
Ligation of oligonucleotides to nucleic acids or proteins via disulfide bonds

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ABSTRACT

We have developed general methods for joining together, via cleavable disulfide bonds, either two unprotected polynucleotides or a polynucleotide and a peptide or protein.

To join two oligonucleotides, each is first converted to an adduct in which cystamine is joined to the 5'-terminal phosphate of the oligonucleotide by a phosphoramidate bond. The adducts are mixed and reduced with dithiothreitol. The dithiothreitol is then removed by dialysis. Oxidation by atmospheric oxygen occurs to yield the required dimer.

To join an oligonucleotide to a cysteine-containing peptide or protein, the 5'-cystamine oligomer is first converted to a 2'-pyridyldisulfide adduct and then reacted with an excess of the peptide or protein. If the peptide does not contain a free cysteine residue, it is first treated with iminothiolane to introduce one or more sulfhydryl groups.

We have used these procedures to join a 16mer deoxynucleotide probe and MDV-1 RNA, a substrate of Q β RNA polymerase. This adduct hybridizes with a complementary target DNA. We have also joined a 16mer probe to peroxidase and MDV-1 RNA to human IgG. The probe-peroxidase adduct maintains enzymatic activity and the MDV-1 RNA-IgG adduct binds to a complementary anti-IgG.

INTRODUCTION

Procedures for attaching oligodeoxynucleotides to biopolymers, particularly proteins, are becoming increasingly important in molecular biology and biotechnology (1-5). One approach that has been widely used involves the incorporation of a linker group into a synthetic oligodeoxynucleotide. A suitably protected linker-containing nucleotide-analogue replaces one of the standard monomers in a solid-phase synthetic procedure. After deprotection the oligonucleotide can be coupled, for example to a protein, via the linker. Procedures that introduce a thiol group at the 5'-terminus (6) or the 3'-terminus (7) have been reported, and in one case the product has been coupled to a protein, micrococcal nuclease (4).

These synthetic procedures are not applicable when it is necessary to attach a ligand to an unprotected oligonucleotide or nucleic acid that has been synthesized enzymatically or isolated from natural sources. We have developed an alternative methodology for linking an unprotected oligonucleotide or polynucleotide via a 5'-terminal phosphate to any of a wide range of ligands (8-10). The chemistry is simple and rapid and may be applied to short oligonucleotides, short oligodeoxynucleotides, RNA or DNA.

In this paper we first present a very simple procedure for joining together pairs of

oligonucleotides of any length via cleavable disulfide linkages. We have also used previously described methods for linking together proteins via disulfide bonds (11, 12) to ligate an oligonucleotide or nucleic acid to a thiol-containing peptide or protein. Applications include the linking of an amplifiable reporter, MDV-1 RNA (221 bases) (13), to either a probe oligodeoxynucleotide or an antibody and the formation of a probe-peroxidase adduct. Some of these products could not easily be prepared using standard methods.

MATERIALS AND METHODS

Materials

The following were purchased: calf intestine alkaline phosphatase and peroxidase (Boehringer Mannheim), T4 polynucleotide kinase and single-stranded M13mp18 DNA (New England Biolabs), [γ - ^{32}P]-ATP (Amersham), cystamine (2,2'-dithiobis(ethylamine)dihydrochloride) (C.T.C. Organics), 2,2'-dipyridyldisulfide (Aldrithiol-2) (Aldrich), bradykinin (arg-pro-pro-gly-phe-ser-pro-phe-arg) and fatty acid free bovine serum albumin (BSA) (Sigma), 2-iminothiolane (Pierce), human IgG and affinity purified goat anti-human IgG (Cappel), 3,3'-diaminobenzidine (Bio-Rad), DEAE Sephadex A-50-120 (Sigma), Immulon-2 ELISA removeawell strips (Dynatech).

The deoxyribonucleotide sequence, 5'-CACAATTCACACAAC (16mer), complementary to residues 6170-6185 of the M13mp18 DNA (+) strand, and the 37mer

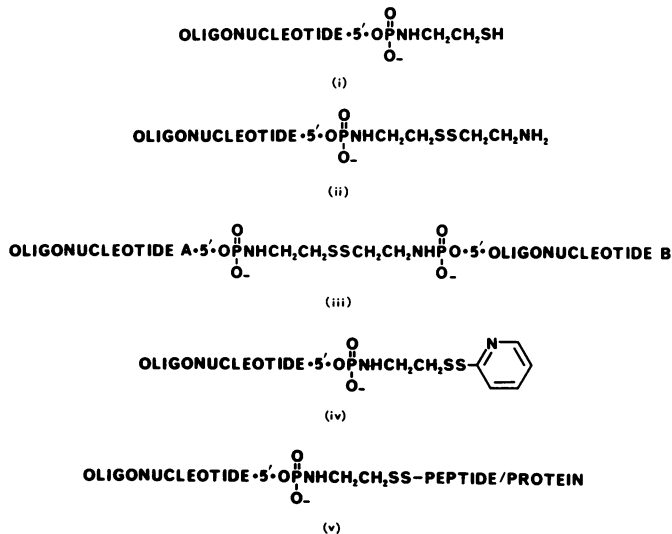


Figure 1: The structures of (i) 5'-thio-ethylamino-P-oligonucleotides, (ii) 5'-cystamine-P-oligonucleotides, (iii) 5'-P-oligonucleotide-ss-5'-P-oligonucleotides, (iv) 5'-(2-pyr)-ss-P-oligonucleotides, (v) 5'-P-oligonucleotide-ss-peptide or protein. The oligonucleotides can have any length and belong either to the ribo- or the deoxyribo- series.

5'-TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTT, which corresponds to residues 6164-6200 of the M13mp18 (+) strand, were synthesized on an Applied Biosystems Synthesizer Model 380. The purification of the 16mer and 37mer and their conversion to the 5'-phosphates has been described (9). 5'-ppp-MDV-1 (+) RNA was a gift from Dr. Fred Kramer, Public Health Research Institute, New York. Its propagation, dephosphorylation, and conversion to the 5'-[³²P]-MDV-1 RNA and 5'-cystamine-[³²P]-MDV-1 RNA (Fig. 1 ii)

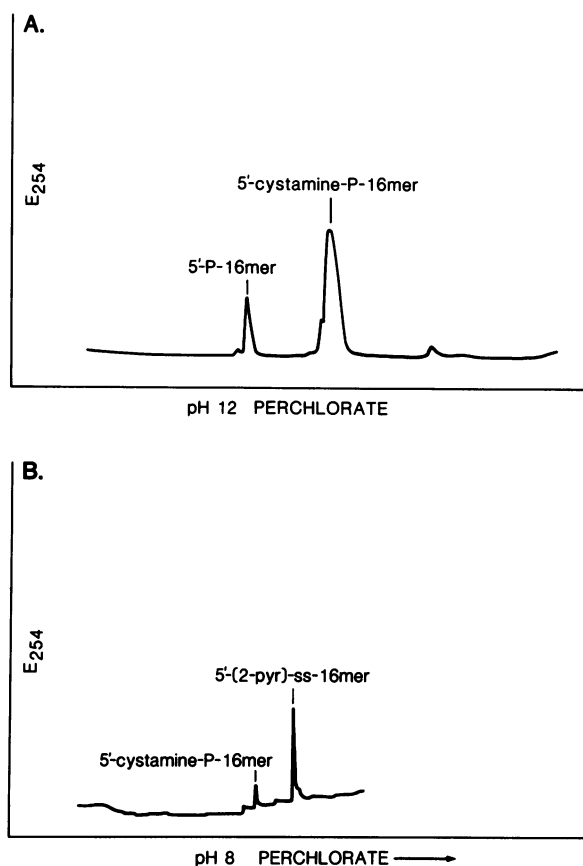


Figure 2: HPLC elution profile of reaction mixtures showing:

A. Conversion of 5'-P-16mer to 5'-cystamine-P-16mer. 5'-P-16mer was treated with 0.1 M 1-methylimidazole, 0.15 M 1-ethyl-3,3'-dimethylaminopropylcarbodiimide, and 0.5 M cystamine for 2 hours at pH 7.2 and 50°C. Oligonucleotides were eluted from an RPC-5 column at pH 12, using 0.026 M-0.066 M perchlorate gradient in 30 mins.

B. Conversion of 5'-cystamine-P-16mer to 5'-(2-pyr)-ss-P-16mer. 5'-cystamine-P-16mer was treated with 5 mM DTT for 1 hour at pH 7.2. 2,2'-dipyridyl-disulfide was then added to a final concentration of 1.6 mM. The reaction mixture was incubated at room temperature for 2 hours. Oligonucleotides were eluted from an RPC-5 column at pH 8, using a 0.024 m-0.40 m perchlorate gradient in 30 mins.

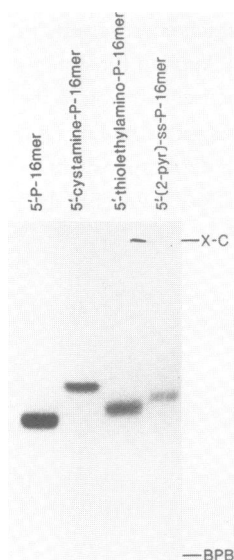


Figure 3: Autoradiogram of denaturing 20% gel showing migration of 5'-[³²P]-16mer, 5'-cystamine-[³²P]-16mer, 5'-thiolethylamino-[³²P]-16mer, and 5'-(2-pyr)-ss-[³²P]-16mer. The markers are bromophenol blue (BPB) and xylene cyanol (X-C).

have been previously described (13). The peptide gly-leu-glu-cys-glun-gly-arg-val-asn-leu-ala-gly-tyr (Peptide A) was a gift from Dr. J. Rivier, Salk Institute.

Synthesis of 5'-cystamine adducts of short oligonucleotides (Fig. 1 ii)

0.01-0.5 O.D.U. (260 nm) of 5'-P-16mer or 5'-[³²P]-16mer was reacted with 0.1 M 1-methylimidazole (pH 7), 0.15 M 1-ethyl-3,3-dimethylaminopropylcarbodiimide (carbodiimide) and 0.5 M cystamine (pH 7.2) in a volume of 50-250 μ l at 50°C for 2 hours. The product was separated from reactants by HPLC on RPC-5 (Fig. 2 A). The yield ranged between 75% and 85%. 5'-cystamine-[³²P]-16mer and 5'-[³²P]-16mer are also readily separated by electrophoresis on 20% polyacrylamide containing 7 M urea (Fig. 3). The corresponding cystamine adduct of 5'-[³²P]-37mer was obtained by analogous procedures.

Synthesis of 5'-[³²P]-16mer-ss-5'-[³²P]-16mer or 5'-P-16mer-ss-5'-[³²P]-37mer (Fig. 1 iii)

A 5 μ M solution (25-50 μ l) of 5'-cystamine-[³²P]-16mer (10^4 - 10^6 cpm), or a 5 μ M solution of 5'-cystamine-P-16mer and 10^3 - 10^4 cpm of 5'-cystamine-[³²P]-37mer, were treated with 6 mM dithiothreitol (DTT) in 10 mM Tris buffer containing 1 mM EDTA (Tris-EDTA) at pH 7 for 1 hour at 25°C. The reaction mixture was then dialyzed against one liter of buffer containing 1 mM Tris, pH 7.2, 1 mM EDTA and 0.1 mM DTT for 30 minutes at 4°C. It was next dialyzed against fresh buffer containing 1 mM Tris, pH 7.2, and 1 mM EDTA for a further 30 minutes. After concentration in a speed-vac concentrator, the products were separated from reactants by electrophoresis on 20% polyacrylamide under denaturing conditions (Fig. 4 A, B). Yields of dimerized 16mer ranged from 30% to 40% and of 5'-P-16mer-ss-[³²P]-37mer from 45% to 55%.

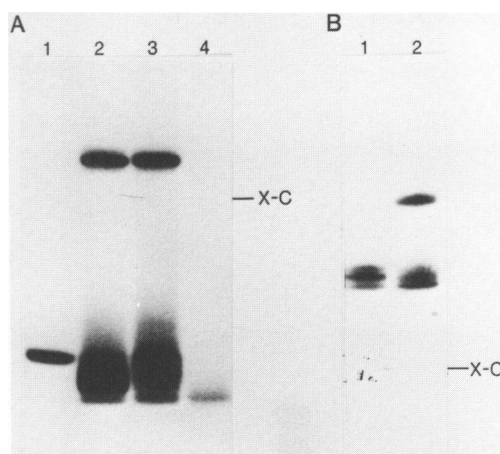


Figure 4: Autoradiograms of denaturing 20% gels showing:

A. lane 1) 5'-cystamine-[^{32}P]-16mer; lane 2) formation of the slower moving 5'-[^{32}P]-16mer-ss-5'-[^{32}P]-16mer by reduction of a 5 μM solution of 5'-cystamine-[^{32}P]-16mer with DTT, removal of thio-ethylamine and DTT by dialysis, and air oxidation for 30 mins; lane 3) same as lane 2, except oxidation allowed to continue for a further 12 hours; lane 4) formation of 5'-thio-ethylamino-[^{32}P]-16mer by treatment of 5'-[^{32}P]-16mer-ss-5'-[^{32}P]-16mer with 5 mM DTT for 1 hour.

B. lane 1) 5'-cystamine-[^{32}P]-37mer; lane 2) formation of the slower moving 5'-P-16mer-ss-5'-[^{32}P]-37mer after reduction of a mixture of 5'-cystamine-[^{32}P]-37mer and 5 μM 5'-cystamine-P-16mer with DTT, removal of DTT and thio-ethylamine by dialysis, and air oxidation for 30 mins. Marker is xylene cyanol (X-C).

Synthesis of 5'-P-16mer-ss-5'-[^{32}P]-MDV-1 RNA (Fig. 1 iii)

10^4 - 10^6 cpm (5-500 ng) of 5'-cystamine-[^{32}P]-MDV-1 RNA (Fig. 1 ii) and a 5 μM solution of 5'-cystamine-P-16mer (total volume 25-50 μl) was treated for 1 hour with 6 mM DTT in Tris-EDTA buffer at pH 7. The reaction mixture was then dialyzed against one liter of buffer containing 1 mM Tris, pH 7.2, 1 mM EDTA and 0.1 mM DTT at 4°C for 30 minutes. The solution was next dialyzed against buffer containing 1 mM Tris, pH 7.2, and 1 mM EDTA for a further 30 mins at 4°C. The reaction solution was then concentrated in a speed-vac concentrator, and the product separated from reactants on 6% polyacrylamide containing 7 M urea (Fig. 5 A). The yield ranged from 45% to 55%.

Synthesis of 5'-(2-pyr)-ss-[^{32}P]-16mer (Fig. 1 iv)

0.02-0.2 O.D. (254 nm) of 5'-cystamine-[^{32}P]-16mer was reacted with 5 mM DTT in 11 μl of Tris-EDTA buffer at pH 7.2 for 1 hour at room temperature. 1.5 μl of buffer containing 500 mM Tris (pH 7.2) and 1 mM EDTA, and 51 μl of a 3 mM solution of 2,2'-dipyridyldisulfide in water were then added. The final reaction mixture contained 0.8 mM DTT and 2.4 mM 2,2-dipyridyldisulfide. It was incubated at room temperature for 2 hours.

The 5'-(2-pyr)-ss-[^{32}P]-16mer was then separated from the starting 5'-cystamine-

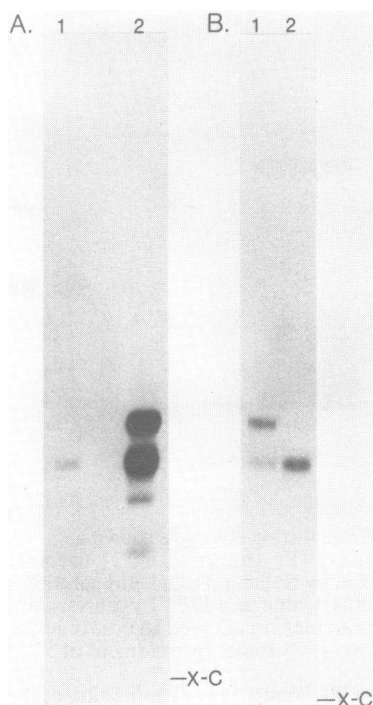


Figure 5: Autoradiograms of denaturing 6% gel showing:

A. lane 1) 5'-cystamine-[32 P]-MDV-1 RNA; lane 2) formation of the slower moving 5'-P-16mer-ss-5'-[32 P]-MDV-1 RNA by reduction of a mixture of 5'-cystamine-[32 P]-MDV-1 RNA and 5 μ M 5'-cystamine-P-16mer with DTT, removal of thio-ethylamine and DTT by dialysis, and air oxidation for 30 mins;

B. 5'-P-16mer-ss-5'-[32 P]-MDV-1 RNA extracted from gel; lane 1) before DTT treatment; lane 2) after treatment with 10 mM DTT at pH 7.2 for 1 hour at room temperature.

[32 P]-16mer either by HPLC on RPC-5 at pH 8 (Fig. 2 B) or by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 3). The yield ranged from 70% to 90%.

Synthesis of 5'-(2-pyr)-ss-[32 P]-MDV-1 RNA (Fig. 1 iv)

10-100 ng of 5'-cystamine-[32 P]-MDV-1 RNA were treated with 5 mM DTT in 4.5 μ l of Tris-EDTA buffer at pH 7.2 for 1 hour at room temperature. 2 μ l of a 1 mM solution of EDTA and 24 μ l of a 3 mM solution of 2,2'-dipyridyldisulfide were then added to the reaction mixture. The resulting solution contained 0.8 mM DTT and 2.4 mM 2,2'-dipyridyldisulfide. After 2 hours at room temperature, the 5'-(2-pyr)-ss-[32 P]-MDV-1 RNA, mixed with unreacted MDV-1 RNA, was separated from reagents by denaturing gel electrophoresis on 6% polyacrylamide. The product was then extracted from the gel and purified as described.

Thiolation of Bradykinin, Peroxidase and Human IgG

200 μ g (0.15 μ mole) of bradykinin in 100 μ l of buffer containing 100 mM phosphate and 0.1 mM EDTA at pH 7.6 was added to 2 mg (14.5 μ mole) of iminothiolane. The final

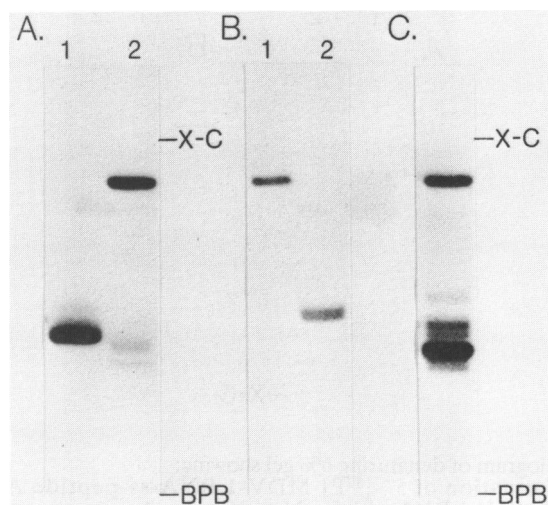


Figure 6: Autoradiogram of denaturing 20% gel showing:

A. lane 1) 5'-(2-pyr)-ss-[³²P]-16mer; lane 2) formation of 5'-[³²P]-16mer-ss-peptide A after reaction of 5'-(2-pyr)-ss-[³²P]-16mer with 3 mM peptide A overnight at room temperature and pH 7.2.

B. 5'-[³²P]-16mer-ss-peptide A extracted from gel: lane 1) before DTT treatment; lane 2) after treatment with 10 mM DTT at pH 7.2 for 1 hour at room temperature.

C. formation of 5'-[³²P]-16mer-ss-bradykinin after reaction of 5'-(2-pyr)-ss-[³²P]-16mer with 1 mM thiolated bradykinin overnight at room temperature and pH 7.2.

concentration of iminothiolane was 0.15 M. The pH was adjusted to 8.5 and the reaction allowed to proceed at room temperature for 1 hour. The thiolated bradykinin was then separated from the reagents by HPLC on a Synchronapak GPC 60 gel filtration column, eluting with 0.1 mM EDTA at pH 6.9.

The thiolation of peroxidase was carried out as described in reference 12. 34 μ g of peroxidase (\sim 1 nmole) in 40 μ l of 50 mM sodium borax buffer, pH 8.5, was added to 2 mg (14 μ moles) of iminothiolane. The final concentration of iminothiolane was 0.3 M. The pH was adjusted to 8.4 and the reaction allowed to proceed at room temperature for 1 hour. The thiolated peroxidase was separated from reagents by HPLC on a Synchronapak GPC 60 gel filtration column, eluting with buffer containing 50 mM sodium phosphate, pH 6.9, and 1 mM EDTA. The product, collected in \sim 0.5 ml of buffer, contained an average of 3 moles of thiol per mole of peroxidase. The thiolated peroxidase was as active as native peroxidase in the H₂O₂/diaminobenzidine assay on nitrocellulose (14).

The thiolation of human IgG followed the procedure described in reference 15. 400 μ g of human IgG (2.5 nmoles) was dissolved in 180 μ l of buffer containing 60 mM triethanolamine, 7 mM potassium phosphate, 100 mM NaCl and 1 mM EDTA at pH 8. The tube was flushed with argon, and 20 μ l of a 10 mM solution of iminothiolane in 1 M triethanolamine at pH 8 was added. The final concentration of iminothiolane in the reaction mixture was 1 mM. The

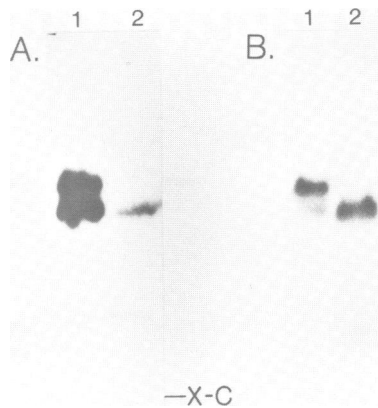


Figure 7: Autoradiogram of denaturing 6% gel showing:

A. lane 1) formation of 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA-ss-peptide A after reaction of 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA with 5 mM peptide A at room temperature overnight at pH 7.2; lane 2) 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA.

B. 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA-ss-peptide A extracted from gel: lane 1) before treatment with DTT; lane 2) after treatment with 10 mM DTT at pH 7.2 and room temperature for 1 hour.

reaction was allowed to proceed at 0°C for 1 hour. The thiolated human IgG was then separated from the reagents by HPLC on a Synchronpack GPC 60 gel filtration column, eluting with 5 mM bis-tris acetate buffer containing 50 mM NaCl and 1 mM EDTA at pH 5.8. The preparation of thiolated IgG contained an average of 1 mole of thiol per mole of IgG.

Synthesis of 5'-(2-pyr)-ss-[³²P]-16mer-ss-peptide A (Fig. 1 v)

A. 2-5 ng of 5'-(2-pyr)-ss-[³²P]-16mer was reacted with 3 mM peptide A in 12 μl of buffer containing 50 mM Tris and 1 mM EDTA (pH 7.2) at room temperature overnight. The product was separated from starting materials by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 6 A). The yield ranged from 70% to 90%.

B. 2-5 ng of 5'-cystamine-[³²P]-16mer was used in place of the 5'-(2-pyr)-ss-[³²P]-16mer in procedure A described above. The yield of 5'-(2-pyr)-ss-[³²P]-16mer-ss-peptide A was only 10%.

C. 30 ng of 5'-cystamine-[³²P]-16mer and a 4 mM solution of peptide A in Tris-EDTA buffer at pH 7.2 was treated with 10 mM DTT for 1 hour at room temperature. The reaction mixture was then dialyzed against 1 mM Tris (pH 7.5) for 1 hour at 4°C and the resulting solution lyophilized. The product was resuspended in 5 μl of Tris-EDTA buffer at pH 7.2 and allowed to stand at room temperature overnight. The product, 5'-(2-pyr)-ss-[³²P]-16mer-ss-peptide A, was separated from starting materials by denaturing gel electrophoresis on 20% polyacrylamide. The yield ranged from 80% to 100%.

Synthesis of 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA-ss-peptide A (Fig. 1 v)

3,000-5,000 cpm (0.1-0.5 ng) of 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA was reacted with 5 mM peptide A in 9 μl of buffer containing 100 mM Tris and 1 mM EDTA at pH 7.2. The reaction proceeded overnight at room temperature. The product was separated from reactants by

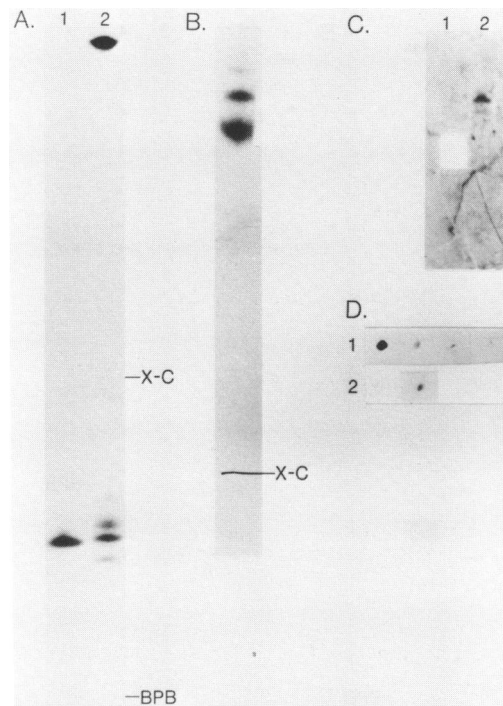


Figure 8: A. Autoradiogram of denaturing 20% gel showing: lane 1) 5'-(2-pyr)-ss-[³²P]-16mer; lane 2) formation of 5'-[³²P]-16mer-ss-peroxidase after overnight reaction of 5'-(2-pyr)-ss-[³²P]-16mer with 15 μ M thiolated peroxidase at pH 7.2 and room temperature.

B. Autoradiogram of denaturing 6% gel showing same reaction as A.

C. Denaturing 6% gel stained with Coumassie blue showing same reaction as A: lane 1) after removal of 5'-[³²P]-16mer-ss-peroxidase band; lane 2) 5 μ g peroxidase marker.

D. Color development of nitrocellulose filters showing peroxidase activity in the presence of diaminobenzidine and 0.003% H₂O₂. Lane 1) 0.06, 0.015, 0.008 and 0.004 pmoles of peroxidase; lane 2) 0.05 pmoles of 5'-[³²P]mer-ss-peroxidase.

denaturing gel electrophoresis on 6% polyacrylamide (Fig. 7 A). The yield of 5'-[³²P]-MDV-1 RNA-ss-peptide A ranged from 40% to 50%.

Synthesis of 5'-[³²P]-16mer-ss-bradykinin

20-30 μ g of thiolated bradykinin in 60 μ l of HPLC buffer (0.1 mM EDTA at pH 6.9) (0.2 mM) was mixed with 1-5 ng of 5'-(2-pyr)-ss-[³²P]-16mer and concentrated in a speed-vac concentrator. The reaction mixture was suspended in 10 μ l of Tris-EDTA buffer at pH 7.2 and allowed to stand overnight at room temperature. The product was purified by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 6 B). The yield of 5'-[³²P]-16mer-ss-bradykinin ranged from 30% to 50%.

Synthesis of 5'-[³²P]-16mer-ss-peroxidase

4-5 μ g of thiolated peroxidase (\sim 0.1 nmole) in 60 μ l of elution buffer (50 mM sodium

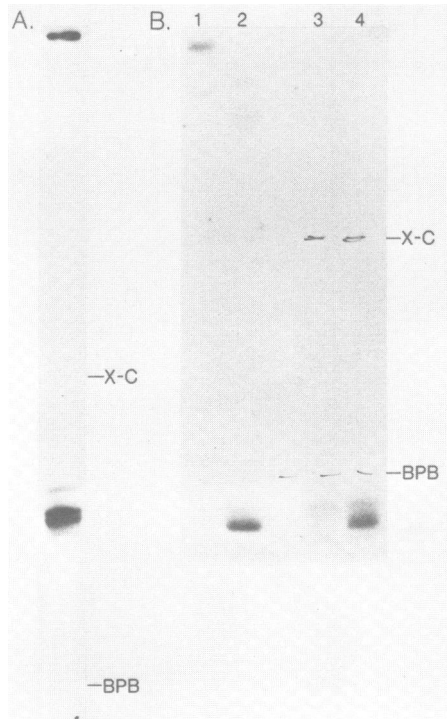


Figure 9: Autoradiograms of A. denaturing 20% gel showing formation of 5' -[³²P]-16mer-ss-IgG after reaction of 5'-(2-pyr)-ss-[³²P]-16mer with ~20 μM thiolated IgG overnight at pH 7.2 and room temperature. The faster migrating band has same mobility as a 5'-(2-pyr)-ss-[³²P]-16mer marker (not shown).

B. 6% denaturing gel showing fractions of the above reaction mixture eluted from a DEAE column: lane 1) fraction eluted with 0.5 M TEAB buffer, pH 7.8 before DTT treatment; lane 2) same as lane 1 after reaction with 10 mM DTT at pH 7.2 for 1 hour; lanes 3) and 4) fractions eluted successively with 1 M TEAB, pH 7.8.

phosphate pH 6.9 and 1 mM EDTA) (~2 μM) were mixed with 3-30 ng of 5'-(2-pyr)-ss-[³²P]-16mer and concentrated in a speed-vac concentrator. The reaction mixture was then suspended in 10 μl of Tris-EDTA buffer at pH 7.2 and incubated overnight at room temperature. An aliquot of the reaction mixture was analyzed by gel electrophoresis on 20% polyacrylamide to determine the yield of product. The product is visualized at the origin (Fig. 8 A). The remaining 5' -[³²P]-16mer-ss-peroxidase was separated from peroxidase and 5'-(2-pyr)-ss-[³²P]-16mer by denaturing gel electrophoresis on 6% polyacrylamide (Fig. 8 B). The electrophoresis was not terminated until the xylene cyanol marker had migrated at least 37 cm from the origin. The band containing 5' -[³²P]-16mer-ss-peroxidase was then removed. Urea was removed from the gel by extracting for several hours with extraction buffer that did not contain SDS. 5' -[³²P]-16mer-ss-peroxidase was extracted with the same buffer containing

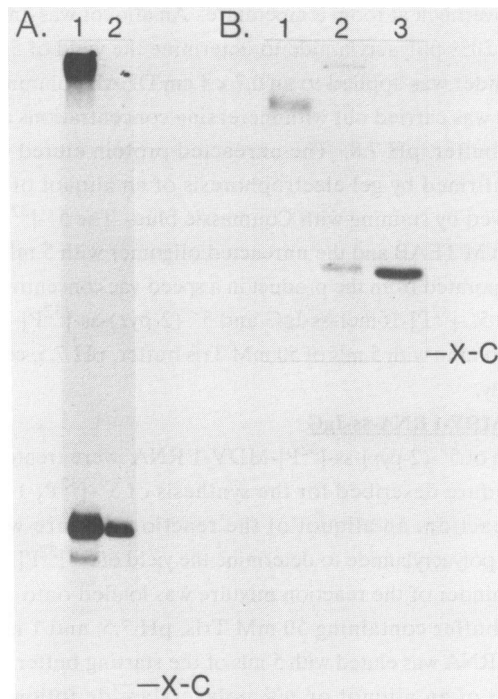


Figure 10: Autoradiograms of denaturing 6% gel showing:

A. Lane 1) formation of $5' \text{-}^{32}\text{P}$ -MDV-1 RNA-ss-IgG after treatment of $5' \text{-}(2\text{-pyr})\text{-ss-MDV-1 RNA}$ with $\sim 20 \mu\text{M}$ thiolated IgG overnight at pH 7.2 and room temperature; lane 2) $5' \text{-}(2\text{-pyr})\text{-ss-MDV-1 RNA}$.

B. Same reaction mixture as lane 1 (above) applied to a DEAE column. Fractions were eluted from the DEAE column with 50 mM Tris-1 mM EDTA (pH 7.5) buffer containing increasing concentration of NaCl; lane 1) Fraction eluted with 0.5 M NaCl, before treatment with DTT; lane 2) same as lane 1 after treatment with 10 mM DTT for 1 hour at pH 7.2; lane 3) fraction eluted with buffer containing 0.6 M NaCl.

0.01% SDS. The remaining 6% gel was then stained with Coumassie blue. $5 \mu\text{g}$ of a peroxidase marker in a neighboring lane that was stained with Coumassie blue had a lower mobility than the product, $5' \text{-}^{32}\text{P}$ -16mer-ss-peroxidase (Fig. 8 C).

Synthesis of $5' \text{-}^{32}\text{P}$ -16mer-ss-IgG

10-100 pmoles of $5' \text{-}(2\text{-pyr})\text{-ss-}^{32}\text{P}$ -16mer (10,000-100,000 cpm) were dialyzed against a liter of buffer containing 1 mM Tris and 0.1 mM EDTA at pH 7.0 for 30 minutes. $40 \mu\text{g}$ of thiolated human IgG in 0.2 ml of HPLC elution buffer (5 mM bis-tris acetate pH 5.8, 50 mM NaCl and 1 mM EDTA) ($1 \mu\text{M}$ IgG) was then added to the $5' \text{-}(2\text{-pyr})\text{-ss-}^{32}\text{P}$ -16mer in the dialysis bag. The mixture was dialyzed against 1 liter of buffer containing 1 mM Tris, 0.1 mM EDTA and 2 mM NaCl at pH 7.0 for 30 minutes and then concentrated in a speed-vac concentrator. The reaction mixture was then dissolved in $10 \mu\text{l}$ of Tris-EDTA buffer at pH 7.2,

and allowed to stand overnight at room temperature. An aliquot was analyzed by denaturing gel electrophoresis on 20% polyacrylamide to determine the yield of 5'-[³²P]-16mer-ss-IgG (Fig. 9 A). The remainder was applied to an 0.7 x 4 cm DEAE column. Elution of product and starting materials was carried out with increasing concentrations of triethylammonium bicarbonate (TEAB) buffer, pH 7.8. The unreacted protein eluted with 5 mls of 0.25 M TEAB. This was confirmed by gel electrophoresis of an aliquot of this fraction on 6% polyacrylamide followed by staining with Coumassie blue. The 5'-[³²P]-16mer-ss-IgG was eluted with 5 mls of 0.5 M TEAB and the unreacted oligomer with 5 mls of 1 M TEAB. The volatile buffer was evaporated from the product in a speed-vac concentrator in the presence of 50 µg of carrier BSA. 5'-[³²P]-16mer-ss-IgG and 5'-(2-pyr)-ss-[³²P]-16mer could also be eluted off the DEAE column with 5 mls of 50 mM Tris buffer, pH 7.5, containing 0.25 M and 0.4 M NaCl, respectively.

Synthesis of 5'-[³²P]-MDV-1 RNA-ss-IgG

20,000-40,000 cpm of 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA were treated with thiolated IgG using the same procedure described for the synthesis of 5'-[³²P]-16mer-ss-IgG. After completion of the reaction, an aliquot of the reaction mixture was analyzed by gel electrophoresis on 6% polyacrylamide to determine the yield of 5'-[³²P]-MDV-1 RNA-ss-IgG (Fig. 10 A). The remainder of the reaction mixture was loaded onto an 0.7 x 4 cm DEAE column packed with buffer containing 50 mM Tris, pH 7.5, and 1 mM EDTA. Protein unattached to MDV-1 RNA was eluted with 5 mls of the starting buffer. This was confirmed by gel electrophoresis of an aliquot on 6% polyacrylamide followed by staining with Coumassie blue. The column was then washed with 5 mls of the same buffer containing 0.2 M NaCl and then again with buffer containing 0.4 M NaCl. 5'-[³²P]-MDV-1 RNA-ss-IgG was eluted with 5 mls of buffer containing 0.5 M NaCl and unreacted 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA with buffer containing 0.6 M NaCl. The yield of 5'-[³²P]-MDV-1 RNA-ss-IgG ranged from 30% to 50%. Fractions containing 5'-[³²P]-MDV-1 RNA-ss-IgG or 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA were pooled and desalted and concentrated with 20 µg BSA carrier in an Amicon 30 microconcentrator.

Methods

High performance liquid chromatography (HPLC) of oligonucleotides was usually performed on RPC-5 at pH 12, using a perchlorate gradient as previously described (16). 5'-(2-pyr)-ss-16mer was separated from 5'-cystamine-16mer by HPLC on RPC-5 at pH 8, using a 0.02-0.4 M perchlorate gradient in 30 mins. The 5'-cystamine MDV-1 RNA was separated from unreacted cystamine by gel electrophoresis or by HPLC on a SynChropak GPC 60 gel exclusion column (Western Analytical Products) by elution with 0.1 mM EDTA at pH 7. DEAE Sephadex was converted to the bicarbonate form by incubation with 1 mM sodium bicarbonate overnight and then repeatedly washed with water. Electrophoresis was carried out on 0.5-1.0 mm thick 6% or 20% polyacrylamide gels, cast and run in 90 mM Tris borate, pH 8.0, and 1 mM EDTA, with or without 7 M urea. Autoradiographs of gels were

obtained by exposure to Kodak X-Omat AR film at -80°C with or without a Du Pont Cronex Lightning Plus intensifying screen.

RNAs were precipitated from solution by the addition of 2 volumes of ethanol at -80°C in the presence of 100 mM NaCl. All RNA and oligonucleotide adducts were extracted from gels with 500 mM ammonium acetate, pH 7.2, 0.1 mM EDTA, and 0.01% SDS, except the 5'-(2-pyr)-ss-adducts, which were extracted with buffer containing 100 mM Tris and 1 mM EDTA at pH 7. They were then purified by passage through a Du Pont Nensorb²⁰ nucleic acid purification cartridge. RNA and 16mer peptide or protein adducts were purified after gel extraction or elution from DEAE by passage through an Amicon 10 or Amicon 30 microconcentrator.

Dialysis tubing (Spectrum) 1000 m.w. cutoff was treated with acetic anhydride for 1 hour and then washed with 2% NaHCO_3 and 1 mM EDTA (17).

Reductions with Dithiothreitol

Adducts linked via a disulfide bond were reduced by reaction for 1 hour at room temperature with 10 mM DTT in Tris-EDTA buffer at pH 7.2. Reduction of disulfide bonds required a minimum concentration of 5 mM DTT and could be maintained in the reduced state with 0.1 mM DTT.

Hybridization of 5'-[³²P]-16mer and 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA to M13mp18 DNA (+) strand

Hybridization procedures described by Meinkoth and Wahl (18) were used to immobilize single-stranded M13mp18 (+) DNA on nitrocellulose filters. Filters containing 100, 10 and 1 ng of single-stranded M13mp18 DNA and 100 ng λ DNA were pre-hybridized for 1 hour at 32°C in hybridization buffer (900 mM NaCl, 6 mM EDTA, 90 mM Tris, pH 7.5, 0.1% SDS) containing 200 $\mu\text{g}/\text{ml}$ homochromatography mix I (randomly cleaved RNA) (19). Hybridization was carried out overnight at 32°C with approximately 7,000 cpm of 5'-[³²P]-16mer (0.0045 pmole or 0.025 ng per ml), 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA (approximately 0.0045 pmole or 0.36 ng RNA per ml), or 5'-[³²P]-cystamine-MDV-1 RNA (0.0045 pmole or 0.34 ng RNA per ml). The filters were then washed several times with 1 x SSPE (180 mM NaCl, 10 mM Na_2HPO_4 and 1 mM EDTA, pH 7.5) containing 0.1% SDS at room temperature. After drying, the filters were autoradiographed.

After removal of the filters, the hybridization fluid containing unhybridized material was passed through an Amicon 30 microconcentrator. An aliquot of the recovered material was analyzed by gel electrophoresis to test the stability of 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA under hybridization conditions.

Hybridization of 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA to a complementary 37mer

0.03 pmole of the target 37mer, or 0.03 pmole of a non-complementary 25mer were heated at 95°C - 100°C for 1 minute and rapidly chilled before addition of 0.02 pmole of 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA or 5'-cystamine-[³²P]-MDV-1 RNA. Hybridization was carried out for 7 hours at 25°C in buffer (10 μl) containing 50 mM Tris at pH 7.2, 1 mM EDTA, 500 mM NaCl and 20 μg randomly cleaved RNA (19). The 5'-P-16mer-ss-

5'-[³²P]-MDV-1 RNA/37mer hybrid was separated from non-hybridized 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA by electrophoresis on a 6% gel under non-denaturing conditions at 120 v. (6 ma) for 15 hours.

Measurement of Thiol Concentrations

Thiol concentrations were measured according to the method of Ellman (20).

Measurement of Peroxidase Activity

Nitrocellulose filters were pre-wetted with buffer containing 10 mM Tris (pH 8) and 150 mM NaCl and then dried. 1 μ l aliquots containing up to 10 ng of peroxidase or thiolated peroxidase were then spotted on the filters. The filters were then submerged in 20 mls of phosphate-buffered saline at pH 7.2 containing 0.5 mg/ml diaminobenzidine and 0.003% H₂O₂ (14). In this assay spots containing 0.5 ng or more of peroxidase were seen as colored brown spots.

Binding of 5'-[³²P]-16mer-ss-human IgG or 5'-[³²P]-MDV-1 RNA-ss-human-IgG to a Complementary anti-human IgG

Antibody-binding assays were carried out by coating wells of an ELISA plate with 100 μ l of goat anti-human IgG (5 μ g/ml) in borate-buffered saline containing 10⁻³ EDTA at pH 8.4. The wells were incubated overnight at 4°C. The anti-IgG solution was then removed and the wells blocked by the addition of 200 μ l of borate-buffered saline containing 1 mM EDTA and 1% BSA. After incubation for 1 hour at room temperature, the wells were emptied and washed twice with 100 μ l of borate-buffered saline containing 1 mM EDTA. 100 μ l of DEAE purified 5'-[³²P]-16mer-ss-IgG, 5'-(2-pyr)-ss-[³²P]-16mer, 5'-[³²P]-MDV-1 RNA-ss-IgG or 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA (300-600 cpm) were then added to the coated wells and the wells incubated overnight at 4°C. The solutions were then removed and the wells washed with 100 μ l of borate buffered saline. The amount of radioactivity contained in the supernatant fraction and the amount attached to the wells were measured in a scintillation counter.

RESULTS AND DISCUSSION

Formation of 5'-cystamine-P-16mer

The 5'-cystamine-P-16mer could be made directly from the 5'-P-16mer in a one-step synthesis in the presence of carbodiimide, 1-methylimidazole and cystamine. The 1-methylimidazole adduct of 5'-P-16mer is formed as a transitory intermediate, and subsequently reacts with cystamine to form a stable product. The yield of the 5'-cystamine-P-16mer from the 5'-P-16mer was 75% to 85%.

To show that carbodiimide does not react with the disulfide group, a sample of 5'-cystamine-P-16mer was made by first isolating the stable 5'-imidazolide adduct of 5'-P-16mer (8) and then treating it with cystamine in the absence of carbodiimide. This procedure gave a product that had the same mobilities both on RPC-5 and gel electrophoresis as the product made by the one-step procedure with 1-methylimidazole.

The 5'-cystamine-P-16mer is converted to 5'-thio-ethylamino-P-16mer (Fig. 1 i) in one hour with 5 mM DTT at pH 7, and can be maintained in the reduced state with 0.1 mM DTT.

The 5'-P-16mer, 5'-cystamine-P-16mer and 5'-thio-ethylamino-P-16mer are readily separated by gel electrophoresis (Fig. 3).

Synthesis of 5'-cystamine-[³²P]-MDV-1 RNA

The procedure given above for the preparation of cystamine adducts of short oligodeoxynucleotides is not applicable to RNA because high molecular weight RNA reacts with carbodiimide at 50°C. The synthesis of 5'-cystamine-[³²P]-MDV-1 RNA has been previously described (13) and requires isolation of the 5'-imidazolide of MDV-1 and removal of the carbodiimide before reaction with cystamine at 50°C. The RNA is separated from excess cystamine either by gel electrophoresis or, more quickly (in 15 minutes), by HPLC on a gel filtration column. The product purified by gel electrophoresis contains a mixture of unconverted 5'-[³²P]-MDV-1 RNA and 5'-cystamine-[³²P]-MDV-1 RNA. The product, if purified by HPLC gel filtration, may also contain 5'-[³²P]-RNA degradation products and their cystamine adducts. The entire synthesis, starting from 5'-[³²P]-MDV-1 RNA, can be completed in 4 hours.

Linkage of 16mer with 16mer, 37mer or MDV-1 RNA via a disulfide bond

An autoradiogram demonstrating the dimerization of 16mer is shown in Fig. 4 A. Dimerization was achieved by reducing a 5 μM solution of 5'-cystamine-[³²P]-16mer with DTT, removing the 2-thio-ethylamine under reducing conditions and finally removing the DTT by dialysis. After the removal of DTT, air oxidation occurs in the dialysis bag within a half hour, resulting in disulfide bond formation between two 16mers. Ligation of 16 mer with 37mer or with MDV-1 RNA was carried out in the same way. Autoradiograms demonstrating the formation of 5'-P-16mer-ss-5'-[³²P]-37mer and 5'-P-16mer-[³²P]-MDV-1 RNA are reproduced in Fig. 4 B and 5 A, respectively. The oxidation reaction was complete in 30 minutes, and the entire ligation procedure required 2 hours. One advantage of this procedure is that all derivatives can be stored as stable 5'-cystamine adducts, rather than as less stable thiol adducts; the thiol adducts do not need to be isolated before the start of the reaction.

When the disulfide-linked adducts of 16mer with 16mer, 37mer or MDV-1 RNA were treated with 10 mM DTT, the S-S links were reduced and the oligonucleotides released as 5'-thio-ethylamino derivatives (Fig. 4 A and 5 B).

Hybridization of 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA to a complementary M13mp18 DNA or 37mer

Figure 11 shows the autoradiographs of nitrocellulose filters containing 100, 10, and 1 ng of single-stranded M13mp18 DNA and 100 ng λ DNA, hybridized with 5'-[³²P]-16mer, 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA, or 5'-cystamine-[³²P]-MDV-1 RNA. No radioactivity could be detected on filters incubated with 5'-cystamine-[³²P]-MDV-1 RNA. The minimum amount of M13mp18 DNA that could be detected with 5'-[³²P]-16mer was 10 ng, whereas the minimum amount of M13mp18 DNA detected with 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA was 100 ng DNA. In these assays the probes were used in very low concentrations. The sensitivity of the assay could be increased greatly by using the probes in substantial excess over the target. In the present context, however, our primary interest is the relative hybridization efficiencies

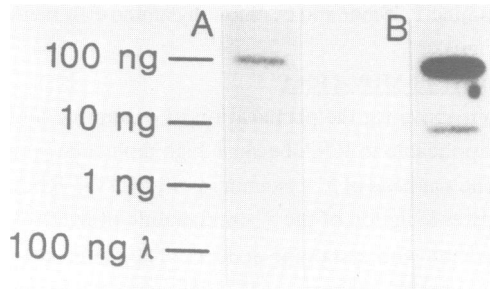


Figure 11: Autoradiogram of blots using 100 ng, 10 ng and 1 ng of single-stranded M13mp18 DNA or 100 ng λ DNA hybridized overnight at 32°C with:
 A. 0.0045 pmole or 0.36 ng/ml 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA (~ 7000 cpm).
 B. 0.0045 pmole or 0.025 ng/ml 5'-[³²P]-16mer (~ 7000 cpm).

of the 16mer and its adduct with MDV-1 RNA. This is best measured at low probe concentration. If we worked at saturating concentration of probe, as is usually done, the difference between the sensitivity achieved with 5'-[³²P]-16mer and 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA would be much reduced. Electrophoresis of the 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA remaining in the hybridization fluid after hybridization showed that the adduct does not decompose under hybridization conditions.

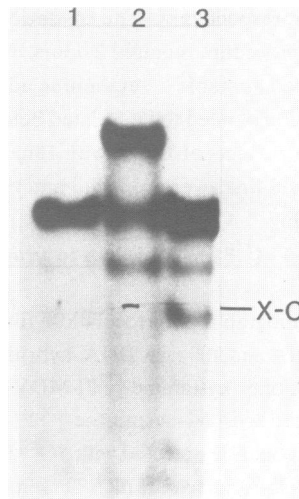


Figure 12: Autoradiogram of a 6% non-denaturing gel showing: lane 1) 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA, lane 2) 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA after hybridization with a complementary 37mer; lane 3) 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA after attempted hybridization with a non-complementary 25mer.

Figure 12 shows the results obtained when 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA was incubated under hybridization conditions with a complementary 37mer or with a non-complementary 25mer, and the products subjected to non-denaturing gel electrophoresis. The 5'-P-16mer-ss-5'-[³²P]-MDV-1 RNA formed a hybrid with the complementary 37mer but not with the non-complementary 25mer. In a separate experiment (data not shown) no hybrid formation occurred between 5'-cystamine-[³²P]-MDV-1 RNA and the 37mer.

The synthesis of 5'-(2-pyr)-ss-[³²P]-16mer and 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA

5'-(2-pyr)-ss-[³²P]-16mer could be synthesized starting from 5'-cystamine-[³²P]-16mer in a one step procedure, by first reducing 5'-cystamine-[³²P]-16mer to 5'-thioethylamino-[³²P]-16mer with 5 mM DTT and then adding a two-fold excess of 2,2'-dipyridyldisulfide to the reaction *in situ*. The product, obtained in 70 to 80% yield, could readily be separated from starting materials by HPLC on RPC-5 at pH 8 (Fig. 2 B) or by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 3). 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA was made from 5'-cystamine-[³²P]-MDV-1 RNA by an analogous procedure and purified by denaturing gel electrophoresis on 6% polyacrylamide. Both the 5'-(2-pyr)-ss-[³²P]-16mer and 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA were stable for several months at pH 7.2 if stored at -20°C.

The linkage of 16mer or MDV-1 RNA to Peptides via Disulfide Bonds

Both 5'-(2-pyr)-ss-[³²P]-16mer and 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA reacted readily with a cysteine-containing peptide (Peptide A). When the peptide was present in 3-5 mM concentrations, overnight incubation with the 5'-(2-pyr)-ss-[³²P]-oligomers gave yields ranging from 70% to 90% for 5'-[³²P]-16mer-ss-peptide A (Fig. 8 A) and 40% to 50% for the 5'-[³²P]-MDV-1 RNA-ss-peptide A (Fig. 9 A). To determine the yield of product obtained by disulfide interchange with a less active disulfide, 5'-cystamine-[³²P]-16mer was reacted with peptide A under identical conditions. The yield of 5'-[³²P]-16mer-ss-peptide A was only 10%. When 5'-[³²P]-16mer or 5'-[³²P]-MDV-1 RNA was reacted with peptide A, no product was formed. Both 5'-[³²P]-16mer-ss-peptide A and 5'-[³²P]-MDV-1 RNA-ss-peptide A were readily converted to the 5'-thioethylamino derivatives by treatment with 10 mM DTT for 1 hour at pH 7.2 (Fig. 6 B and 7 B).

When peptides that do not contain cysteine residues are to be used as ligands, a thiol group is attached to the peptide by reaction with iminothiolane. When bradykinin was thiolated, and reacted with 5'-(2-pyr)-ss-[³²P]-16mer, formation of 5'-[³²P]-16mer-ss-thiolated-bradykinin occurred readily (Fig. 6 C).

The above procedure, using 5'-(2-pyr)-ss-[³²P]-oligomer derivatives, can be used as a general method for attaching nucleic acids to peptides or proteins by a cleavable disulfide linkage, even when a disulfide linkage is already present in the polypeptide. However, if only cysteine groups are present in the peptide, better yields of 5'-[³²P]-16mer-ss-peptide A (ranging from 80% to 100%) could be obtained by reducing 5'-cystamine-[³²P]-16mer with DTT in the presence of the cysteine containing peptide, removing the DTT by dialysis and allowing oxidation to occur in air.

Synthesis and Activity of 5'-[³²P]-16mer-ss-peroxidase

Thiolation of peroxidase was achieved, using the method described in reference 12. An average of 3 moles of thiol per mole of peroxidase was introduced. The activity of the thiolated peroxidase was not significantly different from that of the unmodified enzyme.

A dilute solution of the thiolated peroxidase ($\sim 2 \mu\text{M}$), obtained after purification on a gel filtration column, was concentrated in the presence of 5'-(2-pyr)-ss-[³²P]-16mer and reacted overnight. Figure 8 A shows an autoradiogram of the reaction mixture after electrophoresis on a 20% polyacrylamide gel. This gel separates 16mer protein adducts from 5'-(2-pyr)-ss-[³²P]-16mer and allows an estimate of the yield to be made. The yields varied from 40% to 50%. However, since the thiolated protein contains an average of 3 thiol groups per molecule of protein, some 16mer-peroxidase dimer and trimer adducts were formed as well. 5'-[³²P]-16mer-ss-peroxidase could be separated from these side products, as well as unreacted peroxidase, by lengthy gel electrophoresis on 6% polyacrylamide. Figure 8 B shows the separation of 5'-[³²P]-16mer-ss-peroxidase from higher molecular weight [³²P]-adducts and Fig. 8 C shows a 6% gel after removal of 5'-[³²P]-16mer-ss-peroxidase and staining with Coumassie blue. 5 μg of a peroxidase marker in a neighboring lane that was stained with Coumassie blue had a lower mobility than 5'-[³²P]-16mer-ss-peroxidase.

Figure 8 D shows the peroxidase activity of a sample of purified 5'-[³²P]-16mer-ss-peroxidase in an H_2O_2 /diaminobenzidine assay on nitrocellulose (14). Figure 8 D (lane 1) shows the activity of 0.06, 0.015, 0.008 and 0.004 pmoles of peroxidase (equivalent to 2.4, 0.6, 0.3 and 0.15 ng, respectively). The limit of sensitivity is 0.015 pmoles (0.6 ng). Lane 2 shows the peroxidase activity of a sample of 5'-[³²P]-16mer-ss-peroxidase containing the equivalent of 0.05 pmoles of peroxidase. The color intensity of this sample is equivalent to the color intensity of 0.015 pmoles of peroxidase. Thus 5'-[³²P]-16mer-ss-peroxidase is approximately 3-fold less active than unreacted peroxidase.

The Synthesis of 5'-[³²P]-16mer-ss-IgG and 5'-[³²P]-MDV-1 RNA-ss-IgG

5'-[³²P]-16mer-ss-IgG and 5'-[³²P]-MDV-1 RNA-ss-IgG were synthesized by a procedure similar to that described above for the synthesis of 5'-[³²P]-16mer-ss-peroxidase. However, in this case, after treatment with iminothiolane and purification on a gel filtration column, thiolated IgG was obtained as a very dilute solution ($\sim 1.0 \mu\text{M}$) in elution buffer. To remove the large excess of salt in the buffer, a mixture of the thiolated IgG with the 5'-(2-pyr)-ss-[³²P]-adduct was first dialyzed against a dilute salt buffer. The resulting reaction mixture was then concentrated and the reaction allowed to proceed overnight at room temperature.

Both 5'-[³²P]-16mer-ss-IgG and 5'-[³²P]-MDV-1 RNA-ss-IgG could be separated from starting materials by DEAE chromatography. In the case of 5'-[³²P]-16mer-ss-IgG, it was possible to use a volatile buffer TEAB as eluant. Figure 9 B shows an autoradiogram of the products eluted with 0.5 M and 1.0 M TEAB (pH 7.8) analyzed on 6% polyacrylamide. The product in the 0.5 M TEAB buffer fraction, 5'-[³²P]-16mer-ss-IgG, could be converted, after incubation with 10 mM DTT, to a product with the same mobility as the product eluted with

1 M TEAB, 5'-(2-pyr)-ss-[³²P]-16mer. The reduction product 5'-thioethylamino-[³²P]-16mer and 5'-(2-pyr)-ss-[³²P]-16mer do not separate on 6% polyacrylamide.

It was also possible to separate 5'-[³²P]-MDV-1 RNA-ss-IgG from starting materials with TEAB as eluant. However, because of the excessively high concentrations of TEAB necessary for their elution (1.5 M - 2 M TEAB), the separation was achieved using 0.05 M Tris - 1 mM EDTA (pH 7.5) buffer to elute unreacted protein and the same buffer containing 0.5 M NaCl and 0.6 M NaCl to elute 5'-[³²P]-MDV-1 RNA-ss-IgG and 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA respectively. Figure 10 B shows an autoradiogram of the products eluted from DEAE with 0.5 M and 0.6 M NaCl and then analyzed by gel electrophoresis on 6% polyacrylamide. The product that eluted with 0.5 M NaCl, 5'-[³²P]-MDV-1 RNA-ss-IgG, could be converted to a band with the same mobility as the product eluted with 0.6 M NaCl, 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA, by incubation with 10 mM DTT for 1 hour at pH 7.2 (Fig. 10 B). The product eluted with 0.6 M NaCl had the same mobility as a marker 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA (not shown).

Binding of 5'-[³²P]-16mer-ss-IgG and 5'-[³²P]-MDV-1 RNA-ss-IgG to anti-human IgG

Both 5'-[³²P]-16mer-ss-human IgG and 5'-[³²P]-MDV-1 RNA-ss-human IgG, after purification on DEAE, retained their binding activity to anti-human IgG. When ELISA wells coated with anti-human IgG were incubated with 5'-[³²P]-16mer-ss-IgG or 5'-[³²P]-MDV-1 RNA-ss-IgG, 80% to 100% of the counts were found bound to the wells. Neither adduct bound if wells were coated with human IgG instead of anti-human IgG. Binding of the adducts to anti-human IgG was completely inhibited by the presence of 5 μg of human IgG, but not by the presence of 10 μg of BSA. 5'-(2-pyr)-ss-[³²P]-16mer or 5'-(2-pyr)-ss-[³²P]-MDV-1 RNA, after purification on DEAE, did not bind to wells coated with anti-human IgG under identical conditions. If the DEAE purified 5'-[³²P]-MDV-1 RNA-ss-IgG was first treated with 10 mM DTT and then added to the anti-human IgG coated wells, no radioactive material bound to the well. These results show that MDV-1 RNA linked to IgG through a disulfide linkage is bound specifically to its complementary antigen.

Conclusions

The methodology developed in this paper is very general. It allows any oligodeoxynucleotide or nucleic acid to be ligated via a cleavable disulfide linkage to another oligonucleotide, to a peptide or to a protein. In ligations involving short oligodeoxynucleotides it would be possible to substitute sulfhydryl containing oligodeoxynucleotides obtained by solid-state synthesis for the cystamine adducts that we use. Where ribonucleic acids are the substrates, solid-state synthesis does not provide a viable alternative approach.

The simplest and most efficient procedure for the synthesis of adducts joined by S-S bonds involves oxidation of a mixture of the two sulfhydryl compounds with air. Its major disadvantage is a lack of specificity; homo-dimers are obtained as major products, along with the desired hetero-dimers. In many cases this is not a serious problem, since separation of the three disulfides is straightforward. If this is not the case, or if either of the substrates is

sensitive to oxidation or reduction, it is better to form the S-S bond in a trans-thiolation reaction instead of an oxidation-reduction reaction. Adducts containing an S-S-linked thiopyridine moiety readily exchange with free thiol groups on another molecule to give the desired disulfide under mild conditions.

In general, adducts linked by a cleavable disulfide bond are useful in applications where a detector molecule (DNA probe or antibody) becomes attached to a target immobilized on a solid phase, but the final assay is most conveniently carried out in solution. We have prepared three products that could be used in such assays, as examples. We have not attempted to carry out useful assays under realistic conditions - our intention was only to develop the chemistry of ligation.

The oligodeoxynucleotide-peroxidase adduct allows hybridization to be carried out on a solid support and the final quantitative assay to be performed in aqueous solution. This would make it possible to use accurate spectrophotometric methods to determine the extent of hybridization and hence the concentration of target. The same methodology could also be used to attach DNA probes to enzymes that can be used to perform accurate non-colorimetric assays in solution. In many cases such assays are impossible if the enzyme is attached to a filter. The use of a nuclease or topoisomerase, for example, might permit a very sensitive assay based on the relaxation of supercoiled circular DNA.

We have previously shown that the same cystamine derivative of MDV-1 RNA that is released by the reduction of the disulfide bond in our adducts replicates efficiently in the presence of Q β -RNA polymerase and the four nucleoside triphosphates (13). Our simple scheme for the preparation of Q β -MDV RNA adducts of an oligodeoxynucleotide probe or of an antibody molecule is, therefore, another step towards the development of a very sensitive replication-assisted assay.

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REFERENCES

1. Cheng,S., Merlino,G.T., and Pastan,I.H. (1983) *Nucleic Acids Res.* **11**, 659-669.
2. Jablonski,E., Moomaw,E.W., Tullis,R.H. and Ruth,J.L. (1986) *Nucleic Acids Res.* **14**, 6115-6128.
3. Nitta,N., Kuge,O., Yui,S., Tsugawa,A., Negishi,K. and Hayatsu,H. (1984) *FEBS* **166**, 194-198.
4. Corey,R.D. and Schultz,P.G. (1987) *Science* **238**, 1401-1403.
5. Kremsky,J.N., Wooters,J.L., Dougherty,J.P., Meyers,R.E., Collins,M. and Brown,E.L. (1987) *Nucleic Acids Res.* **15**, 2891-2909.
6. Connolly,B. (1985) *Nucleic Acids Res.* **13**, 4485-4502.
7. Zuckerman,R., Corey,D. and Schultz,P. (1987) *Nucleic Acids Res.* **15**, 5305-5321.
8. Chu,B.C.F., Wahl,G.M. and Orgel,L.E. (1983) *Nucleic Acids Res.* **11**, 6513-6529.
9. Chu,B.C.F. and Orgel,L.E. (1985) *Proc. Nat'l. Acad. Sci. USA* **82**, 963-967.
10. Chu,B.C.F. and Orgel,L.E. (1985) *DNA* **4**, 327-331.
11. Brocklehurst,K. and Little,G. (1973) *Biochem. J.* **133**, 67-80.
12. King,T.P., Li,Y. and Kochoumian,L. (1978) *Biochemistry* **17**, 1499-1506.

13. Chu, B.C.F., Kramer, F.R. and Orgel, L.E. (1986) *Nucleic Acids Res.* **14**, 5591-5603.
14. Tsang, V.C.W., Peralta, J.M. and Simons, A.R. (1983) *Methods in Enzym.* **92**, 377-391.
15. Blattler, W.A., Kuenzi, B.S., Lambert, J.M. and Senter, P.D. (1985) *Biochemistry* **24**, 1517-1524.
16. Bridson, P.K. and Orgel, L.E. (1980) *J. Mol. Biol.* **144**, 567-577.
17. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
18. Meinkoth, J. and Wahl, G. (1984) *Anal. Biochem.* **138**, 267-284.
19. Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1974) *Nucleic Acids Res.* **1**, 331-354.
20. Eilman, G.L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77.