Sp1 and chromatin environment are important contributors to the formation of repressive chromatin structures on the transfected human adenine nucleotide translocase-2 promoter

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The influence of chromatin on the human adenine nucleotide translocase isoform 2 (ANT2) promoter was investigated in transfected cells treated with the deacetylase inhibitors butyrate and trichostatin A (TSA). Both inhibitors activated the expression of reporter plasmids transfected into HeLa cells, indicating that the promoter was suppressed by hypoacetylated chromatin and activated by hyperacetylation. Inhibitor-dependent activation was traced to the two Sp1-activation elements within the proximal promoter region, indicating that the Sp1 elements are repressed by chromatin structure. Repressive chromatin structures were also formed on the promoter integrated into a stable chromatin environment, as shown by the effects of TSA and butyrate on 14 single-cell-derived NIH3T3 clones bearing the stable integrated ANT2 promoter. Both the

basal expression of the luciferase reporter gene and the response to TSA and butyrate varied widely between clones. The range of basal expression (4000-fold) was due partially to variation in the formation of repressive chromatin, since clones with low basal expression were induced by TSA, but those with high basal expression were less effected. These data indicate that chromatin environment surrounding the integrated DNA exerts a strong influence on chromatin-dependent repression of the ANT2 promoter, and that the ability of Sp1 to activate ANT2 expression is compromised in the repressed state.

Key words: butyrate, chromatin organization, gene expression, histone acetylation, trichostatin A.

INTRODUCTION

The adenine nucleotide translocase (ANT) proteins play a central role in maintaining the steady-state levels of cytosolic ATP and, thus, metabolic and growth responses of the cell. ANT is also involved in the initiation of apoptosis via the mitochondrial pathway (see [1,2] for reviews), possibly as a component of the mitochondrial transition pore, which, when open, leads to a decrease in the mitochondrial membrane potential, release of cytochrome *c* and apoptosis-inducing factor, and activation of caspases.

ANTs are encoded in three expressed genes in yeast [3,4] and mammals [5–8]. The human gene products are highly conserved [9], but it is not known how the functions of the individual mammalian isoforms vary. However, ANT isoform 2, ANT2, is expressed in most tissues except muscle, and its expression is growth-activated in a large number of cell lines, suggesting that it might have a major role in maintaining cellular energy homeostasis.

As part of a project to understand the growth control of human ANT2 expression, we have characterized the promoter region thoroughly [10–12]. Maximum expression of the gene is driven from the proximal promoter region that includes two synergistically interacting Sp1-activation elements, and a TATA box [10]. In addition, three suppressor regions have been identified that modulate activity of the promoter. Two of these are upstream of the proximal activation region [10,13] and the third is an Sp1 element that is juxtaposed to the transcription start site [10]. Since much of this analysis was done using classical transient-

transfection assays, it is now important to understand how the function of these regions might be influenced when organized in chromatin, in particular how chromatin might influence the Sp1 sites. Sp1 function is severely affected by nucleosome structure, as indicated by reports that the affinity of Sp1 for its cognate binding site is diminished upon the formation *in vitro* of nucleosomes on naked DNA [14], but is enhanced by the SWI/SNF remodelling complex in *in vitro*-reconstituted nucleosomes [15]. As an initial step in understanding the role of chromatin in regulating ANT2 expression, we have studied the effect of the deacetylase inhibitors, trichostatin A (TSA) [16] and butyrate [17], on both transiently and stably transfected cells. We demonstrate that, in both cases, nucleosomes are formed on the promoters, resulting in transcription repression, and that, at least with transiently transfected promoters, the site of suppression is traced to the two Sp1-activation elements in the proximal promoter. In addition, we demonstrate that formation of repressive chromatin varies greatly between individual stably transfected clonal cell lines, most probably due to the chromosomal environment into which the transfected DNA is inserted.

EXPERIMENTAL

Plasmid preparation

The human ANT2 promoter/chloramphenicol acetyltransferase (CAT) or ANT2/luciferase gene constructs used in this paper have been described elsewhere [10,13].

Abbreviations used: ANT, adenine nucleotide translocase; ANT2, ANT isoform 2; HDAC, histone deacetylase; TSA, trichostatin A; CAT, chloramphenicol acetyltransferase.

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Figure 1 Butyrate and TSA stimulate transcription of transiently transfected human ANT2 promoter fragments

HeLa cells were transfected for 24 h with plasmid DNA (5 μ g) carrying the various deletion fragments of the human ANT2 promoter shown on the left. After transfection, the cells were grown for an additional 24 h in the presence or absence of 5 mM butyrate or 500 ng/ml TSA. CAT activities were normalized to protein content, and are expressed as activity obtained with butyrate or TSA relative to untreated control. All experimental points were run in triplicate. Means $\pm$ S.E.M. are given for at least three separate experiments.

Cell culture

HeLa cells and NIH3T3 cells were grown to subconfluence in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco-BRL), 2 mM glutamine, 50 units of penicillin and 50 μ g/ml streptomycin in a humidified atmosphere containing 5% CO₂.

Transient transfection

HeLa cells (5×10^5) were plated on 60-mm Petri dishes and transfected by the calcium phosphate method described in [18] using 5 μ g of reporter plasmid DNA containing the CAT or luciferase genes. The concentrations of histone deacetylase (HDAC) inhibitors used (5 mM butyrate and 500 ng/ml TSA), the time of their addition (24 h after transfection), and the time of exposure of cells to both drugs before harvesting (24 h) were optimized in pilot experiments (results not shown). Enzymic activities (CAT, luciferase) were measured according to [18]. Protein was estimated using the Bio-Rad Protein Assay kit (Bio-Rad).

Stable transfection

The preparation of stably transfected NIH3T3 diploid fibroblasts bearing the pLuc-ANT2(–1237/+46), pLuc-ANT2(–546/+46), pLuc-ANT2(–235/+46) or pLuc-ANT2(–87/+46) plasmids and measurement of reporter-gene activity was described in [13]. To obtain clones derived from single cells, stable transfectants bearing the pLuc-ANT2(–1237/+46) promoter fragment were cloned by the dilution method [19]. The luciferase activity of 14 individual clones was measured in the absence or presence of butyrate and/or TSA. Optimal conditions for TSA treatment were determined in a series of pilot experiments. Maximum stimulation, obtained with > 30 ng of TSA/ml, reached a plateau 24 h after induction, and lasted for 48–72 h. The effect was independent of plating density or the state of confluency of the cells (results not shown). Luciferase activity was normalized to gene dosage. Gene dosage and intactness of the inserted constructs were estimated by Southern blotting of purified genomic DNA using ³²P-labelled *Hind*III/*Kpn*I and *Pst*I/*Pst*I fragments as probes for the transgene (luciferase) and the human ANT2 promoter regions, respectively. A mouse *Kpn*I/*Hind*III genomic fragment from the *UCP1* gene was used for standardization of DNA loading. All clones were examined

in parallel on one filter in each experiment. Signals from the individual probes were quantified by PhosphorImaging (Fujix Bas1000; Fuji), and gene dosage was estimated for each clone from the ratio of the signals obtained with the transgene and mouse *UCP1* probes. Relative gene dosage was calculated for the 14 clones by setting the clone exhibiting the lowest ratio to unity. These relative values were used to recalculate luciferase activity for each clone.

RESULTS

Butyrate and TSA stimulate the human ANT2 promoter

The effects of butyrate and TSA on the activity of various ANT2 promoter deletion constructs were measured using the transient-transfection assay. Promoter fragments covering region –1237/+46 (numbering is relative to the transcription start site [10]) were fused with either the CAT gene or the luciferase (*Luc*) gene, and transfected into HeLa cells. After a 24-h exposure of cells to 5 mM butyrate or 500 ng/ml TSA, CAT activity of the longer constructs was increased 7–19-fold by butyrate and 11–34-fold by TSA, compared with untreated controls (Figure 1). Induction by TSA and butyrate was significantly reduced in the –674/+46 fragment, which is consistent with findings [10,11] that the –674/–235 region functions as a silencer within the context of the –674/+46 fragment. Data in Figure 1 raise the possibility that the silencer partially prevents activation via TSA and butyrate. Experiments shown in Figure 1 were repeated using luciferase constructs spanning promoter regions –1237/+46, –546/+46, –235/+46 and –87/+46, with similar results (not shown).

Intact Sp1-activating elements (A and B boxes) are required for ANT2 promoter activation by butyrate and TSA. Activation of CAT expression by TSA and butyrate was nearly abolished in cells transfected with the shortest promoter construct (–62/+46); basal activity was stimulated only 1.2- and 1.8-fold by butyrate and TSA, respectively (Figure 1). Moreover, since the –62/+46 fragment retains significant residual activity in HeLa cells [10], which is not induced by the inhibitors (Figure 1), the 25-bp sequence between nt –87 and –62 appears to be all that is required for TSA and butyrate activation. This region contains two Sp1 sites (A and B boxes; Figure 2) that are responsible for activation of the ANT2 promoter in mammalian [10] and insect [12,20] cells.

Figure 2 Intact Sp1 sites at A and B boxes are necessary for activation by butyrate and TSA

HeLa cells were transfected with the ANT2(–87/+8) promoter fragment carrying mutations (marked as crosses) in the A, B or C boxes, as shown on left. The experimental conditions were the same as in Figure 1. The data were normalized to protein content and are expressed as activity obtained with butyrate (**A**) or TSA (**B**) relative to untreated control. All experimental points were run in triplicate. Means $\pm$ S.E.M. are given for at least three separate experiments.

To confirm that Sp1 sites are required specifically to mediate the stimulatory effects of TSA and butyrate, constructs bearing various combinations of mutated Sp1 sites (A, B and C boxes; [10]) were transfected (Figure 2). Mutations in the A and B activating boxes abolished the stimulatory effects of both butyrate (Figure 2A) and TSA (Figure 2B). By contrast, mutation in the C box had no significant effect on butyrate induction (Figure 2A). Thus activation by TSA and butyrate is mediated only via the A and B boxes.

ANT2 promoter is stimulated by butyrate and TSA after integration into the genome

Although nucleosomes are formed on DNA templates introduced into cells by transient transfection, these nucleosomes appear to be in a less repressive state than those formed on stably inserted DNA (reviewed in [21]). To determine if the acetylation/deacetylation machinery can be targeted to the ANT2 promoter, stable transfectants of NIH3T3 cells containing the –1237/+46 promoter region fused to the luciferase reporter gene were prepared. Initial experiments, conducted on non-clonal transfected populations, led to a 2–3-fold activation of ANT2 promoter activity by TSA or butyrate (results not shown). Since these values are considerably lower than those obtained with transiently transfected promoter fragments (Figure 1), we decided to isolate individual clones. The dilution method [19] was used to isolate 14 single-cell-derived clones. Basal expression of the luciferase gene varied considerably between these clones, ranging approx. 4000-fold between clones with the lowest (D7) and highest (G11) activities (see Figure 3). Figure 3 shows the 14 clones sorted in ascending order of their basal luciferase-gene

activities. Variation in basal expression by the 14 clones was not related to gene dosage. Gene dosage (see the Experimental section) varied by a factor of 10 between clones, but the ascending order of clones shown in Figure 3 was not altered significantly when basal activity was corrected for relative gene dosage; only two neighbouring clones switched positions. Thus expression of the stably integrated –1237/+46 promoter region appeared to be strongly influenced by chromosomal environment.

The effects of TSA and butyrate on each of the 14 clones is also shown in Figure 3. There is a striking correlation between the extent of induction and the level of basal expression of the individual clones. In general, clones with low basal luciferase expression (lower than approx. 1000 units) were more highly induced by TSA and butyrate than were clones with high basal luciferase expression, indicating that clones with low basal luciferase expression are more highly repressed. However, inhibitor-induced luciferase activities of clones with low basal expression did not reach the basal luciferase activities of the high-expression clones. Thus chromatin acetylation/deacetylation alone does not account for the wide variation in basal expression of the promoter in different clones.

DISCUSSION

Chromatin structure is a major determinant of transcription activity, and repression/activation of gene expression is dependent on the interplay of several nucleosome-remodelling activities (see [22,23] for reviews). The present work, initiated to establish a model for studying activation and regulation of the human *ANT2* gene, is focused on one chromatin-remodelling activity: core-histone acetylation [24].

Figure 3 Single-cell-derived clones carrying the ANT2(−1235/ ±46)/luciferase construct differ in basal reporter-gene activity and express different levels of activation by butyrate or TSA

The human ANT2(−1235/ +46) promoter region cloned in front of the luciferase reporter gene was integrated into the genome of NIH3T3 cells, and single-cell-derived clones were isolated. Individual clones were plated and grown for 48 h, after which they were grown for an additional 24 h in either the presence or absence of 5 mM butyrate or 500 ng/ml TSA. Data are expressed as luminescence units per μ g of protein. The clones are arranged in three graphs in ascending order of their basal (non-induced) reporter-gene activities. Note the different scales of the three y axes. All experimental points were run in triplicate, and all data are expressed as the means $\pm$ S.E.M. from at least two separate experiments. The table below shows the mean basal activity (luciferase expression in the absence of inhibitor), and the fold activation induced by butyrate or TSA for each clone.

Two HDAC inhibitors, butyrate and TSA, stimulate transcription of transiently transfected ANT2 promoter/reporter constructs. This observation supports two general conclusions. First, hypoacetylated nucleosomes that partially suppress ANT2 promoter activity must be present in the absence of inhibitors. If not, activation by acetylase inhibitors would not occur. Secondly, deacetylases must be associated with the repressed region on the ANT2 promoter, where they help to maintain the hypoacetylated steady state. Therefore, the suppressive nucleosome/deacetylase complex should be physically associated with the ANT2 promoter or must be targeted to the promoter. One can also argue that the inhibitor effect is secondary, being mediated through primary gene products or via distal sites. Thus it is important that the region of the ANT2 promoter through which TSA and butyrate mediate activation was traced, using deletion and mutated constructs, to the two proximal Sp1-activating (A and B) boxes. The finding that HDAC inhibitors do not activate a promoter region lacking the AB-box Sp1 sites, but retaining the TATA element (Figure 1), provides additional proof that the inhibitor effect is targeted to the AB-box Sp1 sites. In HeLa cells, the construct lacking the AB boxes retains significant promoter activity that is 15–20% of the maximal activity obtained after removing the −674/−236 suppressor region, but is equal to the activity of the full-length promoter [10]. Thus we were able to accurately measure both the effects of AB-box deletion or mutations, and activation by HDAC inhibitors using the −62/+46 construct.

The mechanism by which deacetylases are targeted to the ANT2 promoter to suppress transcription remains unclear. Several mechanisms have been discussed [25]. However, it would

appear that targeting in most cases is mediated through a complex of HDAC with repressor proteins such as MAD [26], YY1 [27], Rb (retinoblastoma protein) [28] or Ume6 [29]. In this respect, it is of interest that HDAC1 was shown recently to also form a complex directly with Sp1 [30]. Although in the latter experiments the HDAC1–Sp1 complex led to suppression, presumably as the result of sequestering Sp1, the results raise the possibility that Sp1 might target deacetylases to specific promoters, thus leading to hypoacetylation and gene repression. In such cases, however, additional factors would be needed to target deacetylases to specific Sp1 sites, as suggested by the fact that, although a large number of promoters utilize Sp1 as an activator, only a small percentage of the cellular genes are activated by HDAC inhibitors [31].

To our knowledge, the ANT2 promoter is the third example of a promoter that is activated by deacetylase inhibitors specifically through Sp1 sites. Similar results were reported for both the mouse [32,33] and human [34,35] WAF/Cip1 promoters, and the mouse TK (thymidine kinase) promoter [30]. It is of interest that all three promoters are activated by deacetylase inhibitors via Sp1 sites located in similar positions relative to the transcription start site. Furthermore, these sites are not necessarily the major transactivating elements of the promoter. The deacetylase inhibitors also strongly activate the simian virus 40 early promoter [36], which shares a similar promoter organization. Thus it is tempting to speculate that Sp1 bound to specifically positioned GC elements might play a role in directing chromatin-dependent gene suppression.

TSA and butyrate were also found to activate expression driven from ANT2 promoters integrated stably into the genome

of NIH3T3 cells, indicating that the integrated promoter is also repressed by hypoacetylated nucleosomes. In these experiments, we obtained no direct proof that activation is mediated through the A- and B-box Sp1 sites, although this is most likely since these are the major activating elements used by the human ANT2 promoter [10].

Activation of the ANT2 promoter by the HDAC inhibitors varies considerably between individual single-cell clones. This result could be explained if (i) not all clones are able to form repressive nucleosome structures on which the deacetylase can act or (ii) the deacetylase enzymes are not targeted to all integrated promoters. The first of these explanations is supported by two pieces of evidence. First, the 4000-fold variation in basal luciferase expression in the 14 clones argues for various degrees of 'repression'. Secondly, activation by HDAC inhibitors tends to be strongest in clones with low basal reporter-gene expression. Thus repressed activity of the integrated promoter in this study, and perhaps others [37], is due at least partially to variation in acetylation/deacetylation and nucleosome-dependent repression of the promoter. Our results might also provide a partial explanation for the variation in basal expression observed for other promoters stably integrated into the genome [38,39]. Although the mechanism by which nucleosome organization on a single promoter can vary between cell lines is not known, our findings argue that it is under control of the surrounding chromatin (for a review see [40]). Such long-range effects on nucleosome organization within specific promoter regions would have important implications for gene expression.

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