Repression of the Human Adenine Nucleotide Translocase-2 Gene in Growth-arrested Human Diploid Cells

THE ROLE OF NUCLEAR FACTOR-1*

Katarina Luciakova‡§, Peter Barath‡§, Daniela Poliakova‡§, Annaika Persson**, and B. Dean Nelson‡

From the Departments of ‡Biochemistry and Biophysics and §Cell Biology, Arrenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden and the ¶Cancer Research Institute, Slovak Academy of Sciences, 833 91 Bratislava, Slovak Republic

Adenine nucleotide translocase-2 (ANT2) catalyzes the exchange of ATP for ADP across the mitochondrial membrane, thus playing an important role in maintaining the cytosolic phosphorylation potential required for cell growth. Expression of ANT2 is activated by growth stimulation of quiescent cells and is down-regulated when cells become growth-arrested. In this study, we address the mechanism of growth arrest repression. Using a combination of transfection, in vivo dimethyl sulfate mapping, and in vitro DNase I mapping experiments, we identified two protein-binding elements (Go-1 and Go-2) that are responsible for growth arrest of ANT2 expression in human diploid fibroblasts. Proteins that bound the Go elements were purified and identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry as members of the NF1 family of transcription factors. Chromatin immunoprecipitation analysis showed that NF1 was bound to both Go-1 and Go-2 in quiescent human diploid cells in vivo, but not in the same cells stimulated to growth by serum. NF1 binding correlated with the disappearance of ANT2 transcripts in quiescent cells. Furthermore, overexpression of NF1-A, -C, and -X in NIH3T3 cells repressed expression of an ANT2-driven reporter gene construct. Two additional putative repressor elements in the ANT2 promoter, an Sp1 element juxtaposed to the transcription start site and a silencer centered at nucleotide −322, did not appear to contribute to growth arrest repression. Thus, enhanced binding of NF1 is a key step in the growth arrest repression of ANT2 transcription. To our knowledge, this is the first report showing a role for NF1 in growth arrest.

The adenine nucleotide translocase (ANT)1 proteins catalyze the exchange of mitochondria ATP for cytosolic ADP. In doing so, they play an important role in maintaining the cytosolic phosphorylation potential and therefore normal cell growth and function. In addition, the ANTs have been implicated in early events in initiation of mitochondrion-dependent apoptosis (1).

Three ANT isoforms are encoded in separate genes in mammals (2–5) and yeast (6, 7). Two of these isoforms (ANT1 and ANT2) are differentially expressed in mammalian tissues (8–10) and in differentiating cells (8, 11–13). ANT2 expression is down-regulated in the latter case (8, 11–13). Expression of the ANT2 isoform is also growth-dependent (11). Rapid expression of ANT2 mRNA has been demonstrated in a variety of growth-arrested mammalian cell types activated to enter the G1 phase of cell growth (11, 14–17). ANT2 mRNA expression occurs together with the immediate-early genes required for activation of cell cycle progression and is accounted for solely by the activation of transcription (18). However, unlike the other immediate-early genes, ANT2 expression is maintained throughout out the cell cycle. Expression is down-regulated only as cells become growth-arrested at confluence (18).

The mechanism(s) by which gene expression is repressed in cells entering G0 is poorly understood. Growth arrest-specific genes have been identified in growth-inhibited NIH3T3 cells (19), but these do not participate directly in transcription initiation (20). Transforming growth factor-β induces growth arrest of many cell types, leading to repression of many individual genes via the Smad proteins (21, 22). However, microarray analysis of growth-stimulated human primary fibroblasts (23, 24) revealed up-regulation of relatively few transcription factors, suggesting that modulation of transcription factor expression may not be a commonly used mechanism for regulating G0-specific gene expression.

The mechanism by which ANT2 expression is repressed during growth arrest is not known. We demonstrated previously that removal of a 700-bp upstream region of the human ANT2 promoter prevents growth arrest repression (18). In the present study, we show that growth arrest of ANT2 is mediated by members of the nuclear factor-1 (NF1) family of transcription factors via two DNA elements (Go-1 and Go-2) in the upstream repressor region. The NF1 family consists of four genes, NF1-A, -B, -C, and -X, and a large number of splice variants that can act either as transcriptional activators or repressors (see Ref. 25 for review) depending on the cell context. However, to our knowledge, the repression of ANT2 reported here is the first example of NF1 acting as a growth arrest repressor.

EXPERIMENTAL PROCEDURES

Cell Culture—Human primary diploid foreskin fibroblasts were used in passages 7–17. Diploid fibroblasts and NIH3T3 cells were grown as described (18). For serum starvation, cells were washed twice with phosphate-buffered saline; serum-free medium was added; and incubation...
Plasmids—ANT2-Luc reporter plasmids used in stable transfection experiments were prepared using unique restriction enzyme sites (BglII, XbaI, BamHI, and Snol) in the human ANT2 promoter PstUspI fragment (26). These restriction fragments were inserted into the HindIII/NotI sites of pGL3-basic (Promega). ANT2 promoter fragments bearing mutations in the C-box were prepared as described (26) using the Mut-2 sequence. All clones were checked for fragment size and orientation. An oligonucleotide containing the mutated ANT2 Go-2 element (nucleotides (nt) 822 to 794) was prepared by PCR using a mutated 5′-primer (5′-CCA ATT CCT TAA AAG ATC TTT GTC GAA C-3′) and the GL2 primer (5′-TTT ATT GTC TTC CA-3′) from pGL3-basic. The PstUspI ANT2-Luc reporter plasmid (26) was used as the template DNA. An oligonucleotide with both the Go-2 (nt 822 to 794) and Go-1 (nt 726 to 710) elements modified was prepared by PCR amplification of a short fragment using a set of primers in which the core GGC sequence was changed to TAA. The 5′-primer contained the mutated Go-2 element (5′-CCA ATT CCT TAA AAG ATC TTT GTC GAA C-3′), and the 3′-primer contained the mutated Go-1 element (5′-GGG TGC TGT CCT GGA TTA AGT GAA C-3′). The amplified fragment was then used as the 5′-primer for another round of amplification using the PstUspI ANT2-Luc reporter plasmid (26) as the template and the GL2 primer as the 3′-primer. This amplified fragment of ANT2 was used in turn as the template for amplification with the wild-type Go-2 element primer (5′-CCA ATT CCT GGC AAG ATC TTT GTC GAA C-3′) to obtain a combination of mutations. PCR was performed with Vent DNA polymerase (New England Biolabs Inc.) according to the manufacturer’s recommendations. All clones were verified by sequencing.

Stable transfections were performed as described (18). Resistant colonies (100–200) for each of the luciferase constructs were pooled and grown in the presence of Geneticin (0.4 mg/ml). Luciferase activity measurements were performed as described (18). Protein concentration was measured by the Bio-Rad protein assay.

In Vivo DNase I Footprinting—Nuclear extracts were prepared from human diploid fibroblasts and NIH3T3 cells by the method of Dignam et al. (27). The DNase I protection assay was performed as described by Promega (28). Radioactive probes were prepared by PCR using 32P-labeled chromatin methylated acetytransferase primer, the M13 primer, and pCAT-ANT2 (917–764) as the template.

In Vitro Dimethyl Sulfate (DMS) Footprinting—DMS footprinting of human diploid cells was performed as described (29) with minor modifications. Cells were treated with 0.1% DMS in 2 ml of phosphate-buffered saline for 2 min at room temperature. After washing with phosphate-buffered saline, cells were lysed with 1 ml of lysis solution (50 mM Tris-Cl (pH 8), 300 mM NaCl, 25 mM EDTA, 0.2% SDS, and 200 μg/ml proteinase K). Modified genomic DNA was isolated according to established protocols (29). As a control, a first-strand reverse-transcribed cDNA isolated from cells was exposed to 0.125% DMS—unmodified DNA (29). The column was prepared as described (33). Proteins were eluted in a 400 mM NaCl wash, and active fractions were subsequently eluted in a 400–800 mM NaCl gradient. Fractions were tested for Go element-binding activity by in vitro DNase I protection assay. Active fractions were desalted on PD-10 columns (Amersham Biosciences) and placed in 20 mM Tris (pH 8.0) and 100 mM NaCl and applied to a ResourceQ column (1 ml; Amersham Biosciences). Proteins were eluted by a 100–300 mM NaCl linear gradient. Active fractions were rebuffered to 20 mM HEPES (pH 7.9), 5 mM MgCl2, and 5 mM β-mercaptoethanol, and 70 mM NaCl and applied to a DNA affinity column containing the immobilized Go-2 oligonucleotide (5′-CCA ATT CCT GGC AAG ATC TTT GTC GAA C-3′). The column was prepared as described (33). Proteins were eluted in two salt steps (200 and 500 mM NaCl) in 20 mM HEPES (pH 7.9), 5 mM MgCl2, 5 mM β-mercaptoethanol, 5% glycerol, and 0.1% Nonidet P-40. Protein fractions were stored at −70 °C.

SDS-PAGE and Protein Identification by MALDI TOF/MS—Samples from the DNA affinity column were precipitated for 20 min on ice in 10% trichloroacetic acid, followed by a 15-min centrifugation at 10,000 x g and two washes with ice-cold acetone. Samples were air-dried, dissolved in sample buffer, and separated by 10% SDS-PAGE (34). Proteins were visualized by silver staining (35), and bands of interest were cut out. In-gel tryptic digestion and sample preparation were done as described (35). MALDI-TOF analysis was performed in reflector mode using a Voyager-DE STR MALDI-TOF mass spectrometer from Applied Biosystems (Foster City, CA). Internal calibration was done with autodigested trypsin. Data were analyzed using Moverz software (Proteometrics LLC, Winnipeg, Canada), and data base searches were done with Mascot (36). A search of all NCBI databases was performed allowing one missed cleavage for trypsin, carbamidomethylated cysteine residues, and variable modification of oxidized methionine. Peptide tolerance for monoisotopic values was set to 50 ppm. The different forms of NF1 were identified with significant scores.

Western Blot Analysis—Trichloroacetic acid-precipitated samples from the DNA affinity column were subjected to 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane. Membranes

NYF1 Represses ANT2

Total RNA was isolated from human primary diploid fibroblasts. Lane 1, exponentially growing cells; lane 2, cells serum-starved for 48 h; lane 3, starved cells serum-stimulated for 6 h; lane 4, starved cells serum-stimulated for 24 h; lane 5, cells grown continuously in 10% serum. Total RNA (10 μg) was loaded on each lane, and Northern analysis was performed with 32P-labeled ANT2 cDNA. rRNA was used as a measure of the total RNA loaded.

Acidic guanidium thiocyanate/phenol/chloroform extraction (30). The samples were separated on 1.2% agarose gels containing 2.2% formaldehyde, and RNA was transferred to Hybond-N membranes (Amer- sham Biosciences). Hybridization and membrane washing procedures were performed as described (31). Membranes were analyzed on a Fuji BAS-1000 phosphorimager.
were incubated with antibodies against the N terminus of human NF1 (Santa Cruz Biotechnology) and developed with alkaline phosphatase-conjugated secondary antibodies as described (29).

Chromatin Immunoprecipitation—Chromatin immunoprecipitation of NF1 from growth-arrested and growth-induced human diploid cells was performed as described (38), except that 100 μl of protein A-Sepharose was used instead of *Staphylococcus aureus* cells. Immunoprecipitation was performed with 2 μl of antiserum 8199 prepared against a central domain of the NF1 C-protein (kindly provided by Dr. Tanese). Amplification of immunoprecipitated DNA fragments (2 μl) was performed using primers −982 and −795 (5′-GGTTGCAAGA-GATCTTGCCAGGAATTGG-3′) for the Go-2 element and primers −726 (5′-GGTTTCACTGGGTCACGAGCAGCAAC-3′) and −499 (5′-GGGT-GAGGCAGCGAGAAGGTCATG-3′) for the Go-1 element. PCR was performed for 32 cycles, with 30 s of denaturation at 94 °C, followed by 30 s of annealing at 60 °C and 30 s of extension at 72 °C. The last step included extension for 10 min at 72 °C.

RESULTS

Growth Arrest of Human Diploid Cells Down-regulates ANT2 Transcripts—Growth arrest repression of the ANT2 gene (18) was studied in vivo using human primary foreskin fibroblasts. Exponentially growing cells expressed ANT2 transcripts at high levels (Fig. 1, lanes 1 and 5), but transcripts were barely detectable in growth-arrested cells after 48 h of serum starvation (lane 2). However, ANT2 transcript levels were restored to −30% of the control levels after 6 h of serum induction (lane 3) and to 100% after 24 h of induction (lane 4).

Mapping of Proteins Bound to the ANT2 Promoter in Growth-arrested Diploid Cells in Vivo—Proteins bound to the ANT2 promoter during growth modulation were mapped in vivo in growing and growth-arrested diploid fibroblasts by DMS modification (Fig. 2; summarized in Fig. 3). Transcription of ANT2 is maintained by two adjacent, synergistically acting Sp1 elements in the proximal promoter (26, 39). However, growth arrest repression appears to be located in a distal 700-bp fragment of the promoter (18). In agreement with these findings, a region of protein contact was detected in vivo within the 700-bp repressor region. In this region, protection from DMS modification was observed on C residues in growth-arrested diploid cells (Fig. 2B, S lane) that extended over a stretch of −60 bp (nt −830 to −767) (Figs. 2A and 3A). More importantly, none of these C residues was protected in vivo in serum-activated (Fig. 2A, I lane) or exponentially growing (E lane) diploid cells. Thus, protein binding to the repressor region is detected in diploid fibroblasts in vivo only in the growth-arrested state.

Protein contact is also detected in vivo within or near the Sp1 activation elements (AB boxes) and the Sp1 repressor element (C box) in the proximal promoter (26, 39). Strongly protected nucleotides were found within the A and B boxes under all conditions of growth (i.e. exponentially growing, serum-starved, and serum-induced cells) (Fig. 2B; summarized in Fig. 3B), suggesting that these elements are most likely perma-
A hypersensitive C residue is marked with an asterisk in Fig. 2B. The transcription start site is marked by an arrow. Nucleotides protected under all conditions of growth are marked with triangles. Nucleotides protected from DMS modification in serum-activated human diploid cells did not footprint the promoter in the human ANT2 promoter, stable transfectants of NIH3T3 cells were used. As shown in Fig. 4, repression of luciferase activity was lost in growth-arrested 3T3 cells only when the GoR region was deleted (Fig. 6A). However, mutating the C box (data not shown), as described previously (26, 39), did not significantly alter growth arrest repression. To test this, stable transfectants of NIH3T3 cells were prepared using human ANT2 reporter gene constructs bearing various 5′ deletions with and without a mutated C box. Reporter gene activity was measured in cells approaching confluence. In agreement with data in Fig. 4, repression of luciferase activity was lost in growth-arrested NIH3T3 cells when the GoR region was deleted (Fig. 6C). However, mutating the C box (open symbols) either in the presence (Fig. 6, A and B) or absence (Fig. 6C) of the GoR region did not significantly alter growth arrest repression, showing that the Sp1 C box plays little or no role in this process. However, the C box contributes to constitutive repression of the gene since mutations that prevent Sp1 binding increased the absolute activity of the reporter gene by 2–5-fold (data not shown), as described previously (26, 39).

We have also identified a putative silencer centered at nt −332 in the ANT2 promoter (41). To test the contribution of this region to growth arrest repression, promoter-driven reporter gene constructs were prepared with deletions between the Go element and the AB boxes, some of which (clones Δ−692/−235 and Δ−546/−235) (Fig. 6D) delete the −332 silencer. Furthermore, since ANT2 expression is maintained via Sp1 on the AB boxes and the spatial arrangement of the Sp1 elements in the proximal promoter is critical for optimal gene expression (40), these clones also provide a test of the importance of the spatial relationship between Sp1 and the Go element. Stable transfections of these constructs (Fig. 6D) were made in NIH3T3 cells. None of the deletions prevented repression in cells approaching confluence (Fig. 6D). Thus, neither

![Fig. 4. Identification of a promoter region that is essential for growth arrest repression of ANT2 in confluent cells. Stable transfections of NIH3T3 cells were carried out with luciferase reporter constructs driven by 5′-deletion fragments of the ANT2 promoter: −1248/+48Luc (closed circles), −804/+46Luc (open squares), and −692/+46Luc (open circles). Cells were plated at subconfluence (day 0) and grown to confluence. Confluence was reached on day 4 (18). Luciferase activity was normalized to total protein content. The results represent the means ± S.D. of three experiments, in which each experimental point was determined in duplicate.](http://www.jbc.org/)

![Additional Putative Repressor Elements in the ANT2 Promoter Are Not Involved in Growth Arrest Repression](http://www.jbc.org/)

Identification of Growth Arrest DNA Elements in the ANT2 Promoter—To further define the upstream growth arrest repressor in the human ANT2 promoter, stable transfectants were made with 5′-deletion fragments of the promoter (Fig. 4). Since stable transfection of diploid fibroblasts is hampered by their limited life spans, NIH3T3 cells were used. As shown in Fig. 4 (open circles), deletion of a 112-bp fragment between nt −804 and −692 abolished repression of luciferase activity in confluent cells. This is the region in which protein binding was observed in growth-arrested diploid cells in vitro (see above). For convenience, we refer to this extended 112-bp region as the Go repressor (GoR) region.

To identify proteins that bind the 112-bp GoR region, an overlapping fragment of the promoter (nt −917 to −654) was mapped in vitro with DNase I using nuclear extracts from human diploid fibroblasts and NIH3T3 cells. A 28-bp region (nt −822 to −794) was strongly protected by nuclear extracts from both cell types in the growth-arrested state (Fig. 5). This footprint, referred to as a Go-2 element, overlaps the 5′-end of the GoR region defined by deletion constructs (Fig. 4) and is part of an extended region that includes a second DNA element, Go-1 (see Figs. 7 and 9 below). DNase I protection of the Go-2 element was interrupted in both cell types by a hypersensitive site (Fig. 5, asterisks), suggesting that the same or a similar protein is bound. In agreement with the in vivo DMS mapping experiments (see above), nuclear extracts from serum-activated human diploid cells did not footprint the −822/−794

**Fig. 3. Summary of the DMS modifications of the ANT2 promoter region in diploid cells in vitro.** A, summary of data presented in Fig. 2A. Nucleotides protected from DMS modification in serum-starved cells are marked with triangles. The boldface underlined regions and asterisks indicate footprinted and hypersensitive sites, respectively, found in in vitro DNase I protection assay (see Figs. 5 and 8, respectively). The NF1 half-sites are boxed. B, summary of data presented in Fig. 2B. The transcription start site is marked by an arrow. The TATA box and the Sp1 A, B, and C elements are boxed. C residues protected under all conditions of growth are marked with triangles. A hypersensitive C residue is marked with an asterisk.

**Go-2 element (Fig. 5, left panel, compare S and I lanes).** Thus, either the amount or the binding ability of the DNA-binding protein is modulated by the growth state of the diploid cells. By contrast, nuclear extracts from serum-activated NIH3T3 cells protected the −822/−794 element from DNase I (Fig. 5, right panel, compare S and I lanes). Although it is not clear why DNA binding is retained in nuclear extracts from growing 3T3 cells, the result may provide an explanation for the weaker serum induction of ANT2 expression observed in 3T3 cells (31) compared with human diploid cells.
the silencer centered at nt −332 nor the distance between the Go element and the AB box Sp1 activation sites appears to be influence ANT2 growth repression.

Purification of the Growth Arrest Repressor Element-binding Protein—The above data indicate that repression of ANT2 in growth-arrested cells is achieved through protein(s) binding to
NF1 Represses ANT2

The GoR region. Since the rat liver and HeLa nuclear extracts contain a DNA-binding activity that is indistinguishable from the Go element-binding activity in human diploid fibroblast nuclear extract, both extracts were used for protein purification. The eluted fractions from individual purification steps (see “Experimental Procedures”) were tested for DNA-binding activity by in vitro DNase I protection assay of the −917/−654 GoR region of the ANT2 promoter (see above). Fractions exhibiting protection were pooled and used in the subsequent purification step. Fig. 7 shows the DNase I protection pattern using fractions eluted from the last DNA affinity purification step. The first two fractions from the 500 mM NaCl elution step contained Go element-binding activity. However, binding to DNA was not limited to the Go-2 element (which was used as bait on the DNA affinity column), but was also observed on a sequence located between nt −726 and −701 (summarized in Fig. 3A), which we termed the Go-1 element. Both Go-1 and Go-2 are within the GoR region defined by transfection/deletion studies (Fig. 6; see above). Go-1 was not be detected by DNase I protection assays from rat liver (A) or HeLa cell (B) nuclear extracts were separated by SDS-PAGE and stained with silver. Fractions that eluted from the affinity column in 200 (lanes 1–3) or 500 (lanes 4–6) mM NaCl and a flow-through fraction (F lanes) are indicated. Molecular mass standards are shown in the M lane. The small black boxes between lanes 4 and 5 denote polypeptide bands in which NF1 was identified by MS analysis. Also shown are the results from Western blot analysis of the DNA affinity column fractions from rat liver with antibodies against the N terminus of human NF1 (C).

The above experiments identified members of the NF1 tran-

![Fig. 7. DNA affinity purification of the growth arrest (G<sub>a</sub>) repressor-binding protein. Shown are the results from in vitro DNase I protection analysis of rat liver nuclear extract fractions from the DNA affinity column. The assay was performed on the ANT2 −917/−654 GoR region. Lanes 1–3, fractions eluted with 200 mM NaCl; lanes 4–6, fractions eluted with 500 mM NaCl. DNA was digested with DNase I in the absence of protein (P lane) or in the presence of a flow-through fraction of the DNA affinity column (F lane). The G+A lane contains the G + A sequencing ladder. Only the coding strand is shown. Asterisks denote hypersensitive nucleotides. Nucleotide numbering is relative to the transcription start site.](http://www.jbc.org/)

![Fig. 8. Identification of growth arrest (G<sub>a</sub>) repressor-binding protein as a member of the NF1 family of transcription factors. Proteins affinity-purified from rat liver (A) or HeLa cell (B) nuclear extracts were separated by SDS-PAGE and stained with silver. Fractions that eluted from the affinity column in 200 (lanes 1–3) or 500 (lanes 4–6) mM NaCl and a flow-through fraction (F lanes) are indicated. Molecular mass standards are shown in the M lane. The small black boxes between lanes 4 and 5 denote polypeptide bands in which NF1 was identified by MS analysis. Also shown are the results from Western blot analysis of the DNA affinity column fractions from rat liver with antibodies against the N terminus of human NF1 (C).](http://www.jbc.org/)
NF1 Represses ANT2

Fig. 9. Purified recombinant NF1 and affinity-purified fractions from rat liver and HeLa cell nuclear extracts exhibit identical properties in the DNase I and EMSAs. A, DNase I assay; B and C, EMSA. A, DNA affinity-purified fractions from rat and HeLa cells (fraction 4) (see Fig. 8) and purified recombinant NF1 protein were used for DNase I protection analysis of the ANT2 GoR promoter region. Digested probe (P lanes) is shown. Only the coding strand is shown. Asterisks denote hypersensitive nucleotides. Nucleotide numbering is relative to the transcription start site. B, EMSA was done with HeLa nuclear extracts and an oligonucleotide probe containing an NF1 bipartite consensus binding sequence (probe NF1 WT; Santa Cruz Biotechnology). Wild-type (WT) or mutated (Mut) competitor oligonucleotides containing the NF1 bipartite consensus binding element, the Go-1 element (nt −726 to −701), or the Go-2 element (nt −822 to −794) were added in 50-fold excess. In all mutations, the core NF1-binding sequence (TGGCA) was changed to TTAAA. The sample in the left panel was preincubated with antibody (Ab) 8199. The major shifted complex is marked with an arrow. The asterisk denotes a nonspecifically bound probe. C, EMSA was done with purified recombinant NF1 and the Go-2 element oligonucleotide (nt −822 to −794) as the probe. Competitor oligonucleotides containing the NF1 consensus sequence (NF1 WT, left panel) or the Go-2 element (nt −822 to −794; right panel) were added in 50-fold excess.

Fig. 10. Identification of NF1 as the protein in quiescent human diploid fibroblasts that binds to the ANT2 GoR repressor element. DNase I protection assay of the ANT2 GoR region was carried out with nuclear extracts from quiescent diploid fibroblasts (left and middle panels). Extracts were incubated prior to DNase I digestion with a 10-, 20, or 50-fold excess of competitor oligonucleotide (oligo) containing either a wild-type (WT) or mutated (Mut) NF1 bipartite consensus binding element. The P lanes contain a digest of the probe in the absence of protein. DNase I protection assay of the ANT2 GoR region was carried out with nuclear extracts from growth-arrested (starved) and serum-activated (induced) diploid cells (right panel). The asterisk denotes a hypersensitive site. Nucleotide numbering is relative to the transcription start site.

Fig. 11. Both the Go-1 and Go-2 elements impart growth arrest repression. Stable transfections of NIH3T3 cells were carried out with −822/+46Luc reporter gene constructs with no mutations (closed squares) or with mutated Go-2 (open circles), mutated Go-1 (closed circles), or both mutated Go-1 and Go-2 (open squares). Transfected cells plated at confluence (day 0) were grown for the times indicated. Confluence is reached on day 4. Luciferase activity was normalized to total protein content. The results represent the means ± S.D. of three experiments, in which each experimental point was determined in duplicate.

Mutation of the NF1-binding Element Prevents Growth Arrest Repression.—To further test the functionality of Go-1 and Go-2, stable transfections of NIH3T3 cells were done with constructs in which the putative NF1 half-site (TGGC) in each element was mutated. Reporter gene activity was measured in cells approaching confluence. We found that luciferase activity was repressed upon growth to confluence if either the Go-1 or Go-2 element was mutated (Fig. 11, closed circles, closed
NF1 Represses ANT2

NF1 is bound to the GoR region only in growth-arrested human diploid fibroblasts. Chromatin immunoprecipitation was carried out on formaldehyde-cross-linked human diploid cells. 48-h serum-starved cells (Starved), cells serum-starved and serum-activated for 24 h (Induced), or untreated cells were cross-linked with 0.25% formaldehyde. Chromatin was isolated, and specific DNA-protein complexes were immunoprecipitated with anti-NF1 antibody (Ab) 8199. After reversal of the cross-link, precipitated with anti-NF1 antibody (Ab), DNA was amplified by PCR using primers −795 and −726 and −499, covering the Go-1 element (A), and −8199, total chromatin; −Ab, a negative control without antibodies. PCR also included the negative control without the template (−DNA). As a marker (Marker lanes), a 100-bp gene ruler (MBI Fermentas) was used. The expected sizes of the amplified immunoprecipitated fragments are given on the right.

NF1 Binds to the Go Elements Only in Growth-arrested Diploid Cells—To test the binding of NF1 to the ANT2 promoter in vivo during growth modulation of diploid cells, we performed chromatin immunoprecipitation experiments (Fig. 12). In agreement with in vivo DMS mapping (Fig. 2A) and in vitro DNase I protection (Fig. 5) experiments, NF1 was bound to Go-2 (Fig. 12A) in growth-arrested cells, but not in serum-induced cells. Similarly, NF1 was also bound in vivo to Go-1 (Fig. 12B) in quiescent cells, but not in serum-activated cells. Thus, repression of the human ANT2 gene in growth-arrested human diploid cells is associated with NF1 binding to both Go elements in vivo, and binding is released upon growth activation.

Expression of NF1 Isoforms Represses ANT2 Promoter Activity in NIH3T3 Cells—To assess the effect of NF1 isoforms on ANT2 gene expression, we transfected HeLa and NIH3T3 cells with plasmids expressing mouse NF1-A, -B, -C, and -X proteins (Fig. 13). Expression of all four NF1 isoforms activated the ANT2 promoter from 5- to 10-fold in HeLa cells. In contrast, expression of NF1-A, -C, and -X in NIH3T3 cells repressed the ANT2 promoter by 60–75%. In these cells, NF1-B slightly activated the promoter. This observation extends existing data on the dual role of NF1 in activating and/or repressing gene expression.

DISCUSSION

The ANT2 gene was identified as one of the immediate-early genes expressed in cells stimulated to enter growth and division (11). However, unlike most immediate-early genes, expression of ANT2 is not due to cell cycle-regulated, but rather is down-regulated when cells become growth-arrested (18). Repression of ANT2 in growth-arrested cells is eliminated by deleting an upstream region of the promoter (18), suggesting the presence of an active growth-related repressor. In this study, we identified two protein-binding DNA elements (Go-1 and Go-2) that are necessary for growth arrest repression of ANT2. The proteins binding these elements were purified by DNA affinity chromatography and found to be members of the NF1 family of transcription factors. We further showed, by chromatin immunoprecipitation analysis, that both elements were occupied by NF1 in quiescent human diploid cells in vivo, but not in the same cells stimulated to growth. Our data support the view that NF1 binding to specific ANT2 repressor elements is a necessary event for growth arrest repression of the gene.

That the Go-1 and Go-2 elements described here are responsible for growth arrest repression of ANT2 is strengthened by experiments that exclude the participation of additional repressor or silencer regions reported to be present in the ANT2 promoter (26, 41, 42). One of these is an Sp1-binding element
NF1 Represses ANT2

(C box) that is juxtaposed to the transcription start site (26). Mutating this element increased promoter activity many-fold in several cell types. However, mutating the C box had no influence on growth arrest repression. Furthermore, a silencer element between the Go repressor elements and the Sp1 activator p300/CBP (53) and with histone-3 (54) have been observed, showing the importance of chromatin organization in NF1 function. That nucleosome reorganization might play a role in the growth-regulated expression of ANT2 is supported by experiments from this laboratory showing that the histone deactylase inhibitor trichostatin A activates ANT2 expression in quiescent diploid fibroblasts in vivo, as well as in ANT2 reporter gene constructs stably transfected into chromatin of NIH3T3 cells (55).

Growth arrest repression of ANT2 might also be explained by changes in NF1 binding affinities or by changes in NF1 concentration. NF1 has been reported to undergo phosphorylation (56–59), but in those cases where tested, phosphorylation did not appear to alter NF1 binding properties (56–59). Glycosylation of NF1 has also been reported (60), but no regulatory function has been attached to this modification. To our knowledge, no other post-translational modifications of NF1 are known. Early studies have reported changes in the levels or type of NF1 protein expressed in different growth stages, including studies on quiescent and growth-activated NIH3T3 cells (61) and 3T3-L1 cells (56). In the latter studies, however, it is not clear if the changes observed reflect expression of different isoforms or different phosphorylation states. To date, we have detected no differences in the content of NF1 in quiescent and growth-activated diploid foreskin fibroblasts as judged by Western analysis (data not shown). This result does not, however, exclude the possibility that expression of low abundant isoforms or splice variants is altered.

To our knowledge, this is the first study reporting a direct growth arrest repressor function of NF1. NF1 can act as a repressor (Refs. 51, 62, 63, and 65; see Ref. 25 for review); and in several genes, repression is mediated by NF1 binding directly to a repressor or silencer element in the promoter (63, 66–70), similar to what has been described here for the ANT2 promoter. However, none of these repressed genes is associated with growth arrest. Despite this, NF1 does appear to play a role in growth-related processes. For example, NF1 activates expression of p53 (71) and gadd153 (68), both of which have key roles in growth arrest of damaged or environmentally stressed cells. Paradoxically, NF1 has also been shown to prevent growth arrest. NF1-X1 is one of three genes (together with c-myc and MDM2) that prevent transforming growth factor-β induced growth arrest (37). Finally, studies with transfected 3T3-L1 cells show that reporter gene constructs driven by NF1-dependent promoters are more active in quiescent cells than in growing cells (56), in contrast to our findings with ANT2. However, the same promoters are strongly repressed in quiescent cells by overexpressing Myc. Since NF1 can act as an activator or a repressor of the same gene depending on the cell type or promoter context, the above study does not eliminate the possibility that Myc promotes a repressor function of NF1 in quiescent cells, as observed for ANT2 in this study. Thus, even though ANT2 is currently the only clear example of an NF1-dependent growth-repressed gene, we cannot exclude a broader role for NF1 in the growth arrest process.

Acknowledgments—We thank Dr. Miroslav Pirsel for the kind gift of human primary diploid foreskin fibroblasts, O. Wrange for recombinant NF1, and R. M. Gronostajski and N. Tanese for generous gifts of NF1 isoform expression vectors and anti-NF1-C antibodies, respectively.

REFERENCES

1. Marzo, J., Brenner, C., Zamzami, N., Susin, S. A., Suin, G., Beduicka, D., Remy, R., Zie, Z. H., Reed, J. C., and Kroemer, G. (1998) J. Exp. Med. 187, 1261–1271
2. Nicklin, A., Li, K., Wade, R. P., Shuster, R., and Wallace, D. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7580–7584
3. Houldsworth, J., and Attardi, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3778–3781
4. Cozens, A. L., Runswick, M. J., and Walker, J. E. (1989) J. Mol. Biol. 206, 261–280
5. Ku, D.-H., Kagan, J., Chen, S.-T., Chang, C.-D., Baserga, R., and Warzel, J. (1990) J. Biol. Chem. 265, 16000–16003
6. Kolarov, J., Kolarova, N., and Nelson, N. (1990) J. Biol. Chem. 265, 12711–12716
7. Lawson, J. E., and Douglas, M. G. (1988) J. Biol. Chem. 263, 14812–14818
8. Stepies, G., Torrioni, A., Chung, A. B., Hodge, J. A., and Wallace, D. C. (1992) J. Biol. Chem. 267, 4592–4597
9. Doerner, A., Pauschinger, M., Badorff, A., Neutrias, M., Giessen, S., Schulze, K., Bilger, J., Rauch, U., and Schultheiss, H.-P. (1997) FEBS Lett. 414, 258–262
10. Doerner, A., Olesch, M., Giessen, S., Pauschinger, M., and Schultheiss, H.-P. (1999) Biochim. Biophys. Acta 1417, 16–24
11. Battini, R., Ferrari, S., Kaczmarek, L., Calabretta, B., Chen, S.-T., and Baserga, R. (1987) J. Biol. Chem. 262, 4355–4359
12. Luo, P., Attardi, G. (1991) J. Biol. Chem. 266, 16534–16540
13. Luo, P., Hurko, O., Engel, W. K., and Attardi, G. (1992) J. Biol. Chem. 267, 15267–15270
14. Hirashihom, R., Aller, R., Aller, P., Yuan, Z. A., Gibson, C. W., and Baserga, R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6004–6008
15. Ritting, S. R., Brooks, K. M., Cristofalo, V. J., and Baserga, R. (1988) Proc. Natl. Acad. Sci. U. S. A. 83, 3316–3320
16. Kaczmarek, L., Calabretta, B., and Baserga, R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5375–5379
17. Kaczmarek, L., Calabretta, B., and R. A. B. (1985) Biochem. Biophys. Res. Commun. 133, 410–416A. B
18. Barath, P., Luciakova, K., Hodny, Z., Li, B., and Nelson, B. D. (1999) Exp. Cell Res. 249, 583–588
19. Schneider, C., King, R. M., and Phillipson, L. (1988) Cell 54, 787–793
20. Manfootetti, G., Ruo, M. E., Del Sal, G., Phillipson, L., and Schneider, C. (1990) Mol. Cell. Biol. 10, 2924–2930

K. Luciakova, unpublished data.
Repression of the Human Adenine Nucleotide Translocase-2 Gene in Growth-arrested Human Diploid Cells: THE ROLE OF NUCLEAR FACTOR-1
Katarina Luciakova, Peter Barath, Daniela Poliakova, Annika Persson and B. Dean Nelson

J. Biol. Chem. 2003, 278:30624-30633.
doi: 10.1074/jbc.M303530200 originally published online May 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303530200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 69 references, 38 of which can be accessed free at http://www.jbc.org/content/278/33/30624.full.html#ref-list-1