Studies towards the development of chemically synthesized non-radioactive biotinylated nucleic acid hybridization probes

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ABSTRACT

Non-radioactive nucleic acid hybridization probes have been constructed in which the reporter group is long chain biotin chemically linked to a basic macromolecule (histone H1, cytochrome C or polyethyleneimine). The modified basic macromolecule which carries many biotin residues can, in turn, be covalently linked to nucleic acids (DNA) via the bifunctional cross-linking reagents, glutaraldehyde, 1,2,7,8-diepoxyoctane, bis (succinimidyl) suberate or bis (sulfonosuccinimidyl) suberate. This provides a very sensitive probe by which as little as between 10-50fg of target DNA can be visualized using dot-blot hybridization procedures in conjunction with avidin or streptavidin enzyme conjugates.

INTRODUCTION

Much of the routine diagnosis of plant viruses is by serological methods. However, these do have some limitations and the possibilities of using nucleic acid hybridization methods have recently become apparent (for reviews see 1-6). In this approach nucleic acid from the sample (the target nucleic acid) is immobilized on a solid matrix and is probed with a nucleic acid which has complementary sequences; thus the probe will hybridize to the target.

For detection of the successful hybridization the probe has to have reporter groups. In the original development of the technique the radiolabel ³²P was used as the reporter group. However it is widely recognised that for routine use, radiolabel is a considerable disadvantage. This has led to the development of some non-radioactive reporter groups. Renz and Kurz (7) joined peroxidase and alkaline phosphatase directly to the DNA using a linker arm made up from polyethyleneimine, P-benzoquinone and glutaraldehyde. However, these probes are unstable if

hybridization is at temperatures greater than 45°C and such extensive labelling of the probe directly with the enzyme may interfere with hybridization. Most of the non-radioactive probes are based on biotin which is then detected by its strong affinity to avidin or streptavidin. If an enzyme is linked to the avidin, the reporter group can be detected colorimetrically. Biotin can either be incorporated directly as biotinylated deoxyribonucleotides into the probe (see 8) or linked to the probe by chemical means. It is this latter approach which offers the greatest potential for non-radioactive probes. Kempe *et al.* (9), and Chollet and Kawashima (10) have reported methods in which biotin is attached to the 5'-terminus of the DNA molecule. This has the disadvantage that only one biotin molecule is incorporated per DNA molecule. Foster *et al.* (11) developed a biotin derivative (termed photobiotin) which, because of its photoactive azide group which on irradiation with light produces very reactive nitrenes, can be linked directly to the heterocyclic bases of nucleic acid.

In another approach a biotin derivative is linked to amide groups of basic proteins (cytochrome C and histones) which in turn are linked by formaldehyde or glutaraldehyde to the DNA or RNA probe (12-14).

Thus there are several means by which biotin can be linked to nucleic acid. In this paper we describe the development of various methods of linking biotin to nucleic acid which provide a range of lengths of side arm and of numbers of biotin molecules per side arm. The general structure of the reporter groups we have been constructing are shown in Fig. 1. In this paper we report on the synthesis of a new type of biotin side chain and the use of a new group of basic macromolecules; we also examine variations in four parameters of the reporter group: the cross linker, the basic macromolecule, the biotin side chain and the enzyme.

MATERIALS AND METHODS

Biotinyl-N-hydroxysuccinimide ester (BHSE)

Biotinyl-N-hydroxysuccinimide ester (BHSE) was synthesized by a modification of the method described by Bayer and Wilchek

(15). N,N'-dicyclohexylcarbodiimide (0.4g) (Aldrich) was dissolved in dimethylformamide solution (6ml) containing biotin (0.5g) (Sigma) and N-hydroxysuccinimide (0.3g) (Sigma). The reaction mixture was stirred overnight at room temperature. The product was filtered and the filtrate was evaporated under reduced pressure (coevaporation with ethyl acetate is recommended) to give a crude crystalline product. The product was washed with diethyl ether and recrystallized from isopropanol/water to give a crystalline white product m.p 196-198°C (75% yield).

Biotinylation of histone H1 and cytochrome C

Biotinylation of histone H1 and cytochrome C was carried out in a manner similar to the Renz (14) procedure for biotinylation of histone. Histone H1 (Sigma) and cytochrome C (Sigma) were biotinylated separately with BHSE (short chain) and with sulfonosuccinimidyl 6-(biotinamido) hexanoate (SBAH) (Pierce) (long chain) at different ratios. Short chain biotinylated histone H1 and cytochrome C were prepared as follows. 5µg, 50µg, 250µg, 500µg and 1mg of BHSE were each dissolved in 10µl of dimethylformamide and added to solutions each containing 1mg histone H1 (in 250µl of 50mM NaHCO₃). Similar experiments were performed using cytochrome C instead of histone H1. The solutions were incubated at room temperature for 1h with frequent mixing and agitating and were then dialyzed overnight against 5mM sodium phosphate (pH 6.8); the samples were stored at -20°C.

The long chain biotinylated histone H1 and cytochrome C were prepared similarly but using the long chain sulfonosuccinimidyl 6-(biotinamido) hexanoate. This compound is water soluble and thus was added directly to the carbonate solutions instead of being dissolved in dimethylformamide.

Biotinylation of polyethyleneimine and Polymin G35

Polyethyleneimine (a 50% solution of the macromolecule with average mol wt 60 000 and termed P60) was obtained from Aldrich and Polymin G35 (polyethyleneimine with a mol wt of 1400) (termed PG35) was a gift from BASF. P60 and Polymin G35 were biotinylated using various molar ratios of biotin. 5µg, 50µg, 250µg, 500µg, 1.4mg, 2.8mg, 5.6mg and 11.2mg of the long chain biotin derivative, sulfonosuccinimidyl 6-(biotinamido) hexanoate were added to solutions containing 1mg of polymin (P60 or G35) in

0.5ml of 50mM NaHCO₃. The reaction mixtures were incubated at room temperature for 2h with frequent shaking. The solutions were then dialyzed against 5mM sodium phosphate (pH 6.8) to remove unreacted biotin derivative and the samples were stored at -20°C.

Target nucleic acid and DNA probes

For the development of these methods a 4-10kb cDNA to cowpea mosaic virus (CPMV) cloned into M13 was used. This was the kind gift of Dr. G P. Lomonosoff. Single-stranded and replicative form DNAs were prepared by conventional techniques (16). For negative control calf thymus and salmon sperm DNAs (Sigma) and alfalfa mosaic virus RNA (the kind gift of M.J Brisco) were used.

Cross linking of the biotinylated proteins, PEI and Polymin G35 to DNA

M13 phage ssDNA was diluted with sodium phosphate solution (1pg of DNA + 100µl of 5mM phosphate pH 6.8), denatured by heating (100°C for 5 min) and cooled on ice. Biotinylated histone H1 or cytochrome C solution (4-5µl) or polymin (3-5ml) was added to the denatured DNA solution followed by addition of 20µl of 2.5% of the cross-linking reagent (see Results). The sample was then incubated for 20 min at 37°C with frequent agitation. After the addition of 5µl Orange G (0.025 mg/ml) the product was separated from the unincorporated material by passage through a Sephadex G75 column made in a disposable 1ml pipette; 5mM phosphate was used as eluent. The required product was collected in the excluded volume (about 150-200µl) which eluted ahead of the Orange G marker and was added directly to the hybridization solution.

Dot-blot hybridization

Nitrocellulose filters (BA85 from Schleicher and Schüll) were marked into 1cm² squares, cut to the desired size, soaked in water for 15 min and with 20 x SSC for 10 min, placed on Whatman 3MM paper and spotted (5µl) with various amounts of denatured double-stranded target DNA. Since the target DNA was in the covalently closed circular form it was nicked by depurination and alkali treatment. Depurination was in 0.25N HCl for 15 min at room temperature; alkali treatment was in 0.75N NaOH, also for 15 min. After neutralization in 0.6M Na acetate, pH 6,

the DNA was denatured by heating to 100° for 2 min. Filters were air dried at room temperature for 15-20 min and baked in a vacuum oven at 80°C for 2h. Prehybridization and hybridization was using the conditions of Maule *et al.* (3). A solution of 2.5% dried skimmed milk (Sainsbury plc) (17) was sometimes used as an alternative to Denhardt's solution; it gave similar results. The hybridization solution contained 50-250ng/ml probe.

Washing and colorimetric detection

Filters were washed with a solution containing 1% SDS, 2 x SSC (250ml each wash, 4 times x 10 min). Filters were incubated in 20ml blocking buffer (0.1M Tris-HCl, pH 7.5, 1M NaCl, 2mM MgCl₂, 0.5% tween 20, 0.05% Triton X-100 containing 3% bovine serum albumin) at room temperature for 1h. They were then incubated for a further hour in the above buffer, but containing 0.05% tween 20 and 1µg/ml of avidin-peroxidase or avidin alkaline phosphate conjugate (Sigma) with gentle shaking at room temperature. Filters were washed with constant shaking with buffer made of 1M NaCl, 0.1M Tris-HCl, pH 7.5, 2.5mM MgCl₂, 0.05% Triton, 0.5% tween 20, and 1% BSA, 5 x 5 min), then washed again with a solution of 1M NaCl, 0.1M Tris-HCl, pH 9.5, 10mM MgCl₂ 2 x 10 min.

For colour development, filters which had been incubated with the avidin-peroxidase conjugate, were incubated with 10ml of 0.1M Tris-HCl, pH 7.4, 2ml of ethanol, 6mg of 3,3'-diansidine and 6µl of 30% H₂O₂ or with 100ml of 10mM Tris-HCl, pH 7.4, containing 50mg of 4-chloro-1-naphthol dissolved in 2ml methanol + 6µl of 30% H₂O₂. For detection of alkaline phosphatase, the filters were placed in a solution of 0.1M Tris-HCl, pH 9.5, 10mM MgCl₂ and 0.1M NaCl containing either 0.33mg/ml nitro blue tetrazolium in 70% dimethylformamide and 0.17ng/ml of 5-bromo-chloro-3 indoyl phosphatase in dimethylformamide (18) or 6mg of 5-chloro-2-toluidin diazonium chloride mixed with naphthol As-Mx phosphate (19). All colour development processes were carried out at room temperature in a dark place. Colour reaction was terminated by washing the filters with water; filters can be stored in an envelope of Whatman 3MM

Determination of amount of biotin linked to PG35

Various amounts (see results) of ³H labelled N-hydrosuccin-

nimide biotin (Amersham - specific activity) were linked to PG35 using BHSF (see above). The unincorporated biotin was removed from that incorporated onto the Polymin on a Sephadex G25 column, the relevant fractions being determined by scintillation counting. Trial spectra showed that Polymin and biotin had an absorbance at 250nm of 0.284/mg/ml and 0.111/mg/ml respectively. The amount of biotin in the biotinylated PG35 fraction was estimated by scintillation counting and the amount of PG35 by the A_{250nm} reading after allowing for the absorbance due to the biotin. The ³H labelled biotinylated PG35 was linked to DNA as described above. From scintillation counting and the spectrum of the product the amount of biotinylated PG35 per unit wt of DNA was estimated.

RESULTS

The various features of the reporter group shown in Fig. 1 were examined in most of the possible combinations (Table 1).

Cross linker

Five bifunctional cross-linking agents, formaldehyde (BDH chemicals), glutaraldehyde (Agar, aids), 1,2,7,8-diepoxyactane (Aldrich), bis (succinimidyl) suberate (Pierce) and bis (sulphonosuccinimidyl) suberate (Pierce) were compared. These differ in chain length from formaldehyde (1 carbon atom) through glutaraldehyde (5 carbon atoms) to the other three compounds which

Fig. 1. General structure of the reporter group with its components.

TABLE 1

Cross-linking reagent	Basic polymer	Side chain	Enzyme	Detection end point (pg)	Non-specific reaction
F	cyto.C	short	Per.	500	++
F	cyto.C	long	Phos.	100	++
F	Hist.	short	Per.	500	++
F	Hist.	long	Per.	100	++
G	cyto.C	short	Per.	100	+
G	cyto.C	short	Phos.	50	+
G	cyto.C	long	Per.	100	-
G	cyto.C	long	Phos.	50	-
G	Hist.	short	Per.	10-50	+
G	Hist.	short	Phos.	10	+
G	Hist.	long	Per.	10-50	-
G	Hist.	long	Phos.	1	-
D	cyto.C	short	Per.	10	-
D	cyto.C	short	Phos.	2	-
D	cyto.C	long	Per.	10	-
D	cyto.C	long	Phos.	0.25	-
S	Hist.	short	Per.	10	-
S	Hist.	short	Phos.	2	-
S	Hist.	long	Per.	10	-
S	Hist.	long	Phos.	0.25	-
G	PEI	short	Phos.	0.25	-
G	PEI	long	Phos.	10	-
G	PG35	short	Phos.	1	-
G	PG35	long	Phos.	0.01-0.05	-
D	PEI	short	Phos.	10	-
D	PEI	long	Phos.	2	-
D	PG35	short	Phos.	0.25	-
D	PG35	long	Phos.	0.01-0.05	-
S	PEI	short	Phos.	10	-
S	PEI	long	Phos.	2	-
S	PG35	short	Phos.	0.05-0.25	-
S	PG35	long	Phos.	0.01-0.05	-

F = formaldehyde
 G = glutaraldehyde
 D = 1,2,7,8-diepoxyoctane
 S = bis (sulfonosuccinimidyl) suberate
 cyto.C = cytochrome C
 Hist. = histone HI
 Per. = avidin peroxidase
 Phos. = Avidin alkaline phosphatase
 - = no reaction with non specific sites
 + = reaction with non specific sites

have 8 carbon atoms. It can be seen from Table 1 that the sensitivity of the test increases with the chain length of the cross-linking agent; there were no significant differences between the three compounds with 8 carbon atoms. However bis (succinimidyl) suberate was not soluble in water and had to be dissolved in dimethyl formamide before use.

Proportion of biotin to histone and cytochrome C

Renz (14) showed that when 1mg histone was reacted with 5µg, 25µg and 250µg short chain biotin (BHSF), 2,7 and 20 biotin residues respectively were attached to each histone molecule. Analysis of probes made with different proportions of short chain biotin and either histone or cytochrome C (Table 2) shows that 500µg short chain biotin per mg protein gave the greatest sensitivity without significant non-specific reactions. This proportion was used in subsequent experiments.

No. of biotin molecules incorporated using PG35

The number of biotin molecules which attached to each molecule of PG35 using different ratios of biotin to polymin were estimated using ³H biotin as described in Materials and Methods. Table 3 shows that increasing the ratio of biotin increases the number of biotin molecules attached to PG35.

Analysis of the DNA to which biotinylated PG35 was linked showed that there was one molecule of biotinylated PG35 per 50 nucleotides of DNA. This is slightly more than was found by

TABLE 2
Sensitivity of histone/cytochrome C probes with increasing amounts of biotinylated probes

Amount of biotin ester (µg)	Histone or Cytochrome C (mg)	Sensitivity
5	1	500pg
50	1	10-50pg
250	1	1-5pg
500	1	0.250-0.500pg
1000	1	non-specific binding

Renz (14) (one biotin per 70 nucleotides) for the attachment of biotinylated histone to DNA.

Estimates of the sensitivity of detection using probes containing different amounts of biotin attached to PG35 (Table 3) shows that between 15-20 biotin molecules per molecule of PG35 gave the best results. Ratios higher than this gave non-specific binding.

Basic macromolecule

There was not much difference between the two basic proteins in the sensitivity of the test (Table 1). Of the amine polymers the use of P60 resulted in less sensitivity than that found with the proteins. However incorporation of PG35 into the reporter group gave a greater sensitivity (Table 1).

Biotin side chain

Two side chains supporting biotin were compared. BHSE has 5 carbon atoms whereas SBAH has 11 carbon atoms. Table 1 shows that the length of the side chain had a marked effect on the detection level.

Enzyme

The use of alkaline phosphatase conjugated to avidin proved superior in the sensitivity of detection to peroxidase conjugates (Table 1). The two substrates tested for peroxidase, 3,3'-

TABLE 3
Biotinylated polymin probes and their corresponding sensitivities

	Amount of biotin ester (mg)	Polymin (mg)	No. of biotin attached	Sensitivity (pg)
A	0.005	1	3	5-10
B	0.05	1	7	1-5
C	0.250	1	12	0.05-0.5
D	1.4	1		
E	2.8	1	17	0.01-0.05
F	5.6	1		
G	11.2	1	20	reaction with non specific binding sites

dianisidine, giving a brown product, and 4-chloro-1-naphthol, giving a blue product, did not differ in their sensitivity; the two substrates used for phosphatase, nitro blue tetrazolium giving a purple colour and fast red TR salt giving a red colour also did not differ in their sensitivity (results not shown).

Non-specific reactions

Two forms of non-specific reactions were found. In one the whole of the nitrocellulose filter became coloured. This reaction was found using probes F or G (Table 3). In preliminary experiments this non-specific reaction was shown to also be a considerable problem with nylon membranes (Biodyne Transfer Membrane from Pall) even with probes which gave no background reaction on nitrocellulose. The second type of non-specific reaction was shown by the control non-homologous DNA or RNA spots. Use of more than 250ng/ml of polymin or histone or cytochrome C probes gave colour reactions with the control spots but never to the same dilution as the homologous nucleic acid spots.

An assessment of the non-specific reactions of the different probe constructions is given in Table 1.

In an experiment in which calf thymus tRNA (10mg/ml) was used as the diluent for both the target and control samples, thus giving constant levels of nucleic acid per spot, there were no non-specific reactions. The presence of the non-homologous nucleic acid in the samples did not affect the sensitivity of the test (data not shown),

DISCUSSION

Using a model system we have examined the effects of changes in the various features which make up the reporter group. It is clear that each of the basic features is important.

Two of the features, the cross linker and the biotin side chain, affect the length of the reporter group and from the data shown in Table 1 it can be seen that the longer the reporter group the more sensitive the detection. Brigati *et al.* (20), using biotin derivatives of deoxyribonucleotides which were directly incorporated into the probe found that the length of the biotin side chain did not significantly affect the sensitivity on hybridization on nitrocellulose; it did however on in situ hybridization on tissue samples.

The size of the basic macromolecule also had an effect on detection. The sensitivity using the large polyethyleneimine (mol wt 60000) was much less than that using Polymin G35 (mol wt 1400), cytochrome C (mol wt 12327) or histone (mol wt 23000).

It would seem most likely that the degree of sensitivity is limited by steric hindrance. The larger the cross-linker molecule the more basic macromolecules can be linked per unit length of DNA. Steric hindrance between the basic macromolecules also shows up in the lower sensitivity with the larger macromolecule. There can also be steric hindrance at a second level, that between biotin molecules attached to the basic macromolecules.

Of the enzymes tested phosphatase gave the better detection levels. For each enzyme there was no difference between substrate.

The sensitivities achieved in this study are significantly better than those reported for other systems. Of the biotinylated probes those based on basic protein could detect 5pg (14) and biotinylated deoxyribonucleotides nick-translated into the probe (8) and photobiotin photolinked onto the probe (11) could detect 500fg nucleic acid. Although this photobiotin probe is reported to be very sensitive and highly reliable, the starting material, if used in large quantities, is explosive and the final product (before linking to nucleic acid) is extremely sensitive to light. Non-biotinylated probes such as linking an enzyme directly to the DNA (7) or using antibodies to detect N-acetoxy-N-2-acetylaminofluorene linked to the DNA (21) could detect of the order of 1-5pg nucleic acid. Table 1 shows that some of our probe constructions gave an order of magnitude more sensitivity. These constructions also did not suffer some of the disadvantages of other probe systems. The use of biotinylated deoxyribonucleotides is relatively expensive and requires the process of nick-translation. Photobiotin will decompose on exposure to light and thus has to be kept dark. N-acetoxy-N-2-acetylaminofluorene is a possible carcinogen.

With enzyme colour tests care has to be taken to minimise non-specific reactions. Among the various factors found to be important were the use of the blocking buffer noted in the Materials and Methods, extensive washing after incubation with

the avidin-enzyme conjugate and the use of clean single-stranded DNA in the prehybridization solution; we routinely phenol extract the calf thymus or salmon sperm DNA. Table 1 shows that the long chain biotin produces less non-specific reaction than does the short chain biotin.

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