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2-Acetylaminofluorene-modified Probes for the Indirect Hybridocytochemical Detection of Specific Nucleic Acid Sequences

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A new approach is presented for the indirect hybridocytochemical localization of specific nucleic acid sequences in microscopic preparations. The method is based on the application of probes modified with *N*-acetoxy-2-acetylaminofluorene. After hybridization, the 2-acetylaminofluorene-labelled probes are recognized by antibodies directed against modified guanosine and visualized immunocytochemically. This procedure has been optimized on two model objects: mouse satellite DNA in interphase nuclei and chromosomes, and kinetoplast DNA in *Crithidia fasciculata*. A first application that may be of clinical importance is given by the detection of human cytomegalovirus in infected human lung fibroblasts. Other potentials of this procedure are discussed. Its advantages are: (1) the simple, rapid and reproducible labelling procedure; (2) the high stability of both label and modified probes; (3) the feasibility of labelling both double-stranded (ds) and single-stranded (ss) probes (DNA as well as RNA); (4) the rapid and sensitive detection of hybrids.

In situ hybridization has proven to be a valuable method for the localization of specific cellular or chromosomal nucleic acid sequences (for a review see [1]). The method, originally described by Gall & Pardue [2], involves the annealing of polynucleotide probes to their complementary sequences in denatured microscopic preparations. Until recently, the probes were labelled with a radioisotope and the localization of the specific hybrid was accomplished by autoradiography.

Although this procedure is very sensitive and allows the detection of single copy genes on metaphase chromosomes [3, 4], it has several disadvantages. These include the limited resolution inherent in ^{3}H and ^{125}I autoradiography, due to the track of the decay particle and the thickness of the autoradiographic emulsion, and the relatively long exposure times. This led several investigators to look for alternative means for the detection of the target nucleic acid. So far three other types of label have been used for visualization, viz. fluorochromes like rhodamine (TRITC) and fluorescein (FITC) [5], cytochemically detectable enzymes such as peroxidase and phosphatase [6] for light microscopy, and colloidal gold in electron microscopic procedures [7].

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Various methods have been developed to provide the specific hybrids with these labels. Bauman and co-workers coupled the fluorochrome covalently to the 3′-terminus of the RNA probe [8]. In contrast to this direct approach, others used antibodies for the detection of the hybridized target sequences. For these indirect approaches some structural element in the hybrid has to function as an antigen. The method developed by Rudkin & Stollar [9] and optimized by Van Prooijen-Knegt et al. [10], involves the generation of RNA–DNA hybrids. In this case, the antigen is the hybrid itself, which is immunologically distinct from DNA–DNA and RNA–RNA duplexes. Specific antibodies raised in rabbits, were applied in combination with a fluorochrome-labelled anti-rabbit serum to enable specific detection of RNA–DNA hybrids by fluorescence microscopy. More recently, Ward and co-workers synthesized analogs of dUTP and UTP which contain a biotin molecule covalently attached to the C-5 position of the pyrimidine ring through a 'linker-arm' [11]. These analogs can be incorporated enzymically into the hybridization probes by a procedure similar to that for the production of radioactive probes. Visualization of the hapten is accomplished through an anti-biotin serum [6].

In this paper we describe another indirect procedure for in situ hybridization in which, following an idea originated from Dr P. Tchen, 2-acetylaminofluorenyl (AAF) groups are introduced chemically into the probe, to render it immunogenic. Covalent attachment of AAF groups can be achieved by treatment of the probes with *N*-acetoxy-2-acetylaminofluorene (*N*-AcO-AAF), a reactive derivative of the well-known and widely studied chemical carcinogen [12]. The major substitution product in RNA and DNA after in vitro reaction with *N*-AcO-AAF is the *N*-(guanin-8-yl)-AAF adduct [13, 14]. Under the conditions reported here, the number of AAF groups attached to double-stranded (ds) or single-stranded (ss) nucleic acids can be varied and controlled in a reproducible way. The AAF-labelled probes are stable for prolonged periods of time. The immunocytochemical detection of the hybridized probes is achieved by means of antibodies directed against AAF-modified guanosine (G-AAF) [15] and a fluorochrome-labelled second antibody.

The whole procedure, i.e., modification, hybridization and immunocytochemical detection, was tested and optimized on two objects: mouse satellite sequences in metaphase preparations of a mouse–human hybrid cell line, detected with satellite DNA or its complementary RNA (cRNA); and kinetoplast DNA (kDNA) in the single mitochondrion of the insect hemoflagellate *Crithidia fasciculata*, detected with kDNA or its cRNA. The procedure was subsequently applied to localize human cytomegalovirus (HCMV) in infected human embryonic lung fibroblasts.

MATERIALS AND METHODS

N-AcO-AAF was prepared according to a published procedure [16]. The compound was >95% pure, as judged by thin-layer chromatography on silica (DC-Plastikfolien Kieselgel 60 F254, Merck 5735) in chloroform/methanol (19 : 1).

Production of an anti-AAF Serum

Antibodies directed against the G-AAF adduct were obtained as descrbied [15]; briefly, the hapten G-AAF, formed by reaction of riboguanosine with *N*-AcO-AAF, was coupled to bovine serum albumin (BSA) by means of periodate oxidation, and rabbits were immunized with BSA-G-AAF according to the protocol. Antibody activity in the sera was tested in an enzyme-linked immunosorbent assay, in which in vitro prepared AAF-modified DNA was coated to polystyrene or polyvinylchloride assay cups [17]. The affinity constant of the rabbit antibodies towards dG-AAF was 3.2×10^9 mol^{-1} (this value was obtained in competitive radioimmunoassays carried out by courtesy of Drs Kriek and Van der Laken, Netherlands Cancer Institute, Amsterdam). Cross-reactivity with unmodified guanosine was found to be negligible. The antibodies did not discriminate between AAF-labelled ribo- and deoxyribonucleosides.

Nucleic Acid Preparations

Kinetoplast DNA of *C. fasciculata* was a gift from Mrs F. Fase-Fowler (Netherlands Cancer Institute, Amsterdam). Mouse DNA, purified from isolated liver nuclei, was fractionated by Hoechst 33258/CsCl centrifugation essentially as described [18]. The satellite DNA could be removed as a single fraction and was rerun to enhance its purity with respect to main band contamination. HCMV-DNA, extracted from purified virions, was obtained from Dr J. L. M. C. Geeien (Laboratorium voor Gezondheidsleer, University of Amsterdam).

For some experiments the DNA was degraded prior to the modification reaction by mild DNase I (Boehringer) treatment or by sonication for four times 1 min at 0°C with a Branson B-12 sonifier using the microtip at maximal amplitude. The size of the DNA fragments was determined by horizontal agarose gel electrophoresis. cRNA was synthesized in vitro with *E. coli* DNA-dependent RNA polymerase (Boehringer) [8].

Treatment of Nucleic Acids with N-AcO-AAF

DNA and cRNA probes were treated with *N*-AcO-AAF according to [13], with minor modifications. Incubation occurred in a mixture containing 0.2 mg/ml DNA or cRNA, 1.6 mM sodium citrate of pH 7.0, and 20% (v/v) ethanol. The carcinogen was added from a concentrated stock solution in dimethyl sulfoxide (DMSO) to a final DMSO concentration of 0.8% (v/v). The mixture was kept in the dark at 37°C for 1 h. Purification of the AAF-modified probes was accomplished by extraction with water-saturated phenol (1×) and three times with water-saturated diethylether. The nucleic acids were precipitated with 2.5 vol of ethanol, after addition of 0.1 vol of 2.5 M sodium acetate of pH 5.5. The precipitate was collected by centrifugation, washed in 70% ethanol, and dissolved in 10 mM Tris-HCl, 1 mM EDTA, of pH 7.6. *N.B. N*-AcO-AAF is considered to be a carcinogen and should be handled, stored and discarded with due caution. The treatment of probe nucleic acids with this compound should be carried out in a well-ventilated chemical fume hood. Phenolic and ethereal extracts should be treated as carcinogenic waste. Glassware can be decontaminated by immersion in a bichromate/sulfuric acid solution. Up to the stage when the AAF-modified probes are precipitated, all pipette tips and gloves used should be disposed of properly.

Measurement of the Extent of AAF Modification

Three independent methods were used to determine the degree of AAF modification. Method (*a*) is based on the fact that the UV-spectrum of AAF-modified nucleic acid shows a characteristic shoulder at 305 nm, superimposed on the nucleic acid spectrum which shows a maximum at 260 nm [19]. The ratio A305/A260 allows accurate determination of modification levels down to about 2% AAF-modified bases. Because this method requires relatively large amounts of probe, it was performed on samples of calf thymus DNA (Worthington) which had been modified in parallel incubations.

Method (*b*) involves the addition of a trace amount of *N*-acetoxy-2-acetylamino[7(*n*)-^{3}H]fluorene (*N*-AcO-^{3}H-AAF, 21 Ci/mmol, Amersham) to the coupling mixture to follow the rate of incorporation. Samples, taken at the start of the incubation and from the dissolved DNA or RNA precipitate, were counted in glass vials containing toluene-PPO-POPOP. This simple and rapid procedure permits the measurement of low levels of AAF modification in small quantities (less than 1 μg) of probes. *N.B.* Our batch of *N*-AcO-^{3}H-AAF proved to be rather instable.

The reliability of the optical and radioactive tracer methods was assessed by analysing an enzymic

digest of the control samples of AAF-modified DNA by means of high-performance liquid chromatography (HPLC) (method *c*). The modified DNA was degraded with DNase I, Pl nuclease, and bacterial alkaline phosphatase (Boehringer) as described [20]. The hydrolysate was analysed on an RSil cation exchange column (Alltech; 25×0.6 cm) eluted at 1 ml/min at 60°C with 0.05 M ammonium formate of pH 4.25, containing 20% methanol, in combination with a Beckman 112 solvent delivery system equipped with a 165 variable wavelength detector and a Spectraphysics SP 4100 computing integrator. Under the conditions described here, optimal separation can be achieved between the four common deoxyribonucleosides and dG-AAF.

Preparations of Microscopic Slides

Slides with *C. fasciculata* cells were prepared as in [5]. Metaphase chromosome spreads were obtained from cultures of a mouse–human hybrid cell line PGME-4 as described [10]. This somatic cell hybrid, containing a varying subset of human chromosomes (up to 10) in a mouse background, was kindly supplied by Professor P. L. Pearson (Department of Anthropogenetics, Medical Faculty, University of Leiden). HCMV-infected human embryonic lung fibroblasts, grown on coverslips, were received from Professor J. Versteeg (Department of Medical Microbiology, University of Leiden).

Before the hybridization mixture was applied, slides were pretreated as previously published [10], with minor modifications. After RNase incubation (1 h at 37°C) to remove endogenous RNA, chromosomal DNA was denatured by incubation in 0.15 N NaOH in 70% ethanol for 5 min. Instead of aqueous alkali (0.07 N), alcoholic alkaline was used to keep the DNA fixed during denaturation. We found this step to be critical with respect to preservation of the morphology. Slides were then dehydrated through an ethanol series, air-dried, treated with proteinase K (0.25 μg/ml, 2 mM $CaCl_2$, 20 mM Tris-HCl, of pH 7.4) for 15 min at 37°C and dehydrated again. To obtain optimal results, the concentration of proteinase K was sometimes slightly varied depending on the biological object under investigation.

Hybridization with AAF-modified Probes

The hybridization mixture containing 15 μg AAF-modified DNA and 1.5 mg salmon sperm DNA, per ml of 50% formamide, 2×sodium saline citrate (SSC; 0.15 M NaCl and 15 mM sodium citrate, of pH 7.0), was denatured by incubation at 70°C for 5 min prior to use. Five μl of this solution was placed on a slide and sealed with a coverslip after which hybridization was carried out at 34°C (that is 3°C below the hybridization temperature (T_h) used for unmodified DNA probes) for 12 h in a moist chamber. The slides were then washed three times 20 min in 50% formamide/2×SSC and three times 10 min in 2×SSC at room temperature.

When AAF-modified RNA was used as a probe, the hybridization procedure was carried out with 15 μg RNA and 1.5 mg *E. coli* tRNA per ml of 70% formamide/3×SSC at 31°C (T_h−3°C) for 12 h. In this case the first three washes were performed in 70% formamide/3×SSC, followed by three washes in 3×SSC.

Detection of the Hybridized Probes

After the washings in 2×SSC or 3×SSC, the slides were rinsed in phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, of pH 7.2) for 5 min and incubated with 30 μl of rabbit-anti-AAF serum diluted in PBS (1 : 30) containing 2% non-immune goat serum (NGS; Nordic), for 1 h at 37°C in a moist chamber. Following three washings in PBS for 5 min each, 30 μl of a rhodamine-labelled goat-anti-rabbit serum (GAR-TRITC, Biochemical Corp.), diluted 1 : 80 in PBS containing 2% NGS, was applied and incubated as above. The preparations were washed again with PBS (three times 5 min), dehydrated in 70 and 90% ethanol both containing 0.3 M ammonium acetate and finally in 100% ethanol, and air-dried.

The slides were embedded in 30 μl 10 mM Tris-HCl, pH 7.8, 40% ethanol (v/v) containing per ml 1 μg 4′,6-diamidino-2-phenyl-indole (DAPI) as a DNA counterstain and 50 mg *n*-propyl gallate (Sigma) to reduce fluorescence photobleaching [21]. Microscopic observation was performed with a DIALUX microscope (Leitz) using epi-illumination from an HBO 200 W mercury arc (Osram). The DAPI fluorescence was visualized with a 4 mm UGl excitation and an LP435 emission filter combination with a 400 nm dichroic mirror. For the red rhodamine fluorescence an SP560 + BG38 + 2 mm LP530 excitation and an LP590 emission filter combination with a 580 nm dichroic mirror were applied.

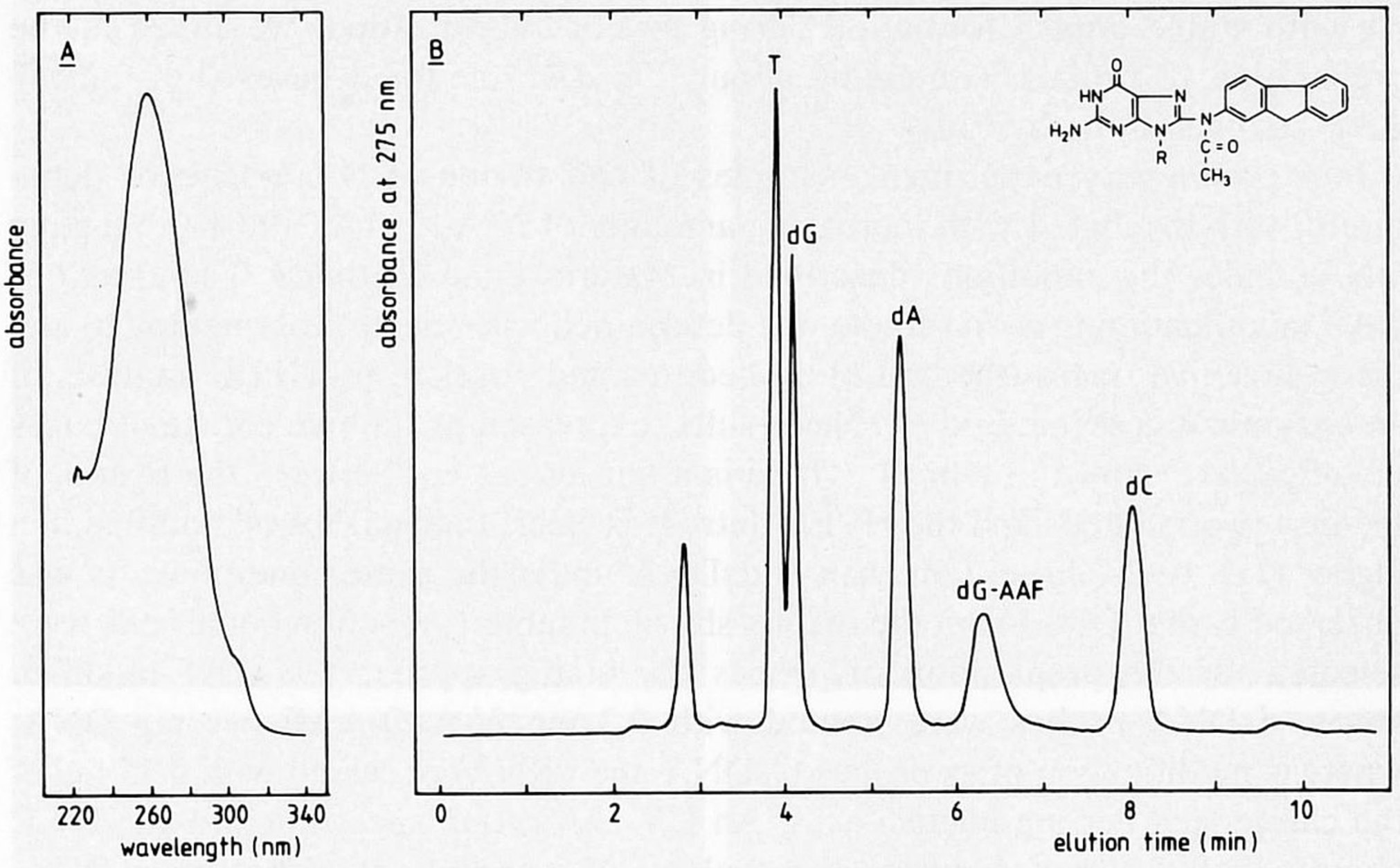


Fig. 1. Analysis of AAF-modified DNA to determine the extent of AAF-modification. Calf thymus DNA was treated with *N*-AcO-AAF (0.3 mg/mg DNA) under conditions described in Materials and Methods. The isolated AAF-modified DNA was heat-denatured (10 min, 100°C) and an UV absorption spectrum was recorded (*A*). The extent of the AAF modification could be determined from the A305/A260 ratio (method *a*). Another aliquot of the AAF-modified DNA was digested with enzymes and analysed on an HPLC cation exchange column (method *c*; see Materials and Methods). (*B*) shows the elution profile, recorded at 275 nm. The relative amount of dG-AAF was calculated from the peak areas, the dC peak being taken as an internal standard. When DNA is treated with excess *N*-AcO-AAF, the dG peak is absent from the HPLC profile of the corresponding enzymic digest (not shown). The dG-AAF peak in this elution pattern was assumed to represent a modification level of 20%. The material eluting at 2.9 min is not retained on the column. Inset in (*B*) shows the chemical structure of dG-AAF (R, deoxyribose).

Photographs were taken on 35 mm Kodak Technical Pan 2415 films, which were pre-exposed to white light to enhance recording capability [22]. Exposure times were 1 sec or less for DAPI fluorescence and up to 4 min for rhodamine fluorescence.

RESULTS

Modification of Nucleic Acids with N-AcO-AAF

Treatment of nucleic acids with *N*-AcO-AAF produces mainly the chemically stable *N*-(guanin-8-yl)-AAF adduct (fig. 1*B*, inset). In native DNA, the presence of this adduct impairs the stability of the double helix. With increasing amounts of modified guanines, a linear decrease of the melting temperature (T_m) is observed [23, 24]. In dsDNA, substitution of 5% of the bases with AAF groups would lead to a decrease in the T_m of about 5°C. Assuming that this level of modification, when present in the probe DNA or cRNA, would still be compati-

ble with stable duplex formation during in situ hybridization, we aimed at the preparation of probes, containing about 5% AAF-modified bases (i.e. ±2.5% AAF adducts in the hybrids).

In a preliminary experiment, samples of calf thymus DNA (native or denatured) were incubated with increasing amounts of *N*-AcO-AAF (0.04–0.6 mg/mg DNA) under the conditions described in Materials and Methods. The extent of AAF modification in each sample was determined with the optical (method *a*) and the radioactive tracer (method *b*) procedures and checked by HPLC analysis of an enzymic digest (method *c*). The results, expressed as the percentage of bases modified, are shown in table 1. There is a fair agreement between the results of the first two methods and the HPLC data. It is clear, that ssDNA is modified to a higher (1.5- to 2-fold) extent than is dsDNA under the same conditions, as was observed earlier [13]. From the results shown in table 1, reaction conditions were selected for the preparation of probes containing approx. 5% AAF-modified bases. dsDNA probes were treated with 0.3 mg *N*-AcO-AAF per mg DNA, whereas modification of ss probes (ssDNA and cRNA) occurred with 0.15 mg of the carcinogen per mg nucleic acid. An UV absorption spectrum and an HPLC elution profile obtained during the analysis of a sample of calf thymus DNA, treated with 0.3 mg *N*-AcO-AAF per mg DNA, are shown in fig. 1 *A*, *B*).

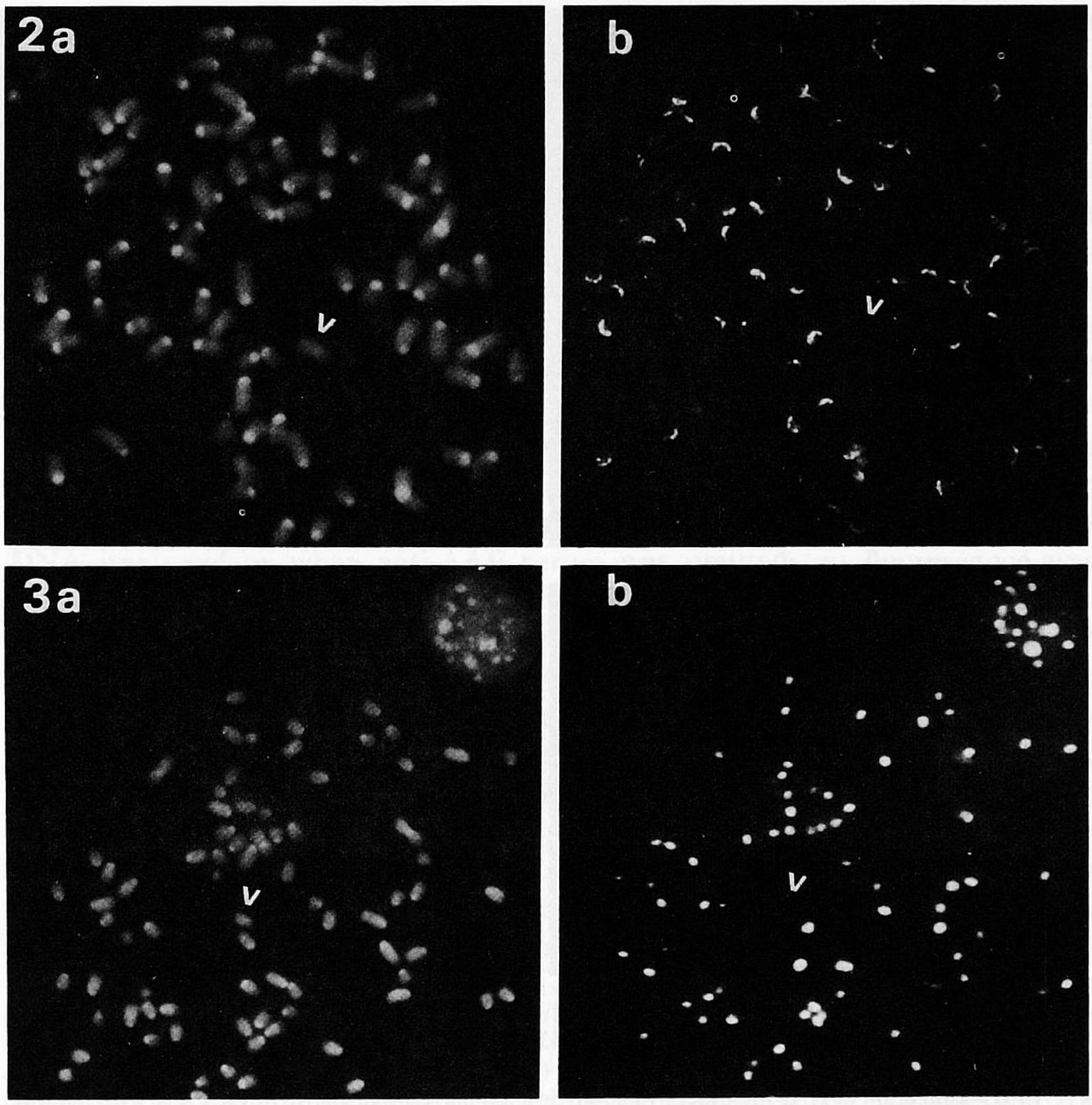
In situ Hybridization and Immunocytochemical Detection

dsDNA probes. Initial experiments with mouse satellite DNA, AAF-labelled in the ds-form to a level of about 5% modification and hybridized with metaphases from the mouse–human hybrid cell line PGME-4, resulted in rather weak signals

Table 1. *Extent of AAF-modification in N-AcO-AAF-treated DNA*

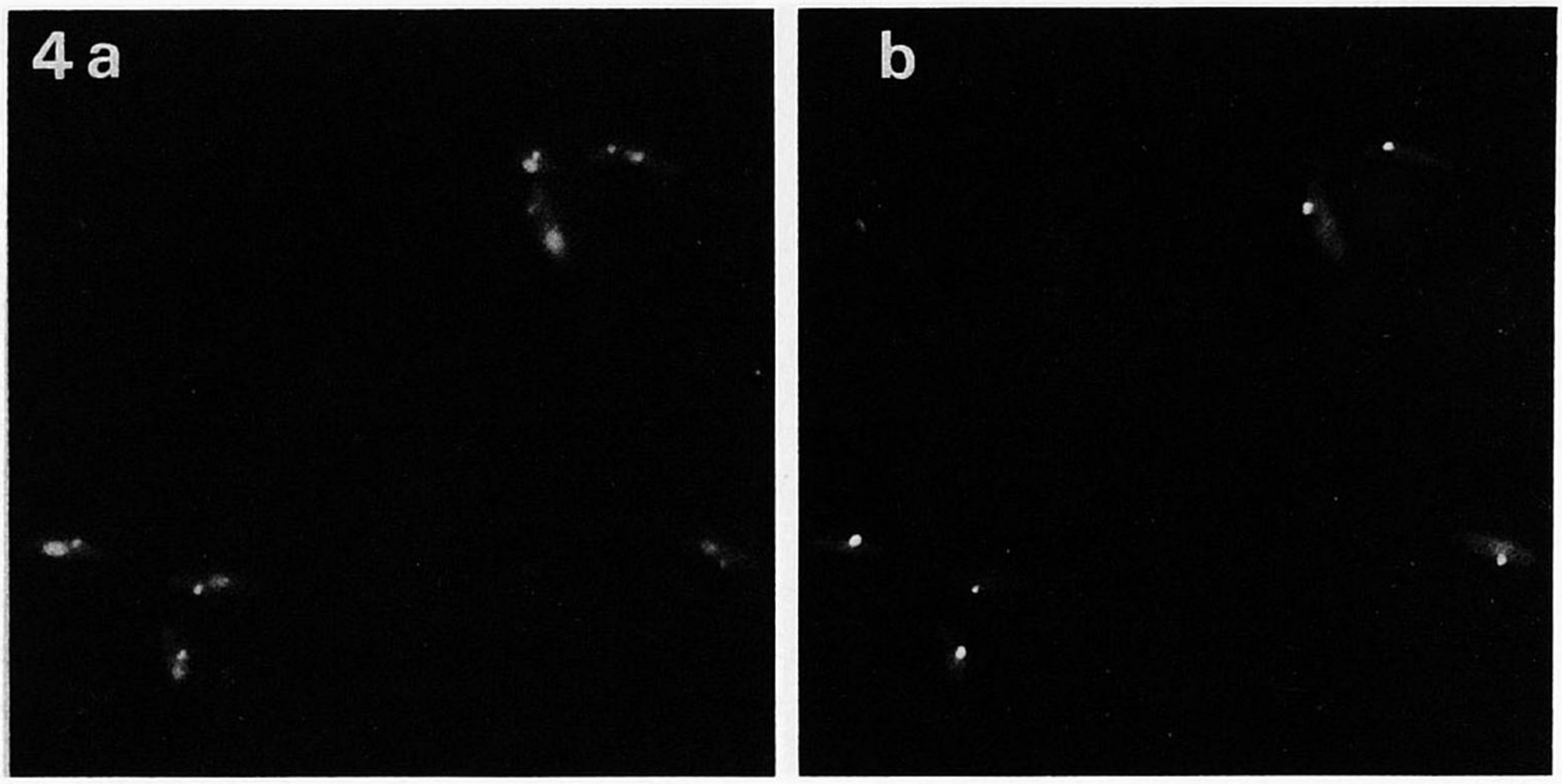
Amount (mg) of *N*-AcO-AAF added per mg DNA[a]	Percentage of AAF-modified bases[b]			
	Native DNA			Denatured DNA[c]
	Method *a*	Method *b*	Method *c*	Method *a*
0.6	12	11	12.8	16
0.3	6	6	5.3	10
0.15	3	3	2.7	5
0.08	ND[d]	1	1.4	3
0.04	ND[d]		0.6	ND[d]

[a] Reaction conditions are given in Materials and Methods.
[b] The percentage of AAF-modified bases was calculated from the A305/A260 ratio of the AAF-modified DNA in single stranded from (method *a*), after the addition of *N*-AcO-^{3}H-AAF as a tracer (method *b*) or from the HPLC elution pattern or an enzymic digest of the AAF-modified DNA (method *c*).
[c] Denatured DNA was obtained by heating the DNA solutions (10 min, 100°C), followed by rapid cooling on ice.
[d] ND, not detectable.



Figs 2, 3. Hybridization of mouse satellite DNA, modified in ds form with *N*-AcO-AAF to a level of about 5%, to metaphase chromosomes from the mouse–human hybrid cell line PGME-4 before (fig. 2) and after (fig. 3) optimalization of the conditions. (*a*) The (blue) DAPI; (*b*) the (red) specific TRITC fluorescence of the same field. *Arrow* indicates one unlabelled human chromosome. *Fig. 2*, ×630; *fig. 3*, ×400.

of specific fluorescence, typically confined to the edges of the target chromosome parts. An example is shown in fig. 2*b*. The total chromosome complement of PGME-4 is visible in fig. 2*a*: the acrocentric mouse chromosomes (except for the Y-chromosome) are easily discernible from the metacentric human chromosomes. The corresponding TRITC fluorescence pattern of the same metaphase pictured in fig. 2*b* shows that only the edges of the mouse chromosomal centromeric regions are stained. Similar results were obtained with kinetoplast DNA, labelled in ds configuration with *N*-AcO-AAF to an extent of 5% and hybridized



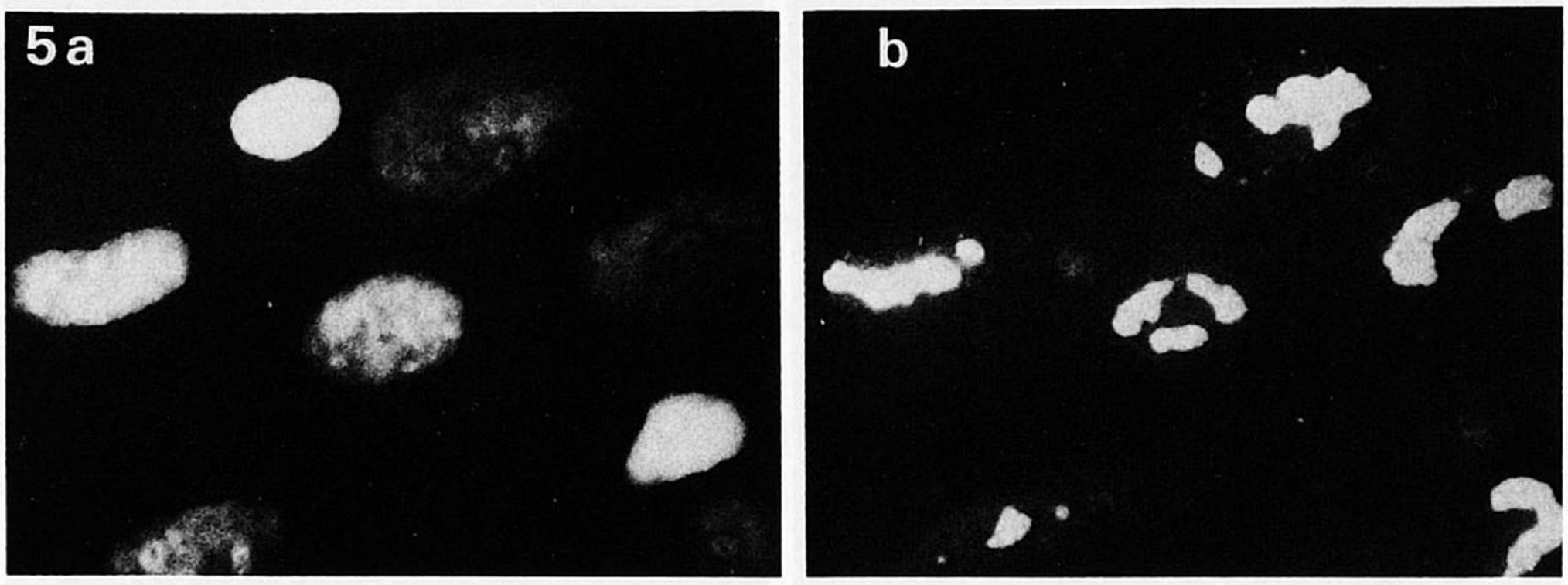


Fig. 4. Hybridization of kinetoplast DNA, modified in ds form with *N*-AcO-AAF to about 5%, to *Crithidia fasciculata* cells. With DAPI, both the kinetoplasts and the nuclei are visible (*a*). Only the kinetoplasts are labelled specifically with TRITC (*b*). ×750.
Fig. 5. Hybridization of AAF-modified (to about 5%) human cytomegalovirus cRNA to a preparation of virus-infected human embryonic lung fibroblasts. With DAPI, the nuclei are all visible (*a*). Some of them are labelled specifically with TRITC (*b*). ×400.

to fixed trypanosomes. Again only the edges of the compact kDNA in the single mitochondrion of these unicellular flagellates showed specific signals (not shown). This weak edge-labelling contrasts sharply with the uniform fluorescence that we obtained with the anti-RNA–DNA hybrid (or the anti-biotin) method performed on the same objects. Moreover, hybridization of AAF-modified cRNA yielded also strong uniform fluorescence at the target sequences (see below). An important difference between the initial hybridization experiments with the AAF-labelled DNA and the other experiments is the (ss) size of the probes: 40–200 nucleotides for cRNA [25] and 300–600 nucleotides for the nick-translated biotin-labelled probes [26] compared with the much larger native DNA used for AAF modification. To see whether the size of the AAF-modified DNA prevented the

probe from penetration into the internal regions of the chromosome, the probe DNAs were treated with DNase I or sonicated prior to the modification reaction. Both treatments resulted in a drastic increase of the specific fluorescence.

To further optimize the conditions for hybridization and immunocytochemical detection, the extent of AAF modification, the concentration of the probe, hybridization temperature and the amounts of anti-AAF serum and TRITC-conjugated second layer antibodies were varied. The specific signals obtained were compared by visual inspection of the fluorescence intensities. These data led to the protocol specified in Materials and Methods. Figs 3 and 4 show the results obtained with AAF-modified mouse satellite DNA hybridized to a PGME-4 metaphase, and AAF-labelled kDNA, applied to fixed trypanosomes, respectively. No significant hybridization is observed with the human chromosomes or with mouse chromosome parts that do not contain satellite sequences (fig. 3) or with the nuclear DNA of *C. fasciculata* (fig. 4). When AAF-modified heterologous DNA was used or when anti-AAF serum was omitted, no fluorescence was observed (not shown).

ssDNA and RNA probes. These optimized conditions were then used for in situ hybridization of *N*-AcO-AAF-treated ss probes (ssDNA and cRNA) modified to a level of about 5%. Both single-stranded nucleic acid probes hybridized specifically to their corresponding cellular or chromosomal DNAs. The intensity of the fluorescence signals was comparable with those obtained with the (size-reduced) dsDNA probes (results not shown).

Detection of HCMV sequences in infected cells. HCMV, a member of the herpes virus group, is associated with a wide spectrum of diseases and a variety of clinical manifestations [27]. Fig. 5 shows the results obtained with this procedure on preparations of human fibroblasts which had been infected in vitro with HCMV. The preparations were hybridized with AAF-labelled (5%) HCMV cRNA. Specific TRITC spots are clearly visible within the blue colored nuclei. Some nuclei remain unlabelled, probably because they are not infected, or contain an amount of virus DNA that is below the detection limit of this procedure. The specificity of the localization of HCMV sequences was tested with non-infected human fibroblasts hybridized with the same probe. Apart from a faint cytoplasmic background fluorescence, caused by non-specific binding of TRITC molecules, no specific signal was observed.

DISCUSSION

In this paper a novel method is described to localize in situ hybridized polynucleotide probes in microscopic preparations. The method combines a simple chemical labelling of probe DNA or cRNA with AAF groups, and the sensitive detection of the hybridized target by means of highly specific anti-AAF antibodies and fluorochrome-labelled second antibodies. We aimed at a compromise

between a high labelling level (in order to optimize immunocytochemical visualization) and a low modification level in order not to hamper stable duplex formation during the hybridization procedure. For the two objects tested an average of 5 G-AAF molecules per 100 nucleotides was found to be optimal: a higher extent of modification (10–12%) did not improve the intensity of the immunofluorescence results, while the use of probes with a lower degree of labelling (±2%) resulted in weaker signals. Because of their chemical stability, AAF-modified probes can be stored frozen for prolonged periods of time without loss of integrity.

The procedure has been optimized on two relatively easy objects: the localization of the highly repeated mouse satellite DNA [28] and the DNA in the kinetoplasts of *C. fasciculata* cells [29]. The results show specific localization with a resolution that is superior to that obtained so far with light microscopic autoradiographic procedures. Its sensitivity in terms of specific signal per unit length of hybridized probe, however, does not yet reach the level obtained with radioactive probes. Attempts are made to enhance the sensitivity of the method through network formation, when cloned material is used as a probe [3]. Work is in progress to refine this catenation procedure. Also at the level of the immunochemical detection, enhancement of the specificity of the signal is pursued through the use of monoclonal antibodies against G-AAF. The unique specificity and purity of such antibodies ensures a low non-specific antibody binding. Recently, we were able to isolate a high-affinity monoclonal anti-G-AAF antibody.

Apart from the potential disadvantages such as a lowered stability (at high modification levels) of the formed duplexes, and the carcinogenicity of *N*-AcO-AAF, which asks for careful experimentation during the modification procedure, this new method possesses a number of advantages over other hybridocytochemical procedures using non-radioactive markers described so far.

(1) The modification procedure is relatively easy and fast. Kinetic studies of the reaction between nucleic acids and *N*-AcO-AAF have shown that the amount of fixed carcinogen reaches a plateau within 20–30 min of incubation [30]. Our experiments show that this plateau value is proportional to the initial concentration of *N*-AcO-AAF (see table 1), which implies that probes with a defined degree of modification can be obtained in a reproducible way. The exact extent of AAF modification can be easily determined optically or by scintillation counting; both these determinations are reliable, as judged by comparison with the results of the HPLC method.

(2) The fact that ssDNA as well as dsDNA and RNA can be modified extends the applicability of the method presented here. In the direct method [8] only RNA probes can be used, because no method is available at present to couple a fluorochrome label to DNA. In the anti-RNA–DNA procedure [10] only one type of nucleic acid probe can be used because the localization of DNA targets requires the use of RNA probes, and vice versa. The indirect method involving

the enzymic production of biotinated ss probes [6] is not as efficient as the labelling of ds probes by nick translation.

(3) The AAF adducts are strong immunogens. Antisera with high affinity constants have been elicited by several groups (for a review see [31]). The relatively easy generation of antibodies against G-AAF contrasts with the more difficult production of the anti-RNA–DNA antibodies. So far only two groups [9, 10] succeeded in obtaining an adequate anti-hybrid serum.

(4) The strong fluorescence obtained with both types of objects—especially in mouse nuclei—allows cytofluorometric measurement of the specific signals and comparison of the results of the four different in situ hybridization procedures involving fluorescence detection. In our hands, the anti-AAF method for both objects proved to be the most sensitive in terms of intensity of the fluorescent spots (unpublished results).

The present hybridocytochemical method can be applied for, e.g., the diagnostic detection of virus infection, as is demonstrated by the results obtained with HCMV-infected human fibroblasts. Preliminary results indicate that this technique can be used not only in routine histopathological diagnosis (Raap et al., in preparation), but that it can become a valuable tool in molecular biology. Possible applications include the processing of Southern blots, colony filter hybridization, and purification of mRNA and (cloned) DNA sequences.

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Note Added in Proof

Tchen et al. recently informed us that they were also able to demonstrate the applicability of AAF modification of probes for hybridization purposes (Proc natl acad sci US, in press).

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