

Oligo(α -deoxynucleotide)s covalently linked to intercalating agents: Differential binding to ribo- and deoxyribopolynucleotides and stability towards nuclease digestion

(intercalation/acridine derivatives/hybridization)

NGUYEN T. THUONG*, ULYSSE ASSELINE*, VICTORIA ROIG*, MASASHI TAKASUGI†, AND CLAUDE HÉLÈNE†

*Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, 45071 Orléans cedex 02, France; and †Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, Institut National de la Santé et de la Recherche Médicale, Unité 201, and Centre National de la Recherche Scientifique, Unité Associée 481, 61, Rue Buffon, 75005 Paris, France

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ABSTRACT An octathymidylate was synthesized with the α anomer of thymidine instead of the naturally occurring β anomer. This oligonucleotide binds to complementary sequences containing β -nucleosides. Binding to ribose-containing oligomers and polymers is much stronger than binding to deoxyribose-containing analogs. A derivative of acridine (9-amino-6-chloro-2-methoxyacridine) was covalently attached either to the 5' phosphate or to the 3' phosphate of the α -octathymidylate. A pentamethylene linker was used to bridge the phosphate group and the 9-amino group of the acridine derivative. In both cases the complexes with the complementary sequences were strongly stabilized due to the additional binding energy provided by intercalation of the acridine ring within the miniduplex structure formed by the oligonucleotide with its target sequence. The acridine-substituted α -oligothymidylates did not lose their discrimination between ribose and deoxyribose-containing complementary sequences. The α -oligothymidylates were much more resistant towards endonucleases than their β analogs, independently of whether they were linked to the acridine derivative. Acridine substitution provided additional protection against the corresponding exonucleases. α -Oligodeoxynucleotides covalently linked or not to intercalating agents represent families of molecules that open possibilities to block mRNA translation or viral RNA expression *in vitro* and *in vivo*.

The regulation of gene expression in living organisms is achieved in most cases by specific proteins that recognize either a defined base (base-pair) sequence or a local conformation, which may result from the folding of the polynucleotide backbone and involve regions that are far apart in the primary sequence (1). It has been recently demonstrated that small RNAs, at least in bacteria, could also be involved in the regulation of gene expression by blocking translation through hybridization with mRNAs (2). Anti-sense RNAs have been used to specifically arrest protein synthesis in prokaryotic as well as eukaryotic systems (2–6). Synthetic oligodeoxynucleotides complementary to mRNAs can play the same role (7–10). The use of oligonucleotides to block mRNA translation *in vivo* is limited, however, by their sensitivity to nucleases and their poor penetration into cells in culture. To overcome these problems different modifications of oligonucleotides have been described. Miller and co-workers have shown that oligophosphonates can penetrate into cells in culture and remain stable with respect to nucleases (11, 12). We have recently reported the synthesis of oligodeoxynucleotides covalently linked to intercalating agents (13–15). The oligonucleotide keeps its binding speci-

ficity towards its complementary sequence, while the intercalating agent provides an additional binding energy that stabilizes the complex. The covalent linkage at the 5' end or 3' end of the oligonucleotide protects it against exonucleases (but not against endonucleases) and facilitates the uptake of the oligonucleotide by cells in culture (15). These modified oligonucleotides can block mRNA translation in a specific way (16).

Here we report the synthesis of oligodeoxynucleotides whose building units are α -deoxynucleotides instead of the natural β anomers. These α -oligonucleotides are much more resistant to nucleases than their β analogs. They form very stable complexes with polyribonucleotides and less stable complexes with corresponding polydeoxyribonucleotides. They can be covalently linked to intercalating agents, which stabilize all complexes without losing the discrimination between ribo- and deoxyribopolynucleotides.

MATERIALS AND METHODS

α -Thymidine was purchased from Sigma. Its purity was checked by thin-layer chromatography. The oligo(α -thymidylate) α -(Tp)₇T[‡] was synthesized by the phosphotriester method exactly as described for β -oligonucleotides (17). Covalent linkage of 6-chloro-2-methoxy-9-(ω -pentylamino)-acridine to the 3' phosphate of oligo(α -thymidylate)s [yielding the product symbolized α -(Tp)₈m₅Acr] was achieved by the procedure previously described (18). Attachment of the acridine derivative at the 5' end [yielding α -Acrm₅(pT)₈] required the introduction of an arylphosphodiester group at the 5'-OH position of the oligo(α -thymidylate). This was achieved with high yield by using arylphosphopyridinium as previously described (ref. 19 and unpublished data). Deprotection of phosphate groups was carried out with a mixture of benzohydroxamic acid and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in aprotic medium. All compounds were purified by high-performance liquid chromatography (HPLC) on an ion-exchange column followed by reverse-phase chromatography. The structure of the synthesized α oligomers is shown in Fig. 1.

Extinction coefficients were determined after digestion of the oligonucleotides by nucleases. During the enzymatic process isobestic points were observed in the UV range for α -(Tp)₇T (285 nm) and in the visible range for the acridine-substituted oligonucleotides (444 nm for 3' substitution and 448 nm for 5' substitution). This allowed us to determine

Abbreviations: Acr, 9-amino-6-chloro-2-methoxyacridine; m₅, pentamethylene.

[‡]Note that this is the deoxy oligonucleotide. Deoxynucleotides other than thymidylate are indicated by the prefix d-, while ribonucleotides are indicated by r-.

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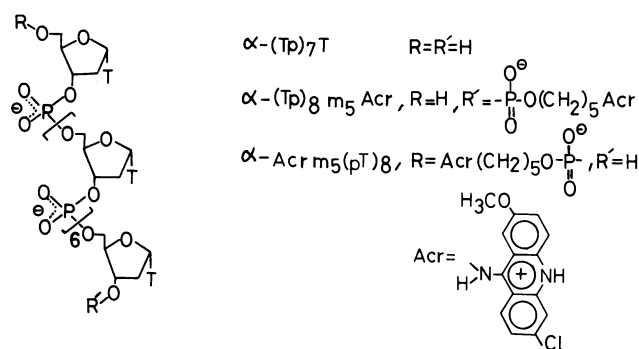


FIG. 1. Structure of the oligothymidylates covalently linked to an intercalating agent. Acr represents 9-amino-6-chloro-2-methoxyacridine. The 9-amino group bears a pentamethylene (m_5) linker.

molar extinction coefficients, taking $\epsilon_{423} = 9750 \text{ M}^{-1}\text{cm}^{-1}$ for the free acridine ring at pH 7 (20) (see Table 1).

Endo- and exonucleases were purchased from Boehringer Mannheim. Polynucleotides and the oligonucleotides $r(\text{Ap})_7r\text{A}$ and $d(\text{pA})_8$ were from P.L. Biochemicals. Absorption spectra were recorded with either a Cary 210 or a Uvicon spectrophotometer.

RESULTS

Binding of $\alpha\text{-(Tp)}_7\text{T}$ to Oligo- and Polyadenylates

Interaction between $\alpha\text{-(Tp)}_7\text{T}$ and poly(rA), poly(dA), $r(\text{Ap})_7r\text{A}$, and $d(\text{pA})_8$ was followed by absorption spectroscopy in the UV range. Adding increasing concentrations of poly(rA) or $r(\text{Ap})_7r\text{A}$ to an $\alpha\text{-(Tp)}_7\text{T}$ solution at 2°C led to a sharp break when absorbance at 268 or 260 nm was plotted versus A/T concentration ratio. The break occurred at a 1:1 ratio, indicating the formation of a double helix in both cases. The calculated hypochromicities for a 1:1 ratio at 2°C were 10% and 9% at 260 nm for poly(rA) and $r(\text{Ap})_7r\text{A}$, respectively. With poly(dA) and $d(\text{pA})_8$ the stoichiometry at the break point was less than 1 (about 0.8 A to 1 T), which suggested either that a mixture of triple (1A:2T) and double (1A:1T) helices was formed or that at 2°C the double helix was partially melted. The analysis of melting curves (see below) seems to favor the second hypothesis, even though there was evidence for triple helix formation at higher ionic concentration (results not shown).

Upon increasing the temperature of the 1:1 (A to T) mixtures formed at 2°C a hyperchromicity was observed. Adenine-containing oligomers and polymers exhibit a hyperchromicity upon increasing temperature due to unstacking of the bases. Plotting the difference in absorbance between the 1:1 mixture and the sum of the two separated compounds gave the melting curves shown on Fig. 2. The corresponding melting temperatures (temperature at which half of the

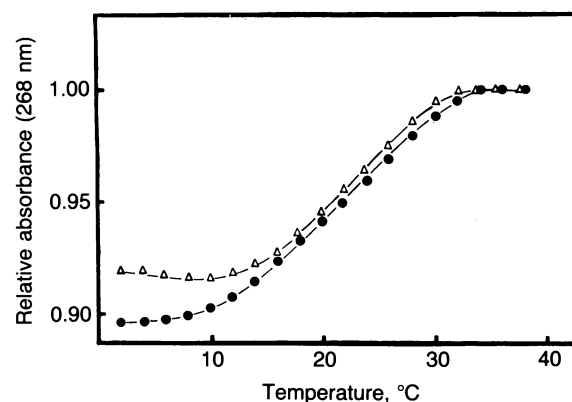


FIG. 2. Temperature dependence of absorbance at 268 nm for a 1:1 (A to T) mixture of $\alpha\text{-(Tp)}_7\text{T}$ with poly(rA) (●) or $r(\text{Ap})_7r\text{A}$ (△). Measurements were carried out in a pH 7 buffer containing 0.01 M sodium cacodylate and 0.1 M NaCl.

dissociation has occurred) are presented in Table 1. The melting temperatures observed with poly(rA) and $r(\text{Ap})_7r\text{A}$ are very close to each other and the widths of the transition are nearly identical. This indicates that binding of $\alpha\text{-(Tp)}_7\text{T}$ to poly(rA) is not cooperative, in contrast to what was previously found for β -oligothymidylates. Also, it should be noted that the melting temperature for $\alpha\text{-(Tp)}_7\text{T}$ bound to its complementary sequence $r(\text{Ap})_7r\text{A}$ is higher than that for $\beta\text{-(Tp)}_7\text{T}$, indicating that the α - β hybrid is more stable than the β - β hybrid. The melting temperatures of the complexes of $\alpha\text{-(Tp)}_7\text{T}$ with poly(dA) and $d(\text{pA})_8$ were lower than those for the corresponding ribopolymers.

Binding of Acridine-Substituted $\alpha\text{-(Tp)}_7\text{T}$ to Oligo- and Polyadenylates

Addition of poly(rA), poly(dA), $r(\text{Ap})_7r\text{A}$, or $d(\text{pA})_8$ to a solution of either $\alpha\text{-Acr m}_5(\text{pT})_8$ or $\alpha\text{-(Tp)}_8\text{m}_5\text{Acr}$ induced a hypochromicity in the visible absorbance band of the acridine derivative. Plotting relative absorbance versus $[\text{A}]/[\text{Acr}]$ led to a stoichiometry of 8, indicating that a double helix (1A to 1T) was formed in all cases. Typical examples of the titration curves are shown in Fig. 3, and the hypochromicities measured at 425 nm for an A to T ratio of 1:1 are given in Table 1.

The changes observed in the absorbance spectra are quite different from those observed with the corresponding β - $\text{Acr m}_5(\text{pT})_8$ and $\beta\text{-(Tp)}_8\text{m}_5\text{Acr}$. The hypochromicities are smaller with the α -oligonucleotides, but the most striking difference comes from the absence of the strong new absorption band at long wavelengths which was induced upon binding of $\beta\text{-(Tp)}_8\text{m}_5\text{Acr}$ to poly(rA) and—to a lesser extent—to $r(\text{Ap})_7r\text{A}$ (13–15). Only in the case of $\alpha\text{-Acr m}_5(\text{pT})_8$ binding

Table 1. Melting temperatures (t_m) and hypochromicities (H) of complexes of oligothymidylates with adenine-containing polynucleotides and oligonucleotides

	$\alpha\text{-(Tp)}_8\text{m}_5\text{Acr}$ ($\epsilon_{425} = 9400$)		$\alpha\text{-Acr m}_5(\text{pT})_8$ ($\epsilon_{425} = 8900$)		$\alpha\text{-(Tp)}_7\text{T}$ ($\epsilon_{268} = 72,400$)		$\beta\text{-(Tp)}_8\text{m}_5\text{Acr}$ ($\epsilon_{425} = 8845$)		$\beta\text{-Acr m}_5(\text{pT})_8$ ($\epsilon_{425} = 8285$)		$\beta\text{-(Tp)}_7\text{T}$ ($\epsilon_{268} = 70,900$)	
	t_m , $^\circ\text{C}$	H , %	t_m , $^\circ\text{C}$	H , %	t_m , $^\circ\text{C}$	t_m , $^\circ\text{C}$	H , %	t_m , $^\circ\text{C}$	H , %	t_m , $^\circ\text{C}$	t_m , $^\circ\text{C}$	
Poly(rA)	38	20	37.5	27	22.5	36.4	45	28.5	35	16.1		
$r(\text{Ap})_7r\text{A}$	29.6	9	35.6	17	23	21.6	29	14.8	24	10		
Poly(dA)	25	35	25	29	14	34.4	43	26.4	31	18.1		
$d(\text{pA})_8$	18.5	12	27	29	13.5	21.9	16	20.0	10	11.2		

Total oligonucleotide concentration was $10 \mu\text{M}$. Measurements were carried out in a pH 7 buffer containing 0.01 mM sodium cacodylate and 0.1 M NaCl. Extinction coefficients at 20°C are given in $\text{M}^{-1}\text{cm}^{-1}$ below each compound. Hypochromicity at 425 nm was measured at 2°C upon addition of the adenine-containing polymer to the thymidine-containing oligomer.

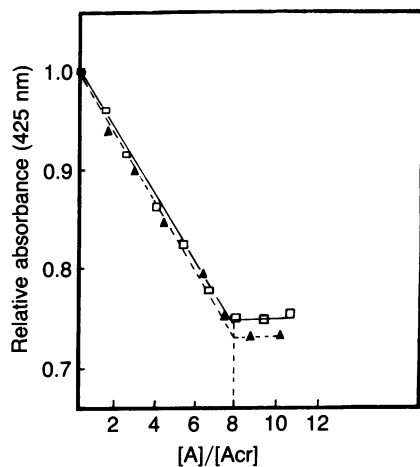


FIG. 3. Change in absorbance at 425 nm of α -Acrm₅(pT)₈ upon addition of poly(rA) (\blacktriangle) or poly(dA) (\square). Measurements were carried out at 2°C. Same conditions as for Fig. 2.

to d(pA)₈ is there any evidence for an increased absorbance on the long-wavelength side (see Fig. 4).

Increasing the temperature of a 1A:1T complex induced a reversal of the spectroscopic effects observed upon adding the adenine-containing polymers to the acridine-substituted

oligothymidylates (Figs. 4 and 5). Temperatures of half-dissociation (t_m) are given in Table 1 at a total oligonucleotide concentration of 10 μ M. Several interesting observations can be made:

(i) Attachment of the acridine derivative to α -(Tp)₇T at either the 5' or the 3' end leads to a stabilization of the complexes, as previously observed with the β analogs (13–15).

(ii) Complexes of α -Acrm₅(pT)₈ and α -(Tp)₈m₅Acr with ribopolymers are more stable than those with deoxyribopolymers, a property that is shared with α -(Tp)₇T not substituted with the acridine derivative (see above).

(iii) α -Acrm₅(pT)₈ forms a complex with an "isolated" complementary sequence [r(Ap)₇rA or d(pA)₈] that has a stability comparable to that of the corresponding complex with a polynucleotide containing multiple binding sites. This result indicates that there is little—if any—cooperativity of binding, in contrast to what was previously found with the β -oligothymidylates (14).

(iv) Complexes of α - and β -(Tp)₈m₅Acr with poly(rA) and r(Ap)₇rA have comparable stabilities, whereas complexes of α -(Tp)₈m₅Acr with poly(dA) and d(pA)₈ are less stable than those of the corresponding β analogs.

(v) Complexes of α -Acrm₅(pT)₈ with "isolated" complementary sequences r(Ap)₇rA and d(pA)₈ are more stable than those formed by all other acridine-substituted oligothymidylates investigated so far. This is especially true for α -

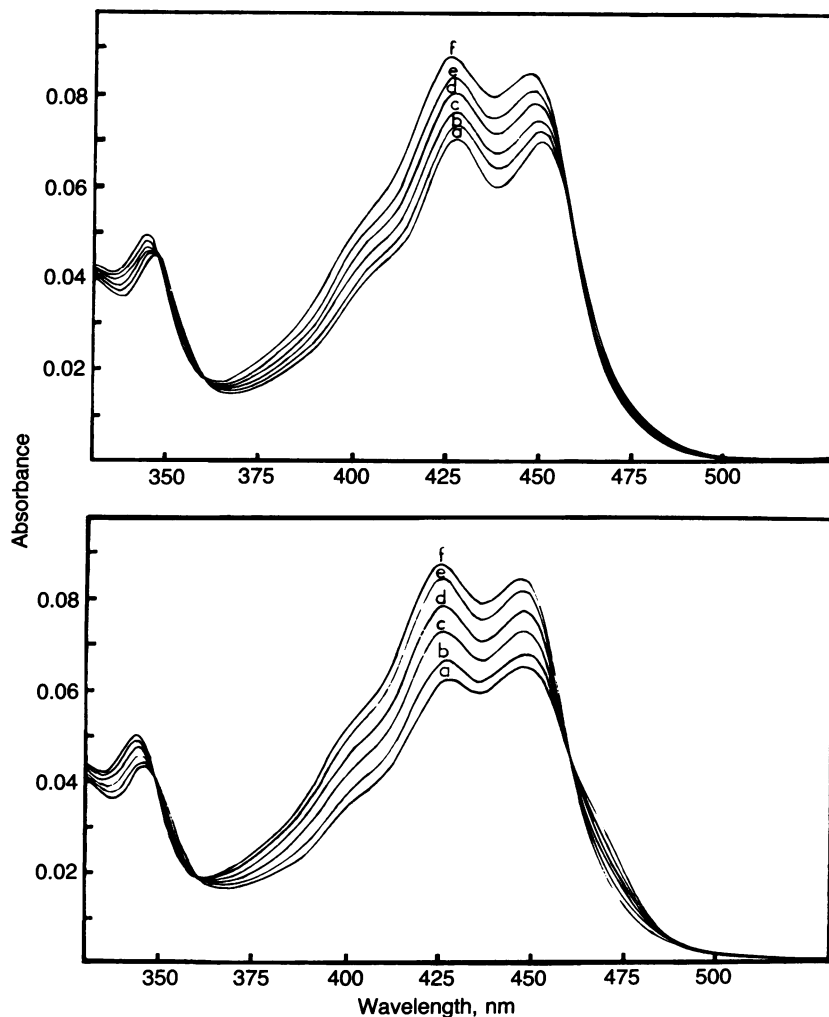


FIG. 4. Change in absorbance spectrum with temperature of 1:1 (A to T) mixtures of α -Acrm₅(pT)₈ with r(Ap)₇rA (Upper) and d(pA)₈ (Lower). Temperatures are (Upper) a, 2.7°C; b, 25.1°C; c, 30.7°C; d, 35.8°C; e, 40.6°C; f, 55°C; (Lower) a, 2.5°C; b, 15.9°C; c, 25.1°C; d, 30.8°C; e, 38.6°C; f, 50°C.

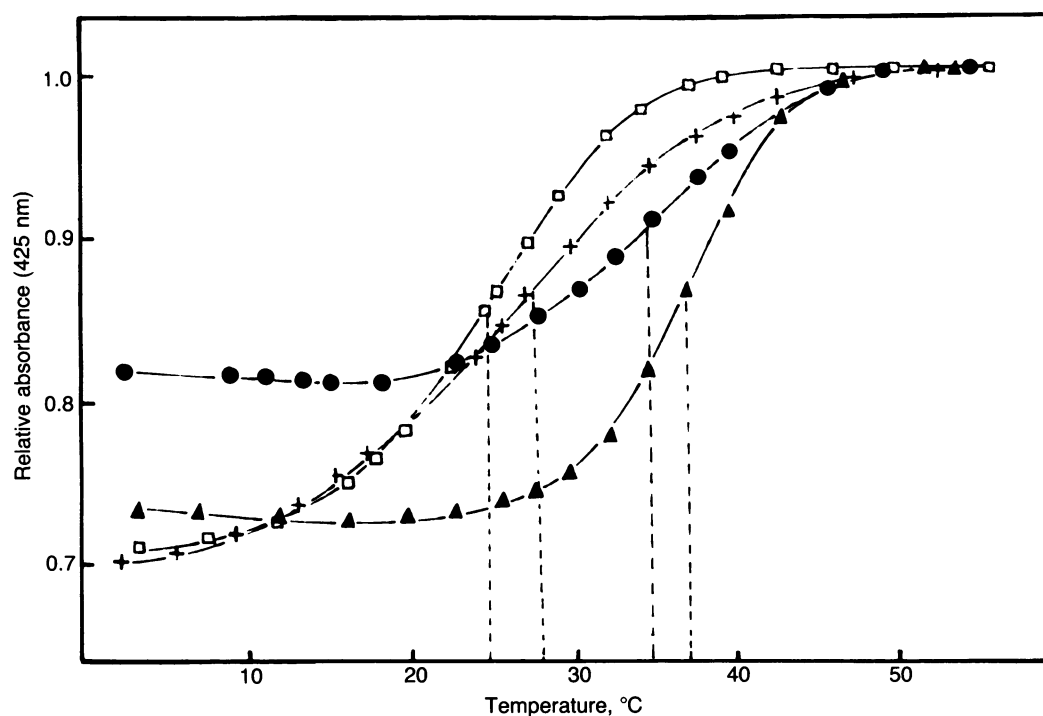
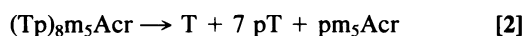
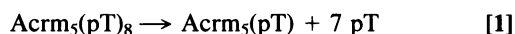


FIG. 5. Melting curves for complexes of α -Acr $_5$ (pT) $_8$ with poly(rA) (▲), poly(dA) (□), r(Ap) $_7$ rA (●), and d(pA) $_8$ (+). Same conditions as for Fig. 2.

Acr $_5$ (pT) $_8$ bound to r(Ap) $_7$ rA, whose melting temperature is quite close to that observed with poly(rA).

Stability of α -Oligothymidylates Towards Nucleases

A comparison was made of the stability of α - and β -oligothymidylates towards endo- and exonucleases. Two endonucleases were used: nuclease P1 from *Penicillium citrinum* and nuclease S1 from *Aspergillus oryzae*. Two exonucleases were used: a 3'-exonuclease extracted from *Crotalus durissus* venom and a 5'-exonuclease extracted from calf thymus. The digestion products were analyzed by thin-layer chromatography as previously described (18). Complete digestion of Acr $_5$ (pT) $_8$ and (Tp) $_8$ m $_5$ Acr by endonucleases P1 or S1 yields the products described by Eqs. 1 and 2, respectively.

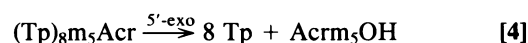


In both cases intermediate species containing oligonucleotides of different lengths were detected during the course of the reaction, Acr $_5$ (pT) $_n$ in case 1 and p(Tp) $_n$ m $_5$ Acr in case 2, with n decreasing from 7 to 1. The release of the acridine with the linker terminated by a hydroxyl group (Acr $_5$ OH) required further incubation with a 3'-exonuclease in case 1 and alkaline phosphatase in case 2.

Endonuclease P1 (3 $\mu\text{g}/\text{ml}$) hydrolyzed the acridine-substituted β -oligothymidylates to about 90% in 7 min at 37°C, independently of the site of attachment of the acridine ring (5' or 3'). With α -Acr $_5$ (pT) $_8$, the same level of digestion was achieved only after 42 hr of incubation. In the case of α -(Tp) $_8$ m $_5$ Acr the terminal phosphodiester linkage was hydrolyzed more rapidly than the internucleotidic bonds. This reaction was, however, 1/5th as fast as in the case of the corresponding β oligomer. The reaction rate for complete degradation of the α -oligothymidylate part was comparable to that observed for α -Acr $_5$ (pT) $_8$.

Similar results were obtained when nuclease S1 was used instead of nuclease P1. The hydrolysis of acridine-substituted α -oligothymidylates was about 1/450th of that of the corresponding β oligomers.

Complete digestion of acridine-substituted oligothymidylates by exonucleases was shown to occur according to Eqs. 3 and 4.



As expected, attachment of the acridine at the 5' or 3' end protected the oligonucleotide (either α or β) against degradation by 5'-exonucleases or 3'-exonucleases, respectively.

With snake venom 3'-exonuclease at a concentration of 50 $\mu\text{g}/\text{ml}$, nearly complete digestion of β -Acr $_5$ (pT) $_8$ was achieved in 5 min. The same reaction required 40 hr with the α oligomer. When calf thymus 5'-exonuclease was used (0.5 $\mu\text{g}/\mu\text{l}$), 25 hr of incubation was required to hydrolyze α -(Tp) $_8$ m $_5$ Acr to the same extent as 5-min incubation for the β oligomer. Therefore the overall stability of the α -oligothymidylates towards exonucleases is roughly 300–500 times higher than that of the β -oligonucleotides.

DISCUSSION

Above we have described the synthesis and some properties of oligothymidylates in which the building units have the α -anomeric configuration instead of the natural β -anomeric configuration of the nucleoside. The synthesis of an α -oligodeoxynucleotide containing both T and C has been recently reported (21). α -Oligothymidylates have been covalently linked to an acridine derivative and the properties of the composite molecules compared to similar compounds of the β configuration.

The α -oligonucleotides differ from the β oligomers in several aspects: (i) They bind much more strongly to complementary sequences containing ribonucleotides rather than

deoxyribonucleotides. (ii) Among all compounds of the same sequence and length studied so far, the 5'-substituted oligothymidylate [α -Acr_m(pT)₈] gives by far the most stable complex with an isolated complementary r(Ap)₇rA sequence. (iii) All the α -oligothymidylates are strongly stabilized against digestion by endo- and exonucleases (reactions slower by a factor of 300–500).

The structure of the complexes formed by α -oligonucleotides with their complementary β sequences is not known yet. Model-building studies (refs. 21 and 22 and unpublished observations) suggest that the two chains can form a double helix provided they adopt a parallel orientation. Further studies are required to characterize these structures, to explain the selectivity of binding to β ribopolymers, and to determine whether this is a general property of α -oligodeoxynucleotides independent of the base sequence. The synthesis of α -deoxynucleosides other than α -dT opens the possibility of testing this possibility (23).

The original aim of synthesizing α -oligodeoxynucleotides was to devise a family of molecules that could regulate gene expression in a specific way by hybridization to messenger RNAs. The observation that RNAs are preferred to DNAs for hybrid formation offers an important advantage in this respect. The second objective in synthesizing α -oligodeoxynucleotides was to confer resistance to nucleases. As a matter of fact, the α -oligothymidylates investigated here are by far more resistant than the corresponding β oligomers. Therefore they should offer a strong advantage when *in vivo* experiments are envisaged. Covalent attachment of an acridine derivative to the 5' or 3' end of the α -oligothymidylate strongly stabilizes the complexes with the complementary sequence as already observed with the β -oligomers. This is attributed to the additional binding energy provided by the intercalation of an acridine ring between the base pairs of the miniduplex structure formed by the oligonucleotide with its complementary target sequence (13–15). The most stable complex is obtained when the acridine is attached to the 5' end of the α -oligonucleotide. It is necessary to synthesize α -oligodeoxynucleotides containing the four bases and complementary to selected mRNAs to test the generality of the observations made with α -oligothymidylates and to assess their potentiality in blocking messenger RNA translation or viral RNA expression *in vitro* and *in vivo*.

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