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Chemically modified nucleic acids as immunodetectable probes in hybridization experiments

(DNA and RNA hybridization/*N*-acetoxy-*N*-2-acetylaminofluorene/antibodies/immunoprecipitation/immunochemical detection)

PAUL TCHEN*, ROBERT P. P. FUCHS†, EVELYNE SAGE‡, AND MARC LENG‡

*Institut de Pathologie Moléculaire, Centre Hospitalier Universitaire Cochin, 24 rue du Faubourg Saint-Jacques, 75014, Biologie Moléculaire du Gène, Centre National de la Recherche Scientifique E.R. 201, Institut National de la Santé et de la Recherche Medicale S.C. 20, Institut Pasteur, and Groupe de Recherche de Génétique Epidémiologique, Institut National de la Santé et de la Recherche Medicale, U. 155, Château de Longchamp, 75016 Paris; †Institut de Biologie Moléculaire et Cellulaire, Strasbourg; and ‡Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, Orléans, France

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ABSTRACT **Guanine residues in nucleic acids can be modified by treatment with *N*-acetoxy-*N*-2-acetylaminofluorene and its 7-iodo derivative in an *in vitro* nonenzymatic reaction. The modified nucleic acids (ribo or deoxyribo, single or double stranded) are recognized by specific antibodies. They can be immunoprecipitated or used as probes in hybridization experiments and detected by immunochemical techniques.**

DNA recombinant technology has suggested new possibilities for the diagnosis of genetic diseases in man (1). The construction of a genetic linkage map by using restriction-fragment-length variants would be of importance to identify specific associations with disease traits (2, 3). Considering the size of the human genome, such a map would be easier to obtain if it were possible to study large genomic domains rapidly. One approach to this problem would be to make immunoprecipitable DNA probes ("immunonucleic" probes) that could be hybridized with large genomic fragments. Long stretches of DNA adjacent to the sequence homologous to a given immunonucleic probe could be immunoprecipitated and either cloned or used as probes to screen a cDNA library. Starting with a probe of known chromosomal location, it would be theoretically possible to obtain sets of clones corresponding to defined chromosomal domains rapidly. With regular immunochemical techniques, such probes could also be used to detect specific gene sequences without radioactive isotopes. Other groups have attempted to develop similar approaches by other methods (see refs. 4–6). In this report, we describe a method based on the chemical modification of guanine residues in ribo- or deoxyribonucleic acids using *N*-acetoxy-*N*-2-acetylaminofluorene (AAF) or its 7-iodo derivative (AAIF). Such modified nucleic acids can be immunoprecipitated, or used to detect homologous sequences by nonradioactive methods (Fig. 1).

MATERIALS AND METHODS

Materials. λ DNA was purchased from New England Biolabs. Ribosomal ribonucleic acid from bovine liver and reagents for alkaline phosphatase demonstration (fast blue RR salt, naphthol AS-MX phosphate) were purchased from Sigma. Nitrocellulose BA-85 filters were obtained from Schleicher & Schüll. Formalin-fixed *Staphylococcus A* (Immunoprecipitin) was from Bethesda Research Laboratories. Nick-translation kits (catalog no. N-5000) and radiolabeled nucleotides ([α-^{32}P]dCTP; 800 Ci/mmol; 1 Ci = 37 GBq) were products of Amersham. AAF and AAIF were synthesized as described (7, 8). These agents are potentially carcinogenic and should be handled cautiously (9). Antisera against DNA modified covalently with AAF (DNA-AAF) and against *N*-2-(guanosin-8-yl)acetylaminofluorene (Guo-AAF) were obtained as described (10–12). Alkaline phosphatase and peroxidase-conjugated antisera were purchased, respectively, from Miles and Institut Pasteur Production. Plasmid 4p7-7 has a 464-base-pair ζ-globin cDNA fragment inserted in pBR322 at the *Pst* I site. The same fragment was also inserted in phage M13 (13). Single-stranded M13 DNA was prepared according to Messing *et al.* (14). Plasmid pWE6 contains a 6.6-kilobase mouse 45S rDNA, inserted in pBR322 at the *Eco*RI site (15, 16).

Modification of Nucleic Acids with AAF and AAIF. The nucleic acid was dissolved in 2 mM sodium citrate buffer (pH 7) at a concentration of 500 μg/ml. It was reduced to fragments of about 1000 base pairs by sonication. After heat denaturation (100°C, 5 min), 1/10th vol of ethanol containing a 3-fold excess (wt/vol) of AAF or AAIF was added and the reaction mixture was incubated at 37°C for 2 hr in the dark. Cross-linking was eliminated by treatment with alkali (0.05 M sodium borate buffer, pH 9, 100°C, 3 min) followed by neutralization (0.1 M Tris·HCl buffer, pH 7). Unreacted fluorene derivatives were removed by five extractions with cold ethyl ether. For single-stranded M13 DNA, the sonication step was omitted. When radioactive DNA was used, the nick-translated DNA was ethanol precipitated, redissolved in citrate buffer, and denatured by heat. Aliquots were treated as indicated above except that the sonication step was also omitted. Control DNA was treated with pure ethanol. After treatment, EDTA was added to a final concentration of 2 mM and modified nucleic acids were kept at +4°C or −20°C.

Determination of the Percentage of Modified Bases. The percentage of modified bases was determined by measuring the absorbance at 305 and 260 nm (for AAF modifications) or 310 and 260 nm (for AAIF modifications) as described (17, 18). When this photometric method could not be used (too small a sample), we spotted on a nitrocellulose filter several dilutions of the test sample and an already measured sample. After immunochemical staining, the color intensities of the dots were compared.

Detection of Modified DNA Fixed on Nitrocellulose Filters. The solution used for saturating the filters with proteins and diluting the antisera was composed of 20% newborn calf serum, 2× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate, pH 7.0), 1% Nonidet P-40. Washing was done with 2× NaCl/Cit/1% Nonidet P-40. Each of the following steps was carried out at laboratory temperature for 1 hr: saturation of the filters with calf serum solution; incubation with a

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Abbreviations: AAF and AAIF, *N*-acetoxy-*N*-2-acetylaminofluorene and its 7-iodo derivative, respectively; DNA-AAF, DNA modified covalently with AAF; DNA-AAIF and RNA-AAIF, DNA and RNA, respectively, modified covalently with AAIF; Guo-AAF, *N*-2-(guanosin-8-yl)acetylaminofluorene; FPG, 0.02% Ficoll 400/0.02% polyvinylpyrrolidone 350/0.02% glycine; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

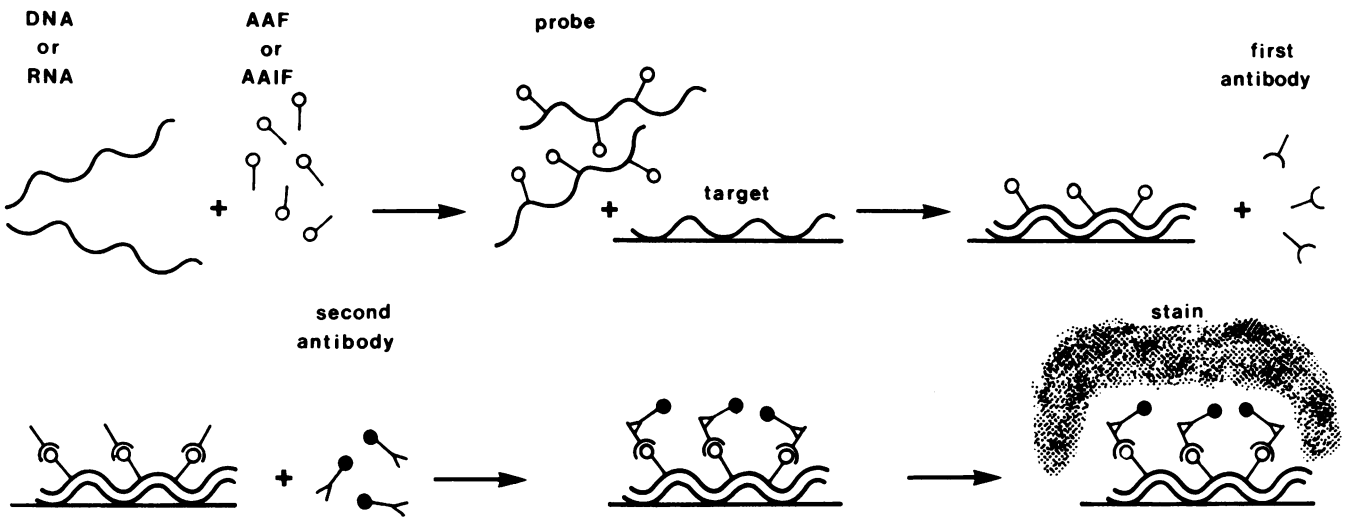


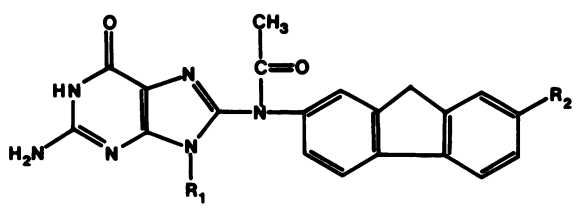
FIG. 1. Procedure for preparation and detection of immunonucleic probes.

1:200 dilution of anti-Guo-AAF rabbit antiserum, washing, incubation with a 1:400 dilution of alkaline phosphatase-conjugated antiserum to rabbit IgG, washing. Then, the filters were rinsed in phosphate buffer (0.1 M, pH 8) rapidly and stained with fast blue as suggested in Sigma Technical Bulletin 85 (fast blue RR salt at 0.5 mg/ml and 0.04% naphthol AS-MX phosphate solution, pH 8.6) at room temperature until proper staining appeared. When staining was done for more than 1 hr, a red deposit formed on the filters. This deposit could be eliminated by washing with 10% $NaDodSO_4$ solution. Stained filters were dried at room temperature. Dried filters protected from light could be kept for months at room temperature without the stain fading. In some experiments, the second antiserum was a 1:400 dilution of peroxidase-conjugated antiserum to rabbit IgG, and the staining was done with 3-amino-9-ethylcarbazol [2 mg dissolved in 0.5 ml of *N,N*-dimethylformamide, 9.5 ml of 0.05 M acetic acetate buffer (pH 5), and 0.05 ml of 30% H_2O_2 aqueous solution].

Hybridization with AAF- or AAIF-Modified Probes. Hybridizations were carried out following standard procedures, except for the Denhardt's solution (19), in which albumin was replaced by glycine. We routinely use a 50× FPG stock solution kept at +4°C (1× FPG is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone 350/0.02% glycine). Filters were prehybridized at 65°C for at least 2 hr in 2× NaCl/Cit/5× FPG. Probe, diluted in water, was denatured by heating at 100°C for 5 min and then chilled. Hybridization was done in 2× NaCl/Cit/1× FPG/25 mM KH_2PO_4/2 mM EDTA/0.5% $NaDodSO_4$, pH 7, usually overnight at 65°C. Sometimes, the hybridization mixture also contained 10% dextran sulfate.

RESULTS

Ribo- or deoxyribonucleic acids react readily *in vitro*, at neutral pH, with AAF and AAIF. Under the conditions described above, the fluorene derivatives are linked mainly at the C-8 position of guanine residues by a covalent bond as shown below (8, 20, 21). The antisera obtained by immunizing rabbits with DNA-AAF and Guo-AAF specifically recognize AAF-modified DNA (10, 12, 22–24). They also recognize AAIF-modified RNA and DNA. The results in Table 1 show that a greater amount of DNA was found in the precipitate when the DNA was AAIF modified. Small quantities of normal DNA were precipitated by anti-DNA-AAF antibodies. For discriminating between modified and unmodified DNA, better results were obtained with anti-Guo-AAF antiserum. Only negligible amounts of unmodified DNA were precipitated by purified anti-Guo-AAF antibodies. These observations suggest that immunonucleic probes can be used for separating specific gene sequences from complex mixtures.



R_1 = 1′-ribosyl or 1′-deoxyribosyl; R_2 = H (AAF) or I (AAIF)

The detection of modified nucleic acids by immunochemical techniques was tested in the following manner: modified DNA was allowed to bind to nitrocellulose filters, and the filters were then treated as indicated in *Materials and Meth-*

Table 1. Percentages of immunoprecipitated DNA

Antibody	DNA (control)	DNA-AAF (≈5% modified bases)	DNA-AAIF (≈5% modified bases)
Rabbit (normal serum)	<0.1	<0.1	<0.1
anti-Guo-AAF			
Whole serum	0.2	44.7	81.9
Purified	<0.1	41.5	71.9
anti-DNA-AAF (purified)	1.0	66.8	93.6

λ DNA was nick-translated to a specific activity of ≈2 × 10^7 cpm/μg and divided into three aliquots. One aliquot was treated with pure ethanol and the others, with AAF or AAIF. The percentage of bases modified was evaluated by the dot method. Four samples of about 4 × 10^5 cpm (10 μl) determined by Cerenkov counting were pipetted into 1.5-ml Eppendorf tubes. The volume was adjusted to 95 μl with 1× NaCl/Cit/2% sarcosyl solution, and 5 μl of whole serum or purified antibody diluted 1:5 in 1× NaCl/Cit/2% sarcosyl was added to each sample (final dilution, 1:100). After 1 night at +4°C, 5 μl of a 10% solution of formalin-fixed *Staphylococcus A* (Immunoprecipitin, Bethesda Research Laboratories) was added to each sample. After 15 min at room temperature, the tubes were centrifuged for 5 min in an Eppendorf centrifuge and supernatants were pipetted out. Pellets were suspended in 200 μl of 1× NaCl/Cit/2% sarcosyl, centrifuged, then suspended in 200 μl of 1× NaCl/Cit/5% sarcosyl and centrifuged again. Supernatants of each sample were pooled. Pellets and supernatants were analyzed by Cerenkov counting. The percentage of immunoprecipitated DNA was calculated by using the formula (cpm of the pellet)/(cpm of the pellet + cpm of the supernatant).

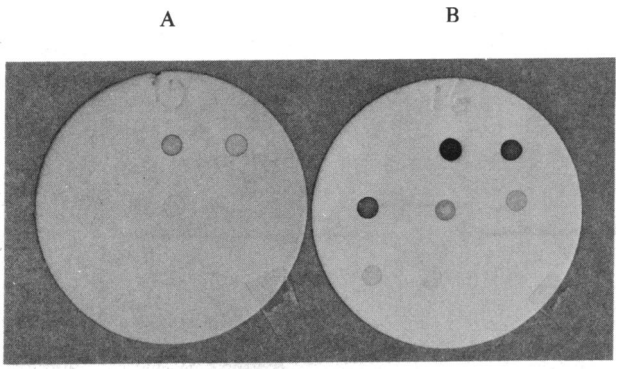


FIG. 2. Nitrocellulose filters were spotted with 1-μl droplets of heat-denatured DNA dissolved in 10× NaCl/Cit. The upper left spot (not visible) was a negative control (unmodified pBR322 DNA at 20 ng/μl). The other spots were made of AAIF-modified DNA solutions (from 64 to 0.5 pg/μl, by sequential 1:2 dilution). Modified DNA was detected by using peroxidase-conjugated (*A*) or alkaline phosphatase-conjugated (*B*) second antibodies.

ods. As the number of parameters to study was considerable, we chose to do all the following experiments with small round filters (25 mm) because they were easier to handle. Using AAIF-modified DNA (≈5% modified bases), we found the sensitivity limit to be <1 pg when the second antisera was conjugated to alkaline phosphatase and ≈8 pg when it was conjugated to peroxidase (Fig. 2). The colored dots appear very rapidly (in seconds for nanogram amounts).

As the DNA melting temperature is slightly affected by the binding of fluorene derivatives, questions could be raised about the usability of modified DNA in hybridization experiments. The decrease of the melting temperature is ≈1.1°C and 0.4°C, respectively, per 1% of bases modified by AAF and AAIF (7, 18, 21, 25, 26). Dot hybridizations were done with radioactive λ DNA. Three kind of probes were used in parallel, the control being treated with pure ethanol and the others with ethanolic solutions of AAF or AAIF. From the signals obtained after autoradiography, no differences could be seen between the control and the AAF- or AAIF-modified probes, whatever the stringency of the washing. After autoradiography, the filters were stained immunochemically. No stain was seen on the filters hybridized with the control probe. The sensitivity attained with the DNA-AAIF probe was consistently higher than that attained with the DNA-AAF probe and depended on the probe concentration. The results obtained at a probe concentration of 150 ng/ml are shown in Fig. 3. In this test, λ DNA was nick-translated to a specific activity of 5×10^6 cmp/μg and divided into two aliquots that were processed in parallel. One aliquot was treated with an ethanolic solution of AAIF, the other with ethanol alone. All the dots were stained on the filter that had been hybridized with an AAIF-modified probe except the pBR322 DNA dot. No stain was visible on the filter that had been hybridized with control DNA. The resolution was much better by immunostaining than by autoradiography. Besides, the immunostaining was much more rapid (a few hours).

Since any nucleic acid can be modified with AAF or AAIF, we have tested the possibility of using single-stranded M13 DNA and RNA immunonucleic probes in hybridization experiments. For hybridization with single-stranded M13 DNA, each filter was spotted with one λ phage DNA control dot and various amounts of plasmid 4p7-7 DNA. Single-stranded DNA of a M13 recombinant phage, with the same 464-base-pair ζ-globin gene insert as 4p7-7, was modified with AAF or AAIF (≈5% modified base pairs). Several probe concentrations, from 125 to 1500 ng/ml, were tried. Hybridization and washing were done at 65°C. Immunostained filters hybridized at a probe concentration of 500 ng/ml are shown in Fig. 4 *A* and *B*. In this case, the concentration of probe hybridizable sequences was ≈33 ng/ml (500 × insert size/total phage DNA size ≈ 33) and the target sequence dots contained 5–660 pg of target sequence. All dots except the negative control were stained. Dot staining was more intense when an AAIF-modified probe was used.

Similar tests were done with AAIF-modified RNA probes.

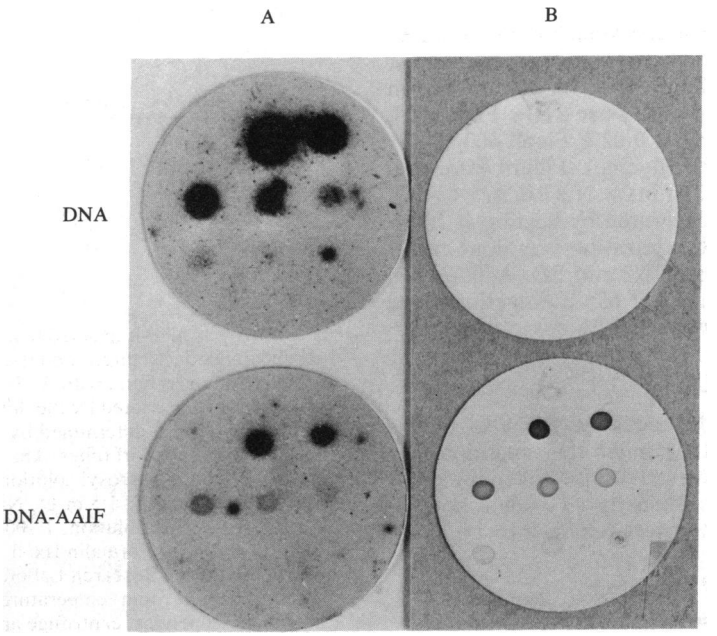


FIG. 3. Nitrocellulose filters were spotted with 20 ng of pBR322 DNA (upper left) and various amounts of λ DNA (from 500 to ≈4 pg by sequential 1:2 reduction). Radioactive probes used (5×10^6 cpm/μg, 150 ng/ml) were unmodified DNA (DNA) and AAIF-modified λ DNA (DNA-AAIF). Hybridization was done overnight at 65°C. Filters were washed at 65°C (1× NaCl/Cit/2× FPG/0.5% $NaDodSO_4$, 10 min, two times; 0.2× NaCl/Cit/0.5% $NaDodSO_4$, 30 min). The DNA-AAIF-hybridized filter was autoradiographed for 44 hr, and the DNA-hybridized filter was autoradiographed for 96 hr. Immunostained dots appear only on the DNA-AAIF-hybridized filter. (*A*) Autoradiography. (*B*) Immunochemical staining.

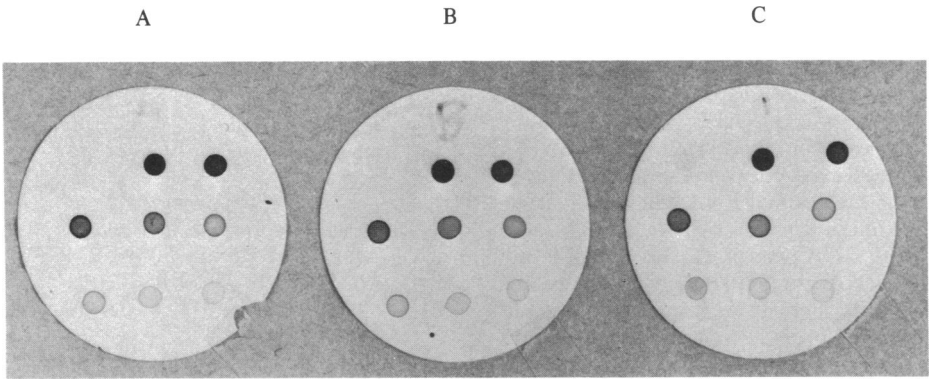


FIG. 4. (*A* and *B*) Nitrocellulose filters were spotted with 25 ng of λ DNA (upper left) and various amounts of 4p7-7 DNA (from 10 ng to ≈80 pg by sequential 1:2 reduction). AAF-modified (*A*) or AAIF-modified (*B*) single-stranded M13 DNA probes were used at a concentration of 500 ng/ml. Hybridization was done overnight at 65°C. Washing was done at 65°C (2× NaCl/Cit/0.5% $NaDodSO_4$, 30 min; 1× NaCl/Cit/0.25% $NaDodSO_4$, 30 min; 0.5× NaCl/Cit/0.125% $NaDodSO_4$, 30 min) and the filters were immunostained. (*C*) The filter was spotted with 100 ng of pBR322 DNA (upper left) and various amounts of pWE6 DNA (from 50 ng to ≈390 pg by sequential 1:2 reduction). AAIF-modified ribosomal RNA was used as a probe at a concentration of 200 ng/ml. Hybridization and washing were done as above, except for the last wash (0.5× NaCl/Cit/0.125% $NaDodSO_4$, 30 min at room temperature).

Ribosomal RNA from bovine liver (Sigma ref. R5502) was dissolved in citrate buffer (2 mM, pH 7), sonicated briefly to reduce it to fragments of about 1000 bases, and modified with AAIF. Filters were spotted with pBR322 DNA (100 ng) to provide the negative control and various amounts of pWE6 DNA, a recombinant plasmid with a 6.6-kilobase insert of mouse 45S rDNA. The amount of hybridizable sequence on the dots ranged from ≈30 ng to 230 pg, and probe concentrations of 200–2300 ng/ml were used. An immunochemically stained filter after hybridization at a probe concentration of 200 ng/ml is shown in Fig. 4*C*. Only slight differences in staining intensities were observable at higher probe concentrations. Filters were not washed at high stringency and there is a slight staining of the negative control dot, which probably results from unspecific hybridization.

When immunonucleic probes were used at high concentrations, the filter background remained very low. This is illustrated in Fig. 5 *A* and *B*, where AAIF-modified DNA was used as the probe at concentrations of 0.5 and 3 μg/ml, respectively. On each filter, the amount of the target DNA ranged from 512 (upper left) to 2 (lower right) pg. The possibility to use high probe concentrations is interesting when one wishes to do rapid hybridizations. A filter hybridized for 1 hr at 65°C with an AAIF-modified M13 DNA probe at 2 μg/ml is shown in Fig. 5*C*. The hybridization mixture contained 10% dextran sulfate. In this case, the concentration of probe hybridizable sequences was ≈132 ng/ml and those of target sequence on the filter were 330–2.5 pg. As the washing was done only to a stringency of 0.5× NaCl/Cit, a slight nonspecific hybridization subsists on the 20-ng DNA control dot (upper left).

DISCUSSION

The properties of AAF- or AAIF-modified nucleic acids make them suitable for detecting specific DNA sequences. They can be hybridized by established procedures and immunochemically detected. As these probes are obtained by a chemical nonenzymatic reaction, it is possible to use ribo- as well as deoxyribo- and single- as well as double-stranded nucleic acids.

Such probes are characterized by high stability and they have retained their properties for more than 2 years at +4°C. AAIF-modified nucleic acids gave consistently better results than AAF-modified ones. This may be explained by higher thermal stability and/or by greater accessibility to the modified site by the antibodies. It has been shown that, unlike AAF residues, which are inserted between neighboring base pairs, the AAIF residues adopt an outside conformation because of the steric hindrance of the iodine atom (7, 17, 18). In our tests, hybridizations were usually done at 65°C, without formamide or dextran sulfate. Experiments have also been done in 50% formamide and 10% dextran sulfate at 37°C with equivalent results (data not shown). Using alkaline phospha-

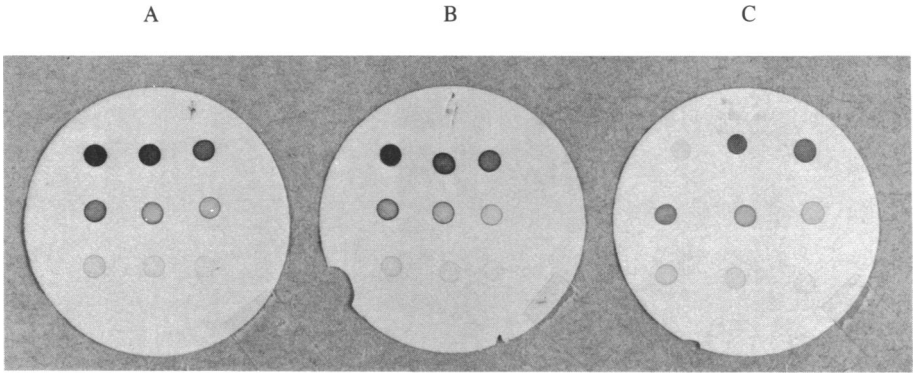


FIG. 5. (*A* and *B*) Nitrocellulose filters were spotted with various amounts of λ DNA (from 512 to 2 pg by sequential 1:2 reduction) and hybridized overnight at 65°C with AAIF-modified λ DNA. The probe concentrations were 0.5 (*A*) and 3 (*B*) μg/ml. Washing was done as indicated in the legend to Fig. 4 and the filters were immunostained. (*C*) The filter was spotted with 20 ng of λ DNA for the control dot (upper left) and 4p7-7 DNA (from 5 ng to 39 pg by sequential 1:2 reduction). It was hybridized at 65°C for 1 hr. The hybridization mixture contained 10% dextran sulfate. Single-stranded M13 DNA with the same insert as plasmid 4p7-7 was modified with AAIF (≈5% modified bases) and used as a probe at a concentration of 2 μg/ml. Washing and immunochemical staining were done as above.

tase-linked second antibodies we found the sensitivity to be in the picogram range. The sensitivity depended on the probe concentration and efficiency. The optimal concentration was found to be ≈500 ng/ml for double-stranded DNA and 40 ng/ml for single-stranded DNA. Tests were also done with bovine rRNA probes hybridized to cloned mouse 45S rDNA. Since divergences exist between bovine and mouse ribosomal sequences and ribosomal RNA can form hairpins on hybridization, this kind of RNA probe should not be compared for efficiency with DNA probes. However, the results obtained indicate that RNA immunonucleic probes can be used to detect homologous sequences. Contrary to radioactive probes, immunonucleic probes can be used at high concentration without significant background and immunochemical detection provides high resolution (Fig. 5). It was not necessary to use carrier DNA to saturate the filters. Techniques accelerating the renaturation rate together with high probe concentrations could prove useful for rapid detection of specific sequences (27–29). In immunochemical detection, we have found that better results are obtained when glycine is used in place of albumin in the Denhardt's solution. Glycine also gives good results with radioactive probes.

We have successfully used immunonucleic probes to screen phage plaques and bacterial colonies and to detect specific DNA sequences on Southern blots (unpublished work). Such probes can potentially be used to enrich specific gene sequences from complex mixtures.

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