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Biotinylation of Double Stranded DNA after Transamination

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The bisulfite catalyzed transamination of cytidine and cytosine has been reported to be single strand specific, but local thermal instabilities of the DNA double helix, coupled with the extreme sensitivity of the Biotin-Avidin revelation methods, allows the random labelling of cytosines in d.s. DNA to detectable levels for those purposes where the overall label can be very low. We have evaluated the use of this reaction to prepare double stranded DNA molecules containing N4-aminoethyl-cytosine (4-aeC). After this step 4-aeC residues can be conjugated to biotinyl-n-hydroxysuccinimide ester yielding biotinylated DNA. This reaction allows the massive production of biotinylated probes. Labelled DNA can serve as molecular weight marker and positive control in Southern-blots. Moreover it can be useful in the study of DNA-protein interaction and in the isolation of d.s. DNA-binding proteins through chromatographic procedures.

Recently, methods for non-radioactive labelling of Nucleic Acids have been reported as useful procedures for the production of probes for molecular biology, and in several other applications (1-25).

In fact, despite a general lower sensitivity of detection in respect of radioactive probes, non-radioactive labelling can allow additional advantages (23) such as gene enrichement (2,24), multiple labelling (10,11), nucleic acid-binding proteins extraction (25), structural studies (3,4,5,14), increase in microscopic resolution and shortening of the time for detection in cytology after in-situ hybridization against DNA or RNA(12).

One of the possibilities to obtain specific chemical modification of nucleic acids has been described by Shapiro and Weisgras (17). The reaction consists in a bisulfite-catalyzed transamination of cytosine and cytidine giving rise to N4 substituted molecules. This path has been followed more recently by Schulman et al. (5) to attach side chains to the N4 position of cytidine residues in E.Coli t-RNA-Met. This transamination reaction has been reported as single strand specific (26-28), and this fact has been used by D. Draper (14) for the attachment of reporter groups to cyti-

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dine residues in single stranded stretches in RNA molecules. For this purpose chemical conditions useful for the derivatization of ribonucleic acids were especially studied . Moreover the reaction has been used recently by Viscidi et al. (20), and by Bignone et al. (18,19) for the production of biotinylated probes to be used in molecular hybridization techniques, respectively through transamination of heat denatured d.s. DNA or transamination above the melting point.

We have investigated in this work the possibility of using the low rate transamination of cytosine in d.s. DNA to obtain intact biotinylated molecules.

MATERIALS AND METHODS

DNA samples

Double stranded DNAs used in these experiments were supercoiled pBR322 and HindIII cut λ phage DNA from Boehringer Mannheim, a pBR322 carrying an ε Globin insert of 2 Kb was a generous gift of Dr.S. Pulciani, and was grown in LB broth in the presence of Ampicillin and purified by cesium chloride procedures (29). Herring Sperm DNA was obtained from SIGMA, further purified by phenol extraction, ethanol precipitated, and sheared by sonication to a mean lenght of 20 Kb. Samples from suppliers were used as directed and pBR322 was digested with single cut Restriction Enzymes (R.E.), EcoRI, BamHI, PstI, from Boehringer Mannheim, and PvuII from Bethesda Research Lab.

DNA electrophoresis, Southern Blots and Dot-Blots

Electrophoresis was performed in 1% Agarose (ME Seakem, Miles), using 1X TAE (0.04 M Tris-Acetate, 0,002 M EDTA pH 8.0) supplemented with 0.5 μg/ml ethidium bromide (E.B.), at 5 volts/cm for 3 hours. Gels were then photographed under U.V. light illumination, denatured with 0.5 M sodium hydroxide, 1.5 M sodium chloride for 1 hour at room temp., neutralized with several passages in 1 M Tris-HCl, 1.5 M sodium chloride pH 8.0, washed for 10 minutes in 1M ammonium acetate, 0.02 M sodium hydroxide and blotted as described (29, 30). Dot-Blots were performed according to Kafatos et al. (30), using 500 ng/dot of Herring Sperm DNA as carrier.

DNA detection

Labelled DNA was detected by the colorimetric assay Biotin Detek I acp from Enzo-Bio, N.Y., based on the Acid Phosphatase-Streptavidin complex, following the protocol suggested by the manufacturer with minor modifications.

DNA labelling

Reactions were carried out in sterile heat sealed glass vials. All reagents were analytical grade, sodium bisulfite, sodium chloride, and DMSO (Dimethyl Sulfoxide), were obtained from Merck; ethylenediammine, Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), EPPS (N-(2-Hydroxyethyl)-piperazine-N'-3-propanesulfonic acid), TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), and Biotinyl-hydroxisuccinimide ester, were from Sigma.

Quantitation of the reaction

The percentage of labelling is time dependent and was monitored both by HPLC or by direct comparison with a standard sample with a dot-blots procedure. For the HPLC determinations DNA samples were digested with DNAseI (1 μg/ml) and Exonuclease III (50 U/ml), in 50mM Tris-HCl pH 7.2, 5 mM magnesium chloride. Each sample was adjusted to pH 8.0 and then digested at 37°C for 20 hours with Alkaline Phosphatase (0.33 U/ml) and Snake Venom Phosphodiesterase ($1.2\ 10^{-3}$ U/ml), all enzymes were from Boehringer Mannheim. Samples were injected in a Waters Nova Pack C 18, 5μm, HPLC column (Waters Associates), and eluted using a Millipore Waters apparatus with a step gradient of: 0.2% acetonitrile for 5 minutes, 1% acetonitrile for 14 minutes, and 5% acetonitrile for 20 minutes, in 0.1 M ammonium acetate pH 5.8, U.V. detector mod. 440.

RESULTS

Transamination reactions

To obtain biotinylated d.s. DNA, ε Globin carrying plasmids were linearized by digestion with BamHI R.E. and transaminated for 1h, 3h, 24h, at 50°C, in order to have a high reaction rate without melting of the double helix (14). The reactions were slight modifications of those reported by Schulman et al. (5). Double stranded DNA (0.5 μg/μl) was allowed to react for the desired time in heat sealed glass vials with nine volumes of 2N sodium bisulfite, 1N ethylenediammine at pH 7.0, at 50°C; after this step each sample was dialyzed for 2 hours against 0.5 M sodium chloride, 10mM Hepes pH 7.0 at 37°C with a subsequent change to 0.1 M sodium chloride in 10mM Hepes pH 7.0 and finally to 10 mM Hepes at 37°C overnight, to convert the adduct back to normal base.

To obtain fast and extensive modification samples to be used as controls were heat denatured and transaminated in boiling water for different times as reported above (18). The reaction for times of: 5, 10, 30, minutes and 1 hour gave after HPLC analysis transamination percentages of 7, 14, 45, 73% (4-daeC/4-daeC+dC). The sample used as control for these experiments was labelled for 20 minutes at 100°C with a percentage of labelling of 28% of the cytosines corresponding to a 7% overall substitution. For the d.s. DNA the precentage of transamination was too low to be detected by HPLC, the samples were thus semiquantitatively evaluated for their labelling using a dot-blots approach, after the biotinylation procedure, by comparison with a known sample (Figure 1).

Biotinylation of Transaminated DNA

After the previous step the samples were lyophilized and resuspended in 0.1 M EPPS buffer (pH 8.5), the introduction of biotin into transaminated nucleic acids was then obtained by addition of one volume of DMSO containing a large excess of bi-

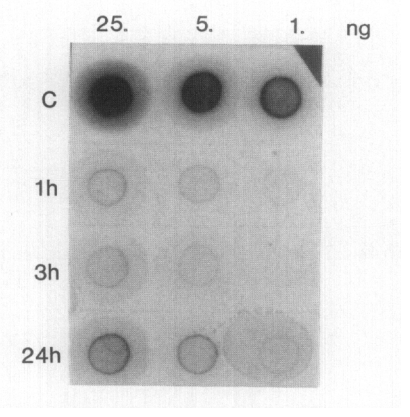
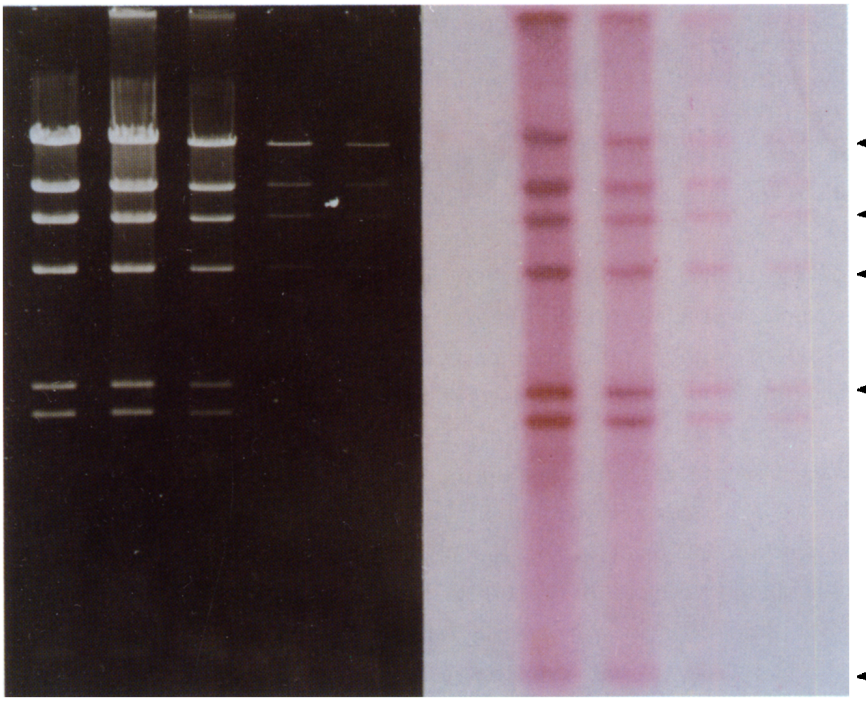
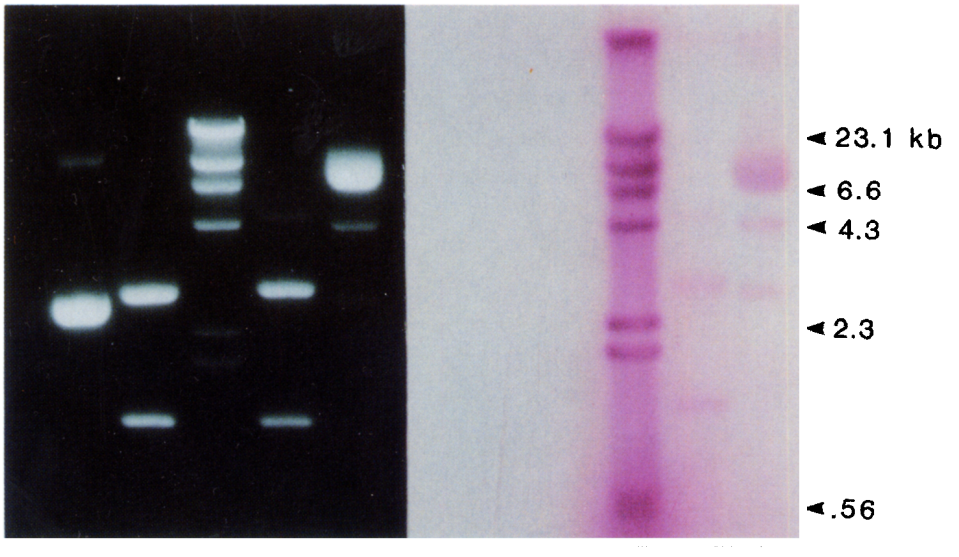


Fig. 1. Comparison of the amount of labelling between transamination performed at 100° C for 20 minutes (control C, 28% of Cytidines modified), and at 50° C, double stranded ε Globin plasmid transaminated for the times shown. Three serial dilutions are presented.



(2)



(3)

otinyl-hydroxysuccinimide ester (20 mg/ml) for 4 hours at 37°C, and then dialyzed against 10 mM TES buffer (pH 6.5), at 4°C with two changes of 1 hour and a final overnight dialysis. In the case of control Herring Sperm DNA samples, the reaction mixture was analyzed by HPLC after complete enzymatic digestion. The peak of 4-daeC disappeared after the reaction, and a new peak with the retention time of Bio-daeC was observed. The biotin labelling was quantitative under these conditions, as checked by disappearance of the 4-daeC peak after biotinylation. Molecular integrity of the ε Globin plasmids was checked after the biotinylation reaction by Agarose Gel electrophoresis in the presence of λ phage DNA molecular weight markers (data not shown).

Biotinylation of HindIII cut λ DNA

In order to ascertain whether the molecular integrity is generally maintained during the d.s. transamination procedure, λ phage DNA molecular weight markers (HindIII cuts), 50 μg of total sample (0.2 μg/μl), were transaminated for 1 hour at 50°C as described above, and afterwards biotinylated following the same procedure.

To check sensitivity of detection and molecular integrity a Southern transfer was performed after Agarose Gel electrophoresis of serial dilutions of the marker in the presence of a non biotinylated sample of the same DNA.

Results are reported in Figure 2. A direct comparison of the same samples before and after the Southern blot and detection procedures is shown. As can be seen there is no modification of the m.w. patterns in respect to the non biotinylated control. The sensitivity of detection shows that an amount of 100-500 ng is more than enough for each run, allowing 500 to 100 runs with a single biotinylated sample of 50 μg.

Supercoiled pBR322 labelling

Supercoiled pBR322 DNA (0.5 μg/μl) was biotinylated as reported above, except that the temperature was kept at 37°C and the transamination reaction was carried out for 1 hour. After this time the plasmid was double digested with PvuII (17h, 3 U/ml) and PstI (20h, 9 U/μl), heated at 65°C for 5', and loaded onto an agarose gel together with unlabelled supercoiled pBR322 cut in parallel under the same conditions and biotinylated λ phage DNA HindIII cut m.w. markers. Electrophoresis was performed in the presence of 0.5 μg/ml Ethidium Bromide, results are shown in Figure 3.

Fig. 2. Agarose Gel electrophoresis and Southern transfer of serial dilutions of a transaminated λ molecular weight marker, HindIII cuts, in the presence of control. From left to right in both A and B: non treated control 1μg, transaminated samples respectively 1 μg, 500 ng, 100 ng, 50 ng.

Fig. 3. Agarose Gel electrophoresis and Southern transfer of transaminated supercoiled pBR322 in the presence of controls. pBR322 in treated samples and controls was double digested with PstI, PvuII R.E.. From left to right in both A and B: supercoiled pBR322 1 μg, pBR322 double cut 1 μg, HindIII molecular weight markers 0.7 μg, biotinylated pBR322 double cut 0.7 μg, biotinylated pBR322 no cut 0.7 μg.

DISCUSSION

We have used a modification of the reaction described by Schulman et al. (5) to attach biotin molecules to d.s. DNA. After the transamination step with ethylenediammine, biotinylated d.s. DNA molecules can be prepared by coupling of the N4-hydroxysuccinimide ester of biotin to derivatized 4-aminoethyl cytosine residues; and these probes are as much effective as those obtained by end labelling with biotinyl-dNTP for the colorimetric detection of nucleic acids after the usual Southern transfer procedures.

Transamination of cytidine residues in nucleic acids by alkyldiammines is a simple and mild reaction which is applicable to mononucleotides as well as to polynucleotides (14,18-21). The reaction of bisulfite to form the initial adduct has been reported to be single strand specific with a difference of at least three orders of magnitude with respect to the reaction with d.s. portions of the molecules (26-28). Conditions giving differential quantitative labelling between single stranded and double stranded portions of the same RNA molecule have been reported by D.E. Draper (14). Because of this, the reaction has ben applied to DNA labelling by previous heat denaturation (20) or reaction above the melting point (18,19).

The reasons for this conclusion are related to the fact that with chromatographic analysis a small percentual amount of transaminated bases in d.s. molecules in practice is not detectable. But the recently developed systems for biotin detection on filters allows the detection of amounts as small as 300fg-1pg of DNA labelled at up to 5-10% of the total bases with Biotin. Thus since the amount of DNA loaded in control lanes in agarose gels is 10^5-10^6 times higher, the detection of very low percentages of labelling becomes possible.

For what concerns the evaluation of the reaction, considering the dot-blots of Figure 1, at 24 hours d.s. DNA is showing a label that is between 25 to 50 times lower than that of a control labelled to 28% (daeC/daeC+dC), samples run in triplicate. Thus in the case of d.s. DNA we can estimate about 1-3 labels per 10^3 bases at 24 hours and 6-10 labels per 10^5 bases at 1-3 hours. Moreover the 2.3 Kb bands of Figure 2 (lane with 50 ng of total loaded sample) show a staining intensity comparable to that of a single copy gene after hybridization with a biotinylated probe. In such case if we suppose to fractionate on an Agarose gel a total loading of 1-5 μg of genomic DNA cutted with a restriction enzyme such as EcoRI, a 2.3 Kb band will contain about 1-5 pg of DNA (corresponding to a single copy gene under the generally accepted assumption that the human genome will give rise to 10^6 fragments)(31); if this amount, supposedly hybridized to completion with a probe labelled at 7% of total bases, can give a staining intensity of the same order of magnitude than the band examined in Figure 2, a crude estimate of about 3-14 labels per 10^5 bases can be done in such case, with a decent agreement between the two sets of data. A more precise evaluation using densitometric analysis of the dot blots scalar dilutions, didn't give more reliable results.

A further point to mention is the fact that in cases in which particular structures are present the prevalence of s.s. versus d.s. reaction in our conditions should not give staining inhomogeneities for fragments of at least a few hundred bases, as it is the case for m.w. markers. To investigate this fact we labelled a plasmid carrying cytidines in the unpaired portion of hairpin loops.

Supercoiled pBR322 is carrying one main and two accessory S1 sensitives sites respectively at positions 3065, 3123, and 3221, which are the result of hairpin looping (32-35), and at these positions a total of six Cytidines are present at the unpaired end of the loops. Cutting the plasmid after transamination and biotinylation with a PstI PvuII double cut, respectively at positions 3613 and 2068, two fragments are obtained respectively of 2.8 Kb and 1.5 Kb, and the latter one is carrying the three sites. As it can be seen in Figure 3 the bulk of the plasmid is showing after transamination a drastic change in the migration rate probably due to a nicking effect, no difference in migration instead is shown between the control non transaminated on the left and the transaminated samples on the right in the case of the cut plasmids. The two bands of 2.8 Kb and 1.5 Kb, obtained after the PstI, PvuII double digestion of the transaminated sample are both labelled.

A close comparison with the intensity of the bands under U.V. illumination could perhaps allow the conclusions that the lower band of the biotinylated pBR322 is carrying a higher percentage of biotin in respect to the upper band, but no strict specificity of the label can be observed. Thus, with the procedure we adopted, there is reaction with portions of the molecule in addition to those at the hairpin loop positions thus masking the labelling of the unpaired part with the overall background, as should be expected on the base of previously published data (5,14,20).

Thus in conclusion, the transamination reaction can be used at temperatures of 50°C with reaction times of few hours for the inexpensive production of labelled intact double stranded DNAs useful as molecular weight markers, and for chromatographic procedures.

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