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Polynucleotide-histone H1 complexes as probes for blot hybridization

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Biotin and/or ^{125}I-labelled histone H1 proteins (21 kd) have been covalently bound to single-stranded DNA. Complexes of equal masses of DNA and modified histone H1 (~1 histone H1 molecule per 70 nucleotides) were used as probes for blot hybridization experiments and found to have hybridization characteristics very similar (or identical) to radiolabelled DNA probes.

Key words: hybridization/non-isotopic labelling/avidin-biotin interaction/DNA-protein interaction

Introduction

Nucleic acid probes for hybridization reactions are usually labelled by enzymatic incorporation of nucleotide triphosphates carrying a radioisotope. Recently a number of nucleotide analogs have been synthesized which contain biotin covalently attached to the pyrimidine or purine ring. These nucleotide derivatives have been shown to be substrates for RNA and DNA polymerases and the resulting polynucleotides hybridize specifically to their complementary sequences (Langer *et al.*, 1981). When used in conjunction with an affinity (avidin-peroxidase) or immunological (biotin-antibody in consort with a second antibody tagged with peroxidase) detector system such probes can be used as an alternative to radioisotopically labelled nucleic acids.

The remarkable stability and specificity of the biotin-avidin complex inspired Davidson and co-workers in 1975 to develop a method of *in situ* hybridization in which biotin residues are covalently attached to RNA *via* cytochrome c bridges by a chemical procedure, and the sites of hybridization were localized in the electron microscope with avidin detector systems (Manning *et al.*, 1975, 1977; Broker *et al.*, 1978; Sodja and Davidson, 1978).

We describe an alternative, simpler and safer method to produce hybridization probes which allows us to introduce large targets, namely protein molecules, and many labels. Our procedure stems from the finding of Manning *et al.* (1975) who showed that a positively charged protein, in their case cytochrome c, binds by electrostatic interactions to a nucleic acid, in their case RNA, at low ionic strength thus favouring formaldehyde cross-linking reactions between the nucleic acid and the protein molecules. However we have used single-stranded DNA and histone H1, as an example of a very lysine-rich DNA-binding protein. The high lysine content of the histone serves two purposes. (i) It guarantees very tight binding to nucleic acids and therefore very efficient cross-linking at low concentrations of glutaraldehyde during short reaction times. Probes produced in this way are not single-stranded DNA labelled with proteins, but rather represent a covalently bound single-stranded DNA-protein entity, with a protein:DNA mass ratio of nearly one (see Results). After hybridization, the complementary sequences can be detected by searching for the protein (e.g., with an antibody). (ii) Proteins can easily be chemically modified at the ϵ-amino group of their lysine residues by cyclic N-succinimide esters. Labelling the lysine NH_2 group should have little influence on the DNA-binding properties of the protein since not all of the lysine residues are needed for strong DNA-protein interactions. In fact, n.m.r. studies on DNA-histone H1 complexes show that, in the C-terminal fragment of histone H1, about one quarter of the lysine residues are not bound to DNA at very low ionic strength (Bradbury *et al.*, 1975). Since we cross-link many histone H1 molecules (now labelled) to one single-stranded DNA, the probe contains a large number of labels which are readily detectable after hybridization.

Blot hybridization experiments show that, under identical reaction conditions, probes labelled according to the outlined protocol (see Figure 1) react with their complementary sequences with a specificity indistinguishable from nick-translated or end-labelled probes.

Results

As indicated in the Introduction, our standard assay is the classical blot hybridization protocol (Southern, 1975) in a modified form (Kafatos *et al.*, 1979). Since quantitative estimations of intensities of bands on nitrocellulose paper are more easily performed by autoradiography than by measuring intensity of colours, most experiments were performed with double labelled probes using the same chemistry for protein labelling. Histone H1 (and poly-L-lysine) were first labelled with biotin and then with ^{125}I.

Use of DNA-histone H1 complexes as probes

To determine whether complexes of polynucleotides and histone H1 were suitable for use as hybridization probes, several DNA species were first digested with different restriction endonucleases, size separated by agarose gel electrophoresis, denatured and chromatographed onto nitrocellulose paper. Replicas were hybridized either with a ^{32}P-labelled nick-translated DNA probe (control experiment) or a probe consisting of a covalently linked complex of the same single-stranded DNA and histone H1 molecules, the latter being labelled with ^{125}I and biotin prior to formation of the complex and cross-linking. As shown in Figure 2b and c the pattern of bands and their intensities on the two autoradiograms are indistinguishable if the ^{32}P-labelled DNA probe or the [^{125}I]protein-DNA probe was used. This result shows that DNA-histone H1 complexes recognize their complementary sequences with the same fidelity as naked single-stranded DNA.

Duplex stability

To measure the stability of duplexes formed between either single-stranded DNA-histone H1 complexes, or single-stranded DNA and their complementary sequences in DNA, melting experiments were performed. Aliquots of single strands of pBR322 DNA were immobilized on two nitrocellulose filters, one of which was hybridized with a ^{125}I-iodinated and biotinylated histone H1-single-stranded pBR322 DNA probe and the other with a ^{32}P 3′-end labelled

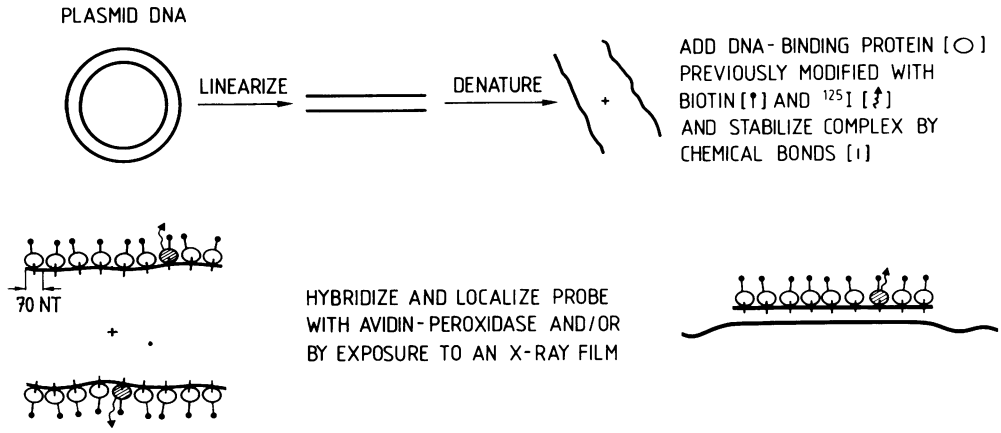

Fig. 1. Procedure for preparing histone H1-DNA probes for hybridization.

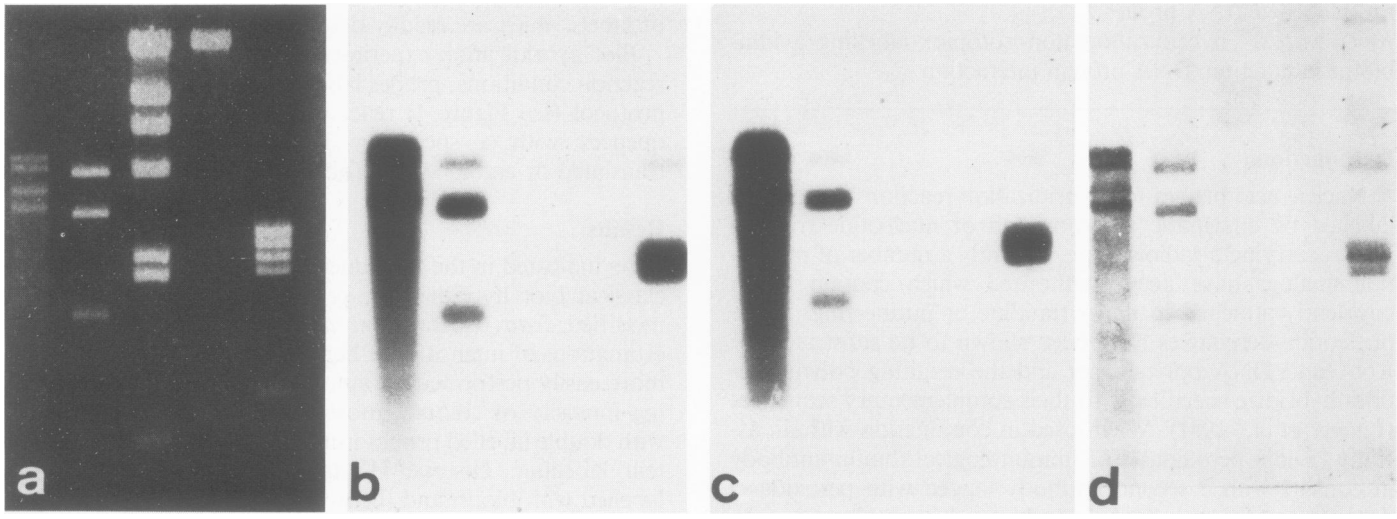

Fig. 2. Blot hybridization experiment with a probe consisting of a complex of single-stranded DNA and histone H1 labelled with ^{125}I and biotin. **(a)** Agarose gel (ethidium bromide stained) after electrophoretic size separation of fragments of the following DNA species (from left to right); pDv101 (80 ng) digested with *Pst*I, pDv131 (60 ng) digested with *Bam*HI, λ DNA (500 ng) digested with *Hind*III, λ DvII (180 ng), pDv1 (100 ng) digested with *Eco*RI and *Pvu*II. **(b)** Autoradiographic exposure of a nitrocellulose paper after transfer of the DNA shown in **(a)** and hybridization with 1 μg of *Pst*I-digested pDv101 DNA cross-linked with 0.8 μg ^{125}I-labelled and biotin-labelled histone H1 (2.4 x 10^6 d.p.m./μg DNA; seven biotin residues/100 nucleotides). Exposure time was 4 h at −70°C (intensifying screen). **(c)** Control experiment: autoradiographic exposure of a replica nitrocellulose paper after hybridization with 0.1 μg ^{32}P-nick-translated pDv101 DNA (2.5 x 10^6 d.p.m.). Exposure time was 3.5 h at −70°C (intensifying screen). **(d)** Nitrocellulose paper used in **(b)** after incubation with avidin-peroxidase, anisidine and H_2O_2. pDv101, pDv131 and pDv1 are pBR322 plasmids containing *D. virilis* DNA inserts.

pBR322 DNA probe. Filters were gradually heated and the remaining radioactivity on the filters was measured (Figure 3). The melting points and the steepness of the two melting curves are similar indicating that there is little if any difference between the two duplexes. Thus, single-stranded DNA covalently bound to histone H1 molecules forms duplexes with the complementary sequence of similar or the same quality as single-stranded DNA free of proteins.

Only ~40% of the ^{125}I radioactivity can be released from nitrocellulose by temperature increase. The failure to release more of the single-stranded DNA histone H1 probe probably reflects non-specific binding between denatured histone H1, produced by the heating, and the filter. It cannot be the result of unspecific binding occurring during hybridization at 37°C otherwise we should observe a much higher background in our blot hybridization experiments (compare Figures 2b and 4c).

Probes labelled with biotin

In the following two experiments, biotin residues introduced into histone H1 proteins have been monitored with an avidin detector system.

In the experiment described in Figure 2, different DNA species have been blotted onto nitrocellulose paper after size separation and hybridized with a biotin and ^{125}I-labelled probe. The nitrocellulose paper, which has been exposed to an X-ray film (Figure 2b), was thereafter treated with avidin covalently linked to horseradish peroxidase, and then with anisidine (colorless) and H_2O_2. After 3 − 5 min, brown colored bands developed (Figure 2d). Comparison of film (^{125}I-label) and nitrocellulose paper (biotin label) shows that the pattern of bands is nearly the same albeit with higher background after biotin labelling. This result indicates that biotin molecules attached to ε-amino groups of lysine residues of histone H1 and then cross-linked to polynucleotides can be recognized by avidin after hybridization to their complementary sequences immobilized on nitrocellulose.

In the second experiment we present an application of our labelling procedure exploiting the fact that different probes can be easily modified with different labels and, after simul-

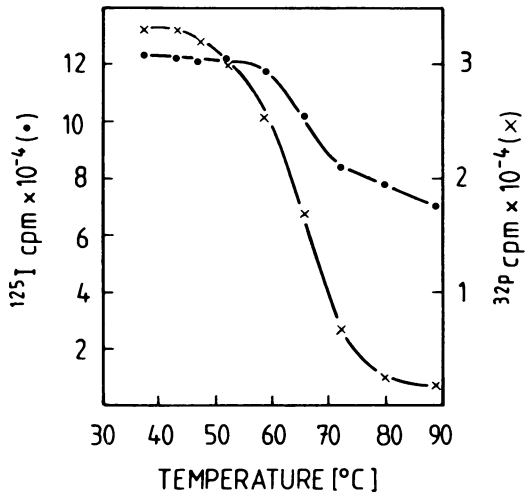

Fig. 3. Melting experiments of dot hybrids with a ^{32}P-labelled DNA probe and a histone H1-DNA probe. Aliquots (100 ng) of *Eco*RI linearized pBR322 DNA were bound to nitrocellulose filters. Replica filters were hybridized for 16 h with probes consisting either of 3′ end-labelled pBR322 (4 x 10^5 d.p.m./μg) or a pBR322-histone H1 complex (0.8 μg ^{125}I- and biotin-labelled histone H1 was cross-linked with 1 μg linearized, single-stranded pBR322 DNA; 2 x 10^6 d.p.m./μg DNA and seven biotin residues/100 nucleotide). After hybridization, each filter was washed and melted in 3 ml melting buffer (50% formamide, 2 x SET) by stepwise increases in temperature. Conditions for hybridization were as described in Materials and methods and for melting as described by Kafatos *et al.* (1979). The melting temperature for both hybrids is ~65°C. (Note that the amount of hybrids formed is very similar in both experiments: 8% for DNA/DNA hybrids and 6% for DNA/DNA-histone H1 hybrids.)

taneous hybridization, visualized by different detector systems. For gene mapping, a λ-clone carrying a *Drosophila virilis* insert was digested with different restriction endonucleases, size separated, blotted onto nitrocellulose paper and hybridized simultaneously with λ-DNA (labelled with [^{125}I]histone H1) and the *D. virilis* DNA sequences (labelled with biotin-histone H1) present in the insert of the λ-clone. After hybridization, the nitrocellulose paper was developed first with avidin-peroxidase (Figure 4b) and later exposed to an X-ray film (Figure 4c). Evaluation of DNA sizes corresponding to individual bands and their intensities is summarized in the map at the bottom of Figure 4.

Sensitivity

As mentioned in the Introduction, the main aim of our studies was to increase the ease and sensitivity of standard hybridization techniques. One of the first experiments, therefore, was a titration in which different amounts of histone H1 (both ^{125}I-iodinated and biotinylated) were covalently bound to DNA. Five probes were made in which increasing amounts of modified histone H1 proteins (between 50 ng and 3.0 μg) were cross-linked to linear single-stranded pBR322 DNA (1.0 μg each) and blot hybridized with replica filters (result not shown). There was a gradual increase in the intensities of the bands on X-ray films exposed to blots without change in the banding pattern (specificity). At histone H1: DNA mass ratios >1, the background increased sharply. These experiments show clearly that a large number of histone H1 proteins can be covalently bound to DNA and used as probes for hybridization and that the optimal mass ratio is 1:1.

Radiolabel

The sensitivity of radiolabelled histone H1 nucleic acid probes is limited by the specific activity of the ^{125}I-iodination reagent and the number of ^{125}I atoms which can be attached to ϵ-NH_2 groups of lysine residues. In the experiments described so far, only about one in 50 histone H1 molecules was labelled with a ^{125}I atom (4.5 x 10^6 d.p.m./μg histone H1). For probe construction usually 0.8 μg of labelled histone H1 has been cross-linked to 1 μg of single-stranded DNA (3.6 x 10^6 d.p.m./μg DNA). If such probes were blot hybridized to 30 ng of complementary DNA ~5000 bases long and immobilized on nitrocellulose, ~7000 d.p.m. (2 ng) were detected in that band giving rise to a strong signal after <1 h exposure to an X-ray film (−80°C, intensifying screen). To increase the sensitivity, histone H1 was radioiodinated to 100 times higher specific activity (~4 x 10^8 d.p.m./ μg histone H1) and used for hybridization. Figure 5a shows an autoradiogram of a titration in which different amounts of pDv1 DNA have been immobilized on nitrocellulose and hybridized with a pBR322-histone H1 complex (3 x 10^8 d.p.m./μg DNA). After a 5 h exposure, 10 pg of DNA (5 kb long) in a band of a blot can be detected. This sensitivity corresponds to that of standard procedures. A further increase in sensitivity might be difficult to achieve with DNA-histone H1 complexes as probes because extensive modification of too many lysine residues leads to a reduction of the DNA-binding capacity of histone H1. If, however, synthetic peptides (e.g., poly-L-lysine) can be used instead of histone H1, probes of such high specific activities probably could be produced. In fact, preliminary experiments using DNA-polylysine complexes as hybridization probes gave similar results to those with DNA-histone H1 complexes although with higher background (results not shown).

DNA-histone H1 complexes can also be used for the detection of single copy genes on genomic blots. *D. melanogaster* DNA was digested with *Eco*RI, size separated, blotted onto nitrocellulose paper and hybridized with a DNA-histone H1 complex containing sequences of the *Drosophila* alcohol dehydrogenase gene (Benyajati *et al.*, 1981). The two bands of the autoradiogram shown in Figure 5c, which have been visualized after a 2 h exposure, result from the polymorphism of the cell line used.

Non-isotopic label

To determine the number of biotin residues introduced into histone H1 molecules, a tritium-labelled biotinsuccinimide ester was used for the biotinylation reaction. Three histone H1 derivatives were synthesized containing ~2, 7 and 20 biotin residues, respectively, and cross-linked with polynucleotides at a histone H1:DNA ratio of ~1 (see Materials and methods). These probes contained ~3, 10 and 30 biotin residues per 100 nucleotides, respectively. After blot hybridization the nitrocellulose papers were treated with avidin-peroxidase, anisidine and H_2O_2. The time after which anisidine became oxidized (appearance of brown colored bands) and the colour intensity in a given band was very similar for all three probes (result not shown). This finding indicates that not many more than ~2 biotin residues in a histone H1 molecule cross-linked to polynucleotides (or ~3 biotin residues per 100 nucleotides) can be recognized by a protein aggregate like avidin-peroxidase (120 kd). Steric hindrance probably prevents recognition of larger numbers of biotin residues; the sensitivity, therefore, cannot be increased by excessive biotinylation.

To test the sensitivity with avidin-peroxidase, a similar titration to that described above was performed. As can be

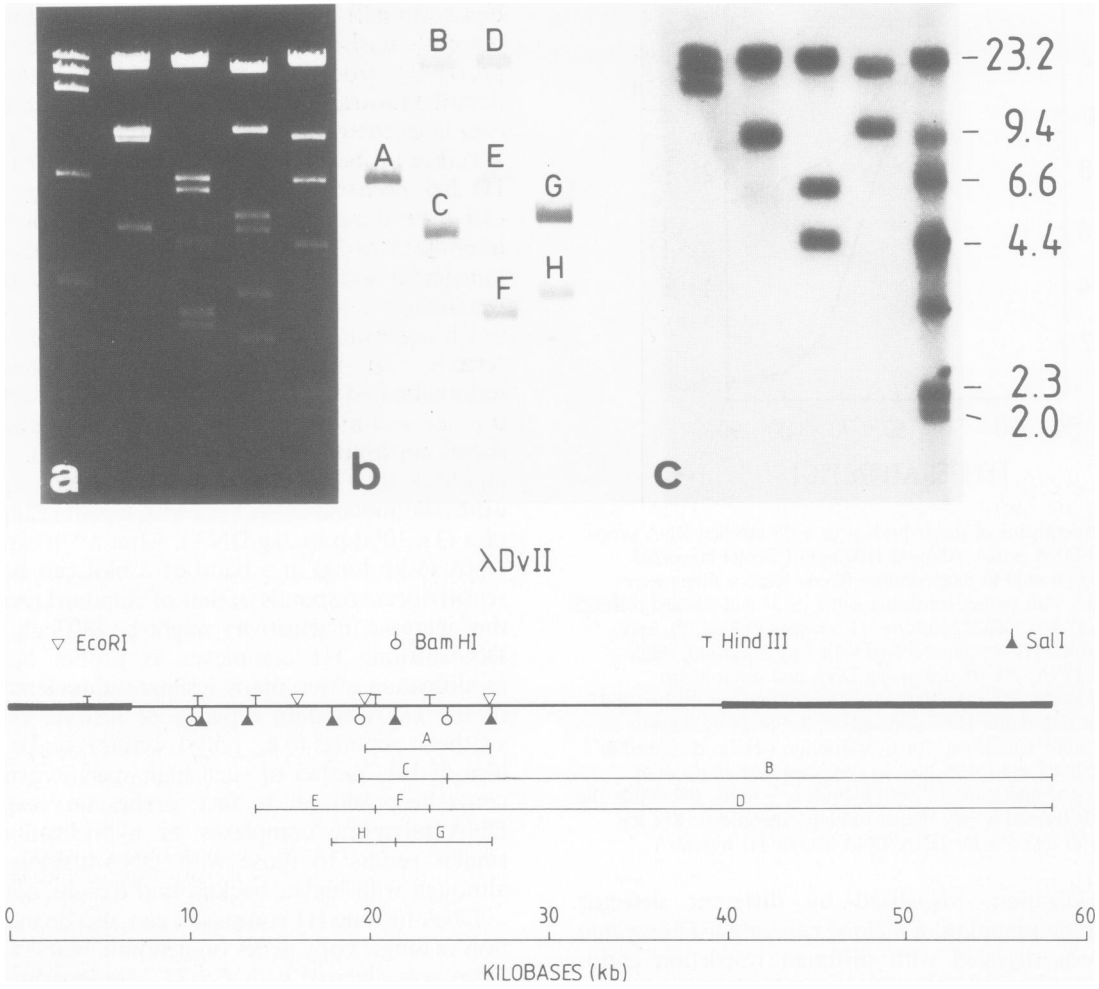

Fig. 4. Simultaneous hybridization with two probes labelled differently. **(a)** Agarose gel (ethidium bromide stained) after electrophoresis of λDvII DNA (230 ng each track) digested with different restriction endonucleases (left to right): *Eco*RI, *Bam*HI, *Hind*III, *Sal*I. The right most lane is a size marker (λ DNA digested with *Hind*III; fragment lengths are indicated in kb). **(b)** Nitrocellulose paper incubated with avidin-peroxidase, anisidine and H_2O_2 after transfer of the DNA shown in **(a)** and simultaneous hybridization with two probes: (i) 1 μg of *Sau*3A-digested pDv1 DNA was cross-linked with 0.9 μg biotinylated histone H1 (nine biotin residues/100 nucleotides) and (ii) 1 μg of *Hind*III-digested λ DNA was cross-linked with 0.9 μg ^{125}I-iodinated histone H1 (4 x 10^6 d.p.m./μg DNA). **(c)** Autoradiographic exposure of the nitrocellulose paper used in **(b)**. λDvII DNA is a clone with a *D. virilis* insert; pDv1 is a subclone of λDvII (fragment A in the *Eco*RI site of pBR322). The thick lines represent the two EMBL 2 λ vector arms.

seen in Figure 5b, the limit of detection of a 5 kb long DNA fragment is close to 100 pg. Single copy genes on genomic blots of species with a similar or higher complexity than that of the *Drosophila* genome can, therefore, not be detected with the biotin detector system.

Discussion

The unexpected finding of our study is that an equal mass of protein can be chemically bound to DNA without detectable loss of hybridization specificity. There is ~1 histone H1 molecule every 70 nucleotides in a polynucleotide-histone H1 complex consisting of equal masses of protein and nucleic acid. We are ignorant about the detailed chemistry of the glutaraldehyde cross-linking reaction between the histone and the polynucleotide chain. Purines and/or pyrimidines might be involved since RNA-histone H1 complexes can be cross-linked similarly and used as hybridization probes (result not shown). If histone H1 is cross-linked to the bases at least one (and more likely a few) out of 70 nucleotides would be modified, probably in such a way that they interfere with the recognition of their complementary bases. Such a low number of base substitutions would have probably escaped detection. To clarify this point, similar DNA sequences (for example polymorphic genes) have to be compared for hybridization signal intensities and melting behaviour.

We would like to emphasize some practical advantages of our labelling method. (1) Since it is a chemical procedure, with a product yield of >95%, no polymerases are needed and separation of precursors and end product is not necesary. Several probes can be labelled in <15 min. (2) Polynucleotides for probe construction do not have to be highly purified because many contaminants (like phenol) do not interfere with the cross-linking reaction. (3) Standard protocols for blot hybridization can be used without change.

However, the concentrations of DNA and histone H1 solutions used for the production of probes must be known, since a mass ratio of histone H1 to DNA of nearly 1 is desirable for maximal specific activity. More histone H1 leads to a higher background. Binding and cross-linking of histone H1 and DNA must be carried out at very low ionic strength where histone H1 binds in a non-cooperative manner. Histone H1 interacts cooperatively with DNA already at 30 mM NaCl and has a strong preference for large and AT-rich DNA

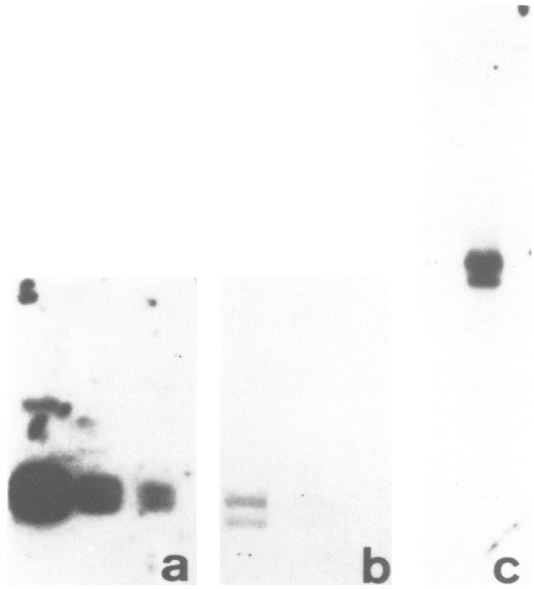

Fig. 5. Titration and genomic blot experiments. **(a)** Autoradiographic exposure of a nitrocellulose paper after transfer of pDv1 DNA fragments shown in the right most lane of Figure 2a and hybridization with 0.1 μg of *Hpa*II-digested pBR322 DNA cross-linked with 0.1 μg ^{25}I-labelled histone H1 (4 x 10^8 d.p.m./μg). The amounts of pDv1 DNA separated by agarose gel electrophoresis were 10, 1 and 0.1 ng, respectively, corresponding to 1000, 100 and 10 pg DNA (from left to right) in each of the two bands assuming 50% transfer efficiency. **(b)** Replica nitrocellulose paper after hybridization with 0.5 μg of *Hpa*II-digested pBR322 DNA cross-linked with 0.4 μg biotin-labelled histone H1 and visualization with the biotin detector system. **(c)** Autoradiographic exposure of a nitrocellulose paper after transfer of 10 μg of *Eco*RI-digested and electrophoretically separated DNA from *D. melanogaster* K_c cells and hybridization with 0.1 μg of a *Hae*III-digested pBR327 clone with a 4.8-kb insert carrying the alcohol dehydrogenase gene (Benyajati *et al.*, 1981) cross-linked with 0.08 μg ^{125}I-labelled histone H1 (4 x 10^8 d.p.m./μg). The DNA fragments in the two bands are 4.8 and 5.0 kb long.

fragments (Renz and Day, 1976). Probes made at higher ionic strengths cannot be expected to be labelled at random with respect to polynucleotide sequences and length.

With biotin-labelled probes, the use of radioactive labels can be completely avoided. The non-isotopic detection procedure using avidin-peroxidase, however, deserves some comments. Avidin is a basic protein and therefore interacts electrostatically with negatively charged polymers like DNA. This is why some unspecific bands are present on the nitrocellulose paper shown in Figure 2d. Track 3 (Figure 2d), for instance, does not contain any sequences homologous to the probe; however, faint bands can be seen. Such 'wrong signals' originate from unspecific DNA-avidin-peroxidase interactions (compare Figure 2a) and are not the result of the specific avidin-biotin binding. We envisaged at least three possibilities to circumvent the interference of the DNA-avidin-peroxidase binding and tested them: (i) use of high salt and non-ionic detergents leads to some improvement but is not sufficient by itself; (ii) replacement of avidin-peroxidase by streptavidin-peroxidase reduces unspecific binding drastically (streptavidin is an almost neutrally charged avidin analogue) but the sensitivity is ~10 times lower compared with avidin-peroxidase; (iii) treatment of nitrocellulose papers with positively charged polymers like poly-lysine to saturate non-specific binding sites prior to the avidin-peroxidase reaction step brings about the best results. Combination of (i) and (iii) is incorporated in our standard procedure (see Materials and methods). To demonstrate the improvement caused by poly-lysine, such treatment was omitted with the nitrocellulose paper shown in Figure 2d as opposed to that of Figures 4b and 5b.

The number of biotin residues per nucleotide (0.07 – 0.1) in the probes used in our experiments was higher as compared with DNA probes labelled by nick-translation using biotin-nucleotide triphosphates (0.05; Langer *et al.*, 1981) and even more biotin residues can be introduced by our labelling procedure (compare Results), but this is of little interest since it is the number of available biotin residues for the detector system (avidin-peroxidase or biotin-antibody) which limits the sensitivity of biotin-labelled probes. Efforts to increase sesitivity should be focused on the development of better detector systems.

Materials and methods

Histone H1 modification

Histone H1 (21 kd) was isolated from calf thymus nuclei by perchloric acid extraction (De Nooij and Westenbrink, 1962). Lyophilized histone H1 was dissolved in water (5 mg/ml) and stored at −20°C.

Biotinylation

Biotinyl-N-hydroxysuccinimide ester was prepared similarly as described (Becker *et al.*, 1971; Bayer *et al.*, 1979). 250 mg biotin (Merck), 250 μCi (1.4 μg) [^{3}H]biotin (NEN), 150 mg N-hydroxysuccinimide (Merck) were dissolved in 3 ml dimethylformamide (DMF) and 200 mg dicyclohexylcarbodiimide were added. The mixture was stirred for 16 h at 20°C. The precipitate was filtered off, and the filtrate was dried under reduced pressure. The residue was washed with ether, crystallized from isopropanol and used for biotinylation reactions. Histone H1 was biotinylated to different extents using increasing amounts of biotinyl-N-hydroxysuccinimide ester (BHSE). 5 μg BHSE (dissolved in 10 μl DMF), 25 μg BHSE (in 10 μl DMF) and 250 μg BHSE (in 10 μl DMF), respectively, were added to solutions containing the same amount of histone H1 (1 mg in 300 μl 50 mM $NaHCO_3$ each) and incubated for 1 h at 20°C. After dialysis against 5 mM sodium phosphate (pH 6.8) the three samples were stored at −20°C. Concentrations of biotinylated histone H1 solutions have been determined by comparing unmodified histone H1 on SDS-polyacrylamide gels after electrophoresis. The number of biotin residues in the three preparations was estimated by making use of the tritium tracer in BHSE and was found to be 2, 7 and 20 per histone H1 molecule respectively.

Iodination

Typically, to 1 mCi of solid mono-iodo [^{125}I]Bolton-Hunter-reagent (2000 Ci/mmol) (NEN) 60 μl of a solution containing 120 – 250 μg of histone H1 (biotinylated or unmodified) and 100 mM sodium borate (pH 8.9) have been added at 0°C and left for 1 h at 0°C. Thereafter, 10 μl of 1 M glycine in 0.5 M sodium borate were added. The solution was gel filtrated (Sephadex G100 in 5 mM sodium phosphate, pH 6.8) through a column (silanized Pasteur pipette) conditioned with 50 μg histone H1. Fractions containing [^{125}I]histone H1 (or [^{125}I]biotin-histone H1) were stored at −20°C; they can be thawed and frozen repeatedly without loss of binding capacity. Concentrations were determined by comparative SDS-polyacrylamide electrophoresis (see 'Biotinylation'). Yields of incorporation were usually ~40% and specific activities between 3 and 4.5 x 10^6 d.p.m./μg protein. Accordingly, every 75th to 50th histone H1 molecule contains a [^{125}I]atom.

To achieve a high specific activity (~4 x 10^8 d.p.m./μg) 4 μg of histone H1 (in 6 μl 100 mM sodium borate, pH 8.9) were allowed to react with 4 mCi [^{125}I]Bolton-Hunter reagent for 4 h and used directly for construction of DNA-histone H1 complexes without further purification.

Probe construction

Circular DNA (plasmids) as well as large linear DNA molecules (phage λ DNA) were linearized or digested with restriction endonucleases to an average size of ~4 kb. During the course of this study it was observed that smaller DNA fragments result in stronger hybridization signals as is also the case with nick-translated probes (Alwine *et al.*, 1979). We therefore digested DNA with enzymes like *Sau*3A or *Hae*III. DNA was used after digestion without further purification. 1 μg DNA (in not more than 20 μl digestion buffer used previously) was diluted with 180 μl freshly prepared 5 mM sodium phosphate (pH 6.8), denatured by heat (100°C, 3 min) and cooled for 3 min on ice. First, histone H1 (0.8 – 1.0 μg in ~5 μl phosphate buffer) was added and

thereafter 20 μl of a 2.5% glutaraldehyde solution. The sample was incubated for 10 min at 30°C and added directly to the hybridization reaction vessel containing nitrocellulose paper and hybridization solution.

DNA binding studies with gel filtration columns (Sephacryl S1000) performed in buffers known to disrupt ionic interactions (1 M NaCl, 50% formamide, 0.2% SDS) showed that >95% of histone H1 molecules are covalently bound to DNA. After cross-linking, non-reacted glutaraldehyde does not have to be removed (e.g., by gel filtration) nor does lysine have to be added to inactivate aldehyde-induced chemically active groups in DNA-histone H1 complexes, since only little, if any, subsequent conjugation to carrier proteins in the hybridization solution or to nitrocellulose was observed.

Hybridization

DNA to be analyzed was digested with restriction endonucleases, fractionated by agarose gel electrophoresis and transferred to nitrocellulose paper (Schleicher and Schull) by the Southern procedure (Southern, 1975). Papers were soaked for 1 h in 10 x Denhardt's solution (Denhardt, 1966), 4 x SET (1 x SET is 0.15 M NaCl, 0.03 M Tris-HCl, pH 8, 1 mM EDTA) at 37°C and transferred to plastic containers containing between 10 and 20 ml blank hybridization mixture (50% deionized formamide, 2 x Denhardt's solution, 4 x SET, 0.1% SDS and 30 μg/ml yeast tRNA) incubated for 1 h at 37°C and then, after addition of the probe, 16–20 h with gentle shaking at 37°C.

Washing

The nitrocellulose papers were washed for 60 min at 37°C with two changes of a solution containing 50% formamide, 0.2% SDS and 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate) and for 40 min at 20°C with two changes of a solution containing 2 x SSC. Filters were drained, autoradiographed and/or developed with a biotin detector system.

Visualization of probes by avidin-peroxidase

Nitrocellulose papers were incubated first for 20 min with a solution containing 10 μg/ml poly-L-lysine HBr, mol. wt. 220 000 (Sigma), 0.1 M Tris-HCl (pH 7.5) and then for 50 min with a solution containing 3% bovine serum albumin (BSA), 0.1 M Tris-HCl (pH 7.4) followed by an incubation for 60 min with the avidin-peroxidase solution (1 M NaCl, 0.1 M Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.1% BSA and 1 μg/ml avidin-peroxidase, E.Y. Laboratories, CA). After washing for 20 min with two changes of a solution containing 1 M NaCl, 0.1 M Tris-HCl, pH 7.4, 0.1% BSA and 0.1% Triton X-100, the papers were incubated with the staining solution (10 ml of 0.1 M Tris-HCl, pH 7.4, 2 ml of ethanol in which 6 mg 3,3'-dianisidine have been dissolved, 6 μl of 30% H_2O_2). Color development (brown bands) takes place in 6–10 min. All incubation steps were carried out at 20°C with gentle shaking.

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