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Novel non-isotopic in situ hybridization technique detects small (1 Kb) unique sequences in routinely G-banded human chromosomes: fine mapping of N-myc and β-NGF genes

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ABSTRACT

A novel in situ hybridization technique is described. This non-radioactive technique combines, for the first time, the high spacial resolution and rapid signal development of the non-isotopic approach with the previously unrivalled sensitivity of autoradiography. The procedure, which employs biotin labelled DNA probes and a streptavidin-alkaline phosphatase based detection system, is compatible with pre G-banding and can be performed on archival material. Unique sequences as small as 1 Kb are detectable. Using this technique, we have mapped the N-myc oncogene and the gene for β-Nerve Growth Factor to 2p24 and 1p13 respectively.

INTRODUCTION

In situ hybridization is increasingly being used to determine the chromosomal location of newly cloned sequences and to investigate the role of chromosomal aberrations in molecular pathology. There is great demand for a technique which combines speed and sensitivity with high resolution. Conventional in situ hybridization techniques using radioactive probes suffer the serious disadvantages of prolonged autoradiographic exposure times (several weeks) and limited spacial resolution. To overcome these problems several non-isotopic methods have been introduced using a variety of different immunogenic, fluorescent and enzymatic labels (1-4). However, the application of such methods has hitherto been restricted by sensitivity limitations to the detection of high copy number and relatively large (20-40 Kilobase; Kb) single copy sequences (5-8).

The success of Leary et al (9) in detecting single copy sequences on Southern blots with biotinylated probes, prompted us to apply a similar approach to the in situ detection of unique sequences in metaphase spreads. The protocol we have developed exploits the specific and strong interaction between biotin and streptavidin ($K_D=10^{-15}M$), together with the extreme sensitivity of alkaline phosphatase when used in conjunction with the

chromogenic substrate mixture NBT/BCIP (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) (10, 11).

Here we describe a simple, rapid, high resolution in situ technique for the detection of unique sequences as small as 1 Kb. Unlike other methods it is entirely compatible with prior Giemsa banding, so permitting in situ experiments on previously G-banded and archival clinical material. The signal is visualized after a single step enzymatic amplification and does not require special reflection contrast microscopy. Using this technique we have localized, more precisely than have autoradiographic studies (12), the position of the N-myc oncogene to 2p24 and mapped the gene for the β-subunit of Nerve Growth Factor (β-NGF) to 1p13.

MATERIALS AND METHODS

Metaphase spreads

To accumulate metaphases, phytohaemagglutinin (Wellcome) stimulated human peripheral blood lymphocyte cultures were incubated for 1 hour at 37°C in 1μg/ml vinblastine sulphate (Sigma). The cells were centrifuged in culture medium at 230g for 5 mins and the pellet resuspended in hypotonic KCl solution (0.075M) for 20 mins at room temperature. Following further centrifugation at 230g for 5 mins the cells were fixed in freshly prepared methanol:acetic acid (3:1) for 40 mins at 4°C. After twice resuspending the cell pellet in fresh fixative, the chromosomes were spread on cleaned glass slides and air dried at room temperature.

Giemsa banding

Slides bearing metaphase spreads were incubated for 15 - 120 sec at 37°C in phosphate buffered saline pH 7.4 (PBS) containing 1 x 10^{-7}% w/v trypsin (Worthington USA). Following a further 1 hour incubation in PBS at 60°C, G-banding was completed by staining the slides for 8 minutes with 2% Giemsa (Gurr) in pH 6.8 phosphate buffer (Gurr; BDH # 33199). G-banded metaphases were located and photographed under pH 6.8 buffer. Giemsa stain was removed with ethanol and the slides treated for 1 hour at 37°C with DNAase-free RNAase (Sigma) at 100μg/ml in 2 x SSC (1 x SSC = 0.15M NaCl, 0.015M Na Citrate). Before hybridization the chromosomes were dehydrated through an ethanol series (70%, 95%, 100%).

Biotinylation of DNA probes

DNA probes were labelled with biotin-11-dUTP (Bethesda Research Laboratories; BRL) by nick translation (BRL kit) and unincorporated nucleotides removed by ethanol precipitation in the presence of 20μg glycogen

(Boehringer). Biotin labelled probes were stored at 100ng/μl in 10mM Tris-Cl pH 7.6, 1mM EDTA at -20°C and remained stable for at least 9 months.

In situ hybridization

50 - 100ng of biotinylated probe in 10μl of hybridization buffer was applied to each slide under sealed glass coverslips. Hybridization buffer contained 50% deionized formamide (Sigma), 10% dextran sulphate (Sigma), 2 x SSC, 0.1 mM EDTA, 0.05mM Tris-Cl pH7.5 and 100μg/ ml denatured sonicated salmon sperm DNA. The chromosomal DNA and probe were then denatured together at 80°C for 10 mins. Hybridization was performed in a humidified box at 42°C for approximately 16 hours and the slides subsequently washed in 2 x SSC for 30 mins, 0.1 x SSC at 42°C for 30 mins, 2 x SSC for 15 mins and finally in TNM-A (0.1M Tris-Cl pH7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05% Triton X-100, 3% bovine serum albumin) for 15 mins. - All washes and incubations were performed at room temperature unless otherwise stated.

Signal detection

Hybridized probe was detected by incubating slides for 20 min with 0.01mg/ml streptavidin conjugated alkaline phosphatase (BRL) in TNM-A. Excess conjugate was removed by washing, 3 x 5 mins, in TNM (TNM-A without albumin) then once in pH 9.5 buffer (0.1M Tris-Cl pH 9.5, 0.1M NaCl, 50mM Mg Cl_2). Incubation with 150μl of chromogenic substrate was performed in subdued light for 4 - 5 hours. The substrate solution (BRL reagents) was made by adding 4.4μl of NBT (at 75mg/ml in 70% dimethylformamide) and 3.3μl of BCIP (at 50mg/ml in dimethylformamide) to 1ml of pH 9.5 buffer. 1mM levamisole (Sigma) was included to inhibit any residual alkaline phosphatase activity (13). Colour development was terminated by washing for 5 mins in 20mM Tris-Cl pH 7.5, 5mM EDTA and the slides mounted in aqueous mountant (Dako). Metaphases were relocated and viewed under phase contrast illumination, and the results were scored by two independent observers. The preparations are permanent and can be viewed repeatedly without fading. Biotinylated pBr322 was used as a negative control in parallel experiments.

RESULTS

For single copy detection of the β-NGF gene a genomic, 7Kb insert probe in pBr322 was used (14). Single strand length of the biotinylated fragments was 300-600 base pairs as determined by glyoxal/agarose gel electrophesis (15). Metaphase spreads were prepared, G-banded and photographed (Fig 1A). Following in situ hybridization and probe detection

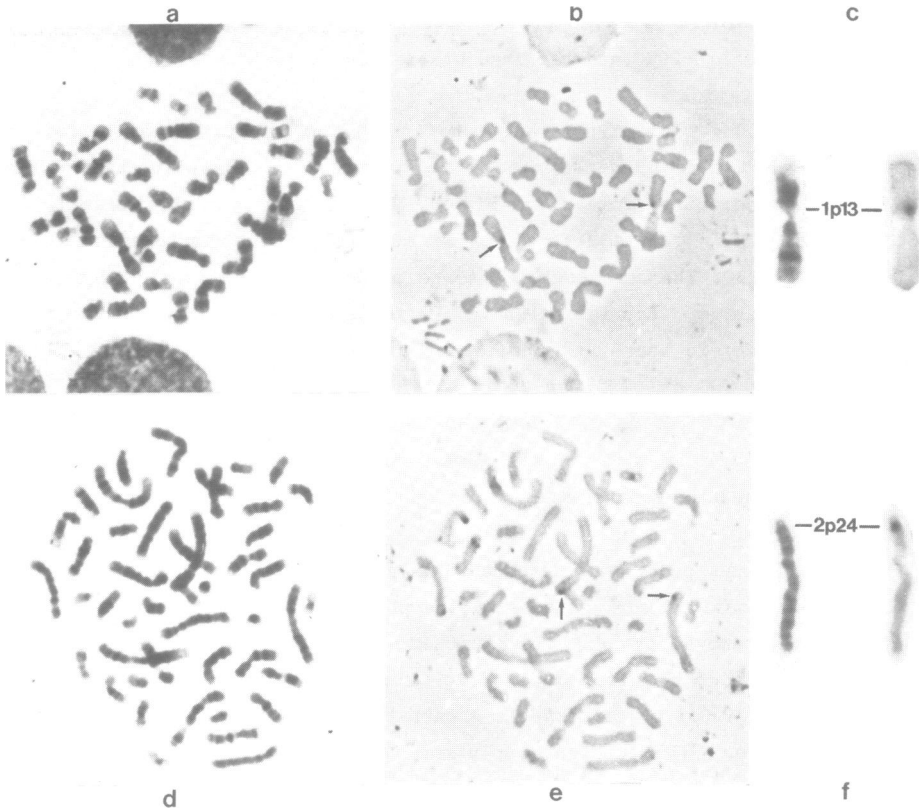

FIGURE 1
Non-isotopic in situ hybridization of β-NGF and N-myc probes to pre G-banded human chromosomes.
a, G-banded metaphase (partial) before in situ hybridization with β-NGF probe.
b, The same metaphase after hybridization with β-NGF; phase contrast illumination. Both chromosome 1 homologues are labelled at band p13 (arrows). No grains are seen on any other chromosome.
c, Higher magnification of chromosome 1 from another metaphase, before and after in situ hybridization with the β-NGF probe.
d, G-banded metaphase before hybridization with the N-myc probe.
e, The same metaphase after in situ hybridization with N-myc; phase contrast illumination. Both chromosome 2 homologues are labelled at band p24 (arrows). Occasional background grains are visible on other chromosomes.
f, Higher magnification of chromosome 2 from another metaphase, before and after in situ hybridization with the N-myc probe.

the previously photographed metaphases were relocated and bright field microscopy revealed discrete deposits of purple precipitate at the sites of hybridization. These deposits, subsequently referred to as "grains", were

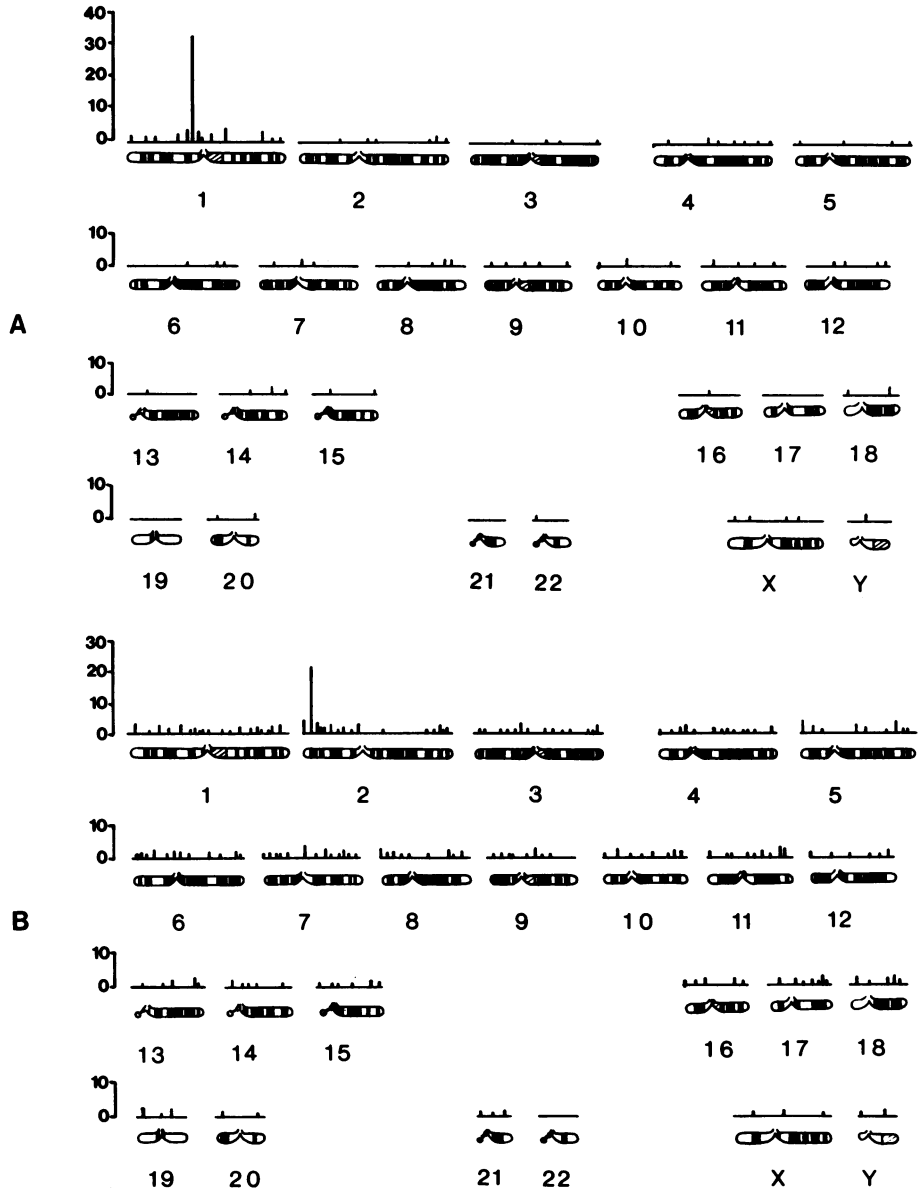

FIGURE 2
Diagrammatic distribution of hybridization signals over normal human metaphase chromosomes. Vertical lines represent the number of signals at each site.
a, Pooled distribution data from 52 metaphases examined in 2 separate experiments with the β-NGF probe. A signal peak is clearly seen at 1p13.
b, Pooled distribution data from 100 metaphases examined in 3 separate experiments (see Table 1) with the N-myc probe. Note the signal peak at 2p24.

TABLE 1 In situ hybridization data for β-NGF and N-myc probes.

	Metaphases with grains at gene locus / total metaphases		Grains at gene locus / total grains		Total grains per metaphase		Expected number of grains per band if random distribution		Signal to noise ratio (observed / expected)	
β-NGF 50ng	13/24	54%	16/49	33%	49/24	2.0	49/311	0.16	16/0.16	100
β-NGF 100ng	14/28	50%	16/83	19%	83/28	3.0	83/311	0.27	16/0.27	59
β-NGF Total	27/52	52%	32/132	24%	132/52	2.5	132/311	0.42	32/0.42	76
N-myc 50ng	9/59	15%	9/196	5%	196/59	3.3	196/311	0.63	9/0.63	14
N-myc 50ng*	6/18	33%	7/60	12%	60/18	3.3	60/311	0.19	7/0.19	37
N-myc 100ng	5/23	22%	5/64	8%	64/23	2.8	64/311	0.21	5/0.21	24
N-myc Total	20/100	20%	21/320	7%	320/100	3.2	320/311	1.03	21/1.03	20

*DNA denatured at 90°C in this experiment.

most clearly vizualized by standard phase contrast illumination (Figs 1B, 1C). Of 52 metaphases examined, 27 (51%) had at least one grain deposited on band p13 of chromosome 1 (Fig 2A). The background staining was minimal with an average of only 2.5 grains per metaphase. Signal to noise ratios were calculated by dividing the total number of grains at 1p13 by the expected number of grains per band, assuming a random grain distribution and using a standard 311 G-band ideogram (16). The optimal signal to noise ratio of 100:1 was achieved with a probe concentration of 50ng/10μl; higher concentrations increased the background only (Table 1).

The high signal to noise ratio obtained with the 7Kb β-NGF target sequence suggested to us that even smaller unique sequences might be detectable by this technique. We therefore proceeded to biotinylate the plasmid pNB-1, which contains a 1Kb EcoR1-BamH1 fragment of the N-myc oncogene in pBr322 (17). The biotinylated probe was hybridized in situ to human metaphase chromosomes as described above. Of 100 metaphases examined, 20 had at least 1 grain deposited on band p24 of chromosome 2 (Figs 1D, 1E, 1F, 2B). The overall signal to noise ratio, although lower than that obtained with the larger β-NGF probe, remained acceptable at approximately 20:1 (Table 1). Optimal probe concentration with biotinylated pNb-1 was somewhat higher (100ng/10μl) than with the β-NGF probe.

Single copy localization was also achieved with a third probe containing a 2.5Kb insert (van den Berghe et al; manuscript in preparation).

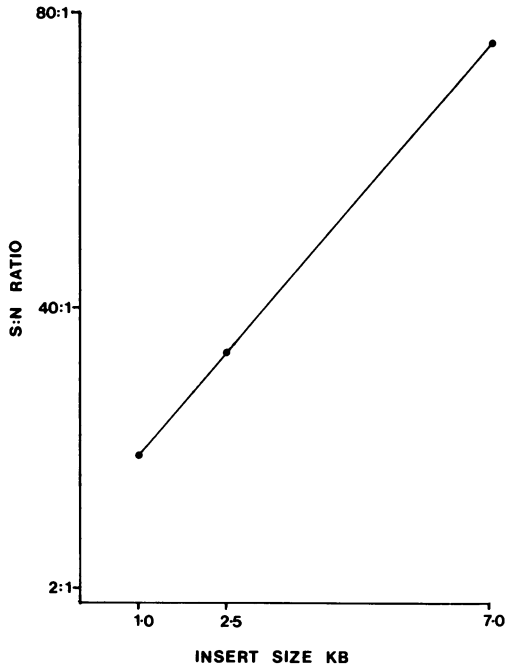

FIGURE 3
Signal to noise (S:N) ratio plotted against size of hybridizing sequence for 3 different biotinylated probes. The S:N ratios represent overall values from several separate experiments as shown in Table 1.

The overall signal to noise ratio obtained in this case was 34:1. Plotting signal to noise ratio against size of the hybridizing sequence of these 3 probes gives an approximately linear relationship over the range 1Kb-7Kb (Fig 3). Although extrapolation beyond this range must remain speculative, the slope of the line suggests that unique sequences even smaller than 1Kb may be detectable.

DISCUSSION

The lower detection limit of this in situ technique appears, unlike earlier non-isotopic systems, to be comparable to that of high specific activity tritiated probes which can detect as little as 0.5 Kb of target sequence (18). We attribute this increased sensitivity to the following factors; 1) the use of an extremely low trypsin concentration for G-banding, 2) the inclusion of vector sequences in the probe, facilitating network formation, 3) the use of alkaline phosphatase in place of peroxidase as the

detection enzyme and 4) the use of phase contrast microscopy for signal enhancement. In addition to its extraordinary sensitivity, streptavidin-alkaline phosphatase detection has the added advantage of being a simple one step procedure, in contrast to alternative antibody based indirect immunoperoxidase methods (3, 19).

The ability to use such short probes for mapping unique sequences eliminates the need for the time consuming subcloning of cosmid DNA and the use of complex probe "cocktails" necessitated by less sensitive non-isotopic techniques (8). To date, optimal chromosome banding and optimal hybridization have generally been regarded as mutually exclusive goals (7). With the present system this generalization does not apply because pre G-banding provides better cytological definition than the inherent R-banding (8), Lipsol banding (20) or post Wrights banding (21) used by other in situ protocols. Prior G-banding reduces the possibility of observer bias inherent in post banding methods and also permits the use of routinely G-banded clinical material for in situ experiments. Even permanently mounted (DPX mountant) archival material is suitable, after coverslip removal by xylene, for in situ hybridization by this technique. This should allow retrospective in situ studies to be performed on a large, previously untapped resource of stored cytogenetic preparations. Employment of standard phase contrast illumination for signal enhancement, rather than much less widely available reflection contrast equipment (8),is another significant advantage of the present system.

The localization of the β-NGF gene to chromosome band 1p13 by this non-isotopic method is consistent with a previous autoradiographic study on normal human chromosomes (22), which reported specific labelling over bands 1p13-1p22. Other studies (23, 24) using rodent-human somatic cell hybrids have mapped β-NGF slightly more distally to 1p21-1p22. Schwab et al (12) using a tritiated probe in situ, have assigned the N-myc gene to the region 2p23-2p24. The present study shows a signal peak at band 2p24 only, thus demonstrating the superior spacial resolution that can be obtained by avoiding the silver grain scatter inevitably associated with autoradiographic emulsion.

In summary, the technique we describe combines for the first time the high resolution and rapid (4 hours as opposed to as much as 4 weeks with tritiated probes) signal development of the non-isotopic approach with the previously unrivalled sensitivity of autoradiography. Furthermore, it is compatible with prior G-banding, can be performed on archival material, uses stable biotinylated probes, requires no special optical equipment and produces

permanent preparations. This combination of advantages should ensure that the technique finds widespread application in mapping unique sequences in both normal and abnormal (25) chromosomes of man and other species.

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