Molecular Biology of the Cell
Vol. 9, 795–807, April 1998

Reprogramming the Cell Cycle for Endoreduplication in Rodent Trophoblast Cells

Alasdair MacAuley*, James C. Cross,† and Zena Werb*‡

*Department of Anatomy, University of California, San Francisco, California 94143-0750; and †Samuel Lunenfeld Research Institute Mount Sinai Hospital and Departments of Obstetrics and Gynecology, and Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5G 1X5, Canada

Submitted October 31, 1997; Accepted January 14, 1998
Monitoring Editor: John C. Gerhart

Differentiation of trophoblast giant cells in the rodent placenta is accompanied by exit from the mitotic cell cycle and onset of endoreduplication. Commitment to giant cell differentiation is under developmental control, involving down-regulation of *Id1* and *Id2*, concomitant with up-regulation of the basic helix-loop-helix factor *Hxt* and acquisition of increased adhesiveness. Endoreduplication disrupts the alternation of DNA synthesis and mitosis that maintains euploid DNA content during proliferation. To determine how the mammalian endocycle is regulated, we examined the expression of the cyclins and cyclin-dependent kinases during the transition from replication to endoreduplication in the Rcho-1 rat choriocarcinoma cell line. We cultured these cells under conditions that gave relatively synchronous endoreduplication. This allowed us to study the events that occur during the transition from the mitotic cycle to the first endocycle. With giant cell differentiation, the cells switched cyclin D isoform expression from D3 to D1 and altered several checkpoint functions, acquiring a relative insensitivity to DNA-damaging agents and a coincident serum independence. The initiation of S phase during endocycles appeared to involve cycles of synthesis of cyclins E and A, and termination of S was associated with abrupt loss of cyclin A and E. Both cyclins were absent from gap phase cells, suggesting that their degradation may be necessary to allow reinitiation of the endocycle. The arrest of the mitotic cycle at the onset of endoreduplication was associated with a failure to assemble cyclin B/p34^{cdk1} complexes during the first endocycle. In subsequent endocycles, cyclin B expression was suppressed. Together these data suggest several points at which cell cycle regulation could be targeted to shift cells from a mitotic to an endoreduplicative cycle.

INTRODUCTION

Progression of the cell cycle requires precise replication of genomic DNA once during each cycle, and its subsequent accurate segregation during mitosis to maintain a euploid DNA content. Aberrations that disrupt this process can lead to aneuploidy and may predispose the cells to oncogenic transformation (Pathak *et al.*, 1994). A variety of models have been proposed to explain the necessary interdependence of mitosis and DNA synthesis, but the molecular mechanisms that regulate the fundamental process of replication and division are only just beginning to emerge. Replication origins in yeast can be primed only during early G_1, when cyclin expression and cyclin-dependent kinase (cdk) activity is low (Cocker *et al.*, 1996). Once the cell is committed to cell cycle by passage through Start, the resultant expression of the cyclins that regulate progression through the remainder of the cell cycle prevents assembly of new replication origins until their degradation during mitosis, limiting the cell to one round of DNA replication per cycle (Basco *et al.*, 1995; Piatti *et al.*, 1996). The destruction of cyclins precipitated by passage through mitosis seems to be the event that separates one cycle from the

‡ Corresponding author: Department of Anatomy, P.O. Box 0750, Third and Parnassus Aves., San Francisco, CA 94143-0750. E-mail: zena@itsa.ucsf.edu.

next and enforces the strict alternation of S and M phases (Piatti *et al.*, 1996).

Normal cell cycle checkpoints are uncoupled in some cell types during development. For example, endoreduplication is a naturally occurring disruption of the mitotic cycle in which rounds of DNA synthesis occur in the absence of mitosis, resulting in polyploid cells. Endoreduplication occurs in a wide variety of embryonic and adult cell types in both animals and plants, including rodent trophoblast (Zybina, 1970; Malinowski and Maszewski, 1994; Hartman and Southern, 1995; Datta *et al.*, 1996; Lilly and Spradling, 1996; Zhang *et al.*, 1996). Both mural trophectoderm and derivatives of polar trophectoderm in rodents are transformed into so-called trophoblast giant cells that have DNA contents up to 1000 times the haploid content as a result of endoreduplication (Zybina, 1970; Zybina and Grishchenko, 1970; Barlow and Sherman, 1972; Hoffman and Wooding, 1993). The mechanism of commitment to giant cell differentiation is poorly characterized, although down-regulation of *Id1* and *Id2*, and up-regulation of the bHLH factor *Hxt* appear to play important roles (Cross *et al.*, 1995). Overexpression of *Id* keeps the trophoblast cells as stem cells, whereas overexpression of *Hxt* drives differentiation to giant cells. Concomitant with this commitment the cells increase their adhesion, a necessary aspect of the implantation process that ensues in vivo (Cross *et al.*, 1994, 1995; Rinkenberger *et al.*, 1997). How these changes are translated into endoreduplication and differentiation remains unclear, because the molecular pathways that produce the phenotypes are not understood. Certainly, differentiation and endoreduplication are very closely linked in trophoblast. Although it has proven to be possible to separate the two events experimentally (Gardner and Davies, 1993), the onset of differentiation is linked to the cells becoming postmitotic, a necessary condition for endoreduplication.

Endoreduplication has been best studied in *Drosophila melanogaster*. It proceeds in cycles with defined S phases separated by gap (G) phases, suggesting that progression through the endocycle is as carefully regulated as progression through the mitotic cycle. Cyclin E appears to play a central role in regulating the initiation of S phase during the endocycle, as it does in the mitotic cycle (Knoblich *et al.*, 1994; Sauer *et al.*, 1995; Lilly and Spradling, 1996), and, in the absence of cyclin A and B, mitosis is not initiated. It is not clear how the transition from the mitotic cell cycle to the endocycle is regulated even in *Drosophila*. For example, it is not clear whether the absence of cyclins A and B is sufficient to uncouple the reinitiation of DNA synthesis from prior passage through M phase, or whether an additional change in regulatory mechanisms is required during the transition from the mitotic cycle to the endocycle.

The control of endoreduplication has been poorly studied in other systems. One of the major hurdles to understanding trophoblast differentiation has been the paucity of material with which to study the early steps of differentiation. However, a rat choriocarcinoma-derived cell line, Rcho-1, is a good candidate in which to address trophoblast differentiation in vitro (Faria and Soares, 1991; Cross *et al.*, 1995). Under the appropriate conditions, Rcho-1 cells undergo a transition from proliferating to postmitotic cells that eventually express trophoblast giant cell-specific markers and also continue DNA synthesis (Shida *et al.*, 1993; Yamamoto *et al.*, 1994; Cross *et al.*, 1995; Hamlin and Soares, 1995; our current research). In this study we have used Rcho-1 cells to examine the regulation of trophoblast giant cell differentiation and the transition in cell cycle structure. We describe the first characterization of the regulation of a mammalian endocycle and propose a mechanism for the abrogation of mitosis and the reinitiation of rounds of DNA synthesis during endoreduplication.

MATERIALS AND METHODS

Culture of the Rcho-1 Cell Line

The Rcho-1 cell line was derived from a rat choriocarcinoma (Faria and Soares, 1991) and was obtained from M. Soares. The cells were maintained in NCTC-135 supplemented with 0.1 mg/ml sodium pyruvate, 2 mM glutamine, penicillin, streptomycin (NCTC-135+), and 20% fetal bovine serum (FBS; Hyclone, Logan, UT). To set up cultures for differentiation, the cells were grown to high density and then replated into the final dishes at high density (roughly a 1:1 transfer) and maintained for 2 d in the NCTC-135+ with 20% FBS. The cells were then treated with trypsin and washed twice with phosphate-buffered saline (PBS) to remove nonadherent cells (Cross *et al.*, 1995). The cells that remained were cultured in NCTC-135+ supplemented with 10% horse serum.

Scanning Fluorimetry

Rcho-1 stem cells, plated for 24 h, or differentiating giant cell cultures, maintained in NCTC+/20% FBS, were fixed in methanol and stained with bisbenzimide (Sigma Chemical, St. Louis, MO). Nuclear DNA content was estimated by fluorescence intensity on 50–100 cells for each population using an imaging system from Compix Inc. Imaging Systems (Cranberry Township, PA). Fluorescent intensity was compared with serum-starved, quiescent HeLa cells shown to be diploid by fluorescence-activated cell sorter analysis.

Measurement of DNA Synthesis by [^{3}H]Thymidine Incorporation

DNA synthesis was estimated by measuring the incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-insoluble material. [^{3}H]thymidine was added directly to the medium to a final concentration of 1 μCi/ml and the cells were incubated for another 4 h. The unincorporated [^{3}H]thymidine was extracted with three washes of 10% TCA and two washes of 95% ethanol, all at 4°C. The TCA-insoluble material was collected with 0.1 N NaOH and the [^{3}H]thymidine incorporation was measured by liquid scintillation spectrometry. The mimosine arrest was achieved by adding mimosine to the medium to 100 μM 3 d after removing the proliferating cells. The block was reversed by washing the cells once in preequilibrated

medium and then continuing the culturing in NCTC+ with 10% horse serum.

Northern Blot Analysis

Total RNA was isolated from proliferating or differentiating Rcho-1 cells using the acid-phenol reagent RNAzol B (Biotecx, Houston, TX). The RNA concentration was calculated from the absorption at 260 nm, and 10 μg from each sample were fractionated on a 1.2% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane, Duralon-UV (Stratagene, La Jolla, CA), and fixed in place by UV cross-linking. The membranes were then hybridized to probes derived from random-primed synthesis using the Rediprime kit and [α-^{32}P]dCTP (Amersham, Arlington Heights, IL). The hybridization was performed using the Quikhyb solution (Stratagene) following the manufacturer's protocol and washing in 2× SSC with 0.1% SDS at 60°C. The blot was then exposed to film (Kodak X-OMAT). The cDNA probes used were as follows: cyclin A1 and A2 were derived by polymerase chain reaction amplification using published sequence information (Sweeney *et al.*, 1996) and a d 7.5 embryonic mouse cDNA library (Stratagene) and cloned in pCRII (Invitrogen, San Diego, CA); mouse cyclin B1 and B2 were a kind gift from D. Wolgemuth (Chapman and Wolgemuth, 1992, 1993); mouse cyclins D1, D2, and D3 were a kind gift from C. Sherr (Matsushime *et al.*, 1991); mouse cyclin E was kindly supplied by J. DeLoia (Damjanov *et al.*, 1994); *Hxt* (Cross *et al.*, 1995); PL-I (Faria *et al.*, 1991; Faria and Soares, 1991); MMP-9 (Reponen *et al.*, 1994); α1 integrin was a gift from A. Sutherland (Sutherland *et al.*, 1993).

Immunoblot, Immunoprecipitation, and Immunokinase Assays

Protein-containing lysates were prepared from proliferating or differentiating Rcho-1 cells by washing the cells with PBS and then scraping them into a modified RIPA buffer that contained 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM β-glycerol phosphate, and 0.1 mM Na_3VO_4. The lysate was clarified by centrifugation at 20,000 × *g*, a sample was removed for determination of protein concentration, and the remainder was frozen at −80°C until use. Using the MicroBCA protein assay (Pierce Chemical, Rockford, IL), equal amounts of protein from each lysate were separated by SDS-PAGE using a 10% separating gel and were transferred to Immobilon-P (Millipore, Bedford, MA). The membrane was treated with PBS containing 0.05% Tween 20 and 10% nonfat milk powder and then it was incubated with the primary antibody indicated for 1 h at room temperature. Antibodies for cyclin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), for cyclin E from J. Roberts, and for cyclin B, p34^{cdk1}, and p33^{cdk2} from PharMingen (San Diego, CA). The membrane was washed with PBS containing 0.05% Tween 20, then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham), washed again, and the immunocomplexes were revealed using the enhanced chemiluminescence reagent (Amersham).

The immunoprecipitations were performed using the same primary antibodies and lysates that were used for the immunoblots. Approximately 1 μg of the primary antibody was added to the lysate and incubated for 1 h on ice, a rabbit anti-mouse IgG antibody was added (ICN, Costa Mesa, CA), and the immunoprecipitates were then collected on protein A-agarose. The pellets were washed three times in RIPA buffer, twice with a washing buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.5% Nonidet P-40), and then, for histone H1 kinase assays, resuspended in 20 μl of the washing buffer lacking detergent, containing 10 mCi of [γ-^{32}P]-ATP and 1 μg of histone H1 (Life Technologies, Gaithersburg, MD), and incubated at 30°C for 5 min. An equal volume of 2× concentrated SDS sample loading buffer was added and the samples were then fractionated on a 12.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue to reveal the histone H1, dried, and exposed to film (Kodak X-OMAT). Anticyclin immunoprecipitates were also subjected to immunoblotting and probed with antibodies against p34^{cdk1} or p33^{cdk2}.

Immunofluorescence

Differentiating Rcho-1 cells were fixed in Carnoy's solution (methanol:chloroform:glacial acetic acid = 6:3:1) for 15 min or incubated with 50 μM bromodeoxyuridine (BrdU) for 2 h and then BrdU-free medium for 5 min before fixation. The samples were incubated with PBS containing 0.1% Tween 20 (PBS.1T) and 10% goat serum and then exposed to one of the anti-cyclin A or anti-cyclin E antibodies (both from Santa Cruz Biotechnology) at 1/100 dilutions in PBS.1T, or the anti-BrdU (Becton Dickinson, San Jose, CA) at 1/1000, or a mixture of the anti-BrdU and one of the anti-cyclin antibodies for 1 h at room temperature. The samples were washed with PBS.1T and then incubated for 30 min with the appropriate secondary antibodies conjugated to either Texas red or fluorescein isothiocyanate to reveal the retained primary antibodies. The samples were washed again with PBS.1T and then counterstained with bisbenzimide.

RESULTS

Synchronized Differentiation of Rcho-1 Trophoblast Giant Cells

Rcho-1 cells differentiate in vitro into cells that express several markers characteristic of terminally differentiated trophoblast giant cells including P450 (Yamamoto *et al.*, 1994) and members of the placental lactogen/prolactin (PL) family (Shida *et al.*, 1993; Hamlin *et al.*, 1994). We found previously that an early event in the commitment to giant cell differentiation was a change in cell adhesiveness, so that brief trypsinization allowed removal of the proliferating stem cells and gave a highly purified population of cells that were committed to giant cell differentiation (Cross *et al.*, 1995). We were interested in whether these cells underwent synchronized cell differentiation. To study this, proliferating Rcho-1 stem cells were grown for 2 d to reach confluence and the population was then trypsinized to remove the stem cells, leaving only cells that had committed to the giant cell fate. The trypsin-resistant cells assumed a morphology typical of giant cells with enlarged nuclei that expanded with time (Figure 1A-C). Designating the day of trypsinization as d 0, the expression of *PL-I* mRNA was detectable as early as d 1, peaked at d 4, and declined thereafter (Figure 1D). The transient expression of *PL-I* in the differentiating Rcho-1 cells recapitulates its developmental expression in vivo, which is also transient (Faria *et al.*, 1991; Faria and Soares, 1991; Carney *et al.*, 1993; Hamlin *et al.*, 1994). *Hxt* (Cross *et al.*, 1995), gelatinase B/MMP-9 (Alexander *et al.*, 1996), and α1 integrin (Sutherland, *et al.*, 1993), genes that are expressed in trophoblast giant cells in vivo, were also developmentally regulated (Figure 1D).

Endoreduplication Accompanies Differentiation of Rcho-1 Cells

After selection, the trypsin-resistant Rcho-1 cells showed no discernible increase in cell number during

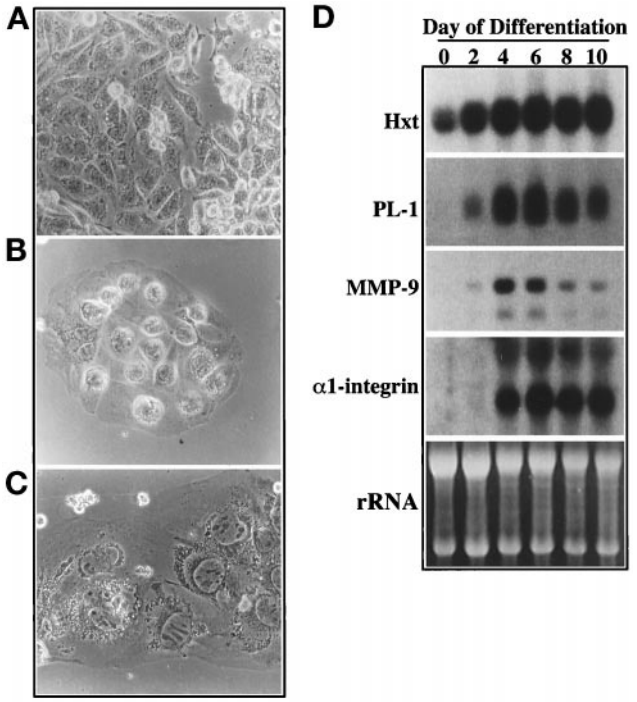

Figure 1. Morphology and gene expression during synchronized Rcho-1 cell differentiation. (A–C) Phase contrast photomicrographs of proliferating (A) and differentiating (d 0, B; d 4, C) Rcho-1 cells are shown. Photomicrographs were taken at the same magnification. (D) Expression of trophoblast marker genes during differentiation. Total RNA was harvested from proliferating or differentiating Rcho-1 cells on d 0, 2, 4, 6, 8, and 10 after trypsinization to remove the proliferating cells. Each sample contains 10 μg of total RNA separated on a 1.2% agarose gel, and the membrane was probed for expression of *Hxt*, PL-I, MMP-9, or α1 integrin as indicated.

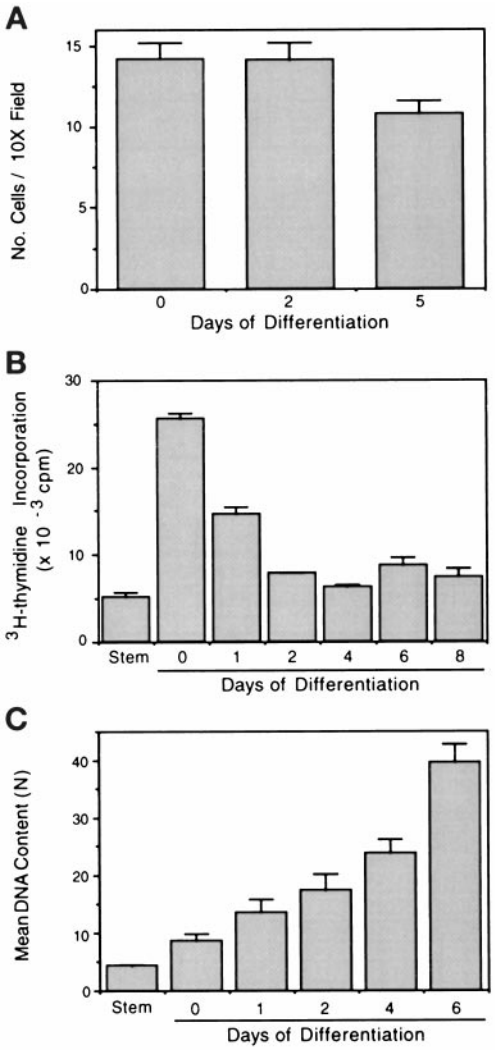

Figure 2. Endoreduplication in Rcho-1 cells. (A) Differentiating Rcho-1 cells are postmitotic. The number of differentiated Rcho-1 cells was estimated at various times after the removal of the proliferating cells and plotted. Data represent means ± SE. (B) [^{3}H]thymidine incorporation during Rcho-1 differentiation. The incorporation of [^{3}H]thymidine into TCA-precipitable material was measured in Rcho-1 stem cells and in giant cells at d 0, 1, 2, 4, 6, and 8 after the removal of the proliferating cells. (C) DNA content of differentiating Rcho-1 cells measured by scanning cytometry. The DNA content of Rcho-1 cells was measured in the stem or giant cells on the indicated d of differentiation, and the average DNA content was expressed relative to the estimated haploid DNA content using serum-starved HeLa cells as a normal control for diploid DNA content.

the first 4–6 d of differentiation, and a gradual decline at later times (Figure 2A). The cells continued to synthesize DNA at levels comparable to those seen in proliferating Rcho-1 stem cells, as revealed by [^{3}H]thymidine incorporation (Figure 2B, and up to d 12 in other experiments). The continued synthesis of DNA in the absence of mitosis suggested that the Rcho-1 cells were undergoing endoreduplication. The Rcho-1 giant cells were analyzed for DNA content as a more direct assay for the increase in ploidy that should accompany endoreduplication. By flow cytometry, the Rcho-1 stem cells had 4–8 N DNA contents, indicating that they are tetraploid (Nakayama, Scott, and Cross, unpublished data). This was confirmed by karyotype analysis. Nuclei of Rcho-1 giant cells appeared to be too fragile to prepare for flow cytometry, since only fragments with DNA contents of up to 8–10 N could be isolated. To avoid subjecting the nuclei to manipulation and the attendant fragmentation, the DNA content of Rcho-1 giant cells was determined by scanning laser fluorimetry of cells that were fixed in situ. Confluent, serum-starved HeLa cells were used as a standard for diploid DNA content. Using this technique, the Rcho-1 stem cells showed DNA contents from 4 N to 8 N (mean 4.29 N ± 0.25), as would be expected for the G_1, S, and G_2 contents of a tetraploid cell line (Figures 2C and 3). Analysis of the committed giant cells at d 0 showed an 8 N or G2 peak (Figures 2C and 3). Cells with DNA contents equivalent to 16 N or greater appeared by the second day of differentia-

tion, DNA contents of up to 32 N between the third and fourth day, and cells with up to 64 N appeared by the sixth day (Figures 2C and 3). Interpolation with these data showed that the Rcho-1 cells were capable of at least three rounds of endoreduplication during the 6 d of analysis, suggesting an estimate of 2 d for the length of the endocycle.

The endocycles in the Rcho-1 cells appeared to consist of alternating S and G phases, based on the clustering of DNA content around twofold increases. In addition, after a brief incubation with [^{3}H]thymidine and autoradiography, both labeled and unlabeled nuclei were apparent. In d 0 giant cells, the labeling index was 60–70% after a 1-h [^{3}H]thymidine pulse, indicating that the cells proceeded through the first round of endoreduplication relatively synchronously. The synchrony of the population decayed over time until the labeling index reached 18–20% on d 4 of differentiation and thereafter. To further examine the structure of the endocycle, a partial synchronization was achieved by incubating d 3 giant cells in mimosine for 16 h to prevent progression through S phase (Lalande, 1990; Watson *et al.*, 1991). The mimosine was removed and DNA synthesis was determined by measuring [^{3}H]thymidine incorporation at later times. Incorporation of [^{3}H]thymidine was detectable 1 and 2 h after removing mimosine and remained elevated relative to controls for 8 to 10 h, providing an estimate of S phase of 8–10 h. Combining the length of S phase with the short-term labeling index of 20% gives an estimated endocycle length of 40–50 h, similar to the estimate derived from the DNA content analysis.

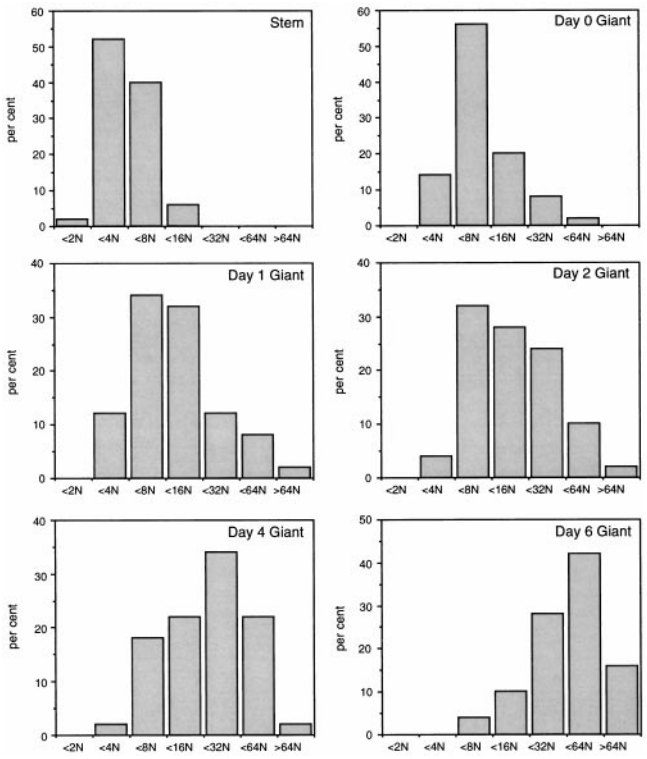

Figure 3. DNA content distribution of Rcho-1 cells at various d during differentiation. Cells were fixed at the indicated times during differentiation and stained with bisbenzimide, and the nuclear fluorescence intensity was measured as indicated in MATERIALS AND METHODS.

G_1-S Control Is Altered during the Endocycle

The transition from a mitotic cycle to an endocycle requires changes in the function of cell cycle checkpoints, for example, dissociation of the initiation of DNA synthesis from prior passage through mitosis. To assess the status of other cell cycle checkpoints during endoreduplication in trophoblast, the effect of treatments that impede the progression of cells through the mitotic cell cycle was compared in proliferating and endoreduplicating giant cells (Table 1). DNA synthesis in Rcho-1 giant cells was relatively resistant to ionizing irradiation when compared with the proliferating cells (Table 1). Likewise, incorporation of [^{3}H]thymidine by Rcho-1 giant cells was considerably less sensitive to treatment with the DNA alkylating agent, mitomycin C, than that of the Rcho-1 stem cells (ED_{50} = 160 versus 31 ng/ml, respectively). These results suggest that the checkpoint that normally inhibits DNA synthesis in response to DNA damage is suppressed in the Rcho-1 giant cells compared with the stem cells.

In replicating cells, the G_1 transition is sensitive to the presence of growth factors, which induce the transcription of D-type cyclins. We tested whether this was true during the Rcho-1 endocycle as well. Incubation of stem cells in serum-free medium for 16 h reduced the incorporation of [^{3}H]thymidine to background levels, and addition of medium containing 20% FBS stimulated incorporation approximately 70-fold by 20 h after serum addition (Table 1). In contrast, [^{3}H]thymidine incorporation by Rcho-1 giant cells was largely independent of the presence of serum, decreasing only twofold in the absence of serum (Table 1). We next examined the expression of cyclin D mRNAs (Figure 4A). Cyclin D2 mRNA was expressed at relatively low levels in Rcho-1 cells at all time points. The expression of cyclin D3 was high in proliferating Rcho-1 stem cells, but significantly decreased with the onset of differentiation (Figure 4A). In contrast, cyclin D1 mRNA was expressed only at low levels in the proliferating Rcho-1 cells, but was induced with the onset of differentiation. Interestingly, the cyclin D1 mRNA levels did not fluctuate during the subsequent endocycles (Figure 4A). In combination, these data indicate that several G_1-S checkpoint functions are altered with commitment to giant cell differentiation and onset of endoreduplication.

Table 1. Effect of serum starvation, radiation, and mitomycin C treatment on DNA synthesis in Rcho-1 stem and giant cells

Treatment		Conc. (^{3}H]thymidine incorporation (% of control) (mean ± SE)		Statistical significance ($p < 0.05$)
		Stem cells	Day 2 giant cells	
Serum (%)	20	100 ± 30	100 ± 34	
	0.5	1 ± 0.2	49 ± 15	*
γ-Radiation (cGy)	0	100 ± 0.1	100 ± 0.0	
	100	75 ± 0.1	104 ± 0.0	*
	300	57 ± 0.0	106 ± 29	*
	1000	34 ± 0.0	105 ± 0.1	*
Mitomycin C (μg/ml)	0	100 ± 0.8	100 ± 26	
	0.01	79 ± 1.0	113 ± 50	
	0.1	30 ± 1.3	54 ± 2.7	*
	1	0.4 ± 0.0	2 ± 0.0	*

Rcho-1 stem and d 2 giant cells were cultured in 20% fetal bovine serum except as noted throughout. For serum starvation experiments, the cells were starved for 24 h before addition of serum, and cells were incubated an additional 20 h. For DNA damaging experiments, cells were treated with a single dose of γ-radiation or with mitomycin C overnight. DNA synthesis was measured by incorporation of [^{3}H]thymidine into acid-insoluble material over an 8-h period. Values are normalized to the controls (20% serum). Differences between stem and giant cells were assessed by paired *t* test, and statistically significant differences are indicated by *.

S Phase Cyclin/CDK Activities during Endoreduplication

The transition from the mitotic cycle to an endocycle was accompanied by other changes in the expression of the G_1-S phase cyclins and their associated kinase activities. The mRNA for cyclin A1 was not detectable at any point in the analysis. Cyclins A2 and E mRNAs were readily detectable in Rcho-1 stem cells (Figure 4A). They were also detectable in d 0 giant cells and both were down-regulated by d 1, suggesting a rapid inhibition of the expression of these genes. The expression of cyclin A2 and cyclin E mRNAs remained low, though detectable, relative to the expression levels in Rcho-1 stem cells. Despite the significant reductions in abundance of the mRNAs, both proteins were readily detectable by Western blotting in Rcho-1 giant cells on d 0 and d 1 (Figure 4B). The cyclin E protein was present at all points during differentiation and, although some fluctuation in the amount of cyclin E was seen during the time course, it followed no obvious pattern in different experiments. The cyclin A2 protein showed two peaks in abundance on d 1 and d 3 of differentiation, with subsequent declines to barely detectable levels (Figure 4B). A similar pattern of accumulation and degradation over a 2-d time course was seen in other experiments. The level of expression of the cyclin A2 and E proteins were not closely linked to the expression of their respective mRNAs, suggesting that the turnover of these proteins differs between the mitotic and endoreduplicative cycles. Cyclin immunoprecipitates were next assayed for cyclin A2- or cyclin E-associated H1 kinase activity (Figure 4C). The fluctuations in associated kinase activity during giant cell differentiation followed the changes in abundance of the respective cyclin as determined by immunoblot analysis. The cyclin E-associated kinase activity remained relatively constant throughout the differentiation time course. The cyclin A-associated kinase activity showed two peaks that coincided with the peaks of cyclin A protein expression.

Despite the continued abundance of the cyclin A and cyclin E proteins, it was notable that the kinase activity associated with both cyclins dropped significantly after the onset of differentiation compared with stem cells. This reduction was not the result of down-regulation of either $p34^{cdk1}$ or $p33^{cdk2}$ (Figure 7A). Coimmunoprecipitation of $p34^{cdk1}$ and $p33^{cdk2}$ by the anti-cyclin A or E antibodies revealed that these two cdks were associated with the cyclins in both the endoreduplicative (Figure 4D) and mitotic cycles in several independent experiments. The continued association suggested that both enzymes contributed to the cyclin-associated kinase activity detected in the differentiating Rcho-1 cells. However, the amount of $p34^{cdk1}$ associated with cyclin A showed a sharp decrease at the onset of differentiation, but there was no notable, similar decrease in the cyclin E-associated $p34^{cdk1}$. The amount of $p33^{cdk2}$ associated with the two cyclins did not change significantly with the onset of endoreduplication (Figure 4D).

Both Cyclin A and Cyclin E Are Expressed during S Phase of the Rcho-1 Endocycle

By indirect immunofluorescence, both cyclin A and cyclin E were expressed in only a fraction of giant

cells, indicating that the expression of these proteins did change during the endocycle. The expression of cyclin A and E relative to the timing of DNA synthesis was analyzed in d 0 giant cells that were pulse labeled with BrdU. Cyclin A was located predominantly in the nucleus (Figure 5), although a few cells also displayed cyclin A in the cytoplasm. Colocalization of cyclin A and BrdU showed that cyclin A was only detectable in S phase cells; however, 14% of the BrdU-positive cells were cyclin A negative, indicating that cyclin A was not expressed at all times of the S phase. Analysis of cyclin E expression in a parallel sample of cells revealed that only 65% of the cyclin E-positive cells were colabeled for BrdU, but essentially all (94%) of BrdU-positive cells were also positive for cyclin E. These data indicate that cyclin E appears in S phase but also during a portion of the G phase (Figure 5). In BrdU-positive cells cyclin E was found exclusively in the nucleus, but in BrdU-negative cells it was found either in the cytoplasm or in both the nucleus and cytoplasm. Together these data suggest that cyclin E initially accumulated in the cytoplasm and was translocated to the nucleus before the onset of DNA synthesis, where it remained through the rest of S phase.

Expression of the Mitotic Cyclins Changes with the Onset of Endoreduplication

Endoreduplication occurs, by definition, in the absence of mitosis; therefore, we examined the fate of B-type cyclins after the transition to the endocycle during Rcho-1 differentiation. The mRNAs for the cyclins B1 and B2 were readily detectable in the Rcho-1 stem cells, but were undetectable by d 2 of differentiation (Figure 6A). The mRNA expression of the two B-type cyclins then remained undetectable throughout differentiation, indicating that transcription was suppressed. The cyclin B1 protein product was readily detectable in the proliferating Rcho-1 stem cells by immunoblot analysis. Curiously, it was also detectable in giant cells at d 0 and d 1 of differentiation despite the loss of mRNA, but disappeared between d 1 and d 2 (Figure 6B) and remained essentially undetectable thereafter.

The cyclin B1-associated kinase activity was analyzed in immunocomplex histone H1 kinase assays and was readily detectable in the proliferating Rcho-1 cells (Figure 6C). Surprisingly, despite the persistence of the cyclin B1 protein, very little cyclin B1-associated kinase activity was detectable in the Rcho-1 giant cells on d 0 and d 1. The down-regulation of cyclin B-associated kinase activity was not achieved by elimination of its potential kinase partner, because the abundance of $p34^{cdk1}$, the kinase most strongly associated with the B-type cyclins, did not change significantly during differentiation of the Rcho-1 cells (Figure 7 A). However, the amount of $p34^{cdk1}$ that was

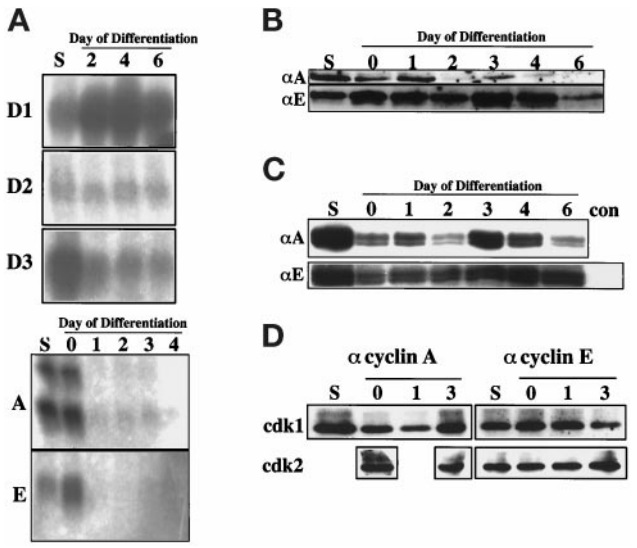

Figure 4. Expression of G_1- and S- phase cyclins in differentiating Rcho-1 cells. (A) Analysis of mRNAs for the D-type cyclins, cyclin A2, and cyclin E. Total RNA was isolated from proliferating (S) and differentiating Rcho-1 cells at various times after removal of the proliferating cells by trypsinization. Each sample contains 10 μg of total mRNA separated and hybridized to the cyclin probe indicated on the left of each panel. (B) Immunoblot analysis of cyclin A and E proteins in differentiating Rcho-1 cells. The samples were normalized for amount of protein. The proteins were separated on a 10% polyacrylamide gel, transferred, and the proteins were detected by using the primary antibody indicated in the panels (αA = α-cyclin A antibody; αE = α-cyclin E antibody). (C) Determination of kinase activity associated with the cyclins A and E. The lysates used for the immunoblot analysis were subjected to immunoprecipitation using the anti-cyclin antibodies. The immunoprecipitates were assayed for cyclin-associated histone-kinase activity and analyzed by separating the products on a 12.5% polyacrylamide gel. Autoradiographs of the phosphorylated histone H1 are shown with the immunoprecipitating antibody indicated on the left of each panel. A negative control, performed by omitting the primary antibody and using the stem-cell lysate in a mock immunoprecipitation–kinase reaction, is included as the right-most lane shown in the panel with the cyclin E-immunokinase assays. (D) Coimmunoprecipitation of $p34^{cdk1}$ and $p33^{cdk2}$ with cyclin A and E. The immunoprecipitates collected with the anti-cyclin A or E antibodies were subjected to gel electrophoresis and were immunoblotted using anti-$p34^{cdk1}$ or anti-$p33^{cdk2}$ antibodies. The lysates used for the immunoprecipitates are the same as those used for the histone H1 kinase assays; however, only lysates from the proliferating cells and differentiating cells at d 0, 1, and 3 were analyzed, as these contain the greatest amounts of cyclin-associated kinase activity.

associated with cyclin B1 was significantly reduced with the transition to the endocycle (Figure 7B). This was confirmed in experiments in which the anti-cyclin B1 immunoprecipitates were formed in antigen excess (Figure 7B). Under these conditions, a small amount of $p34^{cdk1}$ that was complexed with cyclin B1 was detectable in lysates from d 1 giant cells despite the low kinase activity. These data indicate that after the transition to the first endocycle, cyclin B-associated CDK activity remains low due primarily to reduced

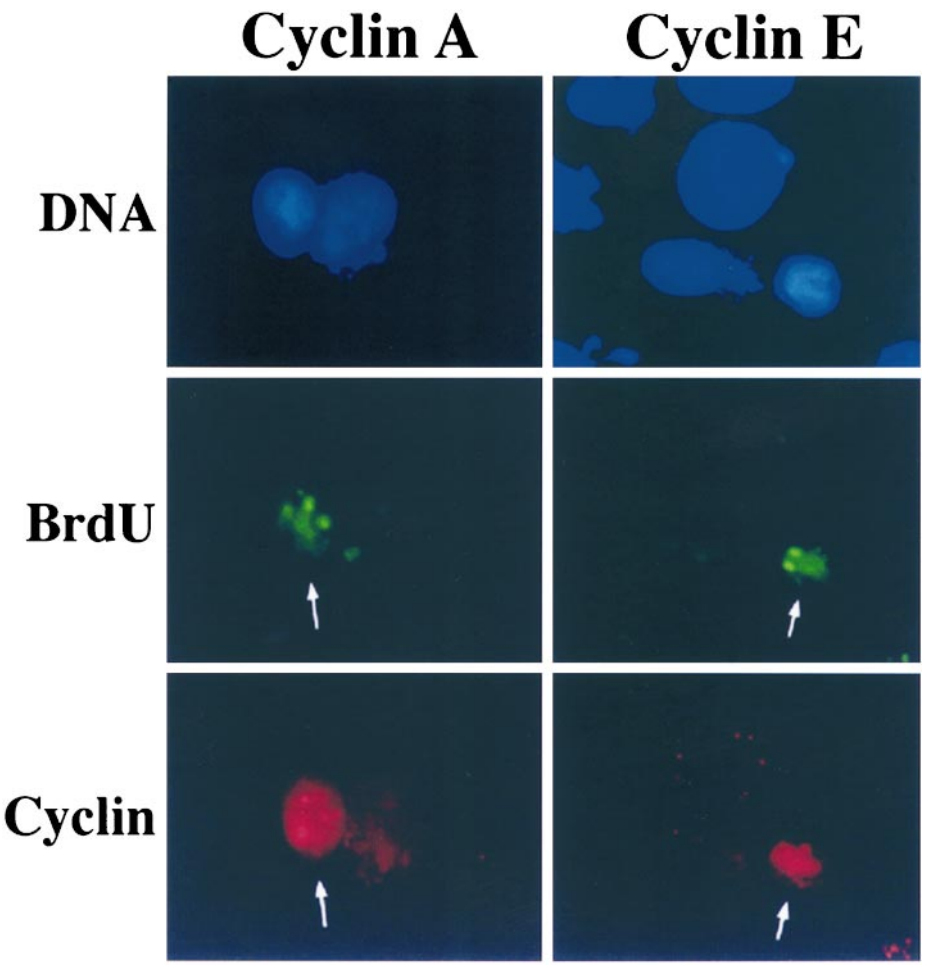

Figure 5. Colocalization of cyclin A and E expression with respect to BrdU incorporation. Cultures of Rcho-1 cells on d 1 of differentiation were labeled with BrdU, fixed, and processed for immunofluorescence as described in MATERIALS AND METHODS. The cultures were costained for the expression of either cyclin A or cyclin E as indicated at the top of each column and for BrdU as a marker of DNA synthesis, and counterstained for DNA. Each column is composed of pictures of the same field of cells.

$p34^{cdk1}$ association, but also to a decrease in specific activity.

DISCUSSION

The intimate interdependence of S and M phases is essential for maintaining the normal euploid state of a cell, and its disruption appears to be an early event leading to the aneuploidy associated with tumorigenesis (Pathak *et al.*, 1994). Understanding endoreduplication should help to define the possible routes by which such a disruption might occur, because it represents a natural dissociation of S from M. The cessation of mitosis and the initiation of endoreduplication in Rcho-1 trophoblast cells were closely linked to the onset of differentiation. The cell cycle changes were detectable from the earliest time at which differentiated cells could be isolated and they preceded the changes in expression of four molecular markers of trophoblast giant cell differentiation. The first indication of commitment to giant cell differentiation is markedly increased adhesion, which we used to obtain synchronized cell populations (Cross *et al.*, 1995). Although trophoblast differentiation appears to be positively regulated by *Hxt* and negatively regulated by *Id1* and *Id2* (Cross *et al.*, 1995), the role of these factors in the cell cycle changes, as opposed to the differentiation changes, has yet to be determined. Indeed, *Hxt* increases more closely paralleled differentiation than the exit from the mitotic cycle. Trophoblast differentiation and endoreduplication can be dissociated (Gardner and Davies, 1993), and it seems likely that the temporal separation distinguishable in the Rcho-1 cells reflects the normal regulatory pathways seen in trophoblast. We found that the transition from the mitotic cell cycle to the endocycle involved several changes in cell cycle control, including altered expression of cyclins and alterations in checkpoint controls. Endoreduplication represents an interesting conflict for a cell as it is a terminally differentiated phenotype that retains key features of proliferation, events that appear to be mutually exclusive in other cell types. It will be of interest to discover how the regulation of

differentiation is insulated from active cyclin A and E complexes. Endoreduplication may allow the growth of cells beyond the limit defined by the nuclear/cytoplasmic ratio that normally restricts the size of diploid cells, and would thus allow considerable growth in postmitotic cells. Other variant cycles in which dissociation of growth from proliferation occurs are the endomitotic cycles of megakaryocytes (Datta *et al.*, 1996; Zhang *et al.*, 1996) and the formation of syncytia in muscle and trophoblast of several species, including humans. The diversity of variant cycles during development suggests the complexity of the repertoire of cell cycle regulation on which the developing organism can draw and the versatility of growth regulation as a tool in morphogenesis.

Rcho-1 Trophoblast Cells Undergo Synchronized Endoreduplication

The induction of trophoblast giant cell markers in differentiating Rcho-1 cells has been previously reported, and it was also suggested that the Rcho-1 cells were capable of endoreduplication (Shida *et al.*, 1993; Yamamoto *et al.*, 1994; Cross *et al.*, 1995; Hamlin and Soares, 1995). The lack of synchrony within the cell population prevented more detailed analyses of the regulatory mechanisms. The ability to select a relatively synchronous cell population allowed us to analyze the temporal aspects of the regulation of the commitment to differentiation and endoreduplication. We demonstrated that the Rcho-1 cells were able to achieve DNA contents of up to 64 N by d 6 of differentiation. DNA synthesis continued past d 12 of differentiation, suggesting that the Rcho-1 cells are capable of reaching DNA contents of up to 128–256 N, close to the ploidies of 500-1000 N that have been reported for murine trophoblast in vivo (Zybina, 1970; Zybina and Grishchenko, 1970; Barlow and Sherman, 1972). Endoreduplication appeared to proceed in cycles that contained gap phases separating rounds of DNA synthesis in which DNA was replicated to completion, revealing an underlying regulation of initiation and progression.

Altered G_1-S Phase Control during the Endocycle

Our studies revealed several changes in cell cycle control after the transition to the endocycle occurs. The loss of the G_1-S checkpoints does not appear to be an oddity of the immortalized state of the Rcho-1 cell line, because DNA synthesis in trophoblast derived directly from mouse embryos is also relatively resistant to inhibition following X-irradiation (Goldstein *et al.*, 1975) and occurs in the absence of growth factor stimulation (Newman-Smith *et al.*, 1997). The molecular basis of the failure to inhibit DNA synthesis in response to environmental insult is not clear, but reprogramming of the cell cycle by regulators of differentiation may override the checkpoints that normally control cell cycle progression.

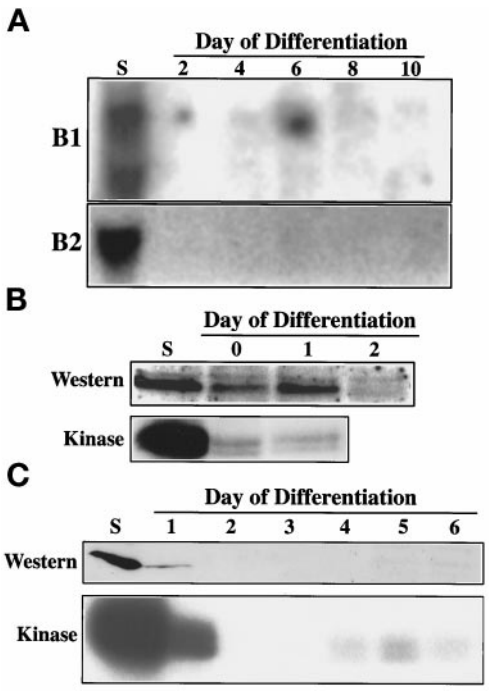

Figure 6. Expression of the B-type cyclins during differentiation of the Rcho-1 cells. (A) Total RNA was prepared from the Rcho-1 cells at the times indicated, and analyzed for cyclin B1 and B2 expression by hybridization to the full-length probes as indicated. (B) Analysis of expression and activity of the cyclin B protein products during the first endocycle. Lysates were prepared in an RIPA buffer from proliferating stem (S) or differentiating giant cells on the d indicated in the panel. Equivalent amounts of the proteins were separated on a 10% polyacrylamide gel, transferred, and the proteins were detected by the electrochemiluminescence system using the anti-cyclin B antibody. The lysates shown in the two panels were derived from two different experiments. Matching samples of the lysates used in the analysis of cyclin B protein expression were subjected to immunoprecipitation using the same anti-cyclin B1 antibody, and the immunoprecipitates were assayed for histone H1 kinase activity. The products were separated on a 12.5% polyacrylamide gel and the autoradiograph is shown. (C) Analysis of cyclin B protein expression and associated kinase over an extended time course. Lysates were prepared and analyzed for cyclin B abundance and associated kinase activity, as described in B from the samples indicated.

With the onset of Rcho-1 differentiation, the expression of the D-type cyclins changes significantly. The commitment of megakaryocytes to the endomitotic pathway requires cyclin D function and is associated with changes in expression of D-type cyclins as well (Wang *et al.*, 1995; Wilhide *et al.*, 1995), suggesting a role for D-type cyclins in regulating the onset of both endomitotic and endoreduplicative cycles. Rcho-1 stem cells expressed cyclin D3, but its expression was suppressed with the onset of differentiation. Cyclin D3 is the only D-type cyclin expressed in the yolk sac and ectoplacental cone of the d 8 mouse embryo (MacAu-

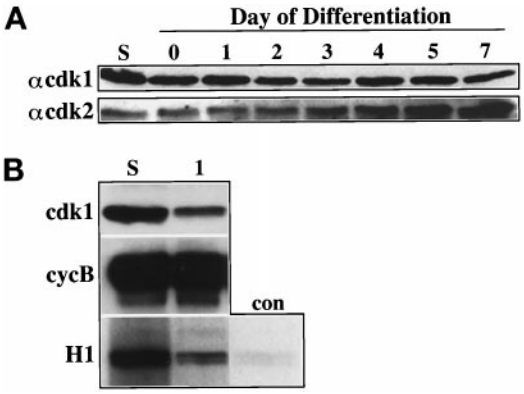

Figure 7. Expression of cdk1 and cdk2 and their association with cyclin B1 during the endocycle. (A) Analysis of of $p34^{cdk1}$ and $p33^{cdk2}$ expression during Rcho-1 differentiation. Protein lysates derived from the differentiating cells were separated and analyzed by immunoblotting for the presence of the two cdks in proliferating (S) and differentiating cells at the time points indicated. The membranes were probed with the antibody indicated to the left of each panel (αcdk1 = anti-$p34^{cdk1}$ antibody; αcdk2 = anti-$p33^{cdk2}$ antibody) and the presence of the protein was revealed by electrochemiluminescence (Amersham). (B) Reduction of $p34^{cdk1}$ and cyclin B1 association with the onset of differentiation. Immunoprecipitates were prepared from lysates of proliferating or differentiating cells on d 1 using the anti-cyclin B1 antibody, in what was estimated to be antigen excess based on previous analyses, and the immunoprecipitates were subjected to either an immunokinase assay (H1) or an immunoblot to determine the amount of cyclin B1 (B) or associated $p34^{cdk1}$. The positions of the cyclin B1 and $p34^{cdk1}$ are indicated on the left of the panels.

ley, unpublished observation), suggesting that giant cells may replace cyclin D3 during the endocycle. The up-regulation of cyclin D1 in differentiating Rcho-1 giant cells suggested that it may function during the endocycle. The cyclin D1 knockout mouse has no obvious defect in the trophoblastic lineage (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). However, if constitutive cyclin D1 expression in giant cells simply renders DNA synthesis independent of serum growth factors or provides a mechanism for overriding inhibitory signals that result from DNA damage, these functions would not necessarily be essential during normal mouse development.

It was surprising that the mRNAs for both the S phase cyclins, A and E, were down-regulated in the differentiated Rcho-1 cells (though still detectable) after d 1 of differentiation. The abundance of the cyclin A and E proteins did not decline as rapidly as the respective mRNAs, but it is not clear whether the slower rate of turnover is related to the lengthening of cell cycle phases during endoreduplication or a change in the regulation of protein turnover. For example, cyclin A degradation normally occurs during mitosis, but in the absence of any mitotic events a new signal must initiate cyclin A destruction at the end of the S phase (Pines and Hunter, 1991; Hunt *et al.*, 1992). The cyclin A- and E-associated kinase activities remained readily detectable during these early stages of differentiation, although the specific activity did not appear to be as high in the differentiating cells as in the proliferating cells. Several potential positive and negative regulatory mechanisms have been described in the mitotic cell cycle (reviewed in Morgan, 1995), but the basis for the lower specific activity of the cyclin/cdk complexes is not clear.

The periodic cycles of accumulation of cyclin A over a time frame similar to that of the doubling of DNA content suggests that cyclin A plays a regulatory role during the endocycle. Similar cycles were not observed for cyclin E, but the shorter gap in cyclin E expression may not have been detectable with the limited degree of synchronization obtainable in these experiments. The colocalization of cyclin A and E to essentially all BrdU-positive cells suggests that both cyclins are required for S phase progression. Cyclin E was found in the cytoplasm of some BrdU-negative cells, suggesting that it was either retained briefly in the cytoplasm after the initiation of synthesis or degraded if it was translocated to the nucleus. It seems likely that the cells that were cyclin E-positive/BrdU-negative cells were before S phase, because this is similar to regulation of the mitotic cycle (Koff *et al.*, 1992). These results indicate that both cyclins play a role in S phase and that, although cyclin E is required for the initiation of S phase, cyclin A is required for its completion. Another conclusion that can be drawn from the colocalization data is that G phase cells lack both cyclin E and cyclin A, supporting the idea that a period of the endocycle must lack cyclin-associated kinase activity to allow the cycle to be reset and the next round of DNA synthesis to occur. It is also apparent from the cyclin/BrdU colocalization data that the cyclin A and E proteins must be rapidly degraded at the end of S phase.

Regulation of the Endocycle

The data presented here outline a pathway that may explain the shift from the mitotic cycle to the endocycle during trophoblast differentiation (model summarized in Figure 8). With commitment to giant cell differentiation, Rcho-1 cells institute a program that prevents activation of mitosis by inhibiting the $p34^{cdk1}$-dependent kinase activity resulting in cells arrested in G_2. Subsequently, both cyclin E and $A/p33^{cdk2}$ kinase activities are reactivated and DNA synthesis is reinitiated. At the end of the first endocycle S phase, cyclins A, E, and B are degraded. Initiation of a new cycle of endoreduplication occurs during a gap phase in which the cyclin A- and E-associated kinase activity are absent. By analogy to the mitotic cell cycle, cyclin degradation presumably allows the reactivation of the origins of replication

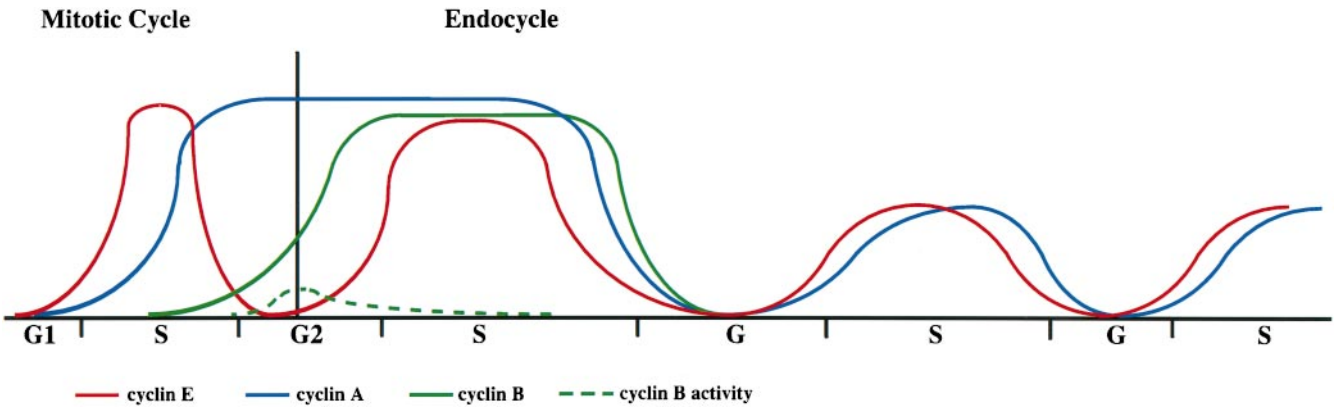

Figure 8. Summary model of the transition from the mitotic to the endoreduplicative cell cycle. This depicts the current interpretation of the analysis of expression of the cyclins A, B, and E and regulation of their associated kinase activities in relation to the cell cycle events involved with the shift from proliferation to endoreduplication. The solid lines indicate the accumulation of the respective cyclin protein. For cyclins A and E, the associated kinase activity is assumed to follow the protein expression fairly closely. The dashed line indicates the cyclin B-associated kinase activity, because it is suppressed during the first endocycle despite the continued expression of the protein.

(Cocker *et al*., 1996). Expression of cyclin E is activated first, leading to its accumulation in the cytoplasm and subsequently in the nucleus. The initiation of DNA synthesis and cyclin A expression appear later. With the completion of DNA synthesis, both cyclin A and E are degraded, signaling the end of one cycle and allowing initiation of the next. Whether the movement of cyclin E into the nucleus is the event that initiates S phase and the expression of cyclin A is unclear, but analogy to the mitotic cycle suggests that this is a likely mechanism.

The outline of the trophoblast endocycle differs somewhat from that described in other systems. In *Drosophila*, entry into each endocycle is driven by a new wave of cyclin E expression in the absence of both cyclin A and B (Knoblich *et al*., 1994; Duronio and O'Farrell, 1995; Sauer *et al*., 1995). The expression of cyclin A in the Rcho-1 cells during endoreduplication may help drive DNA synthesis closer to completion than *Drosophila*, where it appears that as much as 15–20% of the genome is unreplicated during each endocycle, perhaps reflecting limitations in the cdk activity (Lilly *et al*., 1996). Two apparent similarities between the Rcho-1 trophoblast and *Drosophila* are the necessity for a period of little or no cdk activity between rounds of DNA synthesis, and that the B-type cyclins are suppressed during endoreduplication. The regulation of the initiation of the *Drosophila* endocycle by developmental factors may render initiation of each cycle independent of the preceding cycle, so that the termination of one cycle does not act as a signal to initiate the next cycle (Knoblich *et al*., 1994; Sauer *et al*., 1995). Models for the regulation of the endocycle have not been well described in any other system. In corn endosperm, the total cdk activity decreases significantly with the onset of endoreduplication, although there is relatively little decrease in the abundance of the cdk proteins detectable. The loss of cdk activity appears to be an active process (Grafi and Larkins, 1995), perhaps similar to the Rcho-1 endocycle. The level of the S phase-associated cdk activity rises in endoreduplicating tissue, but it is not clear which cyclins are associated with this activity, nor how progression through the endocycle is regulated. Megakaryocytes also become polyploid, but through an endomitotic process in which cyclin B is expressed and is associated with kinase activity (Datta *et al*., 1996; Zhang *et al*., 1996). The expression of the endomitotic events seems to separate rounds of DNA synthesis in a way quite similar to M phase in the mitotic cycle.

The Transition to the Endocycle Involves Unique Regulation of Cyclin B

The Rcho-1 cell system has allowed us to study events that occur at the transition between the mitotic cell cycle and the first endocycle, a step not clearly addressed in other systems. We found that the cyclin B1 protein remained abundant during the first endocycle. The fact that cyclin B was expressed at all during the first endocycle, but not in later endocycles, implies that this first cycle begins in G_2 of a mitotic cycle. The fact that it persists until the end of the first endocycle is presumably due to the failure to activate its rapid degradation, an event usually associated with mitosis (King *et al*., 1994). After the first endocycle, cyclin B was no longer expressed and cyclin B mRNA was not detectable after commitment to giant cell differentiation at d 0, preventing reinitiation of mitosis.

In the mitotic cell cycle, cyclin B transcription is cell cycle regulated, with mRNA induction occurring in

G_2. The fact that cyclin B transcription is not induced during the endocycle implies a fundamental difference in the G_2 phase of the first endocycle. Because cyclin B induction may depend on G_1-S cyclin/cdk activity, it is possible that the abrupt termination of cyclin A- and E-associated kinase activity at the end of endocycle S phase preempts this induction. The failure of the d 0 giant cells to progress into mitosis, despite the presence of cyclin B protein, appeared to derive largely from the inability to form stable, active cyclin B/$p34^{cdk1}$ complexes, as a consequence of a reduction in both the association of cyclin B and $p34^{cdk1}$ and the specific activity of the complexes that did form. The latter effect could be related to $p34^{cdk1}$ phosphorylation, a mechanism well described during the mitotic cycle (Morgan, 1995). The lack of cyclin B association with $p34^{cdk1}$ was, however, somewhat surprising. The amount of $p34^{cdk1}$ associated with cyclin A was also decreased early in differentiation. This suggests that $p34^{cdk1}$ is the target of the inhibition, as might be expected of the cdk most closely associated with regulation of mitosis. In conclusion, it appears that one of the first differentiated functions expressed by trophoblast cells that have committed to giant cell fate is to suppress the regulatory activities associated with mitosis, first by direct inhibition of the kinase activity, and then by inhibition of expression of mitotic cyclins. Although mechanisms involved in cyclin B/$p34^{cdk1}$ complex formation are unclear at present, Hsp70–2 acts as a molecular chaperone that is required for cyclin B1/CDC2 complex formation and kinase activity during meiosis I of mouse spermatocytes (Zhu *et al*., 1997). It is possible that the kinase activity of the cyclin B complex is inhibited by regulation of a chaperone or by a peptide inhibitor that also targets the complex for disassembly. Such novel mechanisms are currently under investigation.

ACKNOWLEDGMENTS

Our sincere thanks to the people who provided reagents, particularly Drs. J. DeLoia, C. Sherr, and D. Wolgemuth for providing plasmids of various cyclins, and Dr. J. Roberts for the anti-cyclin E antibody. We thank O. Behrendtsen for his help with the figures. The work was supported by grants from the National Institutes of Health (HD-26732 and CA-75072) to Z.W. and the Medical Research Council of Canada to J.C.C. J.C.C. is a Scholar of the Medical Research Council of Canada.

REFERENCES

Alexander, C.M., Hansell, E.J., Behrendtsen, O., Flannery, M.L., Kishnani, N.S., Hawkes, S.P., and Werb, Z. (1996). Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. Development *122*, 1723–1736.

Barlow, P.W., and Sherman, M.I. (1972). The biochemistry of differentiation of mouse trophoblast: studies on polyploidy. J. Embryol. Exp. Morphol. *27*, 447–465.

Basco, R.D., Segal, M.D., and Reed, S.I. (1995). Negative regulation of G1 and G2 by S-phase cyclins of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *15*, 5030–5042.

Carney, E.W., Prideaux, V., Lye, S.J., and Rossant, J. (1993). Progressive expression of trophoblast-specific genes during formation of mouse trophoblast cells in vitro. Mol. Reprod. Dev. *34*, 357–364.

Chapman, D.L., and Wolgemuth, D.J. (1992). Identification of a mouse B-type cyclin which exhibits developmentally regulated expression in the germ line. Mol. Reprod. Dev. *33*, 259–269.

Chapman, D.L., and Wolgemuth, D.J. (1993). Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. Development *118*, 229–240.

Cocker, J.H., Piatti, S., Santocanale, C., Nasmyth, K., and Diffley, J.F. (1996). An essential role for the Cdc6 protein in forming the prereplicative complexes of budding yeast. Nature *379*, 180–182.

Cross, J.C., Flannery, M.L., Blanar, M.A., Steingrimsson, E., Jenkins, N.A., Copeland, N.G., Rutter, W.J., and Werb, Z. (1995). Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. Development *121*, 2513–2523.

Cross, J.C., Werb, Z., and Fisher, S.J. (1994). Implantation and the placenta: key pieces of the development puzzle. Science *266*, 1508–1518.

Damjanov, I., Shan, J., Wang, R.F., Damjanov, A., and DeLoia, J.A. (1994). Molecular cloning and characterization of murine cyclin E. Biochem. Biophys. Res. Commun. *201*, 994–1000.

Datta, N.S., Williams, J.L., Caldwell, J., Curry, A.M., Ashcraft, E.K., and Long, M.W. (1996). Novel alterations in CDK1/cyclin B1 kinase complex formation occur during the acquisition of a polyploid DNA content. Mol. Biol. Cell *7*, 209–223.

Duronio, R.J., and O'Farrell, P.H. (1995). Developmental control of the G1 to S transition in Drosophila: cyclin E is a limiting downstream target of E2F. Genes Dev. *9*, 1456–1468.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. *9*, 2364–2372.

Faria, T.N., Ogren, L., Talamantes, F., Linzer, D.I., and Soares, M.J. (1991). Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. Biol. Reprod. *44*, 327–331.

Faria, T.N., and Soares, M.J. (1991). Trophoblast cell differentiation: establishment, characterization, and modulation of a rat trophoblast cell line expressing members of the placental prolactin family. Endocrinology *129*, 2895–2906.

Gardner, R L., and Davies, T.J. (1993). Lack of coupling between onset of giant transformation and genome endoreduplication in the mural trophectoderm of the mouse blastocyst. J. Exp. Zool. *265*, 54–60.

Goldstein, L.S., Spindle, A.I., and Pedersen, R.A. (1975). X-ray sensitivity of the preimplantation mouse embryo in vitro. Rad. Res. *62*, 276–287.

Grafi, G., and Larkins, B.A. (1995). Endoreduplication in maize endosperm-involvement on M phase-promoting factor inhibition and induction of S phase-related kinases. Science *269*, 1262–1264.

Hamlin, G.P., Lu, X.J., Roby, K.F., and Soares, M.J. (1994). Recapitulation of the pathway for trophoblast giant cell differentiation in vitro: stage-specific expression of members of the prolactin gene family. Endocrinology *134*, 2390–2396.

Hamlin, G.P., and Soares, M.J. (1995). Regulation of deoxyribonucleic acid synthesis in proliferating and differentiating trophoblast cells: involvement of transferrin, transforming growth factor-beta, and tyrosine kinases. Endocrinology *136*, 322–331.

Hartman, T.P., and Southern, D.I. (1995). Genome reorganization from polyteny to polyploidy in the nurse cells found in onion fly (*Delia antiqua*) and cabbage root fly (*Delia radicum*) ovaries (*Diptera, Anthomyiidae*). Chromosome Res. *3*, 271–280.

Hoffman, L.H., and Wooding, F.B. (1993). Giant and binucleate trophoblast cells of mammals. J. Exp. Zool. *266*, 559–577.

Hunt, T., Luca, F.C., and Rudermen, J.V. (1992). The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. J. Cell Biol. *116*, 707–724.

King, R.W., Jackson, P.K., and Kirschner, M.W. (1994). Mitosis in transition. Cell *79*, 563–571.

Knoblich, J.A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C.F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. Cell *77*, 107–120.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., and Roberts, J.M. (1992). Formation and activation of a cyclin E–cdk2 complex during the G1 phase of the human cell cycle. Science *257*, 1689–1694.

Lalande, M. (1990). A reversible arrest point in the late G1 phase of the mammalian cell cycle. Exp. Cell Res. *186*, 332–339.

Lilly, M., and Spradling, A.C. (1996). The *Drosophila* endocycle is controlled by cyclin E and lacks a checkpoint insuring S-phase completion. Genes Dev. *10*, 2514–2526.

Malinowski, S., and Maszewski, J. (1994). DNA endoreduplication, RNA and protein synthesis during growth and development of the antheridial basal cell in *Chara vulgaris* L. Folia Histochem. Cytobiol. *32*, 137–142.

Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. Cell *65*, 701–713.

Morgan, D.O. (1995). Principles of CDK regulation. Nature *374*, 131–134.

Newman-Smith, E., and Werb, Z. (1997). Functional analysis of trophoblast giant cells in parthenogenetic mouse embryos. Dev. Genet. *20*, 1–10.

Pathak, S., Dave, B.J., and Gagos, S. (1994). Chromosome alterations in cancer development and apoptosis. In Vivo *8*, 843–850.

Piatti, S., Bohm, T, Cocker, J.H., Diffley, J.F., and Nasmyth, K. (1996). Activation of S-phase-promoting CDKs in late G_1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes Dev. *10*, 1516–1531.

Pines, J., and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. *115*, 1–17.

Reponen, P., Sahlberg, C., Munaut, C., Thesleff, I., and Tryggvason, K. (1994). High expression of 92-kDa type IV collagenase (gelatinase) in the osteoclast lineage during mouse development. Ann. N.Y. Acad. Sci. *732*, 472–475.

Rinkenberger, J.L., Cross, J.C., and Werb, Z. (1997). Molecular genetics of implantation in the mouse. Dev. Genet. *21*, 6–20.

Sauer, K., Knoblich, J.A., Richardson, H., and Lehner, C.F. (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of *Drosophila* embryogenesis. Genes Dev. *9*, 1327–1339.

Shida, M.M., Ng, Y.K., Soares, M.J., and Linzer, D.I. (1993). Trophoblast-specific transcription from the mouse placental lactogen-I gene promoter. Mol. Endocrinol. *7*, 181–188.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell *82*, 621–630.

Sutherland, A.E., Calarco, P.G., and Damsky, C.H. (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. Development *119*, 1175–1186.

Sweeney, C., Murphy, M., Kubelka, M., Ravnik, S.E., Hawkins, C.F., Wolgemuth, D.J., and Carrington, M. (1996). A distinct cyclin A is expressed in germ cells in the mouse. Development *122*, 53–64.

Wang, Z., Zhang, Y., Kamen, D., Lees, E., and Ravid, K. (1995). Cyclin D3 is essential for megakaryopoiesis. Blood *86*, 3783–3788.

Watson, P.A., Hanauske-Abel, H.H., Flint, A., and Lalande, M. (1991). Mimosine reversibly arrests cell cycle progression at the G_1-S phase border. Cytometry *12*, 242–246.

Wilhide, C.C., Van Dan, C., Dispersio, J., Kenedy, A.A., and Bray, P.F. (1995). Overexpression of cyclin D1 in the Dami megakaryocytic cell line causes growth arrest. Blood *86*, 294–304.

Yamamoto, T., Roby, K.F., Kwok, S.C., and Soares, M.J. (1994). Transcriptional activation of cytochrome P450 side chain cleavage enzyme expression during trophoblast cell differentiation. J. Biol. Chem. *269*, 6517–6523.

Zhang, Y., Wang, Z., and Ravid, K. (1996). The cell cycle in polyploid megakaryocytes is associated with reduced activity of cyclin B1-dependent cdc2 kinase. J. Biol. Chem. *271*, 4266–4272.

Zhu, D., Dix, D.J., and Eddy, E.M. (1997). Hsp70–2 is required for CDC2 kinase activity in meiosis I of mouse spermatocytes. Development *124*, 3007–3014.

Zybina, E.V. (1970). Characteristics of polyploidization of trophoblast cells. Tsitologiia *12*, 1081–1094.

Zybina, E.V., and Grishchenko, T.A. (1970). Polyploid cells of the trophoblast in various parts of the placenta of the white rat. Tsitologiia *12*, 585–595.