FAST TRACK

Overexpression of ORC Subunits and Increased ORC-Chromatin Association in Transformed Mammalian Cells

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Abstract The origin recognition complex (ORC) is a conserved heterohexamer required for the formation of pre-replication (pre-RC) complexes at origins of DNA replication. Many studies of ORC subunits have been carried out in transformed human cell lines but the properties of ORC in primary cells have not been addressed. Here, we compare the expression levels and chromatin-association of ORC subunits in HeLa cells to the primary human cell line, WI38, and a virally transformed derivative of WI38, VA13. ORC subunits 2 and 4 were highly overexpressed in both HeLa and VA13, whereas ORC1 levels were elevated in VA13 but considerably higher in HeLa cells. Cellular extraction revealed that the proportion of ORC2 and ORC4 subunits bound to chromatin was similar in all three cell lines throughout the cell-cycle. In contrast, very little ORC1 was associated with chromatin after extraction of primary WI38 cells, whereas the majority of overexpressed ORC1 in both HeLa and VA13 co-fractionated with chromatin throughout the cell-cycle. Although none of the cell lines displayed significant changes in the levels or chromatin-association of ORC during the cell-cycle, the chromatin-associated fraction of ORC1 displayed an increase in apparent molecular weight during S-phase. Similar experiments comparing immortalized CHO cells to an isogenic virally transformed derivative revealed no changes in levels of ORC subunits but an increase in the proportion of all three ORC subunits associated with chromatin. These results demonstrate a complex influence of cellular immortalization and transformation properties on the expression and regulation of ORC subunits. These results extend the potential link between cancer and deregulation of pre-RC proteins, and underscore the importance of considering the transformation status of cell lines when working with these proteins. J. Cell. Biochem. 96: 879–887, 2005. © 2005 Wiley-Liss, Inc.

Key words: DNA replication; ORC; cellular transformation

Replication origins are central to the faithful duplication of the eukaryotic genome. The first step in establishing an origin of replication is the formation of a pre-replicative complex (pre-RC). Pre-RC assembly begins with and requires association of the heterohexameric origin recognition complex (ORC) with DNA at or near the replication origin [Diffley, 2004; Gilbert, 2004]. ORC facilitates loading of the Cdc6 protein, which in conjunction with the Cdt1 protein enables the loading of the minichromosome maintenance (MCM) proteins, forming a pre-RC. In yeast, ORC remains bound to the origin throughout the cell-cycle but in multi-cellular organisms, the fate of ORC and in particular the ORC1 subunit, has not been entirely clear. ORC1 is of particular interest as it contains the only ATP-binding and hydrolysis domain necessary for the DNA binding activity of ORC [Klemm and Bell, 2001]. In experiments with purified subunits, mammalian ORC1 has been shown to interact more loosely with a core complex consisting of ORC2–5 suggesting a more transient, potentially regulatory, role of the ORC1 subunit [Dhar et al., 2001; Vashee et al., 2001].

Studies of mammalian ORC subunits have come to differing conclusions, particularly regarding cell-cycle regulation of the ORC1 subunit. Current models suggest, ORC1 is selectively released from chromatin during S-phase and degraded or otherwise made unavailable for

pre-RC formation to prevent re-replication of the DNA within a single S-phase [DePamphilis, 2003]. However, it has been shown that ORC-depleted *Xenopus* egg extracts can assemble pre-RCs and initiate replication within G2-phase human nuclei [Romanowski et al., 1996], which presumably must require the presence of active mammalian ORC1 during G2-phase. Moreover, since all studies agree that the remaining mammalian ORC subunits bind to origins throughout S-phase, it is hard to reconcile selective removal of ORC1 with the fact that purified yeast, *Drosophila* and human ORC require ORC1 for DNA binding [Klemm et al., 1997; Chesnokov et al., 2001; Vashee et al., 2003]. In fact, some studies have found the majority of ORC1 bound to chromatin throughout S-phase [Rowles and Blow, 1997; Okuno et al., 2001; McNairn et al., 2005] and every study has reported at least 30–50% of the detectable ORC1 is still bound to chromatin during S-phase, and none of these studies have detected the predicted remainder of unbound ORC1 [Mendez et al., 2002; Ohta et al., 2003; Ritzi et al., 2003; Tatsumi et al., 2003]. Recently, it was shown that supplementing cellular lysis buffer with the protease inhibitor MG132 prevents the cell-cycle associated degradation of ORC1 [Ritzi et al., 2003], suggesting that the observed loss of ORC1 occurs during cellular extraction and not in vivo. Indeed, since pre-RC assembly during telophase does not require protein synthesis throughout mitosis [Okuno et al., 2001], a sufficient amount of ORC1 must remain throughout this period.

Most studies of mammalian ORC have utilized cell lines derived from human cancers, which may overexpress pre-RC proteins, since many of these proteins are transcriptionally regulated by E2F [Ohtani et al., 1996; Tsuruga et al., 1997; Yan et al., 1998; Karakaidos et al., 2004; Xouri et al., 2004; Yoshida and Inoue, 2004]. For example, ORC1 is an E2F-regulated gene and E2F deregulation could result in overexpression and degradation of excess ORC1. In fact, overexpression of the related E2F-regulated Cdc6 gene in CHO cells results in nuclear export of the excess Cdc6, while a constant amount of Cdc6 remains chromatin-bound [Okuno et al., 2001; Alexandrow and Hamlin, 2004]. Here, we have compared ORC chromatin-association in the HeLa S3 cancer line, the primary cell line, WI38, and an SV40 transformed WI38 derivative, VA13. In addi-

tion, we have compared non-transformed CHO cells with an isogenic SV40 transformed derivative. Our results demonstrate that ORC subunits are themselves overexpressed in transformed cells, and that transformation can increase both the total level of these subunits and the amount of ORC associated with chromatin.

MATERIALS AND METHODS

Cell Culture and Synchronization

CHO cell lines, CHOC 400, and JH-1 were grown in DMEM supplemented with 5% FBS, penicillin–streptomycin, and non-essential amino acids. HeLa S3 and VA13 cells were grown in DMEM supplemented with 10% cosmic calf serum, penicillin–streptomycin and non-essential amino acids. WI38 cells were grown in DMEM supplemented with 10% FBS, penicillin–streptomycin, and non-essential amino acids. To synchronize cells in mitosis, 0.05 µg/ml nocodazole was added for 4 h, followed by mechanical shake-off [Wu et al., 1997]. Metaphase spread analysis was used to confirm the cells were in mitosis. For all cell lines, greater than 95% mitotic cells were routinely obtained. For S-phase synchrony of immortalized cells, cells were first synchronized either by mitotic shake-off or isoleucine starvation, and released into 5 µg/ml aphidicolin for 12–14 h. In all cell lines, greater than 85% of cells incorporated BrdU within 1 h of release in all cell lines. To synchronize WI38, cells were first grown to confluency and then split 1:4 into fresh media containing 5 µg/ml aphidicolin for 24 h. The cells were then washed and released into fresh media. BrdU analysis was used to confirm the progression of the cells into S-phase [Wu et al., 2005].

Cell Extracts and Immunoblotting

Chromatin enrichment was carried out as described by Mendez and Stillman [2000], with the following modifications, 25 µM MG132 (Calbiochem) and 1 mM ATP was added to all buffers [McNairn et al., 2005]. Cell extracts were normalized to either cell number or by protein content using the BCA protein assay (Bio-Rad). Extracts were run on 8% SDS–PAGE gels and transferred to PVDF membrane (Immobilion, Millipore) by wet transfer using Bolt and Mahoney buffer (40 mM Tris, 10 mM sodium acetate, 2 mM EDTA, 0.05% SDS, 20%

MeOH) at 1A for 45 min [Bolt and Mahoney, 1997]. Blots were blocked with 1% non-fat milk in TBS (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween 20) for 1 h at room temperature. All primary antibody incubations were carried out overnight at 4°C in blocking buffer. The following antibodies were used, anti-CgORC1 NY674 (1:10000), anti-HsORC1 (1:150) (gift of J. Mendez and B. Stillman), anti-ORC2 (1:500) (Santa Cruz), anti-ORC4 (1:500) (BD Transduction Labs), and anti-actin (1:5000) (Chemicon). Detection was with Affinipure goat anti-rabbit HRP, goat anti-mouse HRP, rabbit anti-goat Alexa 633, goat anti-rabbit Alexa 633, or goat anti-mouse Alexa 594 (Jackson Immunolabs), and Supersignal West Pico ECL substrate for all antibodies except anti-HsORC1. For Hs-ORC1, the Supersignal West Dura ECL substrate and associated HRP stabilized goat anti-rabbit antibody was used (Pierce). Fluorescent secondary antibodies were imaged using a Typhoon 9410 (Amersham).

RESULTS

ORC Subunits Are Overexpressed in Transformed Human Cells

To date, all published results reporting ORC1 degradation during S-phase [Mendez et al., 2002; Ohta et al., 2003; Tatsumi et al., 2003; Lidonnici et al., 2004] were obtained with highly transformed human cell lines (HeLa, Raji, Hep-2, 293). The influence of cellular transformation on ORC1 behavior has not been addressed. In fact, ORC1 itself is an E2F-regulated gene and E2F deregulation could result in overexpression and degradation of excess ORC1 [Ohtani et al., 1996]. To determine whether ORC subunits are upregulated in transformed and cancer cell lines, we prepared whole cell extracts from asynchronously growing HeLa S3, WI38 (human primary cell line), and VA13 (SV40 transformed WI38). To prevent the potential for in vitro degradation of protein, all lysis buffers were supplemented with MG132, which inhibits the 26S proteasome as well as many cellular proteases [Mellgren, 1997; Rodgers and Dean, 2003]. The results (Fig. 1A) indicate that ORC subunits, including ORC1, ORC2, and ORC4, are overexpressed in HeLa and VA13 cells relative to WI38 cells. However, while ORC2 and ORC4 were overexpressed to similar levels in HeLa and VA13, ORC1 was overexpressed to considerably higher levels in HeLa cells.

To determine if the upregulation of ORC subunits altered the amount of ORC associated with chromatin, asynchronously growing VA13 and WI38 cells were fractionated according to a widely utilized protocol (Fig. 1B) [Mendez and Stillman, 2000; Ritzi et al., 2003] and each fraction was subject to immunoblotting (Fig. 1C). Little to no ORC1 was detected in the chromatin fraction of WI38, whereas ORC1 was detected in both the soluble and chromatin fractions from VA13 (Fig. 1C). At least some of the ORC1 co-fractionating with chromatin migrated at a higher apparent molecular weight, suggesting post-translational modifications of this fraction of ORC1. For both cell lines, ORC2 and ORC4 subunits were found predominantly in the chromatin fraction (Fig. 1C), however, significantly more soluble and chromatin associated ORC2 and ORC4 were present in VA13 cells, consistent with the overexpression of these subunits. These results indicate that the upregulation of ORC in human cells results in an increase in both soluble and chromatin-associated pools of ORC subunits. However, the proportion of excess ORC1 that co-fractionates with chromatin in transformed cells is higher than that for ORC2 and ORC4.

Changes in ORC-Chromatin Association in Transformed Versus Primary Cells

To determine whether ORC overexpression could result in the reported release and degradation of ORC1 during S-phase in human cells, we examined ORC chromatin association during the cell-cycle in the frequently used HeLa S3 cell line [Mendez et al., 2002; Ohta et al., 2003; Tatsumi et al., 2003] when MG132 is included in the lysis buffer. ORC2 and ORC4 were almost exclusively found in the chromatin fraction throughout the cell-cycle (Fig. 2A). On the other hand, some ORC1 was found in the soluble fraction at all times, with more ORC1 released into the soluble fraction during mitosis, following mitotic synchronization. Mitotic ORC1 remaining in the chromatin fraction exhibited a slightly higher apparent molecular weight than soluble ORC1 or interphase ORC1, consistent with the reported phosphorylation of this subunit during mitosis [Okuno et al., 2001; Li et al., 2004; McNairn et al., 2005]. However, we did not detect any consistent variations in the total amount of ORC1 or the relative amounts of ORC1 in the soluble and chromatin fractions throughout interphase. Slight
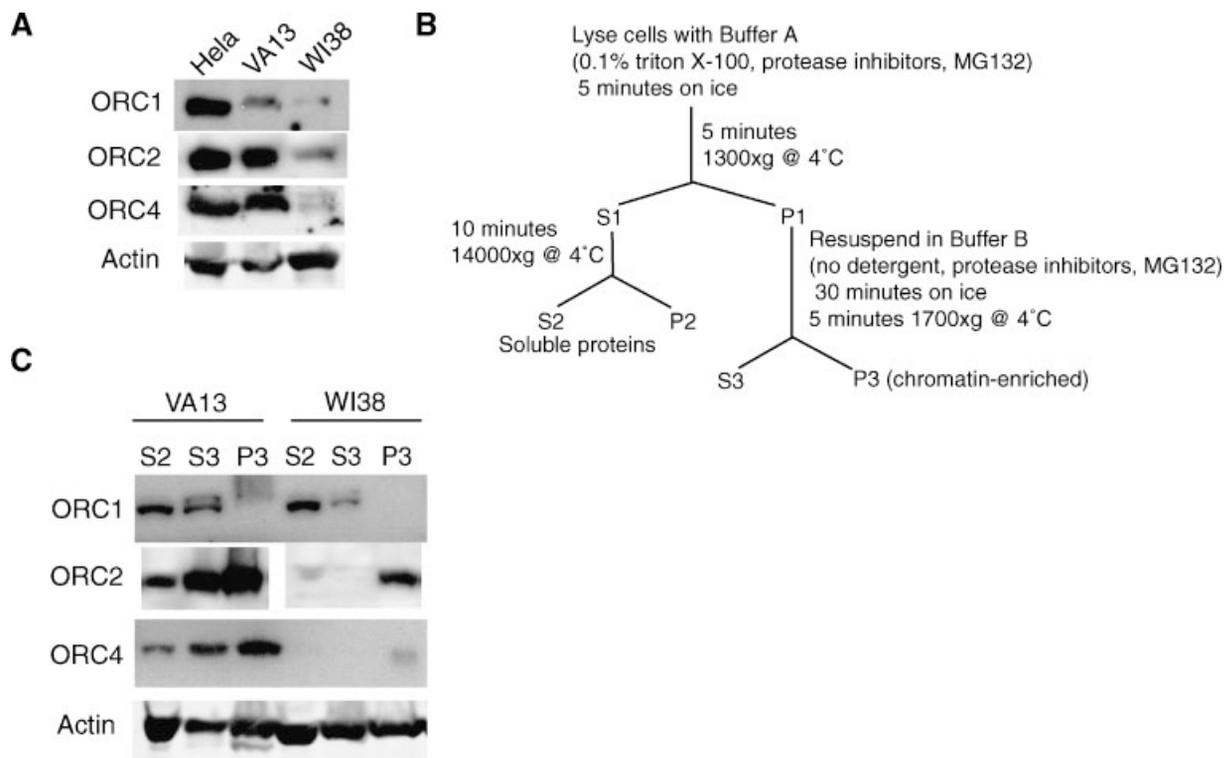
Fig. 1. Comparison of ORC subunit levels in primary, transformed, and cancer cell lines. **A:** Whole cell extracts were prepared from asynchronously growing WI38, VA13, or HeLa S3 cells, and equal amounts of protein (150 µg) were loaded onto an 8% SDS–PAGE gel. The extracts were immunoblotted with anti-human ORC1, ORC2, ORC4, and actin. **B:** Flow diagram of cell fractionation protocol. **C:** Asynchronously growing WI38 and VA13 cells were subject to the extraction protocol shown in (B). Equal amounts of protein (150 µg) from each fraction were immunoblotted as described in (A).

variations seen between time points in any given experiment were no more significant than variations seen between the same time points in different experiments. To verify that cells progressed through S-phase, cells were pulse-labeled with BrdU and the temporally specific spatial patterns of DNA synthesis were quantified (Fig. 2B). These spatial patterns have been well-characterized in the cell lines used here [Dimitrova and Gilbert, 1999; Panning and Gilbert, 2005; Wu et al., 2005]. As the cells passed through S-phase, there was a detectable increase in the apparent molecular weight of ORC1 present in the chromatin as compared to the soluble fractions (Fig. 2A), consistent with the smear for ORC1 seen in Figure 1B with VA13. We conclude that the steady state levels and chromatin-association properties of ORC1 in HeLa S3 cells do not fluctuate during S-phase when MG132 is included in the lysis buffer, as was recently demonstrated for human A39 and Raji cell lines [Ritzi et al., 2003].

In order to address whether transformation affects ORC:chromatin association, a human primary cell line, WI38, was synchronized at the

G1/S-phase boundary and fractionated at 0, 1, 4, 8, and 11 h after release (Fig. 3A). Consistent with Figure 1C, the majority of detectable ORC1 was present in the soluble fractions whereas ORC2 and ORC4 largely co-fractionated with chromatin throughout S-phase (Fig. 3A). Progression through S-phase was monitored by BrdU staining (Fig. 3B). A minor fraction of ORC1 that was associated with chromatin appeared to migrate at a slightly higher molecular weight, suggesting that this modification is not unique to the transformed state. This small amount of ORC1 found in the chromatin fraction was not specific to any time point during S-phase, but showed more variability from experiment to experiment than between time points. Hence, we do not detect any major changes in the properties of ORC subunits during S-phase progression.

To directly assess whether the differences in ORC-chromatin association seen between HeLa and WI38 are due to cellular transformation, we compared results with WI38 to those with an isogenic SV40-transformed cell line, VA13. VA13 cells were synchronized at the G1/S-phase
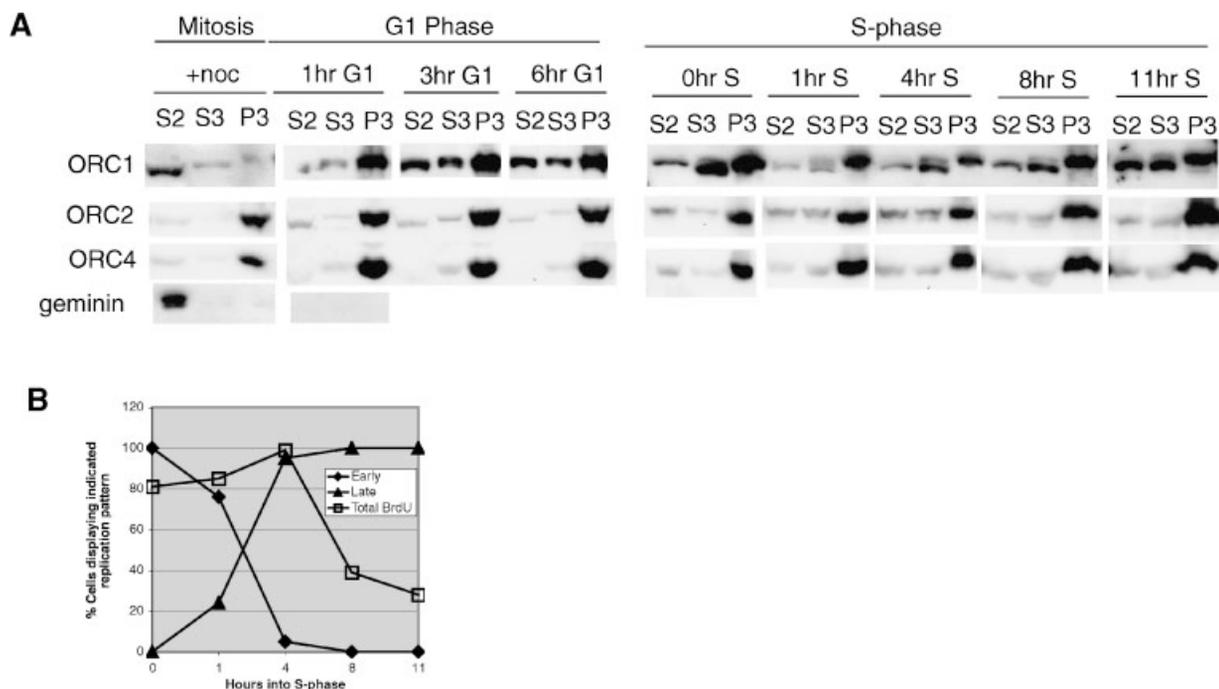
Fig. 2. ORC is stable and chromatin associated in HeLa cells. **A**: HeLa S3 cells were synchronized in mitosis and released into G1-phase as described in Materials and Methods. Aliquots of the same cells were arrested at the G1/S boundary with aphidicolin, and then released into S-phase. Cells were fractionated and immunoblots were performed as described in Figure 1B, except that equal proportions of each fraction were loaded onto SDS–PAGE gels prior to immunoblotting. The experiment was performed four times with similar results (three times with aphidicolin and once with mimosine). **B**: S-phase progression of HeLa cells from (A) was monitored by BrdU analysis. Cells plated on coverslips were pulsed for 30 min with BrdU, fixed with 70% ethanol and immunostained using anti-BrdU and a fluorescent secondary antibody. The percentage of cells displaying the indicated replication pattern was scored.

boundary and fractionated at 0, 1, 4, 8, and 11 h after release (Fig. 3C). BrdU analysis of replication patterns verified S-phase progression (Fig. 3D). ORC1 was found in both the soluble and chromatin-associated fractions throughout S-phase. In addition, there was a detectable decrease in the amount of ORC1 co-fractionating with chromatin during S-phase in this cell line and an increase in the apparent molecular weight of ORC1, selectively in the chromatin fraction. Although the less than twofold decrease in ORC1 found in the chromatin fraction was reproducible, comparisons of whole cell extracts from different times during S-phase did not reveal any changes in total ORC1 (not shown). While ORC2 and ORC4 were predominantly present in the chromatin fractions, both subunits also exhibited detectable soluble fractions, similar to WI38 (Fig. 3A,C). These results suggest that ORC is regulated differently in primary cells versus transformed lines. Moreover, although transformation has a greater effect on the overall expression levels of core subunits ORC2 and ORC4 than ORC1 (Fig. 1A), it selectively affects the extractability of ORC1 throughout the cell-cycle.

ORC-Chromatin Association in Isogenic Transformed Hamster Cell Lines

The above results with primary cell line WI38 differ from our previous observations using immortalized Chinese Hamster CHO cells, in which a significant proportion of ORC1 was bound to chromatin. To investigate the degree to which results might be different in CHO cells, we took advantage of an SV40 T-antigen derivative of our CHO cell line, JH-1 [Wu et al., 1998]. Unlike CHO cells, JH-1 enters S-phase in a serum- and adhesion-independent fashion. In contrast to SV40 transformed primary human cells, similar amounts of ORC subunits were detected in both cell lines (not shown). The amounts of all three subunits did not fluctuate throughout the cell-cycle in either cell line (not shown). Cellular extraction experiments also revealed similar proportions of soluble and chromatin-associated populations
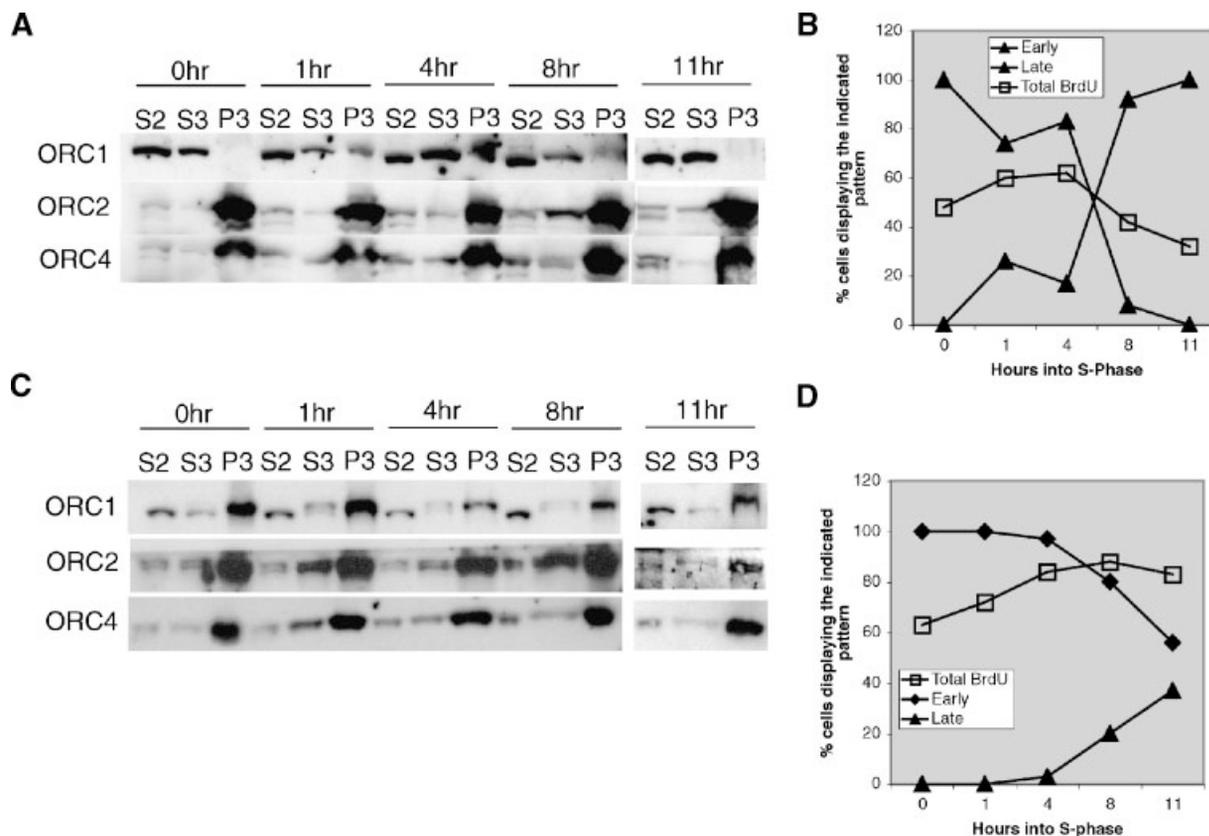
Fig. 3. ORC-chromatin association during S-phase in isogenic human primary and transformed cell lines. **A:** WI38 cells were synchronized at the G1/ S-phase boundary as described in Materials and Methods. Cells were fractionated as described in Figure 1B at 0, 1, 4, 8, and 11 h into S-phase and extracts were immunoblotted with anti-HsORC1, ORC2, and ORC4 as in Figure 2. **B:** Cells from (A) were pulse-labeled with BrdU at the indicated timepoints and the percentage of cells displaying early or late replication patterns was scored as in Figure 2B. **C:** VA13 cells were synchronized in mitosis and released into media containing 5 μg/ml aphidicolin for 14 h. Cells were then released and collected at the indicated timepoints. Cell extracts were immunoblotted as in (A). **D:** Cells from (C) were pulse-labeled with BrdU and the percentage of cells displaying early or late replication patterns was scored as in Figure 2B.

in both cell lines during mitosis and G1-phase (not shown). However, all three ORC subunits were detected almost exclusively in the chromatin fraction in JH-1 cells, whereas a significant proportion of all three subunits were detected in the soluble fraction of the untransformed CHO cells (compare Fig. 4A,C). Moreover, all three subunits were found to be stable throughout S-phase in JH-1(not shown), as previously reported in CHO cells [McNairn et al., 2005]. We conclude that transformation of immortalized CHO cells does not result in the overexpression of ORC subunits but does alter the chromatin-association properties of ORC1, ORC2, and ORC4.

DISCUSSION

Virtually all studies of mammalian ORC subunits have been performed in highly trans-

formed human cancer cell lines. To determine whether cellular transformation affects the regulation of ORC, we have directly compared the expression and chromatin-association of ORC subunits in primary and transformed human cell lines, as well as isogenic transformed hamster cell lines. Our findings indicate that human ORC subunits are overexpressed as a result of cellular transformation, which results in increased ORC-chromatin association. Similarly, viral transformation of immortalized hamster cells also increased the amount of ORC subunits co-fractionating with chromatin, although the overall expression levels were not affected in this case. These results indicate that cellular transformation significantly affects properties of ORC that may be important for the regulation of S-phase.

It is interesting to note that the levels of ORC2 and ORC4 were substantially increased in
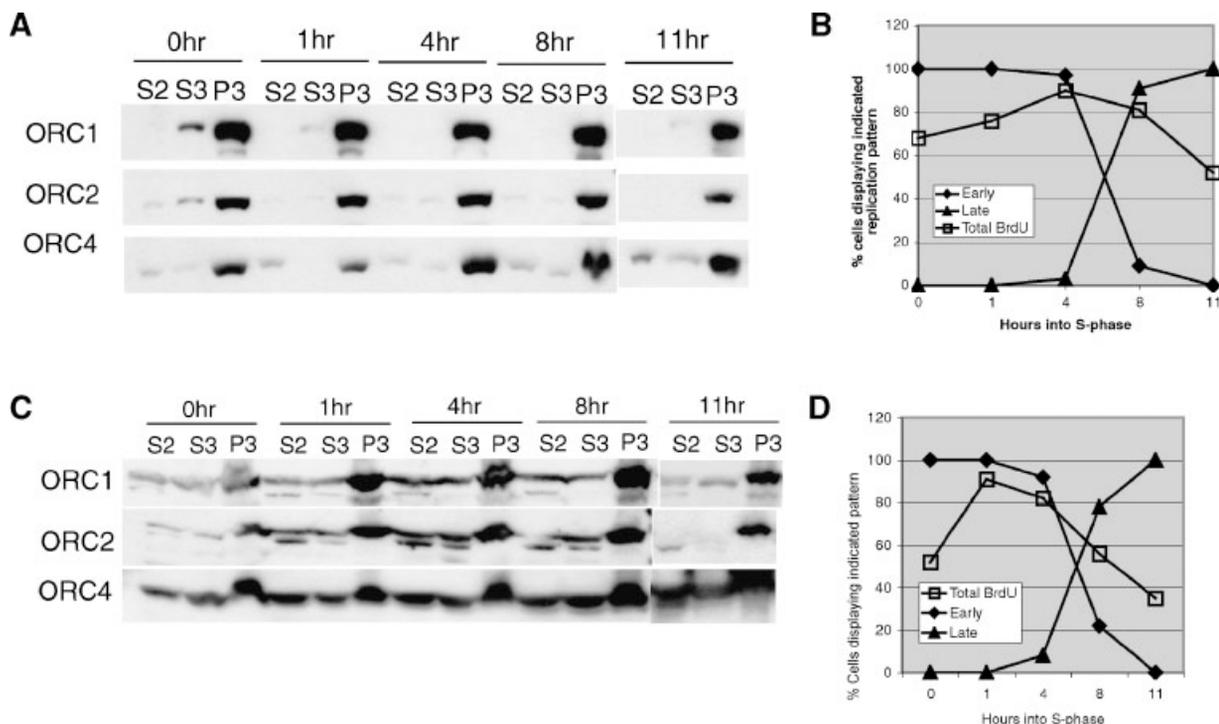
Fig. 4. ORC-chromatin association in isogenic hamster cell lines (**A**) JH-1 cells were synchronized at the G1/S-phase boundary, released, and collected at 0, 1, 4, 8, and 11 h. Fractionated extracts were immunoblotted with anti-ORC1, ORC2, and ORC4 as in Figure 2, except that an antibody specific to hamster ORC1 was used. The experiment was repeated three times with similar results. **B**: S-phase progression of JH-1 cells from (A) was monitored by BrdU analysis as described in Figure 2. **C**: CHO cells were synchronized and fractionated extracts analyzed as in (A). The experiment was repeated at least three times with similar results [McNairn et al., 2005]. **D**: S-phase progression in (C) was determined as described in Figure 2B.

response to transformation of primary WI38 cells, whereas ORC1 protein levels were increased to a lesser extent (Fig. 1). This was surprising, since ORC1 is E2F-regulated, and SV40 transformation is known to deregulate such genes [Nevins, 1992; Zwicker et al., 1999], whereas ORC2 and ORC4 are not E2F-regulated [Ohtani et al., 1996; Springer et al., 1999]. On the other hand, HeLa cells harbor significantly more ORC1 than the SV40-transformed primary cells, but the levels of ORC2 and ORC4 are similar between these lines. Finally, SV40 transformation of immortalized CHO cells had no effect on the levels of any of the ORC subunits, but only affected their association with chromatin. Since the experimental manipulation of the levels of some ORC subunits can affect the levels of the other subunits [Ohta et al., 2003; Prasanth et al., 2004; Machida et al., 2005], the possible mechanisms regulating the levels of the different subunits are likely complex, acting at both transcriptional and post-transcriptional levels. Moreover, there have now been several reports demonstrating

that other proteins regulating the assembly of pre-RCs, including Cdc6, Cdt1, and geminin, are upregulated in transformed and cancer cells [Karakaidos et al., 2004; Xouri et al., 2004]. The extent to which the levels of these proteins affect each other has not been explored. The simplest interpretation of these results is that different aspects of the transformation process can affect the levels and properties of ORC subunits in different ways. Further investigation of these pathways is warranted.

Our results shed some light upon the discrepancies in the literature concerning the cell-cycle regulated behavior of ORC1. Given the complex relationship that our results reveal between cellular transformation, immortalization, and the properties of ORC, it is not surprising that different groups have obtained different results. Our results suggest that, when ORC subunit levels are low, as is the case in primary cells, ORC1 is readily solubilized by cellular extraction methods, whereas cellular transformation increases the levels of chromatin-association. In addition, it is apparent that

chromatin-associated ORC1 migrates at a slightly higher molecular weight. This post-translational modification may result in the preferential degradation of a sub-population of ORC1 following cellular lysis, which would provide one source of variability. By including MG132 in the lysis buffer, this degradation is averted [Ritzi et al., 2003]. MG132 is an inhibitor of the 26S proteasome, but it also inhibits many other cellular protease such as cathepsin B and calpains [Mellgren, 1997; Rodgers and Dean, 2003]. Even in the presence of MG132, we find the amounts or ORC1 detected in the chromatin-containing fraction to be quite variable and we note that existing reports always detect 30–50% of ORC1 remaining with chromatin [Mendez et al., 2002; Ohta et al., 2003; Ritzi et al., 2003; Tatsumi et al., 2003]. Hence, a combination of variable release of ORC1 from chromatin during extraction under these commonly used conditions, the potential lability of ORC1 in cellular lysates, and the effects of cellular immortalization and transformation on the chromatin-association of ORC subunits can easily account for the variable results reported.

It is important to note that the absence of a protein from a chromatin preparation in no way implies that the protein is not associated with chromatin in vivo, as this possibility cannot be distinguished from the removal of a protein during extraction. For example, histone H1 is found in a soluble fraction under the same conditions used to examine ORC1 chromatin association [Li and DePamphilis, 2002; Li et al., 2004]. Moreover, live cell photobleaching of GFP-tagged ORC subunits did not indicate any change in the residence times of ORC1 and ORC4 subunits throughout the cell-cycle [McNairn et al., 2005], even though a detectable proportion of both subunits was solubilized by extraction. In short, while cellular extraction protocols can measure changes in the properties of proteins within a controlled set of experiments, they cannot rule out in vivo interactions.

In summary, we demonstrate that the properties of ORC subunits can vary dramatically in different cell lines. Using isogenic cell lines, we demonstrate that different aspects of transformation can result in either overexpression, increased association with chromatin, or both. These results extend the potential link between cancer and deregulation of pre-RC proteins, and

underscore the importance of considering the transformation status of cell lines when working with these proteins.

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