Recruitment of transcription factors to the target site by triplex-forming oligonucleotides

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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 hairpin-TFO able to recruit transcription factors to a target DNA. The designed oligonucleotide contains a triplex-forming sequence, linked through a nucleotide loop to a double-stranded hairpin including the SRE enhancer of the c-fos gene promoter. We show here that this oligonucleotide can specifically recognise its DNA target at physiological salt and pH conditions. The stability of the triplex formed under these conditions is very high: >90% of the triplex remains intact after 24 h of incubation. Bound to the double-stranded target DNA, the oligonucleotide retains its ability to interact specifically with transcription factors, recruiting them to the proximity of the target DNA. Our results suggest that this type of oligonucleotide 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 targeted against gene promoters can modulate transcription of the targeted gene (1). Two types of TFOs have been described. Pyrimidine oligonucleotides are composed of thymines and cytosines and bind in parallel orientation to runs of purine acceptors of a duplex DNA through Hoogsteen base pairing. In the second type of TFOs, the oligonucleotides are composed of guanosines and adenines or thymines, and bind in an antiparallel orientation to the purine acceptor strand via reverse Hoogsteen base pairing (reviewed in 2,3).

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 (4), a human chromosome (5) or even in the whole human genome (6), 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. 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 7). However, TFOs potentially could be used for more diverse purposes. For example, TFO-based DNA bending ligands have been designed (8). In the present work we have designed an oligonucleotide able to recruit transcription factors to the target DNA sequence.

These molecules (hairpin-TFOs) are bi-functional oligonucleotides, which contain a hairpin able to bind transcription factors, and a sequence forming a triple helix on a target DNA (see Fig. 1A). The specific 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 SIV) and forms highly stable triple helices (9). This TFO portion is linked, through a nucleotide loop, to a double-stranded hairpin including transcription factor binding sites from the SRE enhancer of the c-fos gene promoter. The SRE enhancer (10) is present in the upstream regulatory sequence of a number of immediate early genes (11,12). The SRE is constitutively occupied by a complex of two proteins, p67Srf (13) and p62Tcf (14). P67Srf recognizes a CArG box in the SRE (15). P62Tcf, either the ELK-1 or the SAP-1 protein, does not bind autonomously to the element but requires the presence of SRF in order to efficiently contact DNA (16,17), thus forming a ternary complex.

Here we show that the bi-functional ‘SRE-TFO’ can specifically recognise its DNA target under physiological salt and pH

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The plasmid pVPX1 containing the polyuridine stretch of the SIV vpx gene was constructed by inserting the oligonucleotides 5'-CTAGACCTGGAGGGGGAGGAGGAGGTCCG-3' and 5'-GATCCGGACCTCCTCCTTCCTCCTCCCTCAGGT-3' into the XbaI–BamHI sites of the vector pBluescript II. All plasmids were purified on Qiagen columns, following the manufacturer’s recommendations.

**Oligonucleotides**

Oligodeoxynucleotides were synthesised using an Applied Biosystems 391A DNA synthesiser and purified by electrophoresis on denaturing 20% polyacrylamide gels. The different oligonucleotides used are presented in Figure 1B.
Figure 2. Binding of the hairpin-TFO to the target sequence. (A) Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of a DMS footprinting experiment carried out with SRE-TFO: lane 1, SRE-TFO; lane 2, control oligonucleotide 5'-GGATTAGTATGAGCCAGG-3'.

RESULTS AND DISCUSSION

Hairpin-TFOs form stable triplexes with target DNA

A model hairpin triplex-forming oligonucleotide (SRE-TFO) was designed as described in Figure 1A. The TFO part binds to the polypurine/polypyrimidine tract of the vpx gene of Simian immunodeficiency virus (SIV) in a highly stable manner (9,24). The TFO part is connected, via a linker consisting of three thymines, to a hairpin containing the sequence of the Serum Responsive Element (SRE), an enhancer from the c-fos gene promoter (Fig. 1B). To check the specificity of the interaction between the hairpin-TFO and the target DNA or transcription factors, we have used hairpin-TFOs with a mutated TFO sequence (SRE-mTFO) or a mutated hairpin sequence (mSRE-TFO) (Fig. 1).

Formation of triplexes between the target DNA and the hairpin-TFOs has been demonstrated by three methods. First, triplex formation with the pVPX1 plasmid containing the target sequence, in a buffer containing 50 mM Na+, was monitored by a DMS footprinting assay (Fig. 2). DMS modifies the N7 position of guanines, leading to phosphate backbone chain cleavage after treatment with piperidine. This reaction does not occur with the purines of the double-stranded DNA within a purine-purine-pyrimidine triplex, due to the formation of Hoogsteen base pairing (25). Figure 2 shows that the guanines located within the target sequences of the SRE-TFO are less sensitive to DMS than are those external to the target sequence, indicating that a triplex is formed with the oligonucleotide under these experimental conditions. The pattern of the protection by SRE-TFO was similar to that observed with the TFO part alone (data not shown).

In parallel, electron microscopy was used to detect the triplex formed by the SRE-TFO on its target. The stem part of the oligonucleotide can easily be seen as a short stick, usually oriented perpendicularly to the axis of the double-stranded DNA on the electron micrograph (Fig. 3). The estimated length of the protruding DNA was 29 ± 4 bp which is in good agreement with the length of the hairpin. The position of the hairpin (85 ± 6 bp from the last nucleotide in the 3' end of the target sequence) coincides with the position of the target sequence (86 bp). When the experiments were performed in the presence of SRE-mTFO, no triplexes were detected on the electron micrographs (data not shown). The high selectivity of this hairpin-TFO was also demonstrated by the fact that, at all levels of target occupancy studied (up to 80%), no non-specific interaction was observed over the entire plasmid sequence.

Finally, the formation of triplexes between the hairpin-TFOs and whole plasmids was checked in co-migration experiments (data not shown). Only the oligonucleotides designed to form a triplex with the vpx sequence (SRE-TFO and mSRE-TFO) migrated with the plasmid pVPX1. No oligonucleotide migrated with the control plasmid pBlueskript SK (data not shown). The stability of the triplex was assayed by cold chase experiments. After triplex formation, using an equimolar mixture of the plasmid and the 5'-32P-labelled SRE-TFO, a 500-fold molar excess of the oligonucleotide 5'-GGAGGAGGAGGGGGGGAGG-3' designed to form a triplex with the same target (26) was added to the mixture. After incubation in 10 µl of a buffered solution (containing 10 mM MgCl2, 150 mM KAc and 20 mM TrisAc, pH 7.5) during various periods of time at 37°C, the
Figure 4. Determination of the stability of the triplex. A triplex was formed between plasmid pVPX1 containing the target sequence and a radiolabeled SRE-TFO. After different times of incubation with an excess of non-labelled competitor TFO, the mixture was analysed by co-migration assays (29). (A) Autoradiogram of a dried agarose gel. Lanes from 1 to 6 correspond to 24, 3, 1, 0.5, 0.25, and 0 h incubation, respectively. Lane 7: sample without competitor oligonucleotide. (B) The same gel as (A) after staining with ethidium bromide.

amount of radioactivity which remains bound to the pVPX1 was evaluated by a co-migration assay. Under the conditions of this experiment >90% of the 5′-32P-labelled oligonucleotide remains bound to the plasmid after 24 h of incubation (Fig. 4).

SRF and ELK-1 bind to the hairpin-TFO

The interaction between the transcription factors and the hairpin-TFO was analysed by EMSA. The SRE-TFO and SRE-mTFO hairpins both contain a CArG box, specifically recognized by SRF. Both oligonucleotides bound SRF as efficiently as a classic double-stranded probe used as a control (Fig. 5A); the ratio of the retarded band to the total radiolabelled oligonucleotide was the same for all three probes (SRE, SRE-TFO and SRE-mTFO). The SRE sequence of the c-fos promoter contains, in addition to the CArG box, an Ets box (Fig. 1). The Ets box is recognised by ELK-1 but only when the adjacent CArG box is occupied by SRF (16,17), thus forming a ternary complex which can be observed in EMSA as a supershifted band as compared with that of the SRE/SRF complex (18). As seen in Figure 5B, lanes 4 and 7, SRE-TFO and SRE-mTFO have the ability to bind SRF and ELK-1 simultaneously. The formation of the ternary complex was specific, since no retarded bands were detected when similar experiments were performed with mSRE-TFO, in which the CArG and Ets boxes are mutated (Fig. 5A, lane 5). Thus, the SRE-TFO is able to specifically interact with the transcription factors.

Formation of a multimolecular complex: target DNA/hairpin-TFO/transcription factors

The formation of a multimolecular complex including the target DNA, the hairpin-TFO, and transcription factors was demonstrated by EMSA. In these studies, an end-labelled Clal–Eco136I fragment (102 bp) from the pVPX1 plasmid which contains the target sequence was prepared. When this probe was incubated with the SRE-TFO or the mSRE-TFO, but not with the SRE-mTFO, a retarded band corresponding to the triplex structure could be observed (Fig. 6A, lanes 2, 3, 6, 7). Addition of the SRF protein resulted in the appearance of a further retarded

Figure 5. Analysis of the interaction between the hairpin-TFO and SRF or ELK-1. SRF and ELK-1 were transcribed and translated in vitro. (A) The interaction was analysed by EMSA using radio-labelled SRE-TFO (lanes 1–3), SRE-mTFO (lanes 4, 5), mSRE-TFO (lanes 6, 7), and SRE (lanes 8, 9) as probes. In the control lanes mock-translated samples were used. (B) ELK-1 forms a ternary complex with SRF and the hairpin-TFO. Equivalent amounts of SRF and ELK-1 were incubated with the SRE-TFO (lanes 1–4), the SRE-mTFO (lanes 5–7), and the SRE (lanes 8–10) as probes. In this experiment the radiolabelled probe is the target DNA to which unlabelled free SRE-TFO triplex formation was performed at equimolar concentration of the target DNA and SRE-TFO. Under these conditions, triplex is formed with only 15% of the target DNA, and can easily be seen on a less exposed gel (data not shown). Because our goal was to see the multimolecular complex formation, the part of the gel presenting duplex DNA and triplex is overexposed; consequently the signal from target DNA partially mask the retarded band for triplex.
Figure 6. Analysis of the target DNA/(SRE-TFO)/SRF multimolecular complex formation. (A) *In vitro* translated SRF was incubated with a mixture containing an \( \alpha^{32}P \)-labelled fragment from the pVPX1 (D*) and the SRE-mTFO (lane 1), the mSRE-TFO (lane 2), or the SRE-TFO (lane 3). In lanes 4–9 SRF was omitted. (B) Competition experiments. The multimolecular complex was formed as in (A), but the indicated excesses of unlabelled SRE probe were added (lanes 1–3). In lanes 9 and 10 (A) or 4 and 5 (B), D* was replaced by radiolabelled SRE probe. The band marked by an ‘X’ most likely represents a dimer of the hairpin-TFO, bound to the target DNA.

Figure 7. ELK-1 forms a multimolecular complex with SRF and the target DNA/(SRE-TFO)/SRF structure. ELK-1 and SRF were transcribed and translated *in vitro*. Equivalent amounts of the translation products were analysed by EMSA in the presence of the target DNA/(SRE-TFO) complex.

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