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Regulation of the MDR1 Gene by Transcriptional Repressors Selected Using Peptide Combinatorial Libraries

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ABSTRACT

The ability to selectively regulate the expression of genes implicated in cancer or other diseases could have important ramifications for both basic research and for therapy. Using peptide combinatorial libraries expressed in yeast, we have screened for novel zinc finger proteins that selectively bind to an overlapping EGR1/SP1/WT1 regulatory site in the promoter of the MDR1 multidrug resistance gene. The novel proteins were only moderately effective in blocking transcription by simple masking of the target site. However, when coupled to mammalian transactivator or repressor domains, they could selectively modulate the expression of reporter genes having promoters containing the MDR1 target site. Moreover, they could also regulate transcription of the chromosomal MDR1 gene. Thus, in K562 cells, 12-*O*-tetradecanoylphorbol-13-acetate-inducible expression of P-glycoprotein, the product of MDR1 gene, was strongly and selectively inhibited by the presence of a repressor protein targeted to the MDR1 promoter. These studies potentially provide a novel alternative approach to the control of multidrug resistance. They also provide important insights into strategies for developing selective regulators of gene expression.

Selective modulation of the transcription of disease-related genes potentially offers an interesting alternative to conventional drug-based therapeutics. Transcription factors generally have modular structures with distinct DNA-binding and transregulator domains. These domains can often be interchanged to generate new artificial activators or repressors of gene expression. An important property of transcription factors is their ability to engage in highly precise DNA recognition. Recently it has become possible to design proteins with novel and specific DNA binding abilities through the use of combinatorial library screening approaches (Choo and Klug, 1994; Choo et al., 1994).

A simple structure for generation of new DNA-binding domains is provided by the Cys_2-His_2 family of zinc fingers. Important members of this family include the three-finger DNA-binding domains of the mouse and human transcription factors Zif268 (Christy et al., 1988) and SP1 (Kadonaga et al., 1987), respectively. Crystallographic analysis indicates that the Cys_2-His_2 zinc finger domain consists of ~30 amino acids, forms a simple fold, and typically binds 3 base pairs of double-stranded DNA sequence (Pavletich and Pabo, 1991, 1993; Fairall et al., 1993; Elrod-Erickson et al., 1996). Only several crucial residues in each finger are involved in base contacts. The DNA binding affinity and specificity of zinc fingers can be altered when these residues are varied, suggesting that zinc fingers can be used for generation of proteins with novel DNA binding specificities. Indeed, use of phage display combinatorial libraries has allowed the selection of novel zinc finger peptides that can bind to target DNA sequences in vitro (Choo and Klug, 1994; Jamieson et al., 1994; Rebar and Pabo, 1994; Wu et al., 1995; Greisman and Pabo, 1997; Isalan et al., 1998). Some of these novel zinc fingers (as three-finger or polyfinger peptides, as well as linked to different repressor or transactivation domains) were also functional in mammalian cells, as indicated by reporter gene assays (Choo et al., 1997; Kim and Pabo, 1997, 1998; Liu et al., 1997; Beerli et al., 1998).

Recently we described a yeast one-hybrid system that could be used as an alternative approach for screening peptide combinatorial libraries for double-stranded DNA recognition (Cheng et al., 1997). In contrast to phage display techniques, this strategy allows direct selection of peptides that are able to recognize DNA in vivo within the cellular context. The creation of proteins with novel DNA-binding affinities, using either phage or yeast-based techniques, may be very useful for the selective modulation of gene expression in experimental biology, as well as in therapeutics. However, many issues need to be resolved before this strategy can be effectively implemented.

In this study, we evaluated the possibility of regulating

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ABBREVIATIONS: 3-AT, 3-amino-1,2,4 triazole; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MDR, multidrug resistance; GFP, green fluorescent protein.

expression of the MDR1 gene by design of artificial transcription factors using the yeast combinatorial library approach. MDR1 was chosen as a challenging and important model for cancer-related genes. The human MDR1 gene encodes the P-glycoprotein, a 170-kDa membrane ATPase that can transport many types of drugs from cells. Increased levels of P-glycoprotein expression in tumor cells results in the phenomenon of multidrug resistance, a significant problem for cancer chemotherapy (Roninson, 1992; Bradley and Ling, 1994; Ambudkar et al., 1999). Multidrug resistance can be modulated by pharmacological agents that interfere with P-glycoprotein function; some of these agents are currently in clinical trials (Ambudkar et al., 1999). However, inhibition of P-glycoprotein expression offers an alternative strategy that may be valuable in some circumstances. Overexpression of P-glycoprotein is associated with increased levels of MDR1 transcription (for low levels of drug resistance) or with MDR1 gene amplification (for high levels of resistance). The promoter of the MDR1 gene can be regulated by several transcription factors. In particular, the SP1 transcription factor binds to the MDR1 promoter, and deletion of an SP1-binding site results in reduction of transcription in drug-resistant cells (Cornwell and Smith, 1993). In some cell types, this site is also involved in regulation by the EGR1 transcription factor and by the Wilms' tumor suppressor protein, WT1 (McCoy et al., 1995; McCoy et al., 1999). These results suggest that the EGR1/SP1/WT1 site is functionally significant for regulation of MDR1 gene expression and represents a potential therapeutic target.

In this study we used the yeast combinatorial library approach to develop a DNA-interacting module that binds selectively to the MDR1 promoter, overlapping the EGR1/SP1/WT1 site. This module might then interfere with MDR1 expression in mammalian cells by mass action competition with endogenous transcription factors; alternatively, it might be used to anchor functional repressor domains, thereby inhibiting MDR1 transcription. Following this concept, we have assembled novel four and five zinc finger modules that specifically bind to the MDR1 promoter, but not to consensus SP1 sites, as indicated by reporter gene assays in yeast and mammalian cells. These proteins were only moderately effective in blocking transcription by simple masking of the target site. However, when linked to a KRAB-A repression domain, they significantly inhibited expression of reporter constructs having promoters containing the MDR1 site. Moreover, a novel five-finger repressor protein was able to inhibit inducible expression of P-glycoprotein from the chromosomal MDR1 gene. These observations suggest that it may be possible to use the yeast combinatorial library strategy to create therapeutically effective regulators of MDR1 and other cancer-related genes.

Materials and Methods

Strains and Cells. Yeast strain yWAM2 (*MATa* Δ*gal4* Δ*gal180 URA3:GAL1-lacZ lys2801*^{amber} *his3-Δ200 trp1-Δ63 leu2 ade2-101*^{ochre} *CYH2*) was provided by Dr. P. Hieter (Johns Hopkins, Baltimore, MD) (Sikorski and Hieter, 1989). Yeast strain yM4271 (*MATa ura-52 his3-200 ade2-101 lys2-801 leu2-3 112 trp1-903 tyr1-501*) was purchased from Clontech (Palo Alto, CA). DH10B *Escherichia coli* competent cells were obtained from Life Technologies, GibcoBRL (Gaithersburg, MD). Mammalian KB-8-5 cells, resistant to colchicine, were a gift of Dr. M. Cornwell (Fred Hutchinson Cancer Research Center, Seattle, WA) (Shen et al., 1986). K562 cells were from the Lineberger Comprehensive Cancer Center (University of North Carolina, Chapel Hill, NC).

Library Construction. The framework for selection of new zinc finger peptides in the yeast one-hybrid system was synthesized by polymerase chain reaction using six overlapping oligonucleotides. It consists of two wild-type fingers from Zif268, ZF2 and ZF3, and a portion of ZF1 (ΔZF1), in which eight residues were deleted, including all residues responsible for base contacts (Fig. 1A). In addition, a few substitutions were introduced into the nucleotide sequence (but not the amino acid sequence) to generate restriction sites for DNA manipulations. This framework was cloned into the *Eco*RI and *Not*I restriction sites of the pXC yeast shuttle vector (Cheng et al., 1997) and the resulting plasmid was named pXCZ23. The pXC plasmid was especially designed for expression of yeast combinatorial libraries and selection of new DNA binding peptides. In that vector, in frame fusions result in expression of chimeric proteins that consist of a GAL4 transactivating domain (N-terminal) connected by a "spacer" domain to library peptides (potential DNA-binding domains).

The placement of the Sp1 site in the MDR1 promoter and the model for interaction of Zif268 with its consensus site are shown in Fig. 1B. The LR1 library consisted of two wild-type zinc fingers, ZF2 and ZF3, linked to ZF1 randomized at key base-contacting positions. Randomized zinc fingers were termed RF1 (Fig. 1C). LR1 was constructed by ligation of double-stranded degenerate oligonucleotides into *Eco*RI and *Bam*HI sites of pXCZ23 and subsequent electroporation of DH10B *E. coli* cells. Double-stranded degenerate oligonucleotides were synthesized using two overlapping single-stranded oligonucleotides: 5′-gctgaattcatggaacgcccatat*g*cttgccctgtcgagtcct*g*cgatcgccgcttttct-3′ and 3′-agcggcgaaaaga*bnsbnsbnsbns*gaa*bns*gcggtatatataggcctaggtgtg-5′, where *b* is G/C/T, *n* is G/C/A/T and *s* is G/C. Thus, the second oligonucleotide has five randomized codons and each of them can encode 16 different amino acid residues. The randomized codons do not encode any stop codons nor Cys, Tyr, Trp, and Phe residues, which are rarely found in these positions in zinc fingers. The maximum number of possible amino acid sequences encoded by this library is 16^5 ($\sim 10^6$). A new zinc finger, selected from the LR1 library and encoded by the pLR1–5 plasmid (Fig. 2B), was termed NF1 (Fig. 1C).

The LR2 library was constructed using pLR1-5 and consisted of ZF2, NF1, and randomized zinc finger peptide RF2 (Fig. 1C). First, the ZF3 finger was removed from pLR1-5 by digestion with *Age*I and subsequent self-ligation. The resulting plasmid was named pLR2–0. Double-stranded degenerate oligonucleotides were ligated into *Eco*RI and *Nde*I sites of pLR2–0, upstream of NF1, and the ligation mixture was used for electroporation of DH10B cells. As with the LR1 library, double-stranded degenerate oligonucleotides were synthesized using two overlapping single-stranded oligonucleotides: 5′-gctgaattcatggaacgcccatacgcttgcgacatttgtgggaggaagtttgccagg-3′ and 3′-cttcaaacggtcc*bnsbnsbns***s**gaa*bns*tccgtataggcctaagtgtggccactcttcggtatacgaacgta-5′. The second oligonucleotide had four randomized codons, and the maximum number of all possible amino acid sequences encoded by the LR2 library is 16^4 ($\sim 6.6 \times 10^4$). From positive clones of the LR2 library we selected the pLR2–1 plasmid (Fig. 2C) for further study; this expresses a new zinc finger that we termed NF2.

Construction of Polyfinger Peptides. Polyfinger peptides were generated from the pLR2-1 plasmid by insertion of additional zinc fingers downstream of ZF2 (see Fig. 3B). To design the four-finger peptide (p4F), ZF3 was inserted. Five-finger proteins were constructed by insertion of zinc fingers 3 and 2 (SF2 and SF3) of the SP1 transcription factor. Two variants of five-finger proteins were designed: 1) in the p5FS construct, the two fingers of SP1 were connected to the other three fingers by a standard (or short) linker, TGEKP, that is conserved in most Zifs found in nature; in a second construct, p5FL, the fingers were connected with a longer linker, TGEKGGGGERP. The glycine residues were introduced to allow free rotation of the two sets of fingers with respect to each other. The

length of this linker was chosen on the basis of previous experience in designing polyfinger proteins by another group (Kim and Pabo, 1998). Each linker starts from the last residue (Thr) of the helical region in one finger, and ends at the proline residue, which is immediately followed by the first β-sheet in the next finger. Detailed descriptions of the construction of these plasmids (as well as all constructions presented below) are available on request.

Yeast Reporter Plasmids. Yeast reporters were generated by insertion of target sites upstream of *lacZ* or *HIS3* reporter genes encoded by the pLG670Z (West et al., 1984) and the pRS315HIS-Km [derivative of pRS315HIS (Wang and Reed, 1993)] yeast shuttle vectors, respectively. Target sites contained multiple copies of an SP1 consensus sequence (W4) or MDR1 sequences (3xMDR) (Fig. 2A) or single copies of SP1, or five (MDR5) or four (MDR4) triplet sequences of MDR1 (Fig. 3A). Reporter constructions were named W4-HIS, 3xMDR-HIS, SP1-HIS, MDR5-HIS, MDR4-HIS, W4-*lacZ*, 3xMDR-*lacZ*, SP1-*lacZ*, MDR5-*lacZ*, and introduced into the yeast strains yWAM2 (HIS-reporters) or yM4271(*lacZ* reporters).

Screening the Libraries. The yeast one-hybrid system was used for selection of peptides able to bind the target sequence. This approach is based on use of *his3*$^-$ yeast cells containing a reporter plasmid with a target site inserted upstream minimal promoter of the *HIS3* gene (Wang and Reed, 1993). The minimal promoter of the reporter is not sufficient to rescue the *his3*$^-$ phenotype. However, cotransfection of the recipient strain with a plasmid expressing the GAL4 activation domain fused to a peptide that can bind to the target sequence will result in transcription of *HIS3*, and selection of *his3*$^+$ positive yeast clones. Screening of the LR1 and LR2 libraries, expressing zinc finger peptides fused to the GAL4 transactivation domain, were performed in *his3*$^-$ yWAM2 yeast cells containing the 3xMDR-HIS reporter. Yeast cells were transformed with LR1 or LR2 libraries and plated onto selective medium lacking histidine, leucine, and tryptophan and supplemented with 3.0 (for LR1) or 0.1 (for LR2) mM 3-amino-1,2,4 triazole (3-AT), which is an inhibitor of the histidine pathway. After two days of incubation, strong positive clones were selected for further study. In experiments where analysis of *lacZ* gene expression was performed, yeast cells were plated onto complete YPD medium supplemented with 0.2 mM X-Gal.

Mammalian Plasmid Constructions. Mammalian constructions (pcZF series) were designed on the basis of the pcDNA3.1(−)/Myc-HisA vector (Stratagene, La Jolla, CA). The resulting plasmids expressed zinc finger peptides flanked at the N termini with a nuclear localization signal [NLS, TAT protein, amino acids 37 through 60 (Ruben et al., 1989)] and at the C termini with a Myc epitope and a polyhistidine tag (Fig. 5A). The N-terminal sequence reads MAS**CFITKALGISY**<u>GRKKR</u>**RQRRRPPQ**EFM$_{30}$. . . , where the TAT portion is bold face, the NLS is underlined, and the zinc finger peptide starts from Met^{30}. Plasmids expressing three-finger peptides (SF3-SF2-SF1, ZF3-ZF2-NF1 and ZF2-NF1-NF2), four-finger peptide (ZF3-ZF2-NF1-NF2), and five-finger peptide (SF3-SF2-long linker- ZF2-NF1-NF2) were named pcSP1, pc3F1, pc3F2, pc4F, and pc5F, respectively.

To create artificial transcription activators, termed pcZF-VP16 constructs, the VP16 transactivation domain [residues 411 through 490 (Triezenberg et al., 1988)] was inserted in frame downstream of the zinc finger peptides (Fig. 5A). Transcriptional repressors were generated by insertion of one or two copies of the KRAB-A repression domain [residues 12 through 53 (Witzgall et al., 1994)] into the N-terminal end of the constructs named pcK1-ZF and pcK2-ZF, respectively (Fig. 5A). Expression of proteins was monitored by Western blot analysis using 9E10 antibodies raised against the Myc epitope (BabCo), and following previously described procedures (Alahari et al., 1996).

The reporters were generated from the pFR-Luc plasmid (Stratagene, La Jolla, CA) that contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding site inserted upstream of the TATATA box of the firefly luciferase gene. The 2xMDR-LUC and 2xSP1-LUC reporters were constructed by replacing the 5xGAL4 DNA binding element with two copies of MDR1 or SP1 target sequences, respectively (Fig. 5B). The −88/+105 reporter was provided by Dr. M. Cornwell (Cornwell and Smith, 1993) and contains that

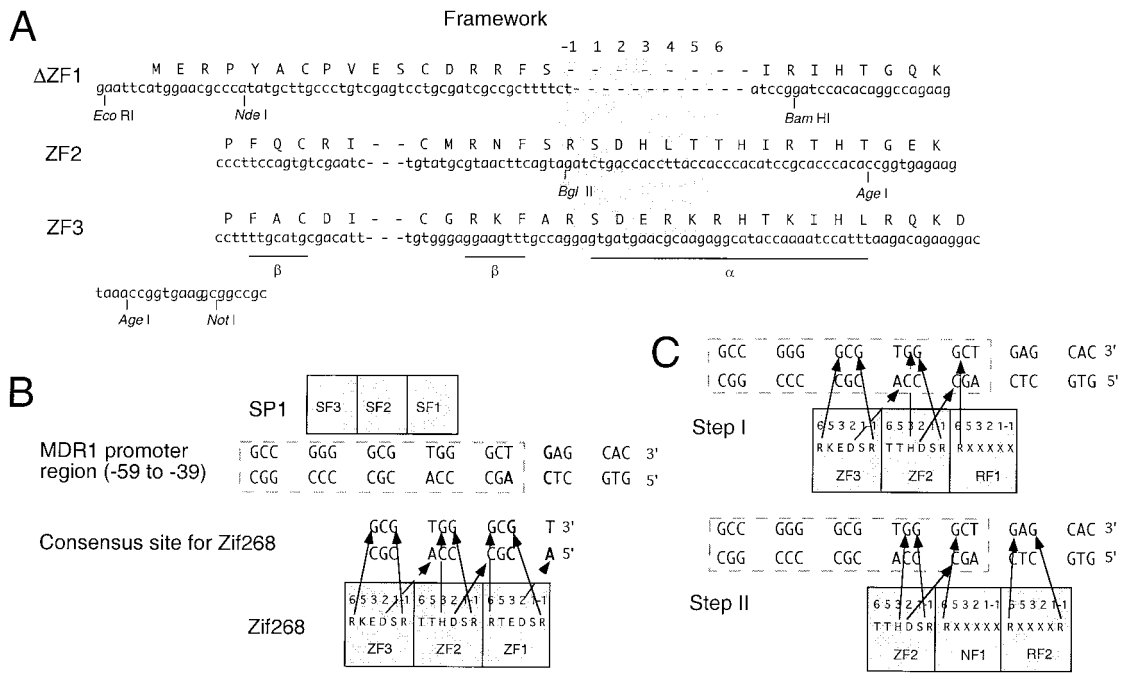

Fig. 1. Strategy for selection of zinc finger polypeptides directed against the MDR1 promoter sequence. A, framework for generation of combinatorial libraries. Elements of secondary structure (antiparallel β-sheets and α-helixes) are indicated below the sequence. Dashes in ΔZF1 indicate deleted residues. Dashes in ZF2 and ZF3 were introduced to align zinc fingers according to the secondary structure. Key residues for DNA recognition are shaded and numbered relative to the first residue in the α-helix. B, model of interactions of SP1 with the MDR1 promoter region and Zif268 with its consensus site (the last according to Elrod-Erickson et al., 1996). The arrows indicate base contacts (solid arrows show key contacts). The overlapping EGR1/SP1/WT1 site is boxed by a dashed line. C, strategy for sequential selection of new zinc fingers targeted to MDR1.

portion of MDR1 promoter (including the SP1-binding site) linked to the luciferase gene (Fig. 5B).

Transient Cotransfection. KB-8-5 cells were cultured under the conditions described (Shen et al., 1986). Cells were cotransfected with 1 μg of reporter plasmid, 0.5 μg of peptide expression plasmid and 0.1 μg of renilla luciferase plasmid (pRL-TK; Promega, Madison, WI) using the LipofectAmine reagent according to the manufacturer's recommendations (Life Technologies, GibcoBRL). To reduce the activation of reporter genes by unknown factors, the concentration of serum was reduced to 1%. K562 cells were transfected by electroporation as described (McCoy et al., 1999) with 10 μg of expression plasmid and 5 μg of pGreen Lantern-1 (Life Technologies, GibcoBRL) expressing green fluorescent protein (GFP) to mark transfected cells. After 18 h of incubation 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added to a final concentration of 16 nM. Flow cytometry analysis was performed after an additional 30 h of incubation.

Flow Cytometry Assay. The flow cytometry assay has been previously described (Alahari et al., 1996). The MRK16 anti-P-glycoprotein antibody (Kamiya Biochemicals, Thousand Oaks, CA) and the MAB1999 anti-$\alpha_5\beta_1$ integrin antibody (Chemicon International, Inc., Temecula, CA) were used as primary antibodies. An R-PE-conjugated goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) was used as the second antibody.

***β*-Galactosidase and Luciferase Assays.** Quantitative determination of β-galactosidase activity was performed with the Luminescent β-galactosidase Genetic Reporter System II (Clontech). Data were normalized per total amount of yeast proteins, determined using the BCA protein assay (Pierce, Rockford, IL). Luciferase activity was determined with the Dual-Luciferase™ Reporter Assay System (Promega), and data were normalized per renilla luciferase activity, used as an internal control. Measurements of all activities were performed on Monolight 2010 instrument (Analytical Luminescence Laboratory, San Diego, CA).

Results

Selection of New Zinc Fingers that Bind to a Sequence Overlapping the SP1 Site in the MDR1 Promoter. To design new zinc finger proteins specific for the MDR1 promoter, we used Zif268 as a framework. We chose Zif268 because the MDR1 promoter has a DNA motif that is highly homologous to the consensus site for Zif268 and overlaps the SP1-binding site. Thus, zinc fingers 3 and 2 of Zif268 (ZF3 and ZF2) recognize two triplets in the 3′-end of the MDR1 SP1 site and can be used as anchors for selection of new zinc fingers (Fig. 1B). The basic strategy, outlined in Fig. 1C, was to use 2 Zif268 fingers to anchor the overall DNA binding module at a site overlapping the SP1 site, and then to use the yeast combinatorial library approach to sequentially select additional zinc fingers that would interact with bases 3′ of the SP1 site in the MDR1 promoter. The newly selected zinc fingers could then be linked in various ways to native zinc finger domains to produce polyfinger proteins having high affinity and specificity. A more detailed description of the strategy and its results follows below.

For screening using the yeast one-hybrid system, various Zif constructs were fused via a spacer domain to the GAL4 transactivating domain and tested for their ability to activate reporters. We have selected two new zinc fingers, which recognize two triplets downstream of the SP1 site in the MDR1 promoter. This selection was performed in two steps using a sequential strategy for assembly of zinc finger proteins. At each step only one finger is added and randomized (optimized), "walking" along desired target. In the first step (Fig. 1C), the wild-type Zif 268 zinc fingers ZF2 and ZF3 were used as anchors and wild-type finger 1 (ZF1) was randomized at five potential base-contacting positions to generate the combinatorial library LR1 comprised of randomized fingers, RF1. The LR1 library consisted of 3.3×10^5 *E. coli* clones. Screening LR1 against the 3xMDR-HIS reporter (Fig. 2A) revealed a large number of positive clones of various size (about 1 positive clone per 300 library clones). For further analysis we picked six large clones, termed pLR1-1 through pLR1-6. These were tested for their ability to grow in the presence of increasing 3-AT concentrations. As shown in Fig. 2B, all clones (except pLR1–3) were able to grow even at 20 mM 3-AT. Similar results were obtained for a positive control comprised of yeast cells transformed with the pXCF3 plasmid that expresses a protein with three SP1 fingers, driving transcription of the W4-HIS reporter (Fig. 2A), which has four SP1 consensus sites (Cheng et al., 1997). A negative control, the pXCZ23 construction (fingers ZF2 and ZF3 only) did not support cell growth in the presence of 3-AT at concentrations higher than 0.5 mM.

To confirm that the *his3*$^+$ phenotype of yeast cells was due to expression of the library peptides and not a result of reverting mutations, we tested plasmids from clones pLR1-1 through pLR1-6 in an alternative reporter system. The library plasmids were recovered from yeast cells and used for transformation of recipient yeast strains carrying the *lacZ* reporter with adjacent W4 or 3xMDR targets. Figure 2B shows that all library plasmids selected in the HIS3 screen positively activated the expression of the *lacZ* gene with the 3xMDR target, but not with the W4(SP1) target, thus demonstrating selectivity. The negative control, pXCZ23, only weakly activated expression of 3xMDR-*lacZ*. Somewhat surprisingly, the positive control pXCF3 strongly activated only W4-*lacZ*, whereas activation of 3xMDR-*lacZ* was similar to that caused by the negative control, this despite the fact that 3xMDR contains three SP1 sites. These latter results indicated that the native SP1 factor interacts with the MDR1 promoter less efficiently than with its consensus site, although the consensus SP1 site and the SP1 sequence of the MDR1 promoter differ only by one base (Fig. 2A).

Sequence analysis of positive clones from screening the first library revealed only partial conservation of residues in the randomized positions of RF1 (Fig. 2B); 50% conservation was observed for Leu at position −1, for Leu and Pro at positions 1 and 5, and for Gly at position 2. The selected residues in clones pLR1-3, pLR1-5, and pLR1-6 showed higher conservation, and the last two clones demonstrated better reporter activation (Fig. 2B and data not shown). We arbitrarily chose the pLR1-5 clone to create the next library, LR2. The newly selected zinc finger encoded by pLR1-5 is henceforth termed NF1.

In the second step of screening (Fig. 1C), ZF2 and NF1 were used as anchors to generate the LR2 library of randomized fingers, RF2. The RF2 proteins had the overall structure of ZF3 but were randomized at 4 potential base-contacting positions {1,2,3,5}. LR2 consisted of 5.4×10^4 *E. coli* clones. Screening of LR2 against the 3xMDR target produced about one positive clone per 2000 library clones. We arbitrarily selected seven positive clones (termed pLR2-1 through pLR2-7, expressing zinc fingers RF1-7) and tested them. Figure 2C shows the summary result. pLR2-0 (anchor fingers ZF2 and NF1, negative control) did not drive *HIS3* nor *lacZ* transcription. All selected LR2 plasmids activated *HIS3* gene

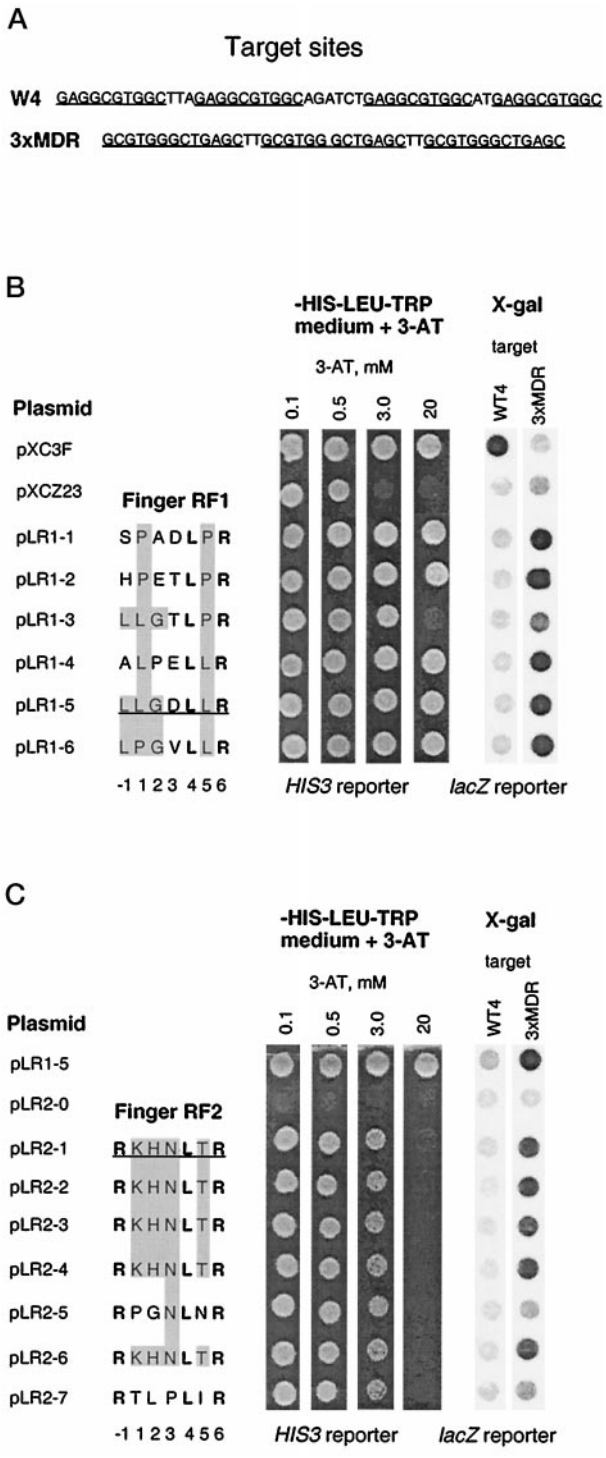

Fig. 2. Selection of new zinc finger proteins that bind to the MDR1 promoter. A, target sites inserted upstream of *HIS3* or *lacZ* reporter genes. B, amino acid sequences and phenotypic analysis of new zinc finger proteins isolated from the LR1 library. C, amino acid sequences and phenotypic analysis of new zinc finger proteins isolated from the LR2 library. Residues that were fixed in combinatorial libraries are bold type, whereas randomized residues are shown in regular style. Conserved residues (≥50%) are shaded. Underlined sequences show clones selected for further studies. The indicated plasmids were used for transformation of yeast cells containing the *HIS3* reporter with the 3xMDR target site (W4 site for pXCF3) or the *lacZ* reporter gene with the 3xMDR or W4 target sites. Transformed cells were plated onto the corresponding selective medium.

transcription, but less efficiently as compared with that of pLR1-5 (they could not grow in the presence of 20 mM 3-AT). All tested LR2 plasmids also specifically activated expression of *lacZ* with the 3xMDR target but not with the W4 target. Sequence analysis of RF2 in selected clones revealed substantial conservation of the residues in the randomized positions. The most conserved residues at positions 1, 2, 3, and 5 were Lys, His, Asn, and Thr, respectively (Fig. 2C). Interestingly, zinc finger proteins selected by phage display against XAX triplets also usually have Asn at position 3 (Wolfe et al., 1999), as found here, thus indicating that our yeast-based selection reflects important themes in protein-DNA recognition. The pLR2-5 and pLR2-7 plasmids, which induced expression of *lacZ* less efficiently, had fewer conserved residues. For the next analysis the pLR2-1 clone was chosen, and the selected RF2 was renamed as NF2.

Analysis of the Specificity of Novel Polyfinger Proteins. We next analyzed the ability of the newly selected zinc fingers to participate in regulation of gene expression in yeast. The newly selected fingers were coupled in various ways with native SP1 or Zif268 zinc fingers and tested against reporters containing MDR1 or consensus SP1 targets (Fig. 3A). The DNA binding affinity of zinc finger proteins can be significantly increased by generation of polyfinger peptides (Beerli et al., 1998; Kim and Pabo, 1998). We have designed novel four- (p4F) and five-finger (p5FS and p5FL) polypeptides to interact with the MDR1 promoter (Fig. 3B). It is known that length of the linker connecting the fingers in polyfinger proteins can significantly affect DNA binding properties (Kim and Pabo, 1998). Thus, we designed two types of linkers for connection of two sets of fingers in five-

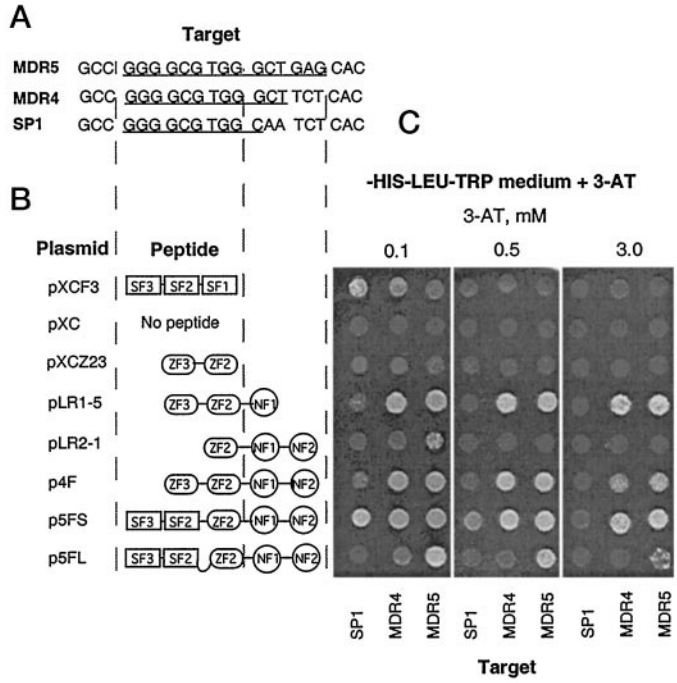

Fig. 3. Analysis of polyfinger proteins. A, target sites; B, schematic illustration of zinc finger peptides encoded by the indicated plasmids. Rectangles, ovals, and circles show SP1, Zif268 and new selected zinc fingers, respectively. C, the plasmids were used for transformation of yeast cells containing a *HIS3* reporter gene with inserted target sites. Transformed cells were plated onto selective medium with different concentrations of 3-AT.

finger proteins. In the p5FS construction, the linker was the canonical sequence (TGEKP) that is usual for native Zif proteins. In another plasmid, p5FL, the linker was extended with several glycine residues to provide additional spacing and flexibility (TGEKGGGGERP).

Figure 3C shows the activation of *HIS3* reporter constructions in the presence of different concentrations of 3-AT. The four finger protein encoded by p4F demonstrated properties similar to the three-finger protein encoded by pLR1-5. It recognized not only the MDR5 target but also MDR4, because its first three fingers match three triplets in that target. However, expression of the SP1-HIS reporter was not significantly activated by p4F or pLR1-5, thus demonstrating selectivity. The five-finger proteins encoded by p5FS and p5FL caused substantial induction of MDR5-HIS expression. However, proteins with different linkers displayed dramatically different properties. Thus, the five-finger protein with the longer linker (encoded by p5FL) was quite specific, and significantly interacted only with the 15-base target, MDR5, and not the 12-base MDR4 target or the 9-base SP1 target. In contrast, the protein with the shorter canonical linker (from p5FS) demonstrated affinity for all targets tested; it could even activate the reporter with the consensus SP1 target site. The basis for this difference is not really understood. Perhaps in the protein with the canonical linker, the SF3-SF2-ZF2 module (refer to Fig. 3B) may act like a unified dominant DNA-binding domain that may efficiently interact at any target where the three appropriate G-rich triplets are in sequence, thus resulting in poor selectivity. In the case of the five-finger protein with the longer linker, the two SP1-derived domains (SF3-SF2) might contribute to binding affinity only after initial recognition of the 3′ portion of the MDR5 target by the ZF2-NF1-NF2 module. Thus, the type of linker and possibly the positioning of the linker within the polyfinger protein may be very important for specificity.

The polyfinger proteins were also tested using a *lacZ* reporter. Figure 4 shows the β-galactosidase activities in cells containing *lacZ* reporters and expressing zinc finger proteins. Except for p5FS, all zinc finger constructs selectively induced β-galactosidase expression from the reporter driven by the MDR5 target as opposed to the SP1 target. The four-finger protein encoded by p4F stimulated MDR5-*lacZ* expression 375-fold above background (activation by pXC empty vector) and 37.5-fold above the level of SP1-*lacZ* expression, indicating a high degree of discrimination between the MDR target and the SP1 site. Other proteins stimulated MDR5-*lacZ* expression 80- to 225-fold above background, and 45- to 160-fold above stimulation of SP1-*lacZ*. Moreover, the levels of SP1-*lacZ* expression stimulated by pLR2-1 or p5FL were about equal to background, indicating that the resultant proteins are highly specific for the MDR target as compared with the SP1 consensus site. A lack of discrimination between MDR and SP1 sites was observed only for p5FS, which encodes the five-finger peptide with the canonical linker; this construct strongly activated reporters driven by both types of sites.

Thus, using the yeast combinatorial library system, we generated new zinc finger polypeptides that can regulate promoters containing the MDR1 target with substantial efficiency and selectivity. In particular, p5FL, which encodes a five-finger protein containing two novel zinc fingers selected from the libraries, provides highly discriminating recognition of a 15-base sequence from the MDR1 promoter. It should be noted that relatively subtle changes in the assembly of polyfinger proteins can have profound effects on their biological properties.

Activation or Repression of the MDR1 Promoter by Artificial Transcription Factors. To determine whether the newly selected zinc finger proteins could modulate transcription driven by the MDR1 promoter in mammalian cells, we subcloned coding sequences for the new zinc finger proteins into eukaryotic expression vectors based on pcDNA3, and fused them with a nuclear localization sequence and with various mammalian effector domain sequences (Fig. 5A). These were cotransfected into human KB-8-5 cells together with luciferase reporters driven by various upstream target sequences (Fig. 5B). Figure 6A shows that the novel zinc finger peptides fused to the VP16 transactivation domain specifically stimulated transcription of a reporter with an MDR1 upstream target (2xMDR) but not a reporter with an irrelevant target (5xGAL4). The levels of activation of 2xMDR-LUC transcription above that of 5xGAL4-LUC were from 210- to 463-fold for the novel zinc finger peptides described above, and 43-fold for SP1 fingers. Compared with the 5xGAL4-LUC reporter, the 2xMDR-LUC reporter was also activated 25-fold by endogenous cellular/serum factors, as evidenced by the expression seen on transfection with the pcDNA3 control vector.

We next tested the ability of the novel zinc fingers selected above to serve as inhibitors of transcription. Initially we evaluated the ability of the novel Zif proteins to inhibit by simple mass action-based competition, presumably interfering with the binding of endogenous SP1 (Cornwell and Smith, 1993). In these experiments, the novel zinc fingers were expressed while linked only to a nuclear localization sequence and to epitope tags. Figure 7, A and B, shows that the selected three-, four-, and five-finger proteins only mod-

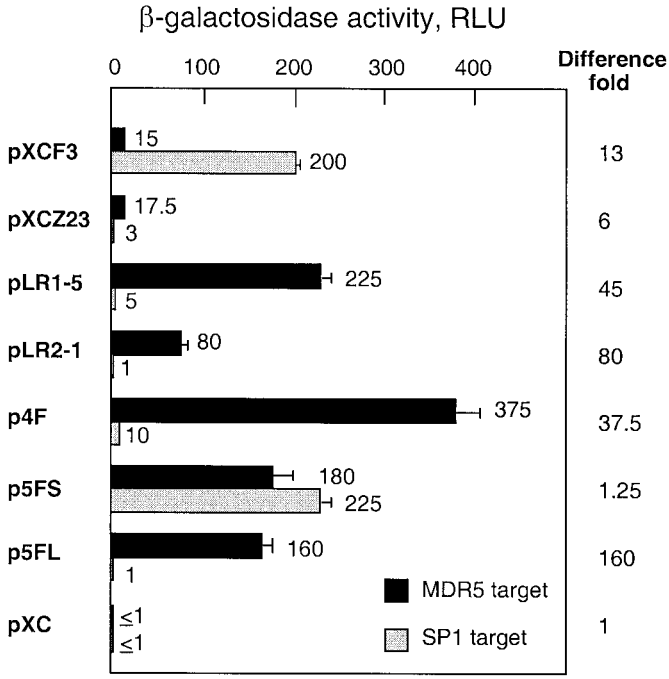

Fig. 4. Activation of *lacZ* expression in yeast cells by zinc finger proteins at a promoter with MDR5 or SP1 targets. Data were normalized per total amount of protein.

estly inhibited luciferase expression from a reporter with the 2xMDR target (up to 3.3-fold inhibition), as well as from a reporter having a portion (−88/+105) of the native MDR1 promoter (up to 1.5-fold inhibition). However, as seen in Fig. 7C, addition of one or two copies of the KRAB-A repression domain resulted in much stronger inhibition of 2xMDR-LUC expression. For example, the pcK2-5F construct, which expresses 2 KRAB-A domains in association with the five zinc finger module having the long linker, produced a 22-fold repression. The four- and five-finger constructs also provided moderate repression of the native MDR −88/+105 reporter (up to 4-fold) (Fig. 7D). Interestingly, the repression properties of three-finger proteins were not improved by fusion to KRAB-A domains, suggesting that they do not permit effective deployment of the repressor; this may possibly be due to their relatively weak binding as compared with the multifinger proteins. We also showed that MDR-targeted proteins with two KRAB-A domains did not repress transcription from the 2xSP1 reporter, whereas SP1 zinc fingers fused to the KRAB-A domains were able to repress, thus demonstrating selectivity (Fig. 7E). Western blotting with an antibody to the Myc epitope included in each of these constructs demonstrated that the newly selected zinc finger proteins are expressed at comparable levels (within 2- to 3-fold) in KB-8-5 cells (data not shown). Thus, the differential repressor actions of the various constructs used are due to differences in innate effectiveness rather than to differences in protein expression levels.

Repression of TPA-Inducible P-glycoprotein Expression from a Chromosomal Gene. Although transactivators and repressors selected by phage library techniques have previously been shown to be able to regulate expression of reporter gene constructs, this approach has not yet been extended to the regulation of endogenous chromosomal genes. This is an important issue, because DNA in an episomal reporter gene target is not likely to be fully organized into the nucleosomes and higher order structures found in chromatin (Kadonaga, 1998). To pursue this issue, we examined the effects of one of our novel transcriptional repressors on P-glycoprotein expression in K562 cells. In these cells the expression of P-glycoprotein from the MDR1 locus can be induced by the EGR1 transcription factor in response to TPA treatment (McCoy et al., 1995, 1999). We transiently transfected K562 cells with the pcK2-5F repressor plasmid, or empty vector, and induced the transcription of MDR1 using TPA. Cell surface expression of the P-glycoprotein was quantitated by antibody staining and flow cytometry, whereas cell surface expression of the integrin $\alpha_5\beta_1$ served as a specificity control. The transfected cells were identified by expression of a cotransfected GFP marker. In K562 cells, TPA treatment resulted in a modest but clear-cut increase in the cell surface expression of P-glycoprotein, as indicated by a right-shift of the flow cytometry profile (Fig. 8A). As seen in Fig. 8B, transfection with the pcK2-5F repressor plasmid very effectively inhibited the TPA-inducible expression of P-glycoprotein. Thus, the P-glycoprotein flow cytometry profile in the pcK2-5F transfected and TPA-induced cells was similar to, or even left-shifted from, the profile seen in the uninduced cells. The few cells that still expressed high levels of P-glycoprotein may reflect a population of cells transfected with the plasmid expressing GFP and not transfected with the plasmid expressing the repressor. Importantly, pcK2-5F did not affect the expression profile of the $\alpha_5\beta_1$ integrin (Fig. 8, C and D), indicating that inhibition of P-glycoprotein expression by pcK2-5F was due to specific repression and not due to any general inhibitory effect on expression of membrane proteins. It is important to note that the promoters for both α_5 and β_1 contain one or more SP1 sites (Birkenmeier et al., 1991; Cervella et al., 1993); thus, the $\alpha_5\beta_1$ integrin is a very suitable specificity control.

Discussion

In this study, we have investigated the possibility of regulating the human MDR1 gene using artificial transcription factors. We designed novel zinc finger DNA binding modules that specifically interact with the MDR1 promoter, overlapping an important regulatory site. These modules are hybrid proteins that consist of native SP1 and Zif268 zinc fingers linked to novel zinc finger peptides selected using a yeast combinatorial library approach. One of the valuable aspects of the yeast library screening system is that it allows direct

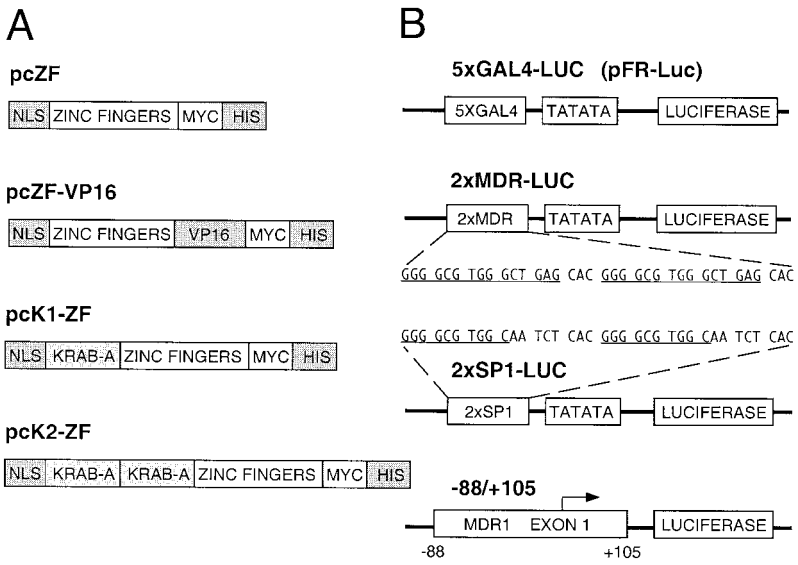

Fig. 5. Constructs for analysis in mammalian cells. A, structure of proteins expressed in mammalian cells: ZF, zinc finger peptide; NLS, nuclear localization signal; MYC, Myc epitope; HIS, polyhistidine tag; VP16, transactivation domain; and KRAB-A, repressor domain. B, schematic illustration of luciferase reporter constructs.

selection of DNA binding modules that can function in a cellular environment, where the DNA target is decorated with histones and other proteins. This approach may confer some advantages over phage display approaches where the selection of DNA binding entities is initially done with a "naked" DNA target; thus the phage-selected DNA binding modules must then be retested for the ability to function in cells. It was initially surprising that, in the absence of any counter-selection system, most of the "hits" that emerged from screening our libraries in yeast represented plasmids coding for proteins that are quite specific in their DNA recognition abilities. This contrasts with phage display, where selection of specific DNA-binding polypeptides is contingent on counter-selection against a background of competing nonspecific DNA. A possible explanation is that in the yeast system plasmids coding for proteins displaying nonspecific DNA interactions result in toxicities due to simultaneous inappropriate activation of multiple genes and are thus lost from the selection process.

The screening processes reported here were done with relatively small libraries (as compared with phage libraries). However, the libraries were sufficiently large to approximate the maximum number of possibilities at the zinc finger residues known to be involved in direct DNA interaction. This was achieved, in part, by using a sequential strategy for selection of zinc fingers, thus walking along the target sequence. Sequential selection is also useful in that it may eliminate problems of steric interference, and yield sets of zinc fingers that can work effectively together (Kim and Pabo, 1998). It should be noted that because a great deal of information is available about specificities of zinc fingers, in some instances it may be possible to design new DNA-binding proteins by directly mixing zinc fingers with known specificities (Beerli et al., 1998). However, it is still difficult to predict subtle effects of protein-protein and protein-DNA interference when combining individual zinc fingers into a polyfinger protein (Kim and Pabo, 1998; Wolfe et al., 1999).

Although sequential selection seems ideal, pragmatic considerations often dictate the use of a mixed strategy, where newly selected zinc fingers derived from library screening are combined with well characterized zinc fingers from native transcription factors, to permit the rapid assembly of multizinc finger proteins that can selectively bind to relatively long stretches of DNA. This modular assembly approach has been used in the present investigation, as well as in previous studies with zinc finger modules derived from screening by phage display (Beerli et al., 1998; Kim and Pabo, 1998; Wolfe et al., 1999). It seems quite clear, however, that relatively subtle changes in the mode of assembly of multizinc finger proteins can result in major differences in biological activity. For example, we found that use of an extended linker region in five zinc finger proteins, as opposed to the canonical linker, can make an enormous difference in terms of selectivity. These observations somewhat resemble previous work indicating that the type of linker used can strongly influence the

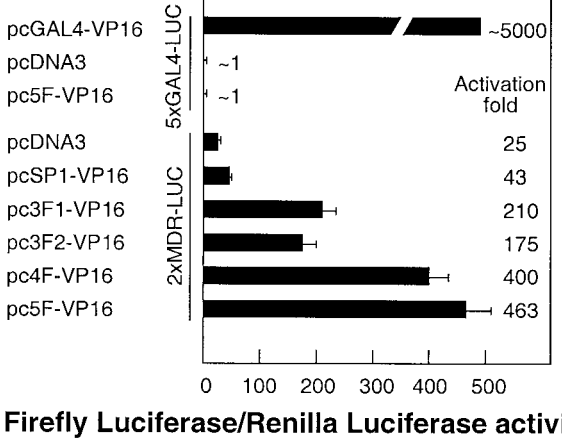

Fig. 6. Transcriptional activation in mammalian cells by newly selected zinc fingers fused to a VP16 transactivation domain. Luciferase activities in KB-8-5 cells were measured 30 h after transfection. With reference to Fig. 3: pcSP1-VP16 includes zinc fingers SF3-SF2-SF1; pc3F1-VP16 includes ZF3-ZF2-NF1; pc3F2 includes ZF2-NF1-NF2; pc4F-VP16 includes ZF3-ZF2-NF1-NF2; pc5F-VP16 includes SF3-SF2-ZF2-NF1-NF2 with the long linker between SF2 and ZF2.

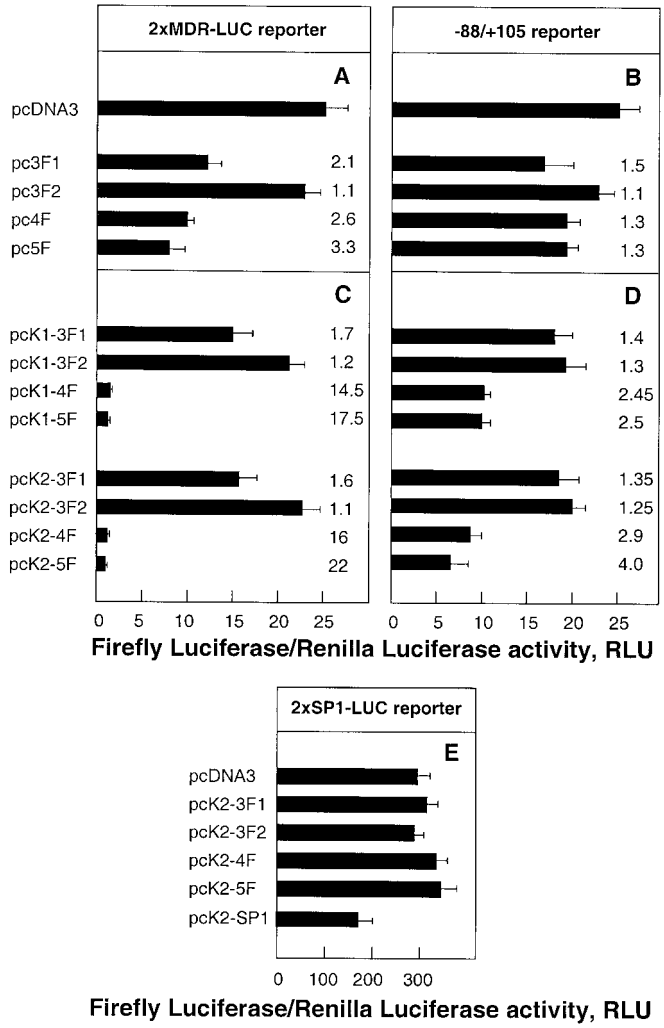

Fig. 7. Transcriptional repression in mammalian cells by newly selected zinc finger proteins. A and B illustrate a passive repression by simple masking of target sites in the native −88/+105 MDR promoter sequence (A) or the 2xMDR sequence with a TATA box (B). C, D, and E illustrate an active repression by chimeric proteins containing one (K1) or two (K2) KRAB-A repressor domains fused to polyfinger proteins including the newly selected zinc fingers. The fold repression is indicated numerically in the bar graph. The zinc fingers associated with the indicated plasmids are the same as described in Fig. 6 and are given within the parentheses below: SP1 (SF3-SF2-SF13F1), 3F1 (ZF3-ZF2-NF1); 3F2 (ZF2-NF1-NF2); 4F (ZF3-ZF2-NF1-NF2); 5F (SF3-SF2-ZF2-NF1-NF2 with long linker). Luciferase activities were measured 48 h after transfection.

overall properties of multizinc finger proteins (Kim and Pabo, 1998). However, in that instance the choice of linker had a major impact on affinity, whereas in our studies the linker mainly affected selectivity. Thus, subtle aspects of protein conformation seem to be able to outweigh simple addition of binding modules.

The DNA target for which we generated novel zinc finger proteins is an important site in the MDR1 promoter that can be regulated by the SP1, EGR1, and WT1 transcription factors in different types of mammalian cells. Several of our novel zinc finger-effector-activator domain chimeras provided strong and highly selective transactivation of reporter genes driven by model promoters containing the MDR1 target site, both in yeast and in mammalian cells. In our experiments on repression of luciferase reporters in mammalian cells, the selected proteins were only moderately effective in blocking transcription by simple mass action competition for promoter sites. However, the addition of the KRAB-A repressor domain resulted in significant enhancement of repression by the novel four and five zinc finger proteins. Expression from model promoters containing TATA boxes was almost completely repressed by our novel DNA-binding proteins fused to KRAB-A. The repression of a reporter driven by a portion (−88/+105) of the native MDR1 promoter, which lacks a TATA box, was less dramatic. This is likely due to the fact that the KRAB-A domain represses most efficiently at promoters where initiation is dependent on the presence of a TATA element (Pengue and Lania, 1996). Thus, although simple masking of the target site in the MDR1 promoter did not result in strong inhibition of its transcription, the use of additional active repressor domains can significantly enhance repression, in agreement with previous reports (Beerli et al., 1998).

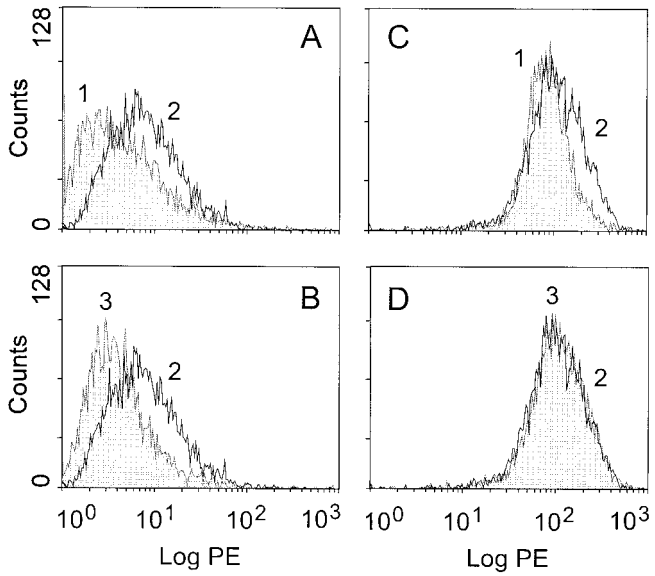

Fig. 8. Flow cytometry analysis of P-glycoprotein expression in K562 cells transfected with a novel repressor. K562 cells were transiently transfected with 10 μg empty vector or repression plasmid and incubated for 18 h. Then TPA was added and cells were incubated for an additional 30 h. Expression of endogenous P-glycoprotein (A, B) or $\alpha_5\beta_1$ integrin (C, D) was quantitated by immunostaining and flow cytometry. The abcissa gives the relative fluorescence intensity using a phycoerythrin (PE) secondary antibody; the ordinate is the number of cells per channel. 1, control cells (pcDNA3 transfected, not induced with TPA); 2, TPA-induced pcDNA3-transfected cells; 3, TPA-induced pcK2–5F-transfected cells. All cells were cotransfected with 5 μg of plasmid expressing GFP for identification of transfected cells.

Although previous studies have successfully used phage techniques to create novel transcriptional regulators that function in mammalian cells, these novel proteins were exclusively evaluated in the context of transient transfection reporter gene assays (Choo et al., 1997; Kim and Pabo, 1997, 1998; Beerli et al., 1998). There are substantial differences in the organization of DNA in episomes, such as reporter constructs, versus the organization of chromosomal DNA. This includes a relative lack of nucleosomes and higher order structures in episomes, and different responsiveness to histone acetylases and deacetylases (Sheridan et al., 1997; Kadonaga, 1998). Thus, it seemed important to evaluate the ability of our novel repressors to regulate expression of an endogenous chromosomal gene. It was significant to find that one of our MDR1 repressor constructs could effectively block TPA-mediated induction of P-glycoprotein expression in K562 cells, without affecting expression of another membrane protein. The success of this initial effort to target a chromosomal gene may be based on the fact that the yeast system inherently selects DNA binding modules that function well in the in vivo setting. The current findings demonstrate the ability to block a TPA-induced increase in P-glycoprotein expression. Actual reversal of multidrug resistance in tumor cells will require a far more prolonged expression of the repressor protein. This could be attained by stable transfection of drug-resistant cells with the repressor plasmid. However, more therapeutically significant approaches might include the development of a viral vector system for gene therapy (Verma and Somia, 1997), or use of technology for the direct intracellular delivery of functional proteins (Nagahara et al., 1998). These approaches are currently under investigation.

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