Chemically synthesized non-radioactive biotinylated long-chain nucleic acid hybridization probes

Ali H. AL-HAKIM* and Roger HULL†

*Agricultural Genetics Co. Ltd., Cambridge Science Park, Cambridge CB4 4BH, U.K., and †Department of Virus Research, John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

A new method for the chemical labelling of nucleic acid with biotin to produce non-radioactive probes has been developed. *NN'*-Bis-(3-aminopropyl)butane-1,4-diamine (spermine) and long-chain diamino compounds (diaminohexane, diaminodecane and diaminododecane) were linked covalently to biotin and the resultant conjugates were attached to nucleic acid by using a cross-linking reagent (glutaraldehyde or diepoxyoctane). Iodoacetylation and biotinylation of the long-chain diamino compounds produced modified biotinylated conjugates that can be linked to DNA without the use of a cross-linking reagent. These types of probes attach one biotin molecule to each linker arm of spermine, diamino and iodoacetylated amino derivatives. Such probes have long linker arms separating the biotin moiety from the hybridization sites of the nucleic acid. These probes can detect 10 pg of target DNA by dot-blot hybridization.

INTRODUCTION

Various methods (Langer *et al.*, 1981; Leary *et al.*, 1983; Renz, 1983; Renz & Kurz, 1984; Tschén *et al.*, 1984; Forster *et al.*, 1985; Al-Hakim & Hull, 1986) have been developed and reported for the synthesis of non-radioactive nucleic acid hybridization probes. Chemically labelled biotinylated nucleic acid probes are becoming very important tools in molecular biology. These probes are stable, safe and sensitive and can be prepared in large quantities.

Biotin can be linked to nucleic acid without affecting its hybridization properties. The resultant probe can then be hybridized to its complementary sequence and the newly formed biotin-labelled hybrid can be easily detected colorimetrically by addition of avidin or streptavidin to which a reporter enzyme has been attached.

The method presented here is an extension of our previous work in which a polybiotin polyimin derivative was attached to DNA by using a cross-linking reagent (Al-Hakim & Hull, 1986). We demonstrated that polyimin compounds could be biotinylated covalently with many biotin molecules.

In the present paper we examine the potential of spermine [(I) in Fig. 1] and diamino derivatives [(II), (III) and (IV) in Fig. 1] as the biotin-carrying linker arm that can be linked to DNA via glutaraldehyde or diepoxyoctane, and of biotinylated iodoacetyl derivatives [(V), (VI) and (VII) in Fig. 1], which do not require a cross-linking reagent for attachment to DNA. The biotinylated spermine derivative can be constructed by the reaction of spermine with biotin *N*-hydroxysuccinimide ester or (biotinamido)hexanoic acid *N*-hydroxysulphonosuccinimide ester. The amino compounds (diaminohexane, diaminodecane and diaminododecane) were also biotinylated in a similar manner, followed by subsequent iodoacetylation to give the biotinylated iodoacetylated amino derivatives [(V), (VI) and (VII) in Fig. 1].

MATERIALS AND METHODS

Materials

Spermine, diaminohexane, diaminodecane, diaminododecane and 4-nitrophenyl iodoacetate were from

Fluka. The rest of the materials were obtained as reported previously (Al-Hakim & Hull, 1986). M13 bacteriophage single-stranded (ss) and double-stranded (ds) DNAs were gifts from Dr. G. Lomonosoff, Dr. J. Donson, Dr. J. Stanley and Miss P. Ettesami.

Synthesis of biotin *N*-hydroxysuccinimide ester was carried out as reported previously (Al-Hakim & Hull, 1986).

Biotinylation of spermine

Spermine (5 mg) (5-fold molar excess) was dissolved in 250 μ l of 50 mM-NaHCO₃ to which 1.34 mg of biotin *N*-hydroxysuccinimide ester in 10 μ l of *NN*-dimethylformamide or 2.20 mg of (biotinamido)hexanoic acid *N*-hydroxysulphonosuccinimide ester also in 10 μ l of *NN*-dimethylformamide was added. The reaction mixture was incubated for 2 h at 30 °C with constant shaking. The solvent was evaporated to dryness and the residue was washed with ethanol. The product was dissolved in a minimum quantity of 5 mM-sodium phosphate buffer, pH 7.0, loaded on to a Sephadex G-25 column and chromatographed with 5 mM-sodium phosphate buffer, pH 6.8, as eluent. The void volume (usually 150–200 μ l) was collected and then stored at –20 °C.

Synthesis of spermine DNA probe

A 1 μ g portion of M13 bacteriophage ssDNA was suspended in 100 μ l of 5 mM-sodium phosphate buffer, pH 7.7, denatured by heat (3 min at 100 °C) and cooled on ice (5 min). Then 10 μ l (or more depending on the volume obtained from the Sephadex column) of the biotinylated spermine was added to the DNA solution, followed by 20 μ l of 3% (v/v) glutaraldehyde solution or 10 μ l of 3% (v/v) diepoxyoctane solution. The solution was incubated at 37 °C for 1 h with constant agitation, and then loaded on to a Sephadex G-75 column and chromatographed with 5 mM-sodium phosphate buffer, pH 7.0, as eluent. The void volume was collected and added directly to the hybridization bag, which contained the nitrocellulose sheet and the hybridization mix.

Synthesis of biotinylated amino derivatives

Synthesis of these derivatives was carried out as

reported previously (Suioh *et al.*, 1984). A 5-fold molar excess of the appropriate diamino compound was dissolved in 2 ml of *NN*-dimethylformamide and for each reaction was added to a solution of biotin *N*-hydroxy-succinimide ester or 6-(biotinamido)hexanoic acid *N*-hydroxysulphonosuccinimide ester in 5 ml of *NN*-dimethylformamide. The reaction mixture was left to stand at room temperature for 3 h. The mixture was then evaporated to dryness and the product washed with ethanol.

Synthesis of biotinylated amino DNA probes

The biotinylated amino derivatives were first dissolved in *NN*-dimethylformamide and then linked to DNA by using glutaraldehyde or diepoxyoctane as described for the synthesis of the biotinylated spermine-DNA probe, except that 15 μ l of these biotinylated amino derivatives was required for probe construction.

Synthesis of biotinylated iodoacetylated amino derivatives

The products obtained in the above experiments were modified further by dispersing 0.1 g of the desired biotinyl amino derivative in 10 ml of *NN*-dimethylformamide followed by addition of 0.1 g of 4-nitrophenyl iodoacetate. The reaction mixture was left to stand at room temperature in the dark for 3 h. The product was precipitated by addition of 25 μ l of diethyl ether. The precipitate was collected and washed several times with ethyl acetate. The product was dried and stored at 4 °C.

Synthesis of biotinylated iodoacetylated amino DNA probe

A 5 μ g portion of the desired biotinylated iodoacetylated amino derivative was dissolved in 20 μ l of *NN*-dimethylformamide and added to 100 μ l of 5 mM-sodium phosphate buffer, pH 7.7, containing 1 μ g of denatured M13 bacteriophage ssDNA followed by addition of 20 μ l of 3% freshly prepared glutaraldehyde solution or 10 μ l of 3% diepoxyoctane solution. The solution was incubated at 37 °C for 1 h with constant agitation, then loaded on to a Sephadex G-75 column and chromatographed with 5 mM-sodium phosphate buffer, pH 6.8. The void volume was collected and added directly to the hybridization mix.

Dot-blot hybridization and detection

Preparation of the nitrocellulose filters and immobilization of the DNA by using dot-blot hybridization were carried out as reported previously (Maule *et al.*, 1983; Al-Hakim & Hull, 1986).

Colorimetric detection was carried out as follows. Filters were washed with 1% (w/v) SDS in 0.3 M-NaCl/30 mM-sodium citrate buffer, pH 7.2 (250 ml each wash, 4 $\times$ 10 min). Filters were incubated in 20 ml of blocking buffer [0.1 M-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl, 2 mM-MgCl₂, 0.5% Tween 20, 0.05% Triton X-100 and 3% (w/v) bovine serum albumin] at room temperature for 1 h. Filters were then incubated for a further 1 h in the above buffer but containing 0.05% Tween 20 and 1 μ g of avidin-alkaline phosphatase or avidin-peroxidase/ml with gentle shaking at room temperature. They were washed with constant shaking at room temperature with 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl, 2.5 mM-MgCl₂, 0.05% Triton X-100, 0.5% Tween 20 and 1% bovine serum albumin

(5 $\times$ 5 min), then washed finally with 0.1 M-Tris/HCl buffer, pH 9.5, containing 1 M-NaCl and 10 mM-MgCl₂ (2 $\times$ 10 min).

For colour development, filters that had been incubated with avidin-alkaline phosphatase conjugate were incubated in 10 ml of 0.1 M-Tris/HCl buffer, pH 9.5, containing 0.1 M-NaCl, 10 mM-MgCl₂, Nitro Blue Tetrazolium (dissolved in 70% *NN*-dimethylformamide) (0.33 mg/ml) and 5-bromo-4-chloroindol-3-yl phosphate (dissolved in *NN*-dimethylformamide) (0.17 mg/ml) (Leary *et al.*, 1983) or 5-chloro-2-toluidine diazonium chloride (0.6 mg/ml) mixed with Naphthol AS-MX phosphate (Bantari & Goodwin, 1985). For detection of avidin-peroxidase, filters were incubated with 10 ml of 0.1 M-Tris/HCl buffer, pH 7.5, 2 ml of ethanol, 6 mg of 3,3'-dianisidine and 6 μ l of 30% (v/v) H₂O₂, or with 100 ml of 10 mM-Tris/HCl buffer, pH 7.4, containing 50 mg of 4-chloro-1-naphthol dissolved in 2 ml of methanol plus 6 μ l of 30% H₂O₂. All colour development processes were carried out at room temperature in the dark. The colour reaction was terminated by washing the filters with water; dried filters can be stored in an envelope of Whatman 3MM paper.

RESULTS

Various biotin-labelled DNA probes were constructed from the corresponding biotinylated spermine, diamino and iodoacetylated amino derivatives (Fig. 1). Comparisons were carried out by using dot-blot hybridization experiments between these probes and the polybiotin polymin probe that was prepared in our previous work (Al-Hakim & Hull, 1986). The same target DNA was blotted on to a separate filter but then hybridized with its complementary strands labelled with monobiotin or polybiotin linkers as shown in Table 1. Detection sensitivity to target DNA increased with the increased number of carbon atoms in the linker arm of the biotinylated DNA probe. For example, the biotinylated spermine DNA probe could detect 10 to 5 pg of target DNA [Fig. 2, blot (b)]. On the other hand, if the slightly shorter-chain biotinylated amino derivatives [(III) and (IV) in Fig. 1(b)] were used in DNA probe construction, the resultant probes gave slightly less sensitive results [Fig. 2, blot (c)]. The very short biotinylated amino derivative [(II) in Fig. 1(b)] produced a DNA probe with low sensitivity [Fig. 2, blot (d)]. When the biotinylated iodoacetylated amino derivatives [(V), (VI) and (VII) in Fig. 1(c)] were used as precursors in DNA probe construction, the resultant biotinylated DNA probes gave similar results to the corresponding biotinylated amino probes. However, the biotinylated iodoacetylated amino derivatives did not require a cross-linking reagent for DNA linkage. In an experiment in which calf thymus tRNA (10 mg/ml) was used as the diluent for both the target and control samples, there were no specific reactions; the presence of the non-homologous nucleic acid in the samples did not affect the sensitivity of the test.

Comparisons also were carried out between the above monobiotin probes and the polybiotin polymin probe. Dot-blot hybridization experiments indicated that the polybiotin probe is more sensitive [Fig. 2, blot (a)], giving faster results than the monobiotin probes [Fig. 2, blots (b), (c), (d) and (e)]. This is due to the presence of many biotin residues at each DNA-attachment site in the

Fig. 1. Monobiotin derivatives

(a) Biotinylated spermine derivative; (b) biotinylated amino derivatives; (c) biotinylated iodoacetylated amino derivatives.

Table 1. Properties of biotinylated amino probes

Cross-linker: +, either glutaraldehyde or diepoxyoctane is required; —, no cross-linker is required. Background: +, reaction with non-specific sites; —, no reaction with non-specific sites.

Biotinylated probe	Quantity	Sensitivity (pg)	Cross-linker	Background
Polymin	2–3 μl	0.05–0.005	+	—
Spermine	10 μl	50–10	+	—
Amino derivatives:				
6 carbon atoms	15 μl	500	+	+
10 carbon atoms	15 μl	50	+	—
12 carbon atoms	15 μl	50–10	+	—
Iodoacetyl compounds:				
6 carbon atoms	15 μl	500	—	+
10 carbon atoms	15 μl	10	—	—
12 carbon atoms	15 μl	100–50	—	—

polybiotin probe, compared with only one biotin residue in the monobiotin probes. It seemed that such extensive labelling of the probe would facilitate the process of biotin–avidin interaction, therefore enhancing and increasing the sensitivity of the final detection process.

DISCUSSION

The method described here demonstrates that spermine and long-chain diamino compounds can be covalently

linked to biotin and that the resultant conjugate can be attached chemically to DNA to produce biotin-labelled DNA hybridization probes. These DNA probes hybridize with their complementary sequence and the resultant hybrids can be detected colorimetrically by the use of the conjugate avidin–alkaline phosphatase. The above types of probes gave slightly less sensitive detection results than the polybiotin polyamine probe (Al-Hakim & Hull, 1986). This is probably due to the linker arm of these amino derivatives carrying only one biotin molecule

Fig. 2. Comparison of hybridization efficiencies of different probes

Equal amounts of target DNA (upper lane) and control DNA (lower lane) were spotted on each lane. The first square of each lane contains 100 ng of DNA, but subsequent portions of 20 ng, 4 ng, 800 pg, 160 pg, 32 pg, 6.4 pg, 1.2 pg, 0.24 pg and 0.05 pg of DNA were spotted along the lanes. Blot (a) was detected with polybiotin polymin probe, blot (b) with spermine probe, blot (c) with long-chain amino probe, blots (d) and (e) with short-chain amino probes (the 6-carbon-atom biotinylated amino and iodoacetylated compounds of Table 1) and blot (f) with spermine probe (prolonged incubation time). Photography enhances the halos and diminishes the colour intensity within the halos. The limits of the detection were from direct observation of the original nitrocellulose and are indicated by double lines.

compared with a cluster of biotin molecules that are present in the linker arm of the polybiotin DNA probe. Between 500 and 10 pg of target DNA can be detected by using these monobiotin linker-arm DNA probes. Although higher sensitivity can be obtained by incubating the filter over a prolonged time in the staining solution or by using a large quantity of DNA probe, a false result can also be obtained together with the appearance of colour deposits [Fig. 2, blot (f)]. We found that it is essential to block all the non-specific sites on the filter in order to eliminate any false results produced from the colorimetric detection system. A combination of bovine serum albumin, Tween 20 and Triton X-100, as described in the Materials and methods section, was found to be a very effective blocking reagent. The avidin-alkaline phosphatase conjugate consistently gave greater sensitivity than did the avidin-peroxidase.

The advantages of these types of monobiotin linker-arm non-radioactive DNA probes are that the starting materials are cheap and readily available and the probes can be constructed in a relatively short time; they are usually stable and have a long storage time at -20°C . In addition, the biotinylated iodoacetylated amino derivatives do not need a cross-linking reagent for DNA linkage. In this case the iodine atom acts as the leaving group that can be replaced by a new bond between the DNA and the biotinylated derivatives. The detailed chemistry of the linkage process between these biotinylated amino derivatives and the DNA involves the formation of a chemical bond between the nucleophilic centres of the DNA bases (e.g. N-7 of purine) and the electrophilic centres of the cross-linking reagent (e.g. carbonyl group of the cross-linking reagent glutaraldehyde or diepoxyoctane) (Baumert *et al.*, 1978; Pinto & Lippard, 1985).

REFERENCES

- Al-Hakim, A. H. & Hull, R. (1986) *Nucleic Acids Res.* **14**, 9965-9976
- Banttari, E. E. & Goodwin, P. H. (1985) *Plant Dis.* **69**, 202-205
- Baumert, G. H., Skold, E. S. & Kurland, C. H. (1978) *Eur. J. Biochem.* **89**, 353-359
- Forster, A. C., McInnes, J. L., Skingle, D. C. & Symons, R. H. (1985) *Nucleic Acids Res.* **13**, 745-761
- Langer, P. R., Waldrop, A. A. & Ward, D. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6633-6637
- Leary, J. J., Brigatti, D. J. & Ward, D. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4045-4049
- Maule, A. J., Hull, R. & Donson, J. (1983) *J. Virol. Methods* **6**, 215-224
- Pinto, A. N. & Lippard, S. J. (1985) *Biochim. Biophys. Acta* **780**, 167-180
- Renz, M. (1983) *EMBO J.* **2**, 817-822
- Renz, M. & Kurz, C. (1984) *Nucleic Acids Res.* **12**, 3435-3444
- Suioh, K., Yamamoto, K. & Wakabayashi, T. (1984) *J. Mol. Biol.* **178**, 323-339
- Tschen, P., Fuchs, R. P. P., Sage, E. & Leng, M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3466-3470