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A complex of single-strand binding protein and M13 DNA as hybridization probe

Ann-Christine Syvänen, Markku Alanen* and Hans Söderlund+

Orion Genetic Engineering Laboratory, Valimotie 7, SF-00380 Helsinki, *Department of Electron Microscopy, University of Helsinki, Mannerheimintie 172, SF-00280 Helsinki, and +Recombinant DNA Laboratory, University of Helsinki, Valimotie 7, SF-00380 Helsinki, Finland

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ABSTRACT

Single-stranded DNA was complexed to the single-strand binding protein (SSB) of *Escherichia coli* in a mass ratio of 30:1. The protein moiety of this complex can be labelled by a number of methods of which we have chosen radio-iodination and biotinylation as examples. The SSB-M13 DNA complexes, labelled to high specific activities, were used as probes in hybridization experiments in which 1.6 x 10^{-18} moles of immobilized target DNA were detected. The stability of the hybrids was not severely decreased by the binding of SSB. Analysis of hybrids by electron microscopy showed that complexing of DNA with SSB could be used to allow its subsequent identification in the hybrids.

INTRODUCTION

Recently efforts have been made to develop non-radioactive nucleic acid probes for DNA hybridization to abolish the inconveniences of radioisotopes. Nonradioactive labelling also offers the possibility to increase the sensitivity of hybridization methods, a need both in basic research and medical diagnosis. Nucleic acids can be labelled enzymatically with biotinylated nucleotide analogues and detected by avidine-enzyme conjugates (1-3). Chemical methods to label polynucleotides with fluorescent compounds (4), with immunodetectable fluorene derivatives (5), or biotin analogues (6) have been described. Another approach is to add a label on the nucleic acid by mediation of a protein. Hybridization probes of nucleic acids crosslinked to radio-iodinated and biotinylated histone H1 (7) or enzymes (alkaline phosphatase and peroxidase) detectable colorimetrically by their substrates (8) have been used.

The *Escherichia coli* DNA unwinding protein (single-strand

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binding protein, SSB) is essential in the synthesis of the chromosomal DNA and of the single-stranded phage DNA of E.coli (9, 10). The SSB protein is a tetramer of 18 500 dalton subunits, which because of its biological function binds cooperatively and specifically to single-stranded DNA (11, 12). In this study we have taken advantage of the natural affinity of SSB for single-stranded DNA. We have constructed a complex of SSB and bacteriophage M13 DNA, which can be labelled by protein labelling methods and used as a hybridization probe. High protein to DNA mass ratio was achieved by reacting the complex with glutaraldehyde. High specific activities of the probe were obtained by introducing a large amount of label via the protein moiety of the molecule. Since the complex is prepared prior to the labelling, no upper limit on the amount of label is set by the biological activity of SSB. Here we have used ^{125}I and biotin as two examples of the various protein labels available. The labelled SSB-DNA complex was successfully used as a probe in dot hybridization experiments.

Binding SSB to single-stranded DNA is a technique commonly used in electron microscopy to distinguish between single-stranded and double-stranded DNA (13). Here we show that complexing with SSB of molecules to be analyzed by electron microscopy after hybridization can be done to allow identification of predetermined specific molecules in the hybrids.

MATERIALS AND METHODS

Plasmid and phage DNA

The plasmid pKTH1220 used as filter bound target DNA is a pBR322 clone with a 9.7 kilobase insert of chromosomal DNA from Chlamydia trachomatis. Single-stranded recombinant phage M13 molecules (mKTH1245 and 1246) obtained by subcloning of fragments from the plasmid pKTH1220 (14) were used for probe construction (Figure 1.).

Construction of the SSB-DNA complex

Single-strand binding protein (SSB) was purchased from PL-Biochemicals. The protein was obtained in a buffer containing 20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiotreitol, 0.2 M NaCl and 50 % glycerol.

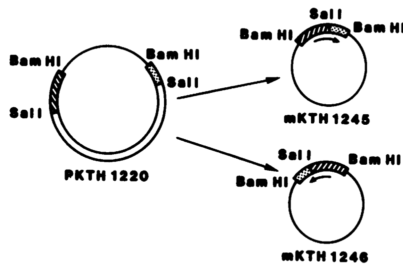

Figure 1.
M13 DNA probe molecules obtained by subcloning of two BamHI-SalI (0.7 kb and 1.4 kb) fragments of the 14 kb plasmid pKTH1220. The mKTH1245 phage contains the ligated 0.7 and 1.4 kb fragments in the BamHI site of M13 mp8. mKTH1246 contains in M13 mp8 the same insert in opposite polarity (14). The single lines represent vector sequences.

The binding reaction of SSB to single-stranded DNA takes place at low salt concentrations (11). SSB (60-500 µg) mixed with mKTH1245 M13 DNA (1-10 µg) at a mass ratio of 50-60:1 in 200-400 µl was dialyzed against 10 mM sodium phosphate, pH 7.5, at room temperature for 2-3 h. After dialyzis the mixture was incubated at 30°C for 20 min. 1 M glutaraldehyde (redistilled and stored at -70°C under N_2) was added to 20 mM to crosslink (13, 15) the SSB with the phage DNA and the reaction was allowed to proceed at 37°C for 15 min. SSB was also bound to M13 DNA omitting the glutaraldehyde reaction step from the procedure. SSB-M13 DNA complexes were separated from unbound SSB by passing the mixture over a 0.7 x 14 cm Sephacryl S-300 column equilibrated with 10 mM sodium phosphate, pH 7.5. Fractions containing DNA eluting at the void volume of the column were pooled and concentrated from 1-1.5 ml to 40-50 µl using a "Centricon 10000" microconcentrator (Amicon). Buffer was changed into 10 mM tricine, pH 7.5, 1 mM EDTA for SSB-DNA samples for electron microscopy and into 0.1 M $NaHCO_3$, pH 8-9, for samples to be biotinylated also using the "Centricon 10000" concentrator. DNA recovery of the process was estimated to 50 %. The protein content of the SSB-DNA complex was determined by the Lowry method (16).

Electron microscopy

The sample molecules together with marker molecules were

spread onto a hypophase according to (17). Circular M13 DNA was used as a single-strand marker and open circular pBR322 as a double-stranded marker. The formamide concentration was adjusted to 50 % in the hyperphase using twice recrystallized formamide. The hypophase was 0.1 mM Tris-HCl, pH 7.5, 0.01 mM EDTA. Samples were picked up onto parlodion (3 %) coated grids with subsequent staining (0.1 mM uranyl acetate for 10 s) and dehydration (90 % ethanol). Additional contrast enhancement was done by Pt-C rotatory shadowing with an electron beam gun (Balzers). The shadowing angle was 8° and the thickness of the metal coating was monitored with a quartz oscillator. Pictures were taken with a JEM100CX at 60 kV. The original magnifications on the negatives varied from 6 600 to 50 000.

Radiolabelling

SSB-DNA complexes (12-15 µg SSB protein) were radiolabelled with 1 mCi ^{125}I ($Na^{125}I$, IMS 30, 17 mCi/µg, Amersham) by the Chloramine T protein iodination method (18). Single-stranded M13 DNA was chemically radiolabelled with ^{125}I (19, Laaksonen,M. personal communication). ^{125}I was measured in a gamma counter. M13 DNA was also labelled with {α-^{32}P}dCTP(PB.10205, 3000 Ci/mmol, Amersham) using a specific primer and Klenow DNA polymerase I (20). ^{32}P was measured by its Cerenkov radiation in a liquid scintillation counter.

Biotinylation

Biotinylation of the SSB-DNA complex was performed essentially as described for proteins in (21). 50 µl of the SSB-DNA complex, (2.5 mg protein/ml in 0.1 M $NaHCO_3$, pH 8-9), was mixed with 50 µl biotin-N-hydroxysuccinimide ester (Betheseda Research Laboratories) in dimethylformamide (14 mg/ml), giving a molar ratio of biotin to SSB of 40 to 1. The mixture was incubated at room temperature for 4 h, and then dialyzed against 10 mM sodium phosphate, pH 7.5, 0.15 M NaCl, at $4^{o}C$ for 18 h. The biotinylation level of SSB-DNA obtained was estimated by comparing with herpes simplex virus DNA (Enzo Biochem) having 28 % of the thymidine residues biotinylated. Serial dilutions of both preparations were spotted on a nitrocellulose filter and visualized as described below.

Hybridization procedure

Plasmid pKTH1220 DNA and sheared calf thymus DNA were immobilized on nitrocellulose filters (0.45 µm, BA 85, Schleicher and Schüll) for dot hybridizations (22) using the Minifold Apparatus (Schleicher and Schüll) and on nitrocellulose filter discs 10 mm in diameter as described in (23).

Filters were presoaked for at least 1 h at 42°C in 10 x Denhardt's solution (Denhardt's solution is 0.02 % Ficoll 400, 0.02% polyvinylpyrrolidone and 0.02 % BSA;(24), 4 x SSCP (SSCP is 0.15M NaCl, 15 mM sodium citrate, 10 mM sodium phosphate, pH 7.6), 0.25 % sodium dodecyl sulphate (SDS), 0.2 mg/ml sheared and denatured herring sperm DNA and 45 % deionized formamide and for 1 h or longer at 37°C in 2 % bovine serum albumin (BSA), 0.1 % Triton X-100, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.5. Hybridization was performed in 2 x Denhardt's solution, 4 x SSCP, 0.25 % SDS, 0.2 mg/ml denatured herring sperm DNA and 45 % formamide over night at 42°C. Filters hybridized with the ^{125}I-labelled SSB-DNA probe were washed twice for 30 min at 50°C in 0.1 x SSC, 0.2 % SDS. Radioactivity was detected by autoradiography at -70°C using Kodak X-Omat film and an intensifying screen or by gamma counting. Filters hybridized with the biotinylated probe were washed for 15 min at room temperature and twice for 15 min at 50°C in 2 x SSC, 0.1 % SDS, followed by three 5 min washes at room temperature in 2 x SSC. Hybridized biotinylated DNA was detected using a streptavidine-acid phosphatase based biotin detection system (Detec 1-acp, Enzo Biochem.).

RESULTS AND DISCUSSION

The SSB-DNA complex

SSB-protein was bound to single-stranded mKTH1245 M13 DNA in 10 mM sodium phosphate buffer, whereafter the complex formed was treated with 20 mM glutaraldehyde to crosslink the SSB. The mass ratio of SSB protein bound to M13 DNA obtained with this procedure varied from 22:1 to 30:1. The maximal mass ratio of 30:1 represents 1300 tetramer molecules SSB (m.w. 74000 daltons) per M13 molecule, which equals to one tetramer SSB per 7.4 nucleotides. The mass ratio of SSB to M13 DNA in

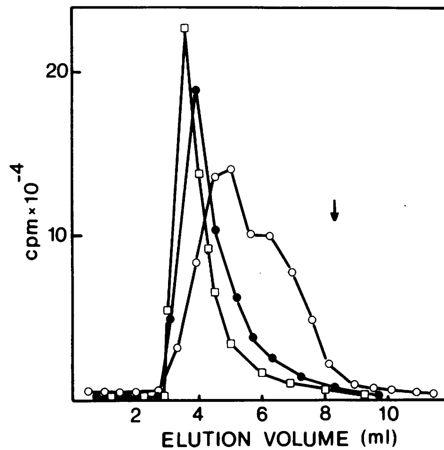

Figure 2.
Gelfitration on a Sephacryl S-300 column (0.7 x 14 cm) of SSB-M13 DNA complexes prepared in the presence (●-●) and abscence (o-o) of glutaraldehyde after incubation in hybridization conditions for 18 h. □-□ shows SSB-M13 DNA fixed with glutaraldehyde without incubation. Non-fixed complexes gave an identical profile. Elution position of free protein is indicated by the arrow.

the complex formed in the absence of glutaraldehyde was 9:1, which is in agreement with reports that DNA in physiological conditions is saturated with SSB at a mass ratio of 8:1 (11, 12). The higher protein: DNA ratio obtained here using glutaraldehyde is probably due to crosslinking of SSB molecules to each other. Whether covalent bonds between the SSB protein and the M13 DNA are produced by the reaction is unclear. The SSB molecules may also be noncovalently bound in the complex as a cap around the M13 DNA molecule. Glutaraldehyde has, however a stabilizing effect on the SSB-DNA complex. This was shown in an experiment where radiolabelled SSB-M13 DNA complexes formed in the presence and in the absence of glutaraldehyde were treated with 0.5 % SDS for 5 min at 22°C and subjected to gel filtration on Sephacryl S-300. 79 % of the radioactivity in the complex reacted with glutaraldehyde eluted at the void volume of the column, while in the non-crosslinked complexes only 13 % of the labelled SSB eluted with the DNA and most at the position of free protein. When the complexes were incuba-

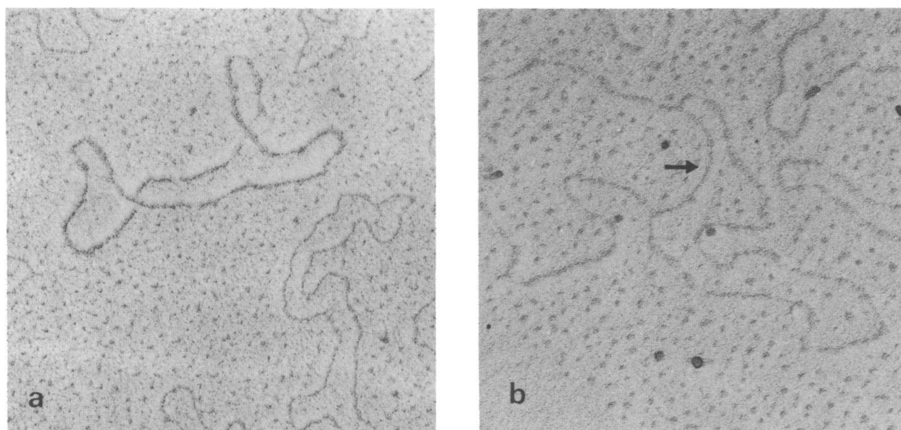

Figure 3.
Electron microscopic visualization of: (a) the complex of SSB protein and mKTH1245 DNA in comparison with naked M13 DNA molecules; (b) complex of SSB protein with a figure eight hybrid of mKTH1245 and mKTH1246 DNA molecules (hybridization was in 4 x SSC for 3 h at 65°C), where the double-stranded region (arrow) is recognized by it's relative thinness when compared to the SSB-covered single-stranded regions. Magnification 80 000.

ted in hybridization conditions for 18 h the glutaraldehyde-fixed preparation was essentially unaltered. The unfixed preparation had dissociated to smaller complexes even if only a small proportion had an elution volume corresponding to protein monomers (Figure 2). Consequently glutaraldehyde-fixed SSB-DNA complexes were used in all further experiments.

As can be seen by electron microscopy (Figure 3) the single-stranded circular mKTH 1245 DNA is completely covered by a shell of SSB protein, clearly distinguishing it from naked single-stranded M13 DNA molecules. In Figure 3b a protein-DNA complex is seen, where two M13 molecules with complementary inserts (mKTH 1245 and 1246) were allowed to hybridize forming a figure eight structure before the binding of SSB. Here the SSB protein covers only the single-stranded regions.

When radiolabelling the SSB-DNA complex with ^{125}I specific activities of 0.8-1.8 x 10^{9} cpm/µg DNA were easily obtained. Radioiodinated SSB-DNA migrates in agarose gel electro-

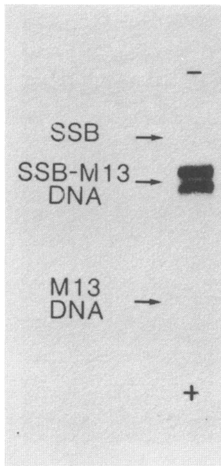

Figure 4.
Electrophoretic mobility of 7 x 10^4 cpm ^{125}I-labelled SSB-DNA complex in a 0.6 % agarose gel shown after autoradiography of the dried gel. The specific activity of the complex was approximately 1.5 x 10^9 cpm/ug DNA and the SSB:DNA mass ratio was 22. The mobilities of the corresponding naked mKTH1245 M13 DNA visualized by ethidium bromide staining and unbound SSB protein visualized by Coomassie brilliant blue staining of the same gel are indicated by arrows.

phoresis as two distinct bands, probably representing circular and linear SSB-M13 DNA molecules (Figure 4). The molecule is not fragmented during crosslinking or radiolabelling contrary to the case in direct chemical radioiodination of M13 DNA, which cannot be labelled to specific activites above 3 x 10^8 cpm/µg without loosing hybridization ability (Laaksonen, M. personal communication).

SSB-DNA complexes in hybridization

The SSB-mKTH1245 DNA complex was incubated with DNA-target sequences (i.e. mKTH1246 or pKTH1220) in annealing conditions. Various conditions were tried (see below) but 45 %

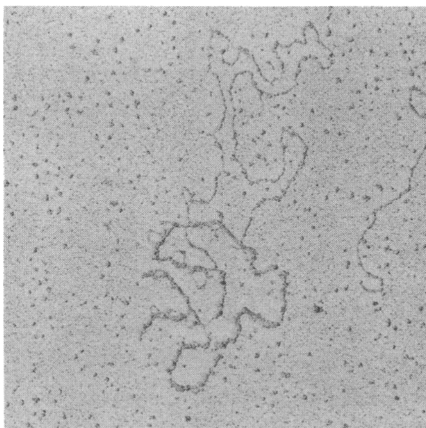

Figure 5.
Electron micrograph of a hybrid between a SSB-mKTH1245 DNA molecule and a naked mKTH1246 molecule. SSB was bound to mKTH1245 DNA and biotinylated prior to hybridizing it with mKTH1246. Hybridization was in 4 x SSCP and 50 % formamide for 4 h at 42°C. Magnification 60 000.

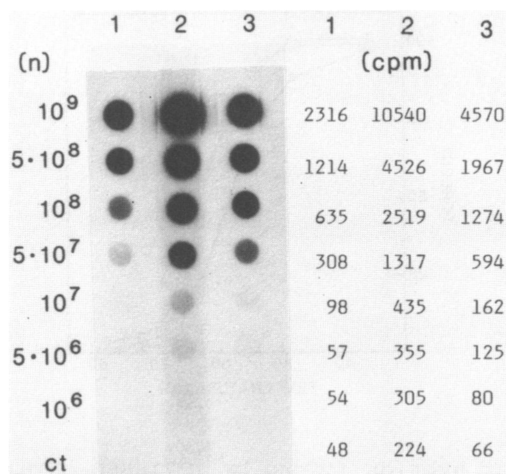

Figure 6.
^{125}I-labelled SSB-mKTH1245 DNA complex used as probe in a dot hybridization experiment. 10^9-10^6 molecules (n) equalling 1.6×10^{-15}-1.6×10^{-18} moles of plasmid pKTH1220 were dotted onto nitrocellulose filters. A dot containing 10 μg calf thymus DNA (ct) was included as a negative control in each lane. The hybridization volume used was 0.75 ml/lane; 10^6 cpm (lanes 1, 2) and 5×10^6 cpm (lane 3) of probe (specific activity 1.3×10^9 cpm/μg) were added to the hybridization mixtures thus yielding probe concentrations of 1 ng/ml and 5 ng/ml respectively. Filters were hybridized for 17 h (lane 1, 3) or 41 h (lane 2) and after washing autoradiographed for 36 h. On the right cpm values for each dot in the autoradiogram, as determined by gamma counting.

formamide in 4 x SSCP were mainly used. That hybrids of SSB-mKTH1245 DNA with mKTH1246 were formed could be observed by electron microscopy (Figure 5). The SSB-DNA complex was also tested for capability to form hybrids with filter bound plasmid pKTH1220 DNA. In this case the SSB protein was labeled with ^{125}I as described in Methods. The results from these two experiments indicated that SSB-DNA complexes even with 30 ug SSB per ug DNA can form hybrids. Presumably some of the protein is released during hybridization exposing regions of free DNA. Another explanation may be that no or very few covalent bonds are present between the SSB protein and M13 DNA. Thus M13 DNA completely covered by SSB can directly be used as probe. Our original intention was to use complexes formed with figure eight DNA in which the probe region is

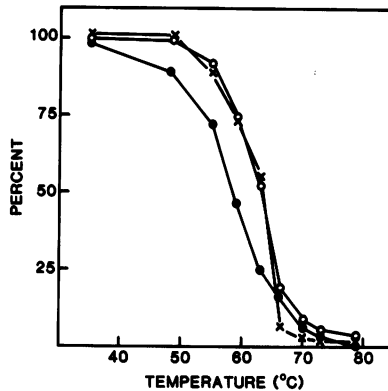

Figure 7.
Melting curves of hybrids formed by ^{125}I-SSB-mKTH1245 DNA (10^5 cpm; specific activity 1.5×10^9 cpm/µg) (● - ●), ^{125}I-mKTH-1245 DNA (10^5 cpm; 1.6×10^8 cpm/µg) (o - o) and ^{32}P-mKTH-1245 DNA (4×10^3 cpm; 2.0×10^7 cpm/µg) (x - x) probes with 5×10^9 molecules of plasmid pKTH1220 DNA immobilized on nitrocellulose filter discs. Hybridization was performed in ampoulles containing 0.3 ml hybridization solution for 18 h using conditions described in Methods. Washing was in 0.1xSSC, 0.2 % SDS for 15 min per increment, raising the temperature stepwise from 38°C to 79°C. Percent radioactivity remaining on filters is shown as a function of temperature. Radioactivity on control filters with 0.4 µg unspecific calf thymus DNA included in each assay was subtracted from the cpm value of the specific filters.

protein-free (Figure 3b). However, SSB-complexes with a single M13 circle are more convenient since the molecules do not have to be denatured and high concentrations can be used without probe reannealing during hybridization.

Conditions for pretreatment of nitrocellulose filters, hybridization and washing to yield optimal signal to noise ratios when using the ^{125}I-SSB-DNA complex as probe were determined by a series of experiments. Since the SSB protein may not be covalently bound to the M13 DNA, conditions affecting the stability of the probe molecule had to be considered in addition to parameters influencing the hybridization kinetics and measures taken to reduce unspecific background radioactivity. Hybridizations were performed at 42°C because the probe molecule apparently precipitated at higher (65°C) hybridization temperatures. 45 % formamide was optimal compa-

red to 50 % normally used at 42°C (c.f. 2) because of the 4.5°C lower T_m-value for the ^{125}I-SSB-DNA hybrids (see below).

The label of the probe being a protein, saturation of the nitrocellulose filter with protein was crucial to reduce unspecific background radioactivity. The best result was obtained when the filter was first preincubated in the presence of carrier DNA and 10 x Denhardt's solution, followed by blocking with a 2 % BSA-solution prior to incubation in the hybridization mixture containing the probe. Unspecific background was reduced by filtering the mixture through a nitrocellulose membrane (12 µm, AE 100, Schleicher & Schüll). The ionic strength (0.1 x SSC - 2 x SSC), the SDS-concentration (0-0.4 %) and the temperature (22°C-50°C) of the washing solution had little effect on the signal to noise ratio.

The applicability of the ^{125}I-labelled SSB-DNA complex as a hybridization probe is shown by the autoradiogram of a dot hybridization presented in Figure 6. Serial dilutions of the plasmid pKTH1220 were immobilized as dots on a nitrocellulose filter and hybridized with the ^{125}I-SSB-DNA probe. After washing, the filters were submitted to autoradiography and gamma counting. At optimal conditions 1.6×10^{-18} moles (10^6 molecules) of plasmid pKTH1220 DNA were detected both visually from the autoradiogram and by measuring the radioactivity hybridized. No unspecific hybridization to unrelated (calf thymus) DNA was observed. Considering the high specific activity of the probe (1.3×10^9 cpm/µg) the signals obtained after hybridization were surprisingly low. Reasons for this could be the unfavorable kinetics of the hybridization reaction due to the reasonably low probe concentration (1 or 5 ng/ml) and the slow diffusion into the nitrocellulose of such a large probe molecule, and a possible partial degradation of the probe complex during hybridization conditions.

The T_m-value of hybrids formed by the SSB-DNA probe molecules with plasmid DNA (pKTH1220) immobilized on nitrocellulose filters was determined by extensive washes at temperatures between 38°C and 79°C. The T_m was 58.5°C in the conditions applied (Figure 7), as compared to 63.0°C for the two other M13 DNA probes labelled by direct chemical iodination

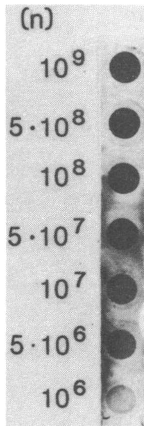

Figure 8.
Biotin-labelled SSB-mKTH1245 DNA complex as probe in dot hybridization. A nitrocellulose filter with dots containing 10^9 - 10^6 molecules of plasmid pKTH1220 was used. Hybridization was performed at a probe concentration of 1.4 µg DNA/ml for 40 h. After washing hybridized, biotinylated DNA was detected using a streptavidine-acid phosphatase conjugate.

and by the primer extension method, respectively. The stability of the hybrids formed by the SSB-DNA probe is reduced surprisingly little considering the fact that the SSB protein also covers the region containing the specific DNA-insert of the M13 molecule. Similarly lowered melting temperatures of hybrids formed by directly biotinylated DNA probes have been observed (1).

Biotinylated SSB-DNA complex as hybridization probe

Biotinylation was chosen as one example of the numerous protein labelling methods possible for attaching a nonradioactive label to DNA via the complex with SSB protein. Figure 5 shows that the SSB-protein remains bound to M13 DNA after biotinylation and that the biotylated SSB-mKTH1245 DNA complex is able to form hybrids. The biotinylation level obtained here was 2-3 biotin residues per SSB-molecule equalling 28-42 biotin residues per 100 nucleotides, which is an amount of biotin that severly interferes with the hybridization properties of the DNA when introduced by direct biotinylation as shown in (1).

The result of a hybridization experiment using the biotinylated SSB-DNA complex as probe and the plasmid pKTH1220 as target-DNA on a nitrocellulose filter is shown in Figure 8. Hybridization conditions were the same as described for the ^{125}I-labelled SSB-DNA probe. Hybridized biotinylated DNA was visualized enzymatically using a streptavidine-acid phospha-

tase conjugate with naphtol AS-MX phosphate as substrate and fast violet B salt to yield a purple precipitate as positive signal. In this experiment 1.6 x 10^{-18} moles (10^{6} molecules) of plasmid pKTH1220 DNA were detected after exhaustive hybridization and colour development for 15 h. The concentration of the biotinylated probe was 10^{3} times higher than the concentration of the ^{125}I-labelled probe (Figure 6). Despite the more favorable reaction kinetics the sensitivity of the method using the biotinylated probe remained approximately the same. This sensitivity is similar to that obtained with directly biotinylated DNA (2). In comparison with biotinylation using nucleotide analogues, which mainly has been used for labelling double-stranded DNA by nick-translation, an advantage of the SSB-mediated method is that single-stranded DNA probes of high specific activity can easily be obtained.

SSB-mediated labelling of hybridization probes offers possibilities to use potentially more sensitive nonradioactive detection methods than the biotin-streptavidine system. In cases where modification of nucleic acids affects the hybridization properties or is chemically difficult, labelling via the SSB protein might well be a method of choice. An example of such a potential new method is the time-resolved fluorometric assay with europium or other lanthanides as label, which so far has been successfully used in immumochemistry (25).

Using ^{125}I and biotin as examples we have shown that more label can be bound to M13 DNA by mediation of the SSB protein than by direct methods without severly affecting the hybridization properties of the molecule. Since the SSB is bound to DNA prior to labelling, preserving the biological activity of the protein is no obstacle for introducing large amount of label into the complex.

We have also shown that SSB-DNA complexes have applications in electron microscopy. In cases when hybrids are analyzed by electron microscopy binding of SSB to specific single-stranded DNA molecules prior to the hybridization reaction can be used to identify the molecules of interest in the hybrids.

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