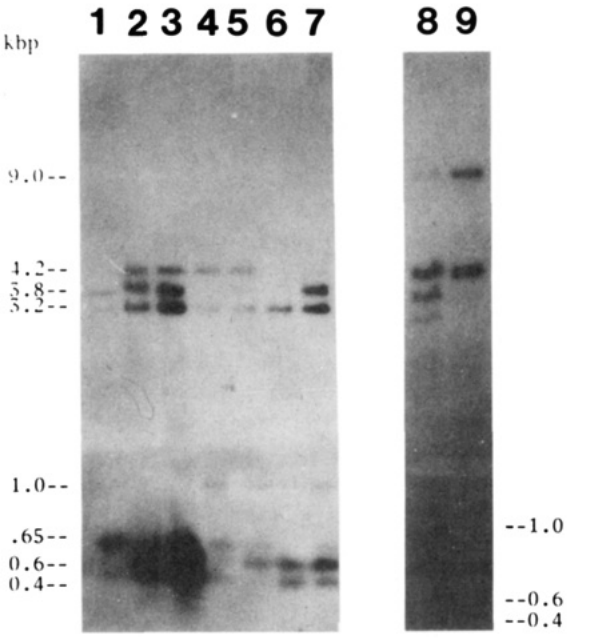

FIGURE 2: GH_3 clonal variation in *Hpa*II methylation status. DNA from seven GH_3 subclones (lanes 1–7), the uncloned GH_3 cell population (lane 8), and GH_3^m (lane 9) was digested with *Eco*RI and *Hpa*II. Electrophoresis was conducted at 30 V for 15 h in 0.7% agarose gels in the Tris–borate buffer system. Twenty micrograms of DNA was run per lane. The gels were stained with ethidium bromide, and then Southern blotting and hybridization reactions were performed as described under Materials and Methods to visualize the GH DNA fragments. The sizes of the restriction fragments were determined with bacteriophage λ DNA digested with *Hin*dIII as the markers.

The methylation-sensitive restriction enzymes *Tha*I and *Ava*I recognize sequences near the GH RNA transcription initiation site (Figure 1). *Tha*I cleaves the sequence CGCG but not CG^{m}CG or ^{m}CGCG (Strobl & Thompson, 1984). *Ava*I cleavage of the sequence CPyCGPuG is inhibited by methylation of the internal cytosine (CPy^{m}CGPuG) (Bird & Southern, 1978). Since the correlation between gene expression and the absence of 5-methylcytosine is strongest in the DNA flanking the 5′ end of many genes (Yisraeli & Szyf, 1984), we thought it important to study the methylation status of the *Tha*I and *Ava*I sites near the GH gene transcription initiation site. In Figure 3, left panel, GH_3 DNA from subclones 1–8 (Table I) digested with *Hin*dIII and *Tha*I is shown. A completely methylated GH gene would migrate as a 5.6-kbp fragment under these conditions, and therefore, none of these subclones is completely methylated at the *Tha*I restriction sites. The 4.2-kbp fragment of the rat GH gene is generated wherever methylation is absent from the *Tha*I site 144 bp upstream of the RNA transcription initiation site. The 2.6- and 1.6-kbp fragments are generated wherever genes also lack methylated cytosines at the internal *Tha*I site. All eight subclones exhibit one or all of these fragments, demonstrating that the DNA at the *Tha*I site immediately upstream of the RNA transcription start site is unmethylated in the GH genes in every GH_3 subclone.

GH_3 subclone DNA is shown in Figure 3, right panel, after digestion with *Hin*dIII and *Ava*I. None of the clones exhibits any completely methylated 5.6-kbp GH genes. DNA restriction fragments of 1.2 and 3.2 kbp are derived from GH genes that lack methylated DNA at the *Ava*I site 10 bp 3′ of the RNA transcription start site, and the 2.8-kbp fragment

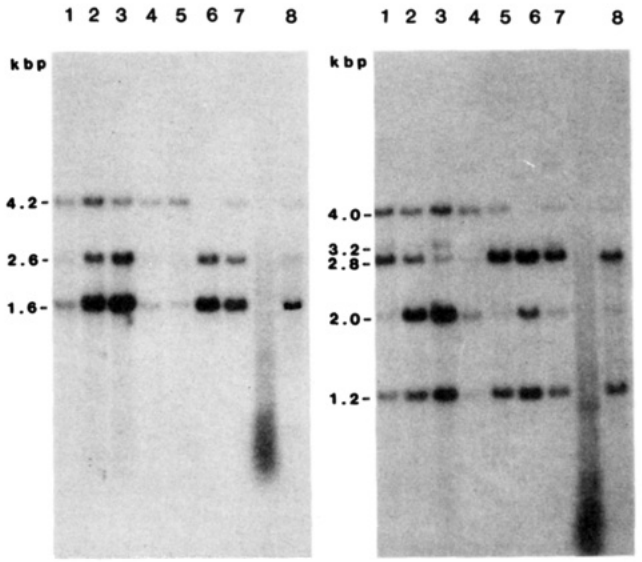

FIGURE 3: GH_3 cell clonal variation in *Ava*I and *Tha*I methylation status. The DNA of eight GH_3 subclones 1–7 corresponding to subclones 1–7 in Figure 2, plus one additional clone, 8, was digested with *Hin*dIII and then either *Ava*I (right) or *Tha*I (left). Electrophoresis and hybridization conditions were exactly as described in Figure 2. The DNA of one additional clone seen in the lane between clones 7 and 8 was degraded and was not considered further.

arises from genes that are methylated at this site. All subclones exhibit some of the 1.2-kbp fragment and some (1, 2, 3, and 7) the 3.2-kbp fragment. However, the 2.8-kbp fragment is equal in intensity or more prominant than the 1.2-kbp fragment in six out of eight subclones, including subclone 1 that produces 2.7 times more GH protein than the uncloned GH_3 population. Thus, the absence of methylated DNA at the *Ava*I site near the site for initiation of transcription is not well conserved among expressed GH genes in GH_3 subclones 1–8 and is not correlated with clonal variations in GH protein production. The third exon *Ava*I site and the first *Ava*I site in the 3′ flanking DNA were more frequently unmethylated than the 5′ *Ava*I site as indicated by the 1.2-, 2.8-, and 2.0-kbp fragments. There was no evidence of a 0.7-kbp fragment indicative of unmethylated *Ava*I sites in the second intron in these eight subclones. Finally, the methylation status of the most 3′ *Ava*I site could not be assessed due to ambiguity in the identification of the origin of the 4.0-kbp fragment.

A comparison of GH gene methylation at *Ava*I and *Tha*I sites in GH_3 and GH_3^m DNA is shown in Figure 4. A clear distinction in the degree of gene methylation is apparent. Fifty percent of the GH genes in GH_3^m (lanes 4–6) and none in GH_3 (lanes 1–3) were completely methylated at all *Ava*I and *Tha*I restriction sites. Thus, the correlation between the extent of GH gene methylation first observed in the *Hpa*II sites in this pair of cell types and the level of GH gene expression extends to the *Tha*I and *Ava*I sites as well.

The data in Figures 2 and 3 show that in the closely related GH_3 subclones 1–8 fluctuations in methylation status occur frequently and randomly at one internal *Tha*I site, at three internal *Hpa*II sites, and at the most 5′ *Ava*I restriction site and exert little influence on GH production levels. However, among these same clones, the absence of methylated DNA in the *Tha*I sequence immediately 5′ of the transcription initiation site is invariant, therefore characteristic of transcribed GH genes. In addition, one *Hpa*II site in the 3′ flanking DNA and two internal *Ava*I sites are characteristically unmethylated in all of the GH_3 subclones examined. In comparisons of the two more distantly related GH_3 cell populations GH_3 and GH_3^m, a clone isolated under selective pressure, the *Hpa*II,

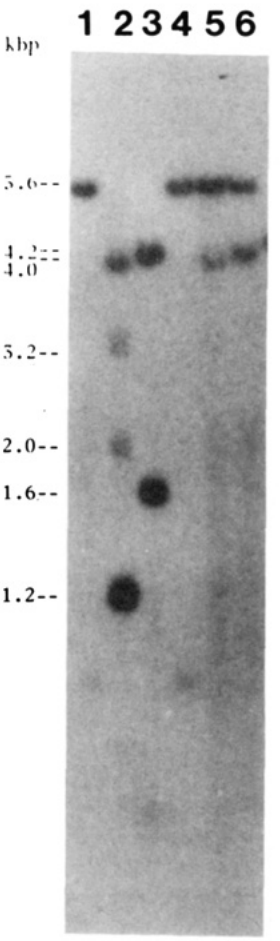

FIGURE 4: GH gene methylation at *Ava*I and *Tha*I in GH_3 and GH_3^m DNA. GH_3 (lanes 1–3) and GH_3^m (lanes 4–6) DNA was digested with *Hin*dIII and then either *Ava*I or *Tha*I. Twenty micrograms of DNA per lane was electrophoresed, blotted, and hybridized as in Figure 2. (Lanes 1 and 4) *Hin*dIII alone; (lanes 2 and 5) *Hin*dIII + *Ava*I; (lanes 3 and 6) *Hin*dIII + *Tha*I.

*Ava*I, and *Tha*I methylation patterns were consistently distinct. At all 11 restriction sites examined, roughly 50% of the GH genes were entirely methylated in the GH_3^m line that expresses 2–3-fold lower levels of GH mRNA and protein than GH_3. None of the GH genes in the GH_3 population were entirely methylated at any of these sites. The distinctions in GH gene methylation and expression in GH_3 and GH_3^m make it clear that extensive methylation of GH genes is associated with a lower level of GH gene expression. The additional information supplied by the analysis of GH_3 subclones 1–8 is that fluctuations in the methylation status of many of these sites, with the exceptions noted above, have little effect on GH gene expression.

Methylation of Nonexpressed GH Genes in Hybrid Cells. Expression of the GH gene in GH_3^m cells is no longer detectable after fusion with GH nonexpressing mouse fibroblast (LB82) cells (Strobl et al., 1982). The $GH_3 \times$ LB82 hybrid cells therefore provide a useful system to investigate the relationship between GH gene methylation and expression. In the following two figures, methylation of the rat GH gene at *Hpa*II, *Ava*I, and *Tha*I restriction sites in GH_3^m and hybrid cell DNA is compared.

Figure 5 shows the DNA hybridization analysis of $GH_3 \times$ LB82 hybrid cell DNA after digestion with *Eco*RI and *Hpa*II. Lane 1 contains DNA from the uncloned, GH_3 cells as a marker to indicate the rat GH bands: 9, 4.2, 3.8, 3.2, 1.0, 0.6, and 0.4 kbp. Lane 2 contains GH_3^m DNA. Lane 3 contains DNA isolated from the mouse fibroblast (LB82) parental cell

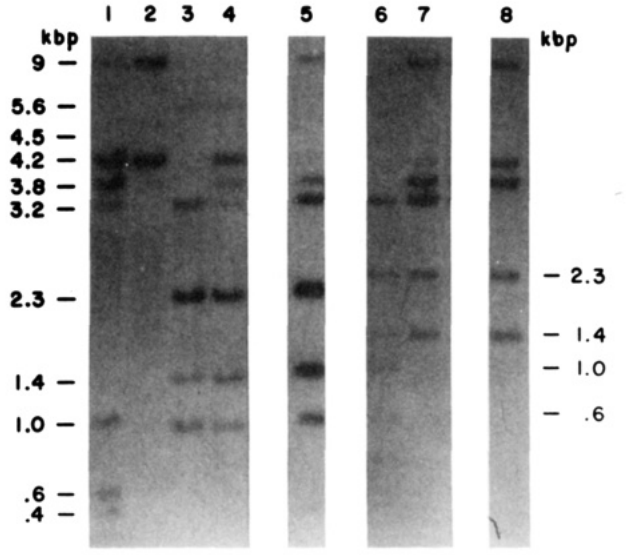

FIGURE 5: Clonal variation in the *Hpa*II methylation status of rat GH genes in GH_3^m × LB82 hybrid cells. All DNA samples (20 μg/lane) were digested with *Hpa*II and *Eco*RI and then subjected to electrophoresis and hybridization as detailed in Figure 2. Lanes 1, 2, 3, and 6 are marker lanes containing respectively GH_3, GH_3^m, LB82, and LB82 DNA. Lanes 4, 5, 7, and 8 contain hybrid cell DNA, clones 3, 3-2, 3-4, and 2, respectively. Note that lanes 1–5 are derived from one gel and lanes 6–8 from another gel that did not run as long. The sizes of the GH gene fragments in the first and second gels are indicated at the left and right of the photograph, respectively.

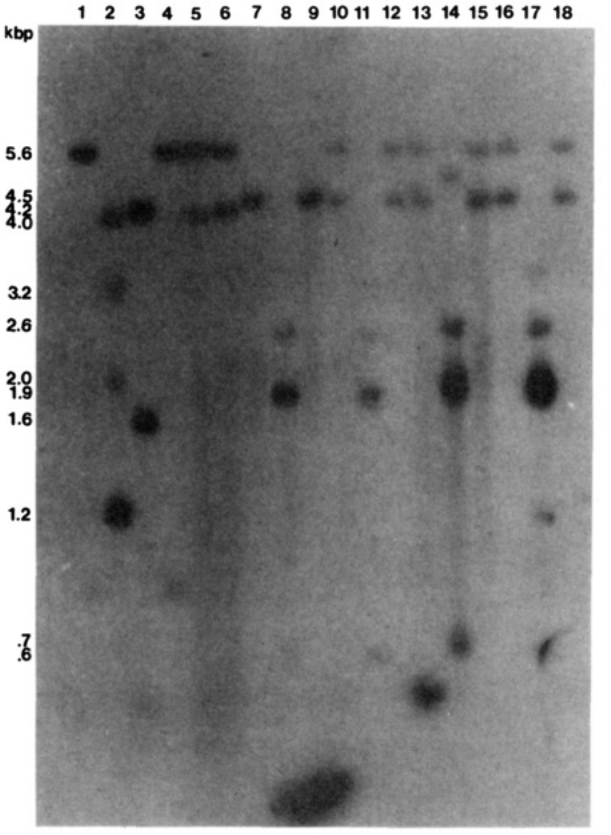

FIGURE 6: Variations in GH gene methylation at *Ava*I and *Tha*I sites in GH_3, LB82, and GH_3^m × LB82 cells. DNA (20 μg/lane) was digested with *Hin*dIII and then either *Ava*I or *Tha*I. Electrophoresis, Southern blotting, and hybridization reactions were performed as described in Figure 2. GH_3 DNA, lanes 1–3; GH_3^m DNA, lanes 4–6; LB82 DNA, lanes 7–9; hybrid cell subclone 3-2, lanes 10–12; hybrid cell subclone 3-3, lanes 13–15; hybrid cell subclone 3-4, lanes 16–18. For each group of three lanes, the first lane was digested with *Hin*dIII, the middle lane with *Hin*dIII + *Ava*I, and the last lane with *Hin*dIII + *Tha*I.

line, indicating the mouse GH gene restriction fragments of 5.6, 3.2, 2.3, 1.4, and 1.0 kbp. The rat and mouse GH genes hybridize well with the rat GH cDNA, but differences in the DNA fragment sizes distinguish these genes in the hybrid cell DNA. Lanes 4, 5, 7, and 8 depict the four methylation patterns that were observed among seven clones of hybrid cells. Each exhibits mouse and rat GH-specific bands. Extinction of GH production in these hybrid cells is not correlated with a single *Hpa*II methylation pattern. Furthermore, the extinguished GH genes in all of the hybrid cells are less methylated at *Hpa*II sites than the parental GH-expressing cell line, GH_3^m. This is seen by comparing lane 2 (GH_3^m parent DNA) with the hybrid cell DNA in lanes 4, 5, 7, and 8. The 9.0-kbp fragment indicative of rat GH genes completely methylated at all four *Hpa*II sites appears equal or reduced in lanes 4, 5, 7, and 8 as compared to lane 2. Thus, extinction of rat GH expression is not correlated with an increase in the extent of methylation at the *Hpa*II site in the 3′ flanking DNA. This is an important observation because *Hpa*II analysis of the GH_3 subclones indicated that an unmethylated *Hpa*II site in the 3′ flanking DNA might be a critical feature of expressed rat GH genes. The hybrid cell data are inconsistent with this hypothesis. Further decreases in the extent of rat GH gene methylation at the internal *Hpa*II sites in the hybrid cells are evident by the appearance of the 3.8- and 3.2-kbp fragments, not present in the parent GH_3^m DNA.

Figure 6 shows the hybridization analysis of the GH genes in hybrid cell DNA after digestion with *Hin*dIII or *Hin*dIII plus either *Ava*I or *Tha*I. Lanes of digested DNA from GH_3 (lanes 1–3), GH_3^m (lanes 4–6), and LB82 (lanes 7–9) mark the restriction fragments containing the rat and mouse GH genes. In the hybrid cells, lanes 11, 14, and 17, methylation of the *Ava*I sites is greatly reduced as compared to the GH_3^m parent (lane 5). In fact, methylated cytosines are rarely present in the *Ava*I recognition sequences of the rat GH genes in three hybrid clones (clones 3-2, 3-3, and 3-4) examined. These three clones are representative of the three *Hpa*II methylation types depicted in Figure 5, lanes 5, 7, and 8. The *Ava*I fragments in the parent GH_3^m cell line (lane 5) are the totally methylated 5.6- and 4.0-kbp partially unmethylated pieces. Both of these are completely absent in the hybrid cells. Instead, lanes 14 and 17 exhibit very strong signals at 1.9–2.0 kbp. These represent rat GH genes unmethylated at the first, third, and fourth *Ava*I sites proceeding 5′ to 3′. In addition, lane 14 contains a 0.7-kbp piece representing rat GH genes unmethylated at the second and third *Ava*I sites as one proceeds 5′ to 3′. The 5.0-kbp band in lane 14 may represent a rat GH gene methylated at all but the most 3′ *Ava*I site. Lane 17 exhibits trace amounts of 3.2- and 1.2-kbp fragments representing less methylated rat GH genes such as seen faintly in the GH_3 cell line but not in the hybrid parent cell line GH_3^m. The bands in lane 11 are faint, but it is clear that the 5.6- and 4.0-kbp heavily methylated genes are absent. Thus, at *Ava*I as well as *Hpa*II sites, the extinguished GH genes in the hybrid cells are less methylated than in the parental GH_3^m line.

In marked contrast, a comparison of *Hin*dIII-digested and *Hin*dIII plus *Tha*I digested DNA shows that the rat GH gene in all three hybrid subclones is completely methylated at all three *Tha*I restriction sites (resulting in the 5.6-kbp fragment, lanes 12, 15, and 18). The 4.5-kbp *Hin*dIII/*Tha*I fragment containing the mouse GH gene (lane 9, LB82 mouse fibroblast cells) and the 4.2-kbp *Hin*dIII/*Tha*I fragment containing the rat GH gene (Lanes 3 and 6, GH_3 and GH_3^m cells, respectively) are clearly distinguishable from the 5.6-kbp fragment indicative of *Hin*dIII cleavage alone. Methylation of the *Tha*I

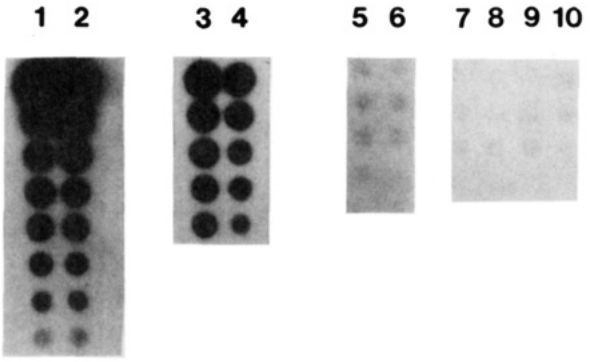

FIGURE 7: Detection of GH RNA by cDNA blot hybridization. RNA was isolated and analyzed by hybridization with ^{32}P-labeled GH cDNA as described under Materials and Methods. (Lanes 1 and 2) Replicate aliquots of 1 μg of total anterior pituitary RNA spotted in serial 1:1 dilutions; (lane 3) 2 μg of total GH_3 RNA and serial 1:1 dilutions; (lane 4) 2 μg of total GH_3^m RNA and serial 1:1 dilutions; (lane 5) 2 μg of tRNA and serial 1:1 dilutions; (lane 6) 10 μg of hybrid subclone 3-2 total RNA and serial dilutions; (lanes 7–10) respectively 10 μg of Sprague-Dawley rat brain, liver, spleen, and kidney total RNA and serial 1:1 dilutions. As described under Materials and Methods, every spot contains 2 μg of carrier tRNA.

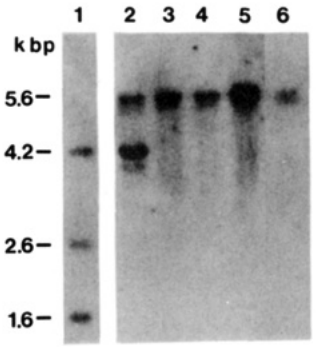

FIGURE 8: Methylation of *Tha*I in normal rat tissue DNA. Twenty micrograms of DNA was digested with *Hin*dIII + *Tha*I, electrophoresed, blotted, and hybridized as described in Figure 2. (Lane 1) GH_3 DNA; (lane 2) anterior pituitary DNA; (lane 3) brain (cortex) DNA; (lane 4) liver DNA; (lane 5) spleen DNA; (lane 6) kidney DNA.

recognition sequences in the region of the rat GH gene therefore correlates with extinction of rat GH gene expression in these hybrid cells.

The GH gene methylation changes seen in the hybrid cells are striking. Upon hybridization with the mouse fibroblast cells, the extensively methylated parental GH_3^m GH genes become markedly less methylated at *Hpa*II and *Ava*I sites while undergoing complete de novo methylation at the three *Tha*I sites. These results show clearly that the methylation state of the *Hpa*II and *Ava*I sites does not correlate with GH gene expression while strengthening the correlation between the methylation state of *Tha*I sites and GH gene expression.

ThaI Methylation in Normal Rat Tissues. To assess the in vivo significance of the correlation between *Tha*I methylation in the rat GH gene in GH_3 cells and GH expression, the methylation state of these sites in rat tissue DNA was determined. RNA dot blot hybridization analysis was used to confirm the absence of GH mRNA in rat brain, liver, spleen, and kidney (Figure 7). The weak hybridization signal in lanes 7–10 was no different than that seen in parallel spots of tRNA alone. DNA from Sprague-Dawley rat brain, liver, spleen, and kidney was digested with *Hin*dIII plus *Tha*I to determine the methylation pattern in these nonexpressed GH genes. The predominant DNA band in these tissues is 5.6 kbp, indicating that all of the *Tha*I sites are methylated (Figure 8). In contrast, roughly 50% of the GH genes in Sprague-Dawley anterior pituitary DNA are present in a 4.2-kbp band. In these genes, the *Tha*I site 5′ of the GH RNA transcription start site is unmethylated. The minor band of 3.8 kbp seen only in anterior pituitary DNA is the size predicted if only the *Tha*I site at the extreme 5′ end of the *Hin*dIII fragment containing the rat GH gene is unmethylated. The high level of expression of the rat GH gene in anterior pituitary (lanes 1 and 2) compared to that in GH_3 RNA (lane 3) is demonstrated in the dot blot analysis in Figure 7. Nevertheless, immunohistochemical analyses have shown that only a subpopulation of anterior pituitary cells produces GH (Daughaday, 1981). This probably explains the presence of the 5.6-kbp band containing completely methylated GH genes in anterior pituitary DNA.

Discussion

GH_3 is a GH-producing cell culture line derived from a rat pituitary adenocarcinoma that has been used extensively in studies of rat GH gene regulatory mechanisms. To determine the relationship between rat GH gene expression and gene methylation, we have analyzed 5-methylcytosine in the rat GH gene region using three methylation-sensitive restriction enzymes. Eight closely related subclones of the GH_3 cell line, GH_3^m, a GH_3 subclone isolated by selection in ouabain, and seven hybrid cell lines isolated from a GH_3^m × LB82 mouse fibroblast fusion experiment were examined. In GH_3 cell subclones that were isolated without selective pressure and that produce similar amounts of GH protein, many restriction sites were heterogeneously methylated. We conclude that many "silent" methylation changes occur in the GH gene in GH_3 cells that exert little or no influence on levels of GH protein produced. Clonal variability in the methylation of tissue culture cell DNA has been documented previously in serially passaged diploid fibroblasts and in permanent cell lines derived from animal tumors (Shmookler-Reis & Goldstein, 1982; Wilson & Jones, 1983). These changes have been attributed to mistakes by the maintenance methylase or to de novo methylation, but neither possibility has been tested directly. Our data demonstrate that the DNA methylating mechanism in GH_3 cells also permits extensive variability.

A notable exception to the clonal variability in GH gene methylation is the CGCG (*Tha*I) sequence 144 bp 5′ of the GH gene transcription initiation site. In 9/9 GH_3 subclones tested, this site was unmethylated. In the GH_3^m line about 50% of the GH genes are entirely methylated at the *Tha*I restriction sites, and this is accompanied by a 2–3-fold reduction in GH mRNA and protein production compared to GH_3. Furthermore, after fusion of GH_3^m with LB82 cells, GH gene expression is undetectable, and GH gene extinction in this hybrid cell is associated with complete de novo methylation of all three *Tha*I sites. Hence, there is a strong correlation between rat GH gene nonexpression and methylation of the sequence CGCG (*Tha*I) upstream of the RNA transcription initiation site in GH_3 cells and their derivatives. The correlation between *Tha*I methylation and GH production is also seen in DNA prepared from GH-producing anterior pituitary and nonproducing (brain, liver, kidney, spleen) rat tissues. We hypothesize that this *Tha*I sequence immediately 5′ of the transcription initiation site exhibits some unique characteristic recognized by the methyltransferase that restricts the interclonal variability in DNA methylation.

Other laboratories have shown that GH production by low-GH-producing GH_3 subclones is stimulated by exposure to the DNA hypomethylating agent 5-azacytidine (Cherington & Tashjian, 1983; Lan, 1984). In GH_3 subclone D6, levels of GH mRNA were increased 3–8-fold after treatment with

8 μM 5-azacytidine. This was accompanied by a modest decrease in the amount of 5-methylcytosine in *Hpa*II sites. In another study, GH_4 C1 cells exposed to 3 μM 5-azacytidine increased their GH protein production 3–5-fold. In both instances, the increased production of GH appeared stable, consistent with a gene-demethylation mechanism. However, the effect of 5-azacytidine treatment on *Tha*I methylation 5′ of the transcription initiation site was not monitored. Our results predict that this site would be a key target for 5-azacytidine's action on GH gene expression.

Several mechanisms have been postulated to explain how 5-methylcytosine might control gene expression. Computer models of B DNA show that the methyl substitution projects into the major groove of DNA (Rich et al., 1984). Binding of regulatory proteins to a single DNA sequence could therefore differ substantially depending upon whether a cytosine were methylated or not. Another consideration is the ability of 5-methylcytosine to stabilize the synthetic polymer poly(dG-dC)·poly(dG-dC) in the Z conformation at physiologic ionic conditions (Behe & Felsenfeld, 1981). In Z DNA the phosphate backbone follows a left-handed helix as the result of the rotation of alternate bases about the glycosidic bond from the anti to the syn conformation (Rich et al., 1984). These and other unique structural characteristics of Z DNA and the B–Z junction could alter the binding of regulatory proteins to a region of DNA. Z DNA binding proteins have been identified, but their role in gene expression has not yet been clarified (Lafer et al., 1985). Alternatively, by reducing local DNA supercoiling, Z DNA formation might directly repress gene transcription. Transcription of supercoiled plasmid DNA in bacteria and *Xenopus* oocytes is reduced when the DNA template is relaxed (Smith, 1981; Pruitt & Reeder, 1984). It has been proposed that unwinding of the DNA helix during the conversion of a closed RNA polymerase complex to an open complex with DNA is facilitated by DNA supercoiling (Saucier & Wang, 1972). Finally, our data do not exclude a passive role of 5-methylcytosine in inactive GH genes. Protein binding has been shown to inhibit DNA methylation in vitro (Kautiainen & Jones, 1985). In light of the general CG sequence specificity of eukaryotic DNA methylases (Hattman, 1981), the pattern of eukaryotic gene methylation may reflect the relative binding strengths of proteins to different DNA regions. A very tightly bound protein 5′ of an actively transcribed gene could protect that sequence from the DNA methylase very efficiently whereas weak or absent protein binding at other sites could lead to variable or complete methylation. At the present time we do not know which of these mechanisms best explains the observed absence of methylation in the *Tha*I site of the rat GH promoter in expressed GH genes.

Registry No. GH, 9002-72-6.

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An Assessment of the Z-DNA Forming Potential of Alternating dA-dT Stretches in Supercoiled Plasmids†

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ABSTRACT: The ability of a stretch of alternating dA-dT to adopt the left-handed Z form has been assessed by examining the behavior of the sequence $d(CG)_6(TA)_4(CG)_6$ contained in the plasmid pBR322. The structural transition occurring within this sequence as a function of negative superhelicity was analyzed by several methods, including (1) the supercoiling-dependent unwinding of the insert as determined by two-dimensional gel electrophoresis, (2) the binding of anti-Z-DNA antibodies to the insert, (3) the sensitivity of the sequence to a single strand specific endonuclease, and (4) the sensitivity of the insert to digestion by a restriction endonuclease that cuts within the $d(CG)_6$ segments when in the right-handed form. These studies have shown that in negatively supercoiled DNA the two $d(CG)_6$ portions of the insert adopt the Z form, while the central $d(TA)_4$ segment forms an underwound structure with a helical repeat that is best approximated as being intermediate between the B form and the Z form. A statistical mechanical treatment of the unwinding of the insert as a function of negative superhelicity provides an estimate of the minimum free energy required to convert an A-T bp from the B form to the Z form, as well as the free energy associated with the conversion of an A-T bp from the B form to the unwound form. These results strongly indicate that Z DNA is an unfavored structural alternative for stretches of $d(AT)_n$ in negatively supercoiled DNA.

It is now apparent that duplex DNA can assume a variety of conformations that differ by degree from the canonical B form. Since these altered structures could act as focal points for important genetic processes, it is of interest to determine which DNA sequences are the most adept at undergoing structural change. The most dramatic and certainly the best characterized structural transition known to date is the conversion of right-handed B DNA to the left-handed Z form [see Rich et al. (1984) for review]. In Z DNA, the bases along each strand of the helix remain Watson and Crick base-paired but alternate regularly between the syn and anti conformation.

Although the rules governing which DNA sequences can adopt the Z form are only partially understood, it is clear that certain sequences can flip to the Z form far more readily than others. Stretches of alternating $d(CG)_n$ and $d(CA)_n$ have been shown to adopt the Z form in negatively supercoiled plasmids under physiological conditions (Singleton et al., 1982; Peck & Wang, 1983; Haniford & Pulleyblank, 1983; Nordheim et al., 1982). The strong Z-forming potential of these alternating purine–pyrimidine stretches has been explained empirically by the observation that purine nucleotides assume the syn conformation more easily than pyrimidine nucleotides (Haschemeyer & Rich, 1967). This observation implies that Z DNA will be formed most stably in an alternating purine–

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