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Expression of the *HsOrc1* Gene, a Human *ORC1* Homolog, Is Regulated by Cell Proliferation via the E2F Transcription Factor

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The initiation of DNA replication in *Saccharomyces cerevisiae* requires the action of a multisubunit complex of six proteins known as the origin recognition complex (ORC). The identification of higher eukaryotic homologs of several ORC components suggests a universal role for this complex in DNA replication. We now demonstrate that the expression of one of these homologs is regulated by cell proliferation. Expression of the human *Orc1* gene (*HsOrc1*) is low in quiescent cells, and it is then dramatically induced upon stimulation of cell growth. In contrast, expression of the *HsOrc2* gene does not appear to be similarly regulated. We have isolated the promoter that regulates *HsOrc1* transcription, and we show that the promoter confers cell growth-dependent expression. We also demonstrate that the cell growth control is largely the consequence of E2F-dependent negative transcription control in quiescent cells. Activation of *HsOrc1* transcription following growth stimulation requires G_1 cyclin-dependent kinase activity, and forced E2F1 expression can bypass this requirement. These results thus provide a direct link between the initiation of DNA replication and the cell growth regulatory pathway involving G_1 cyclin-dependent kinases, the Rb tumor suppressor, and E2F.

The control of cell proliferation is critically important to the normal development and homeostatic maintenance of multicellular organisms, and the loss of this control is central to the development of cancer cells. Much of the understanding of the molecular mechanisms responsible for cell growth control derives from combined genetic and biochemical studies of yeast systems. These studies have led to the identification of a variety of regulatory activities that control the progression of cells through the cell cycle. Particularly important are the events associated with the cellular decision to pass through the G_1 checkpoint known as Start and then enter S phase, initiating the replication of the cellular chromosomes. It is now clear that this decision is governed by the action of the cyclin-dependent protein kinases that control the activity of critical components of the cell cycle.

More recent studies, directed at events involved in mammalian cell growth control, have revealed that regulators of cell cycle progression in yeasts have close counterparts in higher eukaryotic cells. The yeast and mammalian G_1 cyclins and their associated kinases are related in sequence, and indeed, human G_1 cyclins can substitute for the yeast proteins (33). Moreover, like their yeast counterparts, the mammalian G_1 cyclins and associated kinases are also essential for progression through G_1 and into S phase (2, 45, 49). A key target for the action of mammalian G_1 cyclin-dependent kinases is the retinoblastoma protein (Rb), one member of a family of proteins that function to regulate mammalian cell growth (57). Rb phosphorylation, mediated by the G_1 cyclin-dependent kinases, inactivates the growth-regulating activity of Rb (14, 17, 27). This inactivation of Rb function coincides with an inactivation of the capacity of Rb to bind to the E2F transcription factor (9, 27), thus allowing an accumulation of E2F activity during the G_1 phase of the cell cycle.

E2F appears to control the transcription of a group of genes that encode proteins important for S phase as well as cell cycle regulatory proteins (21, 29, 42). The ability of Rb to interact with E2F directly correlates with the ability of Rb to arrest cell growth in G_1, on the basis of analysis of Rb mutants (22, 46, 47). Overexpression of the E2F1 protein induces quiescent mammalian cells to enter S phase (26, 48, 54), and ectopic production of a *Drosophila* E2F homolog induces a substantial increase in the number of cells in S phase in larval imaginal discs (1, 14a), results which are consistent with other work demonstrating a requirement for E2F activity for embryonic S phase (16). Clearly, E2F plays an important role in regulating the transition of cells through G_1 and into S phase.

Although the E2F-mediated activation of genes such as the DHFR, thymidine kinase, ribonucleotide reductase, and DNA polymerase α genes must be an important part of the induction of DNA replication, one might anticipate that these proteins would not be sufficient to allow the initiation of replication. A large body of work with yeast systems, primarily with *Saccharomyces cerevisiae*, has led to the identification of both *cis*-acting sites and *trans*-acting proteins that control the initiation of replication. Originally defined as sequences allowing autonomous replication of plasmids, termed ARS elements, these sequences are now known to represent a component of the origins that are essential for replication of the yeast cell chromosomes (7, 43, 52). These elements are binding sites for a multisubunit complex of proteins known as the origin recognition complex (ORC) that recognizes the ARS element as well as adjacent sequences (5, 12, 50, 51) and that is essential for the initiation of replication (3, 18, 34, 35, 37).

In addition to the ORC components, other proteins are likely involved in the formation of the functional replication complex and thus may determine the cell cycle-specific changes

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in ARS interactions. For instance, the product of the *CDC6* gene, which is related in amino acid sequence to the Orc1 protein, functionally interacts with ORC, as seen by the fact that *CDC6* overexpression can suppress an Orc5 mutation (34), and other experiments have demonstrated a physical interaction between *CDC6* and ORC (34). Moreover, in the absence of *CDC6*, the footprint pattern at ARS sequences changes to a postreplication cell cycle pattern that is normally seen at the end of G_1, prior to S phase (13). A homologous *S. pombe* protein, Cdc18, may function in a similar manner, since Cdc18 is clearly essential for DNA replication (28) and overexpression of the Cdc18 product has been shown to be sufficient for S-phase induction (38, 44). Thus, Cdc18 appears to be a rate-limiting determinant of initiation of DNA replication and serves to couple replication with passage through Start, since transcription of the *cdc18* gene is activated at G_1-S by the *cdc10-sct1* transcriptional activator (6, 36).

Given the role of the E2F transcription factor in regulating cell cycle progression and the fact that overproduction of E2F activity is sufficient in many instances to induce S phase, we have sought to determine if mammalian homologs of the yeast replication proteins might be subject to E2F control. We find that a human homolog of *ORC1* (*HsOrc1*) is tightly regulated as a function of cell proliferation. Moreover, we demonstrate that the control of *HsOrc1* transcription is dependent on E2F.

MATERIALS AND METHODS

Isolation of human *HsOrc1* promoter sequences. A human placenta genomic DNA library (1.5×10^6 clones) (Clontech) was screened with a 580-bp fragment corresponding to nucleotides (nt) 69 to 648 of the 5′ end of the published human *HsOrc1* cDNA as previously described (25). An *HsOrc1* cDNA clone containing the entire coding region (extending 5′ to nt 69 of the published sequence) was positively selected from a Superscript human leukocyte cDNA library (Life Technologies) by using the GeneTrapper system (Life Technologies). The positive selection oligonucleotide corresponded to nt 1771 to 1792 of the published sequence. Sequence information for this region was obtained from a partial *HsOrc1* cDNA clone isolated by standard DNA hybridization methods using an *Eco*RI-*Nco*I fragment of cDNA clone 121313 (GenBank nucleotide sequence accession no. T96858) as a probe.

RNase protection assay. The RNase protection assay was performed as described previously (25). An antisense RNA probe to *HsOrc1* sequences spanned nt +129 to −353.

Gel mobility shift assays. Gel mobility shift assays were performed as described previously (60). Double-stranded oligonucleotides containing overlapping putative E2F sites in the *HsOrc1* promoter (nt −23 to +14) and a mutant version of this sequence were used as probes in some assays or as competitors at a molar excess of 100. Sequences included wild type, 5′-CGGGGCCACGCCGATTGGCGCGAAGTTTTCTTTTCTC-3′, 3′-GCCCCGGTGCGGCTAACCGCGCTTCAAAAGAAAAGAG-5′; and mutant, 5′-CGGGGCCACGCCGATTGGATCGAAGTTTTCTTTTCTC-3′, 3′-GCCCGGTGCGGCTAACCTAGCTTCAAAAGAAAAGAG-5′.

Construction of plasmids. A *Sac*I fragment (nt −8000 to +129) and a *Hin*dIII fragment (nt −1053 to +182) were subcloned into the *Sac*I and *Hin*dIII sites of pGL2 basic (Promega) to make pHsOrc1-Luc(−8000) and pHsOrc1-Luc(−1053), respectively. pHsOrc1-Luc(−353) was made by removal of a *Bgl*II-*Nco*I fragment from pHsOrc1-Luc(−1053) followed by religation after creation of blunt ends with Klenow polymerase. The mutant of putative E2F sites in the pHsOrc1-Luc(−1053) construct, generating pHsOrc1-Luc(E2F-), was made by site-directed mutagenesis which changed ATTGGCGCGAAG (nt −10 to +2) to ATTGGATCGAAG. The E2F1, E2F2, and E2F3 expression plasmids, as well as the pE2F1-Luc(−242) plasmid, have been described previously (25).

Transfection assays. Transfection of REF52 cells and luciferase assays were performed as described previously (26). All assays were done in duplicate.

Northern (RNA) blot analysis. Gel electrophoresis, transfer to nitrocellulose membrane, and hybridization were done as described previously (10) except that the hybridization and wash conditions for the Northern blots shown in Fig. 1 were as described by Johnson et al. (25). *HsOrc1* (nt 88 to 2963) and *HsOrc2* (nt 64 to 2089) cDNAs were cloned by PCR from 293 cell cDNA into the *Eco*RV site of pBluescript SK$^-$ and cutout fragments were used as probes. The E2F1 probe was a *Bam*HI fragment cut out from pDC E2F (26).

Cell culture. Human foreskin fibroblast (HFF) and REF52 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. For Northern blot experiments after serum stimulation, HFF cells were serum starved in Dulbecco modified Eagle medium containing 0.1% fetal calf serum for 48 h, serum stimulated by addition of fetal calf serum to a final concentration of 20%, and harvested at indicated time points.

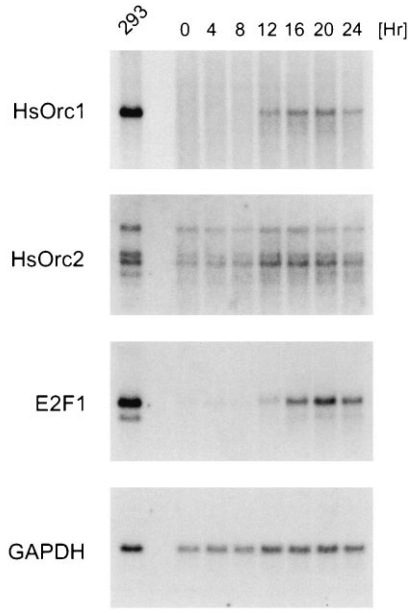

FIG. 1. Expression of *HsOrc1* is regulated by cell growth. Northern blot analysis of *HsOrc1*, *HsOrc2*, and E2F1 mRNA following stimulation of cell growth. Human foreskin fibroblast (HFF) cultures were serum starved and serum stimulated as described in Materials and Methods. Cells were harvested at the indicated times after addition of serum, and poly(A$^+$)RNA was prepared from 170 μg of total RNA from each time point, separated by agarose gel electrophoresis, and then transferred to nitrocellulose. Poly(A$^+$)RNA prepared from 440 μg of 293 cell RNA was used as a positive control. The blot was probed separately with the human *HsOrc1*, *HsOrc2*, and E2F1 cDNAs.

Infection with recombinant adenoviruses. The generation of the E2F1 recombinant adenovirus has been described previously (53). The p21 adenovirus was similarly constructed by ligation of the *Bgl*II-*Bam*HI fragment containing human p21 from CMV-p21 (11) into the *Bam*HI-digested recombination plasmid pGEM-CMV. Ad-E2F2 was constructed by ligation of the *Bgl*II fragment from pCMV-E2F2 (24) into the *Bam*HI-digested pcDNA3 (Invitrogen). The resulting construct (E2F2-pcDNA3) was digested with *Hin*dIII-*Not*I, and the resulting 1,401-bp E2F2 fragment was ligated into the *Hin*dIII-*Not*I-digested pGEM-CMV. Ad-E2F3 was constructed by ligation of the *Eco*RI fragment containing E2F3 from pBSK-E2F3 (32) into EcoRI-digested pcDNA3. The 1,570-bp *Hin*dIII-*Not*I fragment from the resulting clone (E2F3-pcDNA3) was then ligated into the *Hin*dIII-*Not*I-digested pGEM-CMV. Adenoviruses were purified by CsCl gradient centrifugation (41). Density-arrested REF52 cells were infected and replated as described previously (11).

RESULTS

Expression of the human *ORC1* gene is regulated by cell proliferation. The recent isolation of human homologs of two of the yeast genes encoding Orc proteins (19, 39) has provided a means to explore the role of these gene products in the control of DNA replication in mammalian cells. As a start, we have sought to define the expression of these genes in relation to the control of cell growth. For these assays, we utilized human foreskin fibroblast cultures (HFFs) that were brought to quiescence by serum starvation and then serum stimulated to promote synchronous entry into the cell cycle. Cells were harvested at quiescence or at various times following serum stimulation, and poly(A$^+$)RNA was prepared and then analyzed by Northern blot for expression of *HsOrc1* and *HsOrc2*. Under the conditions of this experiment, S phase begins at 16 h following serum stimulation, as measured by bromodeoxyuridine incorporation, and reaches a maximal level at 24 h (data not shown).

As can be seen by the analysis shown in Fig. 1, *HsOrc1* mRNA levels changed dramatically following growth stimulation of the quiescent fibroblasts. Very little if any *HsOrc1* mRNA could be detected in the quiescent cells, and the levels began to increase by 12 h following addition of serum, with peak expression occurring approximately 20 h after serum addition. The kinetics of accumulation are very similar to those of the E2F1 mRNA, as described for previous experiments (23, 25, 55) and as shown in Fig. 1. In contrast, the level of the *HsOrc2* mRNA was relatively unchanged during the course of the experiment. Although there was a modest increase in *HsOrc2* mRNA at the 12-h point, the same change was seen in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. It thus is clear from these results that the expression of at least one gene encoding a human ORC component, likely to be involved in the initiation of DNA replication, is tightly regulated by cell proliferation.

Isolation of the *HsOrc1* promoter. Given the fact that the *HsOrc1* gene is regulated by cell proliferation, we have sought to identify the transcriptional regulatory sequences controlling this expression in order to define the basis for the regulation. To identify sequences containing the *HsOrc1* promoter, a fragment from the 5′ end of the human *HsOrc1* cDNA sequence was used to screen a human placenta genomic DNA library. Four positive clones were isolated from a screen of 1.5×10^6 recombinant phage. An 8.0-kb *Sac*I fragment and a 1.2-kb *Hin*dIII fragment, which hybridized to this probe, were subcloned for further analysis. Sequence analysis of the *Hin*dIII fragment confirmed that this fragment contained the 5′ sequence of the *HsOrc1* cDNA (Fig. 2A).

We used an RNase protection assay to identify the sequences corresponding to the 5′ end of the *HsOrc1* mRNA. By using an RNA probe spanning the upstream sequences of the *HsOrc1* cDNA, protected fragments of multiple sizes ranging from 103 to 174 nt were detected (Fig. 2B). The presence of heterogeneous transcription start sites is not unexpected, given the lack of a recognizable TATA element. These transcriptional start sites are clustered around two potential E2F binding elements arranged in an overlapping fashion (Fig. 2A). That these putative E2F elements do indeed represent E2F binding sites was shown by a gel mobility shift assay in which an oligonucleotide containing the *HsOrc1* E2F sequence competed for E2F binding to a probe containing E2F sites from the DHFR promoter, whereas a mutant form of this sequence did not compete (Fig. 2C). In addition, assays for the ability of E2F to directly bind to an *HsOrc1* probe demonstrated binding to the wild-type sequence but not a mutant that eliminated the E2F consensus site (Fig. 2D). We conclude from these results that there are E2F binding sites in the promoter of the *HsOrc1* gene.

Control of *HsOrc1* promoter activity in relation to cell proliferation. To determine if the 5′ flanking region of the *HsOrc1* gene did indeed possess sequences responsible for the growth-dependent accumulation of the endogenous *HsOrc1* mRNA, we fused these sequences to a luciferase reporter gene and assayed the activity of the promoter in serum-starved and -stimulated REF52 rat embryo fibroblasts. We compared the activities of three constructs containing various amounts of *HsOrc1* 5′ flanking sequence. The *HsOrc1*-luciferase (luc) reporter plasmids were transfected into REF52 cells along with a plasmid containing the β-galactosidase gene under the control of the constitutively active cytomegalovirus (CMV) promoter as an internal control. Transfected cells were brought to quiescence by serum starvation, and then serum was added to stimulate re-entry into the cell cycle. Cells were harvested, extracts were prepared, and assays for luciferase activity and β-galactosidase activity were performed. A representative experiment, whose results are shown in Fig. 3, demonstrates that the activity of the *HsOrc1* promoter was low in quiescent and early G_1 cells and then rapidly increased as cells progressed through G_1. Essentially the same activity was observed with a promoter containing 353 nt of 5′ flanking sequence as was with constructs containing up to 8,000 nt of *HsOrc1* 5′ flanking sequence. In each case, the pattern of expression closely followed the accumulation of the *HsOrc1* mRNA, indicating that the essential regulatory elements are contained within the 353 nt of *HsOrc1* 5′ flanking sequence.

E2F-dependent regulation of the *HsOrc1* promoter. Given the presence of E2F recognition sequences within the *HsOrc1* promoter, we have utilized transient transfection assays to assess the role of E2F in the control of *HsOrc1* promoter activity. The *HsOrc1*-luc plasmids were transfected into REF52 cells together with plasmids expressing E2F products. Following transfection, the cells were grown in medium containing 0.1% serum for 36 h, extracts were prepared, and aliquots were assayed for luciferase activity. As shown in Fig. 4A, *HsOrc1* promoter activity was stimulated ~5 fold by the coexpression of the E2F1 product. In addition, plasmids expressing the E2F2 and E2F3 products were also able to activate transcription directed by the *HsOrc1* promoters. That this is a direct effect involving the E2F sites in the *HsOrc1* promoter was demonstrated by the fact that mutation of the E2F recognition sequences eliminated the E2F1-mediated stimulation of *HsOrc1* promoter activity (Fig. 4B). Interestingly, the activity of the *HsOrc1* promoter lacking E2F binding sites was elevated relative to that of the wild-type promoter, suggesting a negative role for E2F in the control of the promoter.

In view of the cell growth control of the *HsOrc1* gene evident in the Northern blot assays, as well as work that has shown a role for E2F in cell growth-regulated transcription control, we have investigated the role of the *HsOrc1* E2F elements in the cell growth-dependent control of *HsOrc1* expression. The wild-type and the E2F mutant promoter-luciferase constructs were transfected into REF52 cells, the cells were brought to quiescence by growth in 0.1% serum for 36 h, and the cells were then stimulated by the addition of serum to a 20% final concentration. Extracts were prepared at various times after stimulation and assayed for luciferase activity. As can be seen by the data in Fig. 5, mutation of the overlapping E2F sites in the *HsOrc1* promoter abolished cell growth control of promoter activity. The activity of the mutant promoter in quiescent cells was elevated 20-fold relative to the wild-type promoter, attaining a level of activity equivalent to that of the fully stimulated wild-type promoter. We conclude that the activity of the *HsOrc1* promoter is regulated by E2F during a growth response and that this is primarily a negative control, as has been observed in past experiments analyzing both the B-myb promoter (30) and the E2F1 promoter (23, 25, 40). The ability of the various E2F products to activate the *HsOrc1* promoter in quiescent cells, as shown by results in Fig. 4, likely reflects the ability of the overproduced E2F proteins to compete for binding with an E2F-p130 repressor which is found in quiescent cells.

Growth-dependent activation of *HsOrc1* expression requires G_1 cyclin-kinase activation which can be bypassed by E2F overexpression. The finding that control of the *HsOrc1* promoter in quiescent cells involves negative regulation by E2F is consistent with previous results demonstrating a role for E2F-Rb family member complexes in transcription repression (23, 25, 40, 58, 59). The repression of *HsOrc1* transcription likely is due to the E2F4-p130 complex that predominates in the quiescent REF52 cells; activation of transcription would

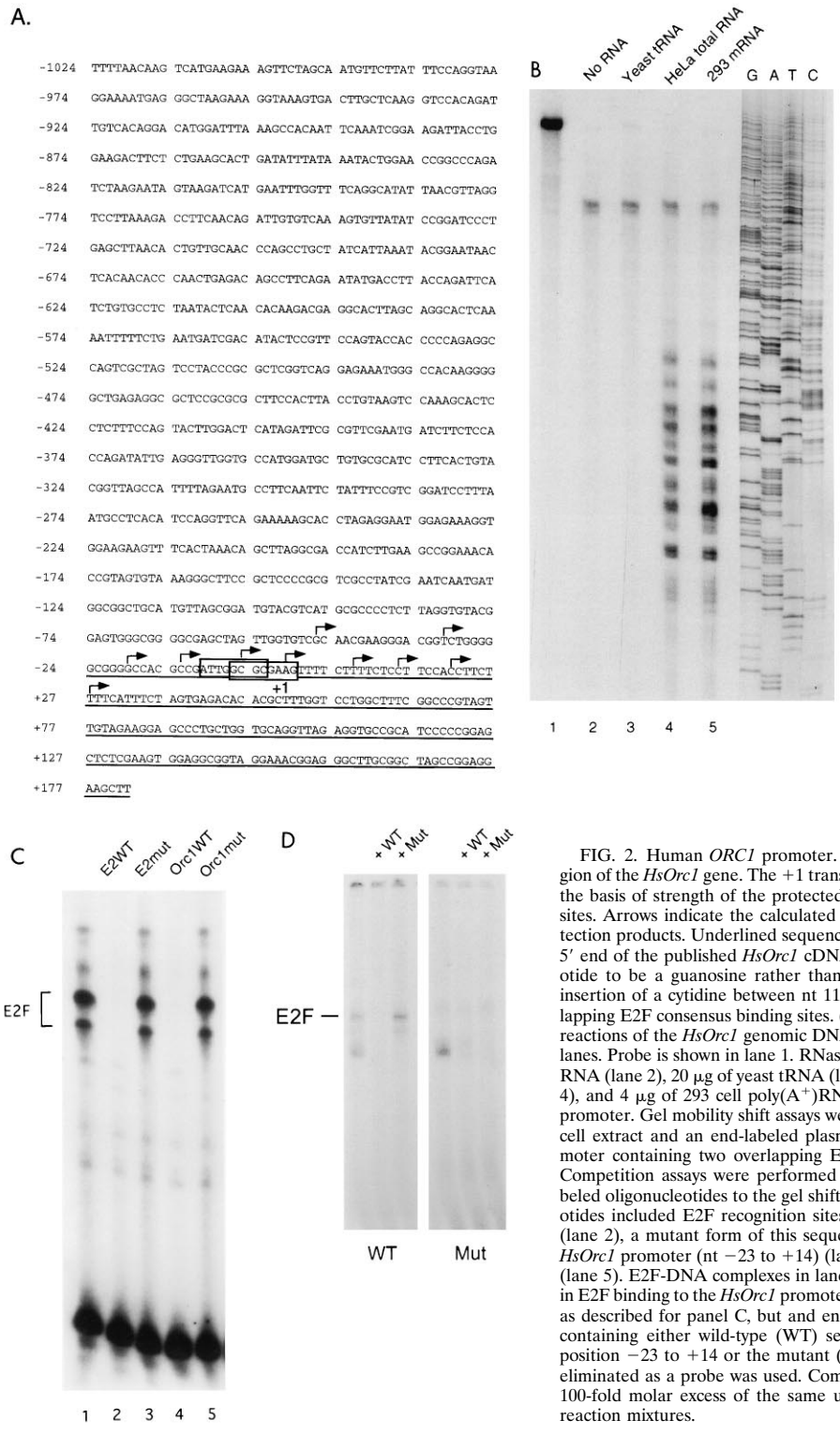

FIG. 2. Human *ORC1* promoter. (A) DNA sequence in the 5′ flanking region of the *HsOrc1* gene. The +1 transcription start site was chosen arbitrarily on the basis of strength of the protected products as well as proximity to the E2F sites. Arrows indicate the calculated ends of each of the 10 major RNase protection products. Underlined sequence matches untranslated sequence from the 5′ end of the published *HsOrc1* cDNA (19), except that we find the first nucleotide to be a guanosine rather than the reported cytidine as well as find an insertion of a cytidine between nt 11 and 12. Boxed sequences represent overlapping E2F consensus binding sites. (B) RNase protection analysis. Sequencing reactions of the *HsOrc1* genomic DNA were run as size markers in the far right lanes. Probe is shown in lane 1. RNase protection reaction mixtures included no RNA (lane 2), 20 μg of yeast tRNA (lane 3), 20 μg of HeLa cell total RNA (lane 4), and 4 μg of 293 cell poly(A$^+$)RNA (lane 5). (C) E2F binds to the *HsOrc1* promoter. Gel mobility shift assays were performed with partially purified HeLa cell extract and an end-labeled plasmid DNA fragment from the DHFR promoter containing two overlapping E2F recognition sites (lane 1) as a probe. Competition assays were performed by adding 100-fold molar excess of unlabeled oligonucleotides to the gel shift reaction mixtures. Competitor oligonucleotides included E2F recognition sites from the E2 promoter (nt −34 to −69) (lane 2), a mutant form of this sequence (lane 3), putative E2F sites from the *HsOrc1* promoter (nt −23 to +14) (lane 4), and a mutant form of this sequence (lane 5). E2F-DNA complexes in lanes 1, 3, and 5 are indicated. (D) Specificity in E2F binding to the *HsOrc1* promoter. Gel mobility shift assays were performed as described for panel C, but and end-labeled double-stranded oligonucleotide containing either wild-type (WT) sequence from the *HsOrc1* promoter from position −23 to +14 or the mutant (Mut) version in which the E2F sites were eliminated as a probe was used. Competition assays were performed by adding 100-fold molar excess of the same unlabeled oligonucleotides to the gel shift reaction mixtures.

occur as the repressor complex decays following growth stimulation. Various experiments suggest that control of Rb family proteins during a growth response is a function of the action of G_1 cyclin-dependent protein kinases that phosphorylate these proteins. Indeed, recent experiments show that the decay of the E2F4-p130 complex following growth stimulation is dependent on G_1 cyclin-dependent kinase activity (56).

To address the role of G_1 cyclin-dependent kinases in the activation of *HsOrc1* transcription, we have made use of a recombinant adenovirus that expresses the G_1 cyclin-depen-

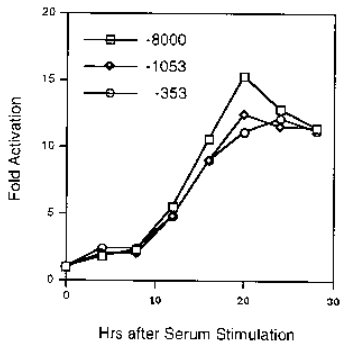

FIG. 3. Cell growth control of *HsOrc1* promoter activity. REF52 cells were transfected with 5 μg of pHsOrc1-Luc(−8000), pHsOrc1-Luc(−1053), or pHsOrc1-Luc(−353) together with 1 μg of a CMV β-galactosidase vector as an internal control. Transfected cells were brought to quiescence and then stimulated with serum for the indicated time. At each time point, cells were harvested, extracts were prepared, and luciferase and β-galactosidase activities were measured as described in Materials and Methods. Luciferase activity was normalized to β-galactosidase activity.

dent kinase inhibitor p21. Since the recombinant virus can infect an entire population of quiescent cells, it is possible to measure the expression of endogenous genes in their normal context. The experimental approach involves the growth of REF52 cells to a density arrest, which thus synchronizes the cells in early G_1. The arrested cells are then infected with the Ad-p21 virus and then released from the growth arrest by replating in fresh medium. The expression of p21 under these conditions effectively inhibits G_1 cyclin-dependent kinase activity and blocks S-phase entry as measured by bromodeoxyuridine incorporation (data not shown). As shown by the analysis in Fig. 6A, this p21-mediated inhibition of G_1 cyclin-dependent kinase activity also eliminated the induction of *HsOrc1* mRNA accumulation that is normally associated with the growth stimulation of quiescent cells. It would thus appear that induction of *HsOrc1* expression, which represents the relief of E2F-mediated repression, is dependent on the action of G_1 cyclin-dependent kinase, consistent with the inactivation of Rb family member proteins by phosphorylation.

We have previously shown that E2F1 expression can bypass a p21-mediated G_1 arrest (11). Presumably, this represents the activation of genes that are normally regulated by E2F accumulation as well as the elimination of E2F-mediated repression. As shown by results of the assays in Fig. 6B, expression of the E2F1 product bypassed the p21-mediated block, resulting in an efficient induction of *HsOrc1* mRNA (Fig. 6B). This E2F-mediated bypass was not due to simply the relieving of the effects of p21 on kinase function, since kinase activity is not restored under these conditions (data not shown). Rather, the induction is likely due to the ability of the overproduced E2F to compete with an E2F repressor, likely an E2F4- or E2F5-p130 complex, for binding to the E2F elements. We thus conclude that the activation of *HsOrc1* expression during a cell growth response is dependent on the action of G_1 cyclin-dependent kinases, likely through the elimination of E2F-dependent repression of the *HsOrc1* gene in quiescent cells.

DISCUSSION

The results we present here provide evidence for a direct link between the events involved in the initiation of DNA replication and the cell growth regulatory pathway involving the G_1 cyclin-dependent kinases, the Rb tumor suppressor, and the E2F family of transcription factors. In particular, these experiments demonstrate that an E2F repressor activity, likely an E2F4-p130 or E2F5-p130 complex, on the basis of the fact that these predominate in quiescent cells, is responsible for repressing the expression of the *HsOrc1* gene. The relief of this repression as cells are stimulated to proliferate requires the action of G_1 cyclin-dependent kinase activity. These results thus demonstrate a role for E2F in the control of various critical cell growth regulatory activities and provide strong evidence for a central role for E2F activity in regulating cell growth progression, including the initiation of DNA replication.

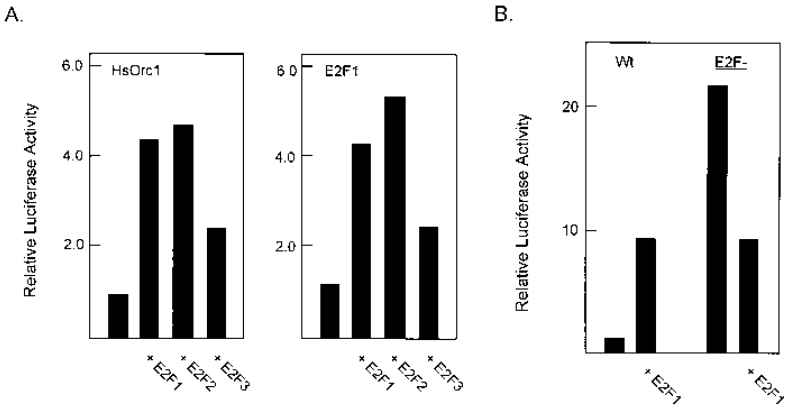

FIG. 4. E2F family members stimulate the *HsOrc1* promoter. (A) REF52 cells were transfected with 5 μg of the *HsOrc1*-luc reporter vector and 0.1 μg of CMV-driven expression vectors for either E2F1, E2F2, E2F3, or a control CMV vector, pCDNA3, as indicated, along with 1 μg of pCMV–β-gal as an internal control. For comparison, a luciferase construct under the control of the human E2F1 promoter (E2F1-luc) was assayed in a similar manner (right panel). Transfected cells were serum starved for 36 h, at which time cells were harvested, extracts were prepared, and luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity, and it is plotted for each cotransfected CMV expression vector as relative activity with the control pCDNA3 level set at 1. A representative experiment is shown. (B) REF52 cells were transfected with 5 μg of the *HsOrc1*-luc reporter vector containing the wild-type E2F sequence (Wt) or a mutant version in which the E2F sites were altered by mutation (E2F−). The cells were cotransfected with the E2F1 vector or a control CMV vector, pCDNA3, as indicated, along with 1 μg of pCMV–β-gal as an internal control. Transfected cells were serum starved for 36 h, at which time cells were harvested, extracts were prepared, and luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity, and it is plotted for each cotransfected CMV expression vector as actual corrected luciferase activity. A representative experiment is shown.

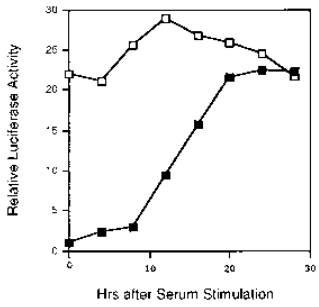

FIG. 5. E2F-dependent control of the *HsOrc1* promoter in response to cell proliferation. REF52 cells were transfected with 5 μg of either the reporter plasmid pHsOrc1-Luc(−1053) (filled squares) or an E2F site mutant vector, pHsOrc1-Luc(−E2F) (open squares), plus 5 μg of pBluescript SK$^-$ as carrier DNA and 1 μg of pCMV–β-gal. Cells were brought to quiescence by serum starvation and then serum stimulated. Cells were harvested at the indicated time points following the addition of serum, extracts were prepared, and luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity.

A role for *HsOrc1* in the control of mammalian cell DNA replication. Studies over the past several years have led to the identification of the multicomponent ORC that recognizes and binds to yeast origins of replication. The identification of homologs of several ORC components from various species (19, 20, 31, 39) suggests the very likely possibility that ORC will be a universal component of DNA replication. Although there is as yet no direct evidence for a role for the human Orc1 protein in initiation of DNA replication, and thus evidence that the absence of *HsOrc1* expression would limit DNA replication in mammalian cells, several observations suggest that this will indeed be the case. The conservation in sequence between the various Orc genes in yeasts, *Drosophila melanogaster*, *Xenopus laevis*, and humans, including the *HsOrc1* gene, is substantial and predicts a conservation of function (8, 19, 20, 39). In addition, various studies have provided evidence for a multicomponent complex containing Orc proteins in *D. melanogaster* (20), *X. laevis* (8), and human cell extracts (19). These findings, when combined with the fact that *ORC1* is an essential gene in *S. cerevisiae* (4) and *S. pombe* (39), strongly suggest that *ORC1* will be an essential component of all replication complexes. As such, the absence of *HsOrc1* expression in quiescent mammalian cells, under the control of the E2F transcription factor, will very likely limit the initiation of DNA replication.

A central role for E2F in the control of DNA replication. Since the initial demonstration that the E2F transcription factor was a target for the retinoblastoma protein, a large body of evidence has accumulated to support the conclusion that E2F plays a critical role in the regulation of genes involved in DNA replication (42). These genes include those encoding proteins such as DHFR, thymidine kinase, thymidylate synthase, and ribonucleotide reductase, all of which contribute to the creation of an environment for DNA replication. Others, including DNA polymerase α and PCNA, participate more directly in the replication process. A role for E2F in the control of such genes is entirely consistent with other observations that the activation of this transcriptional activity, through the inactivation of Rb, appears to be a major function of the DNA tumor virus oncoproteins such as adenovirus E1A, simian virus 40 T antigen, and human papillomavirus E7 (42). The generally accepted view is that each of these viruses has evolved the capacity to induce S phase so as to create an environment suitable for viral DNA replication in their normal host environment, a terminally differentiated quiescent cell.

In addition to the role of E2F in the activation of genes that encode proteins involved in DNA replication, recent experiments now show that additional E2F targets include various gene products that play cell cycle regulatory roles. These include cyclin E, cyclin A, *cdc2*, B-myb, and at least two of the E2F-encoding genes themselves (10). The activation of cyclin E can be viewed as enhancing the accumulation of E2F through the phosphorylation of Rb family proteins, as well as facilitating other aspects of the progression through the cell cycle, as would the *cdc2* kinase. The precise role of B-myb or cyclin A is unclear, but various experiments have demonstrated the importance of these proteins in cell cycle progression. In short, these studies point to a role for E2F that is central in the transition through G_1 and into S phase. Indeed, other experiments have shown that E2F1 overexpression can induce quiescent cells to enter S phase (26, 48, 54). This is not to say that other events do not play a role in the induction of S phase, and indeed, other experiments have indicated a role for the cyclin E-cdk2 kinase activity in the induction of S phase, independent of E2F (15).

Although the E2F-dependent induction of activities such as DHFR and DNA polymerase α must be important for efficient replication, they would not seem to be sufficient for the activation of DNA replication. The finding that transcription of the *HsOrc1* gene is regulated by E2F now provides a direct link to the initiation of DNA replication. Although further experiments will be necessary to establish the role of an ORC in the

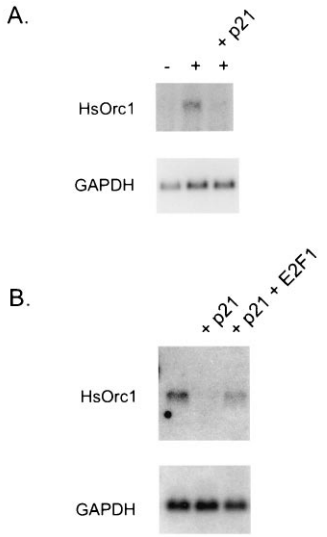

FIG. 6. Activation of *HsOrc1* expression requires G_1 cyclin kinase activity which can be bypassed by E2F overexpression. (A) *HsOrc1* induction is blocked by p21. Density-arrested REF52 cells were either harvested for RNA isolation (lane −) or replated at subconfluent densities (lane +) following infection with the Ad-p21 virus (+p21) or a control virus (Ad-CMV) (lane +) lacking a cDNA insert, each at a multiplicity of virus infection of 100 PFU per cell. Infected cells were harvested at 18 h postinfection, and poly(A+)RNA was prepared and then separated by agarose gel electrophoresis. The RNAs were then transferred to a nylon membrane and probed with either the *HsOrc1* or GAPDH cDNAs as described in Materials and Methods. After hybridization and washing, the filters were exposed to Kodak X-Omat film. (B) E2F bypasses the p21 block of *HsOrc1* induction. Northern blot analysis of *HsOrc1* RNA in REF52 cells infected with the recombinant adenoviruses. Density-arrested REF52 cells were infected with Ad-p21 or Ad-E2F1 and then replated at subconfluent densities. Ad-CMV was used to bring the total multiplicity of virus infection to 100 PFU per cell. Thus, lane 1 represents cells infected with Ad-CMV at 100 PFU per cell, lane 2 contains Ad-p21 at 75 and Ad-CMV at 25 PFU per cell, and lane contains Ad-p21 at 75 and the E2F1 virus at 25 PFU per cell. Cells were harvested and analyzed as described for panel A.

initiation of replication in mammalian cells, the homology with the yeast proteins and the fact that the HsOrc1 protein can be found in a complex with the HsOrc2 protein (19) suggests the likely possibility that a mammalian ORC will direct the initiation of replication. The control of expression of one component of this complex suggests a mechanism for the control of initiation in response to growth-stimulatory events. The fact that *HsOrc1* expression is controlled by the E2F-Rb pathway now suggests that the majority of the critical S-phase activities are under the control of E2F, a situation analogous to the transcriptional control systems in yeasts (*MBF* and *CDC10-SCT1*) that govern the expression of DNA replication activities and which themselves are controlled by G_1 cyclin-dependent kinase activity.

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