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Gene activation by triplex-forming oligonucleotide coupled to the activating domain of protein VP16

Svetlana Kuznetsova, Slimane Ait-Si-Ali[1], Irina Nagibneva[1], Frederic Troalen[2], Jean-Pierre Le Villain[2], Annick Harel-Bellan[1] and Fedor Svinarchuk[3,4,*]

Laboratory of Nucleic Acid Chemistry, Lomonosov Moscow State University, Leninskie gory, 119899, Moscow, Russia, [1]Laboratoire 'Oncogénèse, Différenciation et Transduction du Signal', CNRS UPR 9079, CNRS, Institut Fédératif sur le Cancer (IFC-01), 7 rue Guy Moquet, 94801 Villejuif Cedex, France, [2]Unite des Marqueures Biologiques et Moleculaires, Institut Gustave Roussy, Villejuif, France, [3]Laboratoire Hematopoièse et cellules souches, INSERM U362, PR 1, Institut Gustave Roussy, rue Camille Desmoulins, 94805 Villejuif Cedex, France and [4]Department of Biochemistry, Novosibirsk Institute of Bioorganic Chemistry, 8 Prospect Lavrenteva, Novosibirsk, 630090, Russia

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ABSTRACT

Triplex-forming oligonucleotides (TFOs) are generally designed to inhibit transcription or DNA replication but can be used for more diverse purposes. Here we have designed a chimera peptide-TFO able to activate transcription from a target gene. The designed hybrid molecule contains a triplex-forming sequence, linked through a phosphoroamidate bond to several minimal transcriptional activation domains derived from Herpes simplex virus protein 16 (VP16). We show here that this TFO–peptide chimera (TFO-P) can specifically recognise its DNA target at physiological salt and pH conditions. Bound to the double-stranded target DNA in a promoter region, the TFO-P is able to activate gene expression. Our results suggest that this type of molecule may prove useful in the design of new tools for artificial modulation of gene expression.

INTRODUCTION

Triplex-forming oligonucleotides (TFOs) represent a new approach to artificially regulate gene expression through direct interaction with DNA. Indeed, TFOs directed against gene promoters can modulate transcription of the targeted gene (1).

TFOs can bind specifically to their target sequences even if these targets are present as a single copy in a DNA molecule as long as a yeast chromosome (2), a human chromosome (3) or even in the whole human genome (4), which makes them very attractive compounds for gene-targeted therapy. They have been designed mainly to bind in the vicinity of transcription factor target sites and to act as competitors for these proteins. Alternatively, they can introduce a lesion in a sequence-specific manner which will prevent DNA/RNA polymerase movement through the target template or could be used for gene targeted mutagenesis. In all these cases, TFOs play a negative role, inhibiting a biochemical process by preventing normal interactions between the targeted DNA and protein factors (reviewed in 5). However, TFOs could potentially be used for more diverse purposes. For example, TFO-based DNA bending ligands have been designed (6) as have hairpin-TFOs which are able to recruit transcription factors to the target DNA (7).

Oligonucleotide–peptide conjugates have several applications, mainly as potential inhibitors of gene expression, with the peptide helping intracellular delivery of the antisense oligonucleotides (8,9), directing oligonucleotides to specific compartments within the cell (10,11) or having dual binding capability for a designed RNA (12). In the present work we have designed a TFO–peptide chimera (TFO-P) which is able to activate a promoter containing the target sequence.

These molecules (TFO-Ps) are bi-functional chimeras, which contain several repeats of a peptide transcriptional activation domain, linked through a phosphoroamidate bond to an oligonucleotide which forms a triple helix on a specific target DNA sequence (Fig. 1). The oligonucleotide that we have used in this study contains a homopurine triplex-forming sequence which is targeted to the *vpx* gene of HIV-2 and forms highly stable triple helices (13). The peptide part includes several repeats of a minimal transcriptional activation domain derived from Herpes simplex virus protein 16 and which possess a high transactivation potential.

Here we show that this TFO-P can specifically recognise its DNA target at physiological salt and pH conditions. Bound to the double-stranded target DNA in a promoter region, the TFO-P is able to activate gene expression. Our results suggest that this type of molecule may prove useful in the design of new tools for artificial modulation of gene expression.

*To whom correspondence should be addressed at: Laboratoire Hematopoièse et cellules souches, INSERM U362, PR 1, Institut Gustave Roussy, rue Camille Desmoulins, 94805 Villejuif Cedex, France. Tel: +33 1 42 11 54 68; Fax: +33 1 42 11 52 40; Email: fedorsvi@igr.fr

MATERIALS AND METHODS

Oligonucleotides

Oligodeoxynucleotides 5′-pGGAGGAGGAGGAGGGGGAGG-3′ (TFO-5′) and 5′-GGAGGAGGAGGAGGGGGAGGp-3′ (TFO-3′) were purchased from GenSet (France) and purified by electrophoresis in denaturing 20% polyacrylamide gels. The 5′ end of the oligonucleotides and TFO-P were radiolabelled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs) as per the manufacturer's instructions. Oligonucleotides were labelled at a specific activity of 200 Ci/mmol.

Peptides

Peptides GGGPADALDDFDLDMLPADALDDFDLDML and GGGPADALDDFDLDMLPADALDDFDLDMLPADALDDFDLDML, containing, respectively, two and three repeats of the minimal transcriptional activation domain derived from Herpes simplex virus protein 16 and three G residues at the amino end were synthesised by conventional FMOC chemistry using Applied Biochemistry 432A peptide synthesiser (Perkin Elmer). The structure of the resulting peptides was verified by mass spectroscopy.

TFO-P synthesis

For the synthesis of the oligonucleotide–peptide conjugate we used *N*-hydroxybenzotriazole (HOBT) ester (14,15) activation of the 3′ or 5′ ends of oligonucleotide phosphates. Active HOBT esters of oligonucleotides were synthesized in an aqueous buffer solution with excess of HOBT under the action of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) as previously described (14,15). The reaction routinely proceeds with a quantitative yield within 2–3 h. After synthesis HOBT esters of the oligonucleotides were precipitated by adding 200 µl of lithium perchlorate solution and 1 ml of acetone, and were further reprecipitated three times by resuspension in 2 M $LiClO_4$ and addition of 10 vol acetone.

Synthesis of oligonucleotide–peptide conjugates was performed as follows: 1–50 nmol of HOBT esters of the TFO-5′ and TFO-3′ were incubated with 1.5 µmol of peptide in 5–30 µl of 0.4 M *N*-methylimidazole pH 8.0, 0.2 M NaCl for 12 h at 8°C. After incubation, oligonucleotide–peptide conjugates and remaining oligonucleotides and peptide were recovered from reaction mixtures by precipitation as described above, followed by separation by polyacrylamide gel electrophoresis. The most retarded bands expected to contain the oligonucleotide–peptide conjugates were cut out and the product was eluted with 2 M $LiClO_4$ and desalted by acetone precipitation.

Plasmids

The plasmid pVPX1 containing the polypurine stretch of the SIV *vpx* gene was constructed by inserting the oligonucleotides 5′-CTAGACCTGGAGGGGGAGGAGGAGGAGGTCCG-3′ and 5′-GATCCGGACCTCCTCCTCCTCCCCCTCCAGGT-3′ into the *Xba*I–*Bam*HI sites of the vector pBluescript II. The plasmid pVPX-fos(–40)Luc containing the polypurine stretch of the SIV *vpx* gene followed by minimal c-fos promoter and luciferase gene was constructed by inserting the oligonucleotides 5′-CCCTCCTCCTCCTCCCCCTCCC-3′/5′-TCGAGGGAGGGGGAGGAGGAGGAGGGGTAC-3′ into the *Kpn*I–*Xho*I sites of the vector pFos(–40)Luc (16). All plasmids were purified on Qiagen columns, following the manufacturer's recommendations.

Electroporation of the cells

NIH 3T3 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Calf Serum (FCS). The cells were trypsinated and washed with 10 ml of DMEM supplemented with 10% FCS. After centrifugation the pellets were resuspended in RPMI medium containing 5 mM $MgCl_2$ in a volume giving 10^6 cells/ml. Cells (150 µl) were put in a 4-mm electroporation cuvette (Eurogentec), and 20 µl of DNA solution was added to the cells. Ten micrograms (2.7 pmol) of the plasmid pVPX-fos(–40)Luc (or preformed triplex between this plasmid and TFO or TFO-P, the quantity of which is specified in figure legends and in the text) and 50 ng of a plasmid pCMV-βgal as an internal control were used per electroporation. After 30 min of incubation on ice, the cells were electroporated with a single pulse of 200 V/960 µF with a Bio-Rad electroporator system. After the pulse the cells were resuspended in DMEM medium containing 10% FCS and incubated for 12 h before treatment. Luciferase and β-galactosidase were assayed using kits from Promega and Tropix respectively in a Lumat B 9501 luminometer (Berthold).

Gel-retardation assay

In order to prepare a DNA fragment for gel-retardation assay, 10 µg of the pVPX1 plasmid was cut with the *Cla*I restriction enzyme, 3′ labelled with the Klenow fragment of DNA polymerase I in the presence of 15 pM [α-^{32}P]dCTP (3000 Ci/mmol), and digested with the *Sac*I restriction enzyme. A smaller (95 bp) labelled fragment (0.3 pmol) was dissolved in 20 µl volume containing 50 mM MOPS, pH 7.2, 10 mM $MgAcetate_2$ and 50 mM NaAcetate. After addition of the TFO (0.5 pmol), the mixture was incubated at 37°C for 2 h and the products were separated on a non-denaturing 5% polyacrylamide gel in a buffer containing 0.5× TBE and 2 mM $MgAcetate_2$.

Triplex formation and co-migration assay

For triplex formation, the plasmid pVpx1 (1 pmol) was incubated for various times with the [γ-^{32}P] labelled TFO or TFO-P (quantities are specified in the text) (specific activity 20 Ci/mmol). The reaction was carried out in a 15 µl solution containing 10 mM $MgAcetate_2$, 20 mM Tris–Acetate, pH 7.5 at 37°C for 2 h. After incubation of the mixture (at temperatures and times specified in the figure legends) the triple helix formation was monitored by agarose gel electrophoresis.

After triplex formation a 50% glycerol solution was added to bring the solution to a 5% final glycerol concentration, and the samples were loaded on a 1% agarose gel. Electrophoresis was performed at 3.5 V/cm at room temperature for 4 h in the presence of 20 mM Tris–Acetate, 50 mM NaAcetate, 10 mM $MgAcetate_2$ and 1 µg/ml ethidium bromide. The gel was prepared for autoradiography by drying on a glass surface in a heated air flow (60°C).

RESULTS

Synthesis of TFO-P and control of its structure

TFO-P containing two or three VP16 minimal activating domains linked either to the 3′ or 5′ end of the oligonucleotide

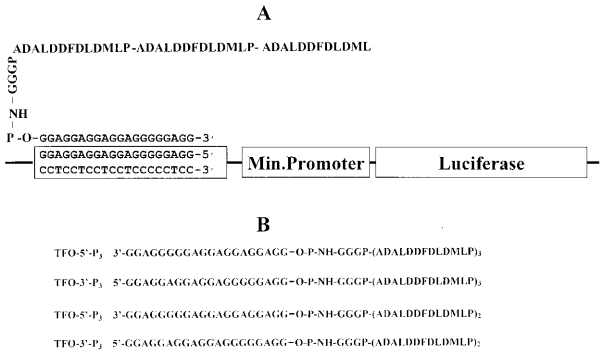

Figure 1. (**A**) Model of the TFO-P and its interaction with the target plasmid. The TFO is oriented antiparallel to the purine strand of the target duplex in the plasmid. The peptide is linked to the TFO by phosphoramidate bond. The peptide sequence is in single letter code. The peptide with three repeats of the minimal activating domain is presented (TFO-5′-P_3). Luciferase gene, minimal promoter [(–40)fos] and the rest of the plasmid are not presented in the same scale. (**B**) Schema of the TFO-Ps used in the study.

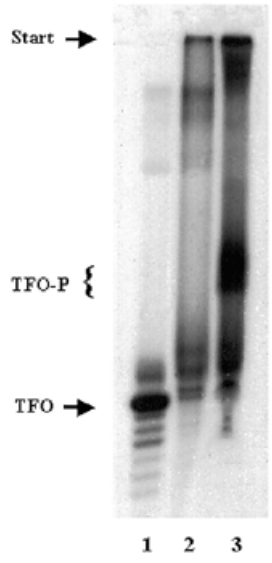

Figure 2. Separation of the oligonucleotide–peptide conjugate (TFO-5′-P_2) and remaining oligonucleotide and peptide by denaturating polyacrylamide gel electrophoresis (lane 3). Lane 1, parental TFO; lane 2, mock reaction (without peptide).

were synthesised. In the activating domain of VP16 only the α-NH_2 group is a suitable nucleophile for the interaction with an activated oligonucleotide phosphate in aqueous media (Fig. 1). We used HOBT ester (14,15) to activate the 3′/5′ ends of the oligonucleotide phosphates. After synthesis the mixture containing conjugates with two (TFO-3′-P_2 and TFO-5′-P_2) or three (TFO-3′-P_3 and TFO-5′-P_3) repeats of the minimal transcriptional activation domain derived from Herpes simplex virus protein 16 and remaining oligonucleotides or peptides were separated by PAGE (Fig. 2). The band with lower mobility compared to the TFO was expected to contain the oligonucleotide–peptide conjugate. The structure of the TFO-P was confirmed by mass spectroscopy: for TFO-3′-P_3, and TFO-5′-P_3 the mass values (calculated versus detected) were 10 991 versus 10 991 ± 2.26 and 9558.5 versus 9558 ± 1 for the TFO-3′-P_2 and TFO-5′-P_2, respectively. The structure of the TFO-P was also verified by acid treatment and proteinase K digestion after 5′ end labelling with [γ-^{32}P]ATP. As expected, treatment of the TFO-P with 50% acetic acid under selective conditions for phosphoramidate cleavage (17) gave rise to a product with the same electrophoretic mobility as non-modified TFO (Fig. 3) which strongly supports the proposed structure of the TFO-P. In addition, removal of the peptidic part by proteinase K digestion gives a product with a mobility slightly lower than the parent TFO (the difference could be due to the conformational inaccessibility of the few amino acids close enough to the TFO for proteinase K digestion), further supporting the TFO-P's structure. The product near the start of the gel (Fig. 2) had given the same pattern of proteinase K digestion and acid treatment as the main product TFO-P. We suggest that this product may present aggregates of the TFO and/or TFO-P. The high G content of the TFO and high negative charge of the peptide favour this hypothesis. Nevertheless we did not use them in further experiments, since we did not know the conditions for the dissociation of these complexes.

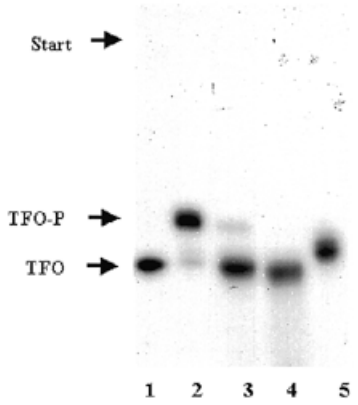

Figure 3. Autoradiogram of a 15% polyacrylamide denaturating gel showing the results of experiments on verification of the TFO-P structure (treatment of the TFO-3′-P_2 in diluted acetic acid in conditions of selective cleavage of phosphoramidate linkage and proteinase K digestion). Lane 1, TFO; lane 2, TFO-P; lanes 3 and 4, treatment by diluted acetic acid 30 and 120 min correspondingly; lane 5, digestion by proteinase K (100 μg/ml, 60 min at 40°C). Start, TFO-P and TFO correspond to the position of the start of the gel, migration of the TFO-P and TFO respectively.

Triplex formation by TFO-P

Triplex formation by TFO-P was verified by two methods: gel-retardation (18) and co-migration (19) assays. Both TFO-3′-P_2 and TFO-5′-P_2 decreased the mobility of the target duplex DNA (97 bp DNA fragment *Cla*I–*Sac*I from pVPX1

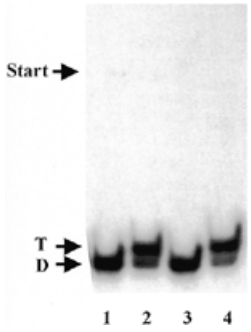

Figure 4. Triplex formation by TFO-Ps with the target DNA (97 bp DNA fragment *Cla*I–*Sac*I from pVPX1 containing the polypurine stretch of the SIV *vpx* gene). Autoradiogram of the gel after gel-retardation assay. Lanes 1 and 3, duplex only; lanes 2 and 4, duplex in the presence of TFO-3′-P_2 and TFO-5′-P_2, respectively. In the presence of both TFO-Ps a band with lower mobility (T) than the target duplex (D) is apparent, which corresponds to the formation of a triple helix structure.

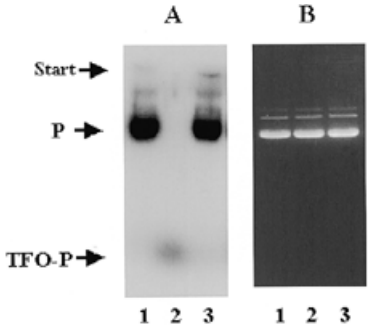

Figure 5. Triplex formation by TFO-P. (**A**) Autoradiogram of the dried agarose gel after co-migration assay with TFO-3′-P_3 (lane 1) and vpx TFO (lane 3). The plasmid pVPX-fos(–40)Luc (lanes 1 and 3) containing the polypurine stretch of the SIV *vpx* gene co-migrates with TFO-3′-P_3 and the TFO. Lane 2, co-migration assay with TFO-3′-P_3 and the vector pFos(–40)Luc. (**B**) The same (not dried) gel stained with ethidium bromide. The experiments were performed at room temperature in a non-thermostated camera and temperature of run was 30–40°C.

containing the polypurine stretch of the SIV vpx gene; 7) suggesting the formation of a triple helix structure (Fig. 4). The same results were obtained for TFO-3′-P_3 and TFO-5′-P_3 (data not shown). In addition, TFO-3′-P_3 was retained by the plasmid pVPX-fos(–40)Luc containing the polypurine stretch of the SIV *vpx* gene and not by the backbone plasmid pFos(–40)Luc in a co-migration assay further demonstrating targeted triplex formation (Fig. 5).

Gene activation by TFO-P

In order to assess the biological activity of the TFO-Ps we used a transient transfection assay. Cells were electroporated with the preformed triplex between pVPX-fos(–40)Luc and all four

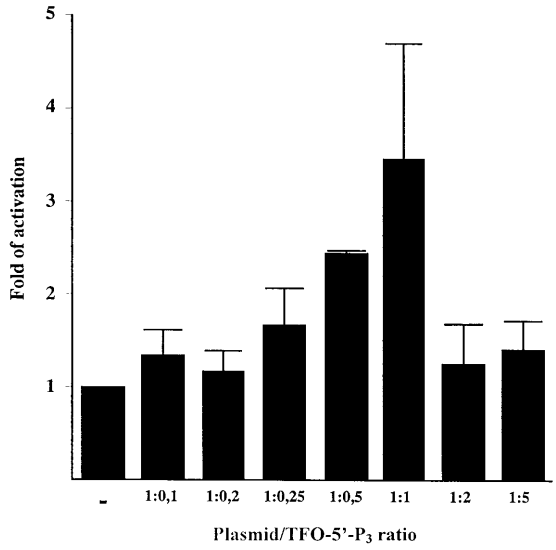

Figure 6. Gene activation by TFO-P. Cells were electroporated with the preformed triplex between pVPX-fos(–40)Luc (2.7 pmol) and different quantity of TFO-5′-P_3. Luciferase activity was normalised by the activity of pCMV-βgal used as a internal control in electroporation. –, Control experiment, where instead of TFO-5′-P_3, 2.7 pmol of TFO and 2.7 pmol of P_3 were used in electroporation. The error bars represent standard error of the mean (SEM) from values obtained in three independent experimental measurements.

types of TFO-P (TFO-3′-P_2, TFO-5′-P_2, TFO-3′-P_3 and TFO-5′-P_3). Intracellular stability of the TFO-P was verified by electroporation of the ^{32}P-labelled TFO-5′-P_3 and analysis of the cellular extracts by PAGE. No degradation of the product was seen after 24 h of cell growth.

The polypurine stretch of the SIV *vpx* gene itself increased the activity of the minimal c-fos promoter (3–10-fold in different cell lines, data not shown). This activating effect of polypurine stretches on promoter activity has been described previously (20). The TFO-P containing two transcriptional activation domains did not have any influence on the promoter activity (data not shown). However, the TFO-5′-P_3 significantly increased luciferase expression (Fig. 6). This effect was dependent on the dose of TFO-P used for triplex formation: luciferase activity increased up to equimolar ratio plasmid/TFO-5′-P_3. Further increase in the ratio between plasmid and TFO-5′-P_3, however, resulted in a decreased luciferase activity (Fig. 6).

DISCUSSION

Current models for transcription activation (21,22) suggest that the recruitment of transcription factors to a gene promoter region plays a pivotal role in gene regulation. In addition, double hybrid experiments demonstrate that transactivation domains are independent of the DNA binding domains and seem to be functionally independent of the way in which they are recruited to the promoter region (23). Thus, a transcriptional activating domain can be recruited, at least in theory, through a

TFO. We therefore created a TFO-P chimera. The TFO part of this molecule is responsible for the specific recognition of a site in the genomic DNA and the peptide serve to activate gene expression. As a model system we choose a TFO targeted to the polypurine stretch of HIV-2, since it has been shown to form a very stable triplex which persists inside cells for at least 3 days (13).

To construct the hybrid molecule a single step strategy, involving the coupling of unprotected peptide to an oligonucleotide bearing previously activated 3′- or 5′-end phosphate groups has been developed. Other than the α-NH_2 group the acidic domain of VP16 transcription factor contains no suitable nucleophilic groups able to react with activated oligonucleotide phosphate in aqueous media. Current methods of activation of oligonucleotide end phosphate groups include carbodiimide (24), imidazolide (25) and HOBT ester (14,15) activation. The first of these includes activation of the phosphate group directly in the oligonucleotide and nucleophile reaction mixture and consequently cannot be used since carbodiimide modifies the carboxylic moieties of peptide (26). The other two methods include preliminary activation of the 3′- or 5′-end of oligonucleotide phosphate followed by the isolation of the active compound from the reaction mixture. This allows the use of unprotected peptide for synthesis of oligonucleotide–peptide conjugates. Since the HOBT ester method was shown to be more effective than that with imidazolide for nucleophilic substitution at the phosphorus atom (15,25), this method was chosen for coupling of the peptides to 3′- or 5′-phosphorylated 20mer TFOs. The two compounds in the resulting product are joined by a phosphoramidate bond. We demonstrated that phosphoramidate bonds are formed most efficiently at pH 8.0 in the presence of *N*-methylimidazole. The structure of the TFO-P was verified by acid treatment, proteinase K digestion and mass spectrometry. The proposed method of synthesis is the first to use a HOBT-activated oligonucleotide phosphate and unprotected peptide in the synthesis of an oligonucleotide–peptide chimera in aqueous solution.

Formation of very stable triplex with the TFO used in this study has been demonstrated by us in previous studies (7,13). In the present work specific triplex formation by TFO-P at the target site was confirmed by gel-retardation and co-migration assays. The biological activity of the TFO-Ps was assessed using transient transfection of preformed triplex between a plasmid including the target sequence and all four types of TFO-P (TFO-3′-P_2, TFO-5′-P_2, TFO-3′-P_3 and TFO-5′-P_3). Of the four constructs tested only TFO-5′-P_3 was able to activate the expression of the reporter gene. Neither the TFO-P with two repeats of VP16 activating domain nor the TFO-P with three repeats of VP16 in 'reverse orientation' were able to activate gene expression. This clearly demonstrates the specificity of TFO-P action. The specificity of TFO-P action was further demonstrated by the absence of the activating activity of the TFO-5′-P_3 in transient transfection assay with the parent plasmid pVPX-fos(–40)Luc (without the polypurine stretch of the SIV *vpx*) which can be considered as the control with mismatched TFO sequence (data not shown). In the concentrations we have used we did not see any effects of the TFO or peptide alone on the luciferase expression. Nevertheless, in all transient transfection experiments we used as a control the mixture of the TFO and corresponding peptide [ratio 1/1/1 of the TFO, peptide and the plasmid pVPX-fos(–40)Luc] (Fig. 6).

The level of activation was substantially lower than expected given the reported efficiency of VP16 transcriptional activator demonstrated using experiments with hybrid protein between the minimal transcriptional activation domain and TetR DNA binding domain (27). In these experiments with the hybrid proteins the level of activation with two and three repeats of the transactivating domain reached 10^3–10^5 times the level of gene expression without the hybrids. In our experiments no activation was observed with two repeats and only 3–4-fold activation was observed in the experiments with TFO-5′-P_3. This low activity was not due to degradation of the TFO-Ps inside cells after electroporation since these chimerical molecules were stable after 24 h of cell culturing (data not shown). The relatively low level of gene activation could be due to a different flexibility between the hybrid proteins and the TFO-P. In the former case the proteins can adapt their conformation to interact with the transcriptional machinery, when the TFO-P could possess more rigid structure and needs to be precisely localised relative to the minimal c-fos promoter containing a TATA box. This hypothesis is supported by the specificity of TFO-P action: only the TFO-5′-P_3, where activating domains are located further from the TATA box, is active and not the TFO-3′-P_3. One possible reason explaining the more rigid structure of the TFO-P could be the interaction between the highly negatively charged structure of the triplex and the negatively charged peptide (15 aspartic acid residues in P_3). Since the stability of TFO-3′-P_3 was not tested, we can not also exclude a possibility that potential degradation of TFO-3′-P_3 could explain its lack of action.

The activating effect was dependent on the quantity of the TFO used for triplex formation: luciferase activity increased up to equimolar ratio plasmid/TFO-5′-P_3. Further decrease in plasmid/TFO-5′-P_3 ratio decreased luciferase activity. We suggest that this effect may be explained by the titration of the cellular transcription factors by excess free TFO-5′-P_3 present in the electroporation solution. Since β-galactosidase activity did not have a tendency to decrease with increasing quantities of TFO-P we suggest that the titrated factors are specific for pVPX-fos(–40)Luc construct.

The optimisation of the distance between the triplex target sequence and the minimal promoter may be one way of improving the activity of the TFO-P. Another possibility could be the augmentation of the number of minimal activating domains of the TFO-P. The results presented here suggest that this type of molecule may prove useful in the design of new tools for artificial modulation of gene expression.

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