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DNA Methylation Patterns Associated with Asparagine Synthetase Expression in Asparagine-Overproducing and -Auxotrophic Cells

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In Chinese hamster ovary cells, the gene for asparagine synthetase, which spans 20 kilobase pairs, was found to contain a cluster of potential sites for CpG methylation in a 1-kilobase-pair region surrounding the first exon. Fourteen of the sites that could be assayed for methylation by *Msp*I-*Hpa*II digestions were found in this region, with an additional nine *Msp*I sites spread throughout the remainder of the gene. The methylation status of the gene was analyzed in a series of cell lines that differed in the amount of asparagine synthetase activity. The level of expression showed a direct correlation with the extent of methylation of a subset of the *Msp*I sites found in the 5′ region of the gene. The rest of the gene was completely methylated in most cell lines. Wild-type cells, which expressed a basal level of asparagine synthetase activity, were partially demethylated in the 5′ region. In contrast, asparagine-requiring N3 cells, which lacked detectable mRNA for asparagine synthetase, were methylated throughout the entire gene. Spontaneous revertants of strain N3, selected for growth in asparagine-free medium, exhibited extensive hypomethylation of the asparagine synthetase gene. The methylation pattern of the gene in cell lines that overproduced the enzyme was also examined. Albizziin-resistant cell lines, which had amplified copies of the gene, were extensively demethylated in the 5′ region. Overexpression of asparagine synthetase in β-aspartyl hydroxamate-resistant lines without amplified copies of the gene was also correlated with DNA hypomethylation.

The pattern of DNA methylation has been proposed to play a role in the expression of tissue-specific genes and, more recently, in genomic imprinting in mammalian cells (32; for reviews, see references 9, 10, and 31). The methylation of cytosines occurring in CpG dinucleotides has also been shown to be correlated with lack of expression of certain X-linked genes (20, 21), and it has been suggested that some nutritional auxotrophic mutants of mammalian cells may have arisen from DNA hypermethylation (14–17, 30). Several cell lines that require asparagine for growth because of a lack of asparagine synthetase (AS) activity have been isolated (24, 34). Harris and colleagues (17, 30) found that rat Jensen sarcoma, CHO-KI, and hamster V79 auxotrophs revert to asparagine independence at a very high frequency after exposure to the methylation inhibitor 5-azacytidine, presumably because of hypomethylation of the AS gene. Thus, changes in DNA methylation may be one mechanism involved in the control of expression of the AS gene. To test this, we have examined the pattern of DNA methylation of the AS gene from wild-type Chinese hamster ovary (CHO) cells and asparagine auxotrophs.

We have also investigated the role of DNA methylation in the expression of AS activity in drug-resistant mutant cell lines that overproduce the enzyme (2, 3, 6). Single-step mutant lines resistant to the amino acid analog β-aspartyl hydroxamate (β-AHA^R) exhibit elevations in AS activity up to 8-fold over that of the parental cells (6, 11), and multistep β-AHA^R lines express as high as 20-fold increases in AS activity (6). On the other hand multistep albizziin-resistant (Alb^R) lines that overproduce the enzyme by 300-fold have been isolated (2). We took advantage of a highly resistant multistep line to clone an AS cDNA and show that the gene was amplified in this line (25).

This study was designed to investigate the role of DNA methylation in the expression of AS. We have isolated the wild-type CHO AS genomic sequences and found that the gene spans 20 kilobase pairs (kbp) and contains 12 exons (I. L. Andrulis, M. Shotwell, S. Evans-Blackler, H. Zalkin, L. Siminovitch, and P. N. Ray, Gene, in press). We report here that the AS gene has 23 sites for CpG methylation which can be analyzed by digestion with *Msp*I and *Hpa*II. Most of these are in the G+C-rich 5′ upstream sequences and the region surrounding the first exon. The methylation patterns of these sites were examined in the genes from wild-type cells, which express a basal level of AS activity, and from nonexpressing cells auxotrophic for asparagine. Spontaneous revertants that do not require asparagine for growth were isolated from the auxotrophs as isogenic expressors. The degree of methylation of the AS gene was also determined in these lines as well as in Alb^R and β-AHA^R mutants containing elevated levels of AS activity.

MATERIALS AND METHODS

Cell lines. Isolation of the Alb^R CHO mutant lines has been previously described (2, 3). Wild-type (Toronto strain) CHO cells and single-step Alb^R mutants (Alb^R 1, 2, 6, 10, and 27) were grown in α complete (α minimal essential medium [28] containing nucleosides and 7% heat-inactivated [56°C for 30 min] fetal calf serum). The multistep line (Alb^R 52) was maintained in selective medium (α complete lacking asparagine) with the glutamine concentration reduced to 200 μM and containing 50 mM albizziin and 7% dialyzed heat-inactivated fetal calf serum. N3 is an asparagine-requiring CHO cell line isolated by Waye and Stanners (34). Spontaneous revertants of N3 (R1, R2, R3, and R4) were obtained by plating freshly cloned N3 cells in selective medium. There was no pretreatment with the mutagen ethyl methanesulfonate (EMS). The revertants arose at a frequency of approximately 10^{-6}.

RNA analysis. Total RNA was isolated as previously

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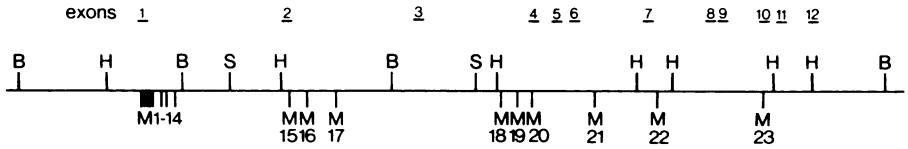

FIG. 1. Genomic organization of the CHO AS gene showing the *Msp*I (M1 to M23) sites contained in the 20 kbp that encompasses the gene. Positions of the 12 exons are depicted above the map. All of the *Bam*HI (B), *Sst*I (S), and *Hin*dIII (H) sites are indicated.

described (25). RNA was denatured with formamide and formaldehyde, run on 1% agarose gels containing formaldehyde, and transferred to nitrocellulose or nylon membranes (22). Hybridizations were performed overnight at 42°C in hybridization solution (5× SSPE [22], 5× Denhardt solution, 0.1% sodium dodecyl sulfate, 100 μg of salmon sperm DNA per ml) containing 50% formamide, with nick-translated (26) inserts of CHO cDNAs (25) used as probes.

DNA analysis. High-molecular-weight DNA was prepared as described previously (5). Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and New England BioLabs, Inc., and used under conditions recommended by the suppliers. The DNAs were digested with restriction enzymes overnight, run on 0.8% agarose gels, blotted by Southern transfer (27) as modified by Wahl et al. (33), and probed with nick-translated CHO cDNAs as previously described (25).

Slot blot analysis. Amounts of DNA and RNA were quantitated by slot blotting (19) of serial dilutions of DNA and RNA and densitometric analysis. The amounts were calculated relative to the copy number and expression of the actin or CAD gene.

RESULTS

Methylation sites in the AS gene. We had previously isolated overlapping bacteriophage containing the CHO genomic sequences and characterized the organization of exons and flanking regions (Andrulis et al., in press). Using subclones of the genomic sequences and Southern blots of wild-type genomic DNA, we mapped restriction sites for *Msp*I, which cuts at CCGG sequences. The AS gene contained 23 *Msp*I sites, 14 of which were clustered within 1 kbp in the 5′ region of the gene surrounding exon 1 (Fig. 1). The other *Msp*I sites were distributed through the remainder of the gene. Not surprisingly, the 5′ cluster occurred in a region with a very high G+C content. We sequenced over 2 kbp of this region and found that there were numerous CpG dinucleotides in addition to the *Msp*I sites. In fact, within an 800-bp region surrounding exon 1, there were 71 CpG dinucleotides. This is peculiar to this region of the gene, as only 16 CpG dinucleotides were found by sequencing 2 kbp further 5′ of the gene (data not shown).

Methylation pattern of the 3′ portion of the AS gene in auxotrophic cells. To investigate whether hypomethylation of AS plays a role in asparagine auxotrophy, we have taken advantage of a CHO cell line (N3) that requires asparagine for growth. This cell line had been isolated from the same strain of CHO cells as were the AS-overproducing cell lines that we had previously described (2, 3, 6). Like other asparagine auxotrophs, N3 cells revert to asparagine prototrophy at a high frequency after treatment with 5-azacytidine (K. S. Worton, R. Kerbel, and I. L. Andrulis, unpub-

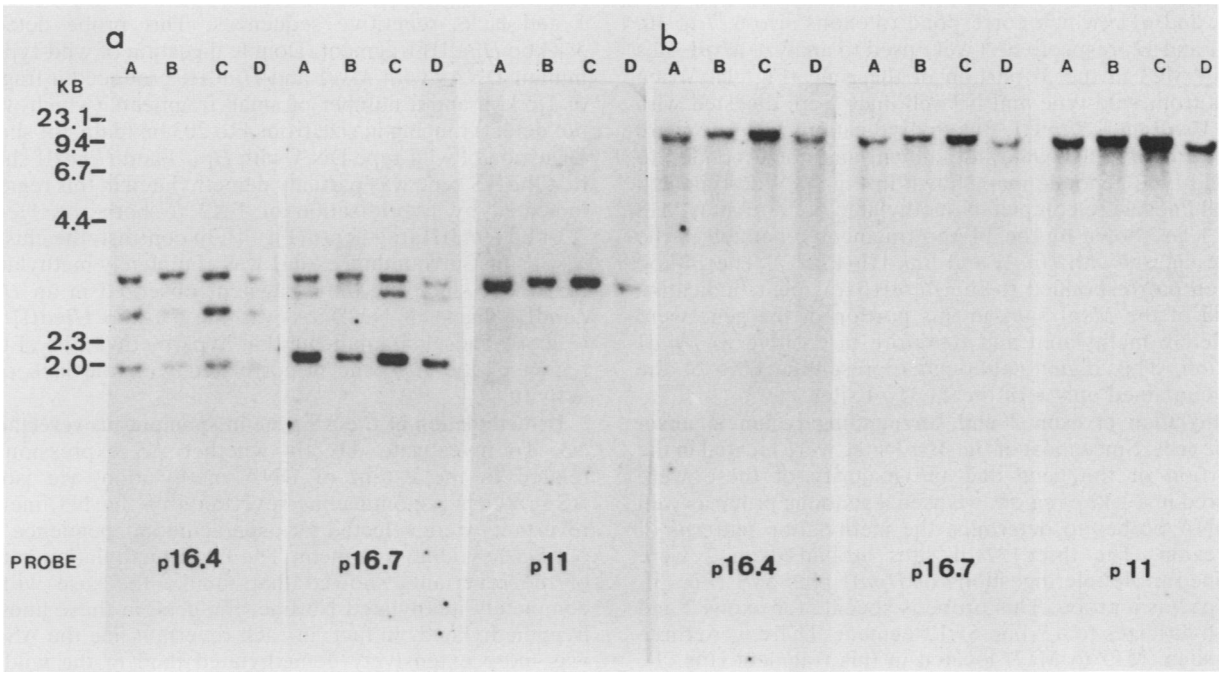

FIG. 2. Complete methylation of most of the AS gene in wild-type and mutant cells. DNAs from wild-type (lanes A), amplified Alb^R 1 (lanes B) and Alb^R 10 (lanes C), and N3 (lanes D) cells were digested with *Msp*I-*Bam*HI (a) or *Hpa*II-*Bam*HI (b). Southern blots were probed with portions of the AS cDNA probes p16.4, p16.7, and p11, corresponding to exons 3 to 12. Amounts of Alb^R 1 and Alb^R 10 DNAs were decreased to normalize the AS copy number to that of wild-type DNA.

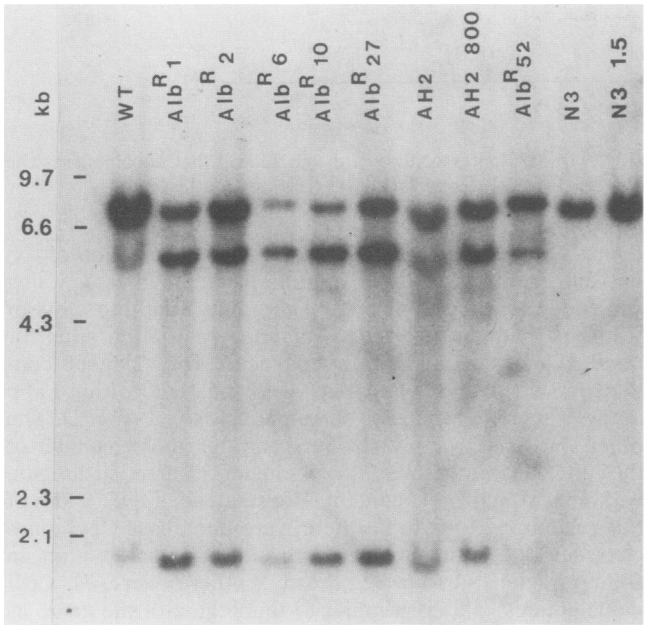

FIG. 3. Detection by an exon 2 probe of differences in the extent of methylation at an *Msp*I site (M15) in wild-type and mutant lines. The indicated DNAs were digested with *Hpa*II plus *Sst*I, and the blot was probed with p12.4. Amounts of DNAs from the amplified single-step lines (Alb^R 1, 2, 6, 10, and 27) and the multistep line (Alb^R 52) were reduced to normalize the copy number to that of wild-type DNA.

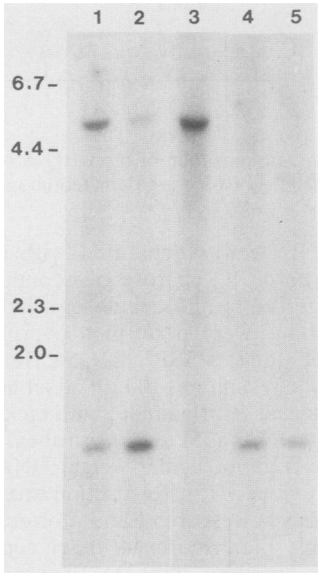

FIG. 4. Hypomethylation of the cluster of *Msp*I sites surrounding exon 1 in DNA from cells expressing AS but complete methylation in DNA from AS-deficient N3 cells. A genomic probe (EX2) extending 300 bp 5′ of exon 1 and including the cluster of 14 *Msp*I sites that surround exon 1 was used to probe *Hpa*II-*Hin*dIII digests of DNA from wild-type (lane 1), Alb^R 1 (lane 2) and asparagine-requiring N3 (lane 3) cells and from spontaneous revertants of N3 (lanes 4 and 5).

lished observations). We examined the pattern of DNA methylation at CCGG sites in the entire AS gene from the N3 cell line.

CHO genomic and cDNA probes were used to examine the methylation status of the AS gene. AS cDNAs p16.4, p16.7, and p11 (which correspond to exons 3 to 6, 7 to 10, and 11 and 12, respectively) were used to analyze *Msp*I sites M18 to M23 in the 3′ portion of the gene (Fig. 1). When DNAs from wild-type and N3 cell lines were digested with both *Msp*I and *Bam*HI, the hybridization patterns were identical (Fig. 2 and data not shown) and corresponded to the wild-type genomic map shown in Fig. 1. Wild-type and N3 cell lines were completely methylated at *Msp*I sites M18 to M23, as shown by the 14-kbp fragment generated in the double digests with *Hpa*II and *Bam*HI (Fig. 2). The 14-kbp fragment corresponded to the *Bam*HI fragment, indicating that all of the *Msp*I sites in this portion of the gene were completely methylated and therefore insensitive to *Hpa*II digestion. This region, although representing 60% of the gene, contained only 6 of the 23 *Msp*I sites.

Methylation of exon 2 and surrounding region in auxotrophic cells. Since most of the *Msp*I sites were located in the 5′ portion of the gene and the majority of these were clustered in a 1-kbp region, we used a genomic probe as well as cDNA probes to determine the methylation patterns of this region. The three *Msp*I sites around exon 2 were examined in double digestions of *Hpa*II plus *Sst*I (Fig. 3), using p12 as a probe. This probe is specific for exons 2 and 3 and hybridizes to a 7-kbp *Sst*I fragment. There were three *Msp*I sites (M15 to M17) located in this fragment (Fig. 1), and all were methylated in the N3 line, as shown by a single 7-kbp band. However, an *Msp*I site within the exon (M15) was partially demethylated in the wild-type cells, to give additional bands of 5.2 and 1.8 kbp.

Hypermethylation of the 5′ region of the AS gene in asparagine auxotrophs. To examine the CpG-rich region surrounding the first exon, we used a genomic probe (EX2) that is 800 bp long and contains the first exon. EX2 includes the cluster of 14 *Msp*I sites (M1 to M14) that surround exon 1 and lacks repetitive sequences. This probe detects a 5.1-kbp *Hin*dIII fragment. Double digestion of wild-type and mutant DNAs with *Msp*I and *Hin*dIII produced a fragment of 1.2 kbp and a number of small fragments (which we did not detect) ranging in size from 4 to 203 bp (data not shown). Digestion of wild-type DNA with *Hpa*II and *Hin*dIII showed that the AS gene was partially demethylated in this region, as indicated by hybridization of EX2 to both the 1.2- and 5.1-kbp *Hin*dIII fragments (Fig. 4). In contrast, the cluster of *Msp*I sites surrounding exon 1 was completely methylated in N3 DNA, since the only fragment observed in an *Hpa*II-*Hin*dIII digest of N3 DNA was the 5.1-kbp *Hin*dIII band (Fig. 4). These data indicate that hypermethylation of the 5′ region of the AS gene is correlated with the lack of AS activity.

Demethylation of the AS gene in spontaneous revertants of N3. To investigate directly whether AS expression was related to the extent of DNA methylation, we isolated AS-producing spontaneous revertants of the N3 line. The revertants were selected for asparagine independence without 5-azacytidine treatment. The DNA methylation patterns of the revertants showed that, unlike the case with the completely methylated N3 line, the gene in these lines was hypomethylated. In fact, in each revertant line the AS gene was more extensively demethylated than in the wild-type line. This can be seen for the cluster of sites around exon 1 (Fig. 4) as the reduction in intensity of the 5.1-kbp band and increase in the amount of the 1.2-kbp fragment, which is diagnostic for demethylation in the 5′ region. Other *Msp*I

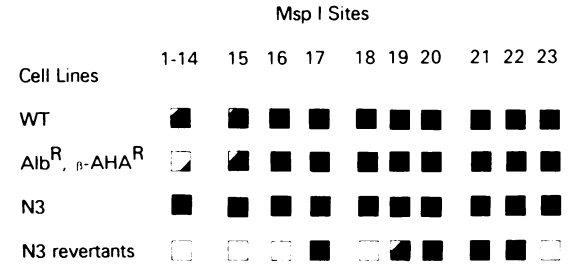

FIG. 5. Summary of the methylation status of the 23 *Msp*I sites in the AS gene from wild-type cells (WT), overproducing mutant lines Alb^R and β-AHA^R, AS-deficient N3 cells, and spontaneous revertants of N3 cells. The extent of methylation ranged from hypomethylated (□) to completely methylated (■).

sites were also demethylated in the revertants (Fig. 5). Since the extent of methylation of the AS gene was directly related to expression of the gene in these lines, we examined whether methylation might also play a role in the increased production of AS in Alb^R and β-AHA^R cell lines.

Molecular analysis of the AS gene in Abl^R mutants. We had previously shown that single-step Alb^R lines exhibited distinguishing biochemical properties, which suggested that there were several classes of mutants (3). These included changes in K_m and K_i, differences in cross-resistance to β-aspartyl hydroxamate, and alterations in AS activity in response to the concentration of asparagine in the medium. However, all of the single-step Alb^R mutants produced levels of AS enzymatic activity 8- to 17-fold greater than those of the parental lines (3). The molecular basis for overproduction of AS enzymatic activity in the mutants was not determined, although we did show that one of the single-step Alb^R mutants contained amplified copies of the gene for AS (25). To examine whether the gene was amplified and overexpressed in the other mutant lines, we examined the copy number of the gene and the levels of AS mRNA in the single-step Alb^R mutant lines. All of the Alb^R lines had amplified copies of AS (Table 1). However, there was not a direct correlation between DNA copy number and the level of specific activity of AS. For example, Alb^R 1, which overproduced AS activity by 17-fold, exhibited only a 3-fold increase in DNA copy number. Since the increase in copy number alone could not account for the elevation in AS activity, we investigated additional mechanisms that may be involved in altering the expression of AS.

Overproduction of AS mRNA in Alb^R and β-AHA^R lines. Northern (RNA) blot analysis showed that the Alb^R mutants overproduced AS mRNA of the same size as the wild-type message (data not shown). The level of AS mRNA overexpression was correlated with, but not equal to, the increase in AS enzymatic activity (Table 1). In the highly amplified multistep lines and in longer exposures of autoradiographs of the single-step lines, a slightly larger species that represented only a minor portion of the total AS RNA was also observed. These results are consistent with the use of one major site for initiation of transcription of the AS gene and other less frequently used cap sites (Andrulis et al., in press). Since the mutant lines had increases in expression of AS mRNA that were greater than the increases in DNA copy number, we examined the gene in the mutants for changes in DNA methylation.

TABLE 1. Overexpression and amplification of the AS gene in β-AHA^R and Alb^R cell lines

Cell line	AS sp act (fold increase)	AS RNA (fold increase)	DNA copy no.
Wild type	1	1	1
Alb^R 1	17	7	3
Alb^R 2	11	6	3
Alb^R 6	12	7	ND[a]
Alb^R 10	12	11	9
Alb^R 27	8	ND	4
β-AHA^R AH2	6	ND	1
β-AHA^R AH2 800	11	6	1

[a] ND, Not determined.

Hypomethylation of the 5′ region of the AS gene in Alb^R and β-AHA^R lines. The mutant cell lines exhibited the same pattern of hypermethylation as the wild type at all of the CCGG sites in the 3′ portion of the AS gene (Fig. 2). However, the amplified lines (Alb^R 1, 2, 6, 10, and 27) exhibited extensive demethylation in the area surrounding exon 2 (Fig. 3) and in the most 5′ region of the gene (Fig. 4 and data not shown). The level of hypomethylation in DNAs from the Alb^R cell lines was much greater than that of the wild-type DNA (Fig. 4 and 5). The same patterns of hypomethylation (Fig. 3 and 5) were observed in DNAs from the β-AHA^R lines AH2 and AH2 800, which did not have amplified copies of the gene but overexpressed AS.

DISCUSSION

We have examined the methylation patterns of the AS gene in CHO cells that lack AS activity. Genetic studies by Harris and colleagues (17, 30) had suggested that asparagine auxotrophs had methylated copies of the AS gene. They were able to revert asparagine-requiring Jensen rat sarcoma cells and asparagine auxotrophs of hamster K1 and V79 cell lines at a high frequency (up to 5% of the cells) by treatment with 5-azacytidine. As well, we had previously found that Jensen rat sarcoma cells into which we had introduced multiple silent copies of transfected AS cDNA sequences could be induced to express very high levels of AS by 5-azacytidine treatment (1).

To examine the molecular basis of the lack of AS expression in auxotrophic cells, we took advantage of a CHO mutant cell line (N3) that was derived from the same parental line as our mutants but requires asparagine for growth. The nature of the mutation in the N3 line has not been determined, although it has been suggested that it is a structural gene mutation (34). However, we have found that asparagine auxotrophy in this line is associated with complete methylation of the gene. We examined all of the potentially methylatable CCGG sites in the AS gene. Analysis of this mutant showed that the entire gene was methylated in N3 cells. To determine whether the methylation state of the gene was important for AS expression, we isolated spontaneous (without 5-azacytidine treatment) revertants of the N3 line. In these lines, there was extensive demethylation of the gene, principally in the 5′ region. These data suggest that at least one level of control of the AS gene involves changes in DNA methylation.

It has been proposed that auxotrophy for proline (15), glutamine (14), and putrescine (29) is also due to DNA hypermethylation, since these lines can be reactivated to prototrophy by treatment with 5-azacytidine. These auxotrophs had been obtained after treatment with the mutagen EMS. It has been suggested from studies on putrescine auxotrophs of CHO cells (29) and prolactin-deficient rat cells (18) that EMS not only acts as a mutagen in the classical

sense but may also inactivate genes by methylation. This may also be the case for the AS gene in the N3 line, since EMS had been used to obtain the auxotroph (34). On the other hand, EMS may also serve to activate genes, as suggested by the increase in frequency of glutamine prototrophs after EMS treatment of a glutamine-requiring cell line (14). Since the Alb^R and β-AHA^R cell lines were isolated after EMS mutagenesis, this may be a contributing factor in the changes in methylation observed in these lines.

We have previously described biochemical studies on AS regulation (4) and genetic characterization of Alb^R and β-AHA^R cells that overproduce AS activity (2, 3, 6). We have begun a molecular analysis of the mutant lines and found that Alb^R cells have amplified copies of the gene for AS, whereas β-AHA^R cell lines with high levels of resistance have no increase in AS copy number. All of the mutants have increases in expression of AS mRNA greater than increases in the gene copy number. An additional mechanism for the overproduction of AS in the mutant cell lines involves changes in DNA methylation.

By examining the DNA methylation patterns in the wild-type and mutant cell lines, we have shown that differences in AS expression are correlated with the methylation pattern of the 5′ region of the gene (summarized in Fig. 5). Methylation of this region most likely plays a role in regulation of the AS gene, since the gene is partially demethylated in wild-type cells that express a low level of AS activity and demethylated in Alb^R or β-AHA^R mutants that overproduce AS. In contrast, the 3′ portion of the gene is completely methylated in all of these cell lines. Most of the *Msp*I sites and numerous other potential sites for DNA methylation are clustered in the 5′ region of the AS gene, a feature shared with other genes that contain CpG islands (for review, see reference 8). Hypomethylation in the 5′ region as observed in the AS gene has been shown to be correlated with gene activity for a number of X-linked and other genes (for review, see reference 8). It has been shown for the human γ-globin gene (23) that this effect is not site specific but region specific and requires a minimum area of hypomethylation around the promoter. Methylation of cytosines in the promoter region has been found to affect DNA-protein interactions (7). We have recently identified (Y. P. Zhang and I. L. Andrulis, unpublished observation) the 5′ region of the AS gene which contains the C+G-rich area as the promoter for the AS gene in the chloramphenicol acetyltransferase assay (12). It is not known which specific proteins are involved in the interactions with methylatable sequences, but it has recently been shown that binding of transcription factor Sp1, a likely candidate with specificity for GC boxes, is unaffected by DNA methylation (13).

In summary, we have shown that expression of the AS gene is affected by the extent of methylation of the gene, principally in the 5′ region. Cells that lacked AS activity exhibited complete methylation of *Msp*I sites, wild-type cells that expressed basal levels of AS were partially demethylated, and AS overproducers resistant to albizziin or β-aspartyl hydroxamate showed extensive hypomethylation of the gene. The data indicate that although the Alb^R lines were isolated in a single step of selection, two events may be involved in the overproduction of AS in these lines. We have shown that increased expression was associated with 5′-regional demethylation as well as amplification of the AS gene in each of these mutants. In contract, β-aspartyl hydroxamate resistance was associated with demethylation of the AS gene without amplification. The results suggest that an increase in transcription of the gene may be a necessary but not sufficient initial event for gene amplification.

ACKNOWLEDGMENTS

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