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DOUBLE LABELLING IN SITU HYBRIDIZATION USING NON-ISOTOPIC AND ISOTOPIC DETECTION*

R. H. SINGER, J. B. LAWRENCE, G. L. LANGEVIN,
R. N. RASHTCHIAN, C. A. VILLNAVE,
T. CREMER[1], D. TESIN[1], L. MANUELIDIS[1]
AND D. C. WARD[2]

Department of Anatomy, University of Massachusetts Medical School, 55 Lake Avenue, North Worcester, Massachusetts 01605 and Section of Neuropathology[1] and Department of Human Genetics[2], Yale University School of Medicine, New Haven, Connecticut 06510, USA

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In situ hybridization yields information that is not readily obtained with other nucleic acid techniques. In particular, complex cell populations may be evaluated for the presence of specific nucleic acid sequences and these molecules detected in selected individual cells to which they may be confined. Thus, the sensitivity of *in situ* hybridization in mixed cell populations can be considerably greater than that achieved with filter blotting assays. Recent developments which enhance hybridization efficiency (16), combined with the high resolution of non-isotopic detection methods (7, 12, 15, 19, 20, 24, 25, 30–32, 34), have made this technique more rapid, precise and convenient. The potential power of *in situ* hybridization can be enhanced still further by the continued development of reliable methods for obtaining additional molecular and biochemical information on the same cells, such as the simultaneous detection of two different nucleic acid sequences or a protein and its cognate mRNA. The ability to describe the relative expression and/or localization of two distinct nucleic acid sequences is important, for instance for the study of the temporal pattern of gene expression during developmental transitions or viral infections, and for investigating gene arrangements in chromosomes or interphase nuclei.

To date, only a limited number of studies on nucleic acids employing such double-labelling techniques have been reported. Haase *et al.* (9) have used two different isotopic probes, labelled with tritium and sulfur-35, in conjunction with two-color autoradiography, to study cells infected with two distinct viruses. Several groups (2, 8, 28, 29, 36, 37) have also reported the simultaneous detection of a protein product (immunologically) and its cognate mRNA (autoradiographically) in tissue sections. A recent publication by Hopman *et al.*, (10) also reports the detection of two species of nucleic acids simultaneously using non-isotopic reagents. In this paper we describe additional applications of double-labelling procedures for the analysis of mRNAs, chromosomal DNA sequences and proteins, which use two non-isotopic

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detection methods or combinations of isotopic and non-isotopic reagents. In all of these samples, we demonstrate this detection at the single-cell level by light and electron microscopy. Several examples of different dual-labelling strategies are presented and some technical parameters especially relevant for the detection of double probes are discussed. These studies are directed towards improving the versatility and simplicity of *in situ* hybridization techniques, both at the light and electron microscopic levels.

MATERIALS AND METHODS

Cell Cultures and Chromosomes

Cells from 11-day chicken embryos pectoralis muscles were grown in tissue culture on gelatin-coated coverslips after dissociation by trypsin. After appropriate times, cells were removed and fixed in 4% paraformaldehyde for 15 min and stored in 70% ethanol at 4°C indefinitely. These cells were used for the analysis of myosin mRNA and protein.

For chromosomal and nuclear DNA studies human chromosome spreads and cell preparations were done as described in detail elsewhere (21, 22). Briefly, chromosome spreads were made from standard acid fixed cells (methanol-acetic acid, 3:1), paraformaldehyde-fixed and detergent-permeabilized cells were used.

Preparation of Hybridization Probes

Cloned DNAs that were used as hybridization probes were as follows: chicken cardiac actin gene (4, 6), chicken myosin heavy chain gene (a full-length clone kindly given to us by Jeff Robbins), a human chromosome 1-specific repeat sequence pUC 1.77 and a human Y-chromosome specific repeat sequence (a gift of Howard Cooke, 5). The DNAs were labelled enzymatically by nick-translation (27) using either bio-*11*-dUTP 3), 1U3H-dXTP or 35S-dXTP.

Aminoacetoxyfluorene and mercury-thiol ligand labelled probes were prepared chemically, essentially as described previously (11, 12).

Hybridization

Hybridization of the chicken muscle cells for mRNA detection has been described in detail previously (16, 17, 31). Briefly, coverslips were removed from the ethanol and rehydrated in a solution containing 0.1 M Tris, 0.2 M glycine and then put into a solution of 50% formamide, 2X SSC at 65°C for 10 min. For each sample, 2 μg/ml of probe was denatured in formamide at 70°C and rapidly mixed with a hybridization solution such that the final concentration of components were 50% formamide, 2X SSC, 1% BSA and 1 mg/ml each of tRNA and salmon sperm DNA. When using ^{35}S-labelled probes, 300 mM of DTT must be added to prevent non-specific sticking. Ten microliters of this solution was added to a coverslip which had been drained of fluid. The coverslip was put upside down on a piece of parafilm and hybridized at 37°C for 4 hr. The coverslip was removed and then washed in 50% formamide, 2X SSC; 50% formamide, 1X SSC; and 2X SSC alone, each for 30 min.

For detection of DNA targets, hybridization was done in 50% formamide, 2X SSC; when mercurated probes were used, the hybridization mixture also contained 5 mM KCN. Probes were nicked with DNase I or sonicated to yield fragments with an average size of 300–400 bp. The probes, used at a concentration of 0.5–2.0 μg/ml in the presence of a 100-fold excess of salmon sperm DNA, were denatured at 80°C in

formamide for ten min simultaneously with the cellular DNA (22). Hybridization reactions were incubated at 37°C overnight and post hybridization washes were essentially identical to those described above for mRNA. Various combinations of non-isotopically labelled probes were evaluated and specific detection protocols are detailed in the figure legends.

Detection of Two mRNA Targets or an mRNA-Antigen Combination

The cells were washed and, if the probe was isotopically labelled, the cells were dehydrated through ethanol and dried for autoradiography. If the probe was biotinylated, goat anti-biotin antibodies (Zymed, affinity purified) were used and detected indirectly with rabbit anti-goat antibodies conjugated to colloidal gold (Janssen), or to a fluorochrome (Zymed, data not shown). When two probes were used, one was labelled with radioactive sulfur using sulfonated nucleotides (New England Nuclear/Dupont) and the other with biotin. Both were hybridized together in the same reaction, and after washing, the biotinylated probe was detected with streptavidin and subsequently biotinylated alkaline phosphatase exposed to a substrate-chromophore system (18, 31). (The reagents were in a kit sold by Bethesda Research Labs, Gaithersberg, Md.) The coverslip was then mounted on a microscope slide, cell side up, using permount, and dried through graded ethanol. Because the chemicals used in the alkaline phosphatase reaction interact with the emulsion to cause chemographic label over cells, the slides must be coated with a thin barrier film by dipping them briefly into a formvar solution used for coating electron microscopic grids, or into a nitrocellulose solution (1% in amyl acetate). After drying the film, the slide was coated with Kodak NTB-2 emulsion and exposed for the radioactive probe. Coincidence of grains and enzymatic staining when using the same probe confirmed the legitimacy of the signal; control slides hybridized to a biotinylated probe but *not* radioactive probe showed no grains, confirming that chemography had been eliminated.

For simultaneous detection of an mRNA and a protein, a monoclonal antibody to chicken skeletal muscle myosin was used (a gift of John Coleman). After hybridization, samples were reacted with the mouse monoclonal antibody followed by a rhodamine conjugated anti-mouse IgG. Samples were then coated with Kodak NTB-2 emulsion for autoradiography after dehydration through graded ethanol.

Important Parameters for Double-Labelling Experiments

In general, sample pretreatments, prehybridization and hybridization are similar for single and double-labelling procedures. However, several additional technical considerations were found to be specifically relevant when two probes are hybridized and detected simultaneously.

First, when ^{35}S or ^{3}H-labelled probes are used, one must take into consideration the fact that the ^{35}S-probe will require a seven to ten-fold shorter exposure time than a ^{3}H-labelled probe of equivalent specific activity. Furthermore, because the energy of the ^{35}S decay is higher than tritium, it is less subject to quenching. This latter point is important for one of the methodologies presented in the Results section.

Second, when non-isotopically labelled probes (e.g., biotin-labelled) are combined with a ^{35}S-labelled probe, colorimetric detection of the non-isotopic probe should precede isotopic detection since the autoradiographic emulsion limits penetration of the reagents necessary for non-isotopic detection.

Third, when a fluorescent label is used in conjunction with either a non-isotopic

probe or a ^{35}S or ^{3}H-probe, for example to detect a protein antigen, the cell or tissue sample can be dried down through ethanol and coated by standard procedures for autoradiography. After the slide is developed, the fluorescent signal is detected using epifluorescence optics while the autoradiographic grains are visualized by dark field, phase contrast or bright field optics.

Fourth, when using a fluorescent label for detection of the non-isotopically labelled probe, counterstaining the cell sample with certain reagents such as eosin should be avoided, since the dye may interfere with fluorescence and increase the background considerably. The samples can, however, be readily counterstained with the fluorescent dye, DAPI, which makes the nuclei fluoresce bright blue. DAPI avoids the fluorescent background and quenching of conventional dyes such as Giemsa or hematoxylin.

Fifth, in cohybridization of isotopic and non-isotopic probes where detection of the non-isotopic probe uses alkaline phosphatase in combination with the substrates NBT and BCIP, the purple colored product results in positive chemography of the autoradiographic emulsion, and therefore, coating the slide with a thin impenetrable film barrier (formvar or nitrocellulose) prior to the addition of emulsion is mandatory. Although such barriers give no background problems, they lengthen the autoradiographic exposure times by approximately three-fold.

RESULTS

A. Simultaneous Detection of Two mRNAs

Figure 1A shows the results of double-labelling experiments on differentiating chicken muscle tissue culture cells using a probe for myosin heavy chain mRNA as well as a probe for beta actin mRNA. The differentiation of muscle is marked by the fusion of mononucleated myoblasts into multinucleated myotubes which express large amounts of muscle specific gene products. The myosin heavy chain gene is expressed in significant amounts in the myotube, but at negligible levels in the mononucleated fibroblasts and myoblasts. In contrast, beta actin is detected in single cells, and the probe also hybridizes to sarcomeric actins found within the myotube. In Figure 1A the actin probe was labelled with biotin and the myosin probe with ^{35}S. The biotinated probe was detected by the alkaline phosphatase, streptavidin method (31). The undifferentiated single cells in the background show alkaline phosphatase staining due to the presence of actin mRNA, the myotube crossing the center of the field also shows intense autoradiographic labelling indicating the presence of the muscle specific myosin RNA sequences.

B. Simultaneous Detection of Protein and RNA

Figures 1B, C and D show the results of double-label experiments where myosin heavy chain protein in the myotubes is labelled with a monoclonal antibody and the message for this protein is detected using a ^{35}sulfur-labelled probe and subsequent autoradiography. It can be seen in 1C (phase) that there is a myotube crossing the field vertically, and some cells in the process of fusing to the left. In Figure 1B, dark field optics illuminate the grains much better allowing the visualization of the high signal of myosin message in the myotube and the beginning of the expression around the fusing nuclei to the left (the nuclei are stained with DAPI and can be easily seen in this figure). Finally in Figure 1D, the same cells are stained with myosin heavy chain

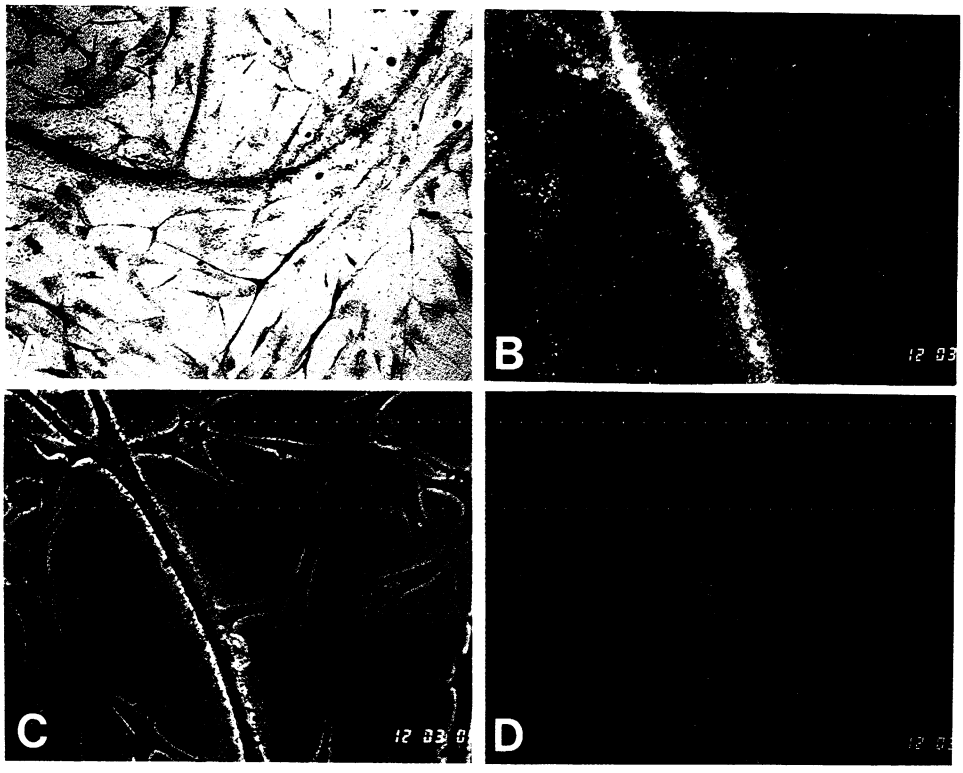


FIG. 1A. Double labelling of two messages in the same differentiating muscle culture. A 2kb beta actin cDNA probe was labelled with biotin and detected by the alkaline phosphatase streptavidin method. A 6kb myosin heavy chain cDNA probe was labelled with ^{35}S and detected subsequent to the non-isotopic probe by autoradiography over a barrier film.

FIGS. 1B–D. A differentiating muscle culture was hybridized with the myosin heavy chain cDNA and then the respective protein detected by monoclonal antibodies. Autoradiography for 7 days followed the antibody labelling. B. Dark field for autoradiography indicating the mRNA detection. C. Phase constrast microscopy. D. Protein fluorescence.

antibody where the same myotube stains intensely and the forming myotube to the left is much lighter. These observations clearly indicate the potential utility of this approach for evaluating transcriptional or translation control processes *in situ*. We have used this approach to study the expression of actin isoforms and myosin heavy chain in differentiating skeletal muscle (Lawrence *et al.*, in preparation).

With regard to the simultaneous detection of mRNA and protein, we have observed that the hybridization procedure often lessens the intensity of antibody fluorescence, presumably by denaturing protein, although this does not markedly interfere with overall detectability. The methods of *in situ* hybridization which we have developed (18, 21–23) utilize paraformaldehyde fixation which is well-suited for immunological techniques and have omitted harsh treatments of the cells (e.g., proteinases, acid, long hybridization times) hence they may be more compatible with pro-

tein immunofluorescence than more denaturing procedures.

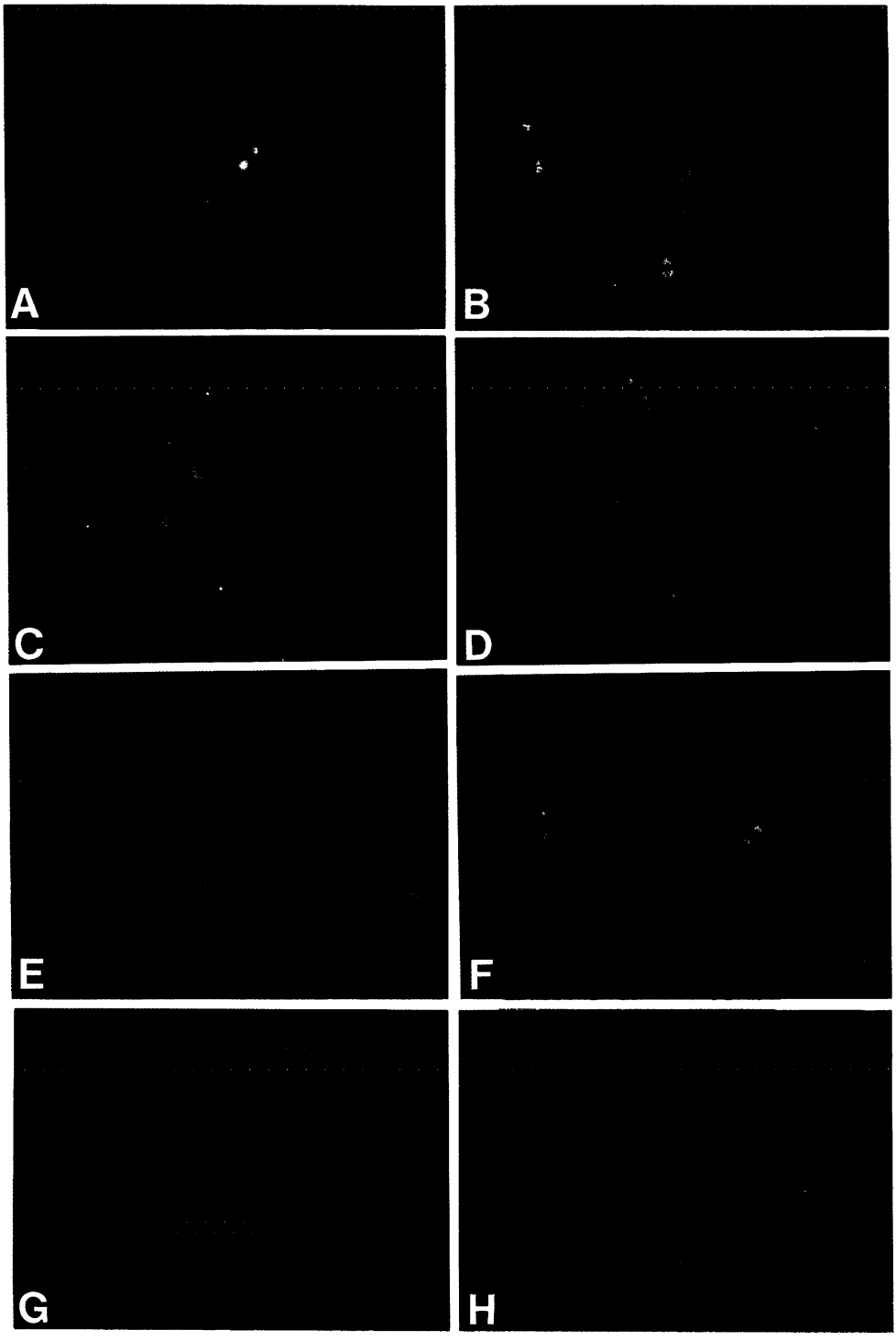
C. Simultaneous Detection of Two DNA Sequence Targets

Figure 2 illustrates the localization of two chromosome-specific DNA sequences in human interphase cells by non-isotopic methods. In panel A, the Y-chromosome specific probe was labelled with biotin and detected with streptavidin-biotinylated alkaline phosphatase, as described above. The Y-chromosome telomeric domain in the nucleus is seen as a single dark blue spot. The alphoid DNA repeat sequence, which under high stringency conditions, hybridized with high specificity to chromosome 1, was labelled with AAF and detected indirectly with FITC labelled antibody. This probe exhibits two bright foci of fluorescence which reflect the position of the two homologous chromosome 1 centromeres in the nucleus. These appear whitish in color because the DAPI counterstaining alters the spectral characteristics of the FITC emission in these double exposures. Panel C shows that essentially identical results are obtained when the Y probe was labelled with biotin and the alphoid probe labelled with mercury. In this case the sample was incubated post-hybridization with a trinitrophenyl modified sulfhydryl oligopeptide to form a haptene-thiol-mercury ligand which was then detected fluorometrically after subsequent exposure to rabbit anti-TNP antibody and fluorescein-labelled goat anti-rabbit IgG.

Interestingly, while we have been able to simultaneously detect probes labelled with biotin and AAF or biotin and TNP-mercury ligands, we have been unable to detect the mercurated probes after they have been cohybridized with an AAF-labelled probe. Although this suggests that these two reporter molecules may be chemically interactive, the cause of this interference and incompatibility is at present unknown.

The results shown in Panels B, D and E suggest that DNA of individual metaphase chromosomes occupy discrete domains within the nucleus of interphase cells and that the homologous chromosomes can be widely separated in the nucleus. Also note 1 centromeres are brightly labelled with the 1 probe in the metaphase plate (1E). Minor (less bright) hybridization sites can be observed on some chromosomes especially at less stringent hybridization conditions. Figure 2D shows the fluorescent signals obtained after hybridization of the biotinylated chromosome 1 probe to human fibroblast cells from an individual found by karyotyping to be trisomic for chromosome 1. Figure 1F is essentially the same as 1A except that rhodamine was used as the antibody detector for the chromosome 1 repeat and there was no Y chromosome hybridization in this picture. These observations indicate that numerical chromosome abnormalities, and possibly chromosome translocations, may

FIG. 2. In situ hybridization to normal human fibroblasts (panels A, B, C, F, G, H), chromosomes (panel E) and amniotic cells from an individual trisomic for chromosome 1 (panel D). The Y-chromosome-specific probe used in panels A and C was labelled with biotin and detected using avidin-alkaline phosphatase with BCIP-NBT substrates (single, dark blue spot). The chromosome 1 specific alphoid repeat was labelled with AAF (A, F) or Hg (B, C, E) and detected with FITC secondary antibodies (to AAF or TNP respectively) or TRIC labelled antibodies (F). In all panels except H, nuclei are counterstained with DAPI and double exposed using two filter sets. In panels G and H, the alphoid DNA was labelled with biotin and detected in cells pulsed with BUdR. Panel G shows the two chomosome 1 centromeres (detected by avidin-alkaline phosphatase) in DAPI stained nuclei while panel H shows the same field after BUdR detection with mouse anti-BUdR IgG and FITC-labelled rabbit-anti-mouse antibodies. Note that only one cell (upper) has incorporated BUdR during the pulse, i.e., is in S-phase.


A
B
C
D
E
F
G
H

be diagnosed directly on interphase cells given appropriate probe subsets. The ability to detect two or more probes hybridized simultaneously to the same cell population would provide the prerequisite internal controls for such analysis. Indeed such methods appear to be useful for detection of specific aneuploidies in human tumor cells (Cremer *et al.*, in preparation).

A second application of simultaneous labelling for two different DNA sequences is the detection of sequence distribution or copy number as a function of the cell cycle. To identify cells in S-phase, cell cultures were pulse labelled with bromodeoxyuridine for one to two hr prior to collection for hybridization. Post-hybridization, newly replicated DNA in S-phase cells was visualized by indirect immunofluorescence using a monoclonal antibody to bromodeoxyuridine as the primary antibody indicator (35; Antibody from Partec, Switzerland). Panels G and H show an analysis of the chromosome 1 repeat sequence in such bromodeoxyuridine-pulsed cells.

The centromeres of both chromosome 1 homologs can be readily distinguished in an S-phase (top) and a G-phase cell (below). The results of these non-radioactive double-labelling experiments are of high resolution and thus offer an improvement over radioactive methods for the assessment of S-phase cell populations (23).

D. Simultaneous Non-Isotopic Labelling of Nucleic Acids and Antigens at the EM Level

The potential resolution of non-isotopic probes is most fully realized at the elec-

FIG. 3. The simultaneous detection of tubulin messenger RNA and tubulin protein. Chicken embryonic myoblasts were grown on an electron microscopic grid for two days and then triton extracted after the method of Pudney and Singer (26), except that extraction was in room temperature isotonic buffer with EGTA added to preserve microtubules. The cells were fixed in 2% glutaraldehyde and dried through the critical point by CO_2. Cells were then hybridized using a probe for chicken alpha-tubulin (a gift of Paul Dobner) and after hybridization, stained with a monoclonal antibody to tubulin (Amersham) as well as a rabbit antibiotin antibody (Enzo biochemical). The secondary antibodies were from Janssen, a 5 nm colloidal gold antimouse IgG; and a 10 nm colloidal gold anti-rabbit IgG. Small arrowheads indicate the position of stained microtubules. The large arrowhead indicates the messenger RNA detected by a cluster of colloidal gold particles iterated along the double stranded hybrid.

tron microscopic level. This is made possible by the detection of biotinylated individual probe molecules hybridized to target mRNA or DNA molecules and then detected using colloidal gold-conjugated antibodies to rabbit IgG after rabbit antibiotin antibodies (Enzo). Protein or a second nucleic acid target can be simultaneously detected using a combination of these reagents or second antibody labelled with a different size colloidal gold particle. A detailed description of the protocols, will be included in Singer *et al.* (submitted for publication). A different approach to ultrastructural detection has been published by Binder *et al.* (1).

For example, Figure 3 shows the visualization of a single tubulin messenger RNA molecule hybridized to a biotinylated tubulin probe and detected by 10 nm colloidal gold antibodies. The cell has been treated with triton to allow penetration of the electron beam and visualization of the insoluble matrix to which the messages are bound (26). The cells were also reacted with monoclonal antibodies to tubulin and then rabbit anti-mouse antibodies, labelled by 5 nm gold. The two antibodies labelled with different sized gold are easily distinguished. The cluster of 10 nm particles denote a single message, whereas the 5 nm particles show microtubules (arrows in Fig. 3). Note, however, that the message is not situated near the microtubules.

DISCUSSION

A detailed molecular analysis of gene expression or nuclear structure in intact cells or tissues on a single cell basis will be advanced by the development of sensitive and reproducible methods for simultaneously detecting two or more target sequences. Other biological problems for which such techniques would be useful include gene mapping on chromosomes, studies of viral pathogenesis and clinical diagnostics. Double-labelling techniques at the EM level, as demonstrated here, will be particularly useful for investigating the interrelationship of specific nucleic acids with other cellular proteinaceous elements or high resolution analysis of cellular substructure using three dimensional reconstruction analysis.

The mixing of isotopic and non-isotopic labels for probes creates problems because of the emulsion and resolution. For this reason it is preferable to have available methods which are totally non-isotopic. Various methods for tagging nucleic acids with a variety of different, and uniquely detectable, reporter molecules have been developed over the past few years (13, 14, 33). The increasing availablity of such reagents should increase still further the convenience and simplicity of the *in situ* methodology. We have demonstrated here that three such probe labelling systems (biotin, aminoacetoxyfluorene and mercury-thiol-TMP ligand complex) can be used effectively for the simultaneous analysis of DNA or RNA sequences as well as with specific proteins. Our current work using these techniques is directed toward a high resolution study of specific mRNA molecules within individual cells and detailed study of the nuclear distribution of chromosomal territories.

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