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Clone pAT 133 identifies a gene that encodes another human member of a class of growth factor-induced genes with almost identical zinc-finger domains

(growth regulation/transcription factors/gene family)

HANS-JOACHIM MÜLLER, CHRISTINE SKERKA, ALEXANDRA BIALONSKI, AND PETER F. ZIPFEL*

Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 2000 Hamburg 36, Federal Republic of Germany

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ABSTRACT **We report the structure and regulation of a gene represented by clone pAT 133, which is induced upon transition from a resting state (G_0) through the early phase of the cell cycle (G_1). The pAT 133 gene is immediately induced, with FOS-like kinetics, in human T cells and in fibroblasts. Primary structure analysis showed that the encoded protein contains three tandem zinc-finger sequences of the type Cys_2-Xaa_{12}-His_2. This zinc-finger region, which is thought to bind DNA in a sequence-specific manner, is similar (>80% on the amino acid level) to two previously described transcription factors pAT 225/EGR1 and pAT 591/EGR2. Except for the conserved zinc-finger domains, the amino acid sequences of the three proteins are distinct. This structural similarity suggests that the pAT 133 gene encodes a transcription factor with a specific biological function. Comparing the regulation of these related zinc-finger-encoding genes showed coordinate induction upon mitogenic stimulation of resting T lymphocytes and of resting fibroblasts. However, upon transition from a proliferating (G_1) to a resting state of the cell cycle the three genes were differently regulated. In human histiocytic U937 cells mRNA of clone pAT 133 was constitutively expressed, whereas mRNA of pAT 225/EGR1 was induced upon induction of terminal differentiation. In contrast mRNA representing pAT 591/EGR2 was not expressed in these cells. This difference in gene regulation suggests distinct biological roles in the control of cell proliferation for the respective proteins.**

Mitogenic activation of resting peripheral blood T lymphocytes is triggered *in vivo* by antigenic peptides associated with molecules of the major histocompatibility complex (1). *In vitro* this activation can be mimicked by agents like phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). Upon activation T cells respond by initiating sequential transcriptional reactions, which eventuate in cell proliferation and expression of effector function (2, 3).

The genetic program induced in resting T cells upon mitogenic stimulation is closely related to that induced by growth factors and by other extracellular ligands in a variety of cells, including fibroblasts. A large number of genes, identical in T cells and in fibroblasts, is immediately activated, and several of these have been cloned from activated T cells and from serum-stimulated fibroblasts, including genes that encode transcription factors (4–14). Some of these transcription factors contain zinc-finger structures that are thought to bind to DNA in a sequence-specific manner (15, 16).

Previously, >60 distinct cDNA clones have been identified, which represent genes that are induced upon mitogenic stimulation of human T lymphocytes (6, 17, 18). Two of these clones, pAT 225 and pAT 591, represent DNA-binding proteins with almost identical zinc-finger domains (ref. 14; C.S. and P.F.Z., unpublished work). Identical cDNA sequences termed EGR1 and EGR2 were isolated from human fibroblasts (9, 19) and also from rat and mouse cells (termed egr-1, zif268, NGFI-A, Krox-24, egr-2, and Krox-20, respectively) (7, 8, 10, 12, 20). The product of the *zif268* gene, which is the murine homolog of the pAT 225/*EGR1* gene, has been shown to bind to its own promoter and to that of other growth factor-induced genes (21). The Krox-20 protein—i.e., the murine pAT 591/EGR2 product—binds to the promoter of the murine homeobox containing gene *Hox-1.4* (22).

We report the nucleotide sequence and the regulation of a cDNA clone pAT 133, the product of which represents another DNA-binding protein with zinc-finger structure.† The nucleotide sequence of this clone displays three tandem zinc-finger regions. These regions are almost identical to the zinc-finger regions of the two human transcription factors pAT 225/EGR1 and pAT 591/EGR2, suggesting a similar function for the pAT 133 product. Despite the sequence similarity of the DNA-binding regions, the three proteins display distinct NH_2- and COOH-terminal sequences, suggesting distinct biological roles. The three genes were coordinately induced and regulated upon mitogenic stimulation but were regulated differently during monocytic differentiation of histiocytic U937 cells.

MATERIAL AND METHODS

T Cell Isolation. Human peripheral blood T cells were obtained from buffy coats from healthy donors of the Blood Bank (University Hospital, Hamburg-Eppendorf) and were isolated as described (6). Briefly, after Ficoll/Hypaque centrifugation, T cells were enriched on nylon/wool columns and were resuspended in RPMI 1640 medium/10% heat-inactivated fetal bovine serum (FCS) at a concentration of 2×10^6 cells per ml.

Cell Culture and Stimulation. Peripheral blood T lymphocytes were stimulated with PHA (Burroughs Wellcome) at 1 μg/ml, PMA (Sigma) at 25 ng/ml, 200 nM calcium ionophore A23187 (Sigma), or with a combination of these stimuli in the presence or absence of cycloheximide (Sigma) at 10 μg/ml. U937 cells were cultivated in RPMI medium/10% FCS/penicillin/streptomycin/gentamicin in a humidified atmosphere at 37°C. Differentiation of U937 cells was induced at 2×10^5 cells per ml with PMA at 25 ng/ml with or without cycloheximide at 10 μg/ml. Human fibroblasts MRC-5 (passage 18–25) were grown in Eagle's minimal essential medium/10% FCS/penicillin/streptomycin/gentamicin. After

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Abbreviations: PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X60104).

reaching confluence the cells were kept in growth medium/0.5% FCS for 3–4 days. To stimulate cell growth the medium was replaced by fresh medium/20% FCS with or without cycloheximide (10 μg/ml).

RNA Isolation and Blotting. Total cellular RNA was extracted with guanidinium isothiocyanate and isolated by centrifugation (23). The RNA concentration was determined spectrophotometrically, and 8 μg of total cellular RNA was separated by electrophoresis in a formaldehyde/agarose gel and subsequently transferred to a nylon membrane (Pall).

Southern Blot Analysis. Human DNA (10 μg) isolated from U937 cells was digested to completion with *Eco*RI, *Pst* I, or *Bam*HI, separated in an agarose gel (1.0%), and transferred to a nylon membrane (Pall).

Labeling and Hybridization. For hybridization the following inserts or fragments were used: (*i*) a pAT 133 cDNA fragment that covered the 3′ region of the sequence, (*ii*) a pAT 225/EGR1 cDNA probe representing a 429-base-pair (bp) *Sph* I–*Pvu* II fragment of the COOH-terminal part (position 1388–1817), and (*iii*) a pAT 591/EGR2 cDNA probe covering the specific region of the sequence (position 32–367). Inserts were excised, labeled with ^{32}P by random priming (Amersham), and used for hybridization at 42°C [5× Denhardt's solution, 5× standard saline citrate, 0.1% SDS, denatured salmon sperm DNA at 250 μg/ml, and 50% (vol/vol) formamide]. After hybridization for 14–18 h the filters were washed at a final stringency at 0.1% standard saline citrate at 55°C or 60°C. The filters were exposed at −70°C by using intensifying screens (Quanta III; DuPont).

Isolation of Additional pAT 133 cDNA Clones and Sequence Analysis. The cDNA clone pAT 133 was originally derived from a subtracted cDNA library (6). This fragment was used to isolate additional clones from a cDNA library prepared from human peripheral blood T cells stimulated with PHA/PMA for 4.5 h in the presence of cycloheximide. Two overlapping cDNA clones, pAT 133-15 and pAT 133-17, were sequenced in double-stranded form by the dideoxynucleotide chain-termination method with adenosine 5′-[α-[^{35}S]thio]triphosphate and Sequenase II (United States Biochemical) (24). Clone pAT 133-17 covered position 1–1699, and clone pAT 133-15 started at position 361 and included 25 residues of a poly(A) tail. Various oligonucleotides were synthesized and used as primers to sequence the cDNA clones in both orientations. The inserts were also subcloned into M13, and single-stranded DNA was isolated and sequenced. To resolve G+C-rich regions electrophoresis was done in 8% polyacrylamide gels with 40% (vol/vol) formamide and 7 M urea.

RESULTS

Kinetics of Induction in T Cells. The gene corresponding to cDNA clone pAT 133 was rapidly induced in human peripheral blood T cells upon mitogenic stimulation (Fig. 1). In the presence of the protein biosynthesis inhibitor cycloheximide, pAT 133 mRNA was superinduced. However, in its absence, pAT 133 induction was relatively weak, and mRNA could be detected only after 45 min of PHA/PMA treatment and upon prolonged exposure (≈1 week). This kinetic analysis shows that pAT 133 mRNA is induced rapidly in human peripheral blood T cells and that *de novo* protein biosynthesis is not required for induction.

Nucleotide Sequence of pAT 133. The nucleotide sequence of two overlapping cDNA clones was determined, and, based on the estimated size of the mRNA from Northern blot analyses, the cDNA sequence is virtually full length. The sequence of 2169 nucleotides is rather G+C-rich (65%) and revealed a single long open reading frame. The ATTAAA motif, starting at position 2138, appears to represent a polyadenylation signal because after 11 nucleotides it is followed by a long stretch of adenine residues. The putative translation start site at position 167–169 shows a good match (7 of 9 nucleotides including the ATG) to the consensus sequence for initiation sites (25) and is followed by an open reading frame.

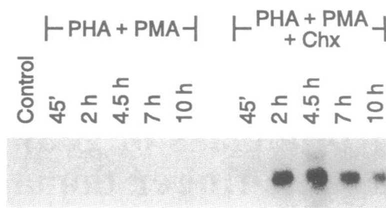

FIG. 1. Kinetics of pAT 133 expression in human peripheral blood T cells upon activation. Northern (RNA) blot of total cellular RNA derived from unstimulated T cells (control) or cells stimulated for the indicated times with PHA and PMA with or without cycloheximide (Chx). RNA was separated on a denaturing gel, blotted onto a nylon membrane (Pall), and probed with a ^{32}P-labeled pAT 133 cDNA probe covering the 3′ untranslated region. Equal amounts of RNA (8 μg) were applied as confirmed by hybridizing the filters with a β_2-microglobulin probe (data not shown).

Predicted Amino Acid Sequence Derived from pAT 133 cDNA. The predicted product of the pAT 133 gene contains 486 amino acids with a calculated molecular size of 50.6 kDa (Fig. 2). The deduced pAT 133 protein is rich in proline (14.0%), alanine (12.8%), leucine (10.3%), serine (9.5%), and glycine (9.7%) residues. The high content of serine and the nine tyrosine residues suggests that the pAT 133 protein could be phosphorylated. The protein contains three tandem zinc-finger regions of the type Cys_2-Xaa_{12}-His_2. The cysteine and histidine residues may bind a Zn^{2+} ion, providing a structure that can interact with DNA in a sequence-specific manner; Fig. 3 shows the putative structure of this complex. One potential site for N-linked glycosylation of the sequence Asn-Xaa-Ser can be found at positions 418–420. This site is located within the region of zinc finger II.

Homology Comparison. A homology search indicated that the zinc-finger region of the pAT 133 sequence showed a rather high degree of similarity to the zinc-finger region of other human genes (Fig. 4). Almost identical regions with three zinc fingers appear in the proteins encoded by the genes

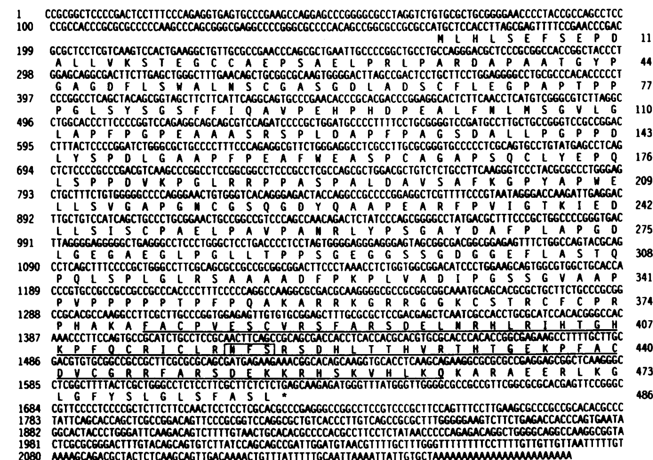

FIG. 2. Nucleotide and derived protein sequence of pAT 133 using a single-letter amino acid code. Nucleotide sequence was obtained from two overlapping cDNA clones pAT 133-15 and pAT 133-17. Clone pAT 133-17 covered nucleotides 1–1699 and clone pAT 133-15 covered nucleotides 361 to the poly(A) tail. Numbers refer to nucleotide sequence (left) and derived protein (right). The putative polyadenylation site ATTAAA at position 2138–2143 is underlined; the amino acid sequence representing the three zinc fingers is also underlined, and the potential site for N-linked glycosylation at positions 418–420 is boxed.

FIG. 3. Schematic representation of the zinc-finger structure. The three zinc fingers in the predicted pAT 133 amino acid sequence are displayed in single-letter code. Amino acids that differ in each finger are shown in boldface, and identical amino acids are presented in white letters. Note the putative site for N-linked glycosylation (N-F-S) located within the second zinc finger. C and H residues that complex zinc ions are enlarged.

FIG. 5. Southern blot analysis of human genomic DNA. DNA (10 μg) isolated from human U937 cells was digested with *Eco*RI, *Bam*HI, or *Sst* I, separated on an agarose gel, and blotted onto a nylon membrane. The filter was probed with a cDNA fragment specific for the 3′ untranslated region of pAT 133. Molecular masses of the markers are shown in kilobases.

of pAT 225/EGR1, pAT 591/EGR2, WT33 and by transcription factor Sp1, with a sequence similarity of, respectively, 83.5%, 82.3%, 61.7%, and 54.3% (refs. 9, 12, 14, 26, 27, and 28; C.S. and P.F.Z., unpublished work). This high degree of similarity may indicate a conserved biological function for these proteins.

Southern Analysis. To determine whether the pAT 133 gene itself is a member of highly related genes, we probed genomic DNA with a fragment specific for the pAT 133 gene. DNA obtained from human U937 cells was digested with various restriction enzymes and subjected to Southern analysis. A cDNA probe specific for the 3′ end of pAT 133 revealed single bands on a Southern blot (Fig. 5), indicating that pAT 133 represents a single-copy gene.

Regulation of pAT 133 Gene. To determine the signals necessary for pAT 133 mRNA induction several different stimuli, or a combination thereof, were used to treat human peripheral blood T lymphocytes for 4.5 h. Induction of mRNA was analyzed by Northern blotting (Fig. 6). In the presence of cycloheximide pAT 133 mRNA was induced by PHA and by calcium ionophore A23187 but not by PMA. mRNA induction was weak, and in this particular experiment only A23187 could induce pAT 133 message in the absence of cycloheximide. By comparison, pAT 225/EGR1 and pAT 591/EGR2 were induced by PHA in the absence of cycloheximide and to an even higher degree by a combination of PHA and PMA. Because mRNA was induced at higher levels in the presence of cycloheximide, induction did not require *de novo* protein biosynthesis. PMA alone could not induce these genes in T cells. However, in combination with PHA or A23187 this agent increased the mRNA levels of both pAT 225/EGR1 and pAT 591/EGR2. For pAT 591/EGR2 induction, mRNA obtained by stimulation with the calcium ionophore A23187 alone was higher than that obtained by PHA. This observation suggests that compared with PHA, A23187 can induce additional intracellular signals enhancing transcription of pAT 591/EGR2.

Expression in Fibroblasts. Upon serum stimulation of resting human fibroblasts pAT 133 mRNA was induced (Fig. 7). The expression was weak as for T cells and could only be detected in the presence of cycloheximide. Comparing the regulation of the three genes showed that pAT 225/EGR1 and pAT 591/EGR2 were induced with kinetics similar to pAT 133, but their mRNAs could be detected in the absence of cycloheximide.

Expression upon Terminal Differentiation of U937 Cells. Is the expression of pAT 133 and the related zinc-finger-encoding genes specific for transition from G_0–G_1, or do these genes play a role in other phases of the cell cycle? To analyze

FIG. 4. Amino acid alignment of related zinc-finger proteins (single-letter code): alignment of zinc-finger regions of human zinc-finger-encoding genes pAT 133 (amino acids 377–466), pAT 225/EGR1 (14, 19), pAT 591/EGR2 (9), the candidate Wilms tumor gene product WT33 (26, 27), and eukaryotic transcription factor Sp1 (27). The amino acid sequence derived from WT33 cDNA has four zinc fingers, of which only fingers II–IV are shown. Dots indicate amino acid identity, and cysteine (C) and histidine (H) residues that complex zinc ions are boxed. Lines indicate zinc fingers I–III of the pAT 133 sequence, and the "spacer-regions" connecting the individual fingers are labeled S_1 and S_2. Amino acids that contact DNA have been determined for the Zif268 product; their corresponding positions in the pAT 133-derived protein sequence are indicated by arrowheads—i.e., Arg-393, Arg-399, Arg-421, His-424, Arg-449, and Arg-455.

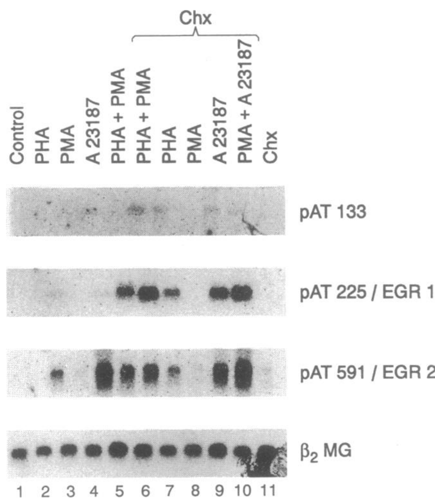

FIG. 6. Regulation of three related human zinc-finger-encoding genes in human peripheral blood T cells. Northern blots of total cellular mRNA derived from unstimulated T cells (control) or from cells treated for 4.5 h with the indicated stimuli without (lanes 2–5) or with cycloheximide (Chx) (lanes 6–10). RNA in lane 11 was isolated from cells incubated with cycloheximide only. Individual filters were probed with ^{32}P-labeled cDNA inserts specific for the indicated genes or with a β_2-microglobulin (β_2MG) probe.

their regulation during transition from a proliferating to a resting state, we used human histiocytic U937 cells, which can be terminally differentiated *in vitro* into monocytic cells. It was found that the three genes are differently regulated (Fig. 8). In proliferating cells mRNA for pAT 133 could be detected, and steady-state levels were neither changed upon induction of differentiation nor affected by cycloheximide. In contrast, mRNA for pAT 225/EGR1 was induced upon induction of differentiation, and in an early phase cycloheximide increased mRNA levels. No mRNA of pAT 591/EGR2 could be detected in U937 cells.

DISCUSSION

We report the molecular characterization of a cDNA clone termed pAT 133, whose mRNA is rapidly and transiently induced upon PHA/PMA stimulation of human peripheral blood T lymphocytes. Sequence analysis indicates that the predicted protein contains three typical zinc-finger sequences of the type Cys_2-Xaa_{12}-His_2, which is characteristic for a class of eukaryotic transcription factors that bind DNA in a sequence-specific manner. Human cDNA clones with almost identical zinc-finger domains termed pAT 225/EGR1 and pAT 591/EGR2 have been isolated from T cells and fibroblasts. Related zinc-finger domains were found in the sequence of the candidate Wilms tumor gene *WT33* and in that of the eukaryotic transcription factor Sp1 (26, 27). Although the amino acid sequences of the zinc-finger regions of pAT 133, pAT 225/EGR1 and pAT 591/EGR2 are almost identical, their flanking regions diverge significantly, indicating related but distinct biological functions. A schematic representation of the three related zinc-finger-encoding genes pAT 133, pAT 225/*EGR1*, and pAT 591/*EGR2* is shown in Fig. 9. The functional relatedness is supported by the coordinate regulation of the three genes upon mitogenic stimulation of resting T cells and of serum-starved fibroblasts.

The coordinate induction of the three related human zinc-finger genes upon mitogenic stimulation of resting cells suggests that their products play a role in the transition from G_0–G_1 phase of the cell cycle.

The mRNA encoding the pAT 133 protein is tightly regulated in T cells and in fibroblasts. In addition the pAT 133

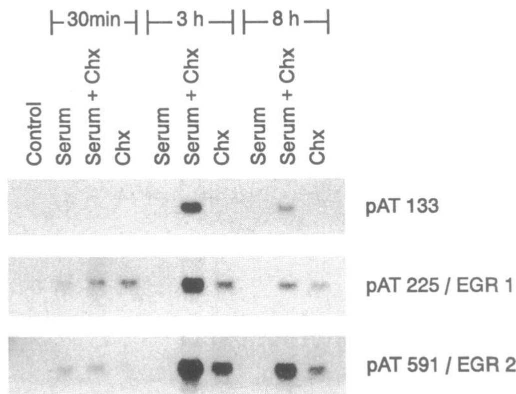

FIG. 7. Regulation of three related human zinc-finger-encoding genes in human fibroblasts. Northern blot of total cellular mRNA derived from resting cells (control) or from cells treated for the indicated time with serum in the absence or the presence of cycloheximide (Chx) or with cycloheximide only. Individual filters were probed with ^{32}P-labeled cDNA inserts specific for the indicated genes. Rehybridization of the filters with a β_2-microglobulin probe showed that equal amounts of RNA were loaded (data not shown).

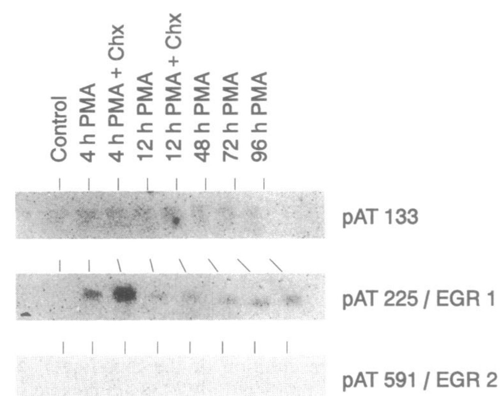

FIG. 8. Expression of three related human zinc-finger-encoding genes during terminal differentiation of the human histiocytic lymphoma cell line U937. Northern blot analysis with total cellular RNA isolated from uninduced cells (control) or from cells treated for the indicated time intervals with PMA or a combination of PMA and cycloheximide (Chx). Individual filters were probed with ^{32}P-labeled cDNA inserts specific for the indicated genes. Rehybridization of the filters with a β_2-microglobulin probe showed that equal amounts of RNA were loaded (data not shown).

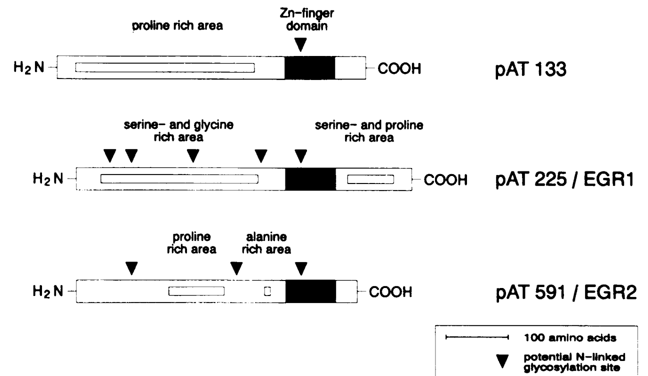

FIG. 9. Schematic representation of the three closely related T cell-induced genes pAT 133, pAT 225/*EGR1*, and pAT 591/*EGR2*. The three proteins have almost identical zinc-finger regions but differ in the amino acid sequences flanking these regions. All are rich in proline residues, and potential N-linked glycosylation sites are indicated by triangles.

protein sequence shows several potential sites for posttranslational modifications, which may be important for the regulation of biological activity. The protein is rich in amino acid residues that can be phosphorylated. The high content of serine and threonine residues may serve as sites for O-linked glycosylation, which plays a role in regulation of the transcriptional activity of Sp1 (29). The putative N-linked glycosylation site of pAT 133 is located within the second zinc finger; this site is conserved in the three closely related genes (Fig. 9). Glycosylation of this particular site might alter or disrupt DNA binding, and in this way the regulatory activity of the proteins may be controlled.

Various levels of regulation have been demonstrated for the related candidate Wilms tumor gene *WT33*. This gene is subject to alternative splicing, in a region between finger III and IV, resulting in two different mRNA species (26, 30). In addition, a mutation that results in the deletion of one of the four zinc fingers encoded by the *WT33* gene abolished its DNA-binding activity (34). As the loss of this DNA-binding seems to contribute to the tumorigenic process, this class of zinc-finger proteins may be important in regulating cell proliferation. Deregulated expression of the pAT 225/EGR1 mRNA has been demonstrated in human T-lymphotropic virus type 1- or type 2-transformed cell lines (14). Although the pAT 133 was constitutively expressed in U937 cells, its mRNA was not generally expressed in all cell lines tested. For example, in the human helper T cell line Jurkat, pAT 133 mRNA could neither be detected, nor induced by stimulation with PHA/PMA in the presence of cycloheximide (P.F.Z., unpublished work).

Although the biological function of pAT 133 is unknown, its expression patterns and its sequence-specific DNA-binding domain suggest a regulatory role of its product in gene expression after mitogenic stimulation. The pAT 133 gene product constitutes one of a family of three proteins with almost identical DNA-binding domains but with distinct NH_2- and COOH-terminal sequences. The Zif268-protein (i.e., the murine homolog of pAT 225/EGR1), the Egr-1 protein, the Krox-20 product (i.e., the murine homolog of the pAT 591/EGR2), as well as WT33 protein bind to the same consensus sequence GCGG/TGGGCG (21, 22, 32–34). However, this binding is specific, as none of the proteins did bind to the related Sp1 consensus sequence (GGGCGG) (21, 34). The amino acids of the individual zinc fingers that contact the corresponding nucleotides have been determined for the Zif268 product (31). These amino acids are conserved in the zinc fingers of all four related proteins (Fig. 4). In the first finger Arg-393 and Arg-399, in the second finger Arg-421 and His-424, and in the third finger Arg-449 and Arg-455 (numbering refers to the amino acids of the pAT 133 gene product) make the primary contact to the target nucleotides.

These target sites have been demonstrated in the promoter of the *zif268/egr-1* gene and in that of other early induced genes (21, 22). However, the effect of binding of each protein may be distinct. One protein may be involved in the down-regulation of the early induced genes, whereas another protein may cause transcriptional activation of genes expressed at a later stage, thereby regulating the cellular response to growth stimulation, which finally leads to DNA synthesis and to cell proliferation. A definition of these target genes as well as an understanding of their biological function and regulation will help elucidate the cellular program that governs cell proliferation.

Note Added in Proof. While this paper was under review, the sequence of the putative rat homolog of pAT 133: NGFI-C has been described (35), and an additional member of this gene family termed EGR3 has been isolated (36).

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