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Methylation of CpGs as a Determinant of Transcriptional Activation at Alternative Promoters for Transforming Growth Factor-β3[1]

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Abstract

The human transforming growth factor-β3 (*TGF-β3*) gene has a typical CpG island, the core of which is centered just upstream of its principle promoter. Activation of an alternative downstream promoter, leading to the production of a truncated mRNA lacking the portion of the 5′ noncoding region responsible for translational inhibition of *TGF-β3* mRNA, is only evident in breast cancer cells. We compared the methylation status of genomic DNA isolated from a panel of breast (SKBR-3 and T47-D) and non-breast cancer (HT-1080, A673, and A375) cell lines by sequencing sodium metabisulfite-treated DNA. In all cell lines, the core of the TGF-β3 CpG island was predominantly unmethylated, irrespective of promoter usage associated with that cell line. In contrast, we observed a marked difference in methylation at 19 CpG sites immediately flanking and downstream of the alternative promoter's transcription initiation site. Specifically, the non-breast cancer cell lines exhibited nearly complete methylation of these CpG sites, whereas in the breast cancer cell lines, these CpGs were predominantly unmethylated. Our data support the hypothesis that methylation of a limited number of CpGs at the periphery of an otherwise unmethylated CpG island underlies the transcriptional repression of the downstream promoter in non-breast cancer cells, thereby serving to regulate the use of alternative promoters for TGF-β3.

Introduction

DNA methylation is becoming an increasingly appreciated epigenetic mechanism of transcriptional control. Methylation of such CpG-rich regions in the 5′ flanking region of certain genes, termed CpG islands, is thought to inhibit transcription by directly impeding the binding of transcription factors to their *cis*-acting sites and/or by promoting the binding of methyl-DNA binding proteins, which restrict access of transcription factors to the DNA (1, 2). In normal adult tissue cells, CpG islands are generally unmethylated, whereas in some neoplastic cells, selected genes with CpG islands undergo *de novo* methylation. In a variety of human cancers, methylation of selected CpG islands has been correlated with repressed transcription of a number of different genes, including the estrogen receptor (3, 4), progesterone receptor (4), retinoblastoma protein (5), E-cadherin (6), *BRCA1* (7), and others.

The human *TGF-β3*[3] gene has a typical CpG island, the core of which is centered just upstream of its principle promoter. The full-length mRNA of *TGF-β3* is expressed as a 3.5-kb transcript in many human cell types (8). Prior work in this laboratory has shown that this transcript includes a 1.1-kb 5′ noncoding region that, largely because it includes multiple small open reading frames, inhibits translation of TGF-β3 (9, 10). One strategy for augmenting production of TGF-β3 protein is the generation of a mRNA transcript that lacks the translational inhibitory sequences in the 5′ noncoding region. This has been observed in breast cancer cells, where activation of an alternative downstream promoter results in the production of a 2.6-kb *TGF-β3* mRNA transcript lacking the most inhibitory portion of the 5′ noncoding region (10). Thus, two promoters have been documented for TGF-β3: the initially characterized upstream promoter (P_1), which directs transcription of the full-length translation-compromised 3.5-kb transcript, and a downstream promoter (P_2), which yields the breast cancer-specific 2.6-kb transcript with enhanced translational capacity.

The work presented here deals with our investigation into whether specific patterns of CpG methylation at the *TGF-β3* gene locus correlate with promoter usage. Using a sodium metabisulfite treatment of genomic DNA to differentiate between methylated and unmethylated CpG dinucleotides (11), we observed a significant difference in the incidence of methylation at 19 CpG dinucleotides within and around the P_2 region when comparing breast cancer cell lines to non-breast cancer cell lines. Our data suggest that the lack of DNA methylation in and around P_2, although limited to a relatively small number of CpGs at the periphery of the CpG island, allows for transcription of the 2.6-kb mRNA TGF-β3 transcript in breast cancer cell lines. In contrast, hypermethylation of these CpG sites is associated with repression of transcription from P_2 in non-breast cancer cell lines.

Materials and Methods

Cell Culture. Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM:Ham's F-12 medium, supplemented with 100 IU/ml of penicillin, 125 μg/ml of streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Cell cultures were maintained in a humidified incubator at 37°C under 5% CO_2.

RNA Isolation and Analysis. Total cellular RNA from adherent cells grown on plastic was prepared by guanidinium thiocyanate-phenol extraction and analyzed by Northern blot with a ^{32}P-labeled 650-bp *Bgl*II fragment from the coding region of TGF-β3 cDNA, as described previously (10).

Bisulfite Modification of DNA. Genomic DNA was isolated from adherent cells using the Puregene DNA isolation Kit (Gentra Systems, Minneapolis, MN), per the manufacturer's instructions. Prior to treatment with bisulfite, DNA was digested with either *Bgl*II (for the analyses shown in Figs. 2 and 3) or *Pst*I (for the analyses shown in Fig. 4). DNA digestions were extracted with buffer-saturated phenol and 24:1 chloroform:isoamyl, precipitated with EtOH, and resuspended in Tris-EDTA buffer.

A slight modification of the method of Raizis *et al.* (11) was used for the bisulfite treatment. In brief, fresh 2.5 M metabisulfite in water was mixed with freshly prepared 500 mM hydroquinone in a 2:1 ratio, and the pH was adjusted to 5.0 with 2 M NaOH. Before treatment with bisulfite, the digested DNA was denatured by incubation in 0.1 ml of 0.3 M NaOH for 15 min at 37°C. Bisulfite treatment was performed by adding 0.9 ml of the sodium bisulfite-hydroquinone solution to the denatured DNA sample. Reactions were then overlaid with mineral oil, wrapped in foil, and incubated at 55°C for 4 h. Following bisulfite treatment, DNA was desalted using the Wizard DNA Clean-Up System (Promega, Madison, WI) as described by the manufacturer's instructions. Desulphonation of sulfonated uracils was effected in 0.3 M NaOH. Finally, the DNA

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[3] The abbreviations used are: TGF-β3, transforming growth factor-β3; UTR, untranslated region.

was precipitated in EtOH, washed with 70% EtOH, and resuspended in TE buffer.

Genomic Sequencing of Bisulfite-treated DNA. Selected regions of the TGF-β3 genomic locus were amplified from bisulfite-modified DNA by nested PCR. To amplify the regions upstream of P_1 (data in Fig. 2) as well as the region flanked by P_1 and P_2 (data in Fig. 3), an initial PCR was performed with the following primers, specific for bisulfite-treated DNA: ATT CGT AAA AGT GAT TTA TCG TTG TGT T (upper primer) and ACC TCC CCA AAT CCC AAA AAC TAA AAC T (lower primer). These primers were designed to be complementary to nucleotides −1206 to −1179 and 985 to 1012, respectively of bisulfite-converted DNA (assigning P_1 transcription initiation site as +1). PCRs included 0.5 μM each primer, 0.2 μM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 2 μl of bisulfite-treated genomic DNA, and 5 units of Taq polymerase (Perkin Elmer Corp., Norwalk, CT) in a final volume of 50 μl. Following initial denaturation at 95°C for 3 min, 38 cycles, consisting of 95°C for 1 min, 54°C for 90 sec, and 72°C for 3 min, commenced. In all PCRs, polymerase was added after the heat block had reached 95°C to effect a hot start of the amplification.

The product of the initial PCR was used as template for a second PCR, using nested primers. To amplify the genomic region upstream of P_1, the following primers were used: GAG TGA GAT GGG GTG GAG CGG TAT TTA TTT (upper primer) and CGT CCG ACC CGA TCT ACT CTC CCT CCT AAT (lower primer), corresponding to nucleotides −1107 to −1078 and −343 to −314, respectively. Similarly, the region of genomic DNA flanked by P_1 and P_2 was amplified using the following nested primers: GGA AGA GGC GTG CGA GAG AAG GAA TAA T (upper primer) and CCA AAA AAC GCT AAC AAC CCT AAA AAC GAA A (lower primer), corresponding to nucleotides 136 to 163 and 906 to 936, respectively. For these nested amplifications, 1 μl of the initial PCR reaction product was used as template, in a PCR with 0.5 μM each primer, 0.2 μM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), and 5 units of Taq polymerase in a final volume of 25 μl. The PCR profile was the same as in the initial PCR, except that 42 cycles were performed.

A different set of initial and nested PCR primers were used to amplify the region flanking the transcription initiation site of P_2 and extending to the start of the first intron. Initial PCR amplification used the following primers: ATT TTA TAT TTT AGT TAA TGA AGA YGA GAG GT (upper primer) and AAC TCC CAA CTC CAA TTC AAA CCC TCC A (lower primer), where "Y" denotes a 50% C-50% T mixture. These primers corresponded to nucleotides 367 to 398 and 1489 to 1516 of the bisulfite-converted sequence, respectively. PCR conditions were as indicated above for the initial PCR, except that only 2 units of polymerase were used in a final volume of 40 μl, and the annealing temperature was 50°C. The nested primers for this region were: TTC GAG GAA GTG TAA ATA AAA GAG AAA GTA TG (upper primer) and CAA ACC CTC CAA CAC AAA CAC CCC AAC R (lower primer), where "R" denotes a 50% G-50% A mixture. These primers corresponded to nucleotides 426 to 457 and 1472 to 1499 of the bisulfite-converted sequence, respectively. For this nested PCR, 1 μl from the initial PCR was used as template, and the annealing temperature was 52°C for 40 cycles.

PCR products from the nested reactions were subcloned using the pGEM-T Vector System (Promega). Miniprep DNA was prepared from multiple clones for each reaction according to the procedure from Zhou *et al.* (12). From the clones containing the anticipated DNA insert, identified by restriction enzyme digests, DNA for sequencing was prepared using the Plasmid Mini Kit (Qiagen, Chatsworth, CA). DNA sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing System (Perkin Elmer Corp.). M13-based sequencing primers, complementary to the plasmid backbone, were used. PCR conditions consisted of denaturing for 30 s at 96°C, annealing for 15 s at 50°C, and extension for 4 min at 60°C. This cycle was repeated 25 times. Sequencing PCR solutions were purified using Centriflex Gel Filtration Cartridges (Advanced Genetic Technologies, Gaithersburg, MD), as described in the manufacturer's instructions. The known genomic DNA sequence (8, 9) was compared to the sequence derived from the bisulfite-treated DNA for each clone. In this way, the methylation status of each CpG was determined because unmethylated cytosines appeared as thymidines in the bisulfite-treated DNA, whereas methylated cytosines were shown as cytosines.

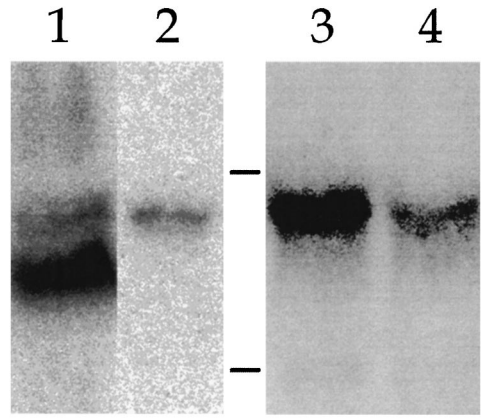

Fig. 1. Northern blot analysis of TGF-β3 expression. Total RNA (25 μg) from cells grown in tissue culture was loaded in each lane. *Lanes 1* (T47-D) and *2* (HT-1080) are from one experiment; *Lanes 3* (HT-1080) and *4* (A375) are from a second experiment. Equivalent loading of RNA was documented by ethidium bromide staining. For the purpose of band intensity comparisons, the exposure and contrast levels of *Lanes 1* and *2* are identical, as are those of *Lanes 3* and *4*. The positions of the 18S and 28S RNA in the gel are indicated by *bars* in the center of the figure.

Results

We have previously reported that human breast cancer cell lines that express TGF-β3 do so by transcription from two promoters, generating two mRNA transcripts which differ in their 5′ noncoding regions (10). As shown in Fig. 1, RNA from the T47-D cell line demonstrates two bands on Northern analysis when a probe from the TGF-β3 coding region is used. In contrast, RNA from two cell lines that transcribe TGF-β3 only from P_1 (HT1080 and A375, sarcoma and melanoma cell lines, respectively) contain only the full-length 3.5-kb transcript (Fig. 1).[4] Initial efforts at mapping the CpG methylation status at the *TGF-β3* gene locus were focused on the main core of the CpG island. Genomic DNA was isolated from cells grown in culture, treated according to a modification of the bisulfite method for distinguishing between methylated and unmethylated cytosines, PCR-amplified, subcloned, and sequenced. Sequence data from multiple clones from each cell line were used to calculate cell type-specific estimates of percentage methylation at each CpG site.

Data from the HT1080 sarcoma cells are plotted in Fig. 2, along with data from two breast cancer cell lines that transcribe from both P_1 and P_2 (SKBR-3 and T47-D). Located at the top of Fig. 2, a scaled map of CpG site locations shows the high-density core region of the island in which we have mapped methylation status. Cumulative data derived from sequence information from multiple clones (n = 4–13 for each CpG site, for each cell line) are plotted in the graph at the bottom of the figure. There were isolated CpGs showing >40% methylation in the upstream half of this region, whereas methylation was minimal or absent in the 3′ portion of the island core. Overall, the majority of CpG sites in this region of the island were only minimally methylated, and there was no evident methylation pattern that was correlated with promoter usage.

A similar series of experiments were performed to map CpG methylation status in a region of genomic DNA flanked by the two promoters. This is a less CpG-dense region, downstream of the high density CpG core of this island complex. As is evident in Fig. 3, most of these CpG sites exhibited minimal methylation in all three cell lines, with the notable exception of the five 3′-most CpG sites. These CpGs, which flank the P_2 transcription initiation site, were nearly

[4] RNA analysis of TGF-β3 expression for the other cell lines used in this work can be found in Ref. 10.

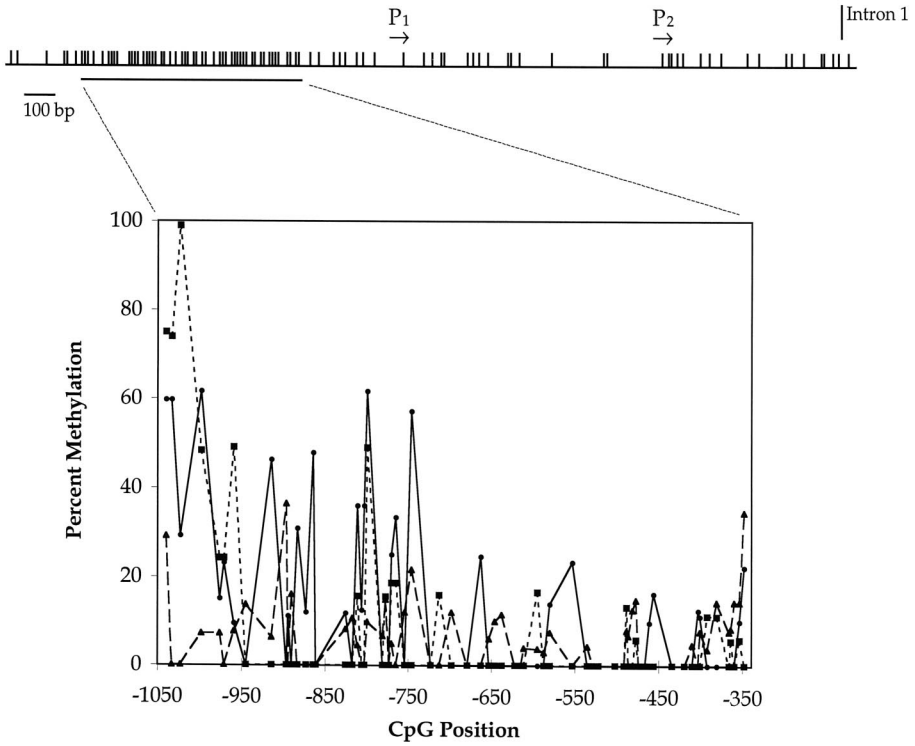

Fig. 2. Methylation within the CpG island core in the TGF-β3 promoter. The locations of CpGs within the genomic region encompassing TGF-β3's two promoters and first exon are indicated by *hatch marks* above the linear representation of genomic DNA, and the region within the core of the CpG island investigated by DNA sequence analysis of bisulfite-treated DNA is indicated by the *line* below. Graphically represented is the percentage methylation at each CpG site within this region observed in DNA from HT1080 cells (●), SKBR-3 cells (■), and T47-D cells (▲). *Data points*, average of multiple sequence determinations for each CpG site for each cell line (n = 4–20).

completely methylated in the HT1080 cells, but remained unmethylated in SKBR-3 and T47-D cells.

The magnitude of the differences at these CpG sites suggested to us that high levels of methylation at selected CpG sites surrounding the downstream promoter (P_2) may be a mechanism by which non-breast cancer cells block activation of P_2 and, therefore, restrict transcription initiation of TGF-β3 to the upstream promoter. To explore that possibility, we broadened our panel of cell lines to include an additional two that use TGF-β3's P_1 only: A375 (melanoma) and A673 (lung cancer). For each of the five cell lines, we mapped the CpG methylation frequency for the region of genomic DNA extending from the CpG at position 525 to the CpG at position 1472, located in the 5′ portion of the first intron. Thus, a total of 22 CpG sites were investigated in this set of experiments. As is evident in Fig. 4, the

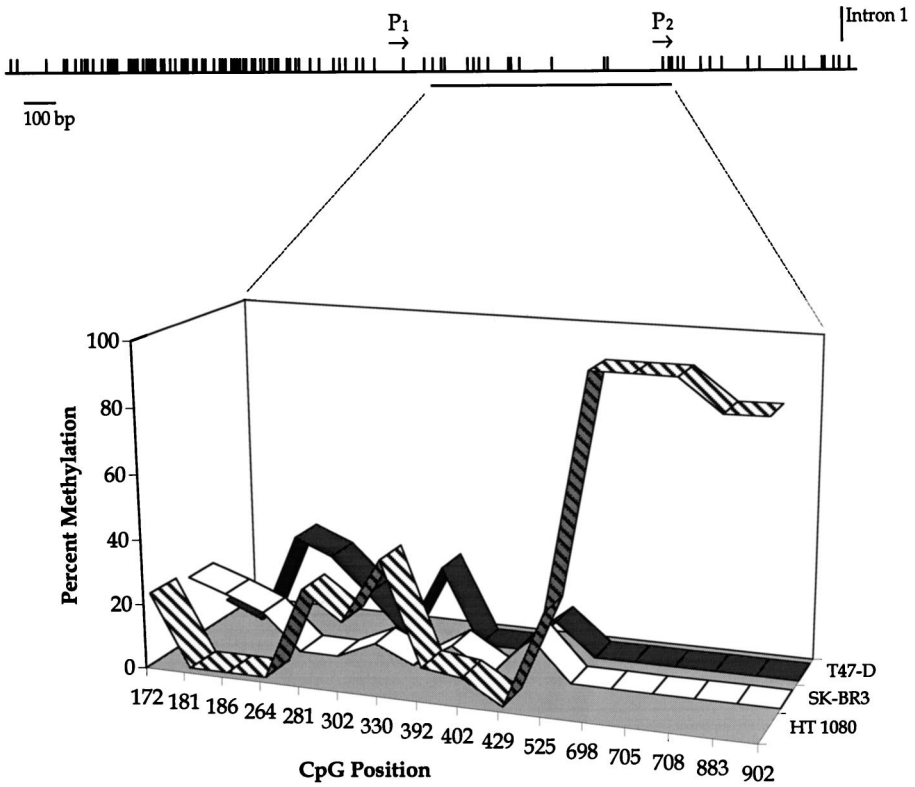

Fig. 3. Methylation at CpG sites flanked by the two transcription initiation sites for TGF-β3. *Top*, the region of genomic DNA investigated in this series of experiments is indicated by the line just beneath the CpG map, as in Fig. 1. Percentage methylation at each of the indicated CpG positions (numbered with +1 = transcription initiation site of P_1) represents the average of multiple sequence determinations from bisulfite-treated DNA from HT1080 cells (▨), SKBR-3 cells (□), and T47-D cells (■; n = 4–18).

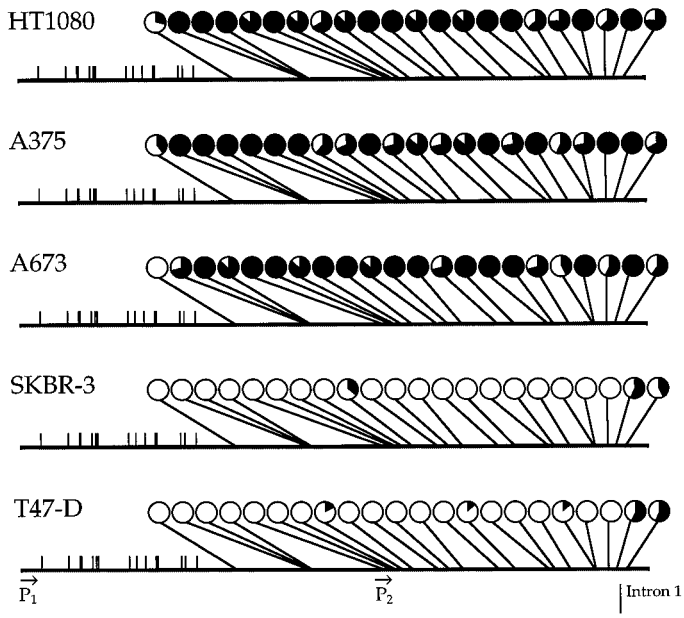

Fig. 4. Methylation at CpG sites flanking the downstream, breast cancer-specific TGF-β3 promoter (P_2). The percentage methylation at the investigated CpG sites is represented by small pie graphs, with the percentage of area filled-in proportional to the frequency of methylation, as determined from sequence analysis of four to eight clones at each CpG site for each of the indicated cell lines. For example, the 3′-most investigated CpG (position 1472) exhibited a 75% incidence of methylation in HT1080 cells and a 43% incidence of methylation in SKBR-3 cells. The investigated CpG sites are located at positions 525, 698, 705, 708, 883, 902, 910, 928, 953, 1014, 1041, 1075, 1161, 1201, 1284, 1297, 1334, 1393, 1398, 1429, 1449, and 1472, relative to P_1.

incidence of methylation in this region is strikingly different when the non-breast cancer cell lines (HT1080, A375, and A673) are compared to breast cancer cell lines (SKBR-3 and T47-D). This difference is most notable for the 19 CpGs from positions 698 to 1429. Within this region, the non-breast cancer cell lines demonstrated high levels of methylation (average = 88%), whereas the breast cancer cell lines exhibited pronounced hypomethylation (average = 1%).

Discussion

One of the well-recognized strategies for regulation of gene expression is the use of alternative promoters in a tissue-specific or developmentally specific pattern (13). In some cases, the different promoters yield mRNA transcripts that share coding regions but differ in their 5′ UTRs. Although the presence of different 5′ UTR sequences in mRNAs encoding the same protein potentially allows for transcript-specific regulation of mRNA localization and stability, the bulk of prior work on the influence of the 5′ UTR suggests that such transcripts might primarily differ in translational capacity (14). In this manner, promoter usage could affect protein levels at the posttranscriptional level.

One example of a gene with two promoters that generate mRNA transcripts with differing translational efficiency but encode the same protein, is *TGF-β3*. Prior work in our laboratory has documented the presence of a downstream promoter, active only in breast cancer cells, that results in a 5′ truncated transcript with enhanced translational capacity (10). Other examples of alternative promoters resulting in differing 5′ UTRs include the insulin-like growth factor-II gene (15) and the neuronal nitric oxide synthase gene (16). An interesting feature of the *TGF-β3* genetic locus, shared by the neuronal nitric oxide synthase gene, is that the two alternative promoters are in close proximity to each other and are associated with a CpG island.

Although methylation of CpG islands associated with the promoter regions of certain genes has been implicated as a mechanism of repressed transcription, there has been little investigation into how methylation may be involved with differential use of alternative promoters. Frequently, the methylation status of a CpG island is determined by characterization of a relatively few number of CpGs within the core of the island. Two commonly used methodologies are methylation-specific PCR, in which primers specific to methylated *versus* unmethylated CpGs are used to amplify bisulfite-treated DNA, and the use of methylation-sensitive restriction enzymes (4, 17, 18). Both approaches provide information regarding a small subset of CpGs within the island complex, and may therefore lack the requisite sensitivity to evaluate the role of CpG methylation in the utilization of alternative promoters.

We have, therefore, approached our examination of the *TGF-β3*

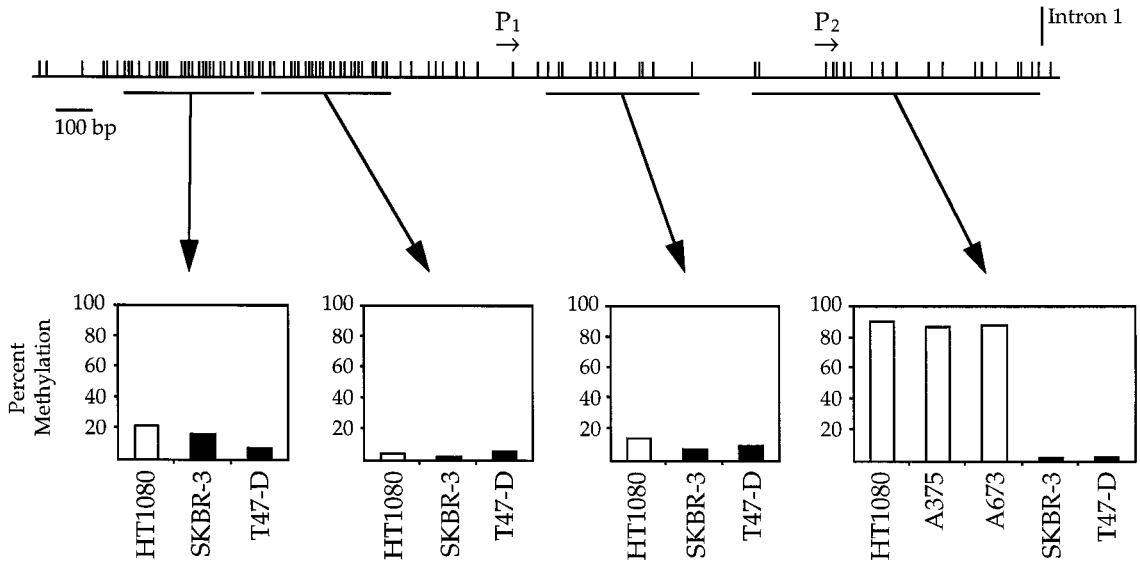

Fig. 5. Summary of cell-specific CpG methylation throughout the TGF-β3 CpG archipelago. The investigated CpGs were divided into four regional groupings, as indicated by the *lines* beneath the CpG map (*top*). The average incidence of methylation for all CpG sites within each region is shown for the indicated cell lines. ■, two breast cancer cell lines (SKBR-3 and T47-D); □, non-breast cancer cell lines (HT1080, A375, and A673).

locus by comprehensive sequence analysis of large regions of the associated CpG island. A composite overview of our findings, including data from multiple cell lines regarding methylation within the indicated CpGs, is presented in Fig. 5. It is clear from our data that dramatic differences in CpG methylation between breast and non-breast cancer cell lines are restricted to a relatively small subset of CpGs that flank the downstream promoter. The hypermethylation at these sites in all of the non-breast cancer cell lines is consistent with the transcriptional suppression of this promoter in these cells. These observations have suggested to us that many CpG islands, such as that associated with TGF-β3, should more appropriately be considered as an island chain or CpG archipelago, composed of many islands of varying density. Such a view would facilitate recognition of the potential importance of methylation that is restricted to one portion of the island chain, which in our case is evident in the 3′ tail of the CpG archipelago.

In summary, we have shown that methylation within most of the CpG island of TGF-β3 does not differ between breast and non-breast cancer cell lines, but that the lack of cytosine methylation in the region proximal to P_2 may underlie its transcription activation in breast cancer cells, thereby yielding a mRNA with enhanced translational capacity.

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