***In vivo* mapping of the human adenine nucleotide translocator-2 (ANT2) promoter provides support for regulation by a pair of proximal Sp1-activating sites and an upstream silencer element**

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Regulatory factors bound to the human adenine nucleotide translocator-2 (ANT2) promoter have been mapped in HeLa cells by *in vivo* DNase I protection and ligation-mediated PCR amplification. Protein binding was detected at only three sites within the extended promoter region (to nt –703). One, starting at nt –61 and covering the TATA box and transcription start, most probably represents occupation by the transcription-initiation machinery. A repeated Sp1 element determined by *in vitro* studies to be the major activation element for the promoter was also protected *in vivo* on nucleotides responsible for strong binding to the zinc fingers. Occupation of two additional upstream Sp1 elements was not observed. The third site occupied *in vivo* was identified previously by *in vitro* studies as a unique

silencer element. Treatment of cells with trichostatin A to induce hyperacetylation released the silencer-binding protein after 1 h, but had no effect on the Sp1-activating elements. Prolonged treatment (24 h) displaced Sp1 from the activating elements. These findings confirm and extend *in vitro* studies indicating that regulation of the ANT2 promoter is most probably exerted through a single pair of proximal Sp1-activating elements and an upstream silencer, and that chromatin organization plays a role in the interaction between the two.

Key words: DNase I protection, promoter regulation, repressor, transcription.

INTRODUCTION

The adenine nucleotide translocator (ANT) proteins catalyse the exchange of mitochondrial ATP for cytosolic ADP, and in doing so play a key role in maintaining cellular homeostasis. ANTs are encoded by three genes in both mammals [1–3] and yeast [4,5]. In mammals, these genes are expressed in tissue- and developmentally specific manners, and they appear to play a central role in initiation of apoptosis via the mitochondrial pathway [6,7]. In addition, expression of the ANT2 isoform is growth-dependent [8,9], and is down-regulated in differentiating cell lines [8,10–12] and in cells leaving the growth cycle [9]. However, the mammalian genes are highly conserved, and the functional roles of the different gene products remain unknown.

In order to understand the factors controlling expression of the human ANT2 gene, we have studied its promoter region in detail [9,13–16]. The results of these studies indicate that the ANT2 promoter could provide an excellent model system to study interactions between regulatory factors and chromatin, since it is relatively simple, yet contains several unique features. Activation of the promoter is simple, involving primarily two adjacent, synergistically acting Sp1 elements in the proximal promoter [13]. However, an interesting feature of activation is that a third Sp1 positioned at the transcription start site appears to partially arrest transcription initiation [13,17], most probably due to direct interaction with Sp1 on the activating sites (A. Zaid and B. D. Nelson, unpublished work). Furthermore, Sp1-dependent activation is enhanced by the histone deacetylase inhibitor, trichostatin A (TSA), suggesting that chromatin organization also plays a role in ANT2 expression [16]. The only additional activating elements identified to date are high-affinity activator protein 2 (AP-2)-binding sites, which, in cells expressing

a relatively high AP-2/Sp1 ratio, could enhance activation by Sp1 [14].

The ANT2 promoter also contains two sites that function in down-regulation of the gene. One of these, located in the distal region of the promoter, has not been characterized, but appears to be necessary for down-regulation of ANT2 expression in cells exiting G₁ [9]. The second site has been characterized extensively [15], and consists of a unique repeated hexanucleotide silencing element centred at nt –332. This element is protected from DNase I digestion *in vitro*, and the binding protein has been purified and partially characterized [15]. Thus, expression of the ANT2 genes seems to be accounted for by two Sp1 sites, the activity of which is modulated by one, perhaps two, upstream repressor or silencer regions and by chromatin organization.

This relatively simple view of the promoter is based on a variety of *in vitro* methods, which, though useful, require confirmation *in vivo*. In the present study we have analysed the endogenous ANT2 promoter in HeLa cells using *in vivo* DNase I protection coupled to ligation-mediated PCR (LMPCR) amplification. The results show that only two sites are occupied *in vivo*: the Sp1-activating sites and the repeated hexanucleotide silencing element. Furthermore, TSA treatment of the cells is associated with the rapid release of protein from the silencing element but not the Sp1-activation element, suggesting that nucleosome organization also plays a role in promoter function.

EXPERIMENTAL

Cell culture

HeLa cells were grown to subconfluence in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated

Abbreviations used: ANT, adenine nucleotide translocator; LMPCR, ligation-mediated PCR; TSA, trichostatin A; AP-2, activator protein 2.

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fetal calf serum (Gibco BRL), 2 mM glutamine, 50 units of penicillin and 50 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂.

DNase I footprinting of permeabilized cells

Cells were permeabilized with 0.0375% lysolecithin (Sigma Chemical Co.) for 1 min at 37 °C in solution I (150 mM sucrose/80 mM KCl/35 mM Hepes, pH 7.4/5 mM K₂HPO₄/5 mM MgCl₂/0.5 mM CaCl₂). After removal of lysolecithin and washing with solution I, cells were treated for 5 min at room temperature with 25 µg/ml DNase I in solution I containing 2 mM CaCl₂ [18]. Cells were lysed with 1 ml of lysis solution (50 mM Tris/HCl, pH 8/300 mM NaCl/25 mM EDTA/0.2% SDS/200 µg/ml proteinase K). Genomic DNA was isolated according to established procedures [18]. As a control, genomic DNA isolated from untreated cells was exposed to DNase I *in vitro*.

LMPCR

For each DNase I-treated sample, 2 µg of DNA was submitted to LMPCR using Vent DNA polymerase (New England Biolabs) as described elsewhere [18]. PCR amplification was for 2 min for the first cycle and was gradually increased to 10 min in the last cycle. A total of 21 cycles was performed for DNA amplification. The third primer was radiolabelled by end labelling using T4 polynucleotide kinase (MBI Fermentas) and [γ -³²P]ATP (Amersham Pharmacia Biotech). Five additional cycles were performed to radiolabel DNA. The final products were analysed on 6% sequencing gels in 1 × TBE (45 mM Tris/borate/1 mM EDTA). The following nested primer sets were used. Primer set –703: primer –733, 5'-TTCGCTGGTTTCACTGGCTCCAGG-3', primer –718, 5'-TGGCTCCAGGACAGCACACGGTCTA-3' and primer –703, 5'-ACACGGTCTAGAGTGGGAAGAGTGA-3'. Primer set –439: primer –469, 5'-ACAAAA-CAAGGGGGCCGGCCAG-3', primer –453, 5'-GGCCAGT-AGGATGTAGTTTGGCCATC-3' and primer –439, 5'-AGT-TTGCCCATACGACTTTTTTAAAG-3'. Primer set –217: primer –263, 5'-CTCAGAGTCCCAGCGTTCAA-3', primer –237, 5'-GTGTTCTCTGGACCCGCCCT-3' and primer –217, 5'-TTCCTCTCGCCGGGTCAGGTG-3'. Linker 11, 5'-GAATTCAGATC-3'; linker 25, 5'-GCGGTGACCCGGGAG-ATCTGAATTC-3'.

RNA purification and Northern-blot analysis

RNA was purified using acid guanidium thiocyanate/phenol/chloroform extraction [19]. The samples were separated on 1.2% agarose gels containing 2.2 M formaldehyde, and RNA was transferred to Hybond-N membranes (Amersham Pharmacia Biotech). Hybridization and membrane washing were performed as in [20]. Membranes were exposed on BAS Imaging plates and analysed on a Fuji BAS-1000 PhosphorImager.

RESULTS

Previous *in vitro* mapping of a 1200 bp region of the human *ANT2* revealed both positive and negative regulatory elements within a 400 bp stretch upstream of the transcription start site [13,15]. To extend this analysis to conditions *in vivo*, the HeLa cell promoter was digested *in vivo* with DNase I and analysed by LMPCR with primers covering a region extending to nt –703. Since *ANT2* expression is associated with cell growth [9], and since HeLa cells used in this study were in the exponential growth phase, we assume that our analysis probably reflects the active state of the promoter.

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-398 TGTATTATCTCGATGACTGAAGGGTCTTTTGGCATCCCTGTAGCAAATGC
-348 GTCTCACCTTAGTCTCTGGTCTCTCAAGGGTTTGTCCAGGCACATC
-298 GTGACCTCACCTTCTCTCCCTCTCCGAGGCTCTCTCAGAGTCCAGCGT
-248 TCAAGTCCCGGTGTCTCTGGACCCGCCCTCTCTCGCCGGGTCCAGG
-198 TCCGAGGGCGAGCAGCGGCGCGGAGAGCAGTCCCGGCCGCCCTCTCC
-148 ACGACTCTCTCTCTCGAGCTGGGCCACTCAGCGGCTCTCGCCGCCAGC
-98 GCGCCGGGGCCAGACCCGCCCGCGGCCGCCCTCCGACGCTCCCGCTC
-48 CAGCTCCGGCTCCCCCTATATAAATCGGCCATTTGCTTCGCTCCGCCGCG
+3 CAGCGCCGGAGTCAAAGCCGGTTCCTCC

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Figure 1 Regulatory features of the human *ANT2* proximal promoter region

Sites footprinted previously *in vitro* by HeLa-cell nuclear extracts are marked with dashed lines and numbers above the sequence. Footprint 2 is part of an extended silencer region, and the double arrow denotes the hexanucleotide repeat required for binding of a silencing complex [15]. *In vitro*-footprinted Sp1 sites are boxed. The major Sp1-activating elements are marked A and B. Box C is a unique suppressor Sp1 element [13]. Nucleotides protected by AP-2 *in vitro* are underlined with dotted lines. The transcription-initiation site is also shown (arrow).

Analysis of protein binding to the promoter *in vivo*

Of the approx. 750 bp of the promoter analysed, only two regulatory regions appear to be associated with the protein *in vivo*; the proximal promoter Sp1-activating A and B box elements and the upstream suppressor elements. The same binding elements were also identified by experiments *in vitro* (see Figure 1). A detailed analysis of these regions *in vivo* is shown in Figures 2 and 3 (see below).

Two major features of the proximal promoter that emerge are seen in Figure 2(A) and are summarized in Figure 2(B). One is an extended protected region starting at nt –61 downstream of the Sp1-activating AB boxes (Figure 2A, compare lanes 1 and 2). Protection covers the TATA box, the Sp1 C box and the transcription start site, and most probably involves the transcription-initiation machinery. The second feature includes DNase I-hyper- and -hyposensitive sites in and around the A and B boxes, indicating protein interactions with these elements (Figure 2A, compare lanes 1 and 2). A major hypersensitive site (Figure 2, marked by ●) is found immediately 3' of the AB boxes at nt –59. In addition, quantitative PhosphorImager analysis of this region (results not shown) reveals hypersensitivity at the G residues in positions –74 and –73. These G residues are located at the centre of a dyad composed of the upstream A element and the downstream B element. Finally, PhosphorImager analysis also reveals that the GC pairs in each of the Sp1 sites are protected against DNase cleavage (Figure 2, △). Together, these data suggest strongly that the Sp1 AB boxes are occupied *in vivo*. There is, however, no evidence indicating specific occupation of the Sp1 C box.

The element centred at nt –332, which was previously identified by experimentation *in vitro* as a suppressor or silencing element [15], is also contacted by protein *in vivo*. Figure 3(A) shows the DNase I footprint in the silencer region. The data show a large footprinted area (Figure 3A, compare lanes 1 and 2, △) preceded by several hypersensitive sites (Figure 3A, lane 2, ●). Of the nine protected nucleotides, eight are located in a region found to be footprinted *in vitro* (Figure 1, region 2), and five of them (Figure 3B, nts –337, –335, –334, –331 and –330) fall into a direct repeat of hexanucleotide GTCCTG that is required for silencing the *ANT2* promoter ([15] and Figure 3B, underlined with arrows). In addition, eight hypersensitive sites

B

Figure 2 *In vivo* mapping of the proximal promoter region of the human *ANT2* gene

(A) HeLa cells grown in the absence of TSA (lane 2) or in the presence of 500 ng of TSA/ml for 1 h (lane 3) or 24 h (lane 4) were permeabilized with 0.0375% lyssolecithin and treated with 0.25 μ g/ml DNase I as described in the Experimental section. Cleaved strands were amplified and labelled by LMP-PCR using the primer set -217. DNA fragments were separated on a 6% polyacrylamide sequencing gel. Digested naked DNA (N) is shown in lane 1. Quantitative analysis was performed by PhosphorImager Analysis (Fuji). Individual nucleotides protected from DNase I are marked with Δ , and nucleotides that were hypersensitive to DNase I cleavage are marked with $\bullet$. (B) summarizes the data in (A). The extended protected region covering the TATA box and transcription start site is shown by a dashed line. Arrows pointing upwards

B

Figure 3 LMP-PCR analysis of the silencer region of the human *ANT2* promoter

(A) Experimental details were as specified in Figure 2. Primer set -439 was used. Lane 1, naked DNA (N); lane 2, no TSA; lane 3, TSA for 1 h; lane 4, TSA for 24 h. Nucleotides protected from DNase I *in vivo* are marked with Δ , whereas hypersensitive nucleotides are marked with $\bullet$. The double arrow identifies the position of the hexanucleotide repeat required for binding of a silencing complex [15]. (B) Summary of the DNase I footprinting pattern in the suppressor region of the human *ANT2* promoter. The boxed region delineates the extended footprint shown in (A) (lane 2). This region was also footprinted *in vitro* (Figure 1, footprint 2). Arrows pointing upwards denote protected nucleotides that were de-protected from DNase I after 1 h of TSA treatment. Other symbols are as in (A).

(Figure 3B, $\bullet$) are found directly upstream of the hexanucleotide repeat. Interestingly, these hypersensitive sites are separated by a single protected nucleotide. The other interesting feature is that the four hypersensitive sites located at the 5' end of the silencer element mark the 3' end of an extended region (Figure 1, region 3) that was footprinted *in vitro* [15]. In summary, these observations are consistent with protein occupying the silencer element in HeLa cells.

TSA alters *in vivo* occupation of the promoter

In attempts to modulate *in vivo* binding to the promoter, cells were treated with the histone deacetylase inhibitor, TSA. TSA-induced hyperacetylation of histones often activates gene ex-

are protected nucleotides (Δ) that were de-protected after 24 h of treatment with TSA, whereas arrows pointing downwards indicate hypersensitive sites ($\bullet$) that became protected by the same treatment. No effects of TSA were seen in this region after 1 h of treatment.

pression, presumably due to nucleosome reorganization. However, as pointed out above, the ANT2 promoter is probably active under the conditions used, and the effects of TSA could be small. This is borne out by the effect of TSA on ANT2 transcript levels measured by Northern-blot analysis (results not shown). Treatment of cells with TSA for 1 h increased transcript levels marginally (by 30–50% compared with untreated controls). By contrast, 24 h of treatment resulted in a greater reduction in transcript levels (30% relative to untreated controls, or 50–60% relative to the 1 h values).

In spite of the relatively small changes in transcript levels, TSA-induced alterations could be observed on both the Sp1 AB boxes (Figure 2) and the upstream silencer region (Figure 3). Lanes 3 and 4 in Figure 3(A) show the effect of TSA on the *in vivo* DNase I footprinting pattern in the proximal promoter. The area covering the transcription-initiation site remained protected in cells treated with TSA for 1 and 24 h (Figure 2). Indeed, this area might be even more strongly protected in the presence of TSA, as suggested by the loss of the faint bands in the region of TATA and C boxes (Figure 2, compare lane 2 with lanes 3 and 4). By contrast, PhosphoImager analysis reveals significant TSA-dependent changes in the DNase I sensitivity pattern of the A and B-box region. These occur, however, only after 24 h of TSA treatment, and are manifested in the reversal of the pattern found in the absence of TSA; i.e. hypersensitivity at G –74 and G –73 is lost, and protected sites, including the GC pairs in the two Sp1 elements, become accessible to DNase I (Figure 3, compare lanes 2 and 4). The results are summarized in Figure 2(B), where the arrows above the symbols show the changes brought about by the TSA. Together, the results suggest that 24 h of TSA treatment weakens Sp1 binding to the A and B boxes, but has no effect on protein binding to the TATA and C box regions. This weakening may explain why RNA levels decrease after 24 h.

TSA also altered binding to the silencer region (Figure 3). However, in contrast with the AB boxes, these alterations occurred rapidly. For example, TSA treatment for 1 h results in disappearance of the clear footprint (Figure 3A, compare lanes 2 and 3) and this pattern does not change with prolonged TSA treatment (Figure 3B, lane 4). PhosphoImager analysis summarized in Figure 3(B) shows that the centre of the protected area (nts –337 to –321) containing the silencer-binding site [15] becomes accessible to DNase I cleavage (arrows above the symbols). Interestingly, nts –337 and –321 remain protected, creating a border for the area affected by the TSA treatment. On the other hand, the hypersensitive sites upstream of the footprinted region were not influenced by TSA. Also not affected was the protected site at –345. Since the nucleotides exposed by TSA treatment are critical for silencer-protein binding [15], we conclude that binding is rapidly weakened by hyperacetylation. Together the above results imply that protein is associated with the silencer element *in vivo*, but is displaced rapidly upon treatment with TSA.

DISCUSSION

In vitro analysis disclosed four probable regulatory regions on the human ANT2 promoter: a promoter proximal pair of Sp1-activating elements [14], a single Sp1 element at the transcription start site that appears to have a suppressor function [13,17], a silencer element centred at nt –332 [15] and two AP-2 sites [14]. The present study provides strong evidence that only two of these sites, the Sp1-activating sites and the upstream silencer element, are occupied in HeLa cells *in vivo*.

The Sp1 A and B boxes are of special interest since they appear to be the major, and perhaps the only, sites through which the promoter is activated [13]. The present findings that the A and B boxes are occupied *in vivo*, whereas two additional Sp1-binding elements 5' of the AB boxes (Figure 1 and [14]) are not, are consistent with this view. Furthermore, the finding that both the A and B boxes are occupied also supports earlier conclusions [13] that promoter activity requires both elements working synergistically. A third Sp1 element located at the transcription start site (Figure 1, the C box) partially represses activation through the AB boxes in both mammalian [13] and *Drosophila* [17] cell lines. The region encompassing the C box is strongly protected in the present experiments, presumably by the transcription machinery, and we are therefore unable to detect specific interactions with the C box. However, ANT2 expression in HeLa cells is partially repressed through the C box [13] and, in view of recent experiments in our laboratory showing that repression involves an interaction between the D domain of Sp1 occupying the C box, and Sp1 on the AB boxes (A. Zaid and B. D. Nelson, unpublished work), we conclude that Sp1 most probably binds to the C box even *in vivo*.

The upstream silencer region, initially identified by transient transfection of deletion constructs [13], extends over a region of 300 bp [15]. However, *in vitro* protein binding was observed only to the 3' half of this region (see Figure 1), and silencing required a specific repeated hexanucleotide element [15], which we now show is occupied *in vivo*. The silencer-binding protein has been purified and partially characterized [15]. Taken together, the present results support the notion that primary regulation of the human ANT2 promoter is exerted through two proximal Sp1 elements that interact synergistically, and an upstream silencer that remains to be fully characterized but which possibly represents a heretofore unrecognized silencing complex [15].

The effects of TSA on DNase I access to the silencer element and the AB boxes implies an involvement of hyperacetylation, most probably of the histones. Although the site(s) of hyperacetylation have not been identified, the result is that specific nucleotides required for Sp1 [13,14] and silencer-protein binding [15] are re-exposed to DNase I. This is especially evident for the AB boxes, in which TSA reverses the protection of several nucleotides that make strong contacts with zinc fingers 3 and 2 of Sp1, and account for strong binding to the GC box element [21]. These results imply that hyperacetylation displaces Sp1 and the silencing protein from their cognate elements. Displacement of the silencing protein appears to be the earlier event. Thus it is tempting to speculate that release of the silencer-binding protein initiates activation of the AB boxes.

An understanding of the interactions between Sp1, the silencer complex and underlying nucleosome organization on the ANT2 promoter requires a full description not only of the nucleosome organization on the promoter, but also of the silencer complex and its interactions with Sp1. We presently have no information concerning the effects of the silencer complex on nucleosome organization, or vice versa. Neither do we know if, or how, interactions between Sp1 and the silencer complex are influenced by nucleosome organization. It is known that Sp1 is able to form ternary complexes on core nucleosomes [22,23] and to induce translational and rotational positioning of surrounding nucleosomes [24]. Moreover, Sp1 has been shown to bind histone deacetylase and target it to the dihydrofolate reductase (DHFR) promoter [25], raising the possibility that Sp1 occupying the AB or C boxes of the ANT2 promoter could influence repression exerted through the silencer element by targeting histone deacetylase. Whatever the mechanism, the ANT2 promoter appears to provide an excellent model for investigating the role

of chromatin organization on silencing/activation events within the context of a relatively simple promoter structure.

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