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Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides

(antisense therapy/NF-κB)

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ABSTRACT **Modified analogues of antisense oligodeoxynucleotides (ODNs), particularly phosphorothioates ([S]ODNs), have been extensively used to inhibit gene expression. The potential sequence specificity of antisense oligomers makes them attractive as molecular drugs for human diseases. The use of antisense [S]ODNs to inhibit gene expression has been complicated by frequent nonspecific effects. In this study we show in diverse cell types that [S]ODNs, independent of their base sequence, mediated the induction of an Sp1 nuclear transcription factor. The [S]ODN-mediated Sp1 induction was rapid and was associated with elevated levels of Sp1 protein. This induction was dependent on NF-κB activity, since inhibition of NF-κB activity abolished the [S]ODN-induced Sp1 activity. [S]ODN-induced Sp1 activity was seen in mouse spleen cells following *in vivo* administration. Sp1 activity induced by [S]ODNs required the tyrosine kinase pathway and did not have transactivating potential. These results may help to explain some of the nonspecific effects often seen with [S]ODNs.**

The potential usefulness of antisense oligonucleotides to inhibit gene expression has been extensively documented (1, 2). This approach involves introducing oligonucleotides complementary to mRNA into cells (3). The specific inhibition of a target gene's expression by an antisense mechanism is dependent on the formation of an antiparallel duplex by complementary base pairing between the antisense DNA and the target mRNA. Phosphorothioate oligodeoxynucleotides ([S]ODNs), because of their nuclease resistance, offer considerable promise for antisense therapy, and several investigators have reported their usefulness in *in vivo* antisense therapy (4–8).

We have recently shown that antisense inhibition of the RelA subunit of the NF-κB transcription factor causes a pronounced block of cellular adhesion to a substratum *in vitro* and inhibits tumor cell growth *in vitro* and *in vivo* (7, 9, 10). In our efforts to understand the molecular mechanism underlying inhibition of tumor cell growth by RelA antisense [S]ODNs, we observed that the control oligomers but not antisense RelA oligomers induced a nuclear transcription factor which was subsequently identified as Sp1. Further experiments revealed that regardless of their orientation and size, phosphorothioates induced Sp1 activity rapidly in numerous cell types following *in vitro* and *in vivo* treatment. This induction could be prevented by inhibiting NF-κB activity. In the context of Sp1 involvement in the regulation of viral and cellular promoters, these results might explain some of the nonspecific effects often seen with phosphorothioates.

MATERIALS AND METHODS

ODNs. The [S]ODNs and unmodified ODNs used in the study (Table 1) were synthesized as described (7, 9, 11). The double-stranded [S]ODN NF-κB competitive inhibitor has been described (12).

Electrophoretic Mobility-Shift Assays. Cytoplasmic and nuclear extracts were isolated and electrophoretic mobility-shift assays were performed as described (7, 9, 13). An ODN containing the sequence 5′-GTA-GGG-GAC-TTT-CC-GAG-CTC-GAG-ATC-CTA-TG-3′ was used to detect NF-κB activity (14, 15), and an ODN containing the sequence 5′-ATT-CGA-TCG-GGG-CGG-GGC-GAG-3′ was used to detect Sp1 activity. Probes were labeled with [α-^{32}P]dNTP as described (15). Nuclear and cytoplasmic extracts (10 μg) and ^{32}P-labeled probe (1 ng; 50,000 cpm) were used in the binding reactions. Unlabeled as well as mutant or irrelevant competitive inhibitors were used to establish specificity of the gel shift. Antibodies to the NFKB1 and RelA subunits of NF-κB and to Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used in the gel shift assays. Complexes were resolved by electrophoresis in a nondenaturing 4% polyacrylamide gel and autoradiographed.

Western Blotting. Nuclear extracts (10 μg) from K-BALB cells or other cell lines treated with diverse [S]ODNs were analyzed by Western blotting and the ECL detection method (Amersham) utilizing an Sp1 antibody according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Lines and Oligomer Treatment. K-BALB (murine fibroblast), B-16 (murine melanoma), HOS-MNNG (human osteosarcoma), DU-145 (human prostate carcinoma), and T-47D (human breast carcinoma) cells were maintained as described (7). Primary mouse splenocytes were isolated as described (13, 16). [S]ODNs were administered to mice as in prior studies (7, 13). Confluent cultures were treated with concentrations of oligomers or other compounds as shown. Genistein and staurosporine were obtained from Upstate Biotechnology.

RESULTS

[S]ODNs Induce a Nuclear DNA-Binding Activity. During our studies aimed at understanding the molecular mechanism underlying inhibition of tumor cell growth by RelA/NF-κB antisense [S]ODNs (7), we observed that treating cells with antisense RelA [S]ODNs inhibited the expression of the cytokine transforming growth factor β1 (TGFβ1). We reasoned that the TGFβ1 promoter sequence might harbor NF-κB binding sites. Among several double-stranded mobil-

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Abbreviations: ODN, oligodeoxynucleotide; [S]ODN, phosphorothioate ODN; TGF, transforming growth factor.
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Table 1. [S]ODNs used in this study

Gene (source)	Sequence (5′ to 3′)	Size, bp	Orientation*
RelA (M)†	ACC-ATG-GAC-GAT-CTG-TTT-CCC-CTC	24	S
RelA (H)	GCC-ATG-GAC-GAA-CTG-TTC-CCC-CTC	24	S
RelA (M)†	GAG-GGG-AAA-CAG-ATC-GTC-CAT-GGT	24	A
RelA (H)	GAG-GGG-GAA-CAG-TTC-GTC-CAT-GGC	24	A
NFKB1 (M)	ACC-ATG-GCA-GAC-GAT-GAT-CCC	21	S
NFKB1 (M)	GGG-ATC-ATC-GTC-TGC-CAT-GGT	21	A
MCF Env (M)	GAG-AAG-GCT-GGA-CCT-TCC-AT	20	A
HIV Rev	TCG-TCG-CTG-TCT-CCG-CTT-CTT-CTT-GCC	27	A
DCC (M, R, H)	TCT-AAG-ACT-ATT-CTC-CAT-ATT	21	A
MDM2 (H)	AGA-CAT-GTT-GGT-ATT-GCA-CAT-TTG	21	A
E2F (H)	AGG-GGC-CCC-GGC-CAA-GGC-CAT-GAC	24	A
p53 (H)	CCC-TGC-TCC-CCC-CTG-GCT-CC	20	A
TGFα (H)	TCC-AGC-CGA-GGG-GAC-CAT-TTT	21	A
v-Ki-*ras*	GCT-AGT-GGC	9	S
v-Ki-*ras*	GCC-ACT-AGC	9	A
CD11b (H)	AAG-GAC-TCT-GAG-AGC-CAT-GGC	21	A

Phosphorothioate oligomers corresponding to diverse genes as shown were used in the study. MCF Env, mink cell focus-forming retrovirus envelope; HIV Rev, human immunodeficiency virus Rev protein; DCC, deleted in colorectal cancer; MDM2, murine double minute 2 oncogene; E2F, cellular transcription factor; M, murine; H, human; R, rat.
*S, sense; A, antisense.
†Unmodified phosphodiester ODNs were also used for these sequences.

ity-shift probes from the TGFβ1 promoter sequence tested, we detected binding of one of the probes (TGFβ3) encompassing the sequence 5′-GCT-GAA-GGG-ACC-CCC-CTC-GGA-GCC-CGC-CCA-3′ to nuclear extract protein from sense [S]ODN-treated K-BALB cells but not to nuclear extract protein from untreated or antisense [S]ODN-treated cells (Fig. 1). This binding was specific, since it was inhibited competitively by unlabeled double-stranded TGFβ3 DNA but not by NF-κB DNA. These results suggested that the nuclear activity we detected was unrelated to NF-κB activity.

The [S]ODN-Induced DNA-Binding Activity Is Spl. A careful look at the double-stranded TGFβ3 probe revealed an Spl binding site (GGGCGGG) at the 3′ end. Hence we suspected that the sense oligomer-induced nuclear activity might be Spl. We synthesized an authentic Spl mobility-shift oligomer and analyzed the nuclear extracts from confluent K-BALB cells treated with RelA oligomers (Fig. 2*A*). In confluent K-BALB cells no basal level of Spl activity was detectable by electrophoretic mobility-shift assay; subconfluent cultures, on the other hand, showed a higher basal level of Spl activity (data not shown). Treatment of K-BALB cells with control RelA sense oligomers induced Spl activity, but treatment with antisense RelA oligomers failed to induce Spl

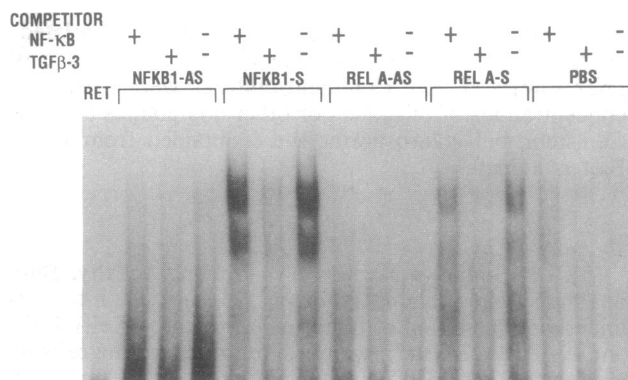

FIG. 1. Nuclear DNA-binding activity is induced by [S]ODN treatment. Nuclear extracts from K-BALB cells treated with phosphate-buffered saline (PBS) or indicated [S]ODNs (20 μM) for 48 hr were analyzed by mobility-shift assay using a double-stranded probe (TGFβ3) in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled double-stranded TGFβ3 or NF-κB competitive inhibitors. RET, rabbit reticulocyte lysate.

activity (Fig. 2*A*). Furthermore, treatment of control (non-oligomer-treated nuclear extracts from K-BALB cells) with [S]ODNs *in vitro* did not cause induction of Spl activity, suggesting that upregulation of Spl activity by [S]ODNs was not an *in vitro* artifact of the gel shift assay. The mobility of sense RelA oligomer-induced Spl activity in the gel was supershifted by the use of an Spl-specific antibody but not by RelA or NFKB1 antibodies (Fig. 2*A*); this suggested that the NF-κB subunits were not components of this complex. The Spl activity induced by the RelA sense oligomers was concentrated in the nucleus, where it was detectable within 30 min, peaked at 6 hr, and stabilized above the basal level by 24 hr (Fig. 2*B*). We reasoned that either the sense oligomers induced Spl activity in a specific manner or that induction of Spl could occur only in the presence of functional NF-κB activity. To clarify this point, we exposed the K-BALB cells to concentrations of RelA antisense [S]ODNs that would not inhibit NF-κB activity, in parallel with sense [S]ODN treatment (Fig. 2*C*). The RelA sense [S]ODN induced Spl activity at 2, 10, and 20 μM (Fig. 2*C*). Surprisingly, RelA antisense also induced Spl activity at 2 μM, but not at higher doses known to inhibit NF-κB activity. These findings suggested that the induction of Spl activity could be dependent on NF-κB activity but not necessarily on the sequence of the inducing oligomer.

Sequence-Independent Induction of Spl Activity by [S]ODNs. To test the universality of induction of Spl activity by [S]ODNs we chose several [S]ODNs in both sense and antisense orientations of varying lengths and targets (Table 1). Treatment of K-BALB cells with any of these [S]ODNs, regardless of their length (9–24 bp) or orientation (sense or antisense), caused induction of Spl activity (Fig. 3*A*). Additionally, [S]ODN homopolymers (sdC_3 and sdC_{15}) as well as unmodified phosphodiester ODNs to murine RelA (sense and antisense) induced this activity. In contrast to murine RelA antisense [S]ODNs, the antisense phosphodiester ODNs did not inhibit NF-κB activity. The ODNs were as potent as the [S]ODNs in inducing Spl activity. This induction was prevented by pretreatment of cells with RelA antisense [S]ODNs. Mechanisms other than antisense that reportedly inhibit NF-κB activity [e.g., double-stranded [S]ODN NF-κB competitive inhibitor (12) or antioxidants related to pyrrolidine dithiocarbamate (17)] also prevented Spl induction by [S]ODNs in various cell lines (J.R.P. and R.N., unpublished data). The induction of Spl activity by [S]ODNs also oc-

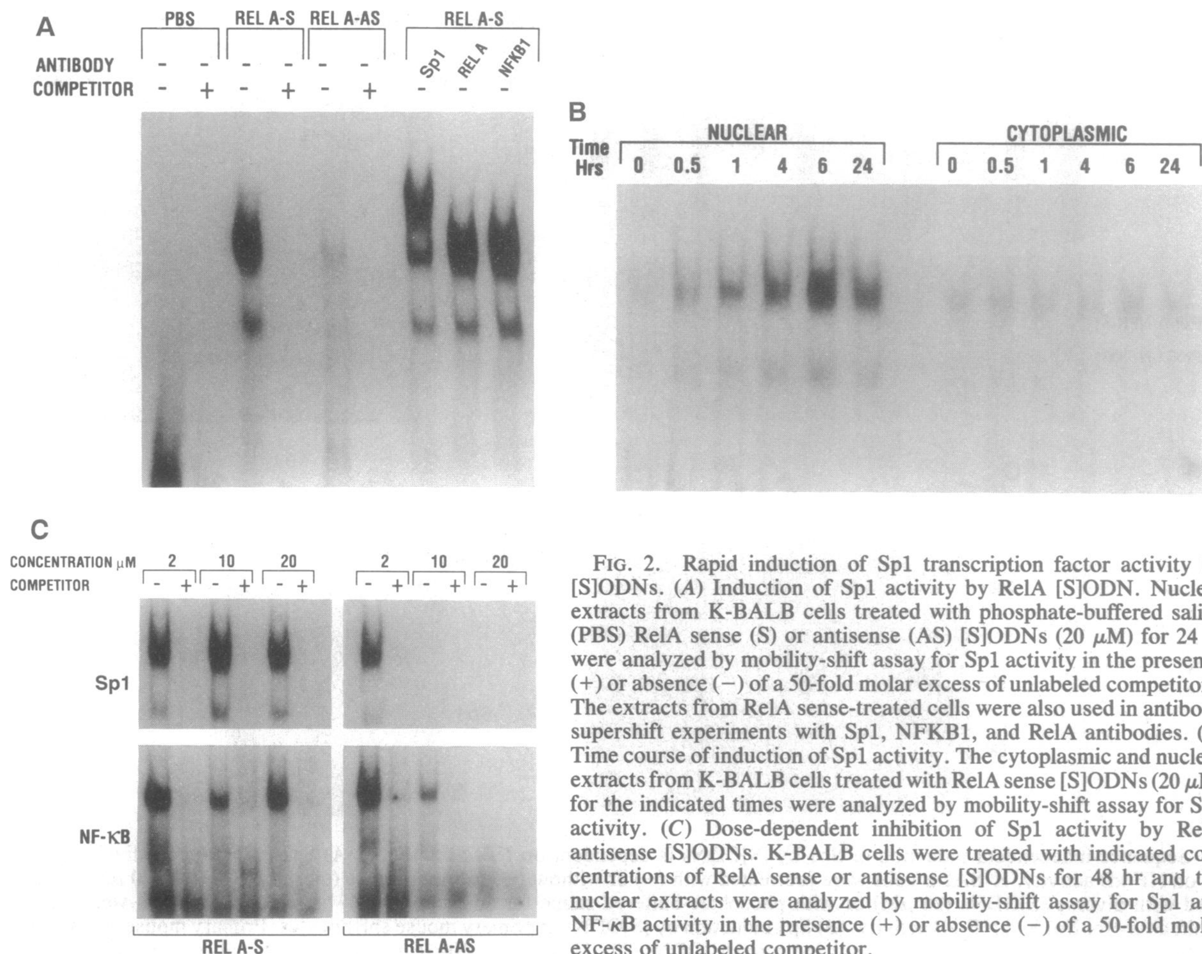

FIG. 2. Rapid induction of Sp1 transcription factor activity by [S]ODNs. (*A*) Induction of Sp1 activity by RelA [S]ODN. Nuclear extracts from K-BALB cells treated with phosphate-buffered saline (PBS) RelA sense (S) or antisense (AS) [S]ODNs (20 μM) for 24 hr were analyzed by mobility-shift assay for Sp1 activity in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled competitors. The extracts from RelA sense-treated cells were also used in antibody supershift experiments with Sp1, NFKB1, and RelA antibodies. (*B*) Time course of induction of Sp1 activity. The cytoplasmic and nuclear extracts from K-BALB cells treated with RelA sense [S]ODNs (20 μM) for the indicated times were analyzed by mobility-shift assay for Sp1 activity. (*C*) Dose-dependent inhibition of Sp1 activity by RelA antisense [S]ODNs. K-BALB cells were treated with indicated concentrations of RelA sense or antisense [S]ODNs for 48 hr and the nuclear extracts were analyzed by mobility-shift assay for Sp1 and NF-κB activity in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled competitor.

curred in primary mouse splenocytes (Fig. 3*B*) as well as in other cell lines, including HOS-MNNG (osteosarcoma), T-47D (breast carcinoma), and DU-145 (prostate carcinoma) (J.R.P. and R.N., unpublished data).

This led us to investigate whether the enhanced Sp1 activity was associated with elevated levels of Sp1 protein. K-BALB cells were treated with [S]ODNs (Fig. 3*C*) and the nuclear extracts were prepared and analyzed by Western blotting for Sp1 protein. In agreement with the gel shift results (Fig. 3*A*), Sp1 protein was induced by treatment with [S]ODNs, the sole exception being the RelA antisense [S]ODN. We next addressed whether the *in vivo* administration of [S]ODNs induced Sp1 activity in mice. The induction of Sp1 activity was seen in spleen cells obtained 24 hr after [S]ODNs were administered subcutaneously (70 mg/kg) to BALB/c *nu/nu* mice; this activity was supershifted by Sp1 antibody (Fig. 3*D*).

Mechanism of Sp1 Induction by [S]ODNs. To begin to understand the molecular mechanism underlying the induction of Sp1 by [S]ODNs, we tested the effects of kinase inhibitors (Fig. 4*A*). Pretreatment with a tyrosine kinase inhibitor (genistein), but not with a protein kinase C inhibitor (staurosporine), inhibited the induction of Sp1 activity by RelA sense oligomers. These results implicated a specific tyrosine phosphorylation-mediated mechanism in the Sp1 induction by [S]ODNs. Furthermore, the induction was blocked by pretreatment with cycloheximide but not actinomycin D, suggesting that new protein synthesis was necessary for this induction but that new mRNA transcription was not (J.R.P. and R.N., unpublished data).

[S]ODNs as well as phosphodiester ODNs bind to cell surface "receptors" (18). The binding can be competitively inhibited by polyanions such as pentosan sulfate (19). Since phosphodiester ODNs also induced Sp1 activity, we reasoned that causing the oligomers to bind to the cell surface receptors might be sufficient for Sp1 induction. Interestingly, we found that pentosan sulfate did not induce Sp1 activity, whereas another polyanion, suramin, did (Fig. 4*B*), suggesting that neither the oligomer binding to the surface nor the sulfated structure is sufficient for Sp1 induction.

DISCUSSION

These studies were undertaken to clarify the molecular mechanisms involved in antisense-mediated inhibition of tumor cell growth *in vitro* and *in vivo* by the RelA subunit of the NF-κB transcription factor complex (7, 9). The NF-κB complex participates in the induction of numerous cellular and viral genes (20–22). NF-κB has two subunits, p50 (NFKB1) and p65 (RelA); both are members of a rapidly growing class of transcription factors that have unique amino-terminal DNA binding and dimerization domains (14, 23).

Unexpectedly, a double-stranded DNA probe from a cellular promoter (that of the TGFβ1 gene) was found to bind specifically to the nuclear extracts from RelA or NFKB1 sense [S]ODN-treated cells but not to untreated or to antisense [S]ODN-treated cells. The nature of this activity was established to be Sp1 transcription factor. Various [S]ODNs caused rapid induction of Sp1 transcription factor activity in diverse cell types, *in vitro* as well as *in vivo*, in a sequence-independent manner. This induction was dependent on NF-κB activity, since inhibition of NF-κB activity prevented the Sp1 induction by [S]ODNs. In addition, the Sp1 induction by [S]ODNs was prevented by genistein, a tyrosine kinase inhibitor, suggesting the involvement of protein phosphorylation in this response. The enhanced Sp1 DNA-binding activity induced by various

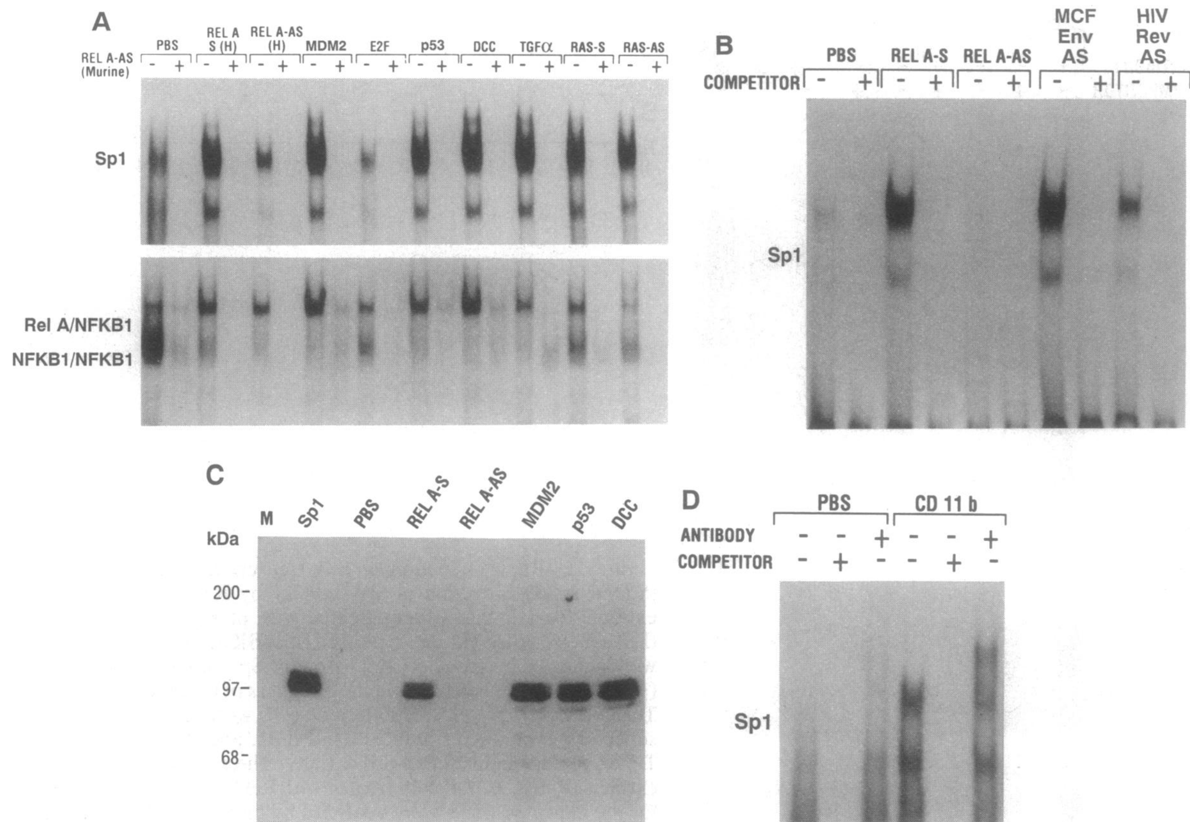

FIG. 3. Sequence-independent induction of Sp1 by [S]ODNs is dependent on NF-κB activity. (*A*) Sp1 induction by diverse [S]ODNs is dependent on NF-κB activity. K-BALB cells were pretreated with (+) or without (−) RelA antisense (murine) [S]ODNs (20 μM) for 24 hr and then treated with diverse [S]ODNs (20 μM) or with phosphate-buffered saline for an additional 24 hr. Nuclear extracts were analyzed by mobility-shift assay for Sp1 and NF-κB activity. (*B*) Sp1 induction by [S]ODNs in primary mouse splenocytes. Primary mouse splenocytes were isolated and cultured as described and treated with indicated [S]ODNs (20 μM) for 48 hr. Nuclear extracts were analyzed by mobility-shift assay for Sp1 activity in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled competitor. (*C*) Elevation of Sp1 protein levels by [S]ODNs. K-BALB cells were treated with PBS or 20 μM [S]ODNs as indicated for 24 hr, and the nuclear extracts were analyzed by Western blot analysis using Sp1 antibody and an ECL chemilluminescence kit (Amersham). Authentic Sp1 protein was used as a positive control. Lane M, molecular size markers (BRL). (*D*) Induction of Sp1 activity *in vivo*. Duplicate mice (BALB/c *nu/nu*) were injected with phosphate-buffered saline (PBS) or a representative [S]ODN, CD11b (1.4 mg/100 μl) subcutaneously, and 24 hr later spleens were isolated and single-cell suspensions were prepared. Nuclear extracts were analyzed by mobility-shift assay for Sp1 activity in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled competitors. Supershifts were performed with Sp1 antibody. Results from representative experiments are shown.

[S]ODNs was also reflected in enhanced levels of Sp1 protein. Further, our observation that phosphodiester ODNs as well as sulfated polyanions such as suramin, but not pentosan sulfate, induced Sp1 activity suggests that the presence of a sulfated structure in these oligomers is not sufficient for Sp1 induction.

The dependence of [S]ODN-induced Sp1 activity upon the RelA subunit of the NF-κB transcription complex is striking. Until the sequence of the Sp1 promoter is known, it will not be possible to clarify whether this dependency on RelA is a primary or transactivational effect. Furthermore, the shift in

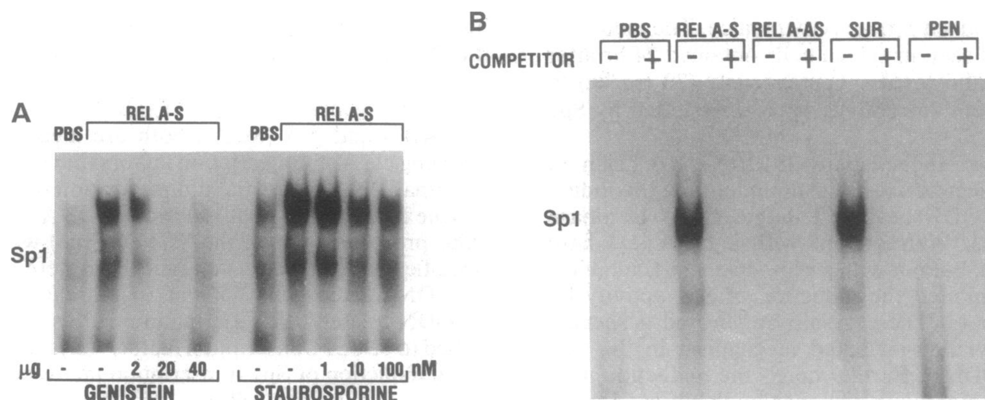

FIG. 4. Mechanism of Sp1 induction by [S]ODNs. (*A*) [S]ODN-induced Sp1 activity is dependent on protein phosphorylation. K-BALB cells were treated with genistein or staurosporine at the indicated concentration for 1 hr before being treated with RelA sense (S) [S]ODNs (20 μM) for 4 hr. Nuclear extracts were prepared and analyzed by mobility-shift for Sp1 activity. (*B*) Differential induction of Sp1 activity by polyanions. K-BALB cells were treated with phosphate-buffered saline (PBS), RelA sense (S) antisense (AS) [S]ODNs (both at 20 μM), suramin (SUR, 300 μM), or pentosan sulfate (PEN, 50 μM) for 48 hr. Nuclear extracts were analyzed by mobility-shift assay for Sp1 activity in the presence (+) or absence (−) of a 50-fold molar excess of unlabeled competitor.

the electrophoretic mobility of the NF-κB complex in nuclear extracts from [S]ODN-treated cells (Fig. 3*A*) suggests that [S]ODNs also may modulate the delicate balance between the homo- and heterodimers of the NF-κB complex. In the context of several recent reports suggesting physical interactions of NF-κB components—in particular the RelA subunit—with various transcription factors, including Sp1 (24–29), our results raise the possibility that the RelA NF-κB complex might play a regulatory role in modulating transcription factors.

The implications of [S]ODN-mediated Sp1 induction *in vitro* and *in vivo* are yet to be established. Originally discovered as a specific factor required for simian virus 40 transcription (30), Sp1 is a general transcription factor that interacts with G·C boxes in the promoter region of several viran and cellular genes (for a review see ref. 31). Although its protein is ubiquitous, the levels of Sp1 mRNA and its protein vary among tissue types and (in at least some cell types) stages of development (32). Other than the present observation, no inducers of Sp1 activity have been described.

Several recent reports have demonstrated the *in vivo* efficacy of antisense [S]ODNs (4–8), and clinical trials of antisense [S]ODNs are already underway (for a review, see ref. 2). Nonetheless, it has frequently been difficult to obtain unequivocal evidence that [S]ODNs are truly sequence specific. Often the control oligomers exert sequence-selective effects (3, 13). In the case of charged phosphorothioates, such sequence-selective effects could be due to their ability to interact or bind to proteins. This complicates attempts to interpret antisense oligomer-based experiments. Activation of transcription factors by these oligomers might at least partially explain their lack of specificity. Since transcription factors are potent regulators of cellular and viral gene expression, it is not unreasonable to exercise caution about activation of such factors by [S]ODNs.

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