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NUCLEIC ACID DETECTION BY *IN SITU* MOLECULAR IMMUNOGOLD LABELING PROCEDURES

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Abstract

We have recently combined immunogold labeling procedures with molecular biology methods to pinpoint the precise locations of nucleic acids in biological material at the ultrastructural level. These new immunocytological approaches involve the incorporation of labeled nucleotides in the nucleic acids present at the surface of ultrathin sections prior to immunogold labeling. The antibodies used recognize a nucleoside analogue (bromodeoxyuridine) or a hapten (biotin) employed to label nucleotides. Examples of high-resolution detection include DNA or RNA present in different substructures of cell nuclei, and in particular, in adenovirus-induced intranuclear regions of HeLa cells. In addition to being highly sensitive and specific, these new methods offer the possibility of studying the spatial distribution of nucleic acids in very well preserved, readily recognizable structures.

Key Words: DNA, RNA, immunogold techniques, ultrastructure, electron microscopy.

Introduction

Different methodological approaches are available to distinguish DNA and RNA from other macromolecules present in biological material at the ultrastructural level (Bendayan, 1984; Gautier, 1976; Moyné, 1980). A powerful strategy for selectively labeling DNA and RNA molecules involves the use of antibodies raised against DNA or RNA (Scheer *et al.*, 1987; Thiry *et al.*, 1991). These techniques exploit the highly specific reaction between antigen and antibody, using labeled immune reagents to locate antigens *in situ*. Unlike autoradiography after thymidine or uridine uptake, moreover, they are less time-consuming. The *in situ* hybridization is a molecular biology technique that has been now successfully applied on ultrathin sections of different biological materials to locate and to map specific nucleic acid sequences (for review, see Morel *et al.*, 1995). To improve the resolution of the *in situ* hybridization technique considerably, a very sensitive immunogold detection procedure was used to detect hybrid molecules. Recently, we have developed *in situ* terminal deoxynucleotidyl transferase (TdT)-immunogold technique, for DNA (Thiry, 1992b) and the *in situ* polyadenylate nucleotidyl transferase (PnT)-immunogold technique, for RNA (Thiry, 1993b) detection, to pinpoint the precise location of nucleic acids on ultrathin sections. The main goal of the present review is to compare these new approaches with the classical immunocytological approaches. Special emphasis is placed on the potential values and limitations of these two new, immunocytological techniques.

Principle of the *in situ* Transferase-Immunogold Techniques

In these new methods for *in situ* detection of nucleic acids, free DNA or RNA ends generated by sectioning are specifically elongated by transferases using labeled nucleotides. Subsequently, these modified nucleotides are visualized by immunogold electron microscopy. Thus, these techniques include two successive steps, the enzymatic reaction followed by an immunocytological

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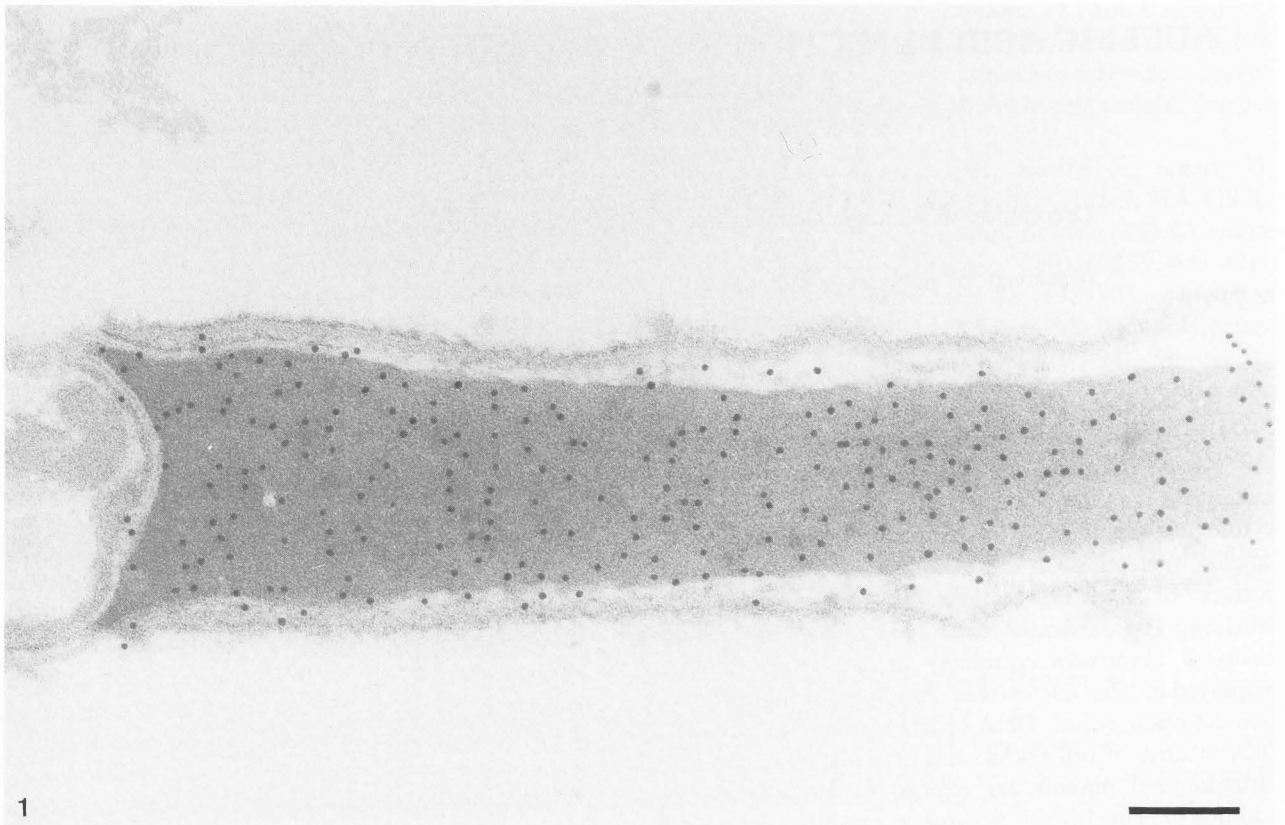


Figure 1. Immunodetection of BUdR triphosphates added by TdT on ultrathin sections in a portion of bull spermatozoa. Intense labeling is found over the condensed chromatin of the nucleus. Glutaraldehyde (1.6 %)/Epon/10 nm. Bar = 0.2 μ m.

(Figures 2 and 3 on facing page)

Figure 2. Location of DNA on Epon sections in the intranuclear fibrillar spots of an Ad5-infected HeLa cell at an intermediate stage of nuclear transformation, by the *in situ* TdT-immunogold technique. Label is found over all the fibrillar, electron-dense spots (stars) located inside the labeled fibrillogranular network (N) near the viral ssDNA accumulation sites (A). No label is present over the interchromatin granule cluster (IG). Bar = 0.2 μ m.

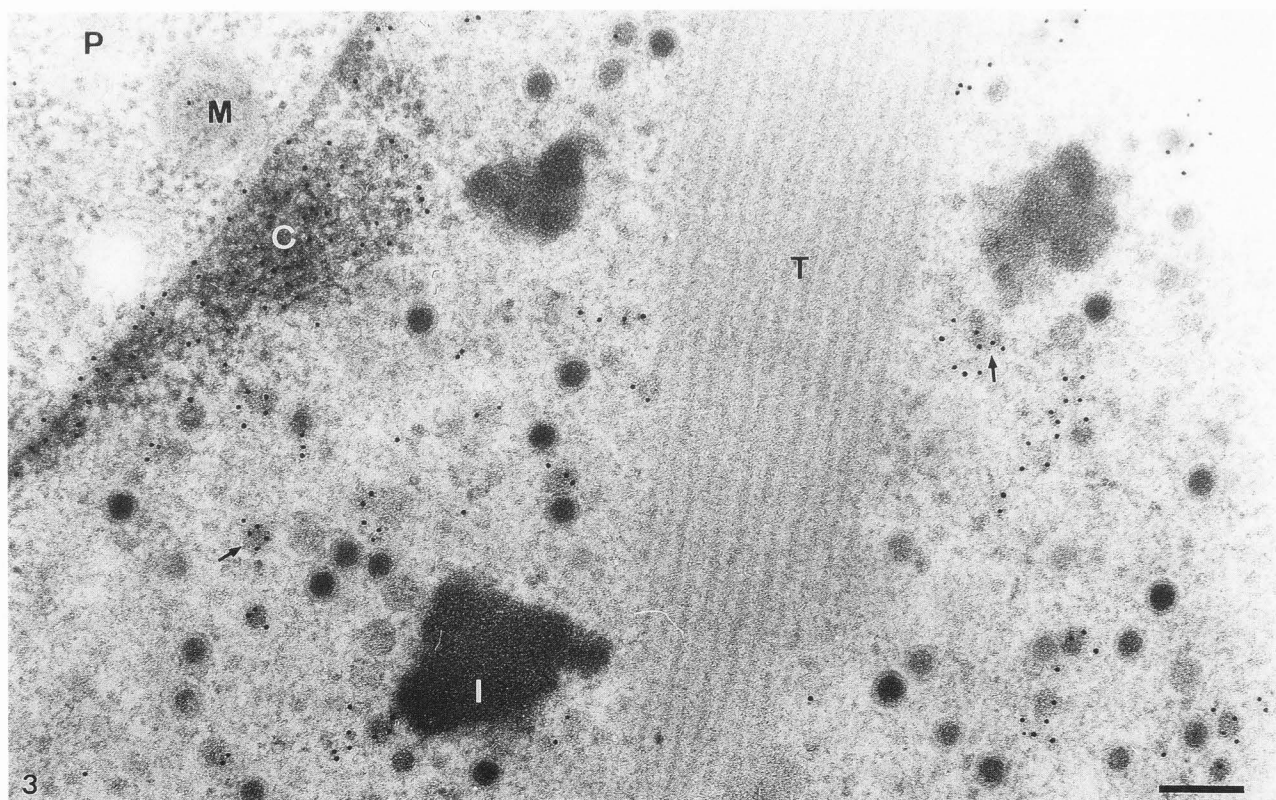
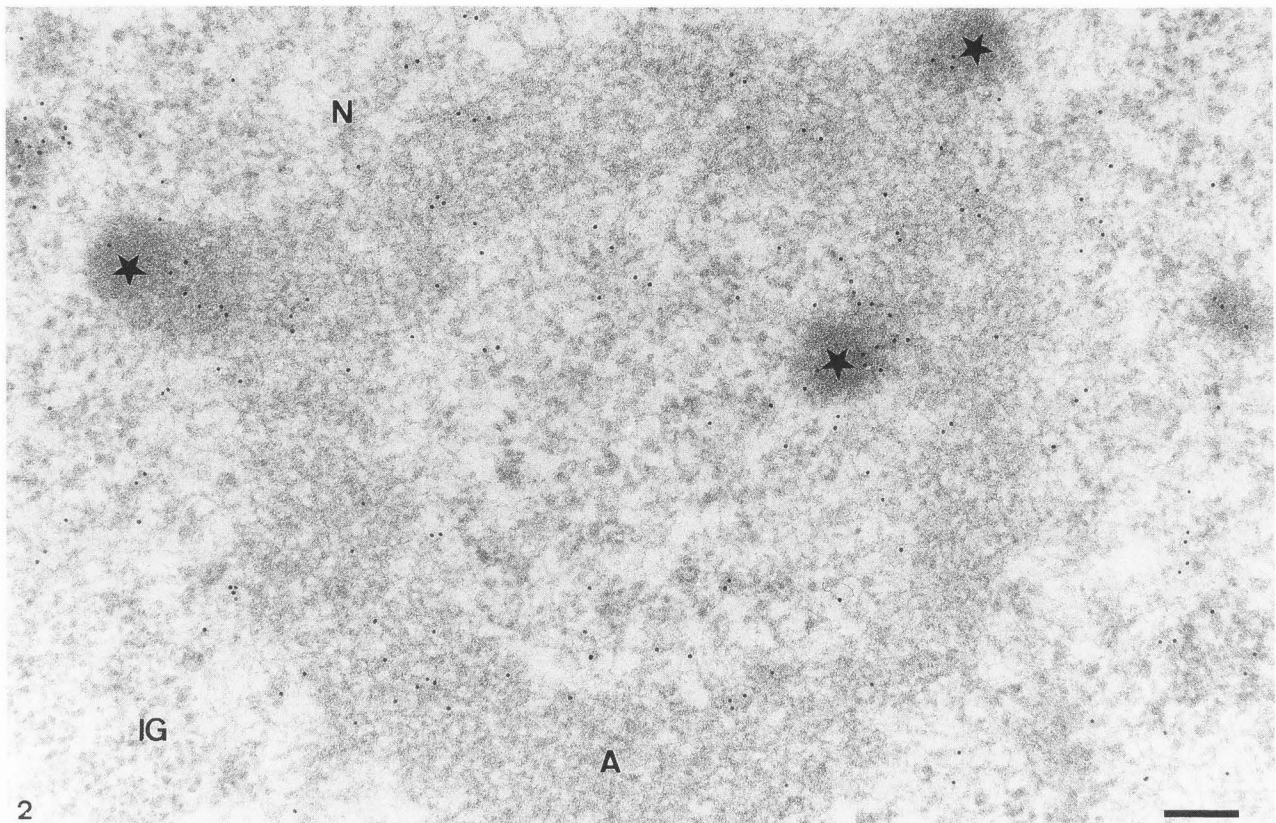
Figure 3. Location of DNA in different virus-induced intranuclear structures of an Epon-embedded HeLa cell at a late stage of nuclear transformation by the *in situ* TdT-immunogold technique. Label is densely distributed over the condensed host chromatin (C). Some viral nucleoids are also labeled (arrows). No label is visualized over the electron-dense amorphous inclusions (I) or the crystalloid (T). Except for some mitochondria (M), the cytoplasm (P) is also gold-free. Bar = 0.2 μ m.

procedure. In the enzymatic reaction for identifying DNA, TdT and biotinylated deoxynucleotides or bromodeoxyuridine triphosphates are employed. TdT is an unusual DNA polymerase which catalyzes a template-independent addition of deoxyribonucleotide triphosphates to the 3'-OH ends of double- or single-stranded DNA (Bollum, 1974; Chang and Bollum, 1986). The labeled nucleotides added by TdT on ultrathin sections are then visualized by an indirect immunogold labeling technique involving either an anti-biotin antibody or a monoclonal anti-bromodeoxyuridine antibody and a

secondary antibody coupled to colloidal gold.

PnT and biotinylated ATP are used to detect RNA. PnT, an *E. coli* enzyme catalyzes the addition of 5'-adenosine monophosphate to the 3'-OH end of single-stranded RNA (Sippel, 1973). All classes of RNA can be used as substrates (Sippel, 1973). The biotinylated ATP bound to the surface of ultrathin sections is then revealed by means of an anti-biotin antibody followed by a second, colloidal-gold-coupled antibody.

Molecular immunogold labeling of nucleic acids



Materials and Methods

TdT method

Cells or tissues are fixed for 30 min at 4°C in 2 % glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4), rinsed in buffer, dehydrated and embedded in Epon, Lowicryl K4M, and London Resin (LR) White.

Ultrathin sections are collected in platinum rings and stored on distilled water until used.

Ultrathin sections are:

- incubated for 10-30 min at 37°C at the surface of the following medium (100 mM Na cacodylate, 2 mM MnCl₂, 10 mM beta-mercaptoethanol, 50 µg/ml bovine serum albumin (BSA), 125 U/ml calf thymus TdT, and 20 µM 5-bromo-2-deoxyuridine triphosphate (pH 6.5-7),
- incubated for 10-30 min at 37°C in the same medium supplemented with 4 µM each of dCTP, dGTP, and dATP.

For labeling, the grids are:

- incubated by floating them face-down in a drop of phosphate buffered saline (PBS) (0.14 M NaCl, 0.006 M Na₂HPO₄, 0.004 M KH₂PO₄, pH 7.2) containing goat normal serum (GNS) diluted 1/30, and 1 % bovine serum albumin (BSA),
- washed with PBS containing 0.2 % BSA (pH 7.2),
- incubated for 4 hours with monoclonal anti-BUDr antibody (Becton Dickinson, Mt. View, CA) diluted 1/50 in PBS containing 0.2 % BSA (pH 7.2), and GNS diluted 1/50,
- rinsed four times with PBS containing 1 % BSA (pH 7.2),
- rinsed one time with PBS containing 0.2 % BSA (pH 8.2),
- incubated for 60 min at room temperature with goat anti-mouse IgG coupled to colloidal gold (5 nm diameter, diluted 1/40 in PBS, pH 8.2, containing 0.2 % BSA),
- washed four times with PBS containing 1 % BSA (pH 8.2),
- rinsed four times in deionized water,
- dried.

Sections are stained with uranyl acetate and lead citrate before examination in an electron microscope.

PnT method

Cells or tissues are fixed at 4°C for 60 min in 1.6 % glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4), dehydrated through graded acetone solutions, acetylated, then processed for embedding in Epon.

Ultrathin sections are either collected in platinum rings and stored on distilled water until used, or mounted on collodion-coated nickel grids.

Ultrathin sections are:

- incubated for 5 min at 37°C with 50 mM Tris

(Figures 4 and 5 on facing page)

Figure 4. Detection of RNA in tangentially sectioned nuclear envelope of acetylated Ehrlich tumor cells by the PnT-immunogold technique. Label is frequently found in the lumen of nuclear pores (P). In contrast, the condensed chromatin blocks are completely devoid of gold particles. T = cytoplasm, M = mitochondria. Bar = 0.2 µm.

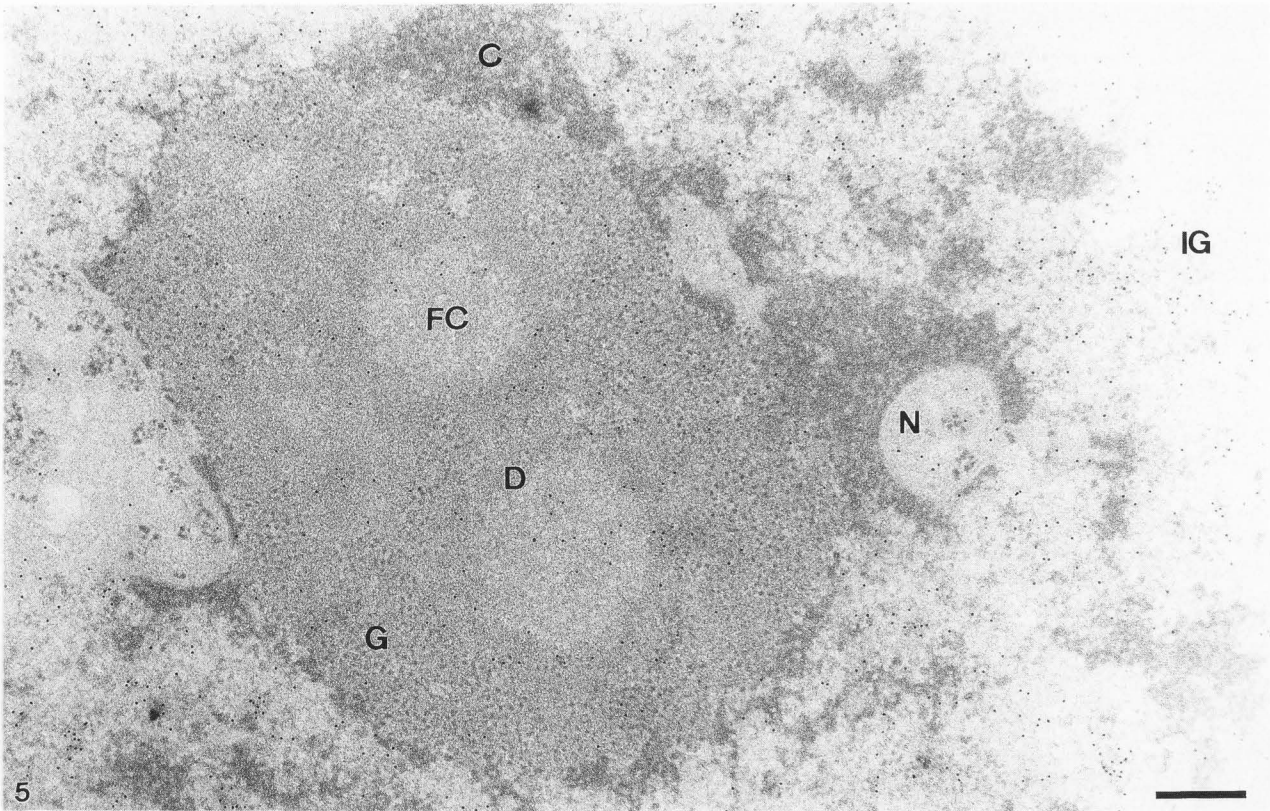
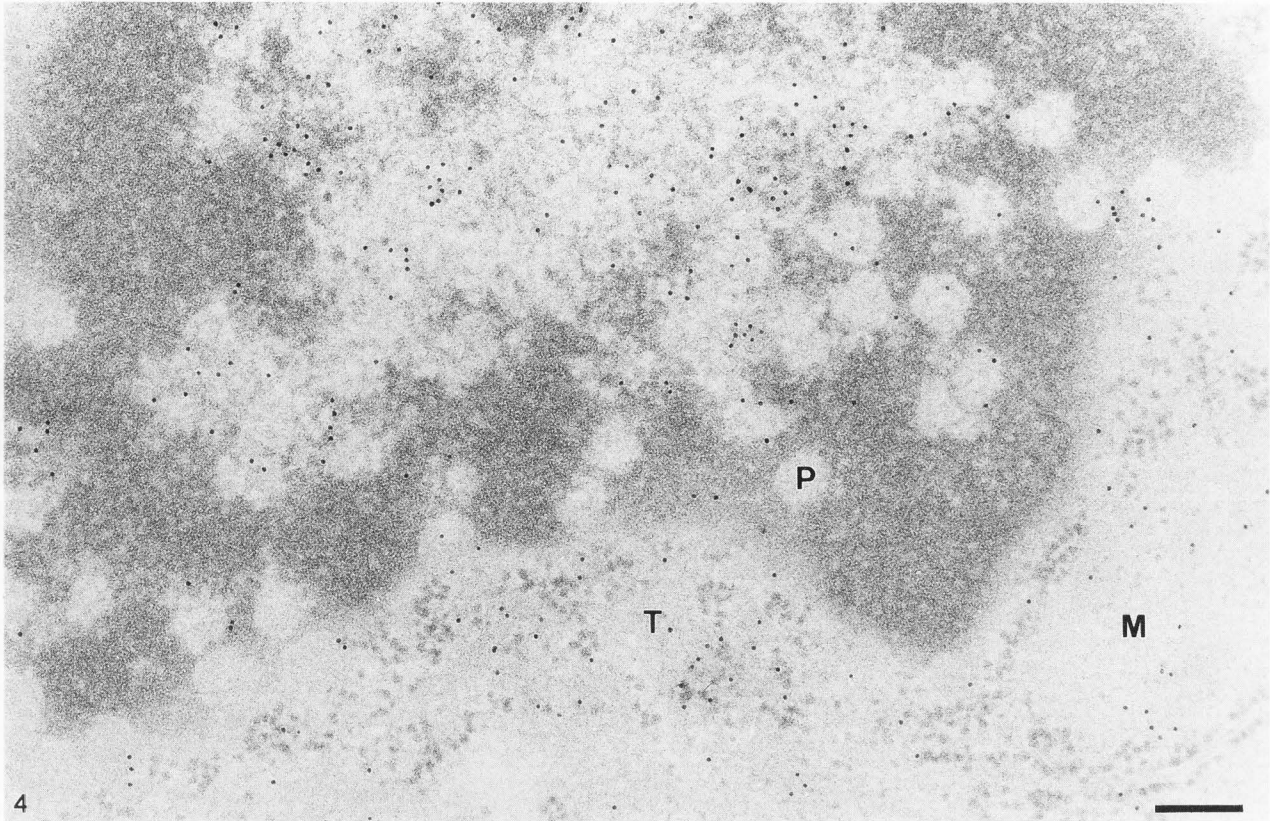
Figure 5. Intranucleolar distribution of RNA in an acetylated Ehrlich tumor cell as visualized with the *in situ* PnT-immunogold method. Gold particles are present in all the three main regions of the nucleolus, including the fibrillar centers (FC). On the contrary, the condensed chromatin (C) associated with the nucleolus is completely devoid of label. Evident label is also detected over an interchromatin granule cluster (IG). D = dense fibrillar component, G = granular component, N = nucleolar canal. Bar = 0.2 µm.

HCl (pH 7.9), 10 mM beta-mercaptoethanol, 10 mM MgCl₂, 2.5 mM MnCl₂, 0.25 M NaCl, 1 mg/ml bovine serum albumin, 25 U/ml *E. coli* PnT and 0.2 mM biotinyl-17-ATP,

- rinsed five times in bidistilled water,
- incubated for 30 min in PBSB (34 mM NaCl, 0.7 mM KCl, 20 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1 % BSA, pH 7.2) containing normal rabbit serum (NRS) diluted 1/30,
- incubated for 60 min at room temperature with goat anti-biotin antibodies diluted 1/500 in PBSB containing NRS diluted 1/50,
- rinsed four times in PBSB (pH 7.2),
- rinsed in PBSB (pH 8.2),
- incubated for 60 min at room temperature with medium containing rabbit anti-goat IgG coupled to gold particles either 5 nm or 10 nm in diameter, respectively diluted 1/50 or 1/200 in PBSB (pH 8.2),
- rinsed four times in PBSB,
- rinsed four times in bidistilled water,
- dried and prestained with salts of uranium and lead.

The *in situ* PnT-immunogold procedure is frequently performed on both sides of ultrathin sections. Once labeled on one side, the sections are mounted on collodion-coated nickel grids and the procedure applied to the second face.

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Potential Values and Limitations

The techniques are based on the highly specific enzyme-substrate reaction, on the one hand, and on the reaction between antigen and antibody, also highly specific, on the other hand. Consequently, highly specific results are obtained by these approaches. An exhaustive series of experimental controls has, moreover, confirmed this specificity (Thiry, 1992a,b, 1993b). As for any other immunogold technique, however, careful quantitative evaluation of the background signals is imperative (Thiry, 1993a,c, 1994; Thiry and Puvion-Dutilleul, 1995; Vandelaer *et al.*, 1993). Because gold particles are highly electron-dense with a perfect round shape and easily recognizable under electron microscope, the resolution of the labeling method is very high. As in the case of all postembedding gold methods, these immunogold labeling procedures are restricted to binding sites exposed at the surface of the sections (Thiry, 1992b). Immunoglobulin-gold complexes do not penetrate into resin sections (Bendayan and Stephens, 1984; Bendayan *et al.*, 1987; Stierhof and Schwarz, 1988; Stierhof *et al.*, 1986) thus revealing only those nucleic acids exposed by the cutting procedure. These constitute a minor but representative portion of the nucleic acids present in biological material sections. While being a limitation of these approaches, this also represents a significant advantage in that it allows for intracellular labeling of DNA and RNA molecules without limitations due to diffusion of reagents, permeation of membrane, and accessibility of nucleic acids. The successful labeling of various nucleic acid-containing structures, some containing very low amounts of nucleic acids (i.e. mitochondria, mycoplasmas, virus) (Thiry, 1992a,b, 1993, 1995a), demonstrates the high sensitivity of these approaches.

As compared with immunocytological techniques involving antinucleic acid antibodies these new approaches present, however, two essential advantages.

First, they show a more general distribution of nucleic acids in biological materials. The *in situ* transferase-immunogold techniques reveal with great precision the specific nucleic acid-containing structures in a wide variety of organisms, from prokaryotes to eukaryotes (Thiry, 1992a,b,c, 1993a,b,c, 1994, 1995a,b; Thiry *et al.*, 1993; Thiry and Puvion-Dutilleul, 1995; Vandelaer *et al.*, 1993). For instance, using the TdT method, label is not only found over condensed chromatin like the metaphase chromosomes or the spermatozoon nuclei (Fig. 1), but also over DNA in a completely extended configuration, like the highly active genes present in the Balbiani rings of *Chironomus tentans* salivary glands (Thiry, unpublished results). In adenovirus infected (Ad5) HeLa cells (Thiry and Puvion-Dutilleul, 1995)

(Figures 6 and 7 on facing page)

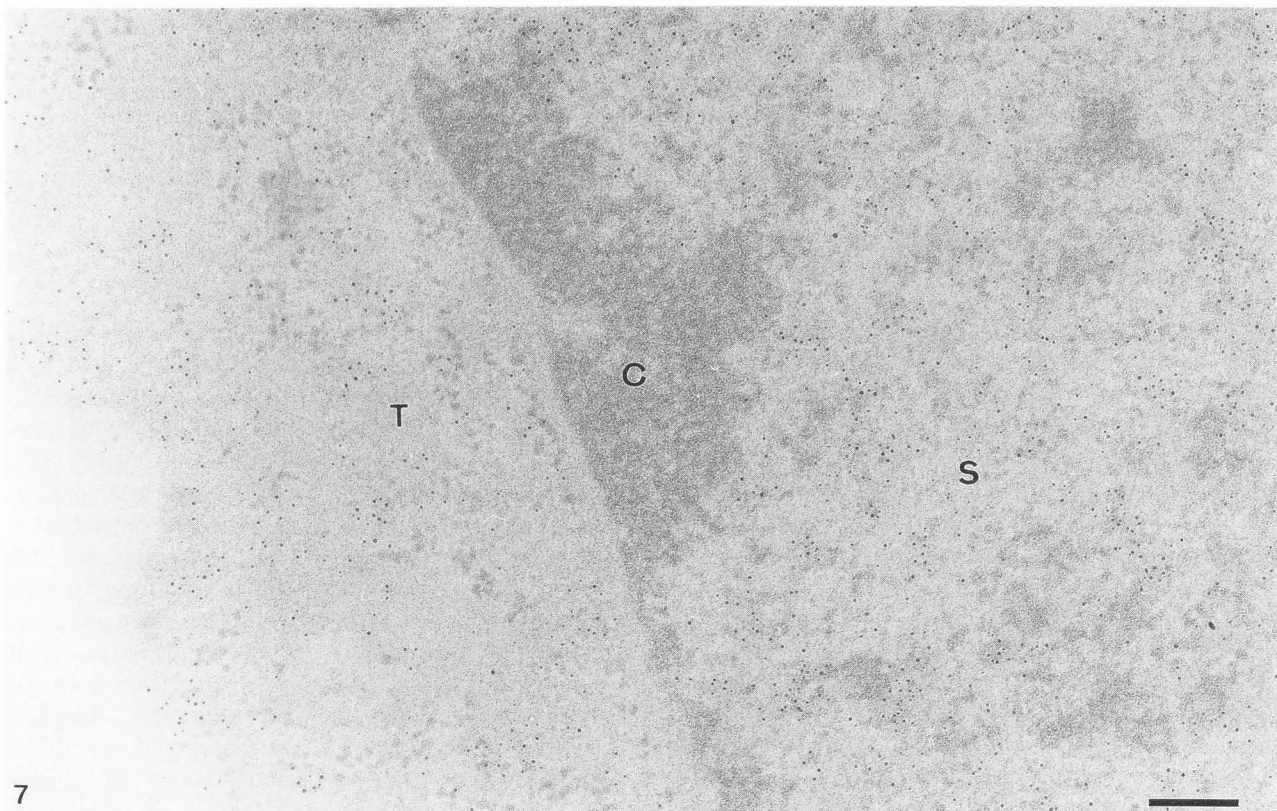
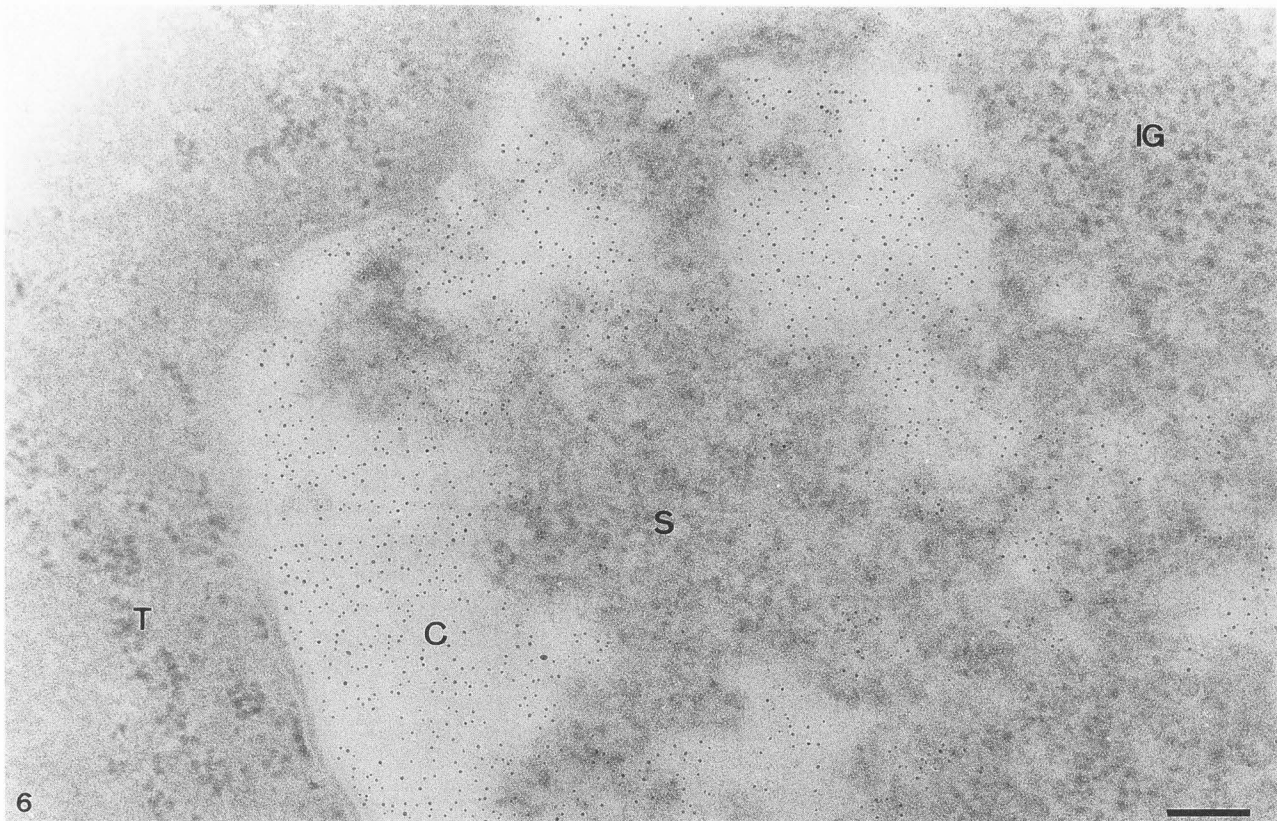
Figure 6. Distribution of DNA in a portion of Ehrlich tumor cell after acetylation and EDTA regressive staining as revealed by the TdT-immunogold technique. Label is preferentially found over the completely bleached blocks of condensed chromatin (C). A few gold particles are also visualized in interchromatin spaces (S). However, the interchromatin granule (IG) cluster is totally unlabeled. T = cytoplasm. Bar = 0.2 μ m.

Figure 7. Immunodetection of biotinylated ATP added by PnT on ultrathin sections of acetylated Ehrlich tumor cells. The gold particle concentration is particularly high over the nucleoplasmic spaces (S) between the heterochromatin blocks. In addition, intense label is also present in cytoplasmic ribosome-rich regions (T). No label occurs over the condensed chromatin (C) associated with the nuclear envelope (NE). Bar = 0.2 μ m.

(Figs. 2 and 3), the labeling reveals not only the condensed host chromatin but also the different types of Ad5 DNA fibers, i.e., active Ad5 genomes, inactive free Ad5 genomes, inactive encapsidated Ad5 genomes and single-stranded Ad5 DNA, in different virus-induced substructures which appear in the centers of infected nuclei. Using the PnT procedure, label is visualized over mitochondria, some nucleoplasmic substructures such as interchromatin granule clusters and coiled bodies, and the three main components of the nucleolus (Figs. 4 and 5). Examples of sensitive, high resolution detection of nucleic acid-containing structures have also been reported with the use of antinucleic acid antibodies (Hansmann *et al.*, 1986; Hayashi-isiharu *et al.*, 1993; Mena *et al.*, 1994; Puvion-Dutilleul and Pichard, 1993; Scheer *et al.*, 1987; Thiry 1992c, 1993c, 1994; Thiry and Puvion-Dutilleul, 1995). However, it is important to remember that no universal antinucleic acid antibody exists. Each antibody recognizes a certain nucleic acid "region" (distinguished by its configuration, sequence, etc.) (Angelier *et al.*, 1986; Lubit *et al.*, 1976; Soyer-Gobillard *et al.*, 1990). In this respect, in adenovirus-infected HeLa cells, by means of the *in situ* TdT method, label was consistently revealed over round fibrillar spots (Thiry and Puvion-Dutilleul, 1995) (Fig. 2). The use of anti-DNA antibodies has not allowed the detection of DNA in this substructure (Puvion-Dutilleul and Pichard, 1993). Consequently, the antibodies to nucleic acids do not constitute an useful mean for studying the general distribution of nucleic acids in biological materials.

The second advantage of the approaches compared with the use of antinucleic acid antibodies is that they are compatible with various fixation and embedding conditions. These new approaches are applicable to

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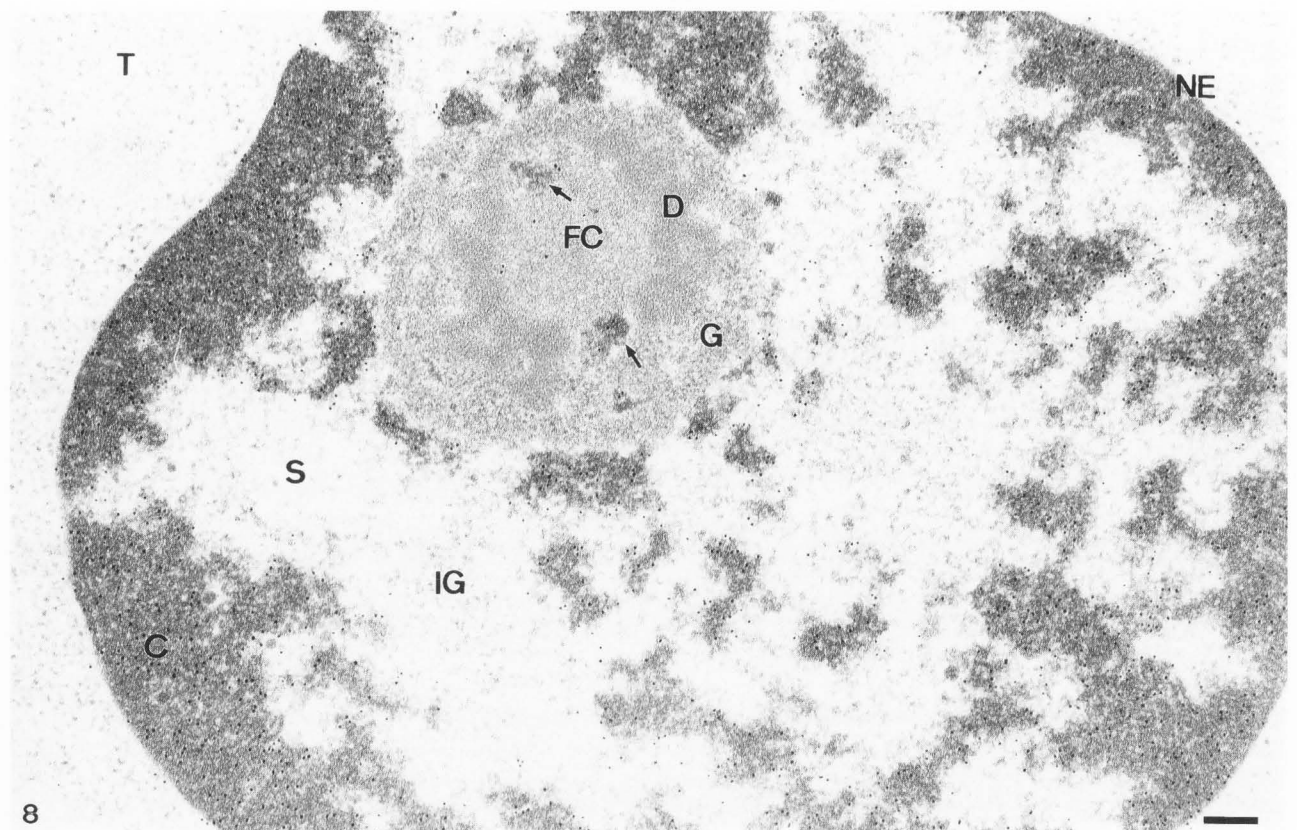


Figure 8. Immunodetection of BUdR triphosphates added by TdT on ultrathin sections of acetylated human resting T lymphocytes. The gold particle concentration is particularly high over the condensed chromatin (C) blocks associated with the nuclear envelope (NE) and the nucleolus. A few gold particles are also found in nucleoplasmic regions (S), however, interchromatin granule (IG) clusters are completely devoid of label. Inside the nucleolus, evident label is exclusively found over the intranucleolar clumps of condensed chromatin (arrows) and over the fibrillar centers (FC), whereas no significant label is detected over the dense fibrillar component (D) and the granular component (G). Except for some mitochondria, the cytoplasm (T) is also gold-free. Bar = 0.2 μm .

sections fixed with glutaraldehyde, formaldehyde, glutaraldehyde/formaldehyde and even glutaraldehyde/osmium tetroxide, whether embedded in Epon, Agar 100, Spurr, Lowicryl K4M or LR white (Thiry 1992a, b, 1993b; Thiry *et al.*, 1993). They can be further combined with cytochemical methods (Thiry 1992a,b, 1993b), such as acetylation or EDTA regressive staining, which intensify the contrast between some of the various nuclear compartments (Figs. 6-7). These methods thus offer the possibility of determining the precise location of nucleic acids in very well preserved, readily recognizable structures.

On the other hand, to respect a perfect compromise among preservation of fine cellular structure, retention of antigenic determinants, and optimal specificity, antibodies to nucleic acids are only applicable to biological material fixed and embedded in a way that does not always optimally preserve the structure to be studied. In

this respect, to facilitate the identification of DNA positive sites within the mammalian cell nucleolus, the *in situ* TdT technique was applied on acetylated material (Thiry 1992a,b, 1993a,b; Thiry *et al.*, 1993; Vandelaer *et al.*, 1993), a cytochemical method allowing an excellent distinction between the various nucleolar components and in particular a high contrast of the condensed chromatin (Thiry and Goessens, 1986; Wassef *et al.*, 1979). Under these experimental conditions (Fig. 8), in a large variety of cell nucleoli, besides the presence of an intense label over the intranucleolar and perinucleolar condensed chromatin, DNA was consistently found in the fibrillar centers whereas no significant label was detected in the dense fibrillar component and the granular component. This analysis also revealed the presence of small clumps of condensed chromatin in close contact with the fibrillar centers and interrupting the layer of the dense fibrillar component.

Such observations cannot be achieved with antibodies to DNA because they are not compatible with acetylation conditions.

Conclusions

The immunocytological approaches represent powerful means of studying and identifying the nucleic acids in biological materials at the ultrastructural level.

The *in situ* transferase-immunogold techniques offer the possibility of studying the spatial distribution of nucleic acids in very well preserved, readily recognizable structures. While the antinucleic acid antibodies do not constitute an useful mean of studying the general distribution of nucleic acids in biological materials, their high elective specificity makes it possible to investigate the precise location and functional role of certain specific nucleic acid regions (i.e., Z-DNA, 5-methylcytosine). The use of antinucleic acid antibodies can therefore provide complementary informations on nucleic acids visualized by the *in situ* transferase-immunogold techniques.

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Discussion with Reviewers

M. Malecki: What is your recommendation for the choice of embedment, i.e., giving the strongest labeling and the lowest background?

Author: The new techniques are compatible with various fixation and embedding conditions. The labeling intensity obtained with the TdT method does not appear to be affected by the fixation and embedding conditions. On the other hand, the *in situ* PnT-immunogold technique is compatible with fewer fixation and embedding conditions than the *in situ* TdT-immunogold technique. In particular, the labeling intensity obtained with the PnT method varies according to the fixation and embedding used. Best results were obtained on 1.6% glutaraldehyde-fixed and Epon-embedded cells.

M. Malecki: Did you attempt to use resinless sections or sucrose infused cryo-sections for derivatized analog incorporation/immuno-gold labeling?

Author: No, nevertheless, it is interesting to note that the TdT method is applicable to cell sections cryofixed, cryosubstituted in acetone, and embedded in Epon.

M. Malecki: Can you explain your preference for using gold conjugated antibodies against biotin rather than streptavidin-gold?

Author: We have not tested different procedures for revealing biotin. The use of anti-biotin antibodies was currently used in our laboratory and provided satisfactory results.

M. Malecki: What is your experience with other analogs in particular those with digoxigenin ?

Author: In our studies, we have commonly used two analogs: biotinylated nucleotides and bromodeoxyuridine triphosphates. In our hands, best results were obtained with the BudR system. Moreover, we have used digoxigenin-ATP as substrate in the PnT method. The labeled sites were subsequently revealed by an anti-digoxigenin antibody and antibodies coupled to colloidal gold. Under these conditions, results were very poor; only a few gold particles were present. These results seemed to indicate that digoxigenin-ATP is not efficiently incorporated into RNA by PnT.

Reviewer II: What is the specificity of TdT and/or PnT

labeling in preserved tissue, and how has it been checked ?

Author: The specificity of the TdT and/or PnT labeling was tested in several ways.

TdT: When TdT or BUdR triphosphate were omitted from the TdT medium, the ultrathin sections were devoid of label. No labeling was observed when BUdR triphosphate was replaced by BUdR monophosphate. When the sections of Lowicryl-embedded cells were incubated with trypsin, pronase, protease, or RNase, the labeling persisted; when DNA was specifically removed from the sections by treatment with DNaseI, the labeling was completely abolished.

PnT: When PnT or biotinylated ATP was omitted from the PnT medium, no labeling occurred. When acetylated cell sections were pre-incubated with RNase A at a high concentration (10 mg/ml), labeling was completely abolished. At a lower RNase A concentration, labeling was only reduced. Labeling was likewise weaker when acetylated cell sections were pre-incubated with RNase T2. No label was detected on acetylated cell sections pre-treated with RNase T2, followed by RNase A at a lower concentration. Preincubation with DNase I, on the other hand, did not prevent labeling. Finally, when the RNase T2 incubation was carried out after the PnT reaction, no labeling occurred. Finally, when the RNase T2 incubation was carried out after the PnT reaction, no labeling occurred.

The immunolabeling specificity was also tested. When the primary antibody was omitted, no labeling occurred. Gold lacking the antibody tag did not bind to the sections. All these experimental controls support the high specificity of these methods.