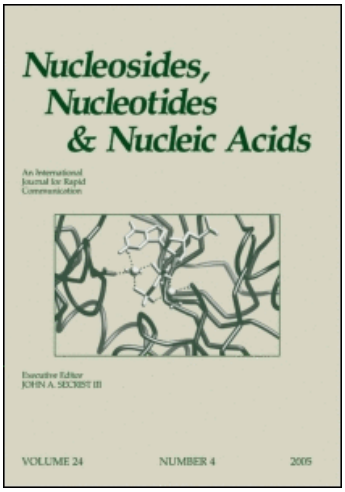

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Effect of Oligomer Length and Base Substitutions on the Cytotoxic Activity and Specific Nuclear Protein Recognition of GTn Oligonucleotides in the Human Leukemic CCRF-CEM Cell Line

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EFFECT OF OLIGOMER LENGTH AND BASE SUBSTITUTIONS ON THE CYTOTOXIC ACTIVITY AND SPECIFIC NUCLEAR PROTEIN RECOGNITION OF GTn OLIGONUCLEOTIDES IN THE HUMAN LEUKEMIC CCRF-CEM CELL LINE.

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ABSTRACT: We have identified phosphodiester oligonucleotides composed of G and T bases, named GTn, which are able to inhibit the cellular growth of human cancer cell lines by recognising specific nuclear proteins. We demonstrated that GTn oligonucleotides require a length of at least 20 nucleotides in order to exert a significant cytotoxic effect and to retain the specific protein binding ability. In addition, we found that GTn cytotoxicity was lost when A or C bases were introduced at either 3' and 5'end or within the GTn sequences.

INTRODUCTION.

Oligonucleotides (ODNs) are able to recognise specific DNA or RNA sequences (antisense[1], triple helix-forming ODNs[2], ribozymes[3]) or proteins (aptamers and decoys)[4-5], thus impairing the expression of genes involved in pathological events such as viral infections, tumor progression and metabolic disorders[6]. Our research deals with ODNs composed of G and T bases, named GTn, that are able to exert a significant dose-dependent growth inhibition effect in different human cancer cell lines by binding to specific nuclear proteins[7]. The main purpose of this work was to investigate the influence that ODN length and base substitutions had on the biological properties of GTn ODNs.

MATERIALS AND METHODS.

ODNs synthesis and purification. The ODNs were automatically synthesised by phosphoramidite method and purified by gel permeation chromatography. After lyophilisation, ODNs were resuspended in saline solution and sterilised on a 0.22 μM filter.

Cell cultures and cytotoxicity assay. The cytotoxic effect of the ODNs was evaluated by seeding $5x10^4$ cells/ml human T-lymphoblastic leukemic CCRF-CEM cells[7] in a 96 well microtiter plate. After 24 h, the ODNs were added to the cells and the cellular growth was evaluated after 72 h of further culture by MTT incorporation, as previously described[7]. The percentage of cellular growth of ODN-treated cells was calculated by considering the absorbance values of control untreated cells as 100%.

UV cross-linking competition assay. Total nuclear extracts were obtained with minor modifications of Dignam procedure[7]. 3 μg of nuclear extracts in 20 mM Hepes buffer, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, containing protease inhibitors were incubated with aspecific competitors (1 μg salmon sperm sonicated DNA and 1 μg $T(CTTT)_6CT$ ODN), 1 ng of [γ-^{32}P]-labelled $T(GTTT)_6GT$ ODN and the specific competitors, when indicated. After 20 min of incubation at room temperature, the samples were UV-irradiated for 10 min at 302 nm, denatured, and then loaded on a 12% SDS-PAGE, according to Laemmli method[8]. The gels were dried and exposed to Kodak XAR-OMAT film.

RESULTS AND DISCUSSION.

We have previously demonstrated that sequences composed of G and T bases, named GTn, with $1 \leq n \leq 7$, exerted a sequence-specific, dose-dependent growth inhibition effect on human cancer cells by the binding to specific nuclear proteins[7].

We investigated the effect that the ODN length and the base-substitutions at 3' and 5'ends or within GTn sequences had on the biological activity of the GTn ODNs. Sequences consisting of GTTT repetitions and the human T-lymphoblastic CCRF-CEM cell line were chosen in this study. We found that the T(GTTT)GT and $T(GTTT)_3GT$ ODNs, having lengths of 7 and 15 bases respectively, did not significantly inhibit the cellular growth, whereas the $T(GTTT)_6GT$ and $T(GTTT)_{12}GT$ ODNs, having lengths of 27 and 51 bases respectively, exhibited a significant dose-dependent cytotoxic activity (FIG.1A). Competition experiments demonstrated that the binding of the labelled $T(GTTT)_6GT$ ODN, having a length of 27 bases, to the nuclear proteins was not affected by the addition of a high molar excess of the non-labelled ODNs having the lengths of 7 and 15 nucleotides, whereas the ODNs having the lengths of 27 and 51 bases efficiently displaced this interaction (FIG.1B). These results suggested that more than 15 nucleotides are required for GTn

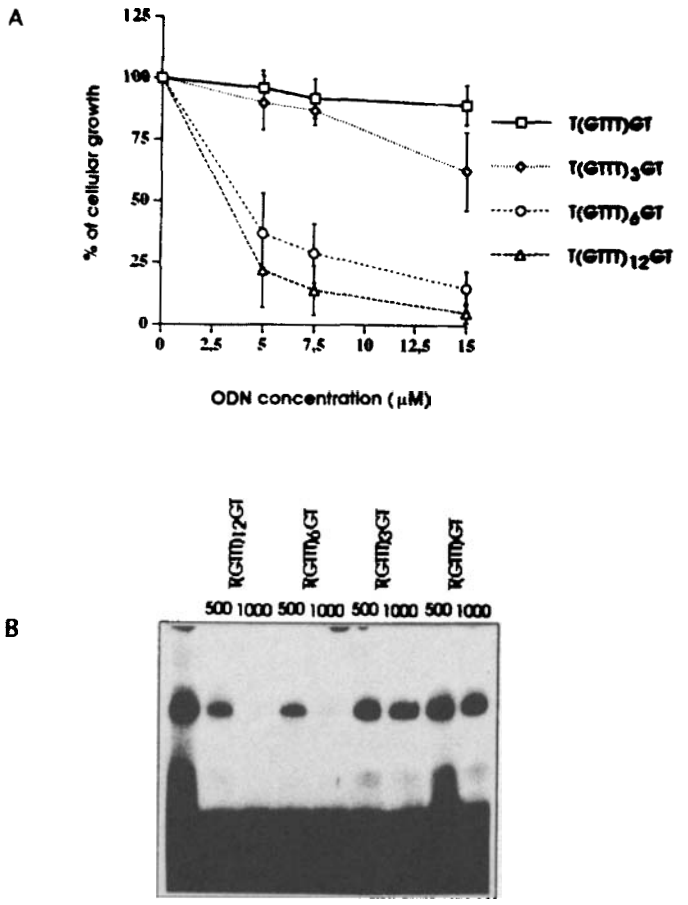

FIG. 1 Effect of the length of GTn ODNs on the cellular growth and nuclear protein binding of CCRF-CEM cells. (**A**) Cytotoxicity assay. $5x10^3$ exponentially growing cells were seeded in 100 μl of complete medium, in a 96 well microtiter plate. The ODNs were added to the medium, at the indicated concentrations, after 24 h of culture. The cellular growth was evaluated after 72 h from the ODN administration by incorporation of MTT, as described in Materials and Methods. (**B**) UV cross-linking competition assay. 3 μg of total nuclear extract were incubated with labelled $T(GTTT)_6GT$ ODN in the absence or presence of a 500 and 1000 fold molar excess of the indicated non labelled ODNs, as described in Materials and Methods.

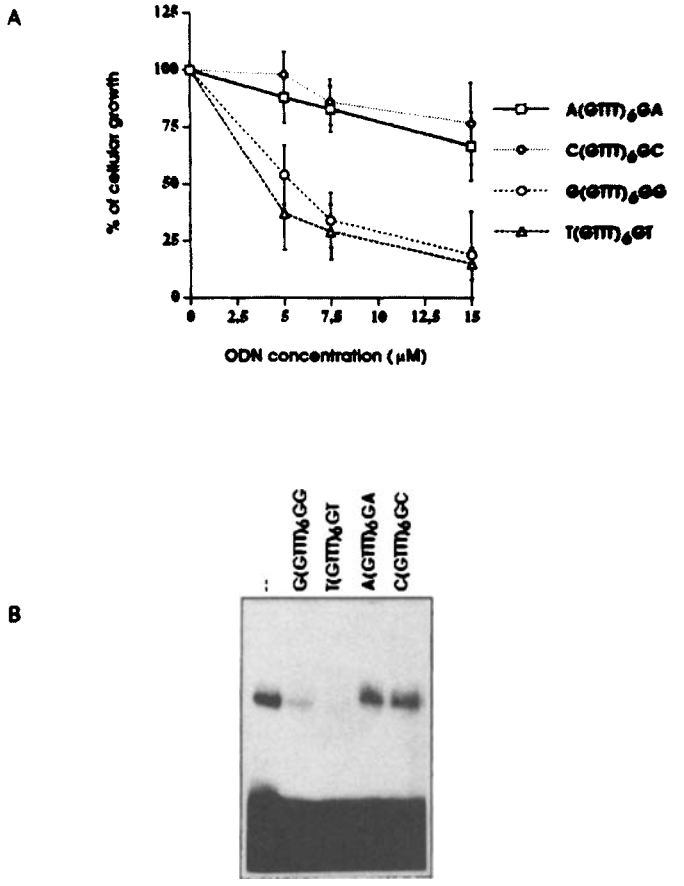

FIG. 2 Effect of base substitutions at the 3' and 5' ends of GTn ODNs on cellular growth and nuclear protein binding of CCRF-CEM cells. (**A**) Cytotoxicity assay. The cells, in exponential growth phase, were seeded at the density of $5x10^4$ cell/ml in a 96 well microtiter plate. The ODNs were added directly to the medium, at the indicated concentrations, after 24 h of culture. The cellular growth was evaluated after 72 h from ODN administration by MTT assay, as described in Materials and Methods. (**B**) UV cross-linking competition assay. 3 μg of total extract were incubated with [γ-^{32}P]-labelled $T(GTTT)_6GT$ ODN in the presence of a 1000 fold molar excess of the indicated non labelled ODNs, as described in Materials and Methods.

sequences to efficiently bind to the specific nuclear proteins and, consequently, exert the cytotoxic activity. Actually, a significant growth inhibition effect and a specific nuclear protein binding activity were obtained utilising GTn ODNs with lengths starting from 20 nucleotides (data not shown). The increase in the cytotoxic ability of longer ODNs may be due to the higher number of potential protein binding sites that they were able to target.

We also investigated the effect of base substitutions at the 3' and 5'ends of the toxic $T(GTTT)_6GT$ sequence. $C(GTTT)_6GC$ (T→C) and $A(GTTT)_6GA$ (T→A) ODNs did not significantly inhibit the growth of CCRF-CEM cells, whereas the $G(GTTT)_6GG$ (T→G) ODN exerted a cytotoxic effect similar to the $T(GTTT)_6GT$ sequence (FIG. 2A). UV cross-linking competition assays demonstrated that the toxic $G(GTTT)_6GG$ sequence efficiently competed with the labelled $T(GTTT)_6GT$ ODN in the binding to the specific nuclear proteins, whereas the non toxic $C(GTTT)_6GC$ and $A(GTTT)_6GA$ ODNs did not (FIG. 2B). We observed, however, that neither the control $T(CTTT)_6CT$ nor the $G(CTTT)_6TG$ ODN, bearing C→G base substitutions at the 3' and 5'ends, affected the cellular growth (data not shown). Furthermore, we also found that the presence of A or C bases within GTn sequences, even when a GTn motif of at least 20 nucleotides was maintained, impaired both the cytotoxic and the nuclear binding activities of these ODNs (data not shown).

These findings demonstrate that the GTn ODNs require G and T bases exclusively and a length of more than 15 nucleotides to elicit the specific nuclear protein interactions and the related cellular growth inhibition effect. Moreover, our results suggest that the introduction of A and/or C bases at the 3' and 5'ends or within the GTn sequence may abolish the GTn ODNs biological activity. A potential therapeutic development of GTn ODNs as anti-cancer drugs should take into consideration these observations to design ODNs with effective pharmacological activity.

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