Gene Expression, Vol. 5, pp. 229-243, 1996
Printed in the USA.

1052-2166/96 $5.00 + .00

From Repression Domains to Designer Zinc Finger Proteins: A Novel Strategy of Intracellular Immunization Against HIV

HANS-JÜRGEN THIESEN

Institut für Immunologie, Schillingallee 70, D-18055 Rostock, Germany

Tissue-specific gene regulation of eukaryotic organisms is to a large extent mediated by transcription factors that interact with genomic DNA sequences in a sequence-specific manner. The purpose of this synopsis is to put forward the potential of designer zinc finger proteins in treating infections of human immunodeficiency virus (HIV). Artificial transcription factors containing designer zinc finger structures fused to activator or repressor domains have been designated Transcription Response Modifiers (TRMs). The principle of engineering TRMs has been derived from the analysis of human Krüppel-type zinc finger genes and their products. Our research efforts encompass two fascinating features that are displayed by the human Krüppel-type zinc finger protein KOX1: 1) the Krüppel-type zinc finger domains display rules of sequence-specific DNA recognition, and 2) the evolutionarily conserved Krüppel-associated box (KRAB) presents one of the strongest transcriptional repressors identified so far in mammalian organisms. The KRAB repressor activity is postulated to be mediated through corepressor molecules, such as Silencing Mediating Protein-1 (SMP-1). Thus, the structural organization and functional analysis of zinc finger proteins revealed principles of zinc finger transcription factors that are applicable for reducing the viral load in individuals infected with HIV. In this article, a novel concept of generating therapeutic proteins is outlined that might be conceptually promising in modulating gene expressions of any kind.

Designer zinc finger proteins Krüppel-type zinc finger genes Repressor domains Activator domains HIV-1

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Pathogenesis of human immunodeficiency virus type-1 (HIV-1) infection is closely linked to the generation of viral RNA particles in vivo (35). Many patients remain healthy for years despite their HIV infection. During this time period the human immunodeficiency virus has been shown to continously replicate in lymphoid organs (20,67). Finally, HIV mutants are hypothesized to accumulate in the body that escape the surveillance of the immune system (34). Thus, the replication of the viral genome might turn out to be a prerequisite for the development of full-blown aquired immunodeficiency syndrome (AIDS). Therapeutic interventions that effect the replication of HIV production should delay or even prevent the onset of AIDS.

Intracellular Immunization: A Novel Approach for Treating HIV

Within the last decade, numerous strategies such as drugs, antisense RNA, RNA decoy, and ribozymes have been developed to interfere with HIV replication (26). To delay HIV production these therapeutic compounds have to be delivered either daily or to be administered by gene transfer techniques (15). In 1988 David Baltimore (2) discussed the term "intracellular immunization" for therapeutic procedures that include gene transfer

Received December 12, 1995; revision accepted January 22, 1996.

of genes designed to modulate viral or endogenous gene expressions. In the case of HIV infections, immune cells have to be transduced with therapeutic genes that are capable of inhibiting HIV replication. At the Cold Spring Harbor Meeting on Gene Therapy in 1992 our concept of generating designer zinc finger proteins designated Transcription Response Modifiers (TRMs) was presented, emphasizing that the frame of zinc finger domains displays an excellent tool to generate DNA binding domains of altered nucleotide specificities. It is challenging to assess to what extent designer zinc finger proteins are capable of reducing the replication of the HIV genome. Whether an approach encompassing gene transfer techniques is finally successful in preventing the outbreak of AIDS symptoms might in particular be dependent on prevalent mechanisms of how the HIV virus finally surmounts immune defense mechanisms. Recently, the life-span of an autologous T cell in HIV-positive individuals has been evaluated in identical twin pairs in which one twin is HIV positive and the other HIV negative. Genetically marked T cells survived for at least 10 months (11).

Transcription Response Modifiers

Numerous naturally occurring transcription factors consist of at least two domains: a DNA binding domain and an effector domain. Within the last decade, protein domains have been elucidated that bind to nucleic acids in a sequence-specific manner (10,12,24,42,47,68). Furthermore, individual amino acid residues could be identified that determine sequence-specific DNA–protein interactions (48,49). In particular, by exchanging specific amino acid residues novel DNA target site specificities could be obtained using the zinc finger backbone (32,33,54). In view of effector functions, protein domains have been found that activate or repress transcriptional gene regulation. So far, chimeric transcription factors have been generated by linking DNA binding domains of cloned transcription factors to various *trans*-acting effector domains, such as VP16 (44), steroid binding domains originating from the glucocorticoid receptor (40), or the estrogen receptor (66). However, the binding preferences of these chimeric transcription factors virtually resemble DNA target site specificities displayed by their native factors.

TRMs represent a novel class of artificial transcription factors (58). These factors consist of designed DNA recognition domains engineered to recognize desired DNA target sites and of modified protein domains that are utilized to activate [VP16 (62)] or to repress [KOX1-KRAB (29)] transcriptional gene expression. In comparison to chimeric transcription factors, designer zinc finger molecules can be envisaged to be engineered for any DNA binding site of interest (7,8,37,70).

Thus, the potency of engineered transcription factors is dependent on the affinity and selectivity of how DNA binding domains interact with their corresponding target sites and on the efficiency of how effector domains are capable of turning on or switching off gene expressions. Artificial transcription factors might be engineered that behave like endogenous factors, but the purpose of these factors is to interfere with inappropriate expressions of endogenous genes. Besides modulating transcriptional gene regulation of endogenous genes, HIV expression might be prevented by designer transcription factors of the type outlined in Fig. 1. Concerning TRMs that are designed to abrogate HIV replication, two essential parameters are necesary. First, a DNA binding domain has to be generated that exclusively recognizes HIV-specific sequences without binding to endogenous cellular sequences. In theory, this DNA binding domain at the best should recognize one HIV-specific sequence within the human genome of 2.9 billion base pairs in size. Second, the effector domain applied to prevent the generation of HIV particles has to inhibit transcriptional mechanisms under any physiological stimuli in a manner that the HIV genome under any circumstances is not actively transcribed.

ZINC FINGER PROTEINS

The human genome is estimated to encode several hundred zinc finger genes whose functions

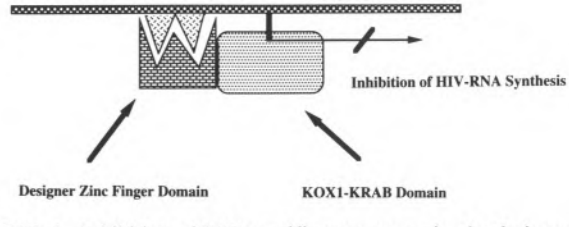

FIG. 1. Inhibition of HIV-specific gene expression by designer zinc finger proteins. This diagram shows the principle of how designer transcription factors can be used to prevent viral replication of HIV particles. A strong repression domain such as KOX1-KRAB is fused to a designer DNA binding domain that interacts with sequences of the HIV genome. The rationale of this approach is based on transcriptional mechanisms described in Fig. 5.

have yet to be determined (1,3,5). Most of these zinc finger proteins contain zinc finger domains with multiple consecutive zinc fingers. One third of all human Krüppel-type zinc finger domains should hold KRAB suppressor domains (4,56). Zinc fingers of the Cys/His-Krüppel type are independently folding domains that are stabilized by zinc (31). In 1990 we reported the cloning and analysis of 30 human Krüppel-type zinc finger proteins designated KOX1-30. Sequence analysis of zinc finger domains derived from 30 KOX zinc finger proteins indicated the presence of common rules for DNA recognition that were hypothesized to lead to the description of a zinc finger-specific recognition code (51). Interestingly, the structural analysis of the protein structure displayed by KOX1 revealed the presence of a leucine zipper-like structure. Later on this protein domain turned out to be present in several hundred zinc finger proteins and was designated Krüppel-associated box (KRAB) (3,56). Target genes regulated by KRAB zinc finger proteins have not yet been identified. The physical localization of KOX genes on human chromosomes revealed that KOX zinc finger genes are clustered in the human genome (22,43,63). However, target genes controlled in their expression by KOX1 are currently searched for in our laboratory at the University of Rostock.

Repression Domains

Several protein domain encoding repression domains have been identified within the last years (36). Several repression domains present in *Drosophila* genes engrailed A and D (19), *Drosophila* even-skipped (19), Krüppel (27,45), and human genes egr-1 (17), Wilms (WT-1) (28,65), and KOX1 (29) are summarized in Fig. 2. Interestingly, the KRAB domain of KOX1 was originally identified because of its resemblance to leucine zipper-like structures (51). The protein KOX1 represents one member of more than 100 human zinc finger proteins that contain KRAB (3,56,57). The KRAB domain of KOX1 has recently been found to actively repress transcription of the HIV (29,39,59) and of the CMV promoter from remote enhancer positions (13). According to our analysis, the KRAB domain turned out to be the strongest human repression domain characterized so far that works in mammalian cells in a distance-independent manner (Thiesen, unpublished). Repressor activities of KRAB containing zinc finger genes have been characterized in man (29,64), mouse (25), and rat (41,69). The repression domain exemplified by KOX1-KRAB has recently been fused to the tetracycline repressor. TetR-KRAB proteins have been applied for switching on and off stably integrated luciferase reporter genes (13).

Repression domains

engrailed (D) (55 aa)	RQQQAAAAAATAAMNLERANFLNCFNP AAYPRIHEEIVQSRLRRSAANAVIPPPM
even-skipped (57 aa)	PYPPYAPAAAAAAAAAAAVGHPSPYGQY RYTPYHIPARPAPPHPAGPHMHHPHMMG
Krüppel (85aa)	VHLDRSMSLSPPMSANTSATSAAAIYPAM GLQQAAAASAFGMLSPTQLLAANRQAAA FMAQLPNSTLAMTLFPHNPAALFGAWAA
engrailed (A) (80aa)	MALEDRCSPQSAPSPITMQMQHLHHQQ QQQQQQQQQMQHLHQLQQLQQLHQQQ LAAGVFHHPAMAFDAAAAAAAAAAAAA
egr-1 (34aa)	QPSLTPLSTIKAFATQSGSQDLKALNTTYQSQLI
wt1 (96aa)	EEQCLSAFTVHFSGQFTGTAGACRYGPF GPPPSQASSGQARMFPNAPYLPSCLES QPAIRNQGYSTVTFDGTPSYGHTPSHHA AQFPNHSFKHED
KOX1 (76aa)	RTLVTFKDVFVDFTREEWKLLDTAQQIVYR NVMLENYKNLVSLGYQLTKPDDVILRLEK GEEPWLV

FIG. 2. Summary of several protein domains with repressor activities. Protein domains are listed that have been documented to repress transcriptional gene expression. Specific characteristics common to these domains have not been elucidated to date. However, the KRAB domains seem to display the strongest repression activities observed to date (Thiesen, unpublished).

The Krüppel-Associated Box

The KRAB domain originally described as a heptad repeat of leucines (51,56) can be subdivided in an A and B box (3) (Fig. 3). In particular, the A box promotes the repressor activity whereas the KRAB B box potentiates the repressor activity of KRAB A (64). The KRAB domain of KOX1 fused to the GAL4-DNA binding domain and to the tetracycline repressor protein (tetR-KRAB) was targeted to GAL4 upstream activating sequences (UAS) and tetracycline operator (tetO) sequences, respectively. UAS and tetO target sequences were utilized proximal and/or distal to SV40, TK, HIV, and CMV promoters.

By inserting two prolines in the highly conserved amino acid residues present in the KOX1-KRAB domain (Fig. 4), the repressor activity of this domain could almost be completely abolished (Fig. 6). KRAB mutations presented in Fig. 4 were preferentially analysed by Margolin et al. (29). In particular, our analysis demonstrated that the KRAB domain actively repressed transcription gene expression of any mammalian promoter that has been studied so far, such as SV40, tk, and CMV promoters (Thiesen, unpublished). In particular, the metallothionein promoter was re-

```
                     KRAB-A box                     KRAB-B box
                                                                          REFERENCE:
          11                                 *     *      *     *      76
KOX1      RTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSL·GYQLTKPDVILRLEKGEEPWLV

ZNF133       MA-R--A----QD--R--SP--RTL--E------S------·ISFS--EL-TQ--Q-K-T-RE

ZNF141    ME-L--R--AIE-S-----C--PD--NL--D------R------·VAISN--LVTC--QRK--YN-

ZNF140    QGS---R--AI--SQ----W-QP--RDL--C------GH-----·LSIS----VSL--Q-K----G

ZNF7      MEV---G--A-H-S----QC--PG-RAL--E-----HSSVAG-A-FLVF--EL-S---Q-----VL

ZNF43           M--AIE-CL---QC--I---NL---------R---F-·-IAVS---L-TC-LQEK---EP

ZNF45     KEA------A-V-SE--LQ---L--RKL--D-----FR-V--V·-H-ST.--GLPQ--RE-KL-MM

ZNF91     MG-L--R--AIE-SP---QC------NL---------R--AF-·-IA-S---L-TY--Q-K---NM

HTF9      MGPLE-R--AIE-SL---HC------NL--D------RH--F-·-IVV----L-TC--Q-KK-FT-

HTF12     MGPL--R--KIE-SL---QC----PGNL--D------R---F-·······················

HPF4      MGPL--R--AIE-SLK--QC-----RNL---------R---F-·-ITVS---LLITTCLL-QGKEA

CONSENSUS:.L.F.DV...F..EEW..LD..Q..LYR.VMLENY...LV..G....KPDL...LE.....W..
                                helix 1                   helix 2
```

FIG. 3. Comparison of several KRAB domains present in human Krüppel-type zinc finger proteins. The consensus of these KRAB domains has been used to identify amino acid residues mediating the repressor activity. Several mutations abolished the repressor activity (29).

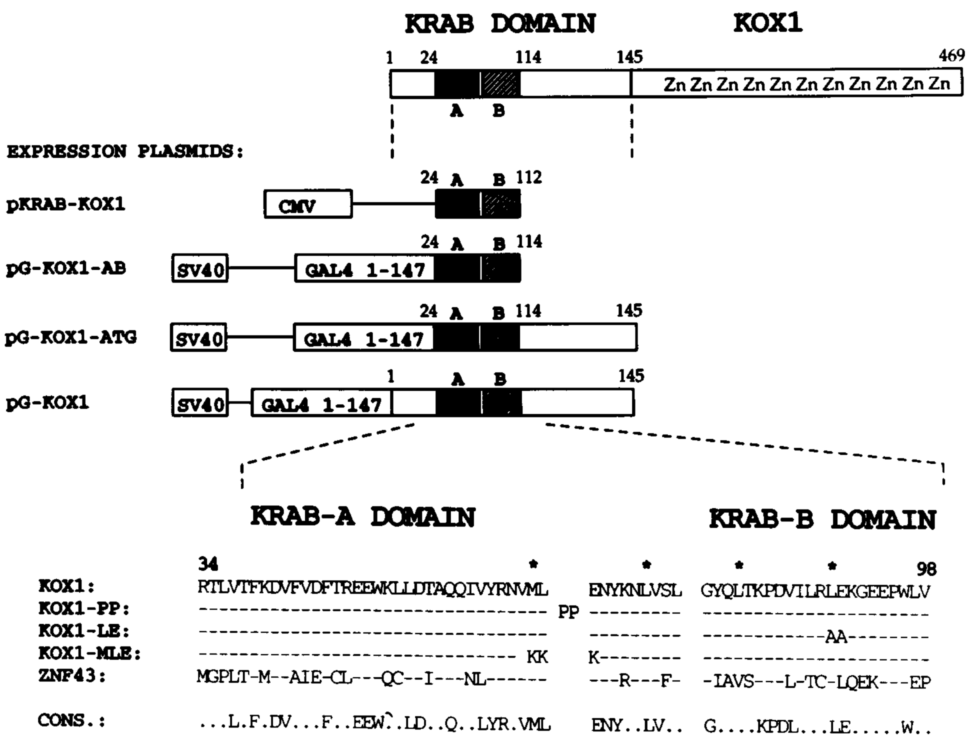

FIG. 4. Description of GAL(1-147)-KRAB(KOX1) fusion proteins. Expression constructs of these fusion proteins are cotransfected with reporter constructs that encode GAL4 DNA binding sites. Furthermore, the KRAB mutants were fused to NFκB and SP1 (Thiesen, unpublished), LexA (Georgiev, unpublished), and tetracycline repressor (13).

pressed in the presence of zinc induction. Endogenous transcription factors activated during addition of zinc were kept functionally inactive due to the presence of coexpressed GAL4-KRAB protein (Georgiev, unpublished). Once strategies have been established to engineer DNA binding domains for given DNA binding domains, the KRAB domain of KOX1 seems to be a potent effector domain to generate transcription factors for repressing endogenous gene expression.

Transcriptional Repressor Mechanisms of Gene Expression

Transcription of eukaryotic genes is regulated by transcription factors that in most cases present a modular structure and consist of at least two different functional domains: a DNA binding domain and effector domains that mediate and/or determine the function of these proteins. These types of transcription factors interact with their cognate DNA binding sites in a sequence-specific manner whereas effector functions are thought to be mediated by domains that interact with factors of the basal transcriptional machinary [for review see Drapkin et al. (14)]. Recently, a factor of around 110 kDa designated Silencing Mediating Protein-1 (SMP-1) has been partially analyzed that most likely mediates the repression exemplified by the KRAB domain of KOX1 (13). SMP-1 presents a candidate for a putative corepressor protein that possibly interferes with transcriptional processes of gene activation in a dominant fashion (Fig. 5). Once KRAB zinc finger proteins

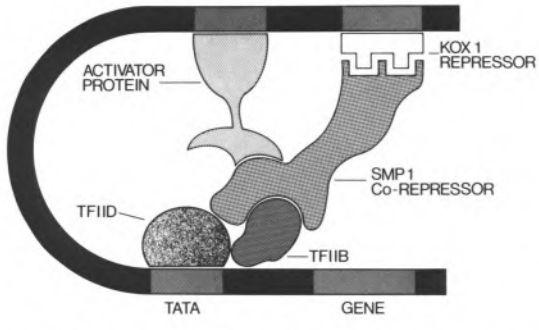

FIG. 5. Model of how KRAB containing zinc finger proteins repress gene expression. This model is based on our experimental data that were obtained during the analysis of stably expressed tetracycline-repressor-KRAB (TetR-KRAB) fusion proteins in HeLa cells (13). In particular, our immunoprecipitation of TetR-KRAB led to the identification of a KRAB-associated protein of 110 kDa designated silencing mediating protein-1 (SMP-1). Interestingly, KRAB zinc finger proteins possibly enhance the local concentration of KRAB-associated proteins at *cis*-acting elements recognized by KRAB zinc finger proteins, exemplified by KOX1.

are selectively bound to specific regulatory elements, effector domains of the KRAB-KOX1 type (13) possibly increase the local concentrations of factors such as SMP-1 in the neighborhood of promoters. It is likely that KRAB–SMP-1 complexes interfere with transcriptional processes of gene activation (Fig. 5).

Interestingly, because KRAB zinc finger proteins constitute a large family of proteins, SMP-1 might be a transcriptional cofactor mediating functions of several, if not all, KRAB zinc finger proteins. However, functional KRAB-specific repressor activities have not been detected in insect and in yeast cells to date (Thiesen, unpublished). Our data indicate that the KRAB domain might have evolved at the time when amphibians evolved on earth.

To determine that the KRAB domain represses transcription in a orientation- and distance-independent manner, GAL4-mutants shown in Fig. 4 were cotransfected with pGL2-promoter constructs (Promega) with and without GAL4 DNA binding sites (44) that were placed in the Bgl II and in the BamH1 restriction sites of pGL2 constructs. Only in cases where reporter constructs harbor DNA target sites were strong silencing activites obtained in a distance- and orientation-independent manner (Fig. 6). If the KRAB domain was coexpressed without any DNA binding domain (KK = KRAB-KOX1), the reporter constructs were hardly effected in their native expression activities.

To assess whether transcriptional repression requires 5 × GAL4 or only one GAL4 DNA binding site, reporter constructs were cotransfected with expression plasmids that expressed GAL4(1–147), GAL4-KRAB(KOX1), and GAL4-WT-1. It is interesting to note that the KRAB domain represses transcription on a single target site more efficiently than does the repression domain of Wilms tumor suppressor protein (WT-1) (Fig. 7).

To investigate whether the repressor activity of the KRAB domain is dominant over the activation mediated by VP16 (62), products of the expression plasmids GAL4-VP16 and GAL4-KOX1 were targeted to the same DNA targets represented by five GAL4 sites (Fig. 8). In ongoing experiments tetO (18), UAS (44), and LexA DNA binding sites (6) were placed in reporter constructs 2000 bp apart from each other and cotransfected with corresponding expression plasmids (Thiesen, unpublished). The KRAB domain even repressed the function of VP16 containing transcription factors when the binding sites were separated by 2 kb. Experiments of this type (Fig. 15) excluded that

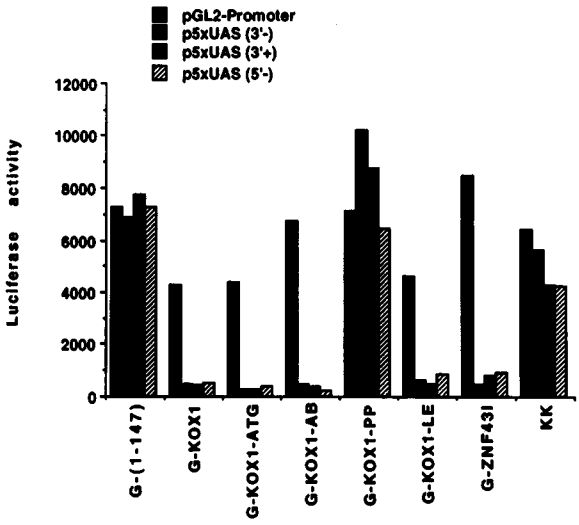

FIG. 6. Silencing of GAL1-KRAB constructs in transient cotransfection assays. GAL1 mutants shown in Fig. 4 were cotransfected with pGL2 promoter constructs (Promega) with and without GAL4 DNA binding sites placed in the Bgl II (p5xUAS 5′ −) and in the BAMH1 restriction sites (5xUAS 3′+ and 3′ −). Only in cases where reporter constructs harbor DNA target sites were strong silencing activities obtained in a distance- and orientation-independent manner. When the KRAB domain was coexpressed without any DNA binding domain (KK = KRAB-KOX1), the reporter constructs were hardly effected in their native repressor activities.

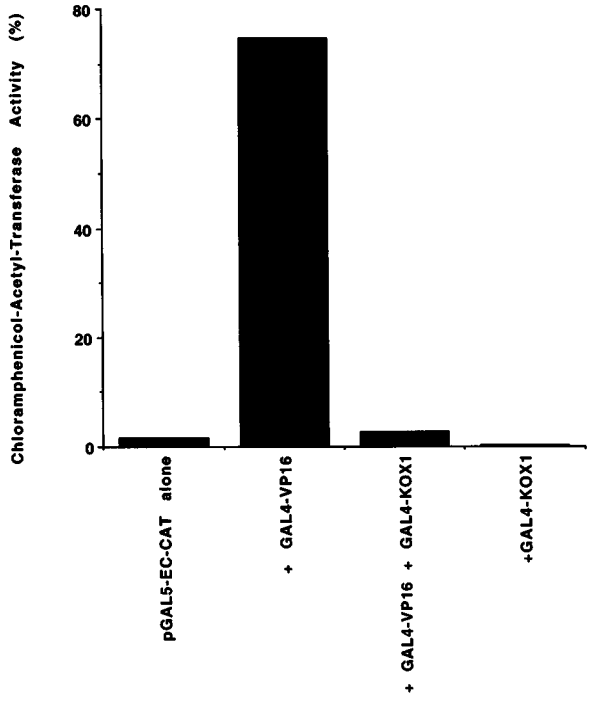

FIG. 8. The repressor activity of the KRAB domain is dominant over the activation mediated by VP16. The products of the expression plasmids GAL4-VP16 and GAL4-KOX1 are targeted to the same DNA targets represented by five GAL4 sites. KRAB-mediated repression is dominant over VP16-mediated activation of gene transcription.

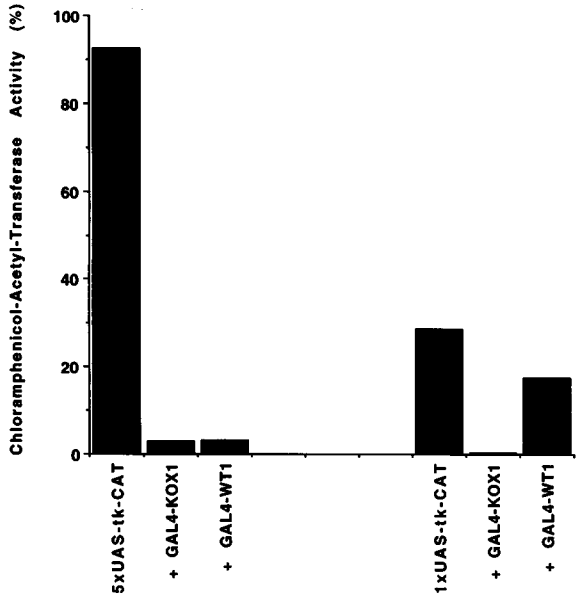

FIG. 7. Comparison of reporter constructs (tk-CAT) that harbor 5 × GAL or only one GAL4 binding site. Reporter constructs were cotransfected with expression plasmids that expressed GAL4(1-147), GAL4-KRAB(KOX1), and GAL4-WT-1. It is interesting to note that the KRAB domain represses transcription on a single target site more efficiently than the repression domain of Wilms tumor suppressor protein (WT-1).

the observed dominance of the KRAB domain was just due to the fact that two transcription factors simultaneously compete for the binding to the same DNA target region.

To analyze whether the repressor activity of the KRAB domain is sufficient to regulate gene expression of stably integrated genes, stable, double-transfected HeLa cells (TIS-10) carrying a chromosomally integrted ptetO7-CMV-L reporter construct and expressing the TetR-KRAB protein (13) were grown in the presence of tetracycline. Tetracycline binds to the tretracycline repressor and releases TetR-KRAB protein bound to tetO7 target sites (18). Addition of tetracycline induced a 50-fold induction of transcriptional gene expression by removing TetR-KRAB proteins from their target sites (13). At day 5 of tetracycline exposure the induction of luciferase expression was hindered because the HeLa cells could no longer be kept at confluency (Fig. 9).

Recently, the phenomen that the KRAB domain totally inactivates the transcriptional activity of VP16 was employed to establish a transient cotransfection assay for comparing transcriptional repressor activities. This assay system has been

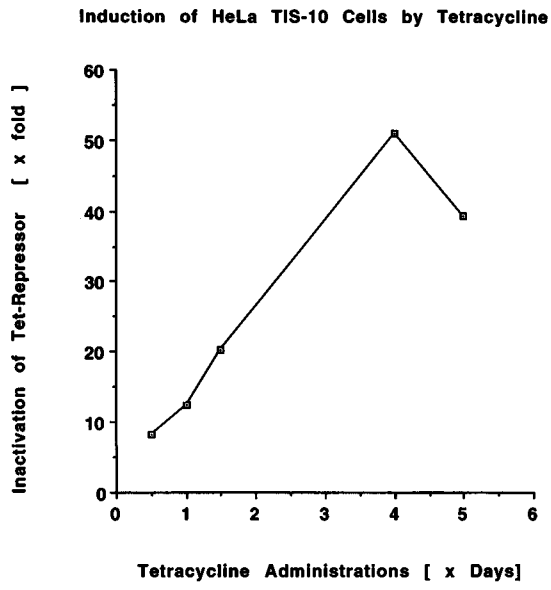

FIG. 9. Induction of luciferase expression by addition of tetracycline. Stable, double-transfected HeLa cells (TIS-10) carrying a chromosomally integrated ptetO7-CMV-L reporter construct and expressing the TetR-KRAB protein (13) were grown in the presence of tetracycline. Tetracycline binds to the tetracycline repressor and releases TetR-KRAB protein bound to tetO7 target sites. At day 5 the induction of luciferase expression did not further increase because the HeLa cells could no longer be kept at confluency.

designated trancriptional repressor assay (TRA). Repressor domains fused to GAL4-VP16 constructs are going to be compared to GAL4-VP16 constructs in transient cotransfection assays with reporter constructs that contain GAL4 DNA binding sites (61).

DNA-Protein Interactions of Krüppel-Type Zinc Finger Domains

The architecture of zinc finger domains identifies the zinc finger itself as an ideal backbone for designing DNA binding domains with DNA target site specificities for DNA target sites of interest. In particular, the Krüppel-type zinc finger domains present in SP1, in KOX1-32, and in more than 150 other human zinc finger proteins display common rules specifying zinc finger-specific DNA-protein interactions. The presence of a zinc finger-specific DNA recognition code was initially put forward by the structural analysis of zinc finger domains present in the KOX gene family (51). Mutagenesis analysis of zinc finger domains demonstrated that DNA binding characteristics of individual zinc fingers can be altered by varying one or more amino acid residues of the α-helical positions (32,54). However, by making use of the Target Detection Assay (50,52), novel target site specificities were determined for mutant SP1 proteins (54,55,58).

Zinc finger domains of zinc finger proteins encompassing zinc fingers of SP1, ZIF268, KROX20, and KOX(1-30) were compared, demonstrating that individual zinc finger were identified to contain almost identical amino acid residues in the helical region of the putative DNA binding portion of the zinc finger. This sequence comparisons supported our hypothesis of the presence of a zinc finger-specific DNA recognition code. Substituting SP1 zinc finger domains by KOX-derived zinc finger domains strengthened our hypothesis that the zinc finger structure fulfills the criteria of a passe-partout: specific amino acids interact with the DNA with variable affinities depending on the amino acids present in the α-helical zinc finger region (51). By subjecting mutant proteins to our target detection assay (TDA), we found that all three zinc finger proteins were involved in contacting cognate SP1 target sites (Schröder, diploma thesis, 1992). Our model of colinear binding of all three SP1 zinc finger is given in Fig. 10. Concomitantly, our working hypothesis was verified by crystal structures of ZIF268-DNA complexes (37). The fascinating feature derived from crystal structures of zinc finger-DNA complexes is that three amino acids from the α-helix contact three adjacent bases (a triplet) of their cognate DNA binding site (37). Our mutagenesis analysis of SP1 indicates that in particular the arginine residues of the second SP1 zinc finger protein most likely interact with the guanine residues of the triplet GCG present in the cognate binding site GGG GCG T/G GG of SP1. Thus, in analogy to the crystal structure of ZIF 268, the following model for SP1-specific DNA recognition has been postulated (Fig. 11). This model guided us in our mutagenesis analysis of SP1 protein in 1990 and 1991. Because SP1 zinc

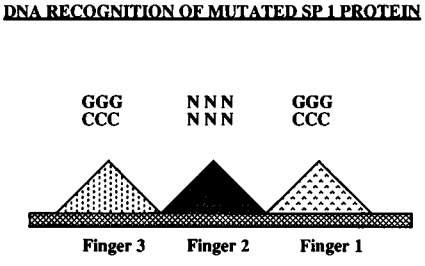

FIG. 10. Model for DNA-protein interactions of zinc finger proteins. This model guided us in our mutagenesis analysis of SP1 protein in 1990 and 1991.

finger 2 resembles zinc finger 1 and 3 of ZIF268, X-ray analysis of ZIF268-DNA cocrystals finally confirmed the exact contact sites between amino acid residues and corresponding nucleic acid residues in ZIF 268 and presumably in SP1 (37).

Determination of DNA Target Sites by the Target Detection Assay

Mutational analysis of SP1 zinc fingers demonstrated that amino acid residues that are not highly conserved in the helical region of individual zinc finger domains determine the specificity of sequence-specific DNA recognition (53,54). To verify our notion of the presence of a zinc finger-specific recognition code, we mutated the third and the second zinc finger of SP1, demonstrating that DNA binding specificities were changed by substituting amino acid residues in the helical region of individual zinc finger domains (53,54). Our mutagenesis of the second SP1 zinc finger domain did not interfere with DNA recognition of SP1 zinc fingers 1 and 3. It is noteworthy that by making use of the TDA (50), novel DNA binding specificities could be detected for mutated SP1 protein. By just mutating the second finger of SP1 the binding specificity of the mutated second SP1 finger could be determined because SP1 fingers 1 and 3 still recognized their cognate DNA binding sites of the consensus sites GGG NNN NGG (54).

A pool of GGG NNN NGG oligonucleotides has been subjected to recombinant SP1 zinc finger domains. The following oligonucleotides were selected regarding the random positions NNNN:

Number:	$N = 1$	$N = 2$	$N = 3$	$N = 4$
6×	G	C	G	T
5×	G	T	G	T
3×	G	A	G	T
2×	G	C	G	G
2×	G	T	G	G
2×	G	A	G	G
1×	G	G	C	G

From the sequence analysis of the selected oligonucleotides we arrived at the conclusion that the SP1 zinc finger protein is positioned by sequence-specific interactions of SP1 zinc finger 2. Based on these results, DNA protein interactions of SP1 protein shown in Fig. 11 have been derived. In particular, the two arginine residues present in the second SP1 zinc finger most likely interact with the guanine residues at nucleotide position $N = 1$ and $N = 3$.

After having mutating the second finger of SP1, recombinant SP1 zinc finger domains were subjected to the TDA. The pool of oligonucleotides encompassed the pattern GGG NNN NGG

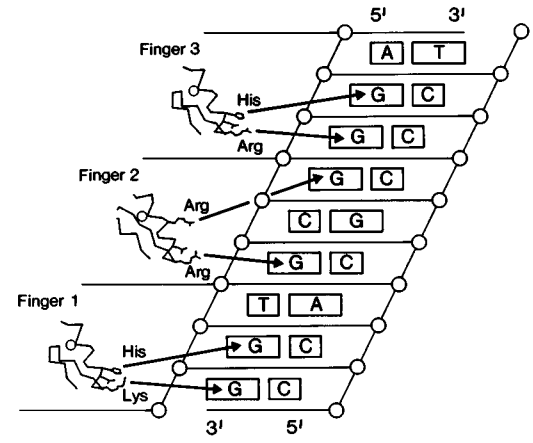

FIG. 11. Model of how SP1 zinc finger proteins interact with their cognate target sites. Because zinc fingers in SP1 and ZIF268 are quite homologous in sequence, X-ray analysis of ZIF268-DNA cocrystals has been used to confirm the exact contact sites between amino acid residues and corresponding nucleic acid residues in SP1 (37). By subjecting the mutant proteins to our target detection assay (TDA), we found that all three zinc finger domains of SP1 were involved in contacting the cognate site of GGG GCG GGG.

(54). TDA selections of mutated SP1 proteins revealed that SP1 zinc fingers 1 and 3 retained their DNA binding specificities (54,58). Thus, finger 1 and finger 3 guide the mutant SP1 protein to bind to a pool of sequences of GGG NNN NGG in which finger 1 binds to NGG and finger 3 binds to GGG. Mutations of SP1 finger 2 did not affect the positioning of the mutant protein on the DNA (Thiesen, unpublished). A similar strategy has been applied for displaying mutant zinc finger domains on bacteriophages (7). ZIF 268 and SP1 contain three consecutive zinc fingers that bind to GCG G/TGG GCG and GGG GCG TGG, respectively. DNA binding specificities of SP1 and ZIF 268 were shown to be altered after having mutated the second zinc finger (7,8,32,54).

Due to these structural constraints of zinc finger proteins a complete affinity matrix of individual zinc finger mutants was established (Bach, diploma thesis, 1991). According to the consensus GGG NNN GGG all 64 possible DNA binding sites were generated. Finally, a complete affinity matrix was established for the DNA-protein interactions of SP1 protein mutants MQ135 (58) and MQ91 (Fig. 12). The helical region of the second SP1 zinc finger RSDE L QR H were replaced by QSSY L IK H in MQ91, in *E. coli* expressed, purified, renatured, and subjected to electromobility shift assays (EMSA). The complete repertoire of binding sites covering the local region contacted by the second zinc finger of SP1 was analyzed (Bach, diploma thesis, 1991). Furthermore, for

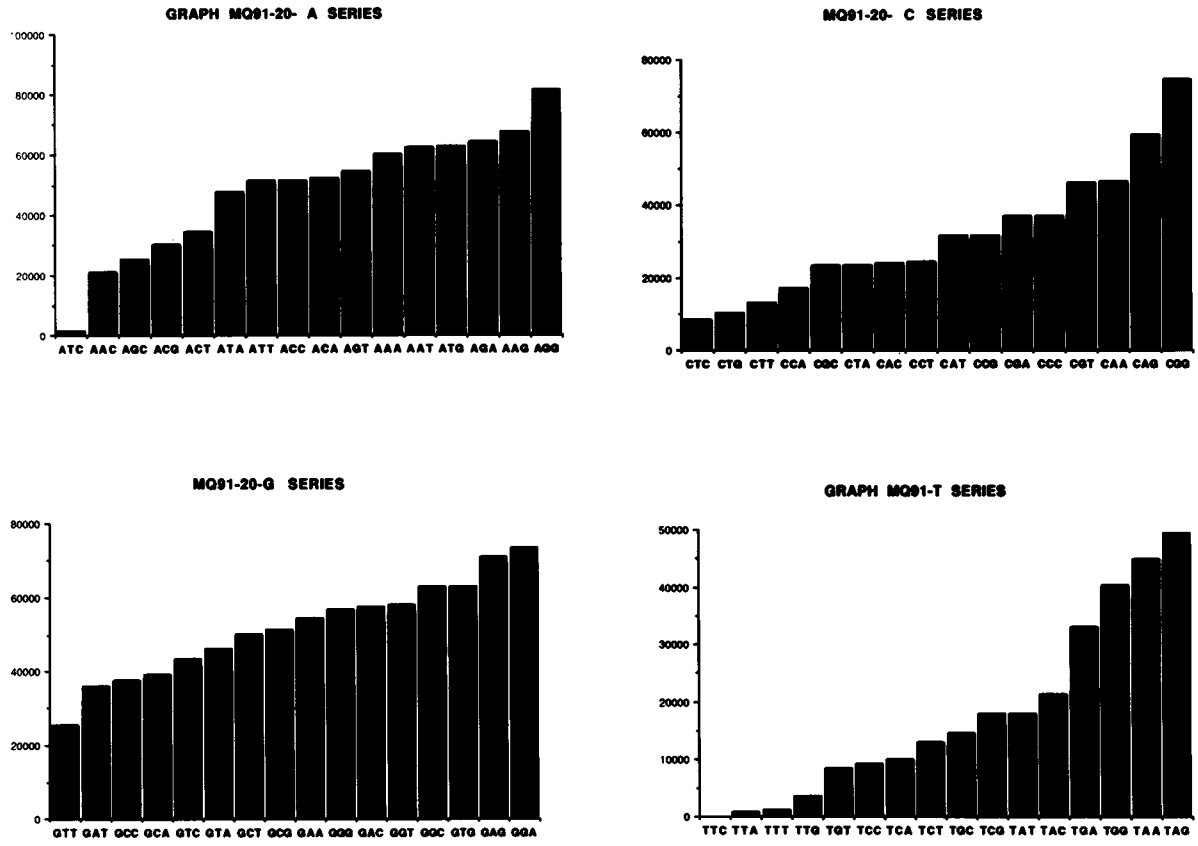

FIG. 12. An affinity matrix was established for the SP1 mutant proteins MQ91. The helical region of the second SP1 zinc finger RSDE L QR H was replaced by QSSY L IKH, in *E. coli* expressed, purified, renatured, and subjected to electromobility shift assays (EMSA). The complete repertoire of binding sites covering the local region contacted by the second zinc finger of SP1 was used.

the first time target sites were determined displaying worst affinities for particular zinc finger domains (58).

In summary, the backbone of ZIF 268 and SP1 zinc finger proteins seem to present ideal structures to engineer zinc finger mutants in the second zinc finger and to select binding specificities to one of the 64 possible DNA binding sites that can be recruited from a triplet of 64 putative nucleotide combinations (43 = 64).

Designer Zinc Finger Domains Selected by Phage Display Techniques

Filamentous bacteriophages have been utilized for displaying peptides or protein domains on their surfaces. By fusing immunoglobulin variable region genes (V-genes) to the phage gene 3 protein (g3p), antigen binding sites with novel antigen specificities have been selected (21,30).

Recently, phage display libraries have successfully been applied to select DNA binding domains of interest. Instead of using specific antigens for selection, double-stranded DNA sequences were used to select individual zinc finger domains specific for particular DNA binding sites (7,8,23). In particular, Choo et al. (9) showed that zinc finger domains can be generated specific for oncogenic sequences by making use of zinc finger domains displayed on filamentous phages. To adapt phage display techniques for selecting zinc finger domains, candidate zinc finger structures derived from ZIF268 were used whose first and third finger both bind to GCG sequences. ZIF 268 mutant proteins with amino acid substitutions in the second zinc finger do recognize the consensus GCG NNN GCG. Based on our own observation, the application of ZIF268 instead of SP1 protein structures might be advantageous because ZIF268 seems to be more rigidly positioned on its cognate consensus binding site than SP1. In general, zinc fingers that recognize GCG motifs are, in our opinon, more selective than zinc finger 3 of SP1 that binds to GGG.

In the phage display procedure of Choo and Klug (7,8), a library of zinc finger domains is generated having mutations in the second finger of ZIF268. Mutant ZIF 268 domains are fused to

the minor coat protein (pIII) of bacteriophage fd. Thus, mutated zinc finger domains are expressed on the tip of the capsid. Nucleotides harboring binding sites for desired triplets are generated in which selected sequences of three nucleotides are surrounded by GCG sequences. These double-stranded oligonucleotides are attached to streptavidin-coated paramagnetic beads and are used to select phages that are bound to these sequences. After three rounds of selection phages are purified and sequences of zinc finger domains selected are analyzed. Each zinc finger selected can be considered to be an independent DNA binding unit recognizing particular DNA triplets. Thus, a DNA binding site of nine consecutive nucleotides can be recognized by three zinc finger domains of which each binds to three nucleotides in a linear manner. Finally, an appropriate zinc finger protein is generated by combining individually selected zinc finger domains present in the pool of ZIF268-derived zinc finger mutants. Thereafter, newly engineered proteins have then to be tested in terms of their DNA binding properties in vitro and in vivo.

Functional Analysis of Designer Zinc Finger Proteins

Biochemical and biophysical properties of engineered designer zinc finger domains have to be characterized prior to their final application. In this respect, the TDA (50) might be ideal for determining DNA binding preferences to exclude that DNA targets are recognized with higher affinities than target sites used for selection. For facilitating a profound biochemical analysis of recombinant designer proteins, procaryotic expression systems might be advantagous to be utilized [see (52)]. In particular, purified zinc finger mutant protein could be subjected to the TDA to determine high-affinity binding sites. The presence of high-affinity binding sites different to target sites selected for can be determined by the TDA analysis (50). The analysis of the repertoire of DNA target sites is required to predict binding preferences of designer proteins under study.

In Vivo Analysis of Designer Zinc Finger Proteins

In transient cotransfection assays, cell lines and primary cell cultures ought to be used to study the effect of stably transfected designer genes. To evaluate whether engineered zinc finger proteins recognize desired DNA target sites in vivo, transient cotransfection assay systems might be suitable to start with. Reporter constructs should at least contain the desired DNA target sites in a triple copy number. The designer zinc finger domains could be fused to effector domains, such as VP16 or KRAB(KOX1) to determine functionally active DNA binding affinities. Once reasonable long-term expressions have been observed in tissue cultures, transgenic mice constitute an informative model to finally determine the in vivo function and to assess side effects of these designer constructs on endogenous cellular functions (16). If transacting functions are mediated via the interactions of an engineered DNA binding domain with its prospective binding site, a factor fulfilling the classification of TRM would have been generated.

APPLICATION OF TRANSCRIPTION RESPONSE MODIFIERS

First of all, it has to be admitted that our understanding of KRAB zinc finger proteins and their in vivo functions is still at its infancy. However, it is noteworthy to propagate ideas that outline the feasibility of designer protein in gene therapy and in particular in the management of HIV infections. The first question that has to be answered is whether TRMs consisting of designed zinc finger domains can be engineered that specifically recognize HIV sequences. The second question would be whether TRMs stably transfected into human T cells render these cells resistant to HIV replication. However, the most important question would be whether functionally active TRMs against HIV sequences do not interfere with T-cell function and with lymphoid differentiation.

Because most of human zinc finger proteins have not been studied in detail, it is possibly too early to make profound predictions about the prospects of designer transcription factors in general. Though the application in human gene therapy might be the ultimate goal, a serious evaluation has to take into account that an engineered DNA binding domain of three individual zinc fingers should only recognize nine nucleotides. In theory, an artificial transcription factor speciic for nine nucleotides would bind to too many genomic target sites in order to be capable of exclusively regulating one particular target gene. Thus, strategies for selecting and engineering zinc finger domains consisting of at least six zinc fingers have to be developed. It is an open question whether DNA binding domains can be generated that are capable of recognizing one sequence within the human genome. In the end of the designer process, gene therapist might discover that their final gene prod-

uct might be immunogenic to the human organism. In this case, sequence information on endogenously occurring zinc finger domains might be advantageous to reduce immunogenicities of particular TRMs by site-directed mutagenesis.

The following applications of TRMs can be foreseen:

1. The expression of endogenous genes might be enhanced to modulate immune functions, such as MHC class I and class II expression.
2. Endogenous oncogenes ought to be suppressed (8).
3. Intracellular immunizations against HIV might be performed.

Perspectives of Artificial Transcription Factors in HIV Therapy

One of the first experiments we conducted was to determine whether the KRAB domain is capable of preventing the HIV promoter from becoming activated in the presence of coexpressed HIV-TAT (59,60). Because zinc finger domains specificly recognizing HIV sequences are not available, we fused the KRAB domain to the TAT protein itself, expecting to inactivate TAT-mediated gene expressions. In Fig. 13, we demonstrate that the KRAB domain fused to TAT protein does not significantly reduce the rate of TAT-mediated activation of gene expression. This finding was surprising because GAL4-KRAB domains targeted distal to the reporter gene in pHIV-CAT were efficiently repressed (Thiesen, unpublished). Furthermore, activation of gene expression by GAL4-VP16 constructs was totally abrogated by GAL4-KRAB (Fig. 8) and TETR-KRAB expression constructs (Thiesen, unpublished). We assumed that mutated TAT would be applicable to target the KRAB domain to the HIV genome. I outlined our opinion to Luigi Lania at the Biotech meeting in Flourence in 1994. In the meantime, his group generated a TAT-KRAB construct using mutated TAT. Their data demonstrated that transduced TAT-KRAB constructs interfere with the transcriptional regulation of the HIV promoter (38). Despite their promising results, I personally think that the KRAB domain should be directly targeted to HIV sequences to utilize the complete potency displayed by the KRAB domain in repressing transcriptional gene expression.

Our model of KRAB-mediated gene regulation (Fig. 5) makes the following predictions. 1) Once the KRAB domain is bound to the DNA, the local concentration of the cofactor SMP-1 is raised and repression of gene expression occurs. 2) Because

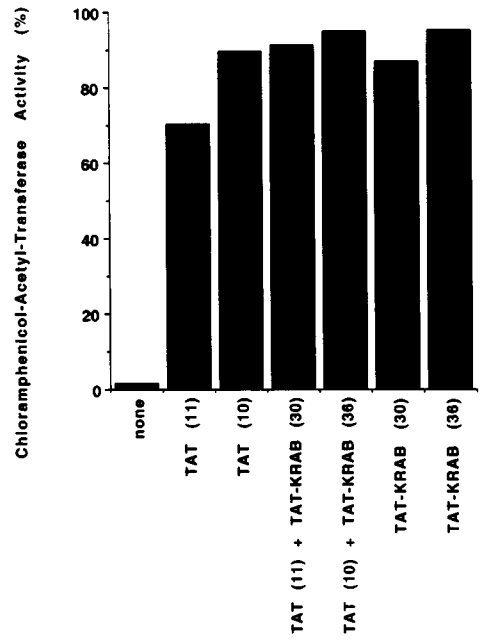

FIG. 13. Transactivation of the HIV promoter by the fusion protein TAT-KRAB. The KRAB domain fused to HIV-TAT did not interfere with the TAT-mediated activation of the HIV promoter as we reported at the Biotech 94 in Florence in 1994. In comparison to Fig. 14, we concluded that the TAT-mediated activation cannot be prevented by the KRAB domain once the KRAB domain is fused to wild-type TAT. The numbering of expression constructs used indicates that different plasmid preparations were used. HIV-CAT was used as reporter construct.

numerous KRAB zinc finger proteins are expressed in one cell (51,57), the SMP-1 protein should be quite abundantly expressed to serve for several KRAB zinc finger proteins. 3) Local changes in the concentration of SMP-1 molecules determine whether a promoter is going to be activated or to be inactivated.

Based on these assumptions above, it is likely that the SMP-1 protein is a much stronger repressor than the KRAB domain itself once tethered to DNA binding domains. Therefore, I favor replacing the KRAB domain with the repression domain of SMP-1 (Fig. 5) to enhance the efficacy of TRM constructs. However, the secret of interfering with endogenous gene expression is to direct these factors voluntarily to regulatory sequences of therapeutic importance. One possibilty would be to design DNA binding domains specific for sequences that naturally occur at genes of clinical relevance.

However, if artificial transcription factors have to compete with transcription factors binding to the same target sites, significant regulatory effects are only obtained when the engineered factor itself is dominant in its function. However, engineered

TRMs would only be of any therapeutic value if they are capable of counteracting regular functions of endogenous transcription factors. The principle of this approach has already been demonstrated on several transdominant-negative transcription factors (46). HeLa cells that stably express TETR-KRAB (13) suppressed the function of transduced GAL4-VP16 constructs. Only in the presence of tetracycline, which relieved the repressor activity of TETR-KRAB, did the activator protein GAL4-VP16 regain its transcriptional activity (Fig. 14).

As long as viral genes that have been incorporated in the genome have to be protected from becoming transcriptionally active, repression domains have to be selected or engineered that only represent one state of regulatory activity. In the case of HIV infection, the repression domain has to prevent transcriptional gene activation under any circumstances. In regard to the DNA binding specificity of TRMs, a strong repressor domain might generate side effects due to DNA-protein interactions of genomic target sites that resemble target sites present in the HIV genome.

Today, with the advent of phage display techniques, a novel and efficient screening method has been adapted for the selection of DNA binding domains specific with selected DNA target preferences (7,8,23,70). In principle, TRMs can be generated that consist of engineered and designed DNA binding domains and of transacting effector domains such as VP16 or KRAB domains. Once DNA binding domains can be designed for any DNA sequence of interest, artificial transcription factors can be utilized for modulating endogenous gene expression. On the way to a therapeutic application of TRMs many hurdles have to be taken.

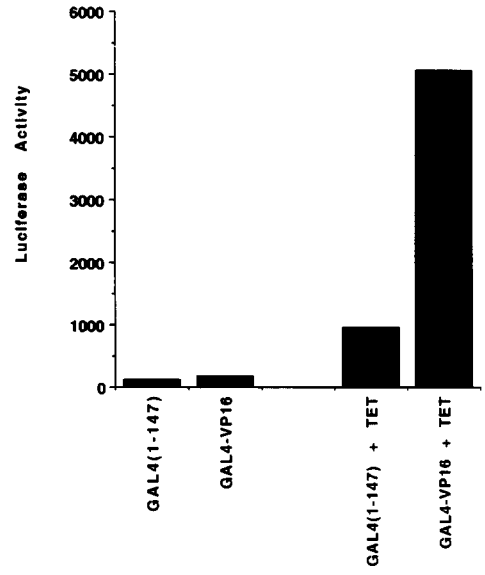

FIG. 14. TetR-KRAB repressors are dominant over GAL4-VP16-mediated activation of gene expression. Reporter plasmids of the pGL2-Control-luciferase family containing tetO7 binding sites and GAL4 binding sites inserted at the Bgl II site were cotransfected in HeLa cells that stably expressed the TetR-KRAB protein (13). Cotransfected GAL4-VP16 (1 μg) did not show any transcriptional activity. However, when tetracycline (1 $\mu g/\mu l$) was added the reporter construct was activated from 175 to 5067 of relative luciferase units. The addition of tetracycline to the reporter construct without cotransfecting GAL4-VP16 confirms that the strong activation displayed by GAL4-VP16 in the presence of tetracycline is due to the combined effect of transcription factors binding to the SV40 promoter and to the activation domain of GAL4-VP16.

Many problems might not even be obvious to us at the moment. Definitely, research projects concerned with designing molecules for modulating transcriptional gene regulation will at least improve our understanding of gene regulation in mammalian organisms.

REFERENCES

1. Abrink, M.; Aveskogh, M.; Hellman, L. Isolation of cDNA clones for 42 Kruppel-related zinc finger proteins expressed in the human monoblast cell line U-937. DNA Cell. Biol. 14(2):125-136; 1995.
2. Baltimore, D. Gene therapy: Intracellular immunization. Nature 335:395; 1988.
3. Bellefroid, E. J.; Lecocq, P. J.; Benhida, Q.; Poncelet, D. A.; Belayew, A.; Martial, J. A. The human genome contains hundreds of genes coding for finger of the Krüppel type. DNA 8:377-387; 1989.
4. Bellefroid, E. J.; Poncelet, D. A.; Lecocq, P. J.; Revelant, O.; Martial, J. A. The evolutionarily conserved Kruppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. Proc. Natl. Acad. Sci. USA 88:3608-3612; 1991.
5. Bray, P.; Lichter, P.; Thiesen, H.-J.; Ward, D. C.; Dawid, I. B. Characterization and mapping human zinc finger protein-encoding genes. Proc. Natl. Acad. Sci. USA 88:9563-9567; 1991.
6. Brent, R.; Ptashne, M. An eukaryotic transcriptional activator bearing the DNA specificity of a procaryotic repressor. Cell 43:729-736; 1985.
7. Choo, Y.; Klug, A. Toward the code tor the interactions of zinc fingers with DNA: Selection of randomized fingers displayed on phage. Proc. Natl. Acad. Sci. USA 91:11163-11167; 1994.
8. Choo, Y.; Klug, A. Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. Proc. Natl. Acad. Sci. USA 91:11168-11172; 1994.

9. Choo, Y.; Sanchez-Garcia, I.; Klug, A. In vivo repression by a site-specific DNA. Nature 372:642–645; 1994.
10. Cook, W. J.; Mosley, S. P.; Audino, D. C.; Mullanay, D. L.; Rovelli, A.; Stewart, G.; Denis, C. L. Mutations in the zinc-finger region of the yeast regulatory protein ADR1 affect both DNA binding and transcriptional activation. J. Biol. Chem. 269(12): 9374–9379; 1994.
11. Crystal, R. G. Transfer of genes to humans: Early lessons and obstacles. Science 270:404–410; 1995.
12. Desjarlais, J. R.; Berg, J. M. Length-encoded multiplex binding site determination: Application to zinc finger proteins. Proc. Natl. Acad. Sci USA 91(23): 11099–11103; 1994.
13. Deuschle, U.; Meyer, W. K.-H.; Thiesen, H.-J. Tetracycline reversible silencing of complex eukaryotic promoters. Mol. Cell. Biol. 15:1907–1914; 1995.
14. Drapkin, R.; Merino, A.; Reinberg, D. Regulation of RNA polymerase II transcription. Cell Biol. 5: 469–476; 1993.
15. Dropulic, B.; Jeang, K.-T. Gene therapy for human immunodeficiency virus infection: Genetic antiviral strategies and targets for intervention. Human Gene Ther. 5:927–939; 1994.
16. Furth, P. A.; Onge, L. St.; Böger, H.; Gruss, P.; Gossen, M.; Kistner, A.; Bujard, H.; Henninghausen, L. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc. Natl. Acad. Sci. USA 91:9302–9306; 1994.
17. Gashler, A. L.; Swaninathan, S.; Sukhatme, V. P. A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. Mol. Cell. Biol. 13:4556–4571; 1993.
18. Gossen, M.; Freundlieb, S.; Bender, G.; Muller, G.; Hillen, W.; Bujard, H. Transcriptional activation by tetracyclines in mammalian cells. Science 268(5218):1766–1769; 1995.
19. Han, K.; Manley, J. L. Functional domains of the *Drosophila* Engrailed protein. EMBO J. 12(7): 2723–2733; 1993; 1993.
20. Ho, D. D.; Neumann, A. U.; Perelson, A. S.; Chen, W.; Leonard, J. M.; Markowitz, M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123–126; 1995.
21. Hoogenboom, H. R.; Griffith, A. D.; Johnson, K. S.; Chiswell, D. J.; Hudson, P.; Winter, G. Multisubunit proteins on the surface of filamentous phage: Methodologies for displaying (Fab)heavy and light chains. Nucleic Acids Res. 19(15):4133–4137; 1991.
22. Huebner, K.; Druck, T.; Croce, C. M.; Thiesen, H.-J. Twenty-seven nonoverlapping cDNAs from human T cells encoding zinc finger structures map to nine different chromosomes with apparent clustering. Am. J. Hum. Genet. 48:726–740; 1991.
23. Jamieson, A. C.; Kim, S.-H.; Wells, J. A. In vitro selection of zinc fingers with altered DNA-binding specificities. Biochemistry 33:5689–5695; 1994.
24. Jones, K.; Kadonaga, J.; Luciw, P. A.; Tijan, R. Activation of the AIDS retrovirus promoter by the cellular transcription factor Sp1. Science 232:755–759; 1986.
25. Lange, R.; Christoph, A.; Thiesen, H.-J.; Vopper, G.; Johnson, K. R.; Lemaire, L.; Plomann, M.; Cremer, H.; Barthels, D.; Heinlein, U. A. O. Developmentally regulated mouse gene NK10 encodes a zinc finger repressor protein with differential DNA binding domains. DNA Cell Biol. 14:971–981; 1995.
26. Larder, B. A. Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. Gen. Virol. 75:951–957; 1994.
27. Licht, J. D.; Ro, M.; English, M. A.; Grossel, M.; Hansen, U. Selective repression of transcriptional activators at a distance by the *Drosophila* Kruppel protein. Proc. Natl. Acad. Sci. USA 90(23):11361–11365; 1993.
28. Madden, S. L.; Cook, D. M.; Morris, J. F.; Gashler, A.; Sukhatme, V. P.; Rauscher, J. F., III. Transcriptional repression mediated by the WT1 Wilms tumor gene product. Science 253:1550–1553; 1991.
29. Margolin, J. F.; Friedman, J. R.; Meyer, W. K.-H.; Vissing, H.; Thiesen, H.-J.; Rauscher, F. J. Kruppel-associated boxes are potent transcriptional repression domains. Proc. Natl. Acad. Sci. USA 91: 4509–4513; 1994.
30. McCafferty, J.; Griffith, A. D.; Winter, G.; Chiswell, D. J. Phage antibodies: Filamentous phage displaying antibody variable domains. Nature 348: 552–554; 1990.
31. Miller, J.; McLachlan, A. D.; Klug, A. Repetitive zinc-binding domains in the protein transcription factor III! from *Xenopus* oocytes. EMBO J. 4: 1609–1614; 1985.
32. Nardelli, J.; Gibson, T. J.; Vesque, C.; Charnay, P. Base sequence discrimination by zinc-finger DNA-binding domains. Nature 349:175–178; 1991.
33. Nardelli, J.; Gibson, T. J.; Vesque, C.; Charnsy, P. Zinc finger-DNA recognition: Analysis of base specificity by site-directed mutagenesis. Nucleic Acids Res. 20:4137–4144; 1992.
34. Nowak, M. A.; May, R. M.; Phillips, R. E.; Rowland-Jones, S.; Lalloo, D.; McAdam, S. Klenerman, P.; Köppe, B.; Sigmund, K.; Bangham, C. R. M.; McMichael, A. J. Antigenic oscillations and shifting immunodominance in HIV-1 infections. Nature 375:606–611; 1995.
35. Nowak, M. A.; McMichael, A. J. How HIV defeats the immune system. Sci. Am. 273:58–65; 1995.
36. Numoto, M.; Niwa, O.; Kaplan, J.; Wong, K. K.; Merrell, K.; Kamiya, K.; Yanagihara, K.; Calame, K. Transcriptional repressor ZF5 identifies a new conserved domains in zinc finger proteins. Nucleic Acids Res. 21:3767–3775; 1993.
37. Pavletich, N. P.; Pabo, C. O. Crystal structure of a

five-finger GLI-DNA complex: New perspectives on zinc fingers. Science 261(5129):1701–1707; 1993.
38. Pengue, G.; Caputo, A.; Rossi, C.; Barbanti-Brodano, G.; Lania, L. Transcriptional silencing of human immunodeficiency virus type 1 long terminal repeat-driven gene expression by the Kruppel-associated box repressor domain targeted to the transactivating response element. J. Virol. 69:6577–6580; 1995.
39. Pengue, G.; Calabro, V.; Bartoli, P. C.; Pagliuca, A.; Lania, L. Repression of transcriptional activity at a distance by the evolutionarily conserved KRAB domain present ina subfamily of zinc finger proteins. Nucleic Acids Res. 22:2908–2914; 1994.
40. Picard, D.; Salser, S. J.; Yamamoto, K. R. A movable and regulatable inactivation function within the steroid binding domains of the glucocorticoid receptor. Cell 54:1073–1080; 1988.
41. Pott, U.; Thiesen, H.-J.; Colello, R. J.; Schwab, M. E. A new Cys_2/His_2 zinc finger gene, rKr is expressed in differentiated rat oligodendrocytes and encodes a protein with a functional repressor domain. J. Neurochem. 65:1955–1966; 1995.
42. Rebar, E. J.; Pabo, C. O. Zinc finger phage: Affinity selection of fingers with new DNA-binding specifities. Science 263: 671–673; 1994.
43. Rousseau-Merck, M.-F.; Duro, D.; Berger, J.; Thiesen, H.-J. Chromosomal location of two KOX zinc finger genes on chromosome bands 7q21–q22. Ann. Genet. 38(2):81–84; 1995.
44. Sadowski, I.; Ma, J.; Triezenberg, S.; Ptashne, M. GAL4-VP16 is an unusually potent transcriptional activator. Nature 335:563–564; 1988.
45. Sauer, F.; Jäckle, H. Dimerization and the control of transcription by Kruppel. Nature 364:454–457; 1993.
46. Shamah, S. M.; Stiles, C. D. Transdominant negative mutations. Methods Enzymol. 254:565–576; 1995.
47. Shi, Y.; Berg, J. M. Specific DNA-RNA hybrid binding by zinc finger proteins. Science 268(5208): 282–284; 1995.
48. Suzuki, M.; Gerstein, M.; Yagi, N. Stereochemical basus of DNA recognition by Zn fingers. Nucleic Acids Res. 22(16):3397–3405; 1994.
49. Suzuki, M.; Yagi, N. DNA recognition code of transcription factors in the helix-turn-helix, probe helix, hormone receptor, and zinc finger families. Proc. Natl. Acad. Sci. USA 91:12357–12361; 1994.
50. Thiesen, H.-J.; Bach, C. Target detection assay (TDA): A versatile procedure to determine DNA binding sites as demonstrated on SP1 protein. Nucleid Acids Res. 18:3203–3209; 1990.
51. Thiesen, H.-J. Multiple genes encoding zinc finger domains are expressed in human T cells. New Biol. 2:263–374; 1990.
52. Thiesen, H.-J. Target detection assay (TDA): A general method to determine DNA binding sites for putative DNA binding proteins. Immunol. Methods IV:61–74; 1990.
53. Thiesen, H.-J.; Schröder, B. Amino acid substitutions in the SP1 zinc finger domain alter the DNA binding affinity to cognate SP1 target site. Biochem. Biophys. Res. Commun. 175:333–338; 1991.
54. Thiesen, H.-J.; Bach, C. Determination of DNA binding specificities of mutated zinc finger domains. FEBS Lett. 283:23–26; 1991.
55. Thiesen, H.-J. Design of DNA binding domains with desired DNA target specificities using the zinc finger specific code determinded by the target detection assay (TDA). J. Cell. Biochem. Suppl. 15G: R345; 1991.
56. Thiesen, H.-J.; Bellefroid, E. J.; Revelant, O.; Martial, J. A. Conserved KRAB protein domain identified upstream from the zinc finger region of KOX8. Nucleic Acids Res. 19:3996; 1991.
57. Thiesen, H.-J.; Meyer, W. Krab domains analysed in human Cys/His type zinc finger proteins KOX1. In: Zinc-finger proteins in oncogenesis: DNA-binding and gene regulation. New York: New York Academy of Sciences; 1993:243–245. (Ann. NY Acad. Sci., vol. 684.)
58. Thiesen, H.-J.; Bach, C. DNA recognition of C2H2-zinc finger proteins: Evidence for a zinc finger specific DNA recognition code. In: Zinc-finger proteins in oncogenesis: DNA-binding and gene regulation. New York: New York Academy of Sciences; 1993:246–249. (Ann. NY Acad. Sci., vol. 684.)
59. Thiesen, H.-J. Transcriptional silencing of the HIV promoter activated by HIV-TAT. Biotech 9(2):94; 1994.
60. Thiesen, H.-J.; Meyer, W. Transcription response modifiers (TRMs)—a promising perspective human gene therapy. Gene Ther. Suppl. 1:S22; 1994.
61. Thiesen, H.-J. Transcriptional gene regulation. In: Lefkovits, I., ed. Immunology methods manual. New York: Academic Press; in press.
62. Triezenberg, S. J. Structure and function of transcriptional activation domains. Genet. Dev. 5:190–196; 1995.
63. Tunnacliffe, A.; Liu, L.; Moore, J. K.; Leversha, M.; Jackson, M. S.; Ferguson-Smith, M. A.; Thiesen, H. J.; Ponder, B. A. J. Duplicated KOX zinc finger gene clusters flank the centromere of human chromosome 10: Evidence for a pericentromeric inversion during primate evolution. Nucleic Acids Res. 21:1409–1417; 1993.
64. Vissing, H.; Meyer, W.K.-H.; Aagaard, L.; Tommerup, N.; Thiesen, H.-J. Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins. FEBS Lett. 369:153–157; 1995.
65. Wang, Z. Y.; Qiu, Q. Q.; Gurrieri, M.; Hunag, J.; Deuel, T. F. WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. Oncogene 10(6):1243–1247; 1995.
66. Webster, N. J. G.; Green, S.; Jin, J. R.; Chambon, P. The hormone-binding domains of the estrogen

and glucocorticoid receptors contain an inducible transcription activation domain. Cell 54:199–207; 1988.

67. Wie, X.; Ghosh, S. K.; Taylos, M. E.; Johnson, V. A.; Emini, E. A.; Deutsch, P.; Lifson, J. D.; Bonhoeffer, S.; Nowak, M. A.; Hahn, B. H.; Saag, M. S.; Shaw, G. M. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373:117–122; 1995.
68. Wilson, T. E.; Day, M. L.; Pexton, T.; Padgett, K. A.; Johnston, M.; Milbrandt, J. In vivo mutational analysis of the NGFI-A zinc fingers. J. Biol. Chem. 267(6):3718–3724; 1992.
69. Witzgall, R.; O'Leary, E.; Leaf, A.; Onaldi, D.; Bonventre, J. V. The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediated transcriptional repression. Proc. Natl. Acad. Sci. USA 91(10):4514–4518; 1994.
70. Wu, H.; Yang, W. P.; Barbas, C. F. 3rd Building zinc fingers by selection: Toward a therapeutic application. Proc. Natl. Acad. Sci. USA 92(2):344–348; 1995.