Positive and negative regulation of endogenous genes by designed transcription factors

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Communicated by Richard A. Lerner, The Scripps Research Institute, La Jolla, CA, December 16, 1999 (received for review November 23, 1999)

Gene regulation by imposed localization was studied by using designed zinc finger proteins that bind 18-bp DNA sequences in the 5′ untranslated regions of the protooncogenes *erbB-2* and *erbB-3*. Transcription factors were generated by fusion of the DNA-binding proteins to repression or activation domains. When introduced into cells these transcription factors acted as dominant repressors or activators of, respectively, endogenous *erbB-2* or *erbB-3* gene expression. Significantly, imposed regulation of the two genes was highly specific, despite the fact that the transcription factor binding sites targeted in *erbB-2* and *erbB-3* share 15 of 18 nucleotides. Regulation of *erbB-2* gene expression was observed in cells derived from several species that conserve the DNA target sequence. Repression of *erbB-2* in SKBR3 breast cancer cells inhibited cell-cycle progression by inducing a G_1 accumulation, suggesting the potential of designed transcription factors for cancer gene therapy. These results demonstrate the willful up- and down-regulation of endogenous genes, and provide an additional means to alter biological systems.

The ability to specifically manipulate the expression of endogenous genes would have wide-ranging applications in medicine and in experimental and applied biology. To accomplish this, a number of promising approaches that aim to control gene expression have been described, operating either at the transcriptional level, such as polyamides, or the posttranscriptional level, such as antisense and ribozymes (1–3). While each of these methods may be applied advantageously in certain circumstances, they are not readily adapted to both gene activation and repression. Nature's control mechanisms center around transcription factors that function to direct the localization of enzymes to specific DNA addresses (4). Exploiting this fundamental principle for imposed control of gene expression is critically dependent on the availability of sequence-specific DNA-binding domains, the design of which has been the subject of intense research for many years. Of the DNA-binding motifs that have been studied, the modular zinc finger DNA-binding domains of the Cys_2–His_2 type have shown the most promise for the development of a universal system for gene regulation. Design studies and phage-based selections have shown that this motif is adaptable to the recognition of a wide variety of DNA sequences, often with exquisite specificity (5–10). Recently, we described a family of zinc finger domains that is sufficient for the construction of 17 million novel proteins that bind the 5′-$(GNN)_6$-3′ family of DNA sequences. Further, we showed that these domains are functionally modular and may be recombined with one another to create polydactyl proteins capable of binding 18-bp sequences with the potential for genome-specific addressing (11, 12). While our early experiments have focused on the regulation of genes transiently introduced into cells, we realized that the willful and specific regulation of endogenous genes with designed transcription factors has remained an unmet challenge in biology.

Herein we target the endogenous *erbB-2* and *erbB-3* genes for imposed regulation. Both genes have been shown to be involved in human cancers (13–15). In particular, the *erbB-2* gene is frequently overexpressed in human cancers, especially breast and ovarian, and elevated ErbB-2 levels correlate with a poor prognosis (13). Moreover, there is increasing evidence that ErbB-3 is also involved in cancer, presumably by acting cooperatively with ErbB-2 (14–16). We show that transcription factors designed to bind in the transcribed regions of either *erbB-2* or *erbB-3* are capable of selectively up- or down-regulating expression of their respective target gene. The results presented herein demonstrate the targeted regulation of endogenous gene expression by using designed transcription factors and provide a foundation for wide-ranging applications of this technology.

Materials and Methods

Antibodies. The ErbB-2-specific antiserum 21N and mAb FSP77 were a gift from Nancy E. Hynes (17, 18). The ErbB-3-specific mAb SGP1 was from Oncogene Research Products. The ErbB-1-specific mAb EGFR1 and the phosphotyrosine-specific mAb PY20 were from Santa Cruz Biotechnologies. Fluorescently labeled, affinity-purified donkey $F(ab')_2$ anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch.

Expression Vectors. For doxycycline (Dox)-inducible expression, the E2C-KRAB and E2C-VP64 coding regions were PCR amplified from pcDNA3-based expression plasmids (12) and subcloned into pRevTRE (CLONTECH) by using *Bam*HI and *Cla*I restriction sites (KRAB, Krüppel-associated box; VP64, tetrameric repeat of herpes simplex VP16's minimal activation domain). Fidelity of the PCR amplification was confirmed by sequencing. For retroviral expression of the E2C and E3 proteins, the E2C-KRAB and E2C-VP64 coding regions were cloned into pMX-IRES-GFP (19) by using *Bam*HI and *Not*I restriction sites (IRES, internal ribosome-entry site; GFP, green fluorescent protein). The E2C coding region was then exchanged for the E3 coding region by *Sfi*I digestion. For transient expression in reporter assays, the various E2C 3 finger-VP64 fusion constructs were assembled in pcDNA3 as described (12).

Retroviral Gene Targeting. The retroviral pMX-IRES-GFP/zinc finger constructs were transiently transfected into the amphotropic packaging cell line Phoenix Ampho by using Lipofectamine Plus (GIBCO/BRL) and, 2 days later, culture supernatants were used for infection of target cells in the presence of 8 μg/ml Polybrene. Three days after infection, cells were harvested for analysis.

Flow Cytometric Analysis. Cells were trypsinized and washed in fluorescence-activated cell sorting (FACS) buffer [phosphate-

Abbreviations: Dox, doxycycline; UTR, untranslated region; KRAB, Krüppel-associated box; VP64, tetrameric repeat of herpes simplex VP16's minimal activation domain; IRES, internal ribosome-entry site; GFP, green fluorescent protein.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.040552697. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.040552697

buffered saline (PBS) containing 0.1% sodium azide and 1% BSA] prior to staining. Approximately 10^6 cells were then resuspended in 100 μl of FACS buffer containing 5 μg/ml of the respective mouse mAb. After incubation on ice for 1 h, cells were washed twice in FACS buffer. Bound antibodies were stained with fluorescently labeled anti-mouse secondary antibody. Finally, the cells were washed twice in FACS buffer, resuspended in 500 μl of FACS buffer, and analyzed for their fluorescence with a Becton Dickinson FACScan.

Luciferase Assays. These assays were performed as described (12).

Construction and Characterization of E3 Protein. For the construction of the E3 six-finger protein, DNA recognition helices from the Zif268 finger 2 variants pmGGA, pGCC, and pGTC were utilized (10). Initially, two three-finger proteins binding each of the 9-bp half-sites of the 18-bp target sequence were constructed, by grafting the appropriate DNA recognition helices into the framework of the three-finger protein Sp1C. DNA fragments encoding the two three-finger proteins were assembled from six overlapping oligonucleotides as described (12). The six- finger protein coding region was then assembled in the bacterial expression vector pMal-CSS. E3 and E2C zinc finger proteins were expressed in the *Escherichia coli* strain XL1-Blue, purified, and analyzed by ELISA and electrophoretic mobility-shift assays as described (10).

Generation of Stable HeLa cell Clones. The pRevTRE/E2C-KRAB and pRevTRE/E2C-VP64 constructs were transfected into the HeLa/tet-off cell line (20) by using Lipofectamine Plus reagent. After 2 weeks of selection in hygromycin-containing medium, in the presence of 2 μg/ml Dox, stable clones were isolated and analyzed for Dox-dependent regulation of ErbB-2 expression.

Western Blotting, Immunoprecipitations, and Northern Blotting. These procedures were carried out essentially as described (21).

Cell Cycle Analysis. Retrovirus-infected cells were stained with mAb FSP77 in combination with fluorescein-conjugated secondary antibody as described above. Cells were then fixed and permeabilized in PBS/4% paraformaldehyde/0.1% saponin for 10 min at room temperature. After two washes with PBS/0.1% saponin, cells were stained with 7-aminoactinomycin (5 μg/ml in PBS/0.1% saponin) for 30 min on ice. After two more washes in PBS/0.1% saponin, cells were subjected to flow cytometric analysis. The fluorescence of the retrovirus-encoded GFP, measured in the same channel as fluorescein, was relatively weak and did not interfere with the ErbB-2 staining.

Results and Discussion

Imposed Transcriptional Regulation of the Endogenous *erbB-2* Gene. We have previously described the generation of designed transcription factors capable of specifically regulating an *erbB-2* promoter–luciferase reporter construct (12). Here we target the endogenous *erbB-2* gene for imposed regulation. Endogenous genes are packaged within chromatin and are controlled by a multiplicity of cis- and trans-acting factors (22, 23), making it not known *a priori* whether specific gene regulation imposed with a dominant designed transcription factor is possible.

Thus, the potent transcriptional repressor E2C-KRAB and the transactivator protein E2C-VP64 (12) were tested for their ability to impose a dominant regulatory effect on the endogenous *erbB-2* gene. Both proteins contain the same designed zinc finger protein, E2C, that recognizes the 18-bp DNA sequence 5′-GGG GCC GGA GCC GCA GTG-3′ in the 5′ untranslated region (UTR) of the protooncogene *erbB-2*. This DNA-binding protein was constructed from six Sp1C-based, predefined, and modular zinc finger domains, each recognizing a specific 3-bp subsite (10, 12). The repressor protein contains the Kox-1 KRAB domain (24), whereas the transactivator VP64 contains a tetrameric repeat of the minimal activation domain (25) derived from the herpes simplex virus protein VP16.

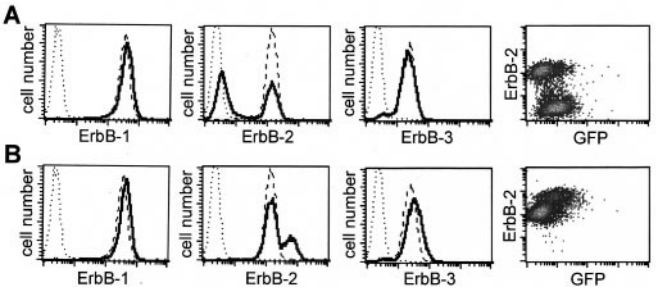

Fig. 1. Retrovirus-mediated *erbB-2* gene targeting. A431 cells were infected with E2C-KRAB- *(A)* or E2C-VP64- *(B)* encoding retrovirus. Three days later, intact cells were stained with the ErbB-1-specific mAb EGFR1, the ErbB-2-specific mAb FSP77, or the ErbB-3-specific mAb SGP1 in combination with phycoerythrin-labeled secondary antibody, and analyzed by flow cytometry. Dotted lines, control staining (primary antibody omitted) of mock-infected cells; dashed lines, specific staining of mock-infected cells; solid lines, specific staining of, respectively, E2C-KRAB- or E2C-VP64-infected cells.

For high-efficiency expression of the E2C-KRAB and E2C-VP64 proteins in various cell lines, their coding regions were introduced into the retroviral vector pMX-IRES-GFP (19). This vector expresses a single bicistronic message for the translation of the zinc finger protein and, from an IRES, the GFP. Because both coding regions share the same mRNA, their expression is physically linked to one another and GFP expression is an indicator of zinc finger expression. Virus prepared from these plasmids was then used to infect the human carcinoma cell line A431. Three days after infection, ErbB-2 expression was measured by flow cytometry. Significantly, about 59% of the E2C-KRAB virus-treated cells were essentially ErbB-2 negative, whereas in about 27% of the E2C-VP64 virus-treated cells ErbB-2 levels were increased (Fig. 1). Plotting of GFP fluorescence vs. ErbB-2 fluorescence revealed that there were two cell populations, one with normal ErbB-2 levels that was GFP negative, and another with altered ErbB-2 levels that was GFP positive (Fig. 1). Specificity of gene targeting was investigated by measuring the expression levels of the related ErbB-1 and ErbB-3 proteins. No significant alterations of the levels of these proteins were detected, indicating that *erbB-2* gene targeting is specific and not a nonspecific result of general alterations in gene expression or overexpression of the effector domains (Fig. 1). The lack of any appreciable regulation of *erbB-3* is particularly remarkable because its 5′-UTR contains the 18-bp sequence 5′-GGa GCC GGA GCC GgA GTc-3′ (26, 27), which presents only three mismatches to E2C's designed target sequence (15-bp identity—lowercase letters indicate differences).

Six-Finger Proteins Are Required for Efficient Imposed Regulation. To evaluate whether targeting 18 bp of DNA sequence with a six-finger protein is necessary for efficient target gene regulation, three-finger proteins were also analyzed. Thus, the two three-finger constituents of the E2C protein, binding either of the 9-bp half-sites of the E2C target sequence, were converted into transcriptional activators by fusion with the synthetic transactivation domain VP64. Significantly, while the E2C-VP64 six-finger fusion protein efficiently activated transcription of an *erbB-2*-luciferase reporter, none of the three-finger fusion proteins had an appreciable effect (Fig. 2). The E2C six-finger protein has a roughly 50-fold higher affinity for the E2C target site than its constituent three-finger proteins, which bind their target DNA with dissociation constants of 25 and 35 nM (12).

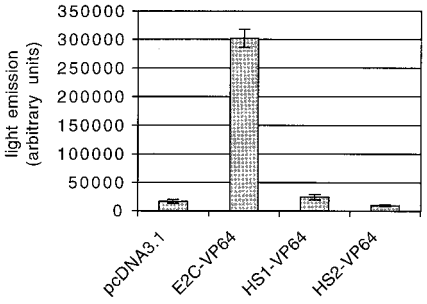

Fig. 2. Luciferase reporter gene assay. HeLa cells were cotransfected with the indicated zinc finger expression plasmids and an *erbB-2* promoter (−758 to −1)-luciferase reporter construct. Luciferase activity in total cell extracts was measured 48 h after transfection. Each bar represents the mean value (± standard deviation) of duplicate measurements. HS1 and HS2, three-finger proteins binding, respectively, half-site 1 or half-site 2 of the 18-bp E2C target sequence. pcDNA3.1 is a control plasmid that does not express a transcription factor.

Thus, these results suggest that not only the increased specificity but also the significantly higher affinity of six-finger proteins may be required to provide a dominant effect on gene regulation.

Regulation of *erbB-2* Expression in Nonhuman Cells. The zinc finger target sequence within *erbB-2*'s 5′-UTR lies within a 28-bp sequence stretch that is conserved in many species (28). To investigate regulation of *erbB-2* gene expression in nonhuman primate cells, African green monkey COS-7 fibroblasts were infected with the bicistronic E2C-KRAB retrovirus and analyzed by flow cytometry. As in human cells, expression of the repressor protein as indicated by the GFP marker correlated well with a loss of ErbB-2 protein (Fig. 3*A*). Similarly, gene targeting in murine cells was evaluated by infection of NIH 3T3 cells with E2C-KRAB- and E2C-VP64-encoding retrovirus. ErbB-2 expression levels were then monitored by Western blotting rather than flow cytometry, because of a lack of reactivity of the mAb with the murine ErbB-2 extracellular domain. Here again, with E2C-KRAB a complete transcriptional knockout upon correction for infected cells was observed (Fig. 3*B*). However, unlike in human cell lines, E2C-VP64-induced ErbB-2 up-regulation was rather modest in NIH 3T3 cells, approximately 1.8-fold upon correction for infection efficiency (Fig. 3*B*). A likely explanation for this discrepancy lies in the different structures of the human and mouse promoters. The mouse *erbB-2* promoter, unlike the human, does not contain a TATA box (28). Transcriptional

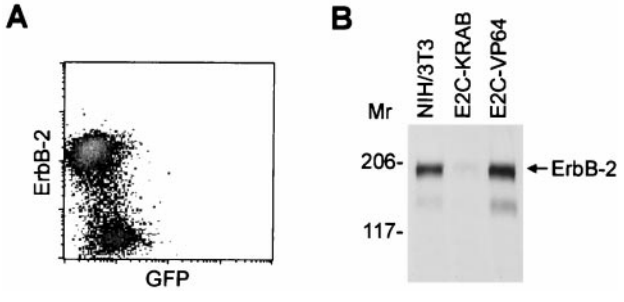

Fig. 3. *erbB-2* gene targeting in nonhuman cells. (*A*) Flow cytometric analysis of ErbB-2 expression. COS-7 fibroblasts were infected with E2C-KRAB-encoding retrovirus. Three days later, intact cells were stained with the ErbB-2 specific mAb FSP77 in combination with phycoerythrin-labeled secondary antibody, and analyzed by flow cytometry. (*B*) ErbB-2 Western blot. NIH 3T3 fibroblasts were mock-infected or infected with E2C-KRAB- or E2C-VP64-encoding retrovirus. Three days later, protein extracts were prepared and subjected to Western blotting with the ErbB-2-specific antiserum 21N. Mr, molecular weight × 10^{-3}.

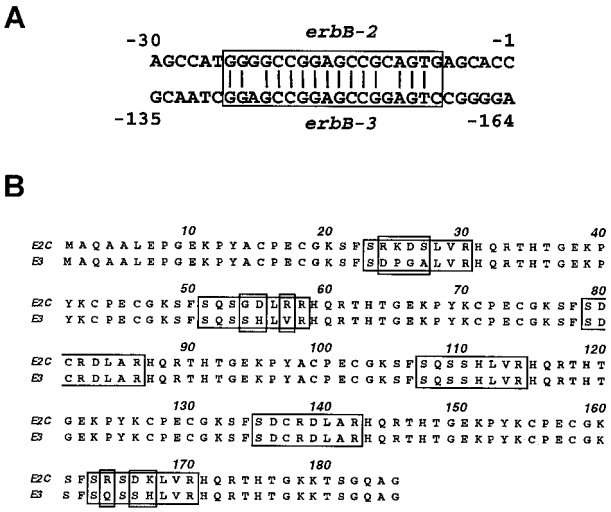

Fig. 4. (*A*) Alignment of E2C target sequence in the *erbB-2* 5′-UTR with the E3 target sequence in the *erbB-3* 5′-UTR. Numbers indicate the distance from the ATG translation initiation codon. (*B*) Amino acid sequence alignment of E2C and E3 proteins. DNA recognition helix sequence positions −1 to 6 of each finger, as well as sequence differences, are boxed.

activation by VP16 is, at least in part, mediated by its interaction with TFIID, a multiprotein complex also containing the TATA-binding protein (29). It is therefore plausible that the E2C-VP64 protein activates transcription less effectively in the absence of a TATA box. These data show that while a DNA-binding site may be conserved with respect to sequence and relative position within a target cell, effector domains may need to be optimized for maximal efficiency due to context effects. Nevertheless, while their potencies may differ, the designed transcription factors described here are capable of imposing regulation of *erbB-2* gene transcription in cells derived from different species, providing a strategy for the study of gene function in a variety of organisms.

Construction and Characterization of a Polydactyl Protein for Regulation of the *erbB-3* Gene. Examination of the *erbB-3* 5′-UTR revealed the presence of an 18-bp sequence that was highly similar to the E2C target sequence in the *erbB-2* 5′-UTR (26, 27). Although they are at different distances and orientations with respect to the ATG initiation codons, the two sequences differ by only three nucleotides (Fig. 4*A*). Thus, we decided to construct a six-finger protein recognizing this sequence to investigate whether transcription factors could be designed to selectively regulate *erbB-3* gene expression.

We have previously described several strategies for the construction of polydactyl proteins from defined, modular building blocks (10, 12). The most successful strategy involved grafting of the amino acid residues of each zinc finger involved in base-specific DNA recognition (a short α-helical region referred to as the "recognition helix") into the framework of the designed consensus protein Sp1C, a derivative of the transcription factor Sp1 (30). Thus, the six-finger protein E3 designed to bind the 18-bp *erbB-3* target sequence was built by using the Sp1C helix grafting strategy, the same method used for construction of the E2C protein described herein. An alignment of the E2C and E3 proteins reveals extensive sequence identity (Fig. 4*B*). In particular, the entire protein framework, as well as three of the six recognition helices, are identical. Only the recognition helices of fingers 1, 2, and 6 were partially different, reflecting the fact that the 3-bp subsites recognized by these fingers differed by 1 nucleotide each.

For a detailed analysis of its binding properties, the E3 protein

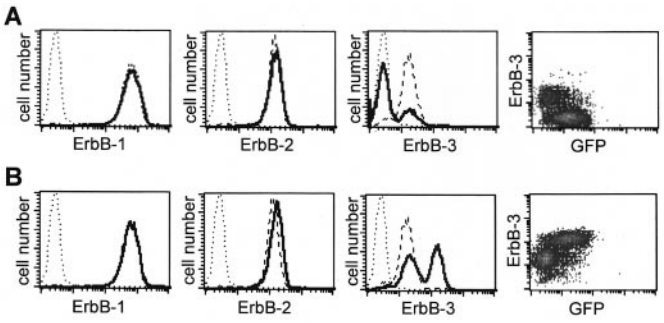

Fig. 5. Retrovirus-mediated *erbB-3* gene targeting. A431 cells were infected with E3-KRAB- (*A*) or E3-VP64- (*B*) encoding retrovirus. Three days later, intact cells were stained with the ErbB-1 specific mAb EGFR1, the ErbB-2 specific mAb FSP77, or the ErbB-3 specific mAb SGP1 in combination with phycoerythrin-labeled secondary antibody, and analyzed by flow cytometry. Dotted lines, control staining (primary antibody omitted) of mock-infected cells; dashed lines, specific staining of mock-infected cells; solid lines, specific staining of, respectively, E3-KRAB- or E3-VP64-infected cells.

was purified as a fusion with the maltose-binding protein. Initially, an ELISA analysis was carried out, revealing specific binding of the E3 protein to its target site, with little or no crossreactivity to various other 5′-$(GNN)_6$-3′ DNA sequences (not shown). A similar observation was made with the E2C protein (12). However, because of the similarity of the DNA sequences recognized, some crossreactivity of the two proteins with each other's target site was detected (not shown). To obtain a quantitative measure for the extent of discrimination between target and nontarget sequence, the affinities of the two proteins to each target sequence was determined by electrophoretic mobility-shift assay (10). These studies revealed high-affinity binding of the E3 protein to its target, with a K_d value of 0.35 nM (±10%), whereas the affinity of binding to the E2C target sequence was about 30-fold lower, with a K_d value of 10 nM (±15%). Similarly, the affinity of the E2C protein to its target was subnanomolar, with a K_d value of 0.75 nM (±15%) as we reported previously (12), whereas binding to the E3 site was significantly weaker, with a K_d value of 11 nM (±30%). Thus, both the E2C and the E3 proteins bind their respective target sequence with very high affinity and are able to discriminate between their cognate and very closely related DNA sequences.

Imposed Transcriptional Regulation of the Endogenous *erbB-3* Gene. Designed transcription factors were generated by fusing the E3 protein to repression or activation domains. In a manner analogous to the E2C fusion constructs, the E3-KRAB protein was produced by fusing the KRAB repressor domain to E3's N terminus, while E3-VP64 was generated by fusing the synthetic VP64 transactivation domain to its C terminus.

To analyze the ability of the *erbB-3*-specific transcription factors to impose a dominant regulatory effect on the native *erbB-3* gene, the E3-KRAB and E3-VP64 coding regions were introduced into the retroviral vector pMX-IRES-GFP. Retroviruses prepared from this vector were then used to infect A431 cells. Three days after infection, expression levels of various members of the ErbB receptor family were monitored by flow cytometry. Dramatic alterations in the levels of ErbB-3 were detected in significant fractions of infected cell populations. Expression was abolished in 74% of E3-KRAB virus-infected cells, whereas almost 8-fold higher ErbB-3 levels were detected in 48% of E3-VP64 virus-infected cells. Plotting of ErbB-3 fluorescence against GFP fluorescence revealed that only GFP-positive, i.e., infected, cells displayed altered ErbB-3 levels (Fig. 5). Thus, E3-based transcription factors are as potent as E2C-based transcription factors in regulating target gene expression.

In contrast to the efficient regulation of ErbB-3 expression, neither E3-KRAB nor E3-VP64 significantly affected ErbB-1 and ErbB-2 expression levels (Fig. 5). Given the similarity of the E3 and E2C target sequences, the lack of a significant effect on *erbB-2* gene expression is yet another demonstration of the exquisite specificity inherent to the zinc finger-based gene switches described here.

Requirements for Imposing Specific Regulation on Endogenous Genes. The extent of discrimination between target and nontarget gene exhibited by the E2C- and E3-based fusion proteins has important implications for the future design of artificial gene switches. In particular, it is possible to make general predictions on the affinity with which a transcription factor has to bind to the promoter of a gene of interest to impose a dominant transcriptional control. We find that binding with K_d values of 10 nM or higher is not sufficient, as evidenced by the lack of *erbB-3* gene regulation by E2C fusion proteins (Fig. 1), the lack of *erbB-2* gene regulation by E3 fusion proteins (Fig. 5), and the lack of *erbB-2* promoter regulation by three-finger fusion proteins (Fig. 2). Binding with a K_d value of around 1 nM, however, appears to be associated with an occupancy of the target site sufficient for imposed gene control, as evidenced by the efficient control of target gene expression exhibited by the E2C- and E3-fusion proteins. Thus, proteins with significantly better affinities may be undesirable because increased binding to nonspecific DNA sequences may lead to significant side effects. Further, the length of the DNA sequence bound is also key to specific regulation. A three-finger protein binding 9 bp of DNA sequence would be expected to find more than 10^4 binding sites in the human genome, whereas an 18-bp binding site targeted by a six-finger protein has the potential to be unique within all genomes. Thus one could expect that any regulatory effect imposed by targeting only 9 bp of sequence would be nonspecific.

Placing Expression of an Endogenous Gene Under Chemical Control. In many circumstances, constitutive up- or down-regulation of a given gene of interest may not be desirable. To evaluate the feasibility of affecting target gene expression in an inducible manner, the E2C-KRAB and E2C-VP64 proteins were expressed from a regulatable promoter. For this purpose, a derivative of the human cervical carcinoma cell line HeLa, HeLa/tet-off, was utilized (20). Because HeLa cells are of epithelial origin they express ErbB-2 and are well suited for studies of *erbB-2* gene targeting. HeLa/tet-off cells produce the tetracycline-controlled transactivator, allowing induction of a gene of interest under the control of a tetracycline response element (TRE) by removal of tetracycline or its derivative doxycycline (Dox) from the growth medium. Thus, the pRevTRE/E2C-SKD and pRevTRE/E2C-VP64 plasmids were constructed and transfected into HeLa/tet-off cells, and 20 stable clones each were isolated and analyzed for Dox-dependent target gene regulation. As a read-out of *erbB-2* promoter activity, ErbB-2 protein levels were initially analyzed by Western blotting. A significant fraction of these clones showed regulation of ErbB-2 expression upon removal of Dox for 4 days—i.e., down-regulation of ErbB-2 in E2C-KRAB clones and up-regulation in E2C-VP64 clones (Fig. 6*A*). ErbB-2 protein levels were correlated with altered levels of their specific mRNA, indicating that regulation of ErbB-2 expression was a result of repression or activation of transcription (Fig. 6*B*). The additional ErbB-2 protein expressed in E2C-VP64 clones was indistinguishable from naturally expressed protein and biologically active, since epidermal growth factor (EGF) readily induced its tyrosine phosphorylation (Fig. 6*C*). The ErbB-2 levels in the E2C-KRAB clone 27, in the absence of Dox, were below the level of detection, as was its EGF-induced tyrosine phosphorylation. Therefore, ErbB-2 expression was also analyzed by flow cytometry, revealing no detectable ErbB-2

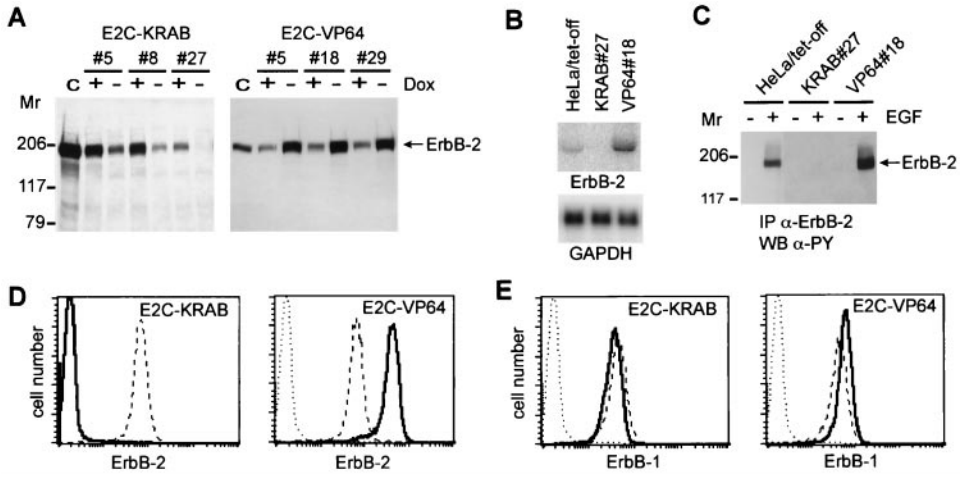

Fig. 6. *erbB-2* gene targeting in stable HeLa cell clones. (*A*) ErbB-2 Western blot. The indicated E2C-KRAB- and E2C-VP64-expressing clones were maintained in the presence or absence of 2 μg/ml Dox for 4 days. Protein extracts from these cells were subjected to Western blotting with the ErbB-2 specific antiserum 21N. Lane C, HeLa/tet-off extract. (*B*) Northern blot. Total RNA extracted from the indicated cell lines maintained in the absence of Dox for 4 days was subjected to Northern blotting with an *erbB-2* specific probe. The membrane was stripped and reprobed with a glyceraldehyde-3-phosphate (GAPDH)-specific probe as a control. (*C*) Epidermal growth factor (EGF)-induced tyrosine phosphorylation of ErbB-2. The indicated cell lines were maintained in the absence of Dox for 4 days, serum starved overnight, and either induced with 100 ng/ml EGF for 10 min at room temperature or left untreated. ErbB-2 was immunoprecipitated (IP) from protein extracts with antiserum 21N and analyzed by Western blotting (WB) with mAb PY20. (*D* and *E*) Flow cytometric analysis of ErbB-2 and ErbB-1 expression. Cells were maintained for 4 days in the absence of Dox, stained with mAbs FSP77 or EGFR1 in combination with phycoerythrin-labeled secondary antibody, and analyzed for their fluorescence in a FACScan (Becton Dickinson). Dotted lines, control staining (primary antibody omitted) of HeLa/tet-off cells; dashed lines, specific stainings of HeLa/tet-off cells; solid lines, specific stainings of, respectively, Dox-deprived KRAB clone 27 and VP64 clone 18.

expression in E2C-KRAB clone 27, in sharp contrast to the dramatic up-regulation (5.6-fold) of ErbB-2 in E2C-VP64 clone 18 (Fig. 6*D*). Thus, the extent of *erbB-2* gene regulation ranged from total repression (E2C-KRAB clone 27) to almost 6-fold activation (E2C-VP64 clone 18). No significant effect on the expression of the related ErbB-1 protein was observed, indicating that regulation of ErbB-2 expression was not a result of general down- or up-regulation of transcription (Fig. 6*E*). In summary, these results show that it is feasible to use designed transcription factors to place the expression of an endogenous gene under control of an exogenous chemical inducer.

Toward a Therapeutic Application of Artificial Gene Switches. Overexpression of ErbB-2 leads to constitutive activation of its intrinsic tyrosine kinase activity (31), and it has been shown that down-regulation of ErbB-2 in tumor cells overexpressing the receptor leads to growth inhibition (32–34). The mechanism of growth inhibition appears to be that progression of the cells from the G_1 to the S phase of the cell cycle is prevented (35). Thus, we investigated whether expression of our designed transcriptional repressor in *erbB-2*-overexpressing tumor cells would lead to a G_1 block. Therefore, SKBR3 breast cancer cells were infected with E2C-KRAB retrovirus and cell-cycle distribution was analyzed in relation to ErbB-2 expression levels by flow cytometry (Fig. 7*A*). Two cell populations were observed: about 40% of the cells were not infected and had normal ErbB-2 levels (Fig. 7*A*, *Left*, cell population 1), whereas the infected cells, ≈60%, displayed approximately 7-fold-reduced receptor levels after 3 days (Fig. 7*A*, *Left*, cell population 2). Compared with cells with normal receptor levels, a significantly larger fraction of cells with decreased ErbB-2 expression levels was in the G_1 phase of the cell cycle (Fig. 7*A*, *Center* and *Right*). To ascertain that the G_1 accumulation observed with SKBR3 cells was specific for ErbB-2-overexpressing tumor cells, a similar analysis was carried out with the T47D breast cancer cell line, which does not display elevated levels of ErbB-2 (Fig. 7*B*). Indeed, when T47D cells were infected with the E2C-KRAB retrovirus and subjected to flow cytometric analysis, cell populations with normal and reduced ErbB-2 levels were found to display indistinguishable DNA contents. Thus, our designed repressor protein is able to specifically induce G_1 accumulation of ErbB-2-overexpressing tumor cells. The ability to inhibit cell-cycle progression, and hence inhibit growth of ErbB-2-overexpressing tumor cells, suggests the potential of designed transcription factors for cancer gene therapy.

Conclusions. In summary, artificial transcription factors can be designed to impose a dominant regulatory effect on the transcription of endogenous genes in their native chromosomal context, and if desired to place them under chemical control. We anticipate that this strategy will find applications in gene therapy and in basic and applied research where modulation of gene transcription can be employed to dissect biological mechanisms or to alter phenotypes of cells and organisms.

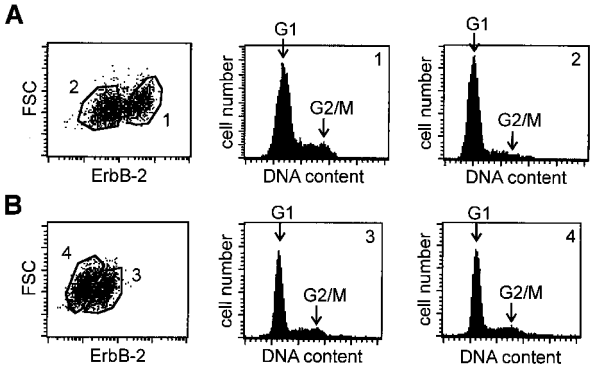

Fig. 7. Flow cytometric cell cycle analysis. SKBR3 (*A*) and T47D cells (*B*) were infected with E2C-KRAB-encoding retrovirus. Three days later, cells were stained with the ErbB-2-specific mAb FSP77 in combination with fluorescein-labeled secondary antibody (*Left*), as well as with 7-aminoactinomycin to show cell cycle distribution by DNA content (*Center* and *Right*), and analyzed by flow cytometry. Cell cycle histograms were generated from cells gated as indicated by numbers. FSC, forward scattered light.

We are very grateful to Nancy E. Hynes for providing us with antibodies, to Harvey Lodish for the pMX-IRES-GFP vector, and to Jody Berry for his critical reading of the manuscript. This study was supported by grants from the National Institutes of Health and the Skaggs Institute for Chemical Biology to C.F.B. R.R.B. was the recipient of fellowships from the Krebsliga beider Basel and the Swiss Cancer League. B.D. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

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