A Detailed Analysis of Cyclin A Accumulation at the G1/S Border in Normal and Transformed Cells

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The temporal relationship between cyclin A accumulation and the onset of DNA replication was analyzed in detail. Five untransformed and nine transformed asynchronously growing cell cultures were investigated using a triple immunofluorescence staining protocol combined with computerized evaluation of staining intensities in individual cells. The simultaneous staining of BrdU, cyclin A, and cyclin E made it possible to determine the cell cycle position of each cell investigated. Cells at the G1/S border were identified on the basis of cyclin E content and were further analyzed with respect to cyclin A and BrdU content. A method was developed to calculate objective thresholds defining the highest staining intensity found in the negative cells in the population. Using the thresholds we could distinguish cells with minute amounts of cyclin A and BrdU from truly negative cells. We show that the onset of cyclin A accumulation and the start of DNA replication occurs at the same time, or deviating by a few minutes at the most. We also show that cyclin A accumulates continuously during S. This study clearly demonstrates that nuclear cyclin A can be used as a reliable marker for the S and G2 phases in both normal and transformed interphase cells.

Key Words: cyclin A; BrdU; DNA replication; immunofluorescence staining; transformation; image cytometry.

INTRODUCTION

Transition events in the cell cycle are controlled by reversible phosphorylations and protein degradation. The phosphorylations are carried out by cyclin-dependent kinases (CDKs). The kinases are regulated by association to cyclins, by phosphorylation and dephosphorylation events, and by binding of CDK inhibitors (CKIs). The cyclins are a group of proteins, originally discovered in the early 1980s, that oscillate throughout the cell cycle [1]. The cyclin levels are controlled by cell cycle-specific protein synthesis, sequestration, and degradation (reviewed in [2, 3]). The cyclins involved in the control of DNA replication are cyclin E and cyclin A. Cyclin E appears late in G1 to associate with CDK2 until it is degraded in early S [4–6]. Cyclin A appears in late G1 or early S to associate with CDK2. It is degraded at metaphase, after participating in the regulation of entry into mitosis [7–9].

The specific role of cyclin A for DNA replication is still unclear but several studies have provided evidence showing that cyclin A regulates the initiation of DNA replication [10–14]. Inhibition of cyclin A activity in cultured mammalian cells through microinjection of antisense cDNA, as well as through microinjection of antibodies directed against cyclin A, inhibits entry into S [10–12]. Overexpression of cyclin A in G1 results in a decreased number of G1 cells in an asynchronously growing population, indicating that G1 cells are forced to prematurely go into S by constitutive cyclin A overexpression [13]. Furthermore, the addition of cyclin E and cyclin A to G1 nuclei has been shown to induce DNA replication [14].

It has also been suggested that cyclin-A-dependent kinase activity has a role in the two-step regulation process, ensuring that the DNA is replicated only once during each cell cycle. CDK activity has been shown to be required for the activation of replication at prereplication complexes (pre-RCs) and to subsequently prevent pre-RCs from reforming until the next cell cycle [15]. This is supported by the recent findings that cyclin A–CDK2 activity regulates the subcellular localization of Cdc6, which is one of the proteins in the pre-RCs [16]. Finally, localization studies using confocal and electron microscopy have shown that cyclin A and CDK2 are localized at sites of DNA replication, indicating that cyclin A might also have a direct role in DNA replication [17, 18].

The aim of this study was to determine the precise temporal relationship between cyclin A accumulation and DNA replication. Is accumulation of cyclin A in late G1 a prerequisite for entry into S, or is initiation of DNA replication a prerequisite for the subsequent accumulation of cyclin A? Previous studies have reported
CYCLIN A ACCUMULATION AT THE G1/S BORDER

FIG. 1. The three alternative models of nuclear cyclin A accumulation. In A cyclin A begins its accumulation at the very start of S phase, and in C cyclin A begins its accumulation first after DNA replication has begun. The expression patterns of the other two antigens stained for, bromodeoxyuridine (BrdU) and cyclin E, have been included in the figure as well. The triple staining makes it possible to decide where in the cell cycle every single cell is located, since cells in the first part of G1 don't contain any of the antigens stained for. Cells around the G1/S transition contain cyclin E, cells in S phase contain cyclin A and BrdU, and cells in G2 contain only cyclin A. Through comparison of the fraction of cells containing cyclin E and cyclin A, but not BrdU, to the fraction of cells containing cyclin E and BrdU, but not cyclin A, it is possible to decide whether cyclin A begins its accumulation before or after DNA replication is begun.

The result clearly shows that cyclin A starts to accumulate at the G1/S border in both normal and transformed cells. DNA replication does not seem to take place before cyclin A starts to accumulate, nor does cyclin A seem to be present in G1 before the cell commences DNA replication. Together with earlier findings, this suggests a pivotal role for cyclin A in the regulation of DNA replication. The results also have important methodological implications, since they clearly demonstrate that cyclin A can be used as a reliable marker for S and G2 in normal as well as transformed cell populations.

MATERIALS AND METHODS

The cell cultures used in the experiment are shown in Table 1. The cultures were chosen to represent a wide range of different tumors and normal tissues and consist of five primary cell strains and nine immortalized cell lines. One of the HDF (human diploid fibroblast) strains was analyzed at two different passages, seeded, and fixed a few weeks apart. The results from this strain have been labeled HDF p19 and HDF p22, respectively.

The cell cultures were kept in an incubator containing a 5% CO2/95% air mix and 100% humidity at 37°C. The cells were transferred to new plastic bottles before reaching confluency. For transfer the cells were treated with a solution containing 0.25% (w/v) trypsin and 0.5 mM EDTA in Puck's modified saline A. For immunocytochemistry the cells were seeded on 0.4-mm-thick coverslips in petri dishes, approximately 3000 cells/cm².

Prior to fixation cells in S phase were labeled with BrdU from Sigma (St Louis, MO). Twenty-four hours after seeding, BrdU was added to each dish to a final concentration of 20 μM. After 5 min, the medium was replaced with BrdU-free medium, and the cells were incubated for an additional 5 min to allow unincorporated BrdU in the precursor pool to become incorporated. The coverslips were then rinsed in Dulbecco's phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4°C.

The following steps were all executed at room temperature unless stated otherwise. Prior to staining the coverslips were washed in washing buffer (0.3 mM NaCl and 0.02% Tween 20 in a buffer consisting of 0.05 mM Tris–HCl at pH 7.6) for 10 min, followed an incubation for 15 min in blocking buffer (1% bovine serum albumin and 0.5% Tween 20 dissolved in PBS) to block nonspecific binding of the secondary antibodies. The coverslips were then rinsed in phosphate-buffered saline (PBS) and fixed in 100% methanol at 4°C for 60 min. The fixed material was stored in 70% ethanol at 4°C.

The antibodies and the detection systems used for staining are summarized in Table 2. The specificity of the cyclin A polyclonal provided by G. Draetta has previously been documented [12]. Denaturation of DNA, in order to expose incorporated BrdU, was done through a 45s incubation in a solution containing 15% methanol, 15% acetone, and 0.07 M sodium hydroxide at 4°C.

The antibodies used were specific to cyclin A, but not BrdU, to the fraction of cells containing cyclin E and BrdU, but not cyclin A, it is possible to decide whether cyclin A begins its accumulation before or after DNA replication is begun.

A triple immunochemical staining protocol was used in order to study the exact timing of cyclin A accumulation and DNA replication, allowing us to simultaneously stain for cyclin A, cyclin E, bromodeoxyuridine (BrdU), and DAPI. The triple staining makes it possible to identify cells at the G1/S border on the basis of their cyclin E content and to then further study the levels of cyclin A and BrdU in each of those cells (see Fig. 1). A novel method using computerized evaluation of nuclear staining was developed to objectively classify cells into positive and negative for cyclin A and/or BrdU. Classifying cells at the G1/S border with respect to content of cyclin A and BrdU is particularly difficult since these cells exhibit low amounts of cyclin A and BrdU, compared to cells in mid or late S phase. However, the combination of computerized evaluation and the triple staining made it possible to study cyclin A accumulation in relation to the G1/S transition in asynchronously growing unperturbed cell cultures.

Our results clearly show that cyclin A starts to accumulate in the nucleus precisely at, or very close to,
All stained coverslips were accompanied by a negative control consisting of an identical coverslip with respect to cell type, fixation, and storage time. The negative controls went through the same staining procedure, only excluding the primary antibodies and using blocking buffer instead. All negative controls exhibited a very low level of nonspecific nuclear staining compared to the stained coverslips.

Three control coverslips received only two of the three primary antibodies but were otherwise stained as described above. All three coverslips exhibited signal levels comparable to those of the negative control in the stain whose primary had been excluded. This rules out any unwanted cross-reactions between the secondary and primary antibodies, as well as any defects in the light excitation and emission filters used.

Images of the cells were obtained using a Delta Vision system, produced by Applied Precision Inc. ( Issaquah, WA ). The system consists of a mercury lamp with a fiber optic illumination system, conventional microscope optics, selective filters for excitation and emission, a Zeiss Plan-Neofluar 40×/NA 1.30 oil immersion lens, and a cooled CCD camera ( Photometrics Ltd., Tucson, AZ ). A binning factor of three was used, which means the measured light intensities from nine pixels were integrated to one pixel, bringing the final resolution of the images from 0.17 to 0.5 μm. The binning was used to increased the light sensitivity and thereby shorten the exposure times. The binning also minimized variations in detected signal caused by variations in focus [ 24 ]. Fields containing 1 to 10 cells were chosen at random and 100 images were acquired from every stained coverslip. Every microscopy field was digitally photographed once with each of the filter sets for detection of DAPI, FITC, Cy-3, and Cy-5, resulting in 4 images from each field. The exposure times were 0.2, 2.0, 0.5, and 20 s, respectively. Twenty images were acquired from each coverslip of the accompanying negative controls. A linear relationship between fluorophore concentration and emitted light can be expected [ 25 ]. The response of the camera is also linear [ 24 ]. The intensity of the detected signal is therefore expected to be directly proportional to the amount of fluorophore present in the specimen.

Individual cell nuclei were found by setting an intensity threshold in the detected fluorescence signal of the DAPI channel. The same threshold was used in all images of cells from the same cell line. All connected pixels above the threshold were considered as belonging to the same cell nucleus and the individual cell nuclei were assigned unique labels. The average fluorescence signal per cell nucleus was directly proportional to the amount of fluorophore present in the specimen. The intensity of the detected signal is therefore expected to be directly proportional to the amount of fluorophore present in the specimen.

### TABLE 1

<table>
<thead>
<tr>
<th>Culture</th>
<th>Origin</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDF p3</td>
<td>Explanted HDF cells from normal adult skin</td>
<td>90% DMEM + 10% NCS</td>
</tr>
<tr>
<td>HDF p19 and p22</td>
<td>HDF cells from circumcisions of newborn babies</td>
<td>45% MEM + 45% F12 + 10% FCS</td>
</tr>
<tr>
<td>Osteoblasts p3</td>
<td>Osteoblasts from explanted head of femur</td>
<td>Alpha-MEM + 10% FCS + 2 mM L-glutamine</td>
</tr>
<tr>
<td>RPE 26 p5</td>
<td>Retinal pigment epithelial cells</td>
<td>90% DMEM + 10% NCS</td>
</tr>
<tr>
<td>WI-38 p27</td>
<td>HDF from fetal lung</td>
<td>90% DMEM + 10% NCS</td>
</tr>
<tr>
<td>3597</td>
<td>Malignant melanoma</td>
<td>90% MEM + 10% FCS</td>
</tr>
<tr>
<td>578T</td>
<td>Ductal breast carcinoma</td>
<td>45% MEM + 45% F12 + 10% FCS</td>
</tr>
<tr>
<td>AA</td>
<td>Malignant melanoma</td>
<td>90% MEM + 10% FCS</td>
</tr>
<tr>
<td>HT29</td>
<td>Adenocarcinoma of the colon</td>
<td>90% MEM + 10% FCS</td>
</tr>
<tr>
<td>MDA231</td>
<td>Cells in pleural effusion from patient with breast carcinoma</td>
<td>45% MEM + 45% F12 + 10% FCS</td>
</tr>
<tr>
<td>SVpgC2a</td>
<td>Experimentally produced oral epithelial cancer</td>
<td>“EMA,” a special serum-free medium</td>
</tr>
<tr>
<td>SK-MEL 28</td>
<td>Malignant melanoma</td>
<td>90% MEM + 10% FCS</td>
</tr>
<tr>
<td>TD</td>
<td>Malignant melanoma</td>
<td>90% MEM + 10% FCS</td>
</tr>
<tr>
<td>U1690</td>
<td>Small cell carcinoma of the lung</td>
<td>90% MEM + 10% FCS</td>
</tr>
</tbody>
</table>

Note. Abbreviations used: MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; NCS, newborn calf serum; F12, Ham's F12 medium; HDF, human diploid fibroblasts; p, the number of passages the strain has passed through since established.

### TABLE 2

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Detection system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin E</td>
<td>Mouse monoclonal HE12 from Santa Cruz Biotechnology ( Santa Cruz, CA ), 0.4 μg/ml.</td>
<td>Biotin-conjugated donkey anti-mouse IgG from Jackson ImmunoResearch Labs Inc. ( PA ), 13 μg/ml.</td>
<td>Cy-5-conjugated streptavidin from Amersham Life Sciences ( Buckinghamshire, England ), 10 μg/ml.</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>Rabbit polyclonal generously provided by G. Draetta, diluted 1:1000.</td>
<td>Cy-3-conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch Labs Inc. ( PA ), 1.5 μg/ml.</td>
<td></td>
</tr>
<tr>
<td>BrdU</td>
<td>Sheep polyclonal from Biodesign Int. ( Kennebunk, ME ), 0.8 μg/ml.</td>
<td>FITC-conjugated donkey anti-sheep IgG from Jackson ImmunoResearch Labs Inc. ( PA ), 3 μg/ml.</td>
<td></td>
</tr>
</tbody>
</table>
and nuclei with condensed chromatin (i.e., cells in M) were manually removed from further analysis. The implementations of the methods described were written in C and integrated in IMP (IMage Processing), which is a general image analysis software for five-dimensional (x,y,z,t,b) image data developed at the Centre for Image Analysis at Uppsala University.

Background fluorescence due to autofluorescence and nonspecific binding of the secondary antibodies and streptavidin was removed by background subtraction. The average nuclear background intensity was calculated from the images of the control coverslips. This background intensity was subtracted from the staining intensities measured in the stained nuclei. Any background staining caused by nonspecific binding of the primary antibody remains, but was minimized through extensive washing between the incubations with the primary and secondary antibodies as described above. The background staining does not, however, influence the analysis presented in this article, since an automatic algorithm was used to classify cells into positive and negative with respect to content of cyclin A, cyclin E, and BrdU. The algorithm uses only the distribution of the staining intensities in the population, not the actual values of the staining intensities, to calculate a threshold.

The algorithm to calculate thresholds was developed in order to objectively determine whether the cell nuclei were positive or negative with respect to the three fluorophores used. The staining intensities varied both between different cell lines and different fluorophores, and therefore individual thresholds were needed for each single cell line and fluorophore. In the process a histogram of the nuclear staining intensities was created for each of the 14 cell lines and three fluorophores (FITC, Cy-3, and Cy-5), resulting in 45 histograms (see example in Fig. 2). The staining intensity of the negative cells can be expected to be normally distributed. The threshold for the negative population should be set at the upper end of the normal distribution of negative cells. This point is located where the change of trend of the bending of the histogram shows a maximum.

The change of trend is represented as the discrete second derivative of the bending of the histogram.

A smoothed approximation of the bending of the histogram was created by least square fitting of a second degree polynomial to a small part, or "window view," of the histogram. The "window" has a specific size and slides along the x axis of the histogram. The polynomial was fitted at each position of the window along the x axis of the histogram, resulting in a smooth curve. The discrete second derivative of this curve shows the change of trend in the histogram. Maxima and minima in the discrete second derivative appear at x values where the trend of the bending of the histogram changes rapidly. The global minima of the discrete second derivative is the sharpest peak of the population, representing the center of the distribution of negative cells, and the largest maximum to the right of the minima marks the end of the population of negative cells. This is the point where the threshold is set (see Fig. 2).

The resulting threshold is dependent on two factors: window size and bin size. The window size is the width of the window that is used when fitting the second degree polynomial to the histogram. A large window will smooth the histogram too much, shifting the threshold to the right. A very small window does not smooth the histogram enough. The bin size is the width of the intervals used when creating histograms from the image data. A very large as well as a very small bin size will distort the curvature of the histogram. Stable thresholds are essential for the accuracy of the final analysis. Therefore, a number of different window and bin sizes were tested for stability. This was done by plotting the value of the calculated threshold against the corresponding bin size for a number of different window sizes. The plots showed in what range of bin and window sizes the thresholds were the most stable. Stable thresholds for all histograms are found when a bin size of one and a window size of 20 intensity units is used. The same bin and window sizes were used for all cell lines and stains, and all thresholds were calculated automatically.
Examples of the resulting thresholds for cyclin A and BrdU are shown as solid lines in Figs. 5 and 7.

**RESULTS**

Cyclin A has been shown to regulate DNA replication [10–14]. However, the temporal relationship between cyclin A accumulation and the onset of DNA replication is still unclear. Therefore, cyclin A accumulation in relation to DNA replication was studied in normal and transformed cells, using a triple immunofluorescence staining for cyclin A, cyclin E, and BrdU. The triple staining made it possible to discern what phase of the cell cycle that every single cell was in on a functional basis. Table 3 shows how many cells were in S and G2 in each of the cell cultures. The cells in G1 and S phase can even be divided into cells being in the early or late part of the phase on the basis of cyclin E content, since cyclin E appears only late in G1 and disappears again in early S.

**Cyclin A Accumulation Begins at the G1/S Transition**

The different possible patterns of cyclin A accumulation are depicted in Fig. 1. Cyclin A begins its accumulation at the very start of S phase in situation A in the figure. If this is the true scenario, then the number of cells positive for cyclin A should be expected to be equal to the number of cells positive for BrdU, among the cells positive for cyclin E (i.e., around the G1/S transition). Figures 3 and 4 were drawn to visualize the correlation between cyclin A and BrdU content at

**TABLE 3**

Data Summary

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Total cells</th>
<th>Cells in G0/1 (%)</th>
<th>Cells in S (%)</th>
<th>Cells in G2 (%)</th>
<th>Cells in late G1 or early S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A+/BU−</td>
<td>BU+</td>
<td>A+/BU−</td>
<td>E+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cells positive</td>
<td>cells positive</td>
<td>cells positive</td>
<td>cells positive/negative</td>
</tr>
<tr>
<td>HDFp3</td>
<td>420</td>
<td>189 (45)</td>
<td>185 (44)</td>
<td>46 (11)</td>
<td>55 0 (0) 4 (1)</td>
</tr>
<tr>
<td>HDFp19</td>
<td>435</td>
<td>339 (78)</td>
<td>87 (20)</td>
<td>9 (2)</td>
<td>68 1 (0) 0 (0)</td>
</tr>
<tr>
<td>HDFp22</td>
<td>363</td>
<td>288 (79)</td>
<td>74 (20)</td>
<td>1 (0)</td>
<td>24 0 (0) 0 (0)</td>
</tr>
<tr>
<td>RPE26</td>
<td>384</td>
<td>227 (59)</td>
<td>62 (16)</td>
<td>95 (25)</td>
<td>41 4 (1) 0 (0)</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>331</td>
<td>315 (95)</td>
<td>14 (4)</td>
<td>2 (1)</td>
<td>15 1 (0) 0 (0)</td>
</tr>
<tr>
<td>WI38</td>
<td>758</td>
<td>592 (78)</td>
<td>134 (18)</td>
<td>32 (4)</td>
<td>44 1 (0) 0 (0)</td>
</tr>
<tr>
<td>3597</td>
<td>486</td>
<td>275 (57)</td>
<td>171 (35)</td>
<td>40 (8)</td>
<td>14 0 (0) 1 (0)</td>
</tr>
<tr>
<td>578T</td>
<td>372</td>
<td>85 (23)</td>
<td>261 (70)</td>
<td>26 (7)</td>
<td>254 9 (2) 3 (1)</td>
</tr>
<tr>
<td>AA</td>
<td>335</td>
<td>99 (30)</td>
<td>173 (52)</td>
<td>63 (19)</td>
<td>18 2 (1) 0 (0)</td>
</tr>
<tr>
<td>HT29</td>
<td>368</td>
<td>137 (37)</td>
<td>214 (58)</td>
<td>17 (5)</td>
<td>140 1 (0) 6 (2)</td>
</tr>
<tr>
<td>MDA231</td>
<td>422</td>
<td>275 (65)</td>
<td>83 (20)</td>
<td>64 (15)</td>
<td>35 16 (4) 0 (0)</td>
</tr>
<tr>
<td>SVpgC2a</td>
<td>536</td>
<td>178 (33)</td>
<td>222 (41)</td>
<td>136 (25)</td>
<td>66 17 (3) 4 (1)</td>
</tr>
<tr>
<td>SKMEL28</td>
<td>511</td>
<td>379 (74)</td>
<td>83 (16)</td>
<td>49 (10)</td>
<td>122 6 (1) 17 (3)</td>
</tr>
<tr>
<td>TD</td>
<td>267</td>
<td>68 (25)</td>
<td>150 (56)</td>
<td>49 (18)</td>
<td>20 5 (2) 1 (0)</td>
</tr>
<tr>
<td>U1690</td>
<td>249</td>
<td>106 (43)</td>
<td>131 (53)</td>
<td>12 (5)</td>
<td>81 4 (2) 3 (1)</td>
</tr>
<tr>
<td>Strains:</td>
<td>2691</td>
<td>1950 (72)</td>
<td>556 (20)</td>
<td>185 (7)</td>
<td>247 7 (0) 4 (0)</td>
</tr>
<tr>
<td>Lines:</td>
<td>3546</td>
<td>1602 (43)</td>
<td>1488 (45)</td>
<td>456 (12)</td>
<td>750 60 (2) 35 (1)</td>
</tr>
<tr>
<td>All:</td>
<td>6237</td>
<td>3552 (55)</td>
<td>2044 (35)</td>
<td>641 (10)</td>
<td>997 67 (1) 39 (1)</td>
</tr>
</tbody>
</table>

Note. Figures in parentheses are the fractions of the investigated cells in percentages. BU ± cells positive/negative for bromodeoxyuridine. Incorporation of bromodeoxyuridine can by definition take place only during S phase. A+, cells positive/negative for cyclin A. Cyclin A is present in the nucleus during S and G2 in normal cells. E+, cells positive for cyclin E. Cyclin E is present in the nucleus only during late G1 and early S in normal cells. Fractions are written in parentheses. The top six cultures are normal strains; the bottom nine are transformed cell lines. Senescent cells and cells in G0 could not be distinguished from the true G1 cells and have thus been counted as such. Cells in M were excluded prior to the analysis through visual inspection of the images. Note the very low number of cells in the two rightmost columns.

FIG. 3. The number of cyclin-A-positive cells plotted against the number of BrdU-positive cells. Every dot represents one cell culture. All measured cells have been included, irrespective of position in the cell cycle. Cultures with short G2 phases have a 1:1 relationship between the number of cells positive for cyclin A and the number of cells positive for bromodeoxyuridine, which are by definition cells in S phase. Cultures with longer G2 have more cyclin-A-positive cells than they have cells in S phase.
cycin A probably starts to accumulate at the same time as DNA synthesis begins, as in scenario A in Fig. 1. Table 3 includes these two “unlabeled” fractions in the columns labeled “A−/E+/BU+” and “A+/E+/BU−.” In the normal strains the two fractions studied consisted of only 11 cells of 2691 studied (556 in S phase), and those 11 cells were evenly distributed between the two fractions.

To allow a calculation of confidence intervals in a suitable unit, we assumed that the S phase in the normal cell strains was approximately 7 h long [26]. Since the total number of S phase cells was 556 (Table 3), each cell proceeding through the cell cycle represents approximately 7 h/556 cells = 0.9 min/cell. Using this figure and the number of cells in the fractions “A−/E+/BU+” and “A+/E+/BU−” to time cycin A accumulation gave that cycin A begins its accumulation less than 3 min from when BrdU incorporation begins, with the 95% confidence interval being less than 10 min in normal cells. Since our analysis is influenced by the calculated thresholds dividing the cell populations into positive and negative with respect to each of the three stains (example in Fig. 2), we performed the same calculation using thresholds shifted up and down by 10% to test the effects on the result. With the altered thresholds cycin A still seemed to begin its accumulation at approximately the same time as BrdU incorporation began, with the 95% confidence interval being less than 15 min. We therefore conclude that cycin A accumulation starts within 15 min from the onset of the DNA replication in normal cells, i.e., the model depicted in Fig. 1A is closer to the truth than the models in Figs. 1B and 1C.

Nine transformed cell lines were also analyzed as described above. They were shown to exhibit a somewhat higher variability with regard to their A−/E+/BU+ and A+/E+/BU− fractions. However, a closer investigation of the cells in those fractions showed at least 3 cells in 578T, 8 in MDA 231, 10 in SVpgC2a, as well as a few cells in some of the other lines were actually G2 cells with aberrantly expressed cycin E, judged from their high content of cycin A and their DNA content typical of G2 cells (data not shown). If these cells were to be subtracted from the data in Table 3 the A+/E+/BU− and A−/E+/BU+ fractions would be similar to those found in normal cells. These results show that cycin A does not start to accumulate during G1 without simultaneous initiation of the DNA replication, nor does DNA replication start before the accumulation of cycin A has begun, in any of the tumor cell lines investigated. The temporal deregulation of cycin E will be further analyzed in future studies.

Nuclear Cycin A Content during G91

Earlier studies have found nuclear cycin A at low levels in G1 cells [19, 20]. Cells negative for cycin A

FIG. 4. The number of cycin-A-positive cells plotted against the number of BrdU-positive cells. Every dot represents one cell culture. Only cells positive for cycin E have been included, i.e., cells in late G1 or early S phase. Note the different scaling in Figs. 4 and 5. All cultures exhibit close to a 1:1 relationship between the number of cells positive for cycin A and the number of cells positive for BrdU, i.e., in S phase. The close correlation between positive nuclear staining for cycin A and BrdU indicates that cycin A appears in the nucleus very close to when DNA synthesis is begun.

the G1 to S transition. Figure 3 shows the number of cells positive for cycin A and BrdU in each of the 14 cultures investigated. The variable length of G2 in the different cultures influences the result, since G2 cells are positive for cycin A but negative for BrdU. This influence was removed when only cells positive for cycin E (i.e., cells in late G1 or early S) were included (Fig. 4). For instance, the rightmost dot in Fig. 3 represents the cell line 578T. It exhibited 261 cells positive for BrdU, but 26 more cells were positive for cycin A. This means these 26 cells were either cells in G2 or cells in G1 expressing cycin A. Also in Fig. 4 578T is represented by the rightmost dot. Here mainly cells in G1 or S are included, and the discrepancy between the number of cells containing BrdU and the number of cells with nuclear cycin A almost disappears. A very strong correlation between the number of cells positive for cycin A and the number of cells positive for BrdU was observed in all of the cell cultures studied when the G2 cells were excluded. This finding is consistent with cycin A accumulation in the nucleus beginning at the same time as DNA synthesis starts, as in scenario A in Fig. 1.

The number of cells that contain only cycin A, but not BrdU, or that contain only BrdU, but not cycin A, was studied in the subpopulation of cycin-E-positive cells (cells in late G1 or early S). If one of these two “unlabeled” fractions would be considerably larger than the other, it would imply that accumulation of cycin A and the onset of BrdU incorporation would not be simultaneous, as in scenarios B and C in Fig. 1. If the two “unlabeled” fractions were equally small, then

FIG. 3. The number of cells containing BrdU and the number of cells expressing cycin A in all cultures investigated. The variable length of G2 in the different cultures influences the result, since G2 cells are positive for cycin A and BrdU in each of the 14 cultures investigated. The temporal deregulation of cycin E will be further analyzed in future studies.
and BrdU, i.e., cells in G\(_1\) or G\(_0\), were therefore investigated with respect to their cyclin A content. They all exhibited uniformly low staining for cyclin A, slightly higher than the average cyclin A stain in the negative controls (cells that never were exposed to the primary antibody), but the difference was not statistically significant. The staining was in the order of 1/60th of the average nuclear cyclin A staining in G\(_2\) cells (data not shown). This finding can be explained by nonspecific binding of the primary antibody, and we therefore conclude that we could not detect any cyclin A in nuclei belonging to cells in G\(_1\) or G\(_0\).

Cyclin A Accumulates throughout S Phase

In order to visualize the pattern of cyclin A accumulation during S phase, Fig. 5 was drawn. It was possible to divide the cells positive for BrdU into five groups ranging from early to late S phase on the basis of integrated DAPI staining intensity, since cells in early S have a lower DNA content than cells in late S. Figure 5 shows that cyclin A is perhaps accumulated a little faster initially, but otherwise the accumulation occurs in an almost linear fashion with respect to the synthesis of new DNA. Transformed cells exhibit a high degree of intercellular variation in DNA content, which made a similar analysis in transformed cells impossible.

The content of cyclin A in S was instead compared with the content of cyclin A in G\(_2\) to investigate whether the relative expression of cyclin A is altered in transformed cells. Figure 6 consists of a scatterplot from two of the cultures, showing how cyclin A content varies with DNA content. In order to investigate how much cyclin A increases from S to G\(_2\), a quota between the average cyclin A content in cells containing cyclin A but not BrdU, and the average content in cells positive for BrdU was calculated. The transformed cell lines exhibited a G\(_2\)/S quota of 1.8, with an SD of 0.6 and the normal cell strains exhibited a quota of 1.7, with an SD of 0.2. Hence we can detect no statistically significant difference between the expression pattern with regard to the levels of cyclin A in S compared to G\(_2\) in the investigated normal and transformed cell lines.

Transformed Cells Exhibit a High Intercellular Variation in BrdU Uptake

In Fig. 7 the amount of DNA synthesized during 5 min, measured as BrdU concentration, has been plotted against DNA content. Cells that incorporate BrdU are by definition cells in S-phase, since BrdU incorporation only occurs during ongoing DNA synthesis. In all normal cell cultures the cells in S exhibited uniformly high concentrations of BrdU, while the G\(_1\) and G\(_2\) populations were entirely BrdU negative. In most of the tumor lines there were many more cells in S phase with intermediate and low amounts of BrdU, especially in SVpgC2a, 3597, 578T, and MDA231. This could indicate a large intercellular variability in the rate of DNA replication in the tumor cell populations compared to the normal cell populations. However, BrdU incorporation might be influenced by several other things than the rate of replication, such as the size of endogenous pools of nucleotides and the function of several enzymes.

DISCUSSION

In the present study we have analyzed in detail the precise temporal relationship between cyclin A accumulation and DNA replication. The data were obtained by a triple immunofluorescence staining procedure applied to unsynchronized cell populations. The immunofluorescence emitted from each fluorophore was measured in each individual cell nucleus. Cells at the G\(_2\)/S border were analyzed in detail. They were identified based on their cyclin E content and analyzed further with respect to their BrdU and cyclin A content. The accuracy of the analysis is dependent on a reliable classification of the cell nuclei into positive and negative with respect to the different fluorophores. This is
important when analyzing cells around the G1/S border, since these cells exhibit low levels of cyclin A and BrdU. Therefore we developed a method to classify investigated cells objectively, based on objectively calculated thresholds (see Fig. 2).

The data presented in this paper clearly show that nuclear cyclin A staining is strictly limited to the S and G2 fractions of interphase cells in both normal and transformed cell cultures. The detailed analysis performed on the subpopulation of cells that was positive for cyclin E, i.e., cells in late G1 and early S, clearly show that very few of these cells were either positive only for cyclin A or positive only for BrdU (<1% of all cells). Thus cyclin A accumulation did not precede initiation of DNA replication, nor did DNA replication precede cyclin A accumulation. A statistical analysis shows that the rise in the nuclear levels of cyclin A and BrdU takes place no more than 10–15 min apart.

![Diagram](image1.png)

**FIG. 6.** Cyclin A concentration on the abscissa, DNA content on the ordinate, arbitrary units. Red dots are nuclei classified as BrdU negative, green as BrdU positive. WI38 is an example of a normal strain and 578T is an example of a transformed cell line. The amounts of DNA roughly corresponding to G0, and G1 have been marked on the ordinate. The horizontal line marks the cyclin A threshold. The group of cells without cyclin A and BrdU and a low DNA content consists of cells in G0 or G1. BrdU-positive nuclei do by definition belong to cells in S phase. Cells negative for BrdU and with a high nuclear cyclin A content are cells in G2. There is also a fairly big group of cells with approximately twice the DNA content of the G1 cells, but with a low cyclin A and BrdU content. These cells are tetraploid cells in G1.

![Diagram](image2.png)

**FIG. 7.** BrdU concentration is shown on the abscissa and DNA content on the ordinate, both in arbitrary units. BrdU concentration is under these experimental conditions probably related to the speed of the DNA replication going on in the nucleus. Red dots represent nuclei classified as cyclin A negative, green represents cyclin-A-positive cells. WI38 is an example of a normal strain and 578T is an example of a transformed cell line. The amounts of DNA roughly corresponding to G0, and G2 have been marked on the ordinate. The horizontal line marks the BrdU threshold.
Previous studies on the accumulation of cyclin A in relation to the $G_1/S$ transition have reported conflicting results. Some studies have indicated that cyclin A, as detected by Western blots, starts to accumulate in late $G_1$, before the onset of DNA synthesis. These studies were based on experiments using synchronized cell cultures [19, 20]. The discrepancy with our results are likely to be of methodological nature and due to the fact that synchronized cell populations never are perfectly synchronized, resulting in contamination with early $S$ phase cells in the $G_1$ cell fraction. Other studies have come to the opposite conclusion, namely, that cyclin A starts to accumulate only after DNA replication has begun. The results are based on traditional visual evaluation of immunostaining experiments [21]. However, visual analysis introduces the problems associated with deciding whether the nuclei are stained strongly enough to be classified as positive or negative. Evaluation of immunostaining experiments requires rigorous determination of thresholds discriminating positive cells from negative cells, as discussed above.

How cyclin A is mechanistically involved in DNA replication is still unknown. Data showing colocalization between cyclin A and replication foci indicate that cyclin A is associated with the replication machinery [17, 18]. Biochemical studies on the function of cyclin A have shown that cyclin A is required for the initiation of DNA replication. It has also been suggested that CDK activity is involved in the two-step regulation process that restricts DNA replication to taking place once and only once during each cell cycle [15]. During late $M$ and early $G_1$, proteins are assembled onto the chromatin at sites where DNA replication is initiated to form pre-RCs [27]. This process, called licensing, is required to render the DNA competent for replication [27–30]. Many of the components of the pre-RC, like Cdc6, Cdc45, and the MCM proteins, are phosphorylated in a cell cycle-specific manner [30–32]. Cdc6 has been shown to be phosphorylated only by cyclin A–CDK2 activity, and not by cyclin E–CDK2. Cdc6 is rapidly exported from the nucleus in response to this phosphorylation and has therefore been proposed be involved in preventing rereplication [16]. Even though it has not been established which CDK activity is responsible for the phosphorylation of the other proteins in the pre-RCs, cyclin-A-dependent kinase activity is a likely candidate. Our finding that the onset of cyclin A accumulation is tightly coupled to the start of DNA synthesis gives further support to the idea that cyclin A–CDK2 phosphorylation of the pre-RCs is essential for initiation of the DNA replication.

Once replication has been initiated, the pre-RC is disrupted since Cdc6 and the MCM proteins are detached from the chromatin and prevented by CDK activity from rebinding to the chromatin. During late $M$ phase and early $G_1$, when CDK activity is low again, pre-RCs are allowed to reform. The finding that cyclin A accumulates continuously in a pattern that is linearly related to the synthesis of new DNA suggests a model in which cyclin A–CDK remains associated with replicated DNA or chromatin at critical sites, possibly to prevent rereplication by keeping Cdc6 and the MCMs away from the RCs. Another plausible function for cyclin-A-dependent kinase activity during $S$ phase is that cyclin A–CDK2 might play a permissive role through regulation of the structural changes of chromatin which are involved in replication.

Overexpression of cyclin A has been found in some tumors [21, 33, 34]. This could be partly explained by an increased proliferative activity in the tumor cell population, as indicated by Ki-67 staining as a proliferation marker [35–37]. Some reports have indicated that transformed cells might have a temporarily aberrant cyclin A expression pattern in the sense that cyclin A is also expressed in $G_1$ [38]. However, with our methodological approach we can show that the presence of cyclin A in the cell nucleus is limited to $S$ and $G_2$ phase in both normal and tumor cells. From a methodological point of view this means that nuclear cyclin A is an excellent marker for interphase cells in $S$ and $G_2$ for methodological purposes in both normal and transformed cell populations.

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