The primary function of a redundant Sp1 binding site in the mouse aprt gene promoter is to block epigenetic gene inactivation

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ABSTRACT

The promoter region of the mouse adenine phosphoribosyltransferase (aprt) gene contains one non-consensus Sp1 binding site at its 5’ end followed by three consensus Sp1 binding sites. The two 3’-most binding sites are sufficient for maximal expression of aprt, suggesting that the non-consensus and consensus binding sites at the 5’ end are redundant. However, the two 3’ sites are not sufficient to block epigenetic inactivation, which led to the hypothesis that the redundant consensus and/or non-consensus 5’ Sp1 binding sites are required to block inactivation events. To test this hypothesis, promoter region constructs were made in which the two 5’ Sp1 binding sites were mutated alone or in tandem, and then each construct was tested for its ability to withstand epigenetic inactivation. A cis-acting methylation center that is normally located 1.2 kb upstream of the promoter was used to induce inactivation. The results demonstrate that the presence of the redundant consensus Sp1 binding site is required to block methylation-associated gene inactivation. Therefore, the Sp1 binding sites comprising the mouse aprt promoter have evolved two distinct functions, one to promote transcription and the other to block epigenetic inactivation.

INTRODUCTION

The mouse adenine phosphoribosyltransferase (aprt) gene is a constitutively expressed housekeeping gene whose product is responsible for adenine salvage. Several features have made it a target for both mutational and epigenetic studies including its relatively small size (2.3 kb), the ability to select for and against cells that lack expression, and the absence of a phenotypic effect for cells that are aprt deficient (1). The aprt promoter is one of the simplest described for a mammalian gene (2). It consists of one non-consensus and three consensus Sp1 binding sites (numbered 1–4; Figs 1 and 2). No other transcription factor binding sites have been identified in the promoter region. TATA and CCAAT boxes are also absent. In vitro binding assays with partially and fully purified Sp1 protein have shown binding to all four binding sites, though with different affinities. Binding sites 2, 3 and 4 exhibited strong binding affinities whereas weak binding was observed for the non-consensus binding site 1 (2). In vivo footprinting confirmed that binding sites 2–4 are occupied in expressing cells and suggested weak binding for site 1. None of these binding sites was occupied in a non-expressing cell line containing a hypermethylated aprt promoter region (3). Several studies have shown that only the two 3’ Sp1 binding sites (3 and 4) are required for maximal promoter activity (2,4). What then is the function(s), if any, of Sp1 binding sites 1 and 2?

We have previously shown that Sp1 binding sites 3 and 4 became sensitive to methylation-associated inactivation when sites 1 and 2 were deleted (4). Methylation was induced by an 838 bp fragment, termed a methylation center (MC) (5), that can signal de novo methylation in embryonic carcinoma cells. The MC fragment, whose 3’ end is normally located 1.2 kb upstream of the aprt promoter (Fig. 2), was unable to inactivate an intact promoter containing all four Sp1 binding sites when it was moved to a position 34 bp upstream of Sp1 binding site 1. The simplest interpretation of our data was that Sp1 binding sites 1 and/or 2 were necessary to block epigenetic inactivation. However, because an 87 bp fragment including these binding sites was deleted to create the sensitive promoter, it was formally possible that deleted sequence information independent of the binding sites was providing the blocking effect. Independent work by Macleod et al. (6) showed that deletion of the entire promoter region, or site-directed mutagenesis eliminating binding activity for Sp1 binding sites 2–4 (Sp1 binding site 1 was not included on the constructs tested), allowed methylation of downstream CpG sites to occur on transgene fragments. Based on their work, the authors also concluded that the Sp1 binding sites can block CpG island methylation and speculated that protein binding may provide the blocking effect. However, the mouse aprt constructs

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examined in their study lacked any transcriptional potential. Because it has been shown that transcriptionally inactive genomic regions serve as non-specific targets for de novo methylation in cultured cells (7), it is possible that the CpG island methylation observed by Macleod et al. (6) was due to the a lack of transcriptional competence for the integrated test fragments. In work with the hamster aprt promoter, Brandeis et al. (8) similarly concluded that Sp1 binding sites can block the spreading of methylation into CpG islands, though the role that these sites play in normal transcription of the hamster aprt gene is unclear (9).

To examine directly the role of Sp1 binding sites 1 and/or 2 of the mouse aprt promoter for blocking epigenetic inactivation, we have used site-directed mutagenesis to eliminate, and in one case increase, Sp1 protein binding affinity at these sites. For all constructs tested, Sp1 binding sites 3 and 4 were left unaltered to preserve transcriptional potential. The results show that the presence of the wild type (i.e. consensus) version of Sp1 binding site 2 is required for the promoter to exhibit maximal resistance to inactivation, but it is not required for transcription.

**MATERIALS AND METHODS**

**Creation of test constructs**

The general scheme for creating the constructs in this study is shown in Figure 2. The promoter and an additional 1.7 kb of downstream sequence were amplified from wild type aprt sequence using primers Sp1a–e with primer 4 as shown in Figure 1. In each case, the 5′ end of primer 1 contained an EcoR1 site (E*) followed by the start of the promoter region at position 702 relative to the E* site. Sp1 binding site 1 begins at position 708. The PCR products were digested with EcoR1 and BstEII and the resultant 0.8 kb fragment was ligated into MC containing constructs obtained from the corresponding wild type aprt promoter region construct (E*) by using primers contained an EcoR1 site (E*) followed by primer 4 to amplify a 1.9 kb PCR product. The Sp1a–e primers contained an EcoR1 site, followed by aprt sequence beginning at position 702 relative to the E* site. Sp1 binding site 1 begins at position 708. The PCR products were digested with EcoR1 (E*) and BstEII (B) and the resultant 0.9 kb E/B fragment cloned in the 664MC plasmid from which the corresponding region was deleted. Closed boxes represent exons; small gray boxes represent the promoter that is expanded into individual Sp1 binding sites at bottom.

**DNA transfection into mouse embryonal carcinoma cells**

The conditions used for DNA transfection into cultured embryonal carcinoma cells have been described previously (11). The recipient cell line used for all transfections is termed DelTG3. This cell line lacks both endogenous aprt alleles and their respective upstream regions and it has the capacity to methylate transfected DNA containing the MC fragment (5,11).

**Southern blot analysis of methylation**

The methods for Southern blot analysis of promoter region methylation have been described previously (4,12).

**aprt assay**

The methods used to determine aprt specific activities in cell-free extracts have been described previously (13).

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**Figure 1.** (A) Mouse aprt promoter region sequence. Sp1 binding sites 1–4 are underlined and the core sequence for each is bolded. CC→AA mutations used to disrupt protein binding for binding sites 1 and 2 are shown, as is a T→C change that strengthens protein binding for binding site 1. A potential Sp1 binding site (5) is underlined with a broken line. No evidence exists to suggest that this site can bind Sp1 protein (see text). (B) Alignment of Sp1 binding sites 1–4 to determine a consensus binding site.
Figure 3. Promoter region mutants tested. Large closed boxes represent wild type consensus Sp1 binding sites (sites 2–4) and small closed boxes represent wild type non-consensus binding site (site 1). Open boxes represent CC→AA mutations used to disrupt Sp1 binding. Stippled version of Sp1 binding site 1 represents T→C mutation used to create a core consensus binding site. The following designations are used: W, wild type promoter; m, CC→AA mutation; w, wild type non-consensus Sp1 binding site; C, T→C mutation for Sp1 binding site 1 to create a consensus binding site.

RESULTS

The mouse aprt promoter region sequence

The sequence for the mouse aprt promoter region is given in Figure 1A. The 6 bp core sequences (CCTGCC) for Sp1 binding sites 2–4 are bolded as is the non-consensus core sequence for Sp1 binding site 1 (CCGCCCT). The central G residues in the core sequences are separated by 17 bp for binding sites 1 and 2 and 18 bp for binding sites 3 and 4. The central G residues in binding sites 2 and 3 are separated by 36 bp. When the regions surrounding the core sequences are aligned, a 12 bp consensus sequence of GCCCCGC-CCTTC is found. Although a potential fifth Sp1 binding site (Sp1–5, Fig. 1B) is located between Sp1 binding sites 2 and 3, no evidence has been obtained to suggest that this site is occupied in vivo (3) or can bind Sp1 protein in vitro (2). Its sequence diverges from the consensus sequence at 5 of 12 positions including a critical C→A change in the core region. In contrast, Sp1 bindings sites 1–4 diverge from the consensus at only 2, 1, 1 and 3 of 12 positions, respectively, and all have been shown to bind Sp1 protein in vitro (though binding at site 1 is weak) (2). Based on the weak binding for site 1 and the lack of observed binding for putative site 5, it appears that the 6 bp core sequence is the most important determinant for Sp1 binding site in the mouse aprt promoter region.

Cloning efficiencies in medium containing azaserine and adenine

We have previously shown that it is possible to distinguish aprt promoters that are sensitive to MC-induced epigenetic inactivation from those that are resistant by determining cloning efficiencies in medium containing azaserine and adenine (AzA medium) (4). This medium permits growth of cells that express aprt, but eliminates aprt-deficient cells (14). To measure promoter region resistance to inactivation, equimolecular amounts of each construct pair (i.e. a given promoter region construct containing or lacking the MC fragment) were transfected into recipient DelTG3 cells and AzA cloning efficiencies determined. DelTG3 cells are mouse embryonic carcinoma cells that lack both endogenous aprt alleles and that contain the capacity for de novo methylation (5,11). Cloning efficiency ratios were defined as the AzA cloning efficiency obtained for a construct containing the MC fragment divided by the AzA cloning efficiency obtained for its paired construct lacking the MC fragment. Ratios of ∼1.0 indicate promoter region resistance to MC-induced inactivation whereas ratios of <0.3 indicate promoter region sensitivity (4).

The first construct pair tested, 702(MC)Wt and (ΔMC), contained the wild type promoter. As expected a cloning efficiency ratio near 1.0 was obtained (see Fig. 3 for a schematic representation of each promoter construct and Table 1 for the results). We next tested a construct pair, 702(MC)mW3 and (ΔMC), containing a mutated Sp1 binding site 1 (i.e. the non-consensus site) followed by wild type versions of the three consensus sites. Once again a ratio near 1.0 was obtained, suggesting that binding site 1 did not normally contribute to promoter region resistance. In contrast, when Sp1 binding site 2 was mutated, either in tandem with a mutant binding site 1 [702(MC)m2W2 and (ΔMC)] or in the presence of a wild type version of Sp1 binding site 1 [702(MC)wmW2 and (ΔMC)], the cloning efficiency ratios obtained ranged from 0.17 to 0.27. Both sets of constructs had wild type versions of Sp1 binding sites 3 and 4. These results suggested that a functional Sp1 binding site 2 is required for the mouse aprt promoter to be resistant to inactivation. To determine if a consensus version of Sp1 binding site 1 could substitute for Sp1 binding site 2, a promoter was created containing a consensus version of Sp1 binding site 1, a mutant version of Sp1 binding site 2, and wild type versions of Sp1 binding sites 3 and 4 [702(MC)wmW2 and (ΔMC)]. When the 702CmW2 construct pair was tested, a cloning efficiency ratio of ∼1.0 was obtained. This result suggested that a consensus version of Sp1 binding site 1 could indeed substitute for the wild type version of Sp1 binding site 2 to create a resistant promoter.

<table>
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<th>Construct*</th>
<th>Cloning efficiency*</th>
<th>Ratio*</th>
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*See Figure 3 for construct designations.

*The average number of azaserine/adenine resistant transfected DelTG3 colonies per 100 000 cells plated. For a given construct pair, each experiment was conducted on the same day and at the same time. Each line represents an independent experiment and 8–10 dishes of 100 000 cells were used for each experiment.

*The number of azaserine/adenine resistant colonies in cells transfected with a construct containing the methylation center (w/MC) divided by the number of azaserine/adenine resistant colonies in cells transfected with the same construct lacking the methylation center (ΔMC).
aprt expression in co-transfectants

AxA medium selectively expands cells that are expressing aprt, and in doing so can mask clones that are actively segregating aprt-deficient cells (as long as some cells in the clone continue to express aprt). Therefore, a second series of experiments was performed in which each construct containing the MC fragment was co-transfected with the bacterial pur (puromycin) gene and cells selected for puromycin resistance. With this approach the different promoter constructs could be introduced into the DelTG3 cells without biasing for or against aprt expression. DNA preparations were obtained from each puromycin resistant clone and examined for the presence of the co-transfected aprt construct. All clones examined that contained aprt transgenes had low copy numbers ranging from 1 to 3. The co-transfectants were assayed for aprt enzyme activities (Table 2).

For constructs containing wild type versions of Sp1 binding sites 2–4 [702(MC)Wt and 702(MC)mW3], which yielded cloning efficiencies ratios of ∼1.0 (Table 1), aprt expression was observed in 7 of 8 and 11 of 11 co-transfectants, respectively. The only exception was one 702(MC)Wt co-transfected that was later found to contain a single integrated construct from which the promoter region was deleted upon integration (not shown). Although a wide range of enzyme activities were observed, the average specific activity (units in nmol adenine converted to AMP/minute/mg protein) was similar; 0.43 U for cells containing the 702(MC)Wt construct and 0.49 U for cells containing the 702(MC)mW3 construct. To determine if the MC was affecting expression, we also determined aprt specific activity for 702(ΔMC)Wt co-transfectants. In this experiment, 10 of 10 co-transfectants were found to express detectable amounts of aprt activity with an average specific activity of 0.56, which is slightly higher than the average level observed for 702(MC)Wt construct (0.43). This difference is attributable to a single co-transfected with a high specific activity level of 1.73.

Co-transfectants containing the 702(MC)m2W2 and 702(MC)wmW2 constructs, which yielded cloning efficiency ratios ranging from 0.17 to 0.27 (Table 1), were also examined for aprt specific activity. Only 1 of 7 co-transfectants containing the 702(MC)m2W2 construct, which contained mutated versions of Sp1 binding sites 1 and 2, and only 4 of 13 co-transfectants containing the 702(MC)wmW2 construct, which contained a mutated version of Sp1 binding site 2, were found to have detectable levels of aprt activity in cell-free extracts (Table 2). The specific activities for the 4 expressing co-transfectants containing the 702(MC)wmW2 construct, which contained a mutant copy of Sp1 binding site 2, were relatively low. The average activity level was only 0.09 U. To confirm that promoter inactivation was due to the presence of the MC fragment, and to further confirm that the aprt promoter only required functional copies of Sp1 binding sites 3 and 4 for expression, co-transfectants were obtained with the 702(ΔMC)m2W2 construct. This construct contained mutant copies of Sp1 binding sites 1 and 2, but lacked the MC fragment. Of 8 co-transfectants obtained, 7 were found to express aprt protein with an average specific activity of 0.37 U. This level is comparable to the average specific activities obtained for constructs with wild type versions of Sp1 binding sites 2–4 [702(ΔMC)Wt, 702(MC)Wt and 702(MC)mW3] (Table 2). The reason for no detectable aprt expression in the remaining 702(ΔMC)m2W2 co-transfected was not determined.

An exception to the tight correlation between the cloning efficiency assay results (Table 1) and the co-transfection assay results (Table 2) was observed with the 702(MC)CmW2 promoter, which contains a consensus version of Sp1 binding site 1, a mutant Sp1 binding site 2, and wild type versions of Sp1 binding sites 3 and 4. The cloning efficiency assay yielded a ratio of ∼1.0 (Table 1) suggesting that this promoter was resistant to MC-induced inactivation. However, only 10 of 15 co-transfectants were found to exhibit detectable aprt specific activities and these activity levels were relatively low, averaging only 0.11 U (Table 2).

<table>
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<tr>
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*nmol adenine converted to AMP/minute/mg protein; averages do not include non-expressing co-transfectants. () indicates standard deviations.
Epigenetic inactivation correlates with promoter region methylation

To determine if inactivation associated with the presence of the MC could be correlated with hypermethylation of the promoter region, we performed a Southern blot analysis for selected co-transfectants that yielded cell-free extracts with or without detectable amounts of the aprt enzyme (see above section). The DNA preparations were digested with HpaII and PstI (MspI and PstI for control lanes) and a 120 bp probe (M3, Fig. 4A) was used to detect methylation of the CpG island. As shown in Fig. 4B, methylation of the CpG island (indicated by hybridization bands ranging from 0.6 to 2.2 kb) was observed for co-transfectants lacking detectable levels of aprt enzymatic activity (lanes 6, 7, 10, 12 and 13). These high molecular size hybridization bands were absent in two co-transfectants which expressed 0.12 (lane 5) and 0.24 (lane 9) activity units, respectively, and were replaced by a 120 bp hybridization band. This hybridization band represents alleles with unmethylated H3 and H4 sites. The same small hybridization band was observed in the absence of the higher molecular size hybridization bands for the 6.3-4-92 co-transfectant (Fig. 4B). This transfecant cell line contains two integrated copies of the pSam6.3 construct (see Fig. 2 for diagram of pSam6.3) which contains a wild type version of the aprt gene and the upstream region. Only the 0.12 kb hybridization band was observed in the expressing 702(MC)WT co-transfectants (data not shown). DNA preparations from the 702(MC)mW3 constructs were not examined. Mixed methylation patterns, indicated by the presence of both high and low molecular size hybridization bands, were observed in a 702(MC)CmW2 co-transfectant expressing 0.06 U of aprt activity (lane 11) and for a non-expressing 702(MC)wmW2 co-transfectant cell line (lane 7) (Fig. 4B). Similar results of mixed methylation patterns were obtained previously for non-expressing constructs in which Sp1 binding sites 1 and 2 were deleted (4). No examples of completely unmethylated, non-expressing transfecants were obtained in either study.

DISCUSSION

The work presented here strongly suggests that the primary function of Sp1 binding site 2 in the mouse aprt promoter is to provide a block against epigenetic inactivation. Three pieces of evidence support this conclusion. Firstly, this Sp1 binding site is not required for transcription of the aprt gene. As shown in Table 2, the co-transfected 702(ΔMC)mW2 construct, which contains mutated Sp1 binding sites 1 and 2 and wild type copies of binding sites 3 and 4, was expressed at an average level similar to that observed for aprt constructs containing inactivation-resistant promoters. It should be noted that considerable variation was observed for aprt-specific activities for all expressing constructs tested. Such variation most likely results from different integration sites, but could also be affected by different numbers of integrated constructs (which were ≤3). A previous study using either the protein coding region of aprt or the bacterial CAT gene as reporters for transcription had also shown maximal expression for constructs containing only Sp1 binding sites 3 and 4 (2). Secondly, a consensus (i.e. wild type) version of Sp1 binding site 2 is required for the promoter to exhibit maximal resistance to epigenetic inactivation induced by the MC fragment. Constructs containing a non-functional version of this binding site were sensitive to inactivation whereas constructs containing a wild type version were resistant. The only exception was for the 702(MC)CmW2 construct, which contained a mutant version of Sp1 binding site 2 in the presence of a consensus version of Sp1 binding site 1. However, resistance to inactivation for this construct was incomplete (see below). Although these results demonstrate the functional importance of Sp1 binding site 2 for blocking epigenetic inactivation, they do not rule out the possibility that the blocking effect also requires the presence of Sp1 binding sites 3 and 4. According to this model, Sp1 binding sites 3 and 4 are required for expression whereas Sp1 binding sites 2, 3, and 4 are required for the blocking function. Thirdly, a Southern blot analysis was used to correlate inactivation with hypermethylation. This result was consistent with previous studies in which epigenetic inactivation of both endogenous (14) and exogenous (i.e. plasmid-derived) (4) aprt genes in embryonal carcinoma cells was associated with promoter region methylation.
The wild type version of Sp1 binding site 1 (i.e. with a non-consensus core sequence) was unable to block epigenetic inactivation in the presence of a mutant Sp1 binding site 2 (the 702wmW2 construct, Fig. 3). However, limited resistance was observed when Sp1 binding site 1 was changed to contain a consensus core sequence (the 702CmW2 construct). Resistance was detected at a high level with the cloning efficiency assay (Table 1). In contrast, resistance was significantly reduced when the co-transfection assay was used (Table 2). An explanation for this discrepancy is based on the different end points for each assay. The cloning efficiency assay examines the ability of cells to grow in azaserine and adenine (AZA). Although this medium eliminates cells that are not expressing aprt, the amount of adenine used in the assay (60 µM) is sufficient to allow growth of cells containing relatively low levels of aprt specific activities (15). Moreover, AZA medium will specifically expand cells that are expressing aprt. Therefore, a clonal population of cells that is actively segregating both expressing and non-expressing cells, which could occur when a marginally resistant promoter is tested, will grow in AZA medium and appear as a clone of expressing cells in the cloning efficiency assay. In contrast, the co-transfection assay allows the recovery of cells without biasing for or against aprt expression.

The results with the sensitive and marginally resistant promoters suggest that two factors are necessary for a redundant Sp1 binding site to provide maximal resistance to MC-induced inactivation. These factors are a consensus sequence (see above) and placement of this sequence. The core G residues of Sp1 binding sites 1 and 2 are 53 and 36 bp upstream of the core G residue of Sp1 binding site 2, respectively. Although this means that Sp1 binding site 1 is only 17 bp further removed from Sp1 binding site 3 than is Sp1 binding site 2, several studies have shown that small changes in the separation of Sp1 binding sites can have significant effects. For example, it has been shown that movement of an Sp1 binding site from 8 bp downstream of a TATA box in the adenovirus E1B early region to 30 bp upstream of the box was sufficient to abolish its transcriptional function (16). Other studies with the E1B early region showed that separation of two Sp1 binding sites by <30 bp did not alter expression levels, but these levels dropped when the separation was ≥40 bp (17). It has also been shown that bending of DNA induced by Sp1 binding relative to a TATA box can be influenced by an insertion as small as 5 bp (18). Though our assays measured the ability of Sp1 binding sites 1 and 2 to block epigenetic inactivation rather than promote transcription, and did so in the context of a TATA-less promoter, the results nonetheless suggest that the location of the redundant consensus Sp1 binding site, relative to the Sp1 binding sites 3 and 4, is an important determinant for achieving maximal resistance function.

For most promoters, deletion or mutation of Sp1 binding sites will reduce the level of gene expression even if additional transcription factor binding sites are available. Examples include the arginine succinase (10), dihydrofolate reductase (19), α2β1 intergins (20), 12-lipoxygenase (21), TGF-β1 (22) and CYP1B1 promoters (23). However, unlike most mammalian gene promoters, which contain two or more transcription factor binding sites and TATA boxes, the mouse aprt gene relies exclusively on Sp1 binding sites to promote transcription. Interestingly, and perhaps surprisingly, a recent study with Sp1-deficient mouse embryonic stem cells has shown that the aprt gene is expressed in the absence of this transcription factor, at least when measured by RT–PCR (24). We have found that these cells are sensitive to 2,6-diaminopurine (not shown), an agent that kills aprt-expressing cells, thereby confirming that relatively high levels of aprt protein are made in the Sp1-deficient cells. Taken together, these results demonstrate that the Sp1 protein is not required for mouse aprt expression or for blocking inactivation, even if the Sp1 binding sites are apparently required for these functions. Several intriguing explanations can account for this finding. One is that there is an unidentified transcription factor binding site in the promoter region. Although there is no evidence from in vivo footprinting data that such a site is normally occupied, it may become occupied in the absence of the Sp1 protein. Arguing against this explanation is our failure to isolate aprt expressing cells when all four Sp1 binding sites were mutated to eliminate protein binding (data not shown). Alternatively, it is possible that a Sp1 family member, or other protein capable of binding to Sp1 binding sites, is compensating for the loss of the Sp1 protein in the deficient cells. Sp1 family members include Sp3 (25), which is constitutively expressed, and Sp4, which is expressed in the brain (26). Although initial evidence suggested that Sp3 acts as a negative regulator of transcription (27), more recent work has shown that it can also promote transcription (28). It is also possible that the putative alternative transcription factor may be the one that normally promotes mouse aprt expression or functions to block inactivation. To distinguish between the above possibilities it will be important examine aprt expression stability, as opposed to absolute levels, in the Sp1-deficient cells.

While the presence of Sp1 binding sites in a promoter region is a good indication that they play a role in promoting gene transcription, there are occasional reports in which these sites do not play such a role. For example, two Sp1 binding sites are present in the minimal regulatory region of the chicken max gene. Both sites were shown to be capable of binding the Sp1 protein in vitro. However, mutation of both sites in tandem was found to have no significant effect on expression of a reporter gene. Interestingly, when one of the two sites was mutated (termed Sp1 binding site ‘b’) a 50% increase in transcription was observed suggesting that it may normally have a negative regulatory role. No such effect was seen when only Sp1 binding site ‘a’ was mutated (29). Perhaps more relevant to the current study is work by She and Taylor (9) which showed that the three Sp1 binding sites found in the hamster aprt promoter region are not required for expression, though all three sites can bind Sp1 protein in vitro. Two sites are adjacent ~90 bp upstream of the strongest transcriptional start site; the third site is located 40 bp downstream of the transcriptional start site. Mutation of the third site had no effect on transcription while mutation of the first site resulted in a small (25%) reduction in transcription. Mutation of the second site, which disrupted Sp1 binding at both the first and second sites, led to an increase in transcription of almost 100%, suggesting that the binding sites act coordinately to dampen expression of the hamster aprt gene. As noted in the Introduction, mutation of the two upstream Sp1 binding sites in the hamster aprt promoter rendered the downstream CpG island sensitive to methylation (8). Therefore, it is possible that the hamster aprt gene has retained Sp1 binding sites in the promoter region to block its inactivation, and perhaps also to act to dampen expression, while acquiring another transcription factor binding site to promote transcription. In contrast, the mouse aprt promoter uses Sp1 binding sites to achieve both functions.
In summary, we have shown that the mouse aprt promoter uses Sp1 binding sites to achieve two distinct functions required for constitutive expression. One function, which is served by binding sites 3 and 4, is to promote transcription. The second function, which is served by binding site 2, is to block epigenetic inactivation associated with DNA methylation. The role of the Sp1 protein for providing both functions remains to be determined.

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