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Replication-Coupled Modulation of Early Replicating Chromatin Domains Detected by Anti-Actin Antibody

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Abstract Evidence is presented for the reversible, cold-dependent immunofluorescence detection of the epitope (hereafter referred to as *epiC*), recognized by a monoclonal anti-actin antibody in diploid human fibroblast cell nuclei and mitotic chromosomes. The nuclear/chromosomal epiC was detected in a cell cycle window beginning in early S phase and extending through S phase, G_2 phase, mitosis until early G_1 phase of the subsequent daughter cells. A small but significant level of co-localization was measured between the nuclear epiC and active sites of DNA replication in early S phase. The level of co-localization was strikingly enhanced beginning approximately 1 h after the initial labeling of early S phase replicating chromatin domains. In contrast, epiC did not co-localize with late S phase replicated chromatin either during DNA replication or at any other time in the cell cycle. We propose a replication-coupled modulation of early S phase replicated chromatin domains that is detected by the chromatin epiC positivity, persists on the chromatin domains from early S until early G_1 of the next cell generation, and may be involved in the regulation and/or coordination of replicational and transcriptional processes during the cell cycle. Further studies will be required to resolve the possible role of nuclear actin in this modulation process. J. Cell. Biochem. 94: 899–916, 2005.

Key words: DNA replication; chromatin; cell cycle; immunofluorescence microscopy; cold-dependent epitope detection; nuclear actin; anti-actin

Unlike prokaryotic genomes, which contain a single circular DNA and a single origin of replication, eukaryotic genomes are segmented into various species-specific numbers of differently sized chromosomes spatially organized in a cell nucleus and containing multiple origins of replication. Replication of eukaryotic genomes takes place during a defined S phase of the cell cycle and is precisely regulated spatio-temporally [Berezney et al., 2000; Berezney, 2002]. During S phase, replication of DNA is accompanied by a process of replication-coupled chromatin assembly, during which newly replicated DNA is packaged initially into nucleosomes and later into higher order chromatin structures resulting ultimately in a complex organization of chromosomal domains within the nucleus [Ridgway and Almouzni, 2001].

The temporal order of genome replication is established in early G_1 phase simultaneously with chromosome nuclear repositioning [Dimitrova and Gilbert, 1999; Gilbert, 2001]. In higher eukaryotes, chromosomal loci containing genes that show, in a given cell type, either constitutive or inducible activation of transcription, generally replicate early in S phase, while chromosomal loci containing genes

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that are constitutively silent as well as transcriptionally inactive heterochromatin replicate late in S phase [Goldman et al., 1984; Holmquist, 1987; Schübeler et al., 2002].

Competence for expressing distinct genes responsible for a given cell phenotype is determined by transient inductive processes during early development and is, in cycling cells, transmitted through mitosis to daughter cells as epigenetically inherited information of the chromosomal state [Muller and Leutz, 2001; Lachner and Jenuwein, 2002]. In contrast to genetic information (stored in DNA and encoded by nucleotide sequences), our understanding of epigenetically inherited information on the chromosomal state (e.g., the gene expression state of the genome) is still in its infancy. It is generally accepted that epigenetically inherited information is stored as a "mark(s)" left on chromatin by developmental and differentiation processes and encoded by modulations of some features, possibly structural, of the chromatin [Turner, 2000; Cavalli, 2002; Johnson et al., 2002; Lachner and Jenuwein, 2002; Vermaak et al., 2003].

In this report, in situ immunofluorescence is used to detect an epitope (epiC), recognized by a monoclonal anti-actin antibody in chromatin of human fibroblast cell nuclei and mitotic chromosomes. The temporal and spatial properties of epiC labeling suggest the presence of replication-coupled modulation of early S phase replicating chromatin domains that persist on newly replicated chromatin from early S phase to early G_1 phase of the next cell cycle.

MATERIALS AND METHODS

Cell Culture

Diploid human embryo lung fibroblast cells (LEP; SEVAPHARMA, Czech Republic) were grown on coverslips in Eagle's minimal essential medium supplemented with 10% fetal calf serum and antibiotics.

Biotin-dUTP Pulse Labeling of Replicating DNA

Biotin-16-dUTP (Roche; 0.2 mM, 20 μl per coverslip) was introduced into the cells by means of a hypotonic shift as described by Koberna et al. [1999]. After biotin-dUTP introduction, the cells were cultivated in a fresh tissue culture medium for various time intervals, fixed and processed for immunofluorescence. Rabbit anti-biotin antibody (Enzo), diluted 1:100 in PBS/1% BSA, was used for the detection of incorporated biotin-dUTP, followed by goat anti-rabbit-FITC or goat anti-rabbit-Cy3 conjugated antibodies (both from Jackson ImmunoResearch) diluted 1:100 in PBS/1% BSA.

Cell Synchronization

Cells were made quiescent (G_0) by cultivation in a serum-free medium for 2–3 days and released from the G_0 state by cultivation again in a medium supplemented with 10% fetal calf serum (Sigma). In order to synchronize cells at the G_1/S border, hydroxyurea (Sigma) was added to the medium at a final concentration of 1 mM. Cells were released, 25 h later, from the G_1/S block by several washes with fresh medium containing 5 μM 2-deoxycytidine (Sigma) to facilitate cell recovery from hydroxyurea block [Bianchi et al., 1986]. At different times following release from G_0 or the G_1/S block, the cells were fixed with methanol and processed for immunofluorescence.

Whenever possible, results obtained with synchronized cells were repeated using unsynchronized cells. This guaranteed that all the cells growing on one coverslip and representing a population of cells at different cell cycle or S phase stages were identically treated during pulse-labeling, cell fixation, permeabilization, digestions, and immunolabeling. It also enabled us to determine the reproducibility of results in the absence of possible perturbations due to the synchronization methods.

For the analysis of early G_1 phase of cycling cells, exponentially growing cells were arrested in metaphase by addition of colcemid (0.1 μg/ml; Gibco) for 2 h. Mitotic cells were collected by the shake-off method, washed with prewarmed fresh medium, and re-plated onto coverslips. At different times after re-plating, the cells were fixed and processed for immunofluorescence.

Cell Fixation and Digestion

Cells were fixed in −20°C methanol for 10 or 30 min and rehydrated in PBS. Similar results were obtained with both variants of methanol fixation. Formaldehyde fixation was performed with 2% or 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS, pH 7.4 for 10–60 min at room temperature (RT) as

described in the text. Cells were permeabilized/postfixed with methanol at −20°C for 15 min and washed in PBS. Digestions were performed at RT for 1 h with either OmniCleave™ endonuclease (Epicentre, Madison, WI), 3,000 U/ml, or with RNase A (Sigma), 1 mg/ml, or RNase-free DNase I (RQ1 DNase, Promega), 500 U/ml.

Immunofluorescence

Fixed cells were washed with PBS, blocked with 1% BSA in PBS for 30–60 min, and incubated with mouse monoclonal anti-actin (Sigma, A4700, IgG2a isotype) diluted 1:200 in PBS/1% BSA at +4°C overnight or at RT for 1–3 h, as described in Results. Three different lots of A4700 anti-actin antibody were used with similar results. Methanol fixation enabled preferential immunodetection of proliferating cell nuclear antigen (PCNA) associated with DNA replication sites [Bravo and Macdonald-Bravo, 1987]. For dual labeling of epiC and PCNA in methanol-fixed cells, mouse monoclonal anti-PCNA antibody (Chemicon, MAB4078, IgMκ isotype) diluted 1:200 was used; in formaldehyde-methanol-fixed cells, rabbit anti-PCNA antibody (Santa Cruz) diluted 1:100 was used. For dual labeling of epiC and biotin-dUTP, rabbit anti-biotin (Enzo) diluted 1:100 was used. No difference in fluorescence patterns was observed following simultaneous or sequential incubations of the two antibodies in these dual-labeling experiments. Secondary antibodies were: FITC conjugated goat anti-mouse IgG-γ chain specific (Sigma), 1:60, Cy3-conjugated goat anti-mouse IgM μchain specific (Jackson ImmunoResearch), 1:200, Alexa Fluor488-goat anti-mouse IgG (H + L) (Molecular Probes), 1:200, Cy3-conjugated goat anti-mouse IgG (H + L) (Jackson ImmunoResearch), 1:200, Cy3-conjugated donkey anti-mouse 1:200 (Jackson ImmunoResearch), and FITC- or Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch) 1:60 or 1:200, respectively, were applied for 40–60 min at RT. For nucleic acid counterstaining, TO-PRO-3 or YOYO-1 fluorescent dyes (Molecular Probes) were used. Coverslips were mounted in Mowiol and viewed using a Leica TCS NT confocal microscope. Optical sections, 0.5 micron, were collected.

Computer Image Analysis

The cross-correlation function [F = F(Δx); van Steensel et al., 1996] was used to describe overlap of red and green fluorescent signals in 2D confocal images. Briefly, F(Δx) was calculated as a Pearson's correlation coefficient for red and green fluorescence channels, while horizontal shift of Δx pixels was applied on the red channel. In all cases, shifts were applied of −30, −29, . . ., 0, . . ., 29, 30 pixels. Pearson's correlation coefficient is often used for matching of different data sets and its values range from −1 to 1. In our case, high positive values indicate a significant overlap (co-localization) of the two fluorescence signals, values around zero reflect random overlap of these signals and negative values demonstrate anti-co-localization ("repulsion") of fluorescence signals. Only pixels corresponding to the area within the cell nuclei were taken into the account. Grayscale images were merged and the nuclear areas were masked using Adobe Photoshop 6 software (Adobe Systems Incorporated, San Jose, CA). The cross-correlation analysis was performed in Matlab 6.5 (The Mat Works, Inc., Natick, MA). Image analysis was performed with the original, not adjusted single optical section images. When required for printing purposes, the image histogram was stretched to 0–255 grayscale values without the loss of image information.

Isolation of Cell Nuclei and Immunoblotting

Cell nuclei were isolated as described [Sahlas et al., 1993]. Lysates of isolated cell nuclei and of whole cells were separated on a 12% SDS–PAGE gel and transferred onto nitrocellulose membrane according to established protocols [Laemmli, 1970; Towbin et al., 1979]. Nitrocellulose membranes were washed with a blocking buffer (5% gelatin (w/v), 0.05% Tween-20 in PBS) overnight at 4°C. Each membrane strip containing proteins, loaded onto one slot, was cut in halves; one half was incubated with anti-actin (Sigma A4700) 1:500, the other half with anti-tubulin antibody Tu-01 [Dráber et al., 1989], a kind gift from P. Dráber, IMG ASCR, Prague), 1:2,000 for 2 h at RT. In parallel, nitrocellulose membranes were blocked for 2 h at RT, and the membrane strips incubated with anti-actin or Tu-01 overnight at +4°C. Tu-01 antibody was used as a marker of cytoplasmic proteins. Goat anti-mouse IgG–HRP (Santa Cruz) 1:10,000 was used as a secondary antibody. Immunoreactive proteins were detected by enhanced chemiluminiscence (ECL kit, Amersham).

RESULTS

Cold-Dependent Detection of Nuclear and Chromosomal EpiC

A mouse monoclonal anti-actin antibody (clone AC-40, Sigma, A4700) was used for immunofluorescence detection of actin in human diploid fibroblast cells (LEP) that were routinely fixed with methanol. This antibody recognizes an epitope located on the C-terminal end of actin that is conserved in all actin isoforms (A4700 data sheet, Sigma) and has been widely used in the past [Gonsior et al., 1999; Chen et al., 2001; Nagy et al., 2003; Batchelor et al., 2004]. Moreover, one such study has reported nuclear actin labeled by A4700 [Okorokov et al., 2002]. Surprisingly, different results were obtained when we compared two commonly used regimes for primary antibody incubation: a RT regime (1–2 h at RT) and a cold regime (overnight at +4°C). While incubation of fixed cells with anti-actin for 1 h at RT resulted in a typical cytoplasmic labeling with no significant fluorescence signal in the nuclear region of interphase cells or over mitotic chromosomes (Fig. 1A), incubation of anti-actin overnight at +4°C yielded, in addition, a bright immunofluorescence labeling of nuclei of some interphase cells (Fig. 1B) and chromosomes of all mitotic cells. The nuclear/chromosomal epitope, which was recognized by anti-actin at +4°C and the detection of which was cold-dependent, is hereafter referred to as "*epiC*."

To exclude the possibility that nuclear epiC immunolabeling resulted from cytoplasmic actin non-specifically attached to the nuclei, we tested for epiC in cells differently fixed with formaldehyde before the subsequent postfixation/permeabilization with methanol for 15 min at −20°C. The cells fixed with 2% formaldehyde for 10 min at RT followed by methanol for 15 min at −20°C exhibited similar immunolabeling of nuclear epiC as did the cells fixed with only methanol (Fig. 1C). With increasing degree of formaldehyde crosslinking, the intensity of epiC immunofluorescence decreased but it was still weakly detectable in cells fixed with 4% formaldehyde for 20 min at RT followed by methanol fixation for 15 min at −20°C (Fig. 1D). No epiC was detected in cells fixed with 4% formaldehyde for 1 h at RT followed by methanol fixation for 15 min at −20°C (Fig. 2A). These results suggest that nuclear epiC labeling detected in cells fixed with only methanol was not due to a non-specific labeling caused by insufficient fixation, and confirm the suitability of methanol fixation for nuclear epiC immunodetection. Fixation by methanol stops biological reactions instantaneously and presumably leaves proteins in their original locations [Nakamura, 2001 and reference therein].

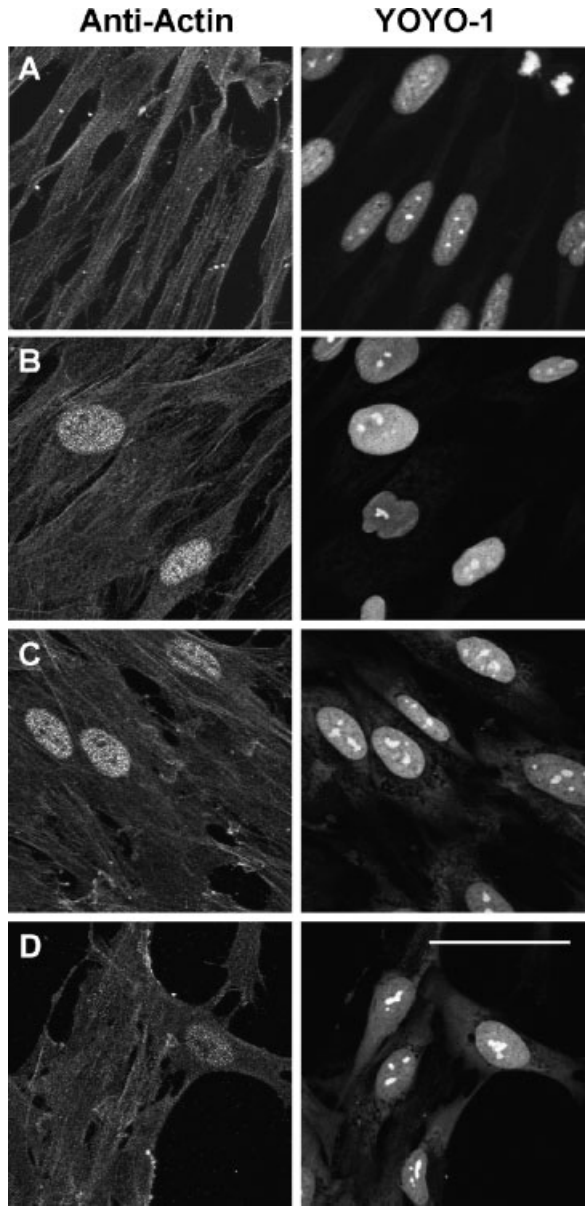

Fig. 1. Effect of the degree of formaldehyde crosslinking on the detectability of epiC in methanol fixed cells. Cells were fixed with methanol for 15 min at −20°C (**A**, **B**), 2% formaldehyde for 10 min at RT followed by methanol for15 min at −20°C (**C**), 4% formaldehyde for 20 min at RT followed by methanol for 15 min at −20°C (**D**). Cells were processed for immunofluorescence with anti-actin under RT detection regime (A), cold (+4°C) detection regime (B–D). Bar, 50 μm.

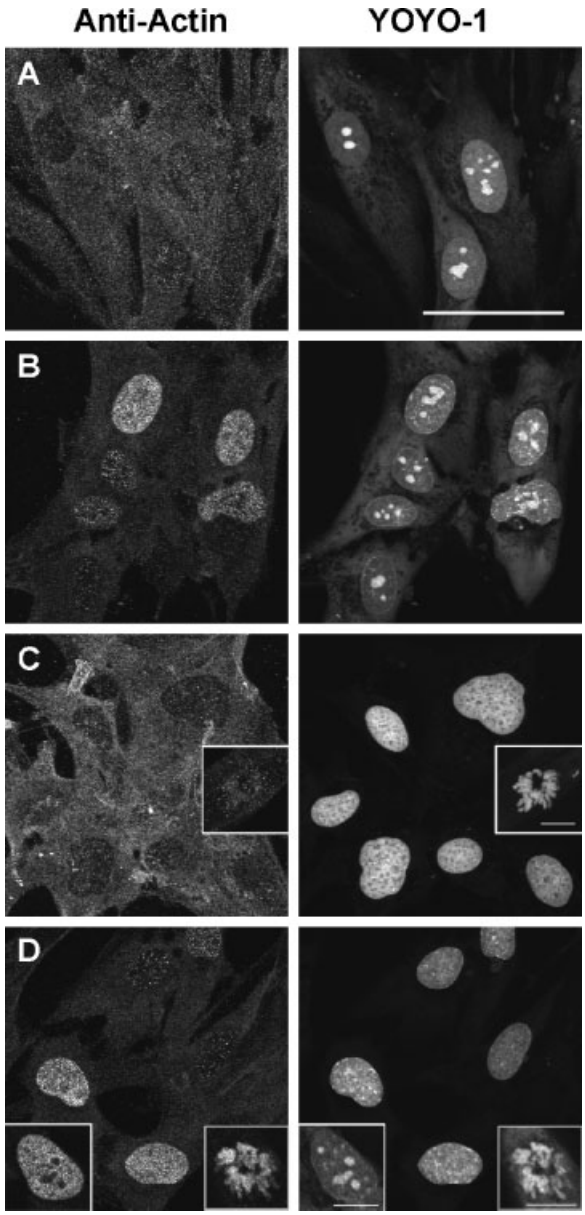

Fig. 2. DNase I or OmniCleave endonuclease but not RNase alone unmasked epiC in highly formaldehyde crosslinked cells. Cells were fixed with 4% formaldehyde for 1 h at RT followed by methanol fixation/permeabilization for 15 min at −20°C. **A**: No digestion, (**B**) fixed cells digested with DNase I, (**C**) fixed cells digested with RNase A, (**D**) fixed cells digested with RNase A as in (C) followed by digestion with DNase I, insets in (D) fixed cells digested with OmniCleave™ endonuclease. Anti-actin, 1:200, overnight at +4°C/Cy3-conjugated goat anti-mouse, 1:200, 1 h at RT, nucleic acids counterstaining YOYO-1. Insets in (C) mitotic cell after RNase A treatment, insets in (D) interphase nucleus and mitotic chromosomes after OmniCleave endonuclease treatment. Bar, 50 μm. Bar in insets, 10 μm.

Since epiC positivity was detected in mitotic chromosomes in addition to interphase nuclei, we considered whether nuclear epiC, by analogy to chromosomal epiC, is also associated with chromatin. We, therefore, tested the possibility that nuclear epiC, if associated with chromatin, might become detectable in highly formaldehyde crosslinked cells following nuclease digestion to make the chromatin more "open" or accessible to binding antibodies. The cells, fixed with 4% formaldehyde for 1 h at RT and postfixed/permeabilized with methanol for 15 min at −20°C (conditions where no epiC is detected, Fig. 1D), were subsequently digested with either RNase-free DNase I or with RNase A and processed for immunofluorescence with anti-actin (Fig. 2). As shown in Figure 2B, digestion with RNase-free DNase I enabled detection of epiC in some nuclei of highly formaldehyde crosslinked cells, while the digestion with RNase A was not effective (Fig. 2C). To exclude the possibility that the lack of epiC detection obtained after RNase digestion resulted from the removal of epiC due to RNase treatment, the cells digested with RNase were further digested with DNase I. The results (Fig. 2D) revealed that subsequent DNase treatment enabled epiC detection and confirmed that epiC remained in the nuclei after the RNase treatment.

A significant increase in YOYO-1 fluorescence was observed in the nuclei of cells treated with RNase compared to those without treatment (Fig. 2C). This suggests that the binding of YOYO-1 to DNA was hindered by some RNA, or RNA-containing structure in non-digested fixed cell nuclei. In addition to DNase I that binds with high affinity to actin and theoretically could, for example, depolymerize actin filaments [Hitchcock, 1980], these results were confirmed by treatment with OmniCleave *E. coli* endonuclease. This enzyme degrades single- and double-stranded DNA and RNA to di-, tri-, and tetranucleotides. As shown in the insets of Figure 2D, OmniCleave endonuclease treatment enables the detection of epiC in both cell nuclei and mitotic chromosomes. The finding that digestion with DNase or endonuclease, but not with RNase alone, effectively unmasked epiC for detection in highly formaldehyde-crosslinked cells, suggests that epiC is located in DNA-containing chromatin structures. In addition, the similarity of results obtained with nuclease treated, highly formaldehyde-crosslinked cells and cells fixed with methanol argue against the possibility that anti-actin labeling of epiC in methanol-fixed cells resulted from the labeling of cytoplasmic actin non-specifically

attached to the nuclei due to insufficient cell fixation.

EpiC Is Detected in an Early S to Early G_1 Cell Cycle Window

Since the epiC labeling was not found in every cell nucleus, we reasoned that epiC positivity might be cell cycle dependent. To test this hypothesis, cells were made quiescent (G_0) by serum deprivation and then stimulated to proliferation either by medium containing serum or by medium containing serum plus hydroxyurea. This latter approach further synchronized the cells at the G_1/S border (see Materials and Methods). At different times after releasing the cells from G_0 or the G_1/S (hydroxyurea) block, epiC was immunodetected with anti-actin. To avoid possible effects of metabolic inhibitors, unsynchronized cells were also used and the progression through S phase was monitored by double immunofluorescence with anti-PCNA antibody. PCNA is a standard replication marker and its labeling pattern, consisting of nuclear fluorescence foci, enables identification of early, mid, and late S phase cell nuclei [Bravo and Macdonald-Bravo, 1987; Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002]. PCNA replication patterns obtained by anti-PCNA labeling of LEP cell nuclei were consistent with DNA replication patterns of typical early, mid, and late S phase cells described by others [Nakayasu and Berezney, 1989; Ma et al., 1998; Leonhardt et al., 2000] and with replication patterns obtained with synchronized LEP cells (data not shown).

The results of epiC labeling during cell cycle progression from G_0 through G_1 until the end of S phase are summarized in Figures 3 and 4. No epiC labeling was seen in serum deprived, quiescent G_0 cells (Fig. 3D). When quiescent G_0 cells were stimulated to cell cycle progression by serum addition, no epiC labeling was observed during the first G_1 phase following stimulation (Fig. 3A). However, epiC labeling was detected in early (Fig. 4A,B), mid (Fig. 4C), and late

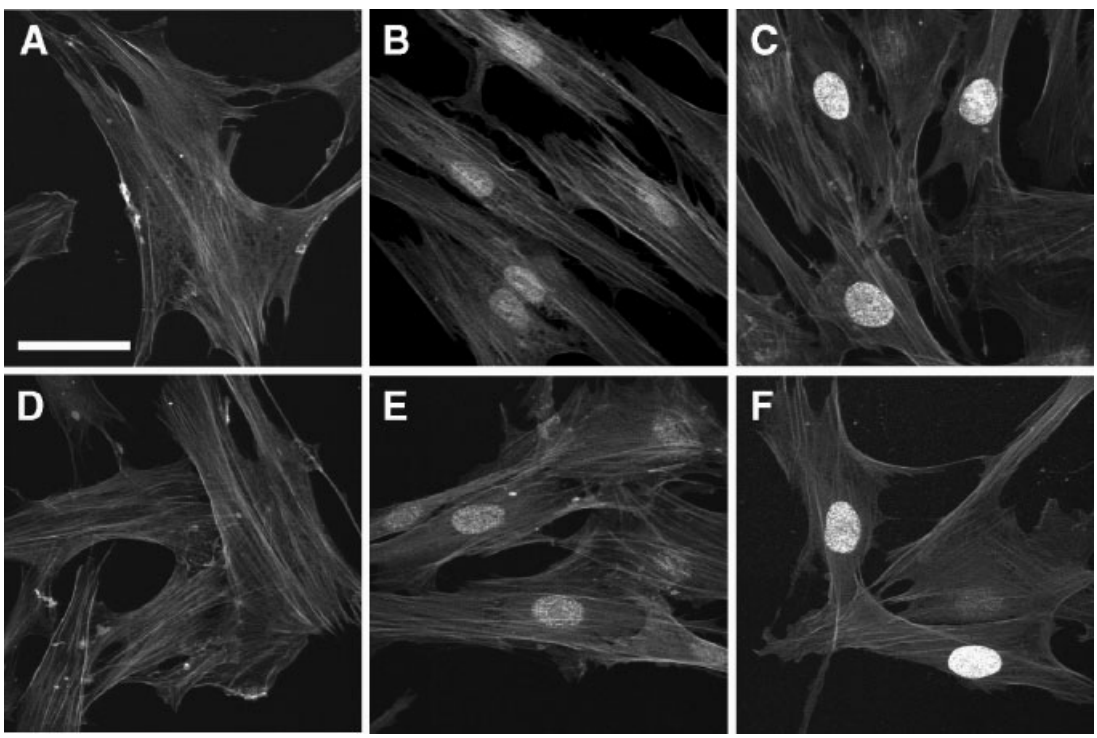

Fig. 3. Immunofluorescence detection of epiC was cell cycle dependent. Cells were made quiescent (G_0) by serum deprivation and then stimulated to proliferation either by serum alone (**A–C**) or by serum containing 1 mM hydroxyurea that stopped cells at the G_1/S border (**E**, **F**). At different times after release from either G_0 or the G_1/S block, the cells were fixed with methanol and immunolabeled with anti-actin at +4°C. EpiC labeling was not detected in G_0 cells (**D**), or during the first G_1 after serum stimulation (A), 8 h after serum stimulation, but was detected in S phase (B), 19 h, (C), 23 h after serum stimulation, (E) 1.5 h, (F) 5 h after releasing cells from hydroxyurea block. Note increasing intensity of nuclear anti-actin immunolabeling during S phase progression (B–C) and (E–F). Bar, 50 μm.

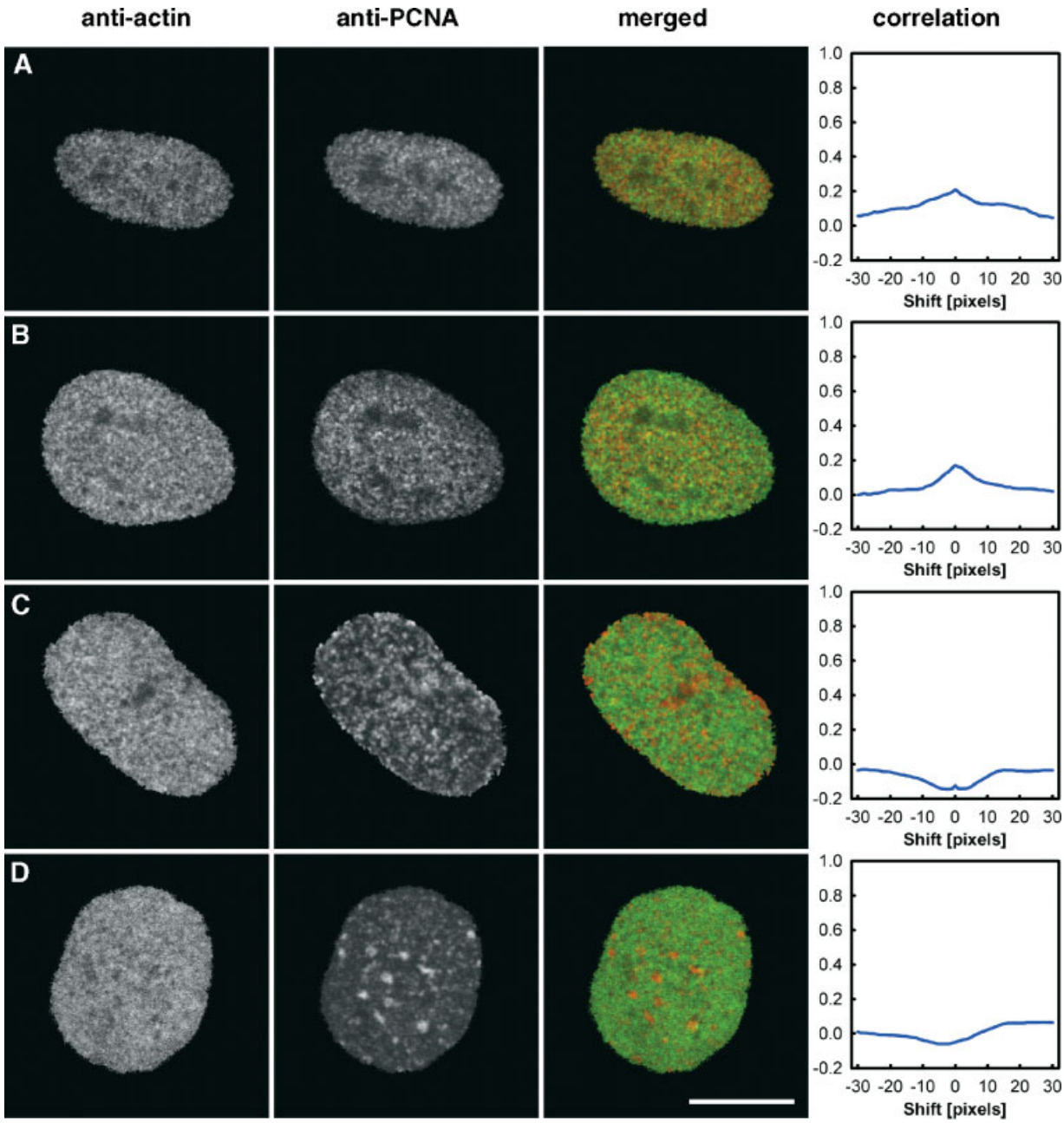

Fig. 4. Cross-correlation analysis of nuclear anti-actin and anti-PCNA immunofluorescence in the course of S phase of unsynchronized cells. The S phase stage was identified by the characteristic fluorescence patterns of the PCNA labeling. **A**, **B**: early S; (**C**) mid S; (**D**) late S. The cross-correlation function (right column) was used to measure the overlap of PCNA (images in the right gray column; in red in the merged color images) and anti-actin (images in the left gray column; in green in the merged color images) fluorescent signal in 2D confocal images (see Materials and Methods for details). The values on the ordinate are the Pearson's correlation coefficients determined for each pixel shift plotted on the abscissa. Only the nuclear area is shown, in which the cross-correlation function was calculated. Bar, 10 μm.

(Fig. 4D) S phase nuclei. During S phase progression, the intensity of nuclear epiC immunofluorescence increased from low in early S (Fig. 3B,E) to the brightest in the mid and late S (Fig. 3C,F). These differences in the intensity of nuclear epiC immunofluorescence (i.e., low in early S and high in mid and late S) were observed during the acquisition of high resolution images of S phase nuclei (Fig. 4A,B and C,D, respectively). Scanning parameters of the LCS confocal microscope, however, were set in order to obtain the maximal dynamic

resolution. Thus the fluorescence intensities of nuclear epiC, shown in the different images of Figure 4, are not directly comparable.

In unsynchronized, dual-labeled cells, larger nuclei were also observed that had the bright, late S phase-like pattern of epiC labeling, but no PCNA labeling (not shown). Since a similar type of nuclei was observed between the end of S phase and the burst of mitosis in synchronized cells (not shown), we conclude that these larger, epiC-positive, PCNA-negative nuclei are in G_2 phase. The epiC positivity in G_2 phase nuclei was directly confirmed by experiments in which the unsynchronized LEP cell nuclei were pulse labeled in late S phase with biotin-dUTP and chased for 2–4.5 h before labeling with anti-actin. All exhibited epiC positivity (results not shown). EpiC positivity was also detected on the chromosomes of unsynchronized cells throughout all stages of mitosis (not shown) and in the newly formed pairs of small, early G_1 nuclei (not shown; nevertheless see Fig. 5A).

The G_1 phase of LEP cells was estimated to last about 15–18 h (results not shown). To investigate the pattern of epiC labeling during early G_1 of cycling cells, cells arrested at metaphase by colcemid were removed by the shake-off procedure, washed with fresh, colcemid-free medium, and re-plated onto glass coverslips. At different times after re-plating, the cells were fixed, and epiC was detected. Exit from mitosis was apparently not synchronous for all the cells. One hour after re-plating the cells into colcemid free medium (i.e., ~1.5 h after colcemid was removed by centrifugation), we observed a mixture of both early G_1 cells and cells still in mitosis. Such an asynchrony was also observed by others [Dimitrova et al., 2002].

The results presented in Figure 5 lead us to propose four types (Types I–IV) of nuclear epiC immunofluorescence during cell cycle progression in early G_1. Types I–III exhibited dispersed focal patterns of nuclear epiC labeling differing in the nuclear density or relative number of epiC foci. Type I had the highest density of epiC foci detected all over the nucleus with the exception of the nucleoli (Fig. 5A,B) and was first observed 1 h and sporadically up to 3 h after cell re-plating. Type II had intermediate levels of nuclear epiC foci (Fig. 5C,D) and was first observed 2 h and sporadically up to 4 h after cell re-plating. Type III had a relatively low density of epiC foci (Fig. 5E,F) and was first observed 3 h and sporadically up to 5 h after cell re-plating. Type IV showed no detectable epiC signal in the nucleus and became progressively the predominating species beginning ~5 h after replating (Fig. 5G,H).

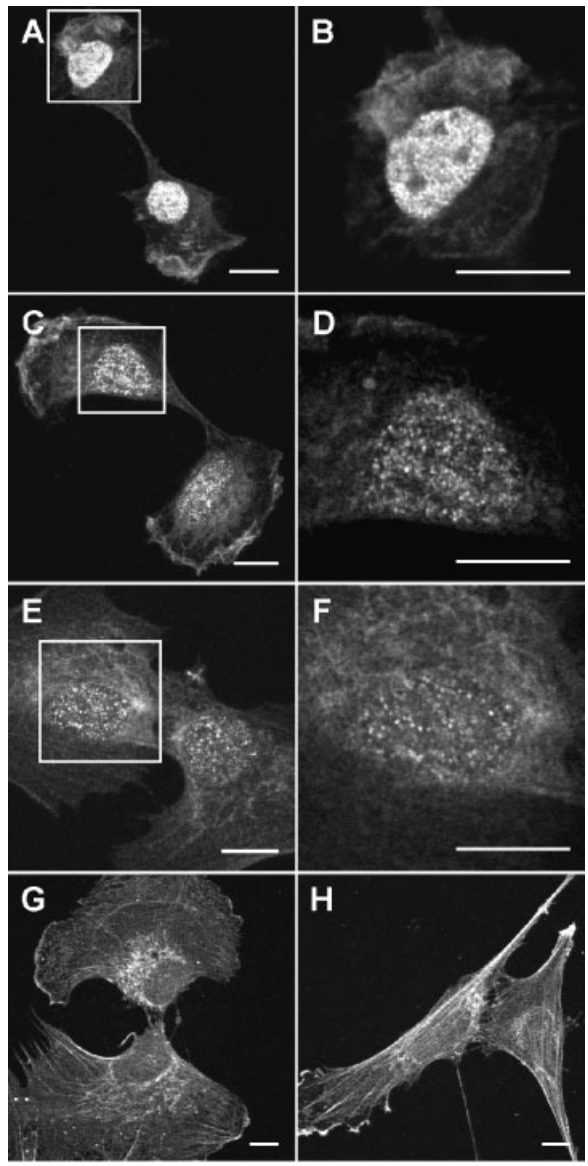

Fig. 5. EpiC positivity disappeared during early G_1 phase progression. Exponentially growing cells were arrested at metaphase by colcemid for 2 h, collected by the shake-off procedure, washed with fresh colcemid-free medium, and re-plated onto coverslips. At different times after re-plating, the cells were fixed and epiC was immunovisualized with anti-actin. Four different types of epiC immunofluorescence were recognized. Type I, (**A**) detail in frame zoomed in, (**B**) was first observed 1 h and sporadically 3 h after cell re-plating, type II (**C**), detail in frame zoomed in (**D**), was first observed 2 h and sporadically 4 h after cell re-plating, type III (**E**), detail in frame zoomed in (**F**), was first observed 3 h and sporadically 5 h after cell re-plating, type IV—no epiC signal in the nucleus (**G**, **H**), was first observed 3 h after cell replating and increased progressively with increasing time. Bars, 10 μm.

The nuclear border decorated by an epiC perinuclear cytoplasmic meshwork (Fig. 5E, G) was first observed 3 h after cell re-plating. Considering the asynchrony of the starting cell population, we suggest that the early G_1 epiC immunolabeling gradually changes to sparsely dotted and finally disappears during the first 3–5 h after mitosis. No epiC labeling was seen either 7 or 10 h after cell re-plating (results not shown). The disappearance of epiC labeling in early G_1 was confirmed with unsynchronized cells, pulse-chase labeled with biotin-dUTP (results not shown). Taken together, these results demonstrate that nuclear/chromosomal epiC positivity occurs in a distinct early S to early G_1 cell cycle window.

Reversibility of Cold-Dependent EpiC Labeling

The early S to early G_1 cell cycle window of epiC positivity provides an explanation as to why only some of the nuclei were labeled by anti-actin in our detection system. Why, however, was the cold detection regime necessary for epiC labeling? Low temperature incubations are commonly used to protect antigens from possible degradation events, which occur at faster rates at RT. The possibility that epiC was degraded by the incubation at RT can be ruled out since incubation of methanol-fixed cells at RT did not affect the subsequent binding of anti-actin to epiC at +4°C (Fig. 6). The cells were first incubated with anti-actin for 2 h at RT, followed by Cy-3-conjugated secondary antibody (Fig. 6A). After washing, the same cells were incubated again with anti-actin at +4°C overnight, followed by Alexa Fluor 488-conjugated secondary antibody (Fig. 6B). Despite the fact that the structures immunovisualized at RT were, in this experimental design, decorated by both fluorochromes (Cy-3 and AlexaFluor 488) giving rise to a yellow-orange color, the appearance of green (AlexaFluor 488) fluorescence in the nucleus after the cold detection regime was striking (Fig. 6C).

To determine the possible reversibility of cold-dependent epiC labeling, the cells, fixed on several coverslips in parallel, were incubated with anti-actin and anti-PCNA sequentially either at +4°C, RT, +4°C, or RT, +4°C, RT, and so on, repeating the sequence of RT and +4°C consecutive shifts three times. Dual labeling with anti-actin and anti-PCNA antibodies was performed to determine whether each examined cell was in S phase and if so, the specific stage of S phase. After each temperature shift, one of the coverslips was processed for immunofluorescence with secondary antibodies. Similar results were obtained with cells fixed with methanol (results not shown) or with 2% formaldehyde and postfixed/permeabilized with methanol (Fig. 7).

In Figure 7, results are shown for mid to late S phase cell nuclei, that is, the S phase nuclei exhibiting the brightest epiC immunofluorescence (Figs. 3C,F and 4C,D), as well as during mitosis. Repeated cycles of incubating fixed cells in the cold and RT resulted in positive nuclear (Fig. 7B,D,F) and chromosomal (Fig. 7B′,D′,F′) labeling in the cold, while negative epiC nuclear (Fig. 7A,C,E) and chromosomal (Fig. 7A′,C′,E′) labeling was always observed at RT. These studies also revealed that 2–3 h incubations

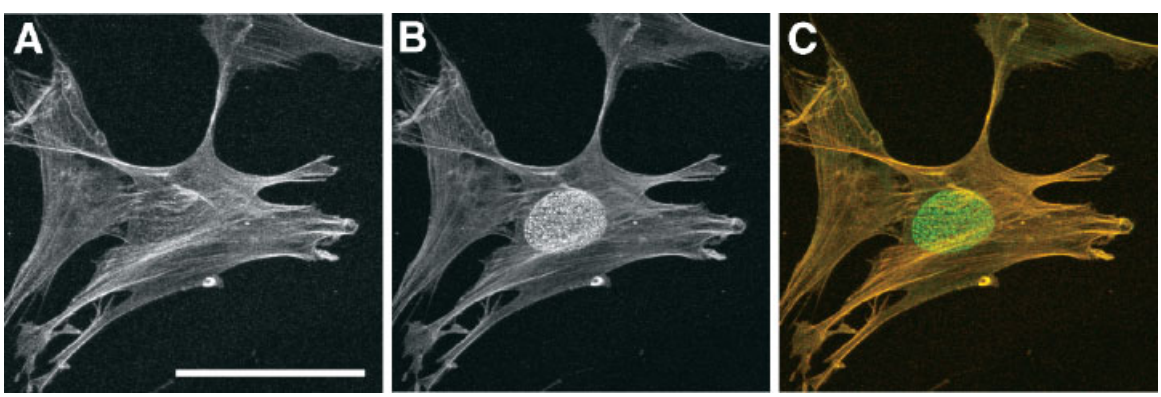

Fig. 6. Cold-dependent epiC labeling. Methanol-fixed cells were incubated with anti-actin for 2 h at RT followed by incubation with Cy3-conjugated goat anti-mouse antibodies (red; **A**) for 40 min at RT. After several washes, the cells were incubated again with anti-actin overnight at +4°C, followed by the incubation with Alexa Fluor 488-goat anti-mouse antibodies (green; **B**). Merging of the two channels is shown in (**C**). Bar, 50 μm.

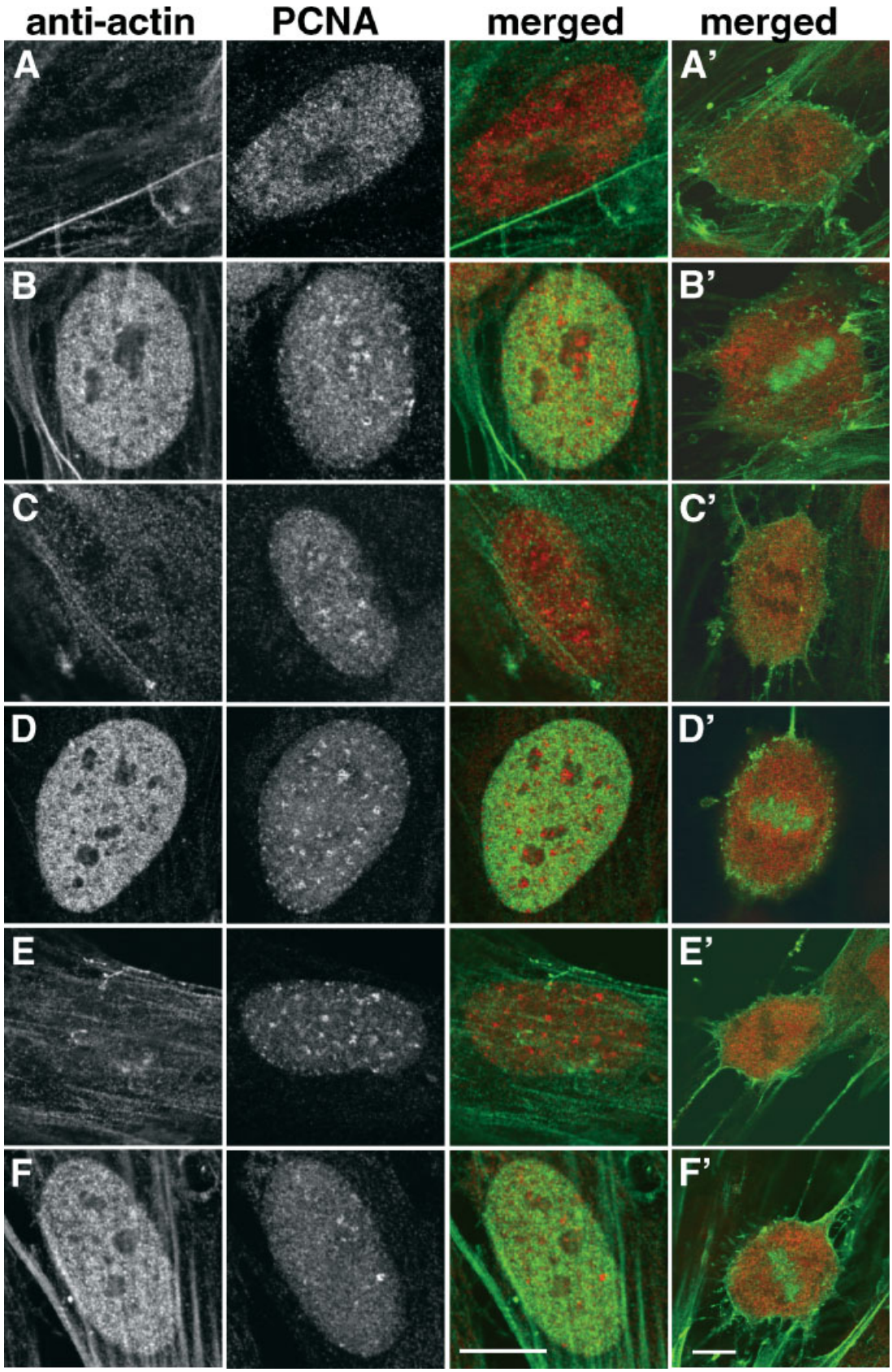

Fig. 7.

with anti-actin at +4°C was sufficient to induce positive labeling of nuclear and chromosomal epiC (Fig. 7D,F). Similarly, 2–3 h incubations at RT were sufficient to revert this positive nuclear and chromosomal epiC labeling back to a negative one (Fig. 7C,E). Reversion to negative epiC immunolabeling was independent of the duration of the previous incubation at +4°C, since similar results were obtained when cells, incubated overnight at +4°C, were shifted to RT (Fig. 7C).

In contrast, an incubation, with secondary antibody after the incubation with anti-actin at +4°C and before incubation with buffer or anti-actin at RT, prevented disappearance of epiC labeling at RT (not shown). This indicated that the binding of secondary antibody stabilized anti-actin-epiC complex created at +4°C and protected it from dissociation during the subsequent incubation for 3 h at RT. Moreover, the binding of anti-actin to epiC at +4°C was not abolished by the subsequent intensive washing of the cells with PBS or PBS/1% BSA for 3 h at +4°C (not shown), indicating that the anti-actin-epiC complex created at +4°C was also stable at +4°C.

These results suggest that either the fixed, epiC positive nuclear and chromosomal structures, and/or the anti-actin antibody possess a specific property that can be reversibly changed by cold and result in cold-dependent epiC labeling.

Nuclear EpiC Labeling Colocalizes With DNA Replicated During Early S Phase

Anti-PCNA-detected foci are widely regarded as markers depicting the sites of ongoing DNA replication [Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002]. Characteristic nuclear patterns of PCNA labeling enable the determination of the early, mid, and late S phase time points [Somanathan et al., 2001]. Since PCNA labels active sites of DNA replication and is released from replicated DNA following replication [Somanathan et al., 2001; Sporbert et al., 2002], we also analyzed cells where the DNA was permanently labeled by biotin-dUTP pulses. The biotin-dUTP, introduced into living cells by means of a hypotonic shift [Koberna et al., 1999], is rapidly utilized within 15–20 min (Koberna, unpublished results) assuring the labeling of a discrete subset of replication sites in the cell.

In contrast to PCNA labeling, the incorporated biotin-dUTP remains in replicated DNA and can be detected later as distinct higher order chromatin domains that are stably maintained throughout the cell cycle including mitosis and into subsequent cell generations [Sparvoli et al., 1994; Jackson and Pombo, 1998; Ma et al., 1998; Zink et al., 1998; Sadoni et al., 1999]. Thus, despite any possible minor structural changes at the microscale level of individual foci, the characteristic nuclear patterns of biotin-dUTP pulse-labeling in early, mid or late S phase enable, after a chase, study and comparison of chromatin domains that replicated their DNA at specific stages of S phase.

We analyzed the spatial distribution of both epiC and PCNA labeling in cells double labeled with anti-actin and anti-PCNA (Fig. 4), and the distribution of epiC and biotin-dUTP labeling in cells pulsed with biotin-dUTP and, after different chase periods, fixed and processed for immunofluorescence with anti-actin and anti-biotin antibodies (Fig. 8). For epiC and PCNA, no preference for dual-labeled pixel populations (co-localization events) was detected when 2D fluorescence intensity histograms were used to describe the distribution of the two (red anti-PCNA and green anti-actin) signals (not shown). Nevertheless, a weak positive correlation between the fluorescence signals was measured by cross-correlation analysis in early S phase nuclei (Fig. 4A,B), while a negative correlation was determined in mid and late S phase nuclei (Fig. 4C,D).

Similar to the PCNA findings, the distribution of biotin-dUTP and epiC labeling after a

Fig. 7. Reversibility of cold-dependent epiC labeling. Cells were fixed with 2% formaldehyde for 10 min at RT and postfixed/permeabilized with methanol for 15 min at −20°C, rehydrated in PBS for 30 min at RT and blocked with 1% BSA/PBS for 1 h at RT. Six coverslips in parallel were incubated with the mixture of anti-actin (1:200) and rabbit anti-PCNA (1:100) in 1% BSA/PBS sequentially at RT (**A**), +4°C (**B**), RT (**C**), +4°C (**D**), RT (**E**), and +4°C (**F**). At time intervals indicated in the scheme below, one of the coverslips was washed with PBS and incubated with a mixture of goat anti-mouse-Alexa Fluor 488 (1:100) and goat anti-rabbit–Cy3 (1:200) for 1 h, washed with PBS and mounted. Scheme of primary antibody incubation: (A) 1 h at RT; (B) as in (A) followed by overnight at 4°C; (C) as in (B) followed by 2 h at RT; (D) as in (C) followed by 2.3 h at 4°C; (E) as in (D) followed by 2 h at RT; (F) as in (E) followed by 3.3 h at 4°C. A–F and **A′–F′** are optical section images of nuclei and mitotic cells, respectively. Bars, 10 μm

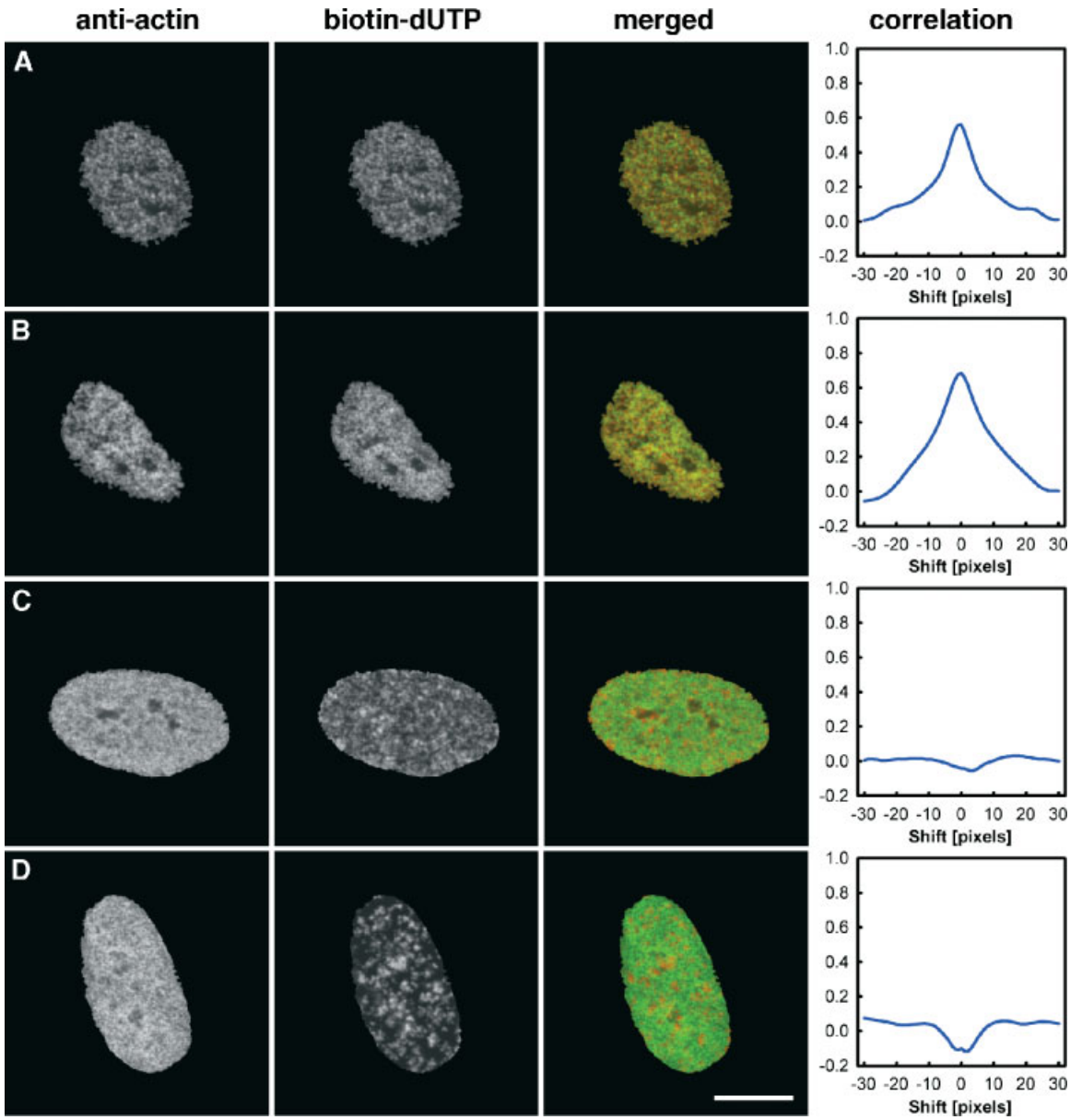

Fig. 8. EpiC labeling colocalized with DNA replicated in early S phase. Unsynchronized LEP cells were labeled with biotin-dUTP as described in Materials and Methods. After a 2-h chase, the cells were fixed with methanol and processed for immunofluorescence with anti-actin and anti-biotin antibodies. Characteristic fluorescence patterns of biotin-dUTP pulse labeling enabled the identification of early (**A**, **B**), mid (**C**), and late (**D**) S phase time points of the previously replicated DNA. The cross-correlation function (right column) was used to measure an overlap of red (biotin-dUTP, right gray column) and green (anti-actin, left gray column) fluorescent signal in 2D confocal images (see Materials and Methods for details). The values on the ordinate are the Pearson's correlation coefficients determined for each pixel shift plotted on the abscissa. Only the nuclear area, in which the cross-correlation function was calculated, is shown. Bar, 10 μm.

10- or 20-min chase exhibited a weak positive correlation in early S phase nuclei but a negative correlation in late S phase nuclei (data not shown). When, however, the chase was increased to 1 and up to 4.5 h, a strong positive correlation was found between the distribution of biotinylated DNA labeled during early S phase and epiC labeling (Fig. 8A,B). In contrast, no significant overlap was detected between late S phase labeled chromatin and epiC over chase periods ranging from 1 to 4.5 h. Following a 4.5-h chase, biotin-dUTP-labeled mitotic cells are detected and some labeled cells begin to enter the G_1 phase.

The continued detection of epiC positivity in late S phase-labeled nuclei following chase period of up to 4.5 h when labeled mitotic cells begin to appear, provides further support for the maintenance of epiC positivity during G_2 phase. Similarly, our previous observations obtained with synchronized cells, which demonstrated the disappearance of epiC labeling in early G_1 phase (see Fig. 5), were independently confirmed with unsynchronized cell. When the chase was prolonged to 6 and 10 h, nuclei with a late S-pattern of biotin-dUTP labeling exhibited epiC positivity after a 6-h chase (early G_1 nuclei), but not after a 10-h chase (later G_1 nuclei, results not shown).

Immunoblot Analysis of Anti-Actin Specificity

To reduce the possibility of in situ nuclear contamination by cytoplasmic actin in our system, we intentionally omitted the use of detergents for cell permeabilization except during the cell nuclei isolation. To determine whether the procedure used for cell nuclei isolation and purification [Sahlas et al., 1993] removed the nuclear epiC-staining structures, immunofluorescence analysis of epiC by anti-actin was performed in isolated cell nuclei washed during the isolation procedure several times with the buffer containing 1% Triton X-100 prior to methanol fixation (Fig. 9A). When nuclei from unsynchronized cells were used, epiC was immunolabeled in PCNA-positive S phase nucleus (right, Fig. 9A,B) but not in PCNA-negative nucleus (left, Fig. 9A,B). This is consistent with a similar cell cycle specific pattern of nuclear epiC labeling in Triton-washed isolated cell nuclei compared to the in situ nuclei of methanol-fixed cells. The results also demonstrate that epiC is detected in the Triton insoluble fraction of the cell nuclei, which contains the chromatin.

Since evidence for the presence of actin in the cell nucleus based on biochemical purification schemes is generally not considered conclusive due to possible cytoplasmic contamination [Rando et al., 2000], we employed SDS–PAGE and Western immunoblotting to determine the in vitro anti-actin specificity at RT and at +4°C. Synchronized LEP cells, in mid to late S phase (5–6 h after the release from hydroxyurea block), were used for immunoblot analysis with anti-tubulin antibody (Tu-01) as a marker of cytoplasmic protein. As shown in Figure 9D, only a single band corresponding to the electrophoretic mobility of an actin standard protein (results not shown) was recognized by anti-actin on immunoblots of SDS–PAGE-separated lysates of both whole cells and isolated cell nuclei.

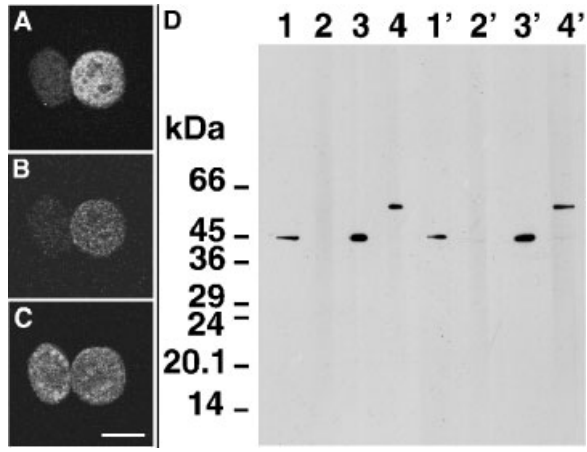

Fig. 9. Anti-actin detected nuclear epiC in isolated cell nuclei. **A–C**: Nuclei were isolated from exponentially growing cells, fixed with methanol, and dual labeled with anti-actin (A) plus anti-PCNA (B) overnight at +4°C. Secondary antibodies were applied for 1 h at RT. DNA was counterstained by TO-PRO-3 (C). Note the epiC- and PCNA-negative nucleus on the left. Bar, 10 μm. **D**: Determination of anti-actin immunoreactivity by Western immunoblotting. Equal protein amounts from lysates of isolated cell nuclei (**lanes 1, 2, 1′, 2′**) and of whole cells (**lanes 3, 4, 3′, 4′**) were separated on a 12% SDS–PAGE gel and transferred onto nitrocellulose membrane. Each membrane strip containing proteins loaded to one slot was cut in halves; one half was incubated with anti-actin 1:500 (lanes 1, 3, 1′, 3′), and another half with anti-tubulin Tu-01, 1:2,000 (lanes 2, 4, 2′, 4′). Tu-01 antibody was used as a marker of cytoplasmic proteins. Similar results were obtained when the membranes were incubated with anti-actin either overnight at +4°C (lanes 1–4) or at RT for 2 h (lanes 1′–4′).

Importantly, no difference in immunoblotting results was found when nitrocellulose membranes were incubated with anti-actin overnight at +4°C or 2 h at RT. Despite possible cytoplasmic contamination, the finding that no other cross-reacting band(s) was detected by anti-actin on the blots of cell nuclei lysates, indicated that either actin or an epiC-possessing protein of electrophoretic mobility similar to actin was being detected by anti-actin immunofluorescence in the nuclei in situ. The possibility, however, that anti-actin bound in situ to a non-sequential epitope [Gonsior et al., 1999] cannot be excluded at the present time.

In summary, these results demonstrate that anti-actin specificity in vitro is not changed by the cold detection conditions. The results did not

exclude, but also did not prove the possibility that nuclear actin was detected by chromatin epiC positivity in situ.

DISCUSSION

Three main findings are presented in this study. First, we have elucidated a new phenomenon of cold-dependent in situ epitope detectability. In contrast to generally known fixation-dependent changes in epitope detectability, we demonstrate specific, cold-dependent detection of an epitope in a new in situ location. Evidence is presented for the cold-dependent immunofluorescence detection of an epitope recognized by a monoclonal anti-actin antibody in chromatin structures of human fibroblast cell nuclei and mitotic chromosomes. The nuclear/chromosomal epitope, which was recognized by anti-actin at +4°C and the detection of which was cold-dependent, is termed *epiC*. Under identical fixation conditions, the epitope detected by anti-actin at RT and epiC are visually distinguishable in the same cell (Fig. 6). Second, under identical detection conditions, epiC positivity is found only in cells within a distinct cell cycle window that extends from early S phase through mitosis until the early G_1 phase of the next cell generation. Third, we found that during S phase, under identical detection conditions, epiC labeling colocalizes with DNA replicated in early S phase. The association of epiC with chromatin domains replicated in early S phase was initiated at low levels during the actual replicative process and was strikingly enhanced 1 h following the pulse labeling of the replicating DNA. In contrast, epiC did not colocalize with late S phase replicated chromatin either during DNA replication or at any other time in the cell cycle.

Cold-Dependent EpiC Detection

The requirement of a cold detection regime for the immunolabeling of epiC was surprising. Reversibility of epiC labeling after shifting the detection temperature from +4°C to RT and back to +4°C (Fig. 7) indicated that reversible, low temperature-induced changes are required for detection. It is known that hydrophobic interactions significantly decrease in magnitude as the temperature decreases [Griko et al., 1989]. This diminishes the probability of a non-specific anti-actin binding to chromatin at +4°C due to "hydrophobic stickiness" [James and Tawfik, 2003], while possible changes in higher order protein structures due to cold-induced weakening of hydrophobic interactions is feasible. Indeed, a phenomenon of low temperature-induced reversible changes (unfolding and refolding) in higher order protein structure, termed *cold denaturation* [Privalov et al., 1986; Griko et al., 1989], has been observed for a variety of globular proteins and investigated in vitro [Jourdan and Searle, 2001; Erez et al., 2002; Kunugi and Tanaka, 2002]. Cold denaturation of anti-actin that would reversibly change its antigen specificity at RT versus +4°C might not be encountered in our case since, in vitro, no differences were found when immunoblot membranes were incubated with anti-actin for either 2 h at RT or overnight at +4°C (Fig. 9). Nevertheless, it cannot be excluded that cold-induced conformational changes of anti-actin reversibly extended its epitope specificity by a non-sequential epitope(s) [James et al., 2003].

Another component that could be affected by the low temperature in situ is the chromatin in fixed cells. Both the native higher order structural interactions in the epitope structural context and the structural changes imposed to these native chromatin structures by fixation treatment could result in epitope "burial" or disappearance. Cold-induced weakening of hydrophobic interactions (cold denaturation), affecting higher order chromatin (protein) structures, might introduce a local structural unfolding manifested finally in the opening of epitope accessibility. Though not very likely, since unfolding rather than compaction of structures is connected with cold denaturation [Privalov, 1990], it cannot be excluded that such cold denaturation-like processes might also create a chromatin structure-specific, non-sequential epitope [Gonsior et al., 1999].

These findings indicate that either the fixed, epiC-positive chromatin domains of the early S-to-early-G_1 cell cycle window or/and the anti-actin antibody itself possess a property that can be reversibly changed by cold and result in cold-dependent epiC labeling. In addition to revealing cell cycle dependent chromatin structural changes, the reversible cold-dependent detection of epiC is, to our knowledge, a newly discovered phenomenon of temperature-induced reversible changes of chromatin and in situ epitope immunodetectability. Similar cold-

dependent epiC labeling was also observed in HeLa, mouse, and rat cells.

Sauman and Berry [1994] previously reported the presence of actin within the cell nucleus of insect cells and on *Drosophila* polytene chromosomes. Consistent with our findings, detection was dependent, under their fixation conditions, on digestion of the nuclear or chromosomal DNA with micrococcal nuclease or DNase I. Incubations with anti-actin antibodies were also performed overnight at 4°C, but the effect of temperature on detection was not studied [Sauman and Berry, 1994].

Co-localization of EpiC Labeling With Chromatin Domains Replicated During Early S Phase

Using dual labeling experiments, we demonstrate that during S phase, epiC co-localizes with chromatin domains previously replicated during early S phase, that is, with chromatin domains representing mainly euchromatic regions of the genome [Dimitrova and Berezney, 2002 and references therein]. In contrast epiC did not co-localize with DNA replicated during late S phase, that is, with chromatin domains representing mainly heterochromatin. The greatly enhanced level of co-localization of epiC with early S replicated chromatin domains observed after chase periods of 1 h or more (Fig. 8A,B) in comparison with co-localization with PCNA, that is, at functional replication sites (Fig. 4A,B), leads us to propose an epigenetic model of replication-coupled modulation of chromatin domains, which is initiated during DNA replication in early S phase and persists on the chromatin domains until early G_1 phase of the next cell generation.

Our Western immunoblotting results (Fig. 9D) are consistent with but also do not prove that nuclear actin is involved in this proposed anti-actin-detected chromatin modulation. It is known that in vitro separated sequences can be in situ structurally compacted into a coherent patch recognized by sequence-specific antibody as an antibody-reactive region [Gonsior et al., 1999]. Although a widely accepted approach, the use of immunoblotting results as evidence for a specific in situ immunoreactive antigen, is certainly not foolproof. It is conceivable that the nuclear component(s) that react(s) in situ with the anti-actin antibody may be unrelated to actin. To determine unambiguously the molecular nature of anti-actin-detected chromatin modulation, in a cellular environment where actin represents the most abundant cellular protein involved in a plethora of mutually interconnected cellular processes and signalling pathways, is not an easy task but a challenge for future research.

Actin was recently shown to be a component of the mammalian SWI/SNF-like BAF and SWI/SNF-related multiprotein chromatin remodeling complexes whose combinatorial assembly could provide a highly complex and functionally specific chromatin environment [Olave et al., 2002; Pederson and Aebi, 2002; Rando et al., 2002]. Considering the possible involvement of actin in the anti-actin-detected modulation of newly replicated euchromatic regions of the genome, it is tempting to speculate that actin, either alone or as a part of some chromatin remodeling complex(es), might function as a "mark" or barrier protecting euchromatin from heterochromatinization during S phase. Later, during chromosome repositioning in early G_1, actin might protect euchromatin from the interaction with nuclear envelope that is known to be associated with heterochromatin.

Replication Timing, Transcriptional Regulation, and EpiC Modulation of Early S Replicated Chromatin Domains

Contrary to late S phase, there is little direct evidence for early S phase specific chromatin assembly activities. [McNairn and Gilbert, 2003]. The long-suspected correlation between gene activity and replication in early S phase in higher eukaryotes [Goldman et al., 1984; Holmquist, 1987] has been demonstrated recently at the genome scale [Schübeler et al., 2002]. Moreover, it was recently reported that exogenous genes, microinjected into early or late S phase rat cell nuclei, become assembled into transcriptionally active or transcriptionally inactive (silenced) chromatin, respectively [Zhang et al., 2002]. Once established, this expression state was maintained after cell division. This suggests that the establishment of epigenetically inherited transcriptional competence is acquired during S phase and is dependent on replication timing but not necessarily on the DNA sequence [Zhang et al., 2002].

Previous studies have indicated that actively replicating chromatin domains in early S phase are not simultaneously active in transcription despite the fact that this region of the genome is highly enriched in actively transcribed genes [Wansink et al., 1994; Wei et al., 1998]. It has,

therefore, been proposed that there is a global switching mechanism in the cell nucleus for the spatio-temporal regulation and coordination of replication and transcription of early S replicated chromatin domains. In this model, chromatin domains are reprogrammed for active gene transcription following completion of replication during early S phase [Berezney and Wei, 1998; Wei et al., 1998; Berezney, 2002]. We, therefore, suggest that the proposed epigenetic modulation of early S replicated chromatin as detected by epiC may be a part of the mechanism for the global reprogramming of chromatin to a transcriptionally competent state.

The cell cycle window, as defined by epiC labeling in this study, begins at early S phase-where a precise spatio-temporal organization of genomic replication takes place in the cell nucleus [Berezney et al., 2000; Berezney, 2002; Dimitrova and Berezney, 2002]—and, after passage through mitosis, ends in early G_1 phase, during which the temporal order of genome replication for the next S phase and the spatial nuclear organization of the genome (chromosome re-positioning) are both re-set [Dimitrova and Gilbert, 1999; Bridger et al., 2000; Gilbert, 2001; Li et al., 2001, 2003; Dimitrova et al., 2002]. Such a scenario further suggests a possible involvement of anti-actin-detected chromatin modulation in transmitting and/or maintaining the epigenetic information on the transcriptionally competent parts of the genome.

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REFERENCES

Batchelor CL, Woodward AM, Crouch DH. 2004. Nuclear ERM (ezrin, radixin, moesin) proteins: Regulation by cell density and nuclear import. Exp Cell Res 296:208–222.

Berezney R. 2002. Regulating the mammalian genome: The role of nuclear architecture. Adv Enzyme Regul 42: 39–52.

Berezney R, Wei X. 1998. The new paradigm: Integrating genomic function and nuclear architecture. J Cell Biochem Suppl 31:238–242.

Berezney R, Dubey DD, Huberman JA. 2000. Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. Chromosoma 108:471–484.

Bianchi V, Pontis E, Reichard P. 1986. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. J Biol Chem 261:16037–16042.

Bravo R, Macdonald-Bravo H. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: Association with DNA replication sites. J Cell Biol 105:1549–1554.

Bridger JM, Boyle IR, Bickmore WA. 2000. Re-modelling of nuclear architecture in quiescent and senescent human fibroblasts. Curr Biol 10:149–152.

Cavalli G. 2002. Chromatin as a eukaryotic template of genetic information. Curr Opin Cell Biol 14:269–278.

Chen D, Xu W, He P, Medrano EE, Whiteheart SW. 2001. Gaf-1, a gamma-SNAP-binding protein associated with the mitochondria. J Biol Chem 276:13127–13135.

Dimitrova DS, Berezney R. 2002. The spatio-temporal organization of DNA replication sites is identical in primary, immortalized and transformed mammalian cells. J Cell Sci 115:4037–4051.

Dimitrova DS, Gilbert DM. 1999. The spatial position and replication timing of chromosomal domains are both established in early G1 phase. Mol Cell 4:983–993.

Dimitrova DS, Prokhorova TA, Blow JJ, Todorov IT, Gilbert DM. 2002. Mammalian nuclei become licensed for DNA replication during late telophase. J Cell Sci 115: 51–59.

Dráber P, Dráberová E, Linhartová I, Viklický V. 1989. Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. J Cell Sci 92: 519–528.

Erez T, Gdalevsky GY, Hariharan C, Pines D, Pines E, Phillips RS, Cohen-Luria R, Parola AH. 2002. Cold-induced enzyme inactivation: How does cooling lead to pyridoxal phosphate-aldimine bond cleavage in tryptophanase? Biochim Biophys Acta 1594:335–340.

Gilbert DM. 2001. Nuclear position leaves its mark on replication timing. J Cell Biol 152:11F–16F.

Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A. 1984. Replication timing of genes and middle repetitive sequences. Science 224:686–692.

Gonsior S, Platz S, Buchmeier S, Scheer U, Jockusch B, Hinssen H. 1999. Conformational difference between nuclear and cytoplasmic actin as detected by a monoclonal antibody. J Cell Sci 112:797–809.

Griko YV, Venyaminov SY, Privalov PL. 1989. Heat and cold denaturation of phosphoglycerate kinase (interaction of domains). FEBS Lett 244:276–278.

Hitchcock S. 1980. Actin deoxyribonuclease I interaction. Depolymerization and nucleotide exchange. J Biol Chem 255:5668–5673.

Holmquist GP. 1987. Role of replication time in the control of tissue-specific gene expression. Am J Hum Genet 40:151–173.

Jackson DA, Pombo A. 1998. Replicon clusters are stable units of chromosome structure: Evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J Cell Biol 140: 1285–1295.

James LC, Tawfik DS. 2003. The specificity of cross-reactivity: Promiscuous antibody binding involves specific hydrogen bonds rather than nonspecific hydrophobic stickiness. Protein Sci 12:2183–2193.

James LC, Roversi P, Tawfik DS. 2003. Antibody multispecificity mediated by conformational diversity. Science 299:1362–1367.

Johnson LM, Cao X, Jacobsen SE. 2002. Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr Biol 12:1360–1367.

Jourdan M, Searle M. 2001. Insights into the stability of native and partially folded states of ubiquitin: Effects of cosolvents and denaturans on the thermodynamics of protein folding. Biochemistry 40:10317–10325.

Koberna K, Staněk D, Malínský J, Eltsov M, Pliss A, Čtrnáctá V, Cermanová S, Raška I. 1999. Nuclear organization studied with the help of a hypotonic shift: Its use permits hydrophilic molecules to enter into living cells. Chromosoma 108:325–335.

Kunugi S, Tanaka N. 2002. Cold denaturation of proteins under high pressure. Biochim Biophys Acta 1595:329–344.

Lachner M, Jenuwein T. 2002. The many faces of histone lysine methylation. Curr Opin Cell Biol 14:286–298.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Leonhardt H, Rahn H-P, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardoso MC. 2000. Dynamics of DNA replication factories in living cells. J Cell Biol 149:271–280.

Li F, Chen J, Izumi M, Butler MC, Keezer SM, Gilbert DM. 2001. The replication timing program of the Chinese hamster {beta}-globin locus is established coincident with its repositioning near peripheral heterochromatin in early G1 phase. J Cell Biol 154:283–292.

Li F, Chen J, Solessio E, Gilbert DM. 2003. Spatial distribution and specification of mammalian replication origins during G1 phase. J Cell Biol 161:257–266.

Ma H, Samarabandu J, Devdhar RS, Acharya R, Cheng P-C, Meng C, Berezney R. 1998. Spatial and temporal dynamics of DNA replication sites in mammalian cells. J Cell Biol 143:1415–1425.

McNairn A, Gilbert D. 2003. Epigenomic replication: Linking epigenetics to DNA replication. Bioessays 25:647–656.

Muller C, Leutz A. 2001. Chromatin remodeling in development and differentiation. Curr Opin Genet Dev 11: 167–174.

Nagy P, Arndt-Jovin DJ, Jovin TM. 2003. Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells. Exp Cell Res 285:39–49.

Nakamura F. 2001. Biochemical, electron microscopic and immunohistological observations of cationic detergent-extracted cells: Detection and improved preservation of microextensions and ultrmicroextensions. BMC Cell Biology 2:10.

Nakayasu H, Berezney R. 1989. Mapping replicational sites in the eucaryotic cell nucleus. J Cell Biol 108:1–11.

Okorokov AL, Rubbi CP, Metcalfe S, Milner J. 2002. The interaction of p53 with the nuclear matrix is mediated by F-actin and modulated by DNA damage. Oncogene 21: 356–367.

Olave IA, Reck-Peterson SL, Crabtree GR. 2002. Nuclear actin and actin-related proteins in chromatin remodeling. Annu Rev Biochem 71:755–781.

Pederson T, Aebi U. 2002. Actin in the nucleus: What form and what for? J Struct Biol 140:3–9.

Privalov PL. 1990. Cold denaturation of proteins. Crit Rev Biochem Mol Biol 25:281–305.

Privalov PL, Griko Y, Venyaminov S, Kutyshenko VP. 1986. Cold denaturation of myoglobin. J Mol Biol 190: 487–498.

Rando OJ, Zhao K, Crabtree GR. 2000. Searching for a function for nuclear actin. Trends Cell Biol 10:92–97.

Rando OJ, Zhao K, Janmey P, Crabtree GR. 2002. Phosphatidylinositol-dependent actin filament binding by the SWI/SNF-like BAF chromatin remodeling complex. Proc Natl Acad Sci USA 99:2824–2829.

Ridgway P, Almouzni G. 2001. Chromatin assembly and organization. J Cell Sci 114:2711–2712.

Sadoni N, Langer S, Fauth C, Bernardi G, Cremer T, Turner BM, Zink D. 1999. Nuclear organization of mammalian genomes: Polar chromosome territories build up functionally distinct higher order compartments. J Cell Biol 146:1211–1226.

Sahlas D, Milankov K, Park P, De Boni U. 1993. Distribution of snRNPs, splicing factor SC-35 and actin in interphase nuclei: Immunocytochemical evidence for differential distribution during changes in functional states. J Cell Sci 105:347–357.

Sauman I, Berry SJ. 1994. An actin infrastructure is associated with eukaryotic chromosomes: Structural and functional significance. Eur J Cell Biol 64:348–356.

Schübeler D, Scalzo D, Kooperberg C, van Steensel B, Delrow J, Groudine M. 2002. Genome-wide DNA replication profile for *Drosophila melanogaster*: A link between transcription and replication timing. Nat Genet 32:438–442.

Somanathan S, Suchyna TM, Siegel AJ, Berezney R. 2001. Targeting of PCNA to sites of DNA replication in the mammalian cell nucleus. J Cell Biochem 81:56–67.

Sparvoli E, Levi M, Rossi E. 1994. Replicon clusters may form structurally stable complexes of chromatin and chromosomes. J Cell Sci 107(Pt 11):3097–3103.

Sporbert A, Gahl A, Ankerhold R, Leonhardt H, Cardoso MC. 2002. DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. Mol Cell 10:1355–1365.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Turner B. 2000. Histone acetylation and an epigenetic code. Bioessays 22:836–845.

van Steensel B, van Binnendijk E, Hornsby C, van der Voort H, Krozowski Z, de Kloet E, van Driel R. 1996. Partial colocalization of glucocorticoid and mineralocorticoid receptors in discrete compartments in nuclei of rat hippocampus neurons. J Cell Sci 109:787–792.

Vermaak D, Ahmad K, Henikoff S. 2003. Maintenance of chromatin states: An open-and-shut case. Curr Opin Cell Biol 15:266–274.

Wansink DG, Manders EE, van der Kraan I, Aten JA, van Driel R, de Jong L. 1994. RNA polymerase II transcription is concentrated outside replication domains throughout S-phase. J Cell Sci 107(Pt 6):1449–1456.

Wei X, Samarabandu J, Devdhar RS, Siegel AJ, Acharya R, Berezney R. 1998. Segregation of transcription and replication sites into higher order domains. Science 281:1502–1506.

Zhang J, Xu F, Hashimshony T, Keshet I, Cedar H. 2002. Establishment of transcriptional competence in early and late S phase. Nature 420:198–202.

Zink D, Cremer T, Saffrich R, Fischer R, Trendelenburg MF, Ansorge W, Stelzer EH. 1998. Structure and dynamics of human interphase chromosome territories in vivo. Hum Genet 102:241–251.