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SYNTHESIS OF DEFINED PEPTIDE-OLIGONUCLEOTIDE HYBRIDS CONTAINING A NUCLEAR TRANSPORT SIGNAL SEQUENCE.

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ABSTRACT : Oligonucleotide-peptide hybrid molecules containing a nuclear transport signal have been synthesized using two different approaches based on the specific reactivity of the thiol group.

The use of oligodeoxynucleotides to specifically inhibit gene expression through intracellular hybridization was first reported some years ago by P. Zamecnik and M. Stephenson (1). Since then, modified and unmodified oligonucleotides have been widely employed to inhibit gene expression (for review, see ref. 2-4). In order to enhance the activity of oligonucleotides and its analogues, these compounds have been covalently linked to intercalating, alkylating, photocrosslinking and radical generating reagents. Besides increasing the affinity for the target sequence, some of these compounds promoted the uptake of oligonucleotides by cells and improved their resistance to nucleases (2-4). Covalent attachment of poly(L-Lysine) to an oligonucleotide complementary to the initiation region of the mRNA encoding the nucleocapsid protein of vesicular stomatitis virus enhanced the antiviral properties of the antimessenger, two orders of magnitude lower than the unmodified oligonucleotide (5).

These results prompted us to study the utility of peptide segments for the in vivo targeting of antisense oligonucleotides. The questions that we want to answer are whether the peptide sequences can or can not direct the transport of the hybrids to the different compartments of the cell and if the hybrid molecules are more or less active as inhibitors of gene expression. In this communication, we describe the preparation of defined oligonucleotide-peptide hybrids containing the SV 40 large-T antigen nuclear transport sequence Pro-Lys-Lys-Lys-Arg-Lys-Val (6-8).

RESULTS AND DISCUSSION

The preparation of oligonucleotide-peptide hybrids presents an interesting challenge because the standard protection schemes are not compatible. For example amide-type protecting groups are used for the protection of nucleic

acid bases in oligonucleotide synthesis. These protecting groups are eliminated by ammonia treatment at 55 °C, conditions that could partially hydrolyse peptide bonds. In the other hand, all standard protection schemes in solid-phase peptide synthesis utilize, at the end of the syntheses, acid treatments that could provoke partial depurination of DNA. Some of these problems have been minimized by modifications of the standard deprotection protocols (9, 10) but, in our opinion, they are not completely solved.

For these reasons, we decided to prepare oligonucleotides and peptides in a separate way but conveniently functionalized to be linked after purification. Specifically, the method developed consists in three basic steps: 1) preparation of oligonucleotides with a thiol function at the 5'-end (11); 2) preparation of peptides with a thiol reactive group (cysteine residue or a maleimido group) in the N-terminal position (12) and 3) reaction of the free thiol group of the oligonucleotide with the activated thiol group or the maleimido group of the peptide (figure 1).

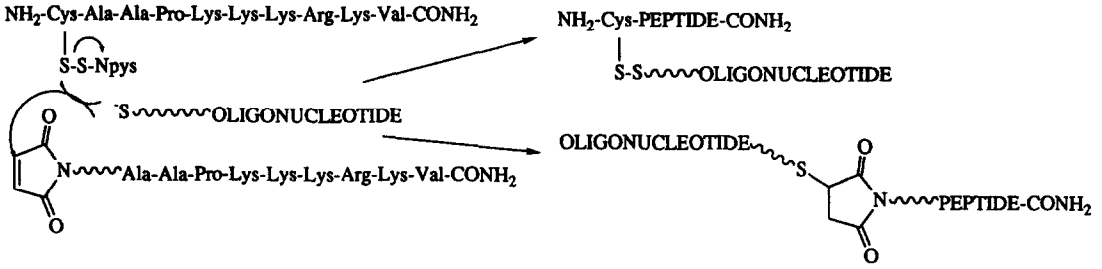

Figure 1 : Synthesis of oligonucleotide-peptide hybrid molecules.

Preparation of peptides containing a thiol-reactive group.

The following sequences were prepared :

I) H-Cys(Npys)-Ala-Ala-Pro-Lys-Lys-Lys-Arg-Lys-Val-$CONH_2$

II) MB-Ala-Ala-Pro-Lys-Lys-Lys-Arg-Lys-Val-$CONH_2$

were Npys stands for 3-nitro-2-pyridinsulphenyl (12) and MB for 3-maleimidobenzoyl.

Both peptides contained the SV-40 large-T antigen nuclear transport sequence. Peptide I has a Npys protected cysteine residue at the N-terminal position and peptide II has at the same position a maleimido group. Both Npys and maleimido groups can react with free thiol functions giving a disulphide and a C-S bond respectively. Two alanine residues were introduced between the transport sequence and the reactive groups. We decided to introduce a C-terminal amide to avoid the presence of an ionizable carboxyl group at the C-end of the peptides. For the preparation of the peptides we used solid-phase methodology on polystyrene supports by the Fmoc/^{t}Bu strategy (13). The acid-labile 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxy-

phenoxy)valeric acid (PAL) handle was used because allows the obtention of C-terminal peptide amides using the same trifluoroacetic acid treatment that deblocks the side chain ^{t}Bu-based protecting groups (14). The resin contained norleucine as "internal reference" amino acid. The following groups were chosen for side chain protection : *t*-butyloxycarbonyl (Boc) for lysine (15), 2,2,5,7,8-pentamethylchroman-6-sulphenyl (Pmc) for arginine (16) and the already mentioned 3-nitro-2-pyridinsulphenyl (Npys) for cysteine (12). Amino acid derivatives were assembled using conventional solid-phase protocols. Syntheses were controlled by the ninhydrin test (17), by measuring the amount of N-(9-fluorenylmethyl)piperidine released during the deprotection step (13) and amino acid analysis of peptidyl-resins hydrolysates. After the addition of the last alanine, the resin was divided in two equal parts and one half was reacted with Boc-Cys(Npys) and the other half with N-hydroxysuccinimidyl 3-maleimido benzoate (MBS). Aliquotes of the peptidyl-resins were treated with trifluoroacetic acid / water 95:5 yielding the desired peptides I and II. Cleavage yields were 75% in both cases and the products were 85% (peptide I) and 80% (peptide II) pure by analytical HPLC analysis measured at 214 nm.

We have observed that the maleimido containing peptide is not completely stable in aqueous solutions. After 3-4 days in neutral aqueous solutions, the purified HPLC peptide II was transformed to a complex mixture of products that we assigned to be reaction products between the side chain amino group of lysines and the maleimido group. To avoid that problem we deprotect the peptidyl-resin just before the coupling with the thiol containing oligonucleotide.

Preparation of thiol-containing oligonucleotides.

The following sequences (A-D) have been synthesized by standard phosphite-triester methodology using an automatic DNA synthesizer.
Conventional 2-cyanoethyl phosphoramidites and controlled-pore glass supports were used (18).

A) 5' trityl-S-$(CH_2)_6$-phosphate-GCATGC 3'

B) 5' trityl-S-$(CH_2)_6$-phosphate-AACGTTGAGGGGCAT 3'

C) 5' AACGTTGAGGGGCAT 3'

D) 5' ATGCCCCTCAACGTT 3'

Oligonucleotides A and B contain a thiol function at the 5'-end protected with a trityl group. We used the commercially available 5'-trityl-6-mercaptohexanol O-2-cyanoethyl-N,N-diisopropyl phosphoramidite for the introduction of the thiol function in these oligonucleotides (11). Hexanucleotide A was prepared to set up the conjugation conditions with peptides I and II. Pentadecanucleotide B contains the complementary sequence of the initiation region of human c-myc oncogene. Oligonucleotide C is the

same as B but without the thiol arm and oligonucleotide D is complementary to C and B.

At the end of the syntheses, the oligonucleotidyl-resins were treated with concentrated ammonia at 55 °C. The resulting DMT-oligonucleotides C and D were purified with OPC^R cartridges. Trityl-S-oligonucleotides A and B were treated with silver nitrate to eliminate the trityl group followed by addition of DTT to precipitate the excess of silver ions (11, 19, 20) and, finally, thiol-containing oligonucleotides were isolated by HPLC. The desired oligonucleotides with free thiol functions were used immediately for conjugation with peptides I and II because they have a strong tendency to form dimers with a disulphide bond.

Preparation of the peptide-oligonucleotide hybrids.

Hexanucleotide A containing a free thiol group at the 5'-end was used to set up the conditions for the conjugation with peptides I and II. HPLC purified A was reacted overnight with HPLC purified peptides I and II and directly with the products obtained after TFA treatment of peptidyl-resins. The reactions were done at pH 6.0-7.0 and peptide-to-oligonucleotide molar ratios were 10 (for peptide) to 1 (for oligonucleotide). Results are shown in figures 2a and 2b. We have obtained identical HPLC profiles using purified or unpurified peptides. HPLC profiles have been recorded at 260 nm where oligonucleotides have a strong UV-absorption while peptides I and II are not visible. In all cases, we have observed that the peak corresponding to the starting thiol-containing oligonucleotide (retention time 14.5 min) completely disappears and a new peak appears with a higher retention time (17.4 min for peptide I and 21.2 min for peptide II). Furthermore, the reaction between oligonucleotide A and peptide I (containing a Cys(Npys) residue) yields an extra product with a lower retention time (12.3 min). All the peaks have a DNA characteristic UV spectra (max. 257 nm), but only the high retention time products (17.4 and 21.2 min) contained the desired peptide by amino acid analysis.

From these experiments we can conclude that peptide-oligonucleotide hybrids can be easily obtained with any of the proposed methodologies but the use of the maleimido approach is highly recommended because no side products were observed.

Afterwards, we have prepared the hybrid molecule between the 15 mer B and the maleimido-containing peptide II. Results are shown in figure 2c. As shown before, the peak corresponding to the starting oligonucleotide (retention time 15.90) disappears to obtain a major peak of higher retention time (17.0 min). The product obtained after HPLC purification was homogeneous by HPLC and was characterized as the desired hybrid molecule by its UV spectrum and

amino acid composition.

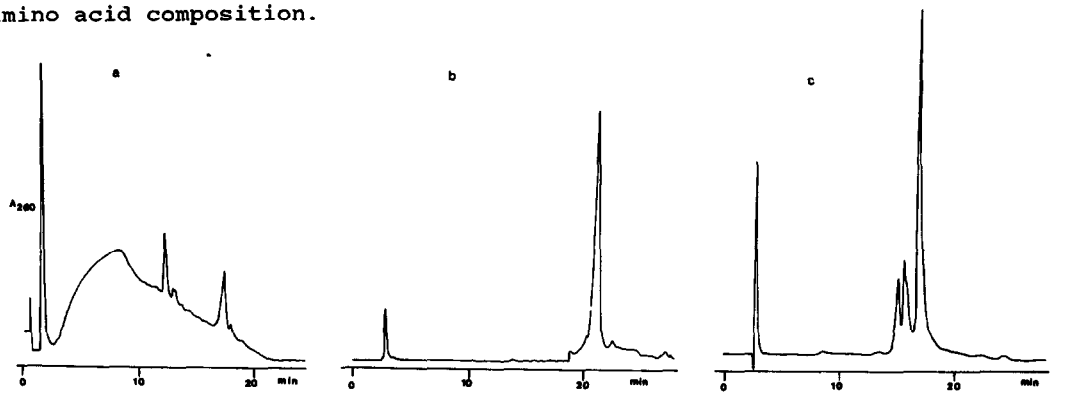

Figure 2 : HPLC profiles of the conjugation reactions. a) Hexanucleotide A with Cys(Npys) peptide (I). b) Hexanucleotide A with maleimido peptide (II). c) Pentadecanucleotide B with maleimido-peptide (II).

Furthermore, we wanted to know the effect of the presence of the peptide molecule in the base-pairing properties of DNA. For that purpose we have measured the melting temperatures of 15mer duplexes with and without peptide.

Table 1 : Melting temperatures of duplexes prepared in this work.

Sequences	Duplex	Tm*	Hiperchromicity
C and D	5'AACGTTGAGGGGCAT 3' 3'TTGCAACTCCCCGTA 5'	56 °C	13%
B and D	Peptide-AACGTTGAGGGGCAT TTGCAACTCCCCGTA	59 °C	12%

* 0.1 M NaCl, 0.05 mM tris-HCl pH 7.5

As it can be seen in table 1 the duplex containing the peptide has a melting temperature 3 degrees higher than the duplex without the peptide. That means that the presence of this peptide does not destabilize the double helix structure, all the contrary, there is a significative stabilization of the double helix structure, probably due to the presence of positive charges of the 4 lysines and arginine present in the peptide that can interact with the negatively charged phosphate groups of the oligonucleotide.

In conclusion, we have shown that define oligonucleotide-peptide hybrids can be synthesized using thiol containing oligonucleotides together with peptides having thiol-specific reagents. The approach that utilizes maleimido peptides gave the best results and it can be of special interest for the preparation of peptide-protein conjugates needed for immunological purposes. Oligonucleotide-peptide hybrid molecules containing the SV-40 nuclear transport sequence are shown to stabilize duplex formation, so ,in principle,

they are good candidates to be studied as potential inhibitors of gene expression. Further work in these directions are currently being done.

EXPERIMENTAL SECTION

Abbreviations : ACN : acetonitrile, Boc : *t*-butyloxycarbonyl, DCC : diciclohexylcarbodiimide, DCM : dichloromethane, DMF : dimethylformamide, DMT : 4,4'-dimethoxitrityl, Fmoc : 9-fluorenylmethyloxycarbonyl, MBS : N-hydroxysuccinimidyl 3-maleimido benzoate, Npys : 3-nitro-2-pyridinsulphenyl, Pmc : 2,2,5,7,8-pentamethylchroman-6-sulphenyl, PTC : phenoxythiocarbonyl, ^{t}Bu : *t*-butyl, TFA : trifluoacetic acid.

Fmoc protected amino acids were from Nova Biochem, Switzerland. Boc-Cys(Npys) (12), MBS and Fmoc-NH-PAL-Nle-polystyrene resin (14) were prepared using previously described protocols. DCC was from Fluka, Switzerland. Reagents for oligonucleotide synthesis were from Applied Biosystems, USA, except for S-trityl-6-mercaptohexanol O-2-cyanoethyl-N,N-diisopropylamino phosphoramidite that was from Cruachem, Scotland. All other solvents and reagents were reagent grade and they were used without further purification. Peptide syntheses were carried out in a manual synthesizer. Oligonucleotide syntheses were performed on an Applied Biosystems 380A automatic DNA synthesizer. Amino acid analyses were done using Pico-Tag^{R} method (Waters Ass.). UV absorption spectra and melting curves were recorded using a Perkin-Elmer Lambda 5 spectrophotometer equipped with a temperature controller.

Solid-phase synthesis of peptides I and II.

Synthesis was carried out on a 0.2 mmols scale (0.5 grs of Fmoc-NH-PAL-polystyrene-resin (14), 0.4 mmols Fmoc / gr) using the following protocol : 1) DMF 4 x 1 min; 2) 20% piperidine / DMF 10 x 1 min; 3) DMF 5 x 1 min; 4) DCM 2 x 1 min; 5) 3 eq. (0.6 mmols) of Fmoc-amino acid + 3 eq. (0.6 mmols) of DCC, shook for 2-3 hrs; 6) DMF 5 x 1 min; 7) DCM 3 x 1 min. At this point, the completion of the coupling reactions was checked by the ninhydrin test (17). If the ninhydrin test was positive, the coupling reaction was repeated with 1.5 eq. (0.3 mmol) of Fmoc-amino acid and DCC. If it was negative, we proceeded to incorporate the next amino acid.

The collected filtrates from treatments 2) and 3) were combined, diluted with ethanol and used for the spectrophotometric determination of N-(9-fluorenylmethyl)piperidine at 301 nm (ϵ 7800) (13).

After coupling of the last alanine, the resin was divided into two equal parts and one half was reacted with Boc-Cys(Npys) and DCC and the second half was reacted with MBS in DMF. The incorporation of Boc-Cys(Npys) needed a double coupling while a ninhydrin test resin was obtained in only 1-hr treatment with MBS.

Amino acid analysis for peptide-I-resin was : Arg 1.2 (1), Ala 1.9 (2), Pro 0.9 (1), Val 1.1 (1), Cystine 0.4 and Cysteine 0.4 (1), Lys 3.9 (4) and for peptide-II-resin : Arg 1.2 (1), Ala 2.0 (2), Pro 1.0 (1), Val 1.1 (1), Anthranilic acid 1.0 (1), Lys 3.8 (4). During the acid hydrolysis, Cys(Npys) is partially hydrolysed giving a mixture of cysteine and cystine. The maleimido benzoyl group gave anthranilic acid. The PTC-derivative of anthranilic acid eluted near the Tyr derivative, so it can quantified together with PTC-derivatives of the natural amino acids.

5-10 mgrs aliquotes of peptidyl-resins were treated with TFA / water 95:5 (1 mL) at room temperature for 4 hours. After filtering the resin, the resin was washed with water and the combined filtrates were evaporated to dryness. Cleavage yield were estimated by hydrolysis and amino acid analysis of the remaining resin that contained norleucine as internal reference amino acid. In both cases we obtained a 75% cleavage yield.

Peptides were analyzed and purified by reverse-phase HPLC. Column : Pico Tag (Waters) 20 x 0.5 cm. Solvent A : 0.2 % TFA in water, solvent B : 0.2 % TFA in ACN / water (7:3). Flow rate 1 mL / min. A 40 min linear gradient from 0% to 100 % B was used. In both cases a major peak was observed with the

expected amino acid composition.

Amino acid analysis of HPLC purified peptide I : Arg 1.3 (1), Ala 1.8 (2), Pro 1.1 (1), Val 0.8 (1), cystine 0.2 and cysteine 0.2 (1), Lys 3.6 (4).

Amino acid analysis of HPLC purified peptide II : Arg 1.2 (1), Ala 1.8 (2), Pro 0.9 (1), Val 0.9 (1), anthranilic acid 1.0 (1), Lys 3.7 (4).

Oligonucleotide syntheses.

Sequences A-D were synthesized on an Applied Biosystems automatic DNA synthesizer using the protocols recommended by the supplier. Standard 2-cyanoethyl phosphoramidite and controlled-pore glass supports were used (18). The efficiency of the coupling reactions were 98% by measuring DMT optical density at 500 nm. Oligonucleotides A and B were synthesized on a 1 μmol scale and oligonucleotides C and D on a 0.2 μmols scale. An extra coupling step was used carried out for oligonucleotides A and B with commercially available S-trityl-6-mercaptohexanol phosphoramidite. This step was analogous to the coupling of a nucleoside phosphoramidite and one of the three additional positions in the synthesizer was used. Following coupling, the phosphite intermediate formed was oxidised in the normal fashion. The protecting trityl group was not removed at this stage. Instead, the cleavage from the resin together with deblocking of the base and phosphate protecting groups was achieved with a conc. ammonia treatment at 55 °C for 6 hrs.

DMT-oligonucleotides C and D were purified using OPC^{R} cartridges (Applied Biosystems). Trityl-S-oligonucleotides A an B were treated with silver nitrate to eliminate the protecting trityl group before HPLC purification. The following protocol was used : About 5 O.D. units at 260 nm of the product obtained after ammonia treatment were dissolved in a 50 μL of a 0.1 M triethylammonium acetate solution pH 7.5. 7.5 μL of a 1 M silver nitrate solution were added and the solution was incubated 30 min at room temperature. In order to precipitate the excess of silver ions and reduce the disulphide bonds, 10 μL of a 1 M dithiothreitol solution were added. After 15 min, the mixture was centrifuged and the supernatant was removed. The precipitated silver salt is washed twice with a 0.1 M triethylamine acetate solution and the combined supernatants were pooled and purified by HPLC. Column : Nucleosil C-18. Solvent A : 20 mM triethylammonium acetate in water, solvent B : ACN / water 1:1. Flow rate : 1 mL / min. A 30 min gradient from 0% to 100 % was used. Hexanucleotide A retention time 14.5 min, pentadecanucleotide B retention time 15.9 min. In both cases, a rather big peak (approx. 50% of the total DNA) eluting before the desired thiol-containing product was observed (retention times were 12.3 min (A) and 14.3 min (B)).

Preparation of oligonucleotide-peptide hybrids.

About 100-200 nmols of HPLC purified peptide or , alternatively, the product obtained after a 4-hr TFA treatment of 3 mg of peptidyl-resin were dissolved in 1 mL of triethylamine acetate buffer pH 7.0 and purified thiol oligonucleotide was added. It should be noted that oligonucleotide solutions contain 1-2 O.D. units at 260 nm (approx. 5-10 nmols). After standing overnight, the reaction mixtures were controlled and purified by HPLC (chromatographic conditions as described above). The products obtained were analyzed by U.V. spectrophotometry and by amino acid analysis.

Hexanucleotide-MB-peptide. Retention time : 21.2 min. U.V. (max) 257 nm. Amino acid analysis : Arg 1.1 (1), Ala 1.8 (2), Pro 1.3 (1), anthranilic acid 1.1 (1), Val 0.8 (1), Lys 3.7 (4).

Hexanucleotide-Cys-peptide. Retention time : 17.4 min. U.V. (max) 257 nm. Amino acid analysis : Arg 1.0 (1), Ala 1.8 (2), Pro 1.2 (1), Val 1.1 (1), cysteine 0.4 and cystine 0.3 (1), Lys 3.8 (4).

Side product from the reaction of hexanucleotide with Cys(Npys) peptide. Retention time : 12.2 min. U.V. (max) 257 nm. No amino acids were detected after acid hydrolysis.

Pentadecanucleotide-MB-peptide. Retention time : 17.0 min. U.V. (max) 260 nm. Amino acid analysis : Arg 1.1 (1), Ala 1.9 (2), Pro 0.9 (1), Val 1.1 (1),

anthranilic acid 1.0 (1), Lys 3.9 (4).

Melting studies.

Duplexes were made by mixing equimolar amounts of the two complementary strands in 0.05 M Tris.HCl pH 7.5 adjusted to 0.1 M sodium concentration with NaCl. Final volume 1.2 mL. Duplexes were annealed by slow cooling from 100°C to room temperature. UV absorption spectra and melting curves were recorded in 1-cm path-length cells. The temperature gradient was constant of 2 degrees / min from 25 to 80°C. Melts were run on duplex concentration of 3 μM at 260 nm.

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