Arylamine *N*-acetyltransferase as a potential biomarker in bladder cancer: fluorescent *in situ* hybridization and immunohistochemistry studies

Michael Stacey, Peter Thygesen, Lesley Stanley, Nada Matas, Angela Risch and Edith Sim

Arylamine *N*-acetyltransferase isoenzymes NAT1 and NAT2 are encoded at two polymorphic loci on human chromosome 8p22. The two loci have previously been identified using chimeric Yeast Artificial Chromosome (YAC) clones encoding either *NAT1* or *NAT2* as probes for metaphase chromosomes using fluorescent *in situ* hybridization. The 8p22 region has been demonstrated to be deleted in highly invasive bladder tumours and since NAT isoenzymes participate in the metabolism of arylamine bladder carcinogens, it is important to determine whether *NAT1* and *NAT2* gene loci are included in the region of deletion. We describe here the application of a cosmid clone for *NAT2* as a biomarker for Fluorescent *In Situ* Hybridization (FISH) on interphase nuclei of exfoliated bladder cells. We also describe a 70kb probe for *NAT1* which is a candidate for a suitable biomarker for use in similar FISH studies. Immunohistochemical staining of bladder tumour sections with a polyclonal anti-peptide antibody specific for the NAT1 isoenzyme as a biomarker for NAT1 protein expression is also shown.

Keywords: *arylamine N-acetyltransferase, bladder epithelial cells, fluorescent* in situ *hybridization, chromosome 8.*

Introduction

In humans, arylamine *N*-acetyltransferases, NAT1 and NAT2, catalyse the acetylation of a range of arylamines and hydrazines (Grant 1993). NAT2 is responsible for the classical acetylator polymorphism demonstrated for example with isoniazid (Price-Evans 1960) and the relationship between multiple alleles at the *NAT2* locus and functional activity has been identified (Blum *et al.* 1991, Deguchi 1992, Hickman *et al.* 1992). NAT1 has also been demonstrated to show functional variation (Cribb *et al.* 1991, Ward *et al.* 1992, Weber and Vatsis 1993) although it is not yet certain whether this is as a result of inheritance of specific allelic variants (Vatsis *et al.* 1995) of *NAT1* which have been identified. The two functional isozymes are 85% identical at the amino acid level (Vatsis *et al.* 1995) but nevertheless show differences in substrate profile and in tissue expression. All of the substrates of NAT2 which have been identified are xenobiotics and include isoniazid, procainamide and sulphamethazine (Ohsako and Deguchi 1990) as well as aromatic and heterocyclic arylamine carcinogens (Hein *et al.* 1993). NAT2 is found in liver (Ohsako and Deguchi 1990), probably in hepatocytes (Coroneos and Sim 1993). There have also been reports of NAT2 expression in other cell types including liver phagocytes (Govier 1965), intestinal epithelium (Turesky *et al.* 1991) and bladder cell lines (Kloth *et al.* 1994). NAT1 has a much wider tissue distribution. It is found in liver (Ohsako and Deguchi 1990) and has also been demonstrated in both red blood cells (Ward *et al.* 1992) and peripheral blood leucocytes (McQueen and Weber 1980, Cribb *et al.* 1991), as well as homogenates of many tissues (Pacifici *et al.* 1986) and in cell lines (Coroneos *et al.* 1991). NAT1 catalyses the acetylation of arylamines including *p*-aminobenzoic acid (*p*-aba) and sulphamethoxazole (Cribb *et al.* 1993) and, in common with NAT2, arylamine carcinogens such as benzidine and aminofluorene. It has been suggested that the range of acetylation activities which are catalysed by the two human NATs differ in that with certain hydroxyarylamine substrates only NAT2 catalyses the *O*-acetylation to form reactive *N*-acetoxyesters (Hein *et al.* 1994, Wild *et al.* 1995). An interesting observation in view of the widespread tissue distribution of NAT1 is that it may also catalyse the acetylation of an endogenous substrate. A breakdown product of folate, a vitamin which is found in all tissues, is *p*-aminobenzoylglutamate (*p*abaglu) (Minchin 1995, Ward *et al.* 1995). This compound has been demonstrated to be *N*-acetylated by NAT1 but not by NAT2 (Minchin 1995) and the product *N*-acetyl*p*abaglu is a naturally occurring breakdown product of folate which is found in urine (McPartlin *et al.* 1993). Therefore there may be subtle differences in the roles of NAT1 and NAT2 as xenobiotic metabolizing enzymes.

There has been a wide interest in the role of the genetic variation in an individual in response to a xenobiotic challenge. In colorectal cancer it has been reported that fast acetylators for the NAT2 isozyme are at increased risk and recent studies have suggested that this is particularly the case for individuals who also carry a mutation in the non-coding region of *NAT1* (Bell *et al.* 1995). Polymorphism at the *NAT2* locus has been shown repeatedly to be important in bladder cancer where the tissue specificity of tumour development following arylamine exposure has been very well documented for over a century (Rehn 1895). There is now general agreement that in Caucasian individuals exposed to arylamines, either occupationally (Cartwright *et al.* 1982, Risch *et al.* 1995) or from smoking (Risch *et al.* 1995), that individuals with slow NAT2 activity are at increased risk of bladder cancer. The situation in different ethnic groups, particularly orientals, may be different (Hayes *et al.* 1993). There have been attempts also to correlate exposure through identification of adducts in blood and urine with slow acetylation to compile a profile of risk (Vineis *et al.* 1994).

There has been speculation on the molecular basis of the tissue specificity of arylamine-induced bladder carcinogenesis which may rely on a competition between glucuronidation and acetylation favouring glucuronidation in slow acetylators (for review see Smith *et al.* 1995). In the bladder, DNA damaging arylitrenium ions may be generated following spontaneous acid

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hydrolysis or beta-glucuronidase attack on the glucuronide conjugate. From other approaches, researchers looking for tumour suppressor genes have searched for regions of deletion in tumours using the loss of individual copies of microsatellite markers now mapped throughout the genome. From these studies it is clear that bladder tumours demonstrate deletions in several different chromosomal regions, as would be expected from a multi-factorial disease (Sandberg and Berger 1994). However, it is intriguing that one of the regions of deletion is around 8p22 (Knowles *et al.* 1993, Spurr *et al.* 1995) where *NAT1* and *NAT2* genes are located. In order to investigate the relationship between the *NAT1* and *NAT2* loci and the deletion already found at 8p22, we describe here the application of tools which can be used in these studies and also describe NAT-isotype specific antibodies which can be used in parallel studies on bladder tumour sections.

The advent of FISH has revolutionized analysis of the genetic composition of cells (Buckle and Rack 1993). Fluorescently labelled DNA specific probes constructed from cosmid or YAC vectors can be used to localize specific chromosomes or genes within a cell *in situ*. Cells can be at interphase or metaphase, but because of difficulties in obtaining good divisions from tumour material, interphase cells are more often used, where both aneuploidy and gene rearrangement may be identified (Reid *et al.* 1992, Stock *et al.* 1994).

FISH has recently been used to help identify specific chromosomal aneuploidy with the progression of bladder cancer in exfoliated bladder cells (Sandberg 1992). The approach described here will allow investigation of the link between the staging of bladder tumours and the presence of specific *NAT* genes. The level of protein expression encoded by *NAT* genes can be compared in parallel.

MATERIALS AND METHODS

Preparation of interphase cells

Human peripheral blood mononuclear cells were prepared by conventional cytogenetic techniques as previously described (Stacey *et al.* 1995). Urothelial cells were prepared from 50 ml of fresh urine by centrifugation at 1500 *g* for 10 min at 4 °C. Cells were washed in 75 mM KCl, pelleted and finally resuspended in 400 µl 75 mM KCl. A 200 µl sample of this suspension was loaded on to a Shandon cytospin II centrifuge, and the cells spun on to APES (3-aminopropyltriethoxysilane) coated microscope slides at 1500 rpm for 5 min. Slides were air dried and fixed in a solution of 70 % 75 mM KCl: 30 % fixative (3:1 methanol: glacial acetic acid) for 5 min. Fresh fixative was added to excess and the slides dried at 65 °C. Slides were stored desiccated at – 20 °C until use.

Fluorescence in situ hybridization (FISH)

Cosmid DNA was prepared from *Escherichia coli* transfected with *NAT2* cosmid (Franke *et al.* 1994). *NAT2* cosmid DNA (500 ng) was nick translated in the presence of Biotin-16-dUTP (Boehringer, Mannheim) following the manufacturer's instructions. The reaction was stopped by heating the mixture to 70 °C for 10 min. The biotinylated probe was stored at – 20 °C.

Exfoliated bladder cells were hybridized in situ with biotinylated *NAT2* cosmid and biotinylated human chromosome 8 DNA simultaneously as follows. Slides were dehydrated through an ethanol series, denatured in 70 % formamide/0.3 M NaCl, 0.03 M trisodiumcitrate (2XSSC) at 70 °C for 2 min, dehydrated through an ice-cold ethanol series and prewarmed to 37 °C. *NAT2* cosmid DNA (90 ng), human chromosome 8 DNA (1.0 ng) (Oncor) and human cot DNA (2 µg) (Gibco BRL) were precipitated together and resuspended in 11 µl hybridization fluid (50 % formamide, 2XSSC, 10 % Dextran sulphate). Probe DNA was denatured at 70 °C for 5 min and applied directly on to prewarmed slides. Hybridization took place at 37 °C for approximately 20 h. Post-hybridization washes were at 45 °C in 50 % formamide/2XSSC (3X), 4XSSC/0.05 % Tween 20 (1X) and one wash in 4XSSC/0.05 % Tween 20 at room temperature. The biotinylated probes were detected using fluorescein isothiocyanate (FITC) conjugated avidin (2.5 µg ml^{-1}) (Vector Laboratories) and amplified with biotinylated anti-avidin (5 µg ml^{-1}) (Vector Laboratories) followed by a further round of FITC conjugated avidin DCS, each for 20 min. Cells were counterstained with propidium iodide (1 µg ml^{-1}) (Sigma) and viewed under a Zeiss Axioscope fluorescence microscope.

Restriction mapping of the ICI YAC clone 10Df9 encoding NAT1

YAC DNA was prepared in agarose plugs for the CHEF (Clamped Homogeneous Electric Field) pulsed field gel electrophoresis apparatus (Bio-Rad) using the method of Mendez *et al.* (1994). For restriction endonuclease digestion (Burke *et al.* 1987) agarose plugs, were washed with 5 ml of 10 mM Tris–NCl, 100 mM EDTA, pH 8.0 twice at 50 °C and twice at 20 °C. Each wash was for 30 min. The plugs were then equilibrated in restriction endonuclease buffer at 37 °C, 16 h. Restriction enzymes (30 units) were used to digest the YAC DNA in the plugs in a total volume of 150 µl per plug using the appropriate restriction enzyme buffer (New England Biolabs). The solutions also contained 5 mM spermidine and bovine serum albumin to a final concentration of 100 µg ml^{-1}. Incubation was at 37 °C for 4 h (BssH I, Ksp I, Mlu I) or 16 h (Sal I).

Agarose plugs containing YAC DNA were loaded on a 1 % (w/v) CHEF-grade agarose (Bio-Rad) gel in 45 mM Tris-borate, 1 mM EDTA pH 8.3 (0.5 × TBE) and sealed with low melting point agarose. Digested fragments were separated on a CHEF-DR II pulsed-field electrophoresis system (Bio-Rad) running 20 s–50 s switch times ramped over 24 h at 200 V in 0.5 × TBE at 6 °C. The gel was stained with 1 µg ml^{-1} ethidium bromide in water for 30 min, destained in water for 20 min and the DNA fragments were visualized by UV transillumination. Undigested intact YAC DNA could be identified directly on the gel as a band additional to the yeast chromosomal bands.

Southern blotting of the separated YAC DNA digests

The DNA in the pulsed-field electrophoresis gel was UV-nicked, denatured and blotted on to a nylon membrane (Hybond-N+, Amersham) by capillary transfer for 48 h using 0.4 M NaOH as transfer buffer (Brown 1991). A *NAT1*-specific probe was generated by amplification of genomic DNA in a polymerase chain reaction mixture using Nat-Hu20 and Nat-Hu22 as sense and antisense primers respectively and labelled with [α-^{32}P]dCTP (30 mCi) using random priming (Boehringer) to a final specific radioactivity of 1.6×10^6 cpm ml^{-1} (Hickman *et al.* 1994).

The membrane was prehybridized for 4 h at 65 °C in 30 ml hybridization buffer (5 × SSPE (0.75 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.7), 5 × Denhardt's reagent, 0.1 % (w/v) SDS and sonicated herring sperm DNA 200 µg ml^{-1}). The labelled probe (400 ml, containing 6×10^5 cpm) was denatured and added to 10 ml hybridization buffer and hybridized overnight at 60 °C. The membrane was subsequently washed at 65 °C 2 × 30 min in 4 × SSPE, 0.1 % (w/v) SDS, followed by 2 × 30 min in 2 × SSPE, 0.1 % SDS. Autoradiographs were exposed at – 70 °C for 48 h.

Immunocytochemical analysis of bladder tumours and exfoliated cells from human urine

Slides bearing 10 µm thick sections of formalin-fixed, paraffin-embedded human bladder tumours were kindly provided by Dr T. O'Brien (Department of Clinical

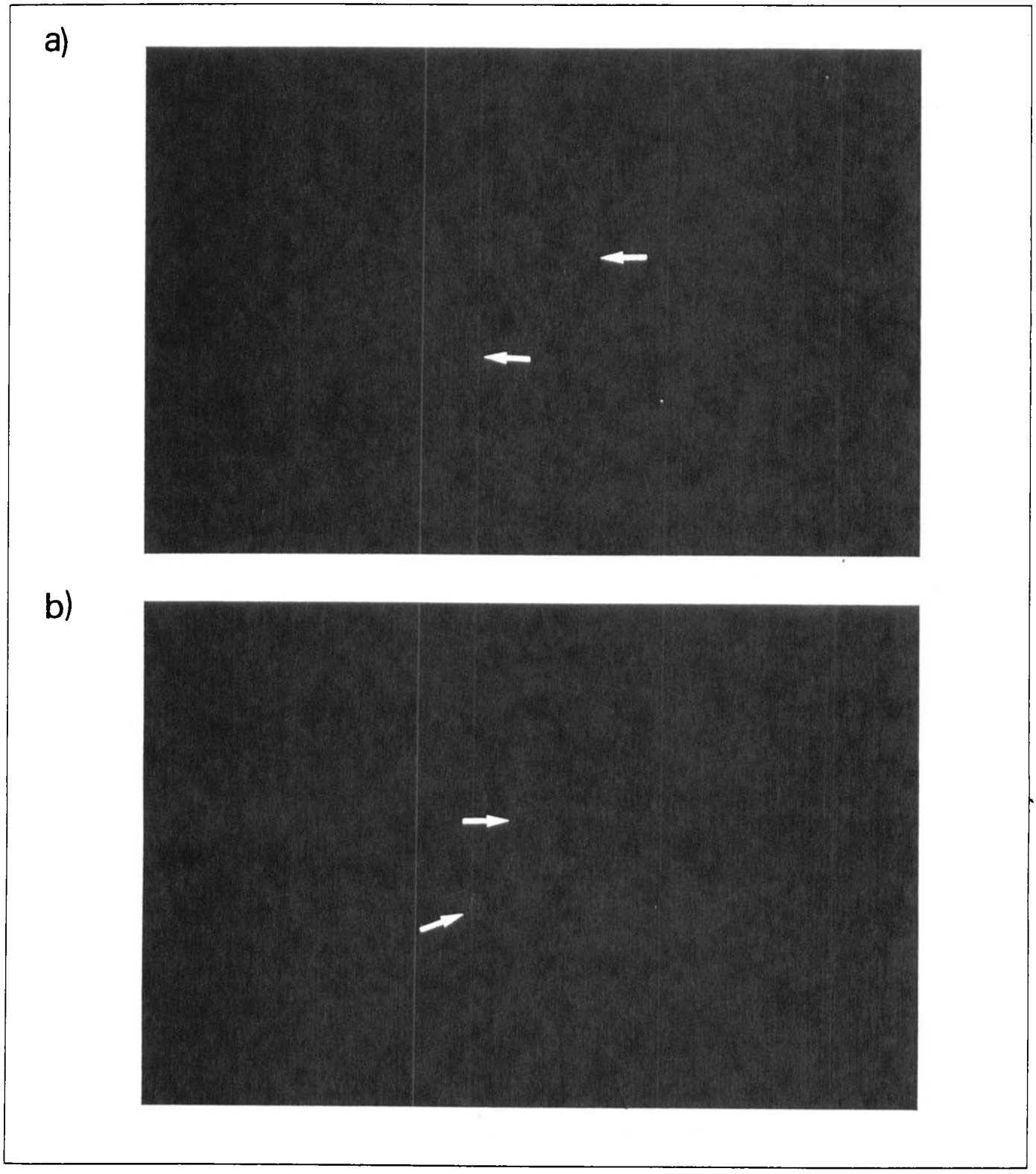

Figure 1. Fluorescent *in situ* hybridization (FISH) of interphase cells (a) human mononuclear peripheral blood leucocyte (diameter 40 μm) and (b) exfoliated human urothelial cell (diameter 12 μm) hybridized with human chromosome 8 centromere and *NAT2* cosmid probes. The arrows indicate the *NAT2* signals.

Oncology, Churchill Hospital, Oxford). Exfoliated urothelial cells from male volunteers were prepared for immunocytochemical analysis from 50 ml of fresh urine. Urine was centrifuged in a Sorvall RT 6000D refrigerated bench-top centrifuge (1500 rpm, 4 °C 5 min), and the pellet was resuspended in 5 ml phosphate-buffered saline. Centrifugation was repeated (1500 rpm, 4 °C, 5 min) and the cell pellet was fixed by resuspending in 2 ml of ice-cold 50 % ethanol. Cells were applied to aminopropyltriethoxysilane (APES) coated slides as described above. Immunocytochemical staining was performed using the Vectastain Elite ABC kit (Vector Labs, Peterborough, UK). The manufacturer's instructions were followed with the following modifications: endogenous peroxidase was inhibited using 1 % (w/v) H_2O_2 in Tris-buffered saline (TBS), sections were incubated with rabbit antiserum at 4 °C overnight and three 10-min TBS washes were performed between each antibody incubation. Rabbit antisera were used at a dilution of 1/1000 in TBS containing 1.5 % normal goat serum. Following immunostaining, slides were lightly counterstained with Gill's haematoxylin and dehydrated. Coverslips were attached using DPX mountant.

Results

Detection of NAT2 genes in human interphase cells

It has previously been demonstrated that *NAT2* is on human chromosome 8 (Hickman *et al.* 1994). However, loss of hybridization signal from interphase cells may be caused by altered hybridization conditions. As a prerequisite to the use of a *NAT2* probe to identify loss of the *NAT2* containing region of chromosome 8, it was therefore necessary to use an additional marker for chromosome 8 to determine that the hybridization conditions are suitable. In this study, a probe specific for human chromosome 8 centromere was hybridized, as a second chromosome 8 marker, together with the *NAT2* cosmid DNA. It was necessary to obtain conditions where cross-reactivity between the two probes did not diminish the hybridization signal. *NAT2* cosmid and chromosome 8 centromere probes were initially used separately to establish good hybridization conditions for both probes. Probes were then pooled to make it possible to show the presence of chromosome 8 centromere and *NAT2* signals together in one cell (Figure 1). These studies provide the tools for identification of a deletion of *NAT2* but with retention of chromosome 8 centromere.

Mapping of the NAT1 gene within the ICI YAC 10Df9

The YAC f9 has been shown by pulsed field gel electrophoresis to be 400 kb. The f9 YAC is chimeric and contains a region of human chromosome 10 as well as human chromosome 8. This is a common occurrence for YAC clones because large pieces of DNA rearrange during the cloning procedures (Anand *et al.* 1990). Since the YAC f9 is chimeric it is not possible to use it as a probe for NAT1. As an approach to obtaining a non-chimeric fragment of the f9 YAC containing the *NAT1* gene for FISH analysis, a series of restriction digestions of f9 have been carried out. The results of these digestions with Mlu1 and Sal1 have demonstrated that there is a fragment of 60 kb which is a suitable size for use as a probe (Figure 2).

Detection of NAT1 protein

Rabbit polyclonal anti-peptide antisera have been raised against the C-terminal nonapeptide of NAT1. Although the NAT1 and NAT2 proteins are highly homologous, their

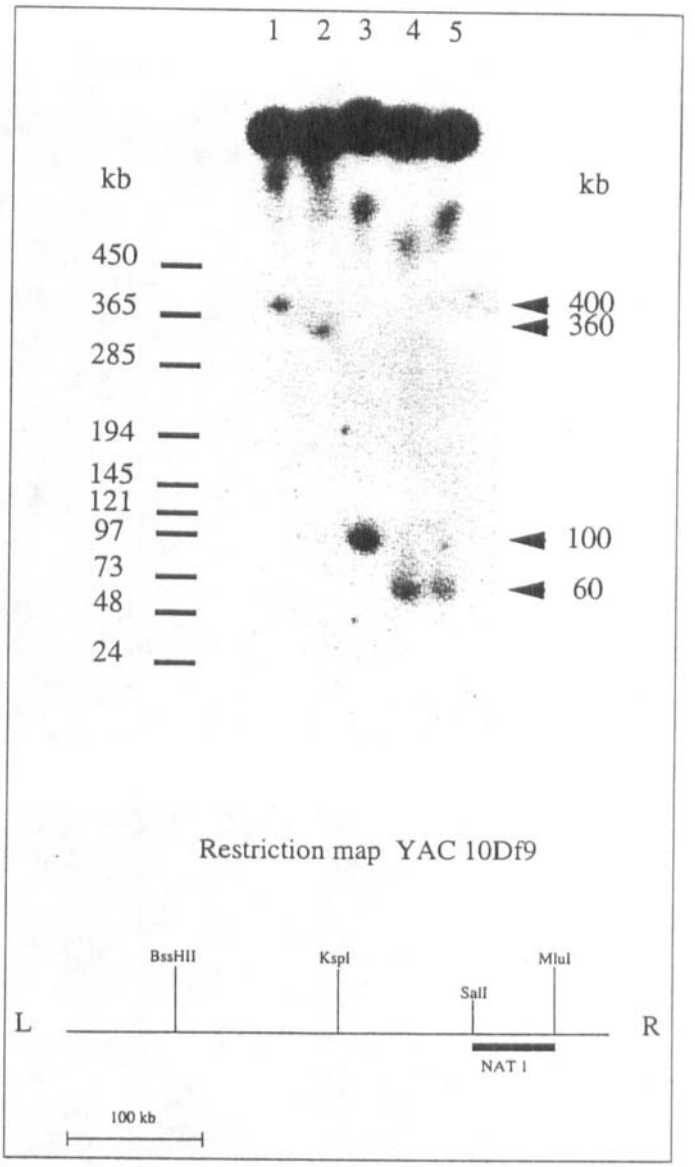

Figure 2. (a) Restriction mapping of the ICI 10Df9 YAC clone. Autoradiograph showing Southern blot analysis of the Mlu1 and Sal1 digestion of the f9 YAC DNA. The probe used was the ^{32}P-labelled 870 bp coding region of *NAT-1*. Lane 1: undigested f9 YAC DNA, lane 2: Mlu1 digested f9 YAC DNA, lane 3: Sal1 digested f9 YAC DNA, lane 4 and 5: Mlu1/Sal1 double digested f9 YAC DNA. Arrowheads indicate the sizes of the intact YAC DNA and the restriction fragments. Markers on the left hand side correspond to sizes of *Saccaromyces cerevisiae* (Bio-Rad) molecular weight markers in kb. (b) Schematic diagram of map of f9 YAC showing sites cleaved by rare cutting restriction endonucleases. L, R indicate left and right arms, respectively.

C-termini are divergent (Ohsako and Deguchi 1990). We have demonstrated by enzyme-linked immunosorbent assay, Western blot and immunohistochemical analysis that the anti-NAT1 antiserum used (#184) is specific to NAT1 and does not cross-react with other human bladder antigens (Stanley *et al.* 1996). Staining of bladder tumour sections and exfoliated cells collected from urine indicated that NAT1 was expressed in the cytoplasm of urothelial cells (Figure 3). In many cases the exfoliated cells which expressed NAT1 were observed to be binucleated (Figure 3(a)), and were therefore identified as

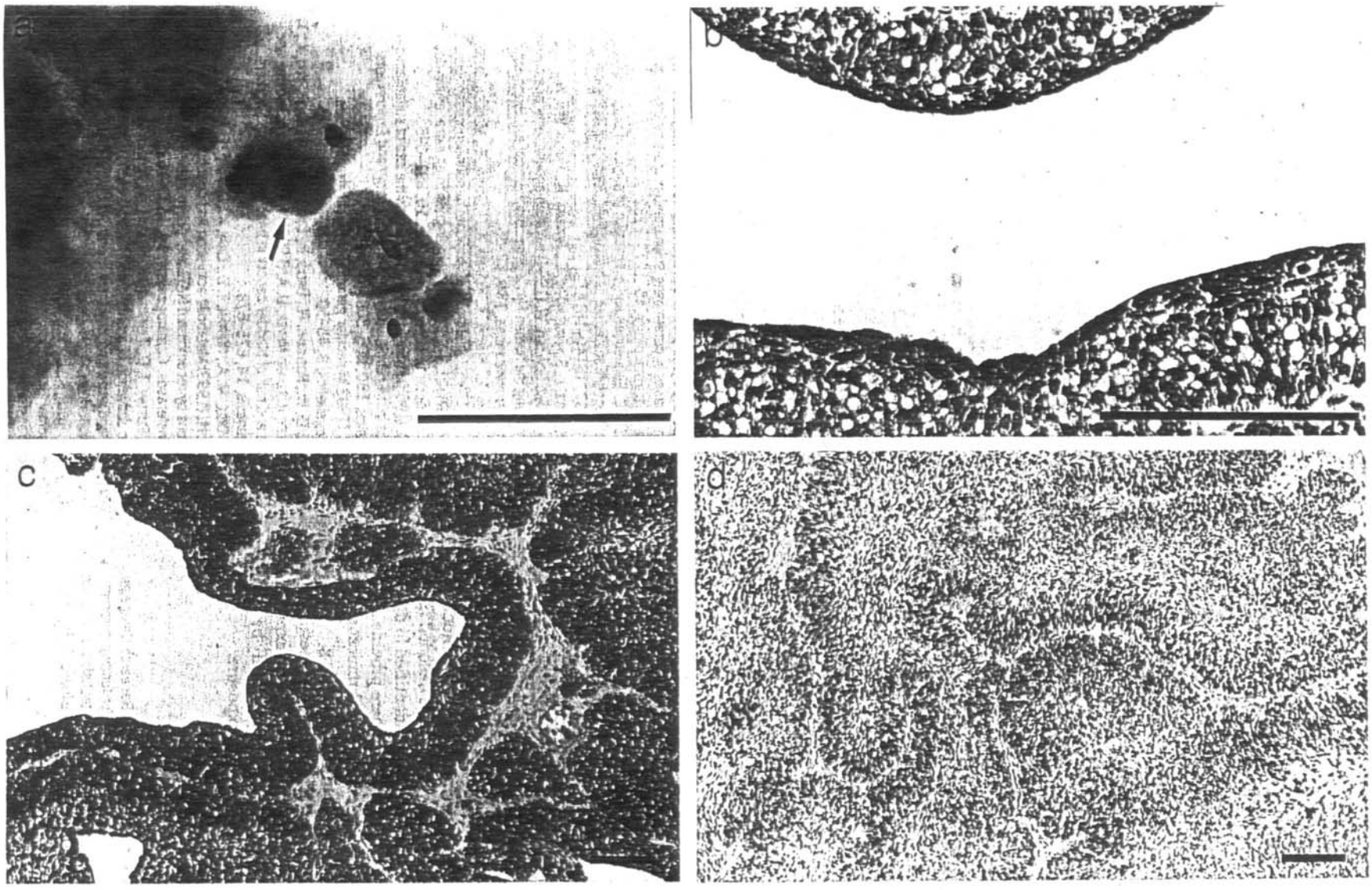

Figure 3. Immunocytochemical localization of NAT1 in human urothelial cells and bladder tumours. Immunocytochemical staining was performed as described. Primary antisera were used at a dilution of 1/1000 in TBS containing 1.5 % normal goat serum. (a) Exfoliated urothelial cells stained with anti-NAT1 antiserum # 177 (original magnification × 400). (b), (c) Well-differentiated region of a transitional cell bladder carcinoma stained with anti-NAT1 antiserum # 184 original magnification, (b) × 400; (c) × 100. (d) Poorly-differentiated region of a transitional cell bladder carcinoma stained with anti-NAT1 antiserum # 184 (original magnification × 100). Scale bars (in black) represent 100 μm.

umbrella cells. Cells at the lumenal surface of human bladder tumours were stained intensely by anti-NAT1 antibodies, again consistent with the presence of NAT1 in umbrella cells (Figure 3(b)). Regions of bladder tumours which exhibited well-differentiated, papillary morphology stained heavily with anti-NAT1, but in less well-differentiated, solid regions little staining was observed (Figure 3 (c and d)).

Discussion

The results presented here show the feasibility of identifying the presence of *NAT2* genes in both peripheral blood mononuclear cells and exfoliated cells collected from urine. These studies of interphase nuclei can now be extended to compare samples of exfoliated cells collected from urine of patients with bladder tumours of different grades and stages. NAT2 protein is unlikely to be expressed in bladder epithelium (Stanley *et al.* 1995) whereas NAT1 protein is expressed in bladder epithelium and enzymic activity has been detected in homogenates of non-malignant bladder epithelium (Coroneos and Sim 1993). Results presented here show that NAT1 protein is expressed both in a well differentiated bladder tumour and in exfoliated cells collected from urine, although the expression appears to be non-uniform in exfoliated cells. NAT1 protein is expressed particularly well in the cells lining the lumen. This study can be extended to correlate the expression of NAT1 protein with tumour grade and stage.

In order to understand any possible role of a change in NAT1 expression with tumour progression, it is important that the presence of the *NAT1* gene is also investigated. We describe a suitably sized 60 kb fragment of DNA encoding *NAT1* for use as a biomarker. A second approach to identifying a suitable *NAT1* probe for FISH analysis is also underway. A flow-sorted chromosome 8 cosmid library (Wood *et al.* 1992) is being screened with a radiolabelled *NAT1* probe prepared as described here for use in Southern blot analysis.

Comparison of the *NAT* genes and proteins as biomarkers could provide information on carcinogenesis and tumour progression. There are likely to be many genes encoded in the 8p22 region, although very few have been identified to date (Fujiwara *et al.* 1995). The tools described here provide a means of comparison of DNA deletions with chromosomal abnormalities detected by other genomic probes (e.g. microsatellite markers). The long term aim is that these chromosomal deletions can then be compared with exposure of an individual to environmental chemicals, ideally by identification of DNA adducts to monitor exposure.

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