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Specific Inhibition of c-*fos* Proto-oncogene Expression by Triple-Helix-Forming Oligonucleotides

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Abstract The promoter region of the c-*fos* oncogene 5′ flanking sequence contains enhancer elements crucial for binding nuclear factors that regulate transcription following cell proliferation and differentiation. Single-stranded deoxyoligonucleotides were chosen for modulation of c-*fos* protooncogene expression because of their high-affinity binding to specific nucleotide sequences. We designed two oligonucleotides that form a triple-helix complex on the retinoblastoma gene product-responsible element of the c-*fos* oncogene.

Modification of the DNA triplex with dimethyl sulfate and affinity cleaving assays demonstrate that the predicted oligonucleotides form a DNA triplex structure with the c-*fos* promoter in a sequence-specific manner. Tumorigenic and non-tumorigenic fibroblasts were transiently transfected with *fos*-CAT plasmid modified with alkylating triplex-forming oligonucleotide reagents. A dramatic depression of CAT activity was found when the cross-linked triple helix complex at the retinoblastoma gene product-related site of the c-*fos* promoter was used.

These experiments suggest that transcription of individual genes can be selectively modulated in cell culture by sequence specific triplex formation in regulatory enhancer sequences.

Key words: c-*fos*, triplex, transcriptional factors, promoter, gene regulation

c-*fos* is an early growth-regulated gene that is expressed during cell proliferation [Lord et al., 1993; Muller et al., 1993], and in the terminally differentiated cell type as in hematopoietic cell progenitors [Yagaloff and Xie, 1995]. Transcriptional stimulation of the c-*fos* gene proceeds in the absence of protein synthesis, suggesting that the transcriptional enhancer and other cis-acting sequences are involved in the regulation of c-*fos* expression [Triesman, 1985]. Several of these factors are located not only 5′ to the start of transcription, but also 3′ in the noncoding region [Prywes et al., 1988; De Belle et al., 1991]. To verify the significance of such *cis*-acting sequences in c-*fos* expression, several methods including DNase I footprinting were used [Lavrovsky et al., 1994]. Recently, oligonucleotides have been discovered as reagents that can bind to specific nucleotide sequences of cellular genes to study the significance of the *cis*-acting elements in regulating gene expression and cell function. Two strategies of oligonucleotide reagents have been used. The best known approach involves antisense oligonucleotides that bind mRNA to inhibit its processing or translation. The second approach is the triplex strategy which employs single-stranded DNA oligonucleotides that bind to the major groove of a double-stranded target DNA to form a triple-helix or "triplex" in a sequence-specific manner [Maher III et al., 1989; Maher III, 1992; Ing et al., 1993; Helene, 1991; McShan et al., 1992]. When triplex-forming oligonucleotides (TFOs) are targeted to a vital promoter region of a test gene, they have been shown to selectively repress transcription and to interfere with the function of sequence-specific DNA binding proteins such as transcription factors, both in vitro and in culture [Duval-Valentin et al., 1992]. For these reasons, oligonucleotides directed to a precise DNA sequence provide an ideal condition to examine the promoter activity of c-*fos*. We have recently identified a novel sequence in the upstream region of the c-*fos* promoter (−611 to −30) to encode nuclear factors which bind to AP-2-like elements [Lavrovsky et al., 1994]. This promoter region contains a homnopurine-homopyrimidine sequence which is expected to form triple helices with corresponding oligonucleotides. Some of these sequences have been shown to overlap with characterized *cis*-ele-

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ments. It therefore provided us with the opportunity to interfere with c-*fos* promoter function by triple helix formation with alkylating derivatives of corresponding oligonucleotides [Knorre and Vlassor, 1991]. In order to better understand the potential of triple-helix-forming oligonucleotide derivatives as site-specific transcription modulators, we developed a cellular system to measure the effects of these oligonucleotide reagents on transcription. A reporter gene strategy coupled with the DNA-affinity modification technique was used in an effort to facilitate oligonucleotide uptake, optimize triple-helix formation and provide convenient biological assays. Moreover, by using the 14-mer oligonucleotides modified with the chloroethylamine group which forms a triple helix, we showed an irreversible and specific inhibition of c-*fos*-CAT activity.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were purchased from Biosynthesis (Texas). The plasmid *fos*-CAT has a double-stranded circular genome 5424 bp long [Lavrovsky et al., 1994]. It was grown in *Escherichia coli,* isolated, purified, and characterized using standard techniques.

Cell Culture, Transient Transfection, and CAT Assay

Cell culture, transient transfection, and CAT assay were performed as previously described [Lavrovsky et al., 1993; Lavrovsky et al., 1994a]. Data were analyzed using Statistica software (Microsoft Corp, Tulsa, OK).

Radiolabeled DNA Substrates

End-labeled DNA fragments of the murine c-*fos* promoter region (−611 to −30, taken from the *fos*-CAT plasmid) containing the triplex target sequence were excised from the plasmid as described below.

The 5′-^{32}P-labeled fragment F1 was prepared as follows: the *fos*-CAT plasmid was cleaved with *Hin*dIII, yielding a 586-bp fragment, and isolated by electrophoresis in 1.5% agarose gel. The DNA fragment was dephosphorylated with calf intestinal alkaline phosphatase and treated with [γ-^{32}P]ATP and T4 polynucleotide kinase to obtain a 5′-end-radiolabeled DNA fragment. After purification procedures, the radiolabeled fragment was cleaved with AvaI endonuclease. The resultant F1 (478 bp) was isolated by electrophoresis in 5% native polyacrylamide gel (PAAG).

The DNA fragment F2 (478 bp) was prepared by digestion of the *fos*-CAT plasmid at the AvaI site. 3′-[^{32}P]-labeling at the ends was performed using the Klenow fragment of DNA polymerase I and the corresponding [α-^{32}P]dNTP. DNA was then cut by the *Hin*dIII restriction enzyme. The labeled fragments were isolated by electrophoresis in a 5% native PAAG. The F5 fragment (586 bp) was labeled at both strands at the *Hin*dIII sites using [α-^{32}P]dATP. The fragment was isolated by electrophoresis in 4% native PAAG and the Klenow fragment of DNA polymerase I.

Oligonucleotide Reagents

The oligonucleotide (5′p-CTTCCCCCCCCTTC) was coupled with the modified alkylating 4-(3-amino)propyl(N-2-chloroethyl-N-methyl) aniline group reagent in which the terminal amino group was conjugated with the $-CH_2CH_2CH_2CH_2NH_2$ spacer [Brossalina et al., 1991]. This modified reagent contains the alkylating group that has more conformational mobility. The reagent was purified by high performance liquid chromatography (HPLC) on a Lichrosorb C_{18} column in a gradient of acetonitrile (0–30%) containing 0.1 M $LiClO_4$ which yielded products of >95% purity.

Affinity Cleaving

Plasmid DNA and the radiolabeled DNA fragments were subjected to affinity cleavage in our experiments. The alkylation conditions were the same as described [Brossalina et al., 1991] with the following modifications:

DNA Fragment Alkylation

In a typical experiment, we used 20 μl of a mixture containing about 10,000 cpm radiolabeled target DNA (about 5 nM), 5 μM of the oligonucleotide reagent, 150 mM NaCl, 50 mM sodium acetate, pH 5.4, and 10 mM $MgCl_2$. The alkylation reaction was performed for 22 h at 22°C. To identify the alkylated sites, the modified DNA was treated with 1 M piperidine at 90°C for 10 min, extracted with ether, ethanol precipitated, lyophilized and resolubilized in formamide loading buffer. The cleavage products were then resolved on the denaturing 10% or 5% polyacrylamide gel and detected by autoradiography.

Plasmid DNA Alkylation

The reactions were carried out in 20 μl (analytical variant) or 300 μl (preparative variant) of the appropriate buffer as described for the fragment DNA alkylation. The concentration was 50 μM for the oligonucleotide reagent and 75 nM for the plasmid DNA. To identify the modification sites after the reaction, DNA was ethanol precipitated, cleaved with *Ava*I and *Hin*dIII enzymes, and labeled at the *Ava*I site. The cleaved DNA was then extracted with phenol/chloroform, followed by ethanol precipitation. To cleave the modified DNA at the alkylated residues, the DNA was incubated for 10 min in 1 M piperidine at 90°C, followed by ether extraction and ethanol precipitation. The products of cleavage were lyophilized and resolubilized in formamide loading buffer and then separated on a polyacrylamide gel, followed by autoradiography detection.

Denaturing Gel-Mobility Band Shift Assay

Triplex formation between the 3′-labeled DNA fragment and the corresponding oligonucleotide reagent, and the reaction of alkylation were performed in a 20-μl reaction mixture as described for DNA fragment alkylation. DNA target and corresponding triplex-forming oligonucleotides are shown in Figure 1. DNA was then ethanol precipitated, lyophilized, and resolubilized in formamide loading buffer. The reaction products were then resolved on the denaturing polyacrylamide gel and detected by autoradiography.

Chemical Sequencing

Sequencing reactions of the uniquely end-labeled DNA fragments were carried out as described [Berger and Kimmel, 1987].

Dimethyl Sulfate Probing

Triplex formation between 3′- or 5′-labeled DNA and the corresponding oligonucleotide (0, 0.0001, 0.001, 0.01, 0.1, 10 μM) was performed in a 20-μl reaction mixture as described for affinity cleaving, unless otherwise stated. Five μl of a 2.5% aqueous solution of DMS and 1 μg of a nonspecific sonicated calf thymus DNA (this carrier DNA were added to maintain constant the level of both single- and double-stranded DNA and facilitate precipitation) was added to the preformed triples. After 2 min, the reaction was terminated by addition of 1 μl of 2-mercaptoethanol, followed by ethanol precipitation. The modified DNA was then treated with 1 M piperidine at 90°C for 10 min. Cleaved DNA was then extracted with ether followed by ethanol precipitation, lyophilization and resolubilization in formamide loading buffer. The piperidine cleavage products were separated on a denaturing polyacrylamide gel and detected by autoradiography.

RESULTS AND DISCUSSION

Target Selection and Design of Triple Helix Forming Oligonucleotides

The murine c-*fos* oncogene promoter contains multiple regulatory *cis*-acting elements [De Belle et al., 1991; Lavrovsky et al., 1994; Maher III et al., 1989; Maher III, 1992; Ing et al., 1993; Helene, 1991; McShan et al., 1992; Duval-Valentin et al., 1992; Lavrovsky et al., 1993]. The plasmid *fos*-CAT contains the reporter gene encoding chloramphenicol acetyltransferase under the control of the murine c-*fos* promoter [Lavrovsky et al., 1993]. The product of the retinoblastoma susceptibility gene RB-1 (Rb) regulates transcription of a wide variety of genes via protein-protein interactions [Nevins, 1992;

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human 5'-CgCgCgCCACCCCTCTggCgCCAC-3'
rat   5'-CgCTTTCC CCCCTCCAgTTCTC-3'
mouse 5'-CgCTT  CC CCCCCC     TTC C-3'
OR      3'-CTT  CC CCCCCC     TTCp-RCL
TFO-1   3'-CTT  CC CCCCCC     TTC-5'
TFO-2   5'-gAA  gg ggggggg    AAg-3'
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Fig. 1. Comparison of the human, rat, and mouse RCE of the c-*fos* promoter region (only upper strand presented). Triplex-forming oligonucleotides (TFO) and oligonucleotide reagent (OR) bearing the reactive group (RCl) are indicated.

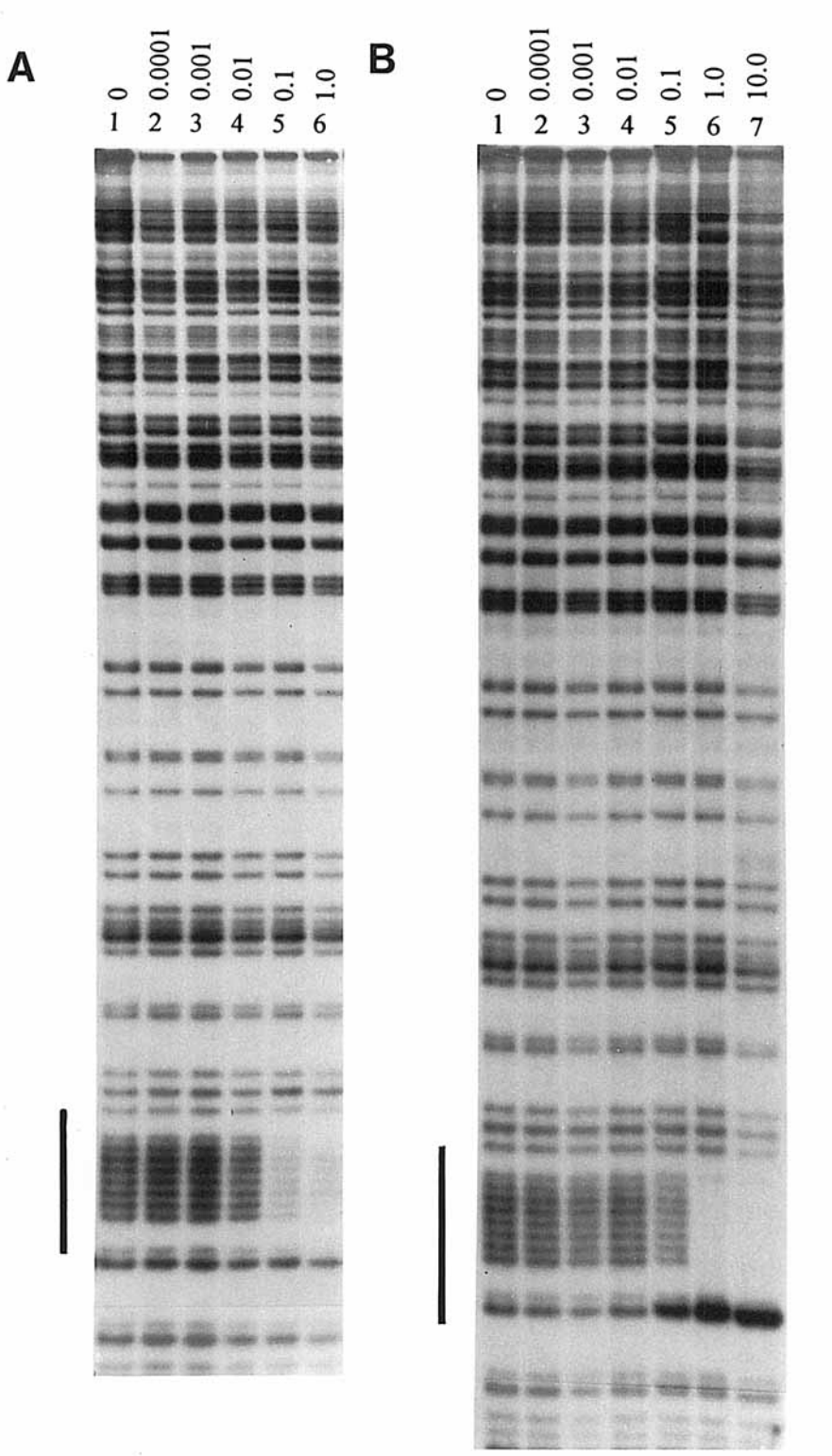
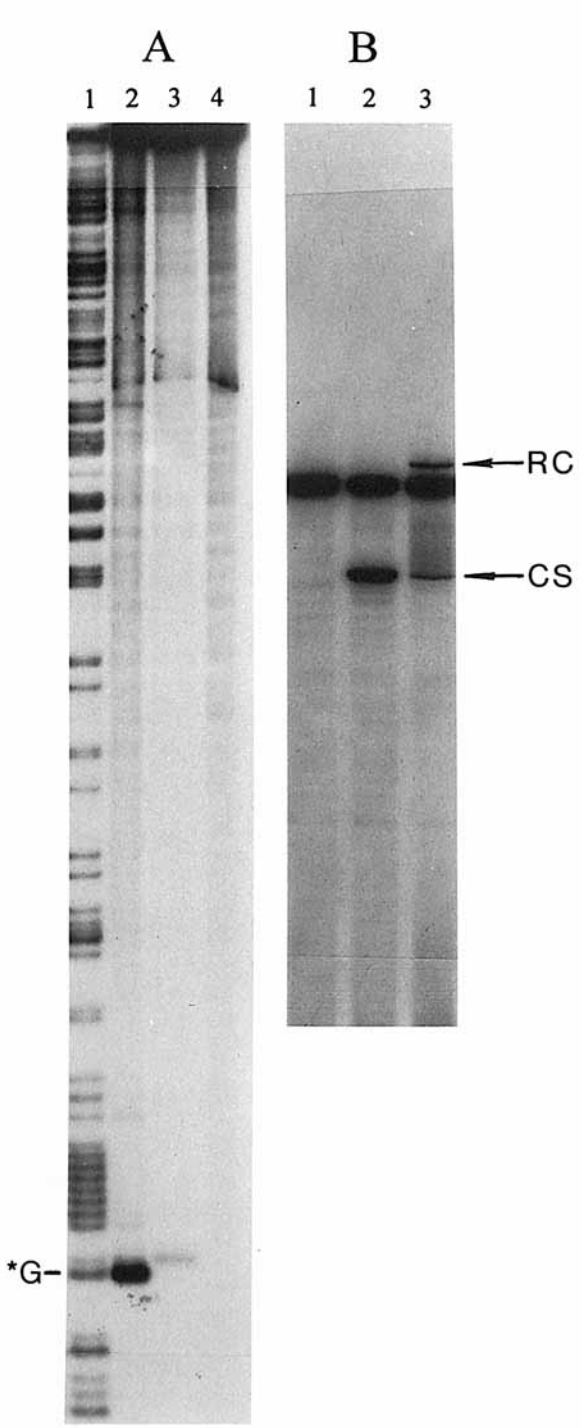


Fig. 2. The chemical probing of the 5′-labeled F1 fragment with DMS. Autoradiogram of the 5% denaturing polyacrylamide gel. The final concentrations of the oligonucleotides added (μM) are shown above each line. The 3′-end is at the top. Vertical line indicates the target sequence 5′-GAAGGGGGG-GAAG. **A:** TFO-1 (5′-CTTCCCCCCCCTTC) used in the triplex-forming buffer (pH 5.4). **B:** TFO-2 (5′-GAAGGGGGGGGAAG) used in the triplex-forming buffer (pH 7.3).

Hamel et al., 1992]. A *cis*-acting element in the c-*fos* promoter that can confer repression by Rb has been reported [Robbins et al., 1990]. We designed triplex forming oligonucleotides targeted to the mouse c-*fos* promoter region which could be considered as a proposed RCE in the context of the murine c-*fos* promoter region. We therefore termed this region the RCE-like site. Homopurine and homopyrimidine oligonucleotides were designed to bind potentially to double-stranded DNA at a selected target contained within the c-*fos* promoter region, as seen in Figure 1 and were designed to bind in a parallel orientation to the purine-rich strand, using a combination of established base triplets (C*GC). By contrast, a purine-rich oligonucleotide was designed to use G*GC triple helix pairing rules [Maher III, 1992; Hanvey et al., 1991; Radhakrishnan and Patel, 1994]. This kind of triplex-forming oligonucleotide (TFO) was designed to bind the target sequence in an antiparallel orientation. Both homopurine and homopyrimidine oligonucleotides and the pairing rules used in their design are shown in Figure 1.



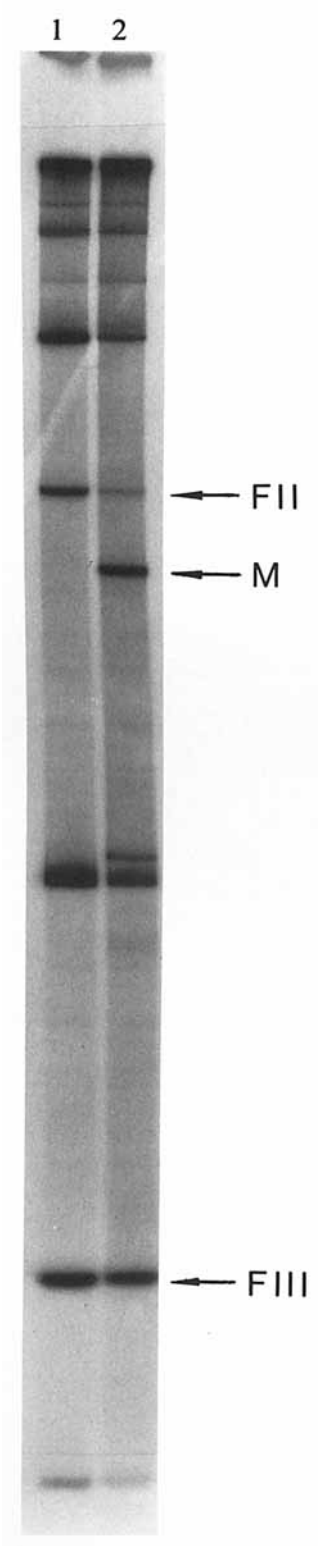
Detection of Triple Helix Formation in the c-*fos* Promoter Region by Chemical Probing with DMS

To detect triple helix formation, we used chemical probing with DMS. Since triplex binding shelters the N7 position of guanosine residues in the duplex, the triplex zone remains unmodified by DMS, while all guanosines outside the zone are modified as usual. As a result, a characteristic DMS footprinting pattern reflects the triplex formation.

For cytosine containing third-strand oligonucleotides, the triple helix formation was carried out under acidic pH values. To form the triple helix DNA structure, the polypurine-rich triplex-forming oligonucleotides were incubated with the DNA fragment possessing the target sequence under physiological pH values. The results of DMS footprinting experiments performed on complexes formed by a symmetrical target and TFOs in magnesium-containing buff-

Fig. 3. Alkylation experiments with target containing fragments. Autoradiogram of the 5% denaturing polyacrylamide gel. **A)** Identification of the alkylation site in the 5′-labeled fragment F1 reacted with oligonucleotide reagent OR at pH 5.4 *Lane 1,* G-specific partial cleavage of the fragment; *lane 2,* F1 treated with 5 μM of OR and cleaved with 1 M piperidine; *lane 3,* F1 fragment treated with 5 μM of OR without piperidine cleavage; *lane 4,* intact F1 control, incubated in the absence of reagent. *G indicates the alkylation site, G (−89), which was attacked by the reactive group, RCl; **B)** Alkylation experiments with the *Aval* site-labeled F2 fragment reacted with OR at pH 5.4. *Lane 1,* intact F2 control, incubated without OR; *lane 2,* treated with OR and cleaved with 1 M piperidine; *lane 3,* denaturing gel mobility bandshift assay. RC indicates the covalent adduct between F2 and OR; CS represents the cleavage site.

ers are presented in Figure 2. It can be seen that both homopyrimidine (Fig. 2A) and homopurine (Fig. 2B) oligonucleotide designs yield clear footprints. For the homopyrimidine-rich TFO-1, a clear footprint appeared at the 0.1 μM concentration of the TFO, whereas TFO-2 produced a clear footprint at a concentration 10 times higher than that of the oligonucleotide. Figure 2B shows the strong reactivity of the flanking guanosine residue toward DMS. It should also be noted that TFO-2 is identical to the target sequence.



Triplex formation can also be monitored by affinity cleavage, in which the RCl-equipped third strand induces site-specific alkylation of the target DNA under described conditions. The alkylated DNA can then be cleaved at the modified residues and the cleavage products can be analyzed by denaturing gel electrophoresis. The results of DMS footprinting experiments carried out on specific triple helix complexes formed by symmetrical RCE-like (target) and appropriate oligonucleotides TFO-1 and TFO-2 showed that both the pyrimidine and purine TFOs had produced clear footprints. First, we studied the reaction of the oligonucleotide derivative OR with the DNA fragments F1 and F2, which possess the target sequence (Fig. 1). Electrophoresis of the purine strand labelled fragment F2 alkylated with the derivative of the oligonucleotide TFO-1 revealed the labelled product RC with decreased electrophoretic mobility (denaturing gel mobility bandshift assay) that apparently was the product of the oligonucleotide coupling with the DNA (Fig. 3B, lane 3). Piperidine treatment resulted in DNA cleavage at the alkylated site resulting in the formation of a shorter labeled fragment, CS (Fig. 3B, lane 2). The modification product, CS, also presented in some amount, even without piperidine treatment. Affinity cleavage of the target performed with the OR derivative allowed us to determine the alkylated site position [G(−89)] and therefore the third-strand orientation toward the purine-rich strand of the target duplex. The third strand oligonucleotide was detected to be aligned parallel to the purine strand of the target (Fig. 3A, lane 2). The modification product was found to be revealed, even without piperidine cleavage

Fig. 4. Alkylation experiments with whole plasmid DNA reacted with OR at pH 5.4. The autoradiogram is of the 5% denaturing polyacrylamide gel. DNA was alkylated, digested with *Ava*I and *Hin*dIII enzymes, labeled at the *Ava*I site, and cleaved with piperidine as described in Materials and Methods. *Lane 1*, the control labeled unmodified plasmid DNA; *lane 2*, alkylated *fos*-CAT plasmid DNA then subjected to digestion, labeling, and piperidine treatment. FII and FIII mark DNA fragments F2 (478 bp) and F3 (108 bp), respectively. M indicates the modification product.

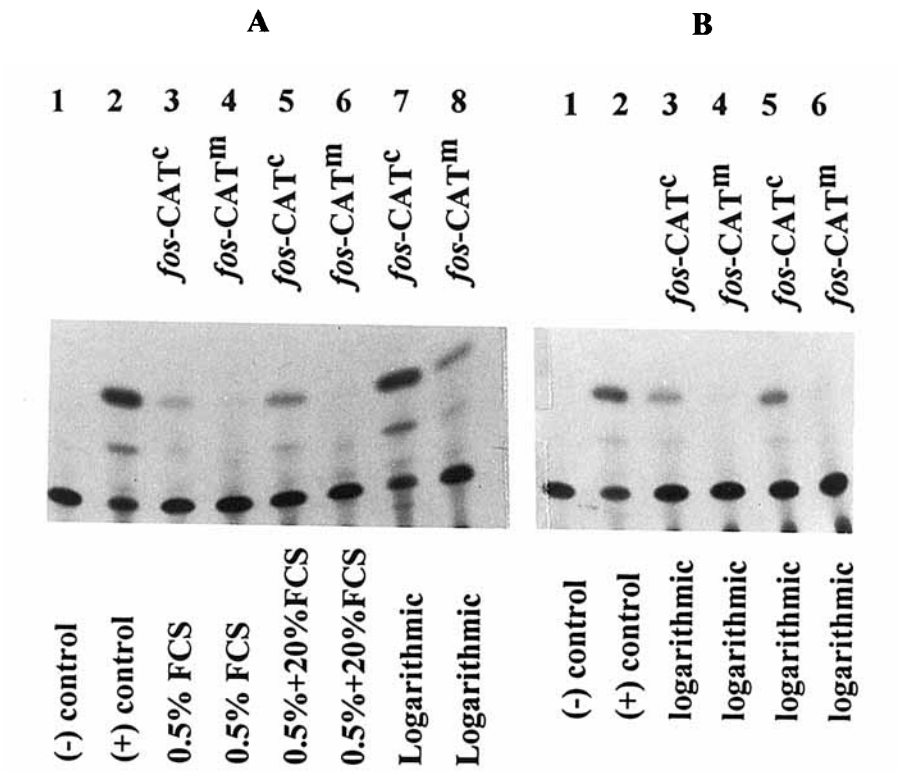


Fig. 5. Representative experiment showing the effects of OR on the expression of the CAT gene driven by the 586-bp c-*fos* proximal promoter fragment. *Lane 1,* no extract (negative control); *lane 2,* tumorigenic clone stably transfected with *fos*-CAT (positive control); *lanes 3,5,7,* unmodified plasmid (20 μg per well); *lanes 4,6,8,* alkylated plasmid (20 μg per well). **A:** Nontumorigenic cells. **B:** Tumorigenic cells; n = 5.

(Fig. 3B, lane 3). An advantage of the alkylating groups is that in the appropriate triple-stranded complexes, the reaction should occur with high accuracy at single target guanosine residues. N-Glycosidic bonds between this guanosine base and its deoxyribose moiety is most susceptible to hydrolysis in DNA. Depurination occurs spontaneously with a relatively high frequency, even under physiological conditions [Lindahl, 1993]. It was demonstrated that by alkylation, the depurination rate is accelerated by 10^6-fold [Loeb and Preston, 1986]. The resultant apurinic sites then led to the apurinic site chain breakage at high temperatures [Suzuki et al., 1994]. We used high-temperature sample incubation in the appropriate 80% formamide loading buffer just before loading onto the denaturing polyacrylamide gel.

The triplex formation followed by site-specific alkylation of the target was supposed to change the transcriptional activity of the modified promoter. For that reason, the whole plasmid DNA was subjected to alkylation by the OR, and then transfected into the cells to estimate the changes in transcriptional activity of the modified promoter in the cell. To test the specificity of interaction of the oligonucleotide derivatives with the target sequence in the whole *fos*-CAT plasmid, the latter was modified with OR under standard conditions. DNA was digested with *Hin*dIII and *Ava*I enzymes, labeled at the *Ava*I sites and then subjected to piperidine treatment. The resultant products were resolved on denaturing polyacrylamide gel. Figure 4 shows the results of these experiments. The modification sites were identified according to the mobility of the restriction fragments with known length: 478-bp and 108-bp fragments, which corresponded to *Ava*I and *Hin*dIII, the DNA restriction products. The modified product was marked with an upper case "M." These experiments were carried out to confirm the precision of the modification address within the whole plasmid construct immediately before performing the transfection experiments.

c-fos Transcription Modulation by OR

Tumorigenic and nontumorigenic fibroblasts were transiently transfected with native (*fos*-CAT^C) and alkylated by OR (*fos*-CAT^M) plas-

mids. Nontumorigenic fibroblasts were used in different phases the cell cycle: quiescent (0.5% FCS for 48 h after transfection), logarithmic (10% FCS), stimulated (0.5% for 48 h after transfection, plus 20% FCS for 3 h before cell extract preparation). Tumorigenic cells could be used only in the logarithmic phase of growth [Lavrovsky et al., 1993; Lavrovsky et al., 1994]. As can be seen in Figure 5, OR was able to decrease *fos*-CAT expression dramatically (compare lanes 3 and 4, 5, and 6, 7, and 8). Tumorigenicity and cell cycle did not play a significant role.

Experiments with transient transfection (Fig. 5) showed that triplex formation in the RCE-like site was crucial for *fos*-CAT expression in fibroblasts and inhibited expression by roughly 70%, $P < 0.05$. This could be due to the modification of binding abilities of Rb-dependent proteins [Robbins et al., 1990]. On the other hand, this site is very close to TATA element and could be involved in basal transcription machinery formation. Therefore, the modification in this site may result in a dramatic *fos*-CAT depression when plasmid DNA incubates with OR.

Oligonucleotide derivatives appear to offer considerable promise as potential gene-targeted drugs such as antivirals and specific inhibitors of oncogene expression. Transcriptional control by triplex-forming complexes for suppression of oncogene expression, as shown in this study, is another example. Since the antisense approach has been quite successful (these reagents having similar structures and binding affinities to TFOs), the success of binding chemically-modified oligonucleotides, such as with the addition of cholesterol [Ing et al., 1993] at neutral pH, is indeed promising. Noonberg et al. have demonstrated that many of the obstacles of this approach, i.e., inefficient and heterogeneous cellular uptake, intracellular sequestration, and rapid intracellular and extracellular degradation can be circumvented. Thus, novel approaches to this technique may be a powerful research tool and with the affinity and specificity of TFOs, such as described here, a stategy may develop into a mechanism by which we can investigate the role of oncogenes in normal and cancerous cells.

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