In Situ Localization of Mitochondrial DNA Replication in Intact Mammalian Cells

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Abstract. Nearly all of the known activities required for mitochondrial DNA (mtDNA) replication and expression are nuclear-encoded gene products, necessitating communication between these two physically distinct intracellular compartments. A significant amount of both general and specific biochemical information about mtDNA replication in mammalian cells has been known for almost two decades. Early studies achieved selective incorporation of the thymidine analog 5-Bromo-2-deoxy-Uridine (BrdU) into mtDNA of thymidine kinase–deficient (TK^{−}) cells. We have revisited this approach from a cellular perspective to determine whether there exist spatiotemporal constraints on mtDNA replication. Laser-scanning confocal microscopy was used to selectively detect mtDNA synthe-

sis in situ in cultured mammalian cells using an immunocytochemical double-labeling approach to visualize the incorporation of BrdU into mtDNA of dye-labeled mitochondria. In situ detection of BrdU-incorporated mtDNA was feasible after a minimum of 1–2 h treatment with BrdU, consistent with previous biochemical studies that determined the time required for completion of a round of mtDNA replication. Interestingly, the pattern of BrdU incorporation into the mtDNA of cultured mammalian cells consistently radiated outward from a perinuclear position, suggesting that mtDNA replication first occurs in the vicinity of nuclear-provided materials. Newly replicated mtDNA then appears to rapidly distribute throughout the dynamic cellular mitochondrial network.

MITOCHONDRIA are semiautonomous organelles that have a resident genome but rely heavily upon the nucleus to encode both proteins and nucleic acids for replication and transcription of mitochondrial DNA (mtDNA).[1] The important discovery that mammalian mitochondria have a genetically distinct (from the major cellular [soluble]) thymidine kinase (TK) enzyme (Berk and Clayton, 1973) greatly facilitated quantitative biochemical studies of mtDNA replication in cultured cells. Early studies showed that cell lines could be developed that are devoid of the cellular TK (Kit et al., 1963); however, these TK(−) cell lines still incorporate the thymidine analog 5-Bromo-2-deoxy-Uridine (BrdU) into mtDNA (Clayton and Teplitz, 1972; Berk and Clayton, 1973). As a result, the mtDNA in these cells can be labeled to a high specific activity to the relative exclusion of nuclear DNA.

Past studies also demonstrated that mtDNA synthesis can occur at random during the cell cycle, displaying no strict phase specificity as is the case with nuclear DNA synthesis (Bogenhagen and Clayton, 1977). In fact, mtDNA can be replicated more than once or not at all during the cycling of a given cell population (Flory and Vinograd, 1973). Yet, mtDNA copy number is maintained remarkably constant through generations of cell growth and division. Several other features typify mtDNA replication, such as the fact that unlike chromosomal DNA synthesis, there does not appear to exist the same extent of repair occurring within the mitochondrion (Clayton, 1991), although some forms of mtDNA repair do occur in mammalian cells (Pettepher et al., 1991; LeDoux et al., 1992; Driggers et al., 1993; Shen et al., 1995). Synthesis of mtDNA is thought to occur within the mitochondrial matrix (Clayton, 1991), enlisting organelle-specific replication and transcription machineries. Accordingly, mtDNA replication is insensitive to inhibitors of nuclear DNA replication such as aphidicolin, but susceptible to inhibition by compounds that have a minimal effect on nuclear DNA replication, such as dideoxycytosine (ddC) and azidothymidine (AZT) (Simpson et al., 1989; Chen et al., 1991; Erikkson et al., 1995).

The significant dependence of mitochondria on nuclear-provided materials for the maintenance of mtDNA copy

number prompted us to revisit the topic of mtDNA replication from a cellular perspective, to determine whether there might exist spatial constraints upon replication of the mitochondrial genome. Indeed, the process of chromosomal DNA replication occurs at specific foci positioned throughout the nuclear interior (Nakayasu and Berezney, 1989). Such an organization suggests that these centers of replicative activity might represent either structural entities or functional aggregates of replication enzymes and their associated cofactors. We were curious to determine whether an analogous arrangement might characterize loci of mtDNA synthesis.

Using antinucleotide antibodies (Gratzner, 1982) and recently developed mitochondrial labeling techniques (Whitaker et al., 1991), we have used confocal microscopic methodology to similarly analyze the process of mtDNA replication in situ in intact mammalian cells. In a manner conceptually analogous to early labeling studies, we have assessed the incorporation of the thymidine analog BrdU into mtDNA of intact cultured mammalian cells whose mitochondria have been specifically labeled with a membrane potential–sensitive dye. Hence, the subcellular location of newly synthesized mtDNA can be readily visualized by virtue of colocalization of BrdU and mitochondria. Our results confirm previous biochemical studies with regard to both qualitative and quantitative aspects of mtDNA synthesis in mammalian cells. Moreover, we report that in several different cell types, the pattern of incorporation of BrdU into mtDNA radiates outward from the nucleus, suggesting that replication of the mitochondrial genome may in fact occur within a spatial proximity of the nuclear-encoded mtDNA replication machinery.

Materials and Methods

Cells and Cell Culture Maintenance

HeLa human cervical carcinoma cells (CCL 2; American Type Culture Collection, Rockville, MD) were grown as monolayer cultures in DME (GIBCO BRL, Bethesda, MD) containing high glucose, L-glutamine, and 110 mg/liter sodium pyruvate, and supplemented with 5% FBS (GIBCO BRL) and 1.25 μg/ml gentamycin (Sigma Chemical Co., St. Louis, MO). 143B (TK[−]) human osteosarcoma cells (CRL 8303; American Type Culture Collection, kindly provided by Dr. Eric A. Shoubridge) were maintained in DME supplemented with 5% FBS, 1.25 μg/ml gentamycin, 50 μg/ml uridine, and 100 μg/ml (333 μM) BrdU (both from United States Biochemical Corp., Cleveland, OH). 143B cells containing mtDNA (ρ+) or lacking mtDNA (ρ°) were cultured under identical conditions. 143B cells were cultured in BrdU-free medium for at least 2 wk (2–3 doubling times) before BrdU pulse labeling and immunocytochemical analysis. PC12 rat pheochromocytoma cells (kindly provided by Dr. Eric M. Shooter) were maintained in DME supplemented with 6% FBS, 6% bovine serum, 1.25 μg/ml gentamycin, 100 U penicillin G/ml, and 100 μg/ml streptomycin. PC12 cell cultures were differentiated with NGF (Harlan Bioproducts, Indianapolis, IN, generously provided by Dr. Eric M. Shooter). Briefly, PC12 cells were cultured on poly-L-lysine (1 mg/ml)–coated glass coverslips, serum starved for 1–2 h, and then cultured in NGF-containing (10 ng/ml) serum-free medium (with 0.1% filtered BSA) for 6–10 d, with NGF treatments every 3 d.

Enucleation

Enucleation of HeLa cells was performed as described previously (Prescott et al., 1971). Briefly, HeLa cells were seeded onto autoclaved 18-mm coverslips (VWR Scientific Corp., Media, PA) in 60-mm plastic dishes (Becton Dickinson Labware, Lincoln Park, NJ) and grown to ~50–60% confluency. Cells on coverslips were placed face down in 50-ml polypropylene conical tubes containing 5 ml prewarmed supplemented DME and cytochalasin B (10 μg/ml) (Sigma Chemical Co.). Cells were centrifuged at 37°C (in a table-top centrifuge [model GS-6KR; Beckman Instrs., Fullerton, CA] warmed by prerunning for ~1 h) for 40 min at 3,000 g. Coverslips were then placed in 60-mm dishes and allowed to recover in a 37°C 5% CO₂ incubator for 1–3 h, followed by in vivo labeling with BrdU and subsequent labeling and immunocytochemical detection steps.

Ethidium Bromide Staining of mtDNA

mtDNA in living HeLa cells was labeled as previously described (Hayashi et al., 1994). Briefly, cells cultured on glass coverslips were weakly trypsinized (1× trypsin, 2–3 min at room temperature) and then stained with ethidium bromide (2 μg/ml, in supplemented DME) for 5 min at room temperature. Cells were then washed repeatedly with supplemented DME and visualized by microscopy.

Platelet Preparation

Human platelets were obtained from peripheral blood by standard methodologies. Briefly, blood was collected into a heparinized tube and centrifuged for 15 min at ~1,500 rpm in a table-top centrifuge to separate plasma from erythrocytes. The plasma and intervening buffy coat were collected, and each was analyzed microscopically for cell content. The plasma was recentrifuged (3,100 rpm [2,300 g]) for 30 min at 4°C. The majority of supernatant was removed, leaving ~1 ml of platelet "pellet." Since the cells were not subjected to a ficoll gradient centrifugation, the platelet preparation was not completely free of contaminating erythrocytes and lymphocytes. Platelets were then labeled with BrdU (2 h, 37°C) and Mitotracker™ (Molecular Probes, Eugene, OR) and subjected to immunocytochemistry as detailed below.

BrdU Labeling and Immunocytochemistry

Monolayers of 143B (TK[−]), HeLa, or PC12 cells were seeded onto autoclaved glass coverslips and cultured in 60-mm plastic dishes in supplemented DME, in the absence or presence of 10–15 μM BrdU for 1–72 h. In some experiments, to inhibit nuclear DNA replication, HeLa cells were pretreated with aphidicolin (Sigma Chemical Co.) as follows: a 30-mM treatment stock of aphidicolin was prepared in sterile DMSO. Aphidicolin was added directly to the existing culture medium in dishes, to a final concentration of 20 μM (7 μg/ml). Cells were pretreated for a minimum of 60 min with aphidicolin, at which point BrdU was added. Aphidicolin was present in the medium throughout the labeling period.

For experiments with ddC, differentiated PC12 cells were treated concurrently with both ddC and BrdU as follows. A 10-mM treatment stock of ddC (Sigma Chemical Co.) was prepared, and cells were treated with variable concentrations (10 nM–10 μM) of ddC simultaneously with BrdU (15 μM). ddC was present throughout the BrdU labeling period.

Before fixation, all cells were labeled in vivo with the mitochondrial membrane potential-sensitive dye Mitotracker™ (Whitaker et al., 1991). Briefly, cells were incubated in the presence of the dye (100 nM, diluted in supplemented DME) for 15 min at 37°C, followed by an identical incubation in DME, except in the absence of dye. Labeling and all subsequent steps were performed in the dark. Cells were then washed twice with PBS, pH 7.4. Cells were first fixed with 4% paraformaldehyde (diluted from freshly prepared 20% paraformaldehyde [Sigma Chemical Co.]) for 10 min at 25°C, and then permeabilized with −20°C acetone. Cells were rehydrated for 3 min (25°C) to 20 h (4°C) in PBS. DNA was denatured by incubation in 2 N HCl at 37°C for 1 h. Acid was neutralized with 100 mM borate buffer, pH 8.5, changing the buffer twice over a 10-min period. Cells were then washed with PBS three times over a 10-min period. Both primary and secondary antibodies were diluted in PBS supplemented with 0.1% BSA at the following concentrations: anti-BrdU, 1:50 (monoclonal; Boehringer Mannheim Corp., Indianapolis, IN); anti-DNA polymerase γ, 1:250 (polyclonal; generously provided by Dr. W.C. Copeland, National Institute of Environmental Health Sciences, Research Triangle Park, NC); anti–mitochondrial transcription factor A, 1:1,000 (polyclonal); <FITC>anti-mouse IgG, 1:500; <Cy5>anti-rabbit IgG, 1:500. Primary antibody incubations were performed for 60 min at 25°C, and secondary antibody incubations were performed for 30 min at 25°C. Cells were washed repeatedly between primary and secondary antibody incubations as well as after the secondary antibody incubation step. Finally, cells on coverslips were mounted onto glass slides with Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ). Microscopy was performed using a laser-scan-

ning confocal microscope (model MRC 1000; BioRad Labs, Hercules, CA) equipped with CoMOS imaging software. mtBrdU in "perinuclear" or "peripheral" cellular regions was experimentally defined as follows. In 143B (TK[−]) and HeLa cells, perinuclear mtBrdU was defined as that which appeared immediately adjacent to the nuclear boundary, and peripheral mtBrdU was defined as the remainder of BrdU incorporation into mtDNA. In differentiated PC12 cells, classification of perinuclear and peripheral mtBrdU was defined as the appearance of nuclear-proximal (cell body–associated) or neurite/growth cone–associated (for perinuclear and peripheral, respectively) mtBrdU. The ratio of subcellular mtBrdU pools in PC12 cells was quantitated visually by counting the number of fluorescent sites of BrdU incorporation in each region. Digital images were colored and merged using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA). Experiments were repeated at least three times, yielding reproducible results.

Results

Detection of mtDNA Replication In Situ in Intact Mammalian Cells

Previous studies have shown that mammalian cell lines can be developed that lack the major cellular TK enzyme that catalyzes the phosphorylation of thymidine (or the thymidine analog BrdU) to thymidine (or BrdU) monophosphate (Kriss and Revesz, 1961). However, cells normally contain two genetically distinct TK enzymes, one of which functions within mitochondria (Berk and Clayton, 1973). Hence, the mtDNA in such cell lines (TK[−]) can be labeled to a high specific activity relative to nuclear DNA through the addition of exogenous sources of thymidine (or BrdU).

We emulated previous biochemical labeling studies using TK(−) cells, using recently developed immunocytochemical methods to detect the incorporation of BrdU into mtDNA (mtBrdU) in intact mammalian cells (Fig. 1). While BrdU incorporation from mtDNA repair cannot be distinguished in this assay from BrdU incorporation consequent to mtDNA replication, repair is unlikely to be so extensive as to contribute significantly to the observed incorporation. Newly synthesized mtDNA was readily detectable in 143B (TK[−]) human osteosarcoma cells after a 2-h pulse of BrdU in vivo (Fig. 1 a, green). Colocalization with dye-labeled mitochondria (Fig. 1 b, red) confirmed the mitochondrial location of BrdU (Fig. 1 c, merged image, yellow). Control experiments with 143B (TK[−]) ρ° cells, which lack mtDNA, revealed no incorporation of BrdU into the mitochondria of these cells (Fig. 1, e and g), although biochemical experiments revealed that these ρ° cells did have functional TK activity measurable in vitro (data not shown). The mtDNA status of 143B (TK[−]) ρ+ or ρ° cells is also indicated by the presence or absence of mtTFA, (blue in Fig. 1, d and h, respectively). mtTFA, a protein whose presence closely mirrors the mtDNA status of the cell, is not detectable in cells that have been either transiently or chronically depleted of mtDNA (Larsson et al., 1994; Poulton et al., 1994; Davis et al., 1996).

The absence of a functional cellular TK enzyme in TK(−) cells permits the exclusive detection of mtBrdU in these cells. Since cells typically contain both TK enzymes, we tested whether or not mtBrdU was detectable in TK(+) cells by similar means. Since mtDNA constitutes <1% of cellular DNA, in TK(+) cells nuclear BrdU incorporation far exceeds that of mtDNA. Although both nu-

clear and mtDNA replication are occurring in these cells, the anti-BrdU antibody is presumably saturated under normal circumstances of nuclear DNA labeling, and only nuclear BrdU could be visualized (Fig. 2 a). However, we observed that mtBrdU was readily detectable in these rapidly dividing cells under circumstances where nuclear DNA replication was pharmacologically inhibited by pretreatment with aphidicolin, a compound that inhibits DNA polymerase α but not mtDNA polymerase γ (Fig. 2 b). Hence, the conditions required for observation of mtBrdU in these TK(+) cells may explain why most reports measuring cell proliferation with anti-BrdU immunocytochemistry fail to detect BrdU incorporation into mtDNA.

Interestingly, we observed that in either 143B (TK[−]) or HeLa cells, newly synthesized mtDNA appeared to be manifest at a perinuclear location (yellow signal adjacent to the nuclear boundary). MtBrdU was not evident in peripheral regions of the cell (yellow mtBrdU signal in mitochondria situated within peripheral cytoplasm) in the concomitant absence of a perinuclear mtBrdU signal, suggesting that perhaps mtDNA synthesis first occurs near the proximity of the nucleus. The results obtained from this microscopic analysis suggest that initial labeling of mtDNA might be preferentially perinuclear, becoming more broadly distributed toward the periphery of the cell upon longer labeling.

Importantly, the distribution of newly replicated mtDNA differs markedly from that of total mtDNA in the cell as assessed with vital staining of mtDNA in living cells with ethidium bromide (Hayashi et al., 1994) (Fig. 2, d and e). Presumably, the ethidium bromide staining pattern observed solely reflects mtDNA, although a contribution by mtrRNA cannot be eliminated. Unfortunately, unlike the case with yeast, mammalian mtDNA is refractory to staining by conventional means, such as with the DNA-specific dye 4', 6-diamino-2-phenylindole (DAPI) (Russell et al., 1975).

Intracellular Localization of BrdU Incorporation into mtDNA

As a test of the hypothesis that proximity to the nucleus is important for the execution of mtDNA synthesis, we next decided to determine whether or not cells could incorporate BrdU into mtDNA in the absence of a nucleus, i.e., in enucleated cells. We predicted that if the nucleus were removed, this function would no longer be supplied, and replication of the mitochondrial genome would cease.

Using cytochalasin B, HeLa cells were enucleated (Prescott et al., 1971) and then labeled in vivo with BrdU. Interestingly, we found that under no circumstances was BrdU incorporated into mitochondria of enucleated cells, even with relatively long BrdU labeling times, which typically resulted in robust levels of mtBrdU. Although a representative example of such experiments is shown, results were consistent over four separate experiments in which at least 20 enucleated cells were analyzed. mtDNA synthesis in enucleated HeLa cells was not detectable either without or with aphidicolin pretreatment (arrowheads in Fig. 3, a and c, respectively). Note that the mitochondria in enucleated cells appear reasonably normal since they retain a
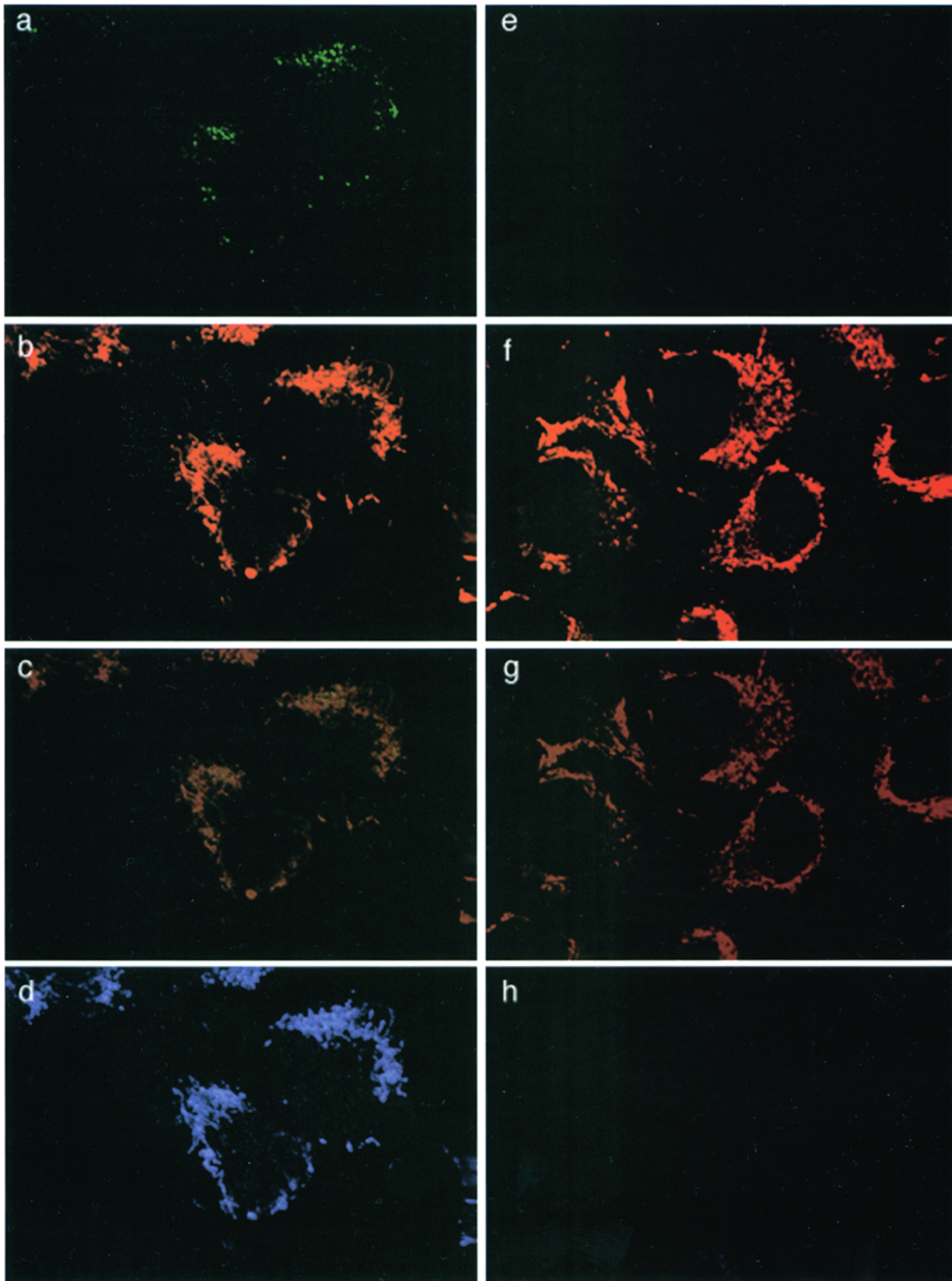
Figure 1. Immunocytochemical detection of newly replicated mtDNA in 143B (TK^{−}) cells. 143B (TK^{−}) cells were analyzed by double-label confocal microscopy as described in Materials and Methods. (*a–d*) 143B (TK[−]) ρ⁺ cells; (*e–h*) 143B (TK[−]) ρ° cells. Cells labeled in vivo with BrdU (10 μM, 2 h) and then stained with anti-BrdU (*a* and *e, green*), anti-mtTFA (*d* and *h, blue*), or labeled with Mitotracker™ (*b* and *f, red*) are shown, as are BrdU-Mitotracker™ merged images (*c* and *g*, colocalization appearing *yellow*).
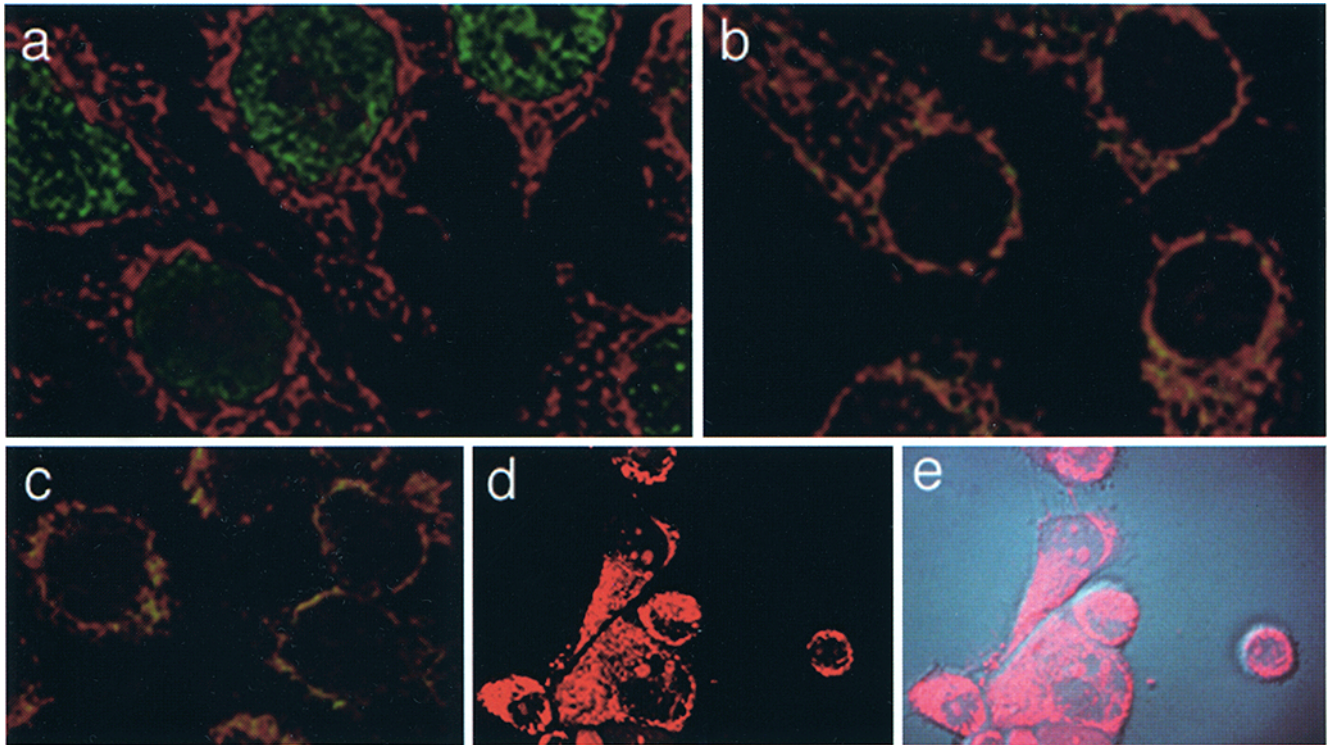
Figure 2. Immunocytochemical detection of BrdU incorporation into mtDNA as a result of aphidicolin pretreatment. Either HeLa or 143B (TK^{−}) cells were labeled in vivo with BrdU (1 or 10 μM, respectively) and analyzed by double-label confocal microscopy as described in Materials and Methods. In *a–c*, all images have been merged; incorporation of BrdU into mtDNA appears yellow, and incorporation of BrdU into nuclear DNA appears green. (*a*) Untreated HeLa cells labeled in vivo with BrdU (1 h); (*b*) HeLa cells pretreated with aphidicolin for 1 h before BrdU labeling in vivo (2 h); (*c*) pattern of BrdU incorporation into mtDNA of 143B (TK^{−}) cells labeled with BrdU for 2 h. (*d* and *e*) Ethidium bromide vital staining (see Materials and Methods) of mtDNA in living HeLa cells (*d*, fluorescent image; *e*, fluorescent image overlaid onto Nomarski DIC image).

membrane potential (Fig. 3, *a* and *c*, *red*) and other mitochondrial components such as mtTFA (Fig. 3, *b* and *d*, *blue*). Also note that the enucleation procedure was never complete throughout the entire cell population; however, this permitted an internal positive control for both nuclear DNA and mtDNA replication (*green* and *yellow* in Fig. 3, *a* and *c*, respectively).

Similar results were obtained with human platelets (Fig. 4), which are anucleate but do contain mitochondria and mtDNA (Shuster et al., 1988). Platelets treated with BrdU in vitro did not incorporate BrdU into mtDNA; the merged images are red due to labeling with Mitotracker™ but no incorporation of BrdU (Fig. 4, *a–c*). Note that a "contaminating" lymphocyte in the platelet preparation does incorporate BrdU into mtDNA (yellow signal), serving as an internal positive control for this experiment. Since lymphocytes in the bloodstream are nondividing cells, they do not replicate nuclear DNA and are devoid of a nuclear BrdU signal. Together, these data indicate that the process of enucleation destroys the ability of mtDNA to replicate.

mtDNA Synthesis in Nondividing Differentiated Cells

To begin to investigate mtDNA replication in situ in different cell types, we next attempted to visualize mtDNA synthesis in situ in differentiated cells. Nondividing, differ-

entiated cells represent the "physiological" absence of nuclear DNA synthesis. For these experiments, we chose PC12 cells as a model system. The PC12 cell line originates from a rat pheochromocytoma and, in the presence of NGF, undergoes differentiation into neuronal-like cells that develop extensive processes (neurites) (Fig. 5 *A*) and synthesize and secrete catecholaminergic neurotransmitters such as dopamine and norepinephrine (Greene and Tischler, 1976). The extensive neurite morphology characteristic of NGF-differentiated PC12 cells provides the opportunity to ascribe more definitively organelles as perinuclear or peripheral since mitochondria in distal neurites and growth cones can be hundreds of microns away from those in the perinuclear cell body region. Such an arrangement provides a quantifiable experimental paradigm for the measurement of mtBrdU in each subcellular region. This is in contrast to the situation with 143B (TK[−]) and HeLa cells, each characterized by a comparatively much more limited cytoplasmic volume and peripheral mitochondrial distribution, thus rendering only a qualitative assessment of the positioning of mtBrdU throughout the cell in those cell types. In addition, differentiated PC12 cells may serve as a convenient model system to investigate some of the drug-induced neuronal toxicities that have been associated with the inhibition of mtDNA replication (Chen et al., 1991; Keilbaugh et al., 1993).

Our results with rat PC12 cells were generally consistent
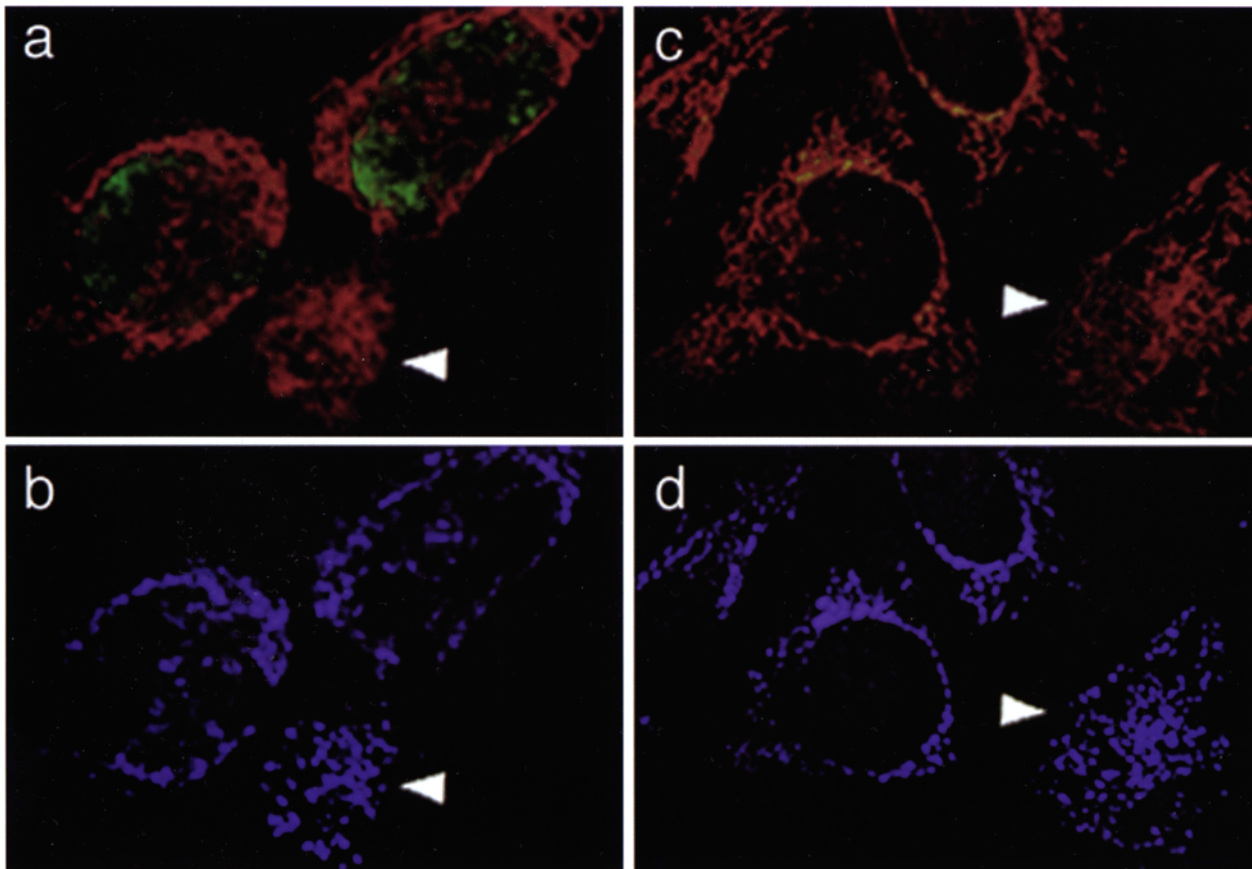
Figure 3. Lack of mtDNA synthesis in enucleated cells. After enucleation with cytochalasin B (see Materials and Methods), HeLa cells were labeled in vivo with BrdU and then analyzed by double- or triple-label confocal microscopy, as described in Materials and Methods. (*a* and *b*) Untreated HeLa cells labeled in vivo with BrdU (1 μM, 18 h); (*c* and *d*) HeLa cells pretreated with aphidicolin for 1 h before BrdU labeling in vivo (1 μM, 18 h). *a*, *b*, and *c*, *d* each represent the same field of cells, showing mtBrdU (*a* and *c*, *yellow*), nuclear BrdU (*c*, *green*), and mtTFA (*b* and *d*, *blue*). In each case, one representative enucleated cell is shown (*arrowhead*).

with those obtained with "undifferentiated," rapidly dividing mammalian (human) cells (both 143B (TK[−]) and (TK[−]) HeLa). Specifically, we observed that relatively short labeling periods (a few hours) resulted in the appearance of mtBrdU solely in the perinuclear (cell body–associated) region of differentiated PC12 cells (Fig. 5 *A*, *b*). With increasing times of exposure to BrdU in vivo, mtBrdU became readily apparent in peripheral neurites and growth cones, but also persisted at a perinuclear location (Fig. 5 *A*, *c* and *d*), our working experimental definitions being nuclear-proximal (cell body–associated) or neurite/growth cone–associated, for perinuclear or peripheral, respectively. Notably, the ratio of perinuclear to peripheral mtBrdU decreased with time (Fig. 5 *B*). These data suggest that, since the fraction of label in peripheral regions increases with time, after synthesis newly replicated mitochondrial genomes are then distributed to peripherally situated mitochondria in the cell through the process of axonal transport. Thus, these results are also consistent with the notion that mtDNA synthesis is initiated, and most likely completed, in a nuclear-proximal position.

Since axonal transport of mitochondria is bidirectional (Morris and Hollenbeck, 1995), we attempted to perturb mtDNA synthesis in progress with the dideoxy chain terminator ddC to localize more definitively the initial sites of mtDNA synthesis. We hypothesized that since increasing amounts of ddC would progressively interrupt mtDNA replication, incompletely replicated mtDNA molecules would be arrested at their site of synthesis and not transported to mitochondria elsewhere within the cell. Chain terminators like ddC and azidothymidine (AZT) are good substrates (and thus inhibitors) for certain polymerases like mtDNA polymerase γ and HIV reverse transcriptase (Simpson et al., 1989; Erikkson et al., 1995), but not others, such as DNA polymerase α, the major nuclear DNA polymerase (Copeland et al., 1992).

NGF-differentiated PC12 cells were treated with increasing concentrations of ddC (10 nM–10 μM), in the simultaneous presence of a constant amount of BrdU. For clarity, only mtBrdU data obtained with untreated cells (BrdU only) compared to 500 nM ddC (and BrdU) are shown (Fig. 6 *A*, *a* and *b*), although a complete range of ddC concentrations was tested. In each case, BrdU with or without ddC exposure was 10 h, at which time peripherally located mtBrdU is reproducibly manifest (Fig. 5 *A*, *c*). In control cells, mtBrdU was readily apparent in both perinuclear and peripheral regions of differentiated PC12 cells (Fig. 6 *A*, *a*, *arrowhead* and *arrows*, respectively). How-
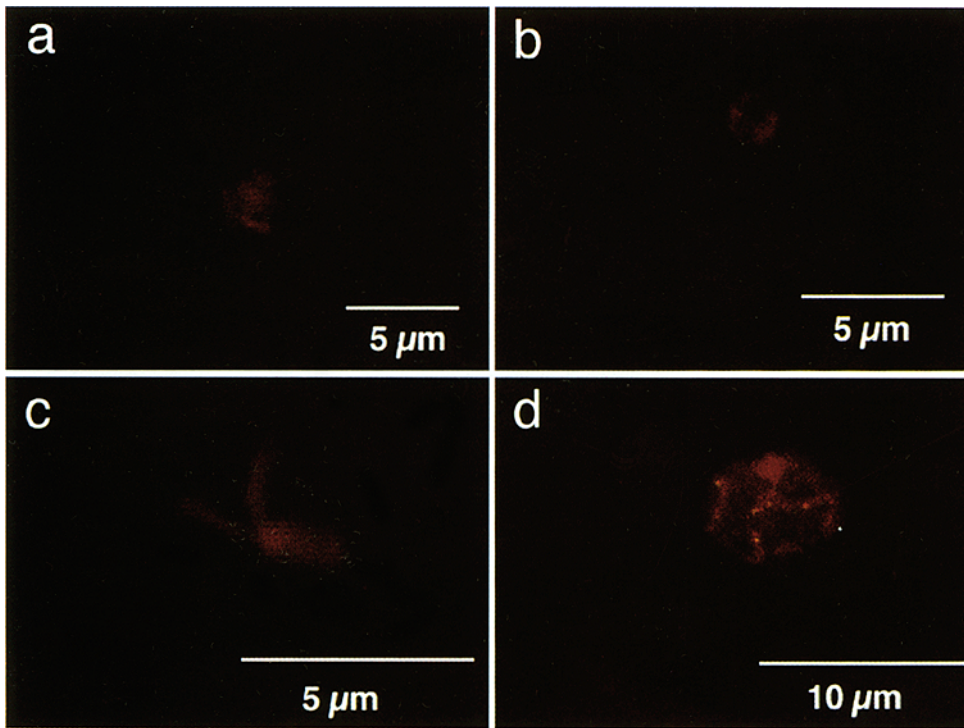
Figure 4. Lack of mtDNA synthesis in anucleate human platelets. Human platelets were isolated from peripheral blood (see Materials and Methods), labeled with BrdU and Mitotracker™, and then analyzed by double-label confocal microscopy, as described in Materials and Methods. All images (*a–d*) have been merged. (*a–c*) Three representative examples of individual platelets. (*d*) "Contaminating" lymphocyte present in the platelet preparation.

ever, as the concentration of ddC was increased, a significantly smaller fraction of mtBrdU was detectable in peripheral neurites, whereas only slightly less mtBrdU was detectable in the cell body region. At high concentrations of ddC (500 nM–1 μM), virtually no mtBrdU was detectable in peripheral mitochondria, yet some perinuclear mtBrdU was still apparent (Fig. 6 *A, b, arrowhead*). The proportion of perinuclear mtBrdU to total mtBrdU increased as the concentration of ddC increased (Fig. 6 *B*), as did the ratio of perinuclear to peripheral mtBrdU (Fig. 6 *C*). These data are consistent with the notion that increasing the relative concentration of ddC during mtDNA synthesis results in chain termination and thus an arrest of mtDNA replication at its site of synthesis within the cell. Consequently, presumably there are insufficient levels of mtDNA synthesized at the cell body to be transported to peripheral organelles.

Very high concentrations of ddC (10 μM) almost completely halted mtDNA synthesis, yet a few remaining perinuclear sites were still detectable (data not shown). Results with these high concentrations of ddC are consistent with previous studies in cultured PC12 cells, which have shown that mtDNA is severely depleted after exposure to 10 μM ddC for 3–6 d in vivo (Chen et al., 1991).
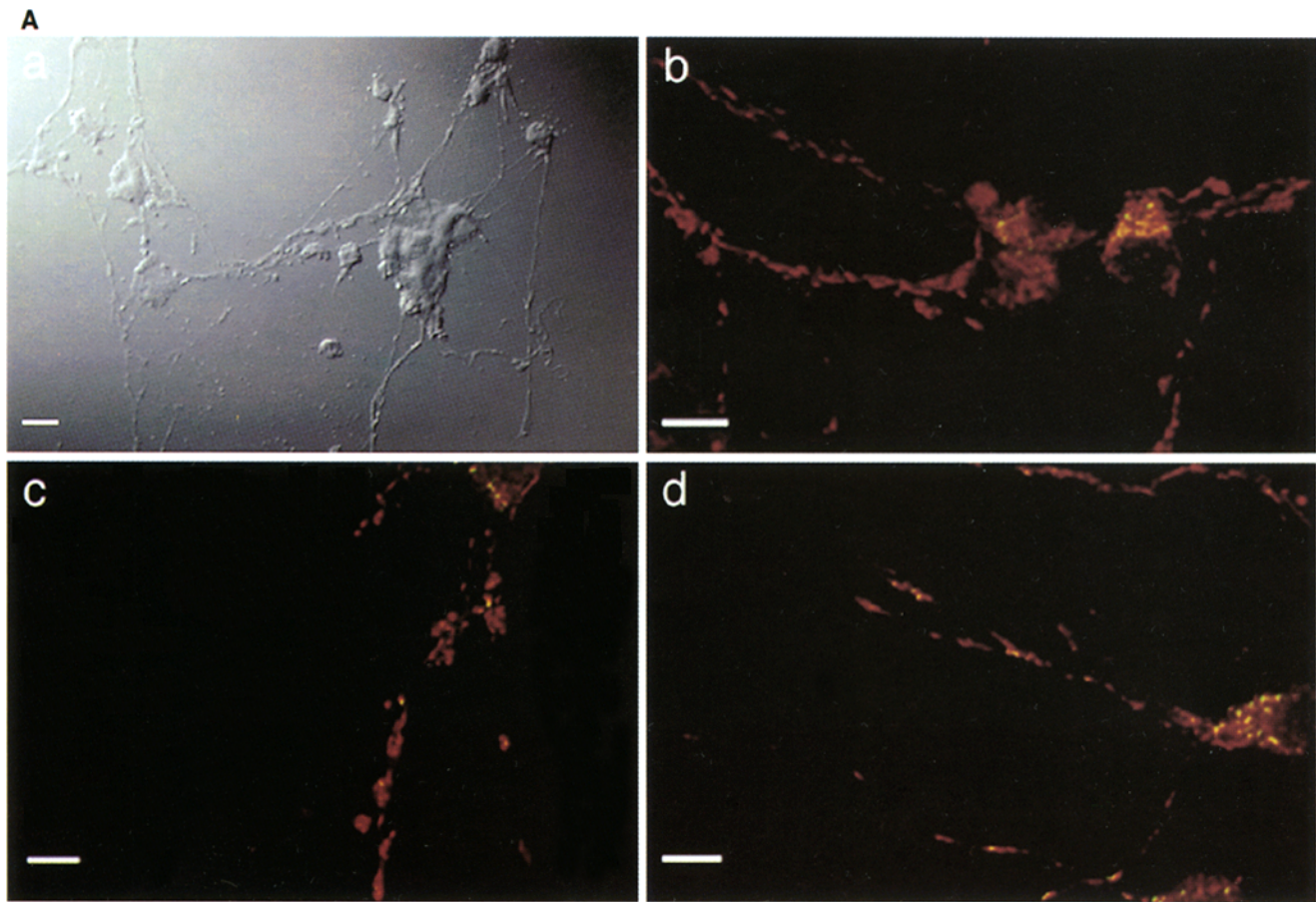
Discussion

We have developed a procedure for localizing mtDNA replication in situ within intact mammalian cells. Visualization of recently synthesized mtDNA (mtBrdU), which we have assessed in this study, offers a unique opportunity to identify spatially the initial locus of mtDNA synthesis, an issue which in previous work was not addressable. Our results are consistent with previous biochemical studies re-

porting characteristics of mtDNA synthesis in mammals. The data obtained and reported here are in good agreement with the amount of time required for synthesis of the mitochondrial genome, as well as the fact that mtDNA synthesis is not linked to nuclear DNA synthesis and may occur at any time during the cell cycle.

Analogous to the case for chromosomal DNA replication, we observed that mtDNA synthesis does not appear to be random with respect to subcellular position. Perhaps the simplest foreseeable scenario would have been that the observed pattern of incorporation of BrdU into mtDNA would not differ from that of the general distribution of all cellular mtDNA, which is present throughout all mitochondria in the cell. Several lines of evidence that we have presented here support the notion that mtDNA is instead preferentially synthesized in the perinuclear region of the cell.

Our observations in several cell types analyzed revealed that newly synthesized mtDNA is manifest in a perinuclear position. We did not observe the presence of mtBrdU in peripherally situated organelles without the coincident appearance of mtBrdU at a perinuclear location, although the distinction between perinuclear and peripheral organelles was not quantifiably distinguishable in "small" cell types, such as 143B (TK[−]) and HeLa cells. To more readily attribute the subcellular location of BrdU incorporation into mtDNA as perinuclear or peripheral, we assessed mtDNA replication in situ in NGF-differentiated PC12 cells, which are considerably larger and, importantly, whose peripheral cytoplasm consists of lengthy neurites and growth cones. Such an analysis permitted a quantifiable assessment of the relative ratio of perinuclear to peripheral incorporation of BrdU into mtDNA, revealing indeed that the relative proportion of peripheral mtBrdU

A

B

increases with the length of the BrdU labeling period. In agreement with our own data (Fig. 2, *d* and *e*), previous studies have demonstrated that mtDNA is distributed throughout the cell's complement of mitochondrial organelles (Hevner and Wong-Riley, 1991; Hayashi et al., 1994; for HeLa cells and neuronal cells, respectively), as are some components of the replication and transcription machineries such as mtDNA polymerase γ and mtTFA (Davis et al., 1996).

One simple explanation to account for the observed appearance of mtBrdU in PC12 cell neurites only at later time points of BrdU exposure is that mtDNA is synthe-

sized first in the cell body area and then transported to the periphery via axonal transport. Interestingly, previous studies have shown that translation and import of nuclear-encoded mitochondrial proteins occur at the cell body region (Hevner and Wong-Riley, 1991), but intraorganellar processing of mitochondrial precursor proteins can also occur in peripheral dendrites and axon terminals (Liu and Wong-Riley, 1994). Perhaps the latter represents one mechanism to provide flexibility with regard to local energy demand throughout large neurons. Although certain compounds such as acrylamide and colchicine have been reported to inhibit axonal transport (Sjöstrand et al., 1970;
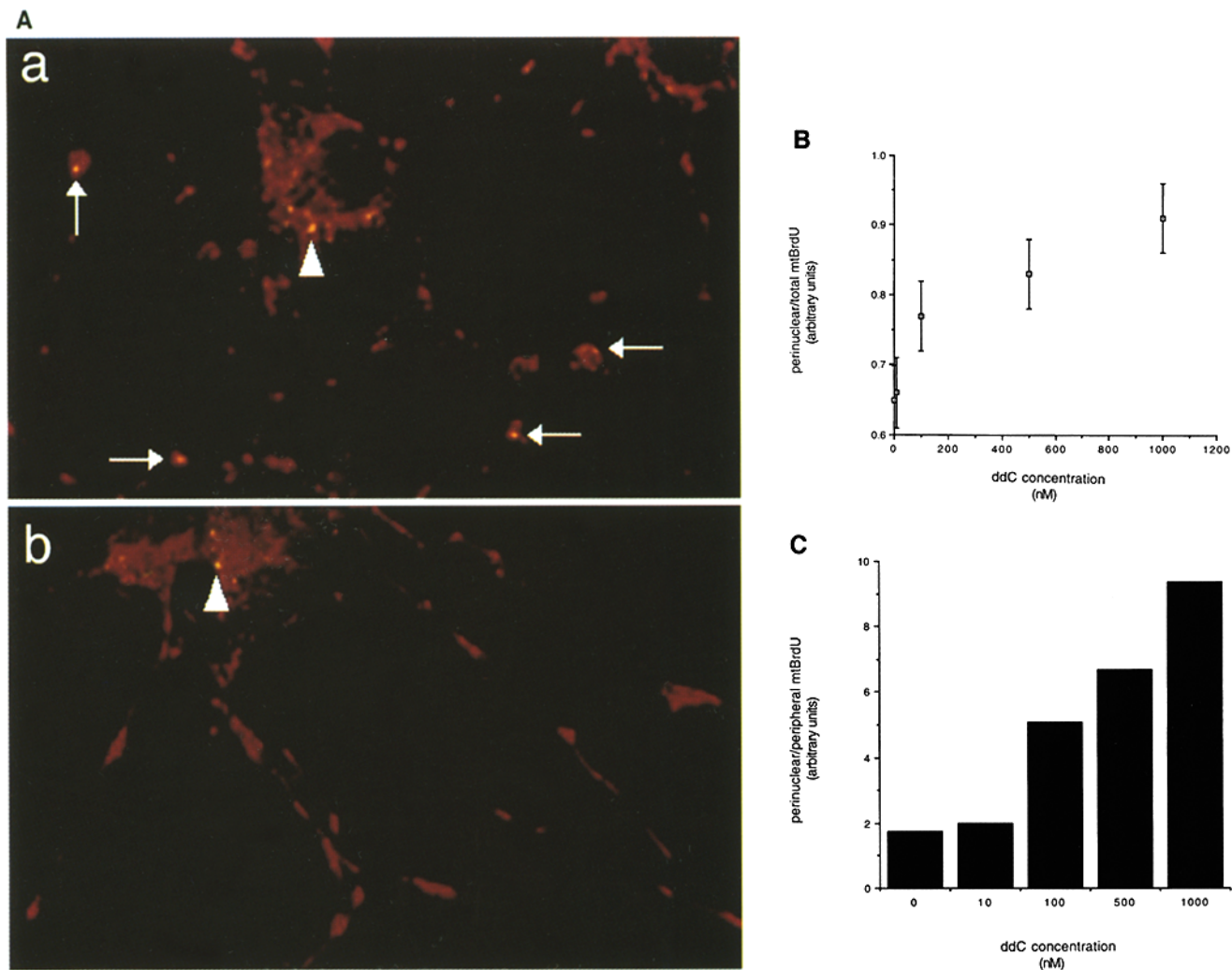
Figure 6. Effects of ddC on mtDNA synthesis in differentiated PC12 cells. Cells were labeled simultaneously in vivo for 10 h with BrdU (15 μM) and increasing concentrations of ddC and then analyzed by double-label confocal microscopy, as described in Materials and Methods. (*A*) Merged confocal microscopic images of mtBrdU incorporation into differentiated PC12 cells as a function of ddC concentration. BrdU incorporation into mtDNA appears yellow. (*a*) Control (no ddC); (*b*) 500 nM ddC. Perinuclear (cell body-associated) mt-BrdU is indicated (*arrowheads*) as is peripheral (neurite-associated) mtBrdU (*arrows*). (*B* and *C*) Quantitation of PC12 ddC concentration-dependency data. (*B*) Shown is the ratio of perinuclear to total mtBrdU as a function of ddC concentration. (*C*) The ratio of perinuclear to peripheral mtBrdU as a function of ddC concentration. Each point is representative of at least three separate experiments.

Sickles, 1991; Brat and Brimijoin, 1993; Harris et al., 1994), experiments in which cells were pretreated with either drug before and throughout several hours of BrdU labeling in vivo proved to be toxic to the PC12 cell cultures, specifically to neurite morphology (data not shown), limiting the use of these reagents.

Our experiments with ddC in PC12 cells represent an alternative strategy to identify initial loci of mtDNA replication, since these studies permitted monitoring the arrest of mtDNA replication in progress. We observed that increasing the relative concentration of ddC in vivo resulted in the absence of peripherally situated mtBrdU despite the persistence of perinuclear mtBrdU, albeit at reduced levels. From these data, we propose that high concentrations of ddC arrest mtDNA synthesis to the extent that insufficient mtDNA levels are produced, and hence not trans-

ported, to the cell periphery. Previous data indicate that there is a tolerance for the accumulation of mtDNA molecules arrested in replication due to pyrimidine dimer–induced blockade of strand elongation (Clayton et al., 1974).

Attempts to chase mtBrdU from its initial site of synthesis (with thymidine) were uniformly unsuccessful with PC12 cells or any cell type examined, with chase times ranging from 30 min to several hours (data not shown). It remains unclear why these experiments failed, but it is possible that the kinetics or lack of cell cycle phase specificity of mtDNA synthesis may have played a role, since similar experiments with nuclear BrdU incorporation revealed that nuclear DNA was successfully chased with thymidine (data not shown).

Finally, it is possible that the cellular distribution of the

mitochondrial TK enzyme is not uniform. Analogous studies with the cellular TK enzyme revealed that it is uniformly distributed throughout the cytoplasm of mammalian cells in G_1- and S-phase (Carozza and Conrad, 1994). However, since the purified enzyme or antibodies against the mitochondrial protein are not presently available, we are unable to address this question directly. Although we consider it unlikely that we are assaying the intracellular localization of mitochondrial TK, it remains possible (perhaps even likely) that some other nuclear-encoded constituent of the mtDNA replication machinery has a restricted localization to the perinuclear region of the cell.

Our inability to detect mtBrdU in enucleated cells is consistent with a previous report which failed to detect any incorporation of [^{3}H]thymidine into enucleated mammalian cells (Follett, 1974), as is our data with physiologically enucleated human platelets (Fig. 4). Together with the remainder of the data presented in this report, these results suggest that mtDNA replication first occurs in the proximity of the cell nucleus either for convenience, i.e., immediate access to certain components of the nuclear-encoded mtDNA replication machinery, or alternatively, that some type of nuclear tethering is required, in which some structural entity inherent to the nuclear membrane maximizes the efficiency of transport of newly replicated mtDNA to peripheral regions of the cell. At present, we are unable to distinguish between these possibilities.

In summary, we have successfully detected recently replicated mtDNA in several cell types, including TK(−) and TK(+) cells, as well as in nondividing, differentiated cells in culture. One previous ultrastructural study achieved detection of BrdU incorporation into mitochondria of rat jejunal intestinal tissue (Thiry, 1992); however these data, obtained with electron microscopic methods, did not preserve the in vivo cellular architecture as we have attempted to maintain here. In addition, using immunocytochemical detection of BrdU, one study in plants (*Nicotiana tabacum*) has shown that mtDNA synthesis appears to occur in a specific region of the cell, near the quiescent center (Suzuki et al., 1992). However, we believe that our results represent the first of such data for mtDNA replication in intact mammalian cells.

It is interesting to note that the results reported here are consistent with earlier studies in amphibian oocytes, in which the incorporation of [^{3}H]thymidine during the period of mtDNA amplification in early oogenesis preferentially occurs close to the large oocyte nucleus (Tourte et al., 1984; Mignotte et al., 1987). Collectively, these data support an as yet unappreciated topological arrangement for replication of the mitochondrial genome in a nuclear-proximal position that may play a role in ensuring that only properly replicated molecules are distributed to all cellular mitochondria.

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