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Direct visualization of single copy genes on banded metaphase chromosomes by nonisotopic *in situ* hybridization

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ABSTRACT

A rapid method is described for non isotopic in situ mapping of single copy genes directly on G-banded chromosomes by "one-step" regular light microscopy. It is based on hybridizing biotinylated probes to metaphase chromosomes. Biotin residues are detected by rabbit antibiotin antibody and anti-rabbit Ig labelled with peroxidase or colloidal gold. The peroxidase reaction product or colloidal gold signals are amplified by silver precipitation. The final product is a black silver dot at the gene locus on a purple G-banded chromosome. N-ras and alpha-1-antitrypsin genes have been mapped using plasmids with inserts of 1.5 and 1.3kb to 1p13.1 and the junction of 14q31/32 respectively. The signal to noise ratio in these experiments ranged from 32:1-46:1. This technology is at least as sensitive as radioisotopic in situ hybridization and gives results within 1 day of hybridization and has much better resolution. Additionally, genes are visualized by regular light microscopy without specialized techniques such as reflection contrast, fluorescence or phase microscopy. This methodology should facilitate more precise chromosomal gene localization.

INTRODUCTION

Assignment of genes on human mitotic chromosomes provides a physical basis for the human genetic map. Traditionally, this has been done by the analysis of somatic cell hybrids carrying different human chromosomes **(1)**. The advent of in situ hybridization, made it possible to visualize genes on mitotic chromosomes **(2,3,4)**. In conventional in situ hybridization, DNA probes labelled with radioisotopes are hybridized with denatured mitotic chromosomes. The radiolabel is subsequently detected by autoradiography. Although radiolabelled probes are sensitive, they have limitations for the microscopic visualization of genes on chromosomes. These include background noise and time-consuming (days-weeks) autoradiography: the track length (^{3}H = 1μm in H_2O) of decaying radioisotope limits the microscopic resolution of the signal; the radioactive signal and the underlying chromosomes cannot be visualized microscopically in the same focal plane; and the use of radioisotopes puts the methodology beyond the reach of many laboratories purely on safety

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grounds. In recent years, several attempts have been made to find suitable non-radioactive reporter molecules. These include fluorescent as well as enzyme labels **(5,6,7)**. However, none of these labels achieve the sensitivity obtained by radioisotopes. Most methods detect only repetitive DNA or very large unique sequences.

Recently, Burns et al **(8)** demonstrated multiple copy DNA sequences on the long arm of the Y chromosome, using a biotin probe which was detected by silver amplification of an immunocytochemical reaction product. Chan et al, using an avidin-alkaline phosphatase detection system, identified picogram quantities of DNA and mRNA on Southern and Northern blots **(9,10)**; the sensitivitiy of this system is equal to overnight autoradiography with ^{32}P probes of specific activity 2-3 x 10^8 cpm **(10)**. These recent developments prompted us to investigate whether biotin-labelled probes could be used for detecting single copy genes on metaphase chromosomes directly by "one-step" regular light microscopy.

We describe a method which is simple and quick, is as sensitive as radioisotopic methods, and detects hybridized probes as small as 1.3kb. The technique makes it possible, for the first time, to observe the signal (a silver dot) directly on banded chromosomes, thereby obviating the need for repeated photography or specialized microscopy such as reflection contrast **(6)**, phase contrast **(11)** or fluorescence **(7)** microscopy. Using DAB/peroxidase, as well as immunogold, detection systems we have mapped N-ras to 1p13 and alpha-1-antitrypsin (AAT) to the junction of 14q31/32.

MATERIALS AND METHODS

Metaphase Spreads. Lymphocyte cultures were set up by adding 0.8ml of heparinized venous blood to 10ml of RPMI 1640 supplemented with 20% foetal calf serum, antibiotics and 1% phytohaemagglutinin (Flow Laboratories). After 72 h at 37°C, cultures were synchronized with 100µg/ml 5'-bromo-deoxyuridine (BUdR; Sigma) for 16 h **(12)**. The cells were washed, resuspended in fresh medium containing 2.5µg/ml thymidine, and incubated at 37°C for a further 6 h. Colcemid (0.06µg/ml; Sigma) was added for the final 30 min. The cells were centrifuged, resuspended for 10 min at 37°C in 0.075M potassium chloride, centrifuged and fixed in chilled methanol/glacial acetic acid (3:1 v/v) for at least 30 min. Air-dried metaphase preparations were made on regular glass slides. Spreads were not treated with RNase before hybridization.

Biotinylation of Probes. DNA probes were labelled with biotin-11-dUTP (Amersham) by nick translation as described in detail **(13)**. Unincorporated

nucleotides were removed by ethanol precipitation. The probes were stored (10ng/µl) in 10mM Tris-HCl, 1mM EDTA (TE) pH 8.0 at 4°C.

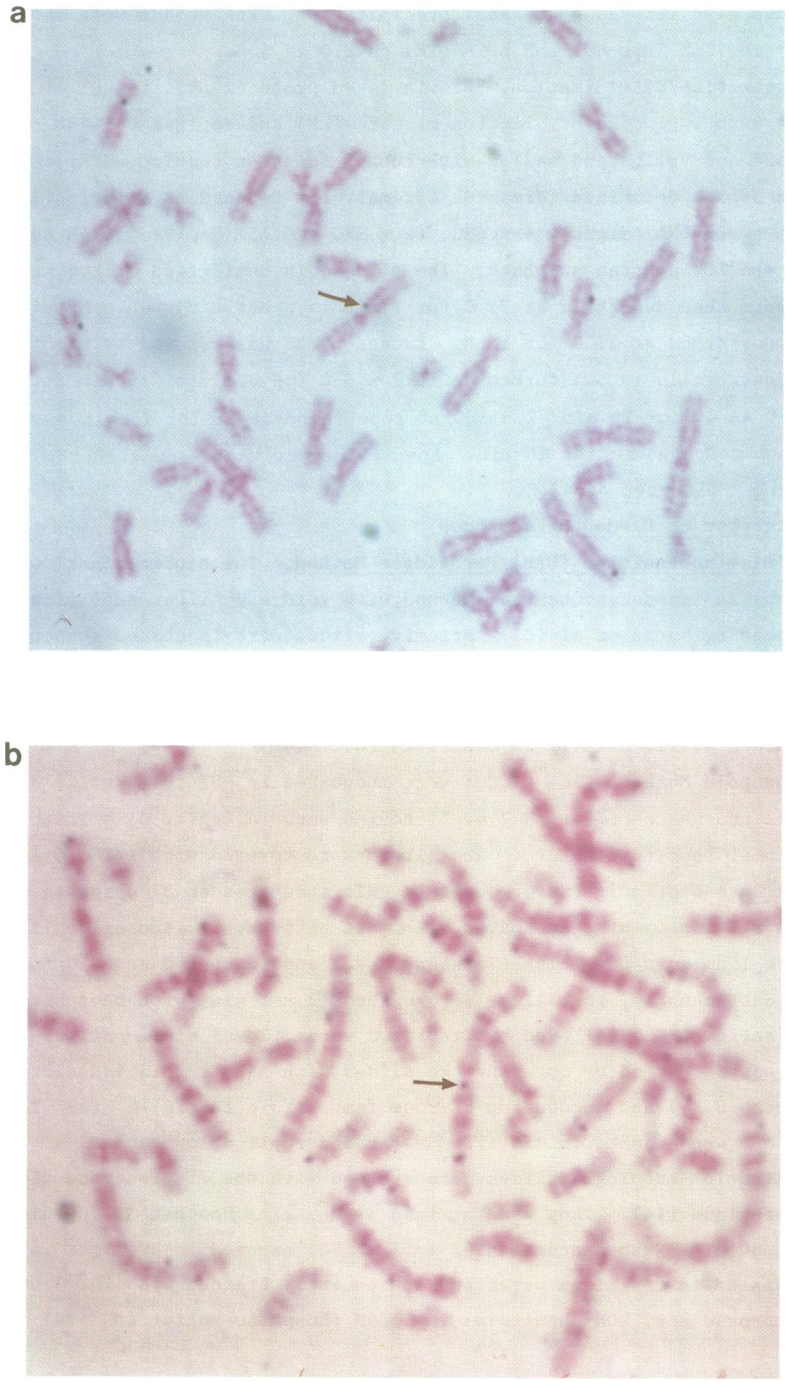
Denaturation/Hybridization. Biotinylated probe (20ng), in 2µl of TE, was mixed with 10µl of hybridization mixture (HM) and applied to each slide under glass coverslips and sealed with rubber solution (Dunlop, U.K.). HM consisted of 55% deionized formamide (Sigma), 1 x Denhardt's solution, 400µg/ml salmon sperm DNA (Sigma), 4 x SSC, (1 x SSC = 0.15M NaCl + 0.015M sodium citrate) and 10% dextran sulphate. The slides, in humidified microtitre plates, were then denatured at 75°C for 7 min in a hot air oven. Hybridization was performed at 37°C for 16 h. Excess probe was removed by washing the slides sequentially in 50% formamide in 2 x SSC (pH 7.4) for 15 min at 42°C, 2 x SSC at 42°C, for 15 min, 2 x SSC at room temperature for 15 min and 0.1 x SSC at room temperature for 15 min. Low stringency washes omitted the 50% formamide/2 x SSC step at 42°C.

Probe Detection on Banded Chromosomes

3,3'Diaminobenzidine (DAB)/Peroxidase Method. The biotin signal was detected by an immunocytochemical method with gold and silver amplification as described by Burns et al **(8)**. Briefly, slides were incubated sequentially with rabbit antibiotin, peroxidase labelled goat anti-rabbit Ig (Dako, U.K.), and the peroxidase detected by DAB/H_2O_2; the reaction product was amplified with gold and silver exactly as described **(8)**.

Immunogold Method. The slides were incubated in PBT (0.15 mol/l NaCl, 0.01 mol/l phosphate buffer pH 7.4, 3% bovine serum albumin, 0.1% Triton X100) for 15 min at room temperature. The slides were covered with 1/200 dilution of rabbit anti-biotin Ig (Enzo, N.Y.) in PBT, incubated at 37°C for 45 min, washed in PBT for 15 min, incubated with goat anti-rabbit immunoglobulin conjugated to 15nm gold particles (Janssen, U.K.) and incubated at 37°C for 45 min. Subsequently, the slides were washed (3 x 5 min) with phosphate buffered saline (PBS) followed by distilled water (2 x 5 min). Silver amplification was performed by covering the slides with silver solution (Intense II, Janssen, U.K.) and incubating at room temperature for 10-15 min. The preparations were washed in distilled water and replication banded.

Replication-banding. Slides were stained with the fluorescence plus Giemsa technique **(14)**. They were stained with 5µg/ml Hoechst 33258 (Sigma, U.K.) for 30 min, washed and mounted in 2 x SSC, exposed to UV light for 60 min, washed in distilled water and subsequently stained with 10% Giemsa (Gurrs improved R66, BDH Chemicals) in 0.06M phosphate buffer (pH 6.8) for


a
b

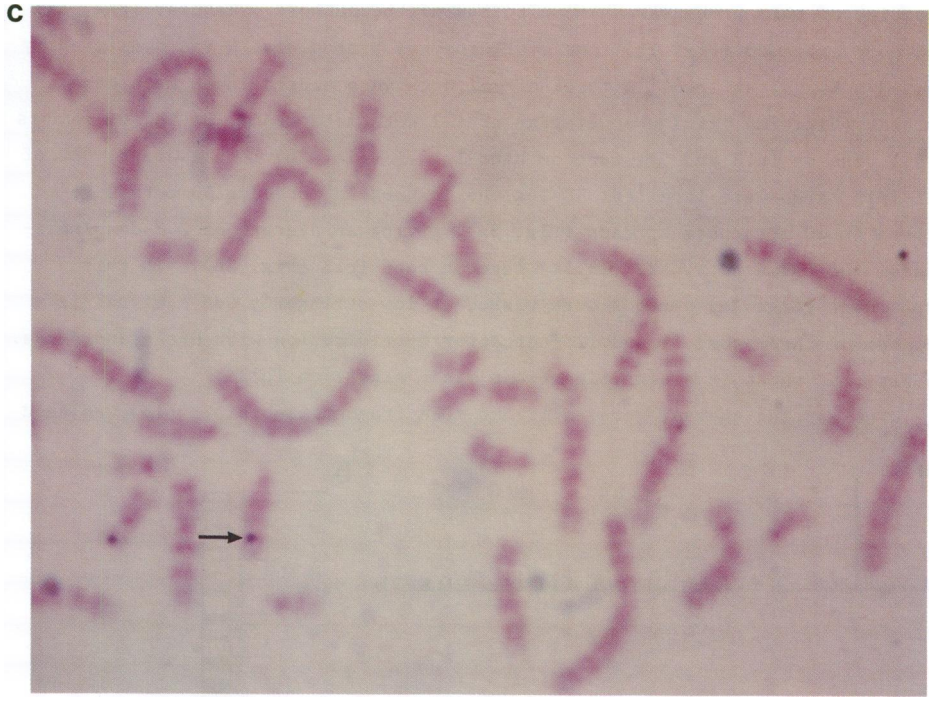


Figure 1. Non-isotopic in situ hybridization of N-ras and AAT probes to human mitotic chromosomes. (a) A representative banded metaphase hybridized with the N-ras probe. A silver dot (arrow) is present on 1p13; DAB/peroxidase detection. (b) More extended chromosomes probed with N-ras. A silver dot is present on 1p13; immunogold method. (c) A metaphase spread probed for AAT showing a silver dot overlying band 14q31/32; DAB/peroxidase detection.

10 min. After washing in distilled water, the slides were air-dried and mounted in DPX (Raymond A. Lamb, U.K.).

Gene Assignment

Genes were assigned by counting silver dots directly on Giemsa banded (G-banded) chromosomes by ordinary light microscopy. The silver dots vary in size (0.13-0.39nm). Dots were assigned to particular chromosome bands when 50% or more of their area lay over that band. AAT was assigned by the detection system of Burns et al **(8)** whereas N-ras was assigned by both the peroxidase and immunogold methods.

RESULTS

The N-ras probe was a 1.5kb fragment of the 3' untranslated region of the N-ras gene in pSP65 vector (Amersham, U.K.). To avoid observer bias, the

identity of this probe was unknown to any of the authors. Regular **(Fig. 1a)** and more extended **(Fig. 1b)** female chromosomes probed with N-ras consistently showed a signal at 1p13. A total of 252 dots were scored in 60 metaphase spreads **(Fig. 2a)**, giving an average of 4.2 dots/metaphase. Of these, 46 dots (18%) were on 1p13 and 57% were on band 1p13.1 **(Fig. 2b)**. On average, 38% of all 1p13 bands were labelled. Using the immunogold method, 168 dots were scored in 60 metaphase spreads **(Fig. 3a)**, giving an average of 2.8 dots/metaphase. Of these dots, 22 (13.1%) were on band 1p13 **(Fig. 3b)**. In this experiment **(Fig. 3a)** spreads were washed at low stringency and a signal is evident on chromosome 11 and 12 indicating hybridization with H-ras and K-ras. In Fig. 2b, there is a significant but minor signal on 1p22.

For mapping the AAT gene, a 1.25kb cDNA fragment in pBR322 **(15)** was used

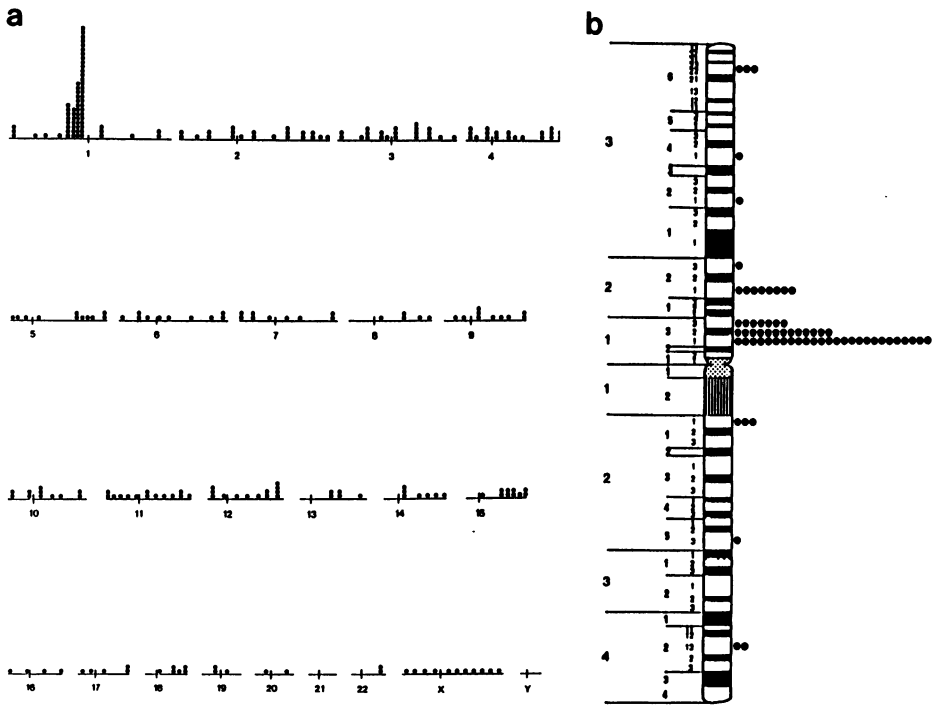


Figure 2. Assignment of the N-ras gene by DAB/H_2O_2 method. Post-hybridization high stringency washing was at 42°C in 50% formamide in 2 x SSC. (a) Histogram showing the distribution of 252 silver dots on 60 metaphase spreads. The major signal is on chromosome 1; silver dots are plotted against location on a 311 band ideogram; (b) The distribution of silver dots on extended chromosome 1. 46 dots were on band 1p13 and of these, 57% were on 1p13.1.

to determine whether cDNA probes could be used effectively. Of 343 silver dots scored in 84 male cells, 26 (7.59%) were on 14q31/32 **(Fig. 4a and 4b)**. On average, there were 4.08 dots per metaphase. In Fig. 1c, the dot shown lies predominantly on band 14q31.

The histograms **(Figs. 2a, 3a and 4a)** show that statistical analysis of the signal to noise (S/N) ratios is not strictly necessary. Traditionally, S/N ratios are calculated by assuming that each band of human G-banded chromosomes has an equal probability of showing a signal if this was a random event. This assumption may be erroneous, because the size of the bands and therefore, the amount of DNA per band, vary widely. We calculated the relative length of band 1p13 as a percentage of the total human genome from the data reported by Francke and Oliver **(16)**. The relative length of band 1p13

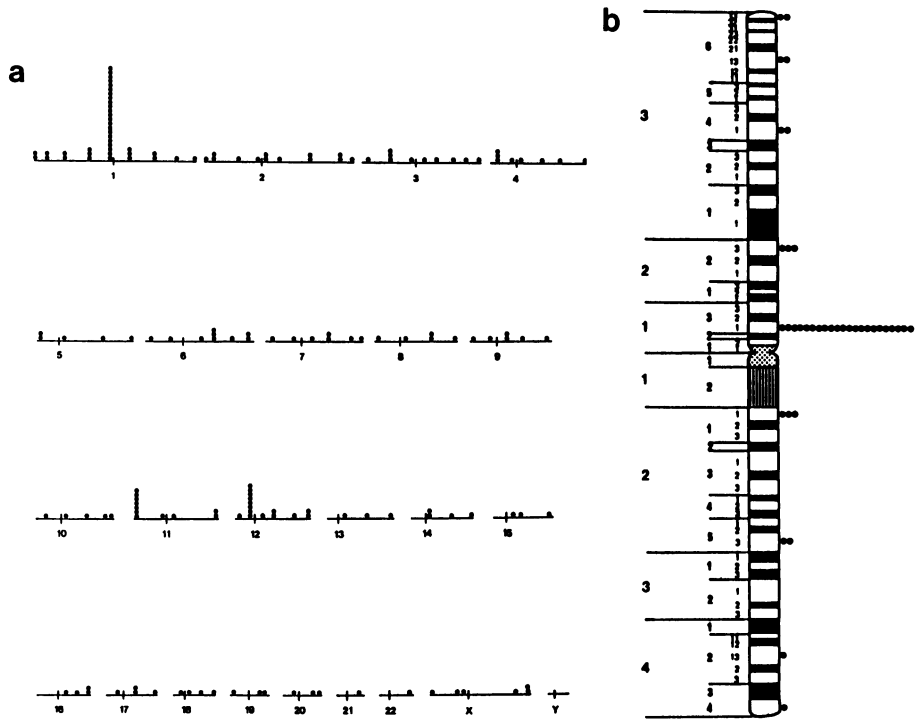


Figure 3. Assignment of N-ras by immunogold method. Post-hybridization low stringency washing was at 22°C in 2 x SSC. (a) Histogram showing the distribution of 168 silver dots on 60 metaphase spreads. The major signal is on chromosome 1 but hybridization with H-ras (11p) and K-ras (12p) is evident at low stringency. (b) The distribution of silver dots on chromosome 1. 22 silver dots (13.1%) were on band 1p13.

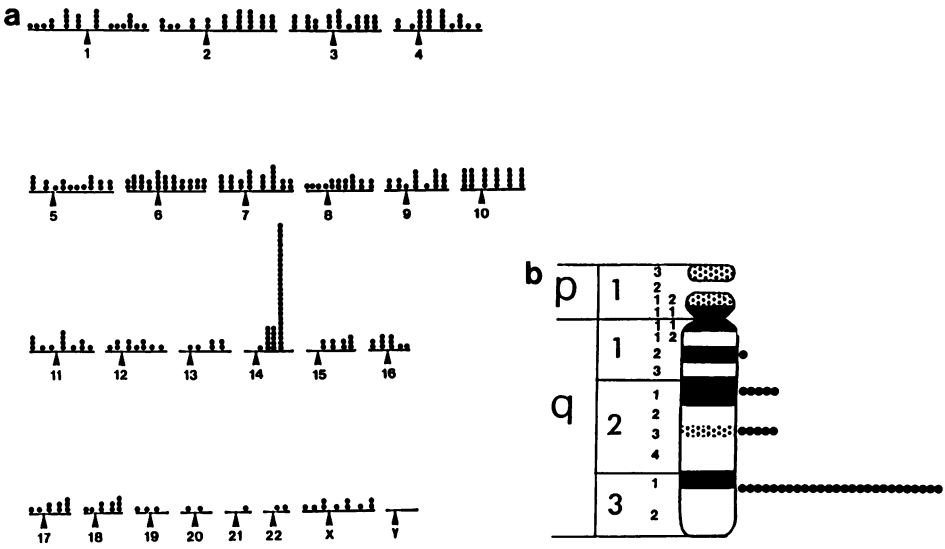


Figure 4. Assignment of the AAT gene. (a) The distribution of 343 silver dots observed in 84 metaphase spreads; silver dots are plotted against location on a 311 band ideogram. (b) The distribution of dots on chromosome 14. 26 dots (7.9%) were at the junction of 14q31/32.

expressed as a percentage of the total human genome was 0.41. Since the observed number of events on 1p13 were 46 and the total number of dots per genome was 252, the random expected frequency on 1p13 is 1.0. Therefore, the S/N ratio is 46. The S/N ratio for N-ras, using the immunogold method was 32.4. Since the AAT gene was localized to the junction of 14q31/32 it is implausible to calculate S/N ratios since the sum of the relative lengths of both 14q31 and 14q32 would be the denominator.

DISCUSSION

We have shown that N-ras maps to 1p13 in 2 subjects by nonisotopic in situ hybridization (NISH). To avoid observer bias, the identity of the probe in these experiments was unknown to the authors until the assignment was complete. N-ras has been previously mapped to 1p11-13 **(17)** by radioisotopic in situ hybridization (RISH). Others have mapped this gene to 1p22 **(18)** by RISH. Although we obtained a small signal (x2 background) at this locus in one subject **(Figs. 2a and b)** this was not confirmed in a second individual **(Fig. 3a)**. We conclude, therefore, that N-ras is located on 1p13 and the presence of homologous sequences at 1p22 remains an open question. Within band 1p13, N-ras is most probably on sub-band 1p13.1 **(see Fig. 2b and**

below). AAT mapped to the junction of 14q31 and 14q32 by NISH. This assignment is more precise than previous studies where AAT was mapped to 14q31-qter by RISH **(19).**

Both N-ras and AAT were mapped with the highest resolution compatible within the limits of the technology described. The resolution of chromosomal gene mapping by NISH is dependant on 2 variables and 1 assumption. Firstly, resolution is dependant on the degree of extention of the chromosomes, and secondly, by the size of the silver dot at the site of hybridization. The first of these variables can be controlled by selecting only extended chromosomes for analysis. The silver dot size in these experiments, however, varied from 0.13-0.39μm (mean = 0.2μm, median = 0.14μm). These sizes were calculated by measuring dot size from projected transparencies of the data shown in Figs. 1a and b. The resolution of gene location in chromosomes by NISH, therefore, cannot be better than 0.2μm. The conditions which determine dot size have not been investigated. In chromosomal assigning of genes, we have assumed that the gene lies in that band over which 50% of the silver dot lies. This is an assumption which is probably incorrect since the initial nidus of growth of the silver dot could lie within any part of the silver dot sphere and this cannot be predicted visually. Although the assumption is not entirely valid it has been adopted here as the best approximation in practice. This may explain the 3 discrete signals obtained within band 1p13 for N-ras **(Fig. 2b).**

Of the recent developments in non-isotopic in situ hybridization, the two most significant are the use of reflection contrast (RC) microscopy for detecting DAB/H_2O_2 products **(6);** and biotinylated probe detection by streptavidin/alkaline phosphatase and phase contrast microscopy **(11).** The disadvantages of the RC system include: low sensitivity which necessitates the use of probe cocktails to increase the effective total probe size to 22kb; and reflection contrast microscopy for signal amplification **(6)** which is available in few laboratories.

The streptavidin/alkaline phosphatase detection system **(11)** entails G-banding chromosomes prior to in situ hybridization and superimposition of the signal onto a photograph of the previously banded chromosomes. The chromosomes themselves are identified after hybridization by phase contrast microcopy because G-banding is destroyed by hybridization. The banding procedure itself causes loss of DNA, which also occurs during the denaturation/reannealing process. The signal generated by alkaline phosphatase substrate is blue which is similar in colour to G-banded chromosomes. By contrast, the

present technique produces a black signal which is easily visualized on G-banded chromosomes. We opted for replication banding because it causes less damage to chromosomal architecture. Both the DAB peroxidase and immunogold methods give similar levels of sensitivity and resolution. The immunogold method is preferred because it eliminates 3 methodological steps namely, DAB/H_2O_2, gold chloride and sodium sulphide incubations **(8)**.

This technology enables simple and rapid detection of single copy genes on chromosomes with a sensitivity that matches that of radioisotopic methods without the manipulative disadvantages and tedium of autoradiography. Furthermore, gene location can be mapped in a single step directly on banded metaphase chromosomes without the need for specialized microscopy. NISH, using silver amplification systems, should facilitate high resolution chromosome gene mapping, and may be useful in analysis of gene deletions and translocations. It is theoretically possible that the resolution offered by NISH can be increased by direct visualization of immunogold particles (15nm) by scanning electron microscopy **(13)** without silver amplification. As indicated in Fig. 3a, gene families may also be identified at low stringency.

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