The transcription factor E2F plays an important role in G1 to S phase transition in the higher eukaryotic cell cycle. Although a number of E2F-inducible genes have been identified, the biochemical cascades from E2F to the S phase entry remain to be investigated. In this study, we generated stably transfected mouse NIH3T3 cells that express exogenous human E2F-1 under the control of a heavy metal-inducible metallothionein promoter and analyzed the molecular mechanism of the E2F-1-mediated initiation of chromosomal DNA replication. Ectopic E2F-1 expression in cells arrested in G0/G1 by serum deprivation enabled them to progress through G1 and to enter S phase. During the G1 phase, mouse cyclin E, but little of cyclin D1, was induced to express, which subsequently activated Cdk2. Experiments using the Cdk inhibitory proteins p27, p18, and p19 proved that the activity of Cdk2, but not of Cdk4, was required for S phase entry mediated by E2F-1. Minichromosome maintenance proteins (MCM) 4 and 7, the components of the DNA-replication initiation complex (RC), were constitutively expressed during the cell cycle, although the MCM genes are well known E2F-inducible genes. However, tight association of these two proteins with chromatin depended upon ectopic E2F-1 expression. In contrast, the Cdc45 protein, another RC component, which turned out to be a transcriptional target of E2Fs, was induced to express and subsequently bound to chromatin in response to E2F-1. Experiments utilizing a chemical Cdk-specific inhibitor, butyrolactone I, revealed that Cdk2 activity was required only for chromatin binding of the Cdc45 proteins, and not for the expression of Cdc45 or chromatin binding of MCM4 and -7. These results indicate that at least two separate pathways function downstream of E2F to initiate S phase; one depends upon the activity of Cdk2 and the other does not.

The initiation of S phase can be considered to be a consequence of all the biochemical reactions performed in G1. From this point of view, the component that functions the furthest downstream of the known G1 regulators would be directly involved in the initiation of chromosomal DNA replication. The candidates for such a factor include transcription factor E2F (1, 2). The expression level of the endogenous E2F protein tran-

Cdk2-dependent and -independent Pathways in E2F-mediated S Phase Induction*

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* This work was supported by grants-in-aid for scientific research and for cancer research from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 81-743-72-5541; Fax: 81-743-72-5649; E-mail: jkata@bs.aist-nara.ac.jp.
‡ The abbreviations used are: ORC, origin recognition complex; MCM, minichromosome maintenance protein; FBS, fetal bovine serum; BrdUrd, 5-bromodeoxyuridine; BLI, butyrolactone I.

†† The paper is available on line at http://www.jbc.org.

Received for publication, August 2, 1999, and in revised form, November 22, 1999
tion origins requires both ORC and Cdc6 proteins (15–17), and phosphorylation of the MCM components by the Cdc7 and Cdc2 kinases is a critical step for the initiation of DNA replication and the dissociation of MCMs from chromatin, respectively (18–22). Thus, the state of MCMs in the nucleus clearly represents the environment around the replication origin. Furthermore, the transcription of all the MCM genes is up-regulated in late G1 by the E2F transcription factor (23–25), suggesting that the MCM genes could be the rate-limiting components in the E2F-mediated induction of S phase.

Another regulatory factor essentially required for replication initiation is Cdc45, which interacts genetically with MCMs and ORC and physically with MCMs and the yeast replication origins (26–28). The amino acid sequences of Cdc45 reveal that it is a unique protein and not a member of the MCM family. Cdc45 is neither a component of the pre-replicative complex nor a requirement for its formation, but it associates with the pre-replicative complex after activation of S-phase-promoting Cdk (28). In addition, a Xenopus Cdc45 homologue has been shown to play a pivotal role in the loading of DNA polymerase onto chromatin under the control of the S-phase-Cdk activity (29). Thus, Cdc45 could be another candidate of the key elements functioning downstream of E2F at the onset of S phase.

In this study, we generated a cell system in which the expression of exogenous E2F-1 can be extracellularly controlled and then analyzed the molecular mechanism of the E2F-mediated induction of S phase. We especially focused on the actions and behaviors of MCMs (MCM4 and -7) and Cdc45. Our results strongly suggest that there are at least two independent pathways functioning downstream of E2F to regulate MCMs and Cdc45, one of which depends upon the activity of Cdk2 and the other does not. Thus, transcription factor E2F and cyclin E/cdk2 kinase seem to cooperate to induce chromosomal DNA replication in mouse fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Mouse NIH3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). The entire coding sequence of human E2F-1 or cyclin E was inserted into an expression vector (pM-CB6+) (30) containing a sheep metallothionein promoter and a neo gene. Cells were transfected with an expression vector by a modified calcium phosphate-DNA precipitation method (31) and selected in medium containing 0.4 mg/ml G418. The nuclear pellet fraction was resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl2, and 0.1% Nonidet P-40) containing phenylmethylsulfonyl fluoride, aprotinin, sodium fluoride, and sodium orthovanadate. The cell lysates were collected and centrifuged at 5000 rpm for 10 minutes at 4 °C. The nuclear pellet fraction was resuspended in a hypotonic lysis buffer, incubated on ice for 5 minutes, collected by centrifugation at 5000 rpm for 10 minutes at 4 °C, and added to SDS sample buffer (40 mM Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 1% SDS, 10% glycerol, and 0.05% bromphenol blue). The samples were boiled for 4 minutes before separation by SDS-polyacrylamide gel electrophoresis.

**Northern Blot Analysis**—Total RNA was purified from mouse fibroblasts using ISO-GEN (Nippon Gene). Thirty μg of RNA per lane was separated on agarose gels under denaturing conditions, transferred to a nylon membrane, and hybridized with radiolabeled cDNA containing mouse E2F-1 or mouse cyclin D1 under high-stringency conditions. For analysis of the Cdc45 gene expression, total RNA was isolated from serum-starved REF52 cells infected with adenovirus vectors and analyzed using human Cdc45 cDNA as a probe.

**Biochemical Analyses**—The pHsCDC45-Luc (−459) reporter plasmid consisting of the human Cdc45 promoter and a luciferase gene was generated by insertion of the genomic DNA fragment containing the 5′-flanking sequence of Cdc45 cDNA (−459 to −21, as 5′ end of the cDNA +1) into the KpnI site of the pGL2-Basic plasmid (Promega). The control reporter plasmid, pHsCdc45-Luc (E2F-2), contains the modified sequence, TTTGATGGGAGT (nucleotides −60 to −49) in place of an E2F-like site, TTTCGGCGGGAT, created by polymerase chain reaction-based site-directed mutagenesis. The methods for REF52 cell transfection and luciferase assay were previously described (4).

**RESULTS**

**Generation of the Human E2F-1 inducible Cell System in Mouse Fibroblasts**—To investigate the molecular action of E2F in S phase induction, we constructed a cell system in which expression of the E2F subunit can be extracellularly controlled. There are 6 members of the E2F gene family, among which E2F-1, -2, and -3 have been shown to be able to induce DNA replication in quiescent fibroblasts (4, 33). We chose E2F-1, the first member of this gene family, to analyze replication induction mediated by E2F transcription factors. We inserted the entire coding sequence of human E2F-1 cDNA into an inducible expression vector containing a heavy metal-inducible metallothionein promoter and a neo gene conferring resistance to G418, and transfected the plasmid into mouse NIH3T3 fibroblasts. After selection in G418, several cell clones (NIH-E2F clones) were isolated and tested for expression of human E2F-1 in response to heavy metal. We present here the results obtained from one representative cell line, but several independent clones behaved similarly. The expression of human E2F-1 was detected in response to heavy metal in a dose-dependent manner (Fig. 1A), which was sufficient to induce entry into S phase.
phase (Fig. 1B). However, addition of more than 40 μM ZnSO₄ to serum-starved cells induced substantial cell death and precluded the precise measuring of the BrdUrd uptake. In the presence of serum, higher doses of ZnSO₄ can induce higher levels of E2F-1 protein expression (Fig. 1A). However, the level of E2F-1 expression induced by 20 μM ZnSO₄ drove a significant portion of the cells into S phase (Fig. 1B) and was sufficient to up-regulate the E2F activity in the cells (Fig. 1C). Therefore, we decided to treat the starved cells with 20 μM ZnSO₄ in the following experiments.

To determine the precise timing of the initiation of S phase, both parental and NIH-E2F cells that had been synchronized in G₁/G₀ by serum starvation were re-stimulated with serum or ZnSO₄ and were pulse-labeled with BrdUrd for 20 min before fixation. Cells that had incorporated BrdUrd into their chromosomal DNA were detected by immunofluorescent staining using antibody to BrdUrd (Fig. 1D). BrdUrd-positive cells began to appear in both serum- and ZnSO₄-stimulated populations of NIH-E2F cells at approximately 10 h post-stimulation and the number of positive cells increased thereafter with similar kinetics except that ZnSO₄ stimulation was slightly more effective. In a control experiment, parental NIH3T3 cells responded to serum but not to ZnSO₄ as expected. To look at the kinetics of induction and the rate of S phase progression, we measured the DNA content of NIH-E2F cells treated as above by flow cytometric analysis (Fig. 1E). Serum-stimulated cells continuously progressed through S phase and the vast majority of cells returned to G₁ within 24 h post-stimulation. Of the ZnSO₄-stimulated cells, however, only 23.3% were in S phase at 16 h post-stimulation although about 50% were BrdUrd-positive at 14 h post-stimulation. These results may sound paradoxical but because we labeled the cells with BrdUrd only for a short time, the intensity of the BrdUrd-positive signals does not necessarily reflect the total sum of newly synthesized DNA. In addition, the measurement of BrdUrd uptake is a far more sensitive assay than the measurement of the DNA content by flow cytometry because a certain amount of DNA needs to be synthesized and accumulated for detection by flow cytometry. Therefore, it may be reasonable to conclude that although ectopic E2F-1 expression effectively fired the origin of DNA replication, the actual rate of DNA synthesis was not very high and not enough newly synthesized DNA to be detected by flow cytometric analysis accumulated. In fact, the increase in DNA content of E2F-1-induced cells during S phase was much slower than in serum-stimulated cells (compare 16 h versus 24 h in Fig. 1E). Thus, it is likely that some other factors induced by serum are required for effective promotion of the normal cell cycle.

**Induction and Activation of G₁ Cyclin/Cdk Kinase by Exogenous E2F-1**—Since the promoters of the G₁ cyclins (cyclins D and E) genes contain the E2F-binding site and the cyclin E gene has been reported as one of the E2F-inducible genes (1, 2, 8), we investigated the expression of G₁ cyclins and the activation of their partner Cdk’s in our E2F-inducible cells. Among three subtypes of cyclin Ds (D₁, D₂, and D₃), cyclin D₁ is the major D-type cyclin expressed in fibroblasts (34). Ectopic human E2F-1 induced much less cyclin D₁ proteins than did FBS. Moreover, E2F-1-mediated cyclin D₁ expression was temporal and occurred in early-to-mid G₁ (Fig. 2A, top panels). In contrast, cyclin E proteins were induced to express in response to ectopic E2F-1 expression. The kinetics of induction and the maximum amount of proteins were very similar between stimulations with FBS and heavy metal (Fig. 2A, middle panels). We occasionally detected an additional band just above that of cyclin E (indicated by an arrowhead in Fig. 2A, middle panel). Although the identity of this is not yet clear, it is most likely that it does not represent alternative forms of cyclin E because (i) we see this band in cell samples in which cyclin E mRNA is not detectable (see time 0 in Fig. 2, A and B, left panels), (ii) detection of this band is not reproducible (see Fig. 2, A, right panel, and Fig. 8D, top panels), and (iii) this band was sometimes observed in blots assayed with antibodies to irrelevant...
proteins (data not shown). The expression of cyclin A proteins occurred several hours after cyclin E, and, once more, we did not see any differences between these two stimulations (Fig. 2A, bottom panels). The mRNA expression of cyclin D1 and E mirrored the protein expression (Fig. 2B), suggesting that G1 cyclins were transcriptionally regulated by the ectopic E2F-1 transcription factor.

An in vitro kinase assay after immunoprecipitation with specific antibodies revealed that the activation of Cdk2 occurred in parallel with cyclin E-protein expression (data not shown). To examine the Cdk2 activation within the cell, we looked at the phosphorylation status of the retinoblastoma (Rb) protein, one of the well known in vivo substrates for the cyclin E-Cdk2 complex, by a mobility shift assay in SDS-polyacrylamide gel electrophoresis. Fig. 2C shows that slower migrating, highly phosphorylated forms of Rb proteins appeared in parallel with cyclin E in response to heavy metal-mediated E2F-1 induction. Interestingly, although the first timings of the appearance of highly phosphorylated Rb proteins were very similar, the total shift of the Rb proteins to the slowest migrating forms was somewhat delayed in heavy metal-treated cells as compared with serum-stimulated cells. This could be because cooperation between cyclin D-Cdk4 and cyclin E-Cdk2 complexes is required for efficient phosphorylation of Rb proteins (35).

To determine whether G1 cyclin-dependent kinase activities are required for E2F-1-mediated induction of S phase in growth factor-deprived mouse fibroblasts, we introduced the Cdk inhibitory proteins by transient transfection and examined their effects on the E2F-1-mediated initiation of DNA replication (Fig. 3). To date, 7 mammalian Cdk inhibitory proteins have been isolated and characterized (36). p27Kip1 belongs to the Cip/Kip family and inhibits a variety of cyclin-Cdk complexes (cyclin D-Cdk4, cyclin D-Cdk6, cyclin A-Cdk2, and cyclin E-Cdk2 complexes). The INK4 family of Cdk inhibitors specifically targets cyclin D-dependent kinases, Cdk4 and Cdk6. DNA replication in serum-stimulated cells was efficiently blocked by all 3 Cdk inhibitors. However, E2F-1-mediated S phase induction was refractory to the INK4 family of Cdk inhibitors and was blocked by the universal inhibitor, p27, indicating that E2F-1 mediated S phase induction requires Cdk2 but not Cdk4 activity. Thus, E2F can be placed downstream of Cdk4 and upstream of Cdk2 in this particular system.

**E2F-1-mediated Binding of MCM Proteins onto Chromatin**—The MCM complex, consisting of 6 different but closely related proteins, is required for the initiation of S phase and functions as the component of the regulatory machinery that allows cells to replicate DNA in S phase only once (11, 12). Although mammalian MCM proteins stay within the nucleus throughout the interphase, their association with chromatin has been shown to

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**Fig. 2. Induction of G1 cyclins in an E2F-1-inducible cell line.** NIH-E2F cells were arrested in G0/G1 by incubation in 0.1% FBS for 48 h, stimulated with 10% FBS (left panels) or 20 μM ZnSO4 (right panels), and harvested at the times indicated on the top. The cell samples were split into half and the total proteins and the total RNA were isolated. The expression of G1 cyclin proteins and mRNA was analyzed by immunoblotting (panel A) and Northern blotting (panel B) techniques. The positions of each cyclin protein and mRNA are indicated by arrows. In the case of immunoblot, a non-reproducible, nonspecific background band is shown by an arrowhead (panel A). Phosphorylation of the Rb proteins in the cell was monitored by the mobility shift of the protein in SDS-polyacrylamide gel electrophoresis (panel C).

**Fig. 3. Cdk2 but not Cdk4 activity is required for E2F-1-mediated S phase induction.** The expression vectors encoding HA-tagged Cdk inhibitors were transfected into NIH-E2F cells, starved in 0.1% FBS for 48 h, and stimulated with 10% FBS (A) or 20 μM ZnSO4 (B). Sixteen hours post-stimulation, cells were pulse-labeled with 100 μM BrdUrd for 20 min and doubly stained with antibodies to an HA epitope and BrdUrd. The BrdUrd-positive cells among HA-positive cells were enumerated. Equivalent expression of the Cdk inhibitors was confirmed by the Western blotting analysis of the transfected cells. The data are mean values from three independent experiments.
be dependent upon the cell cycle: they bind tightly to chromatin at the onset of S phase, and gradually dissociate from it as cells progress through S phase (13, 14). In addition, all six MCM genes are reported to be good E2F-inducible genes (23–25). Thus, we decided to examine the action of MCM proteins in response to E2F-1. For this purpose, we chose MCM4 and MCM7 and looked at their total protein expression and their association with chromatin by Western blotting. MCM proteins were shown to represent alternative or modified forms of Cdc45 proteins because (i) we see this band in cell samples in which no Cdc45 mRNA is detected (see time 0 in Figs. 6 and 7B, left lane; Cdc45 mRNA is below the detectable level in serum-starved cells, data not shown but see Fig. 5C), (ii) detection of this band is not an artifact of the electrophoretic mobility shift assay (data not shown but see Fig. 5C), (iii) preincubation of the antibody with recombinant Cdc45 proteins abolishes the Cdc45 band but had no effect on these three bands (data not shown). The expression and chromatin binding of Cdc45 depends upon both heavy metal stimulations, we saw very little difference in the peak around G1/S transition (Fig. 4B). Comparing serum and heavy metal stimulations, we saw very little difference in the expression of Cdc45 with chromatin was observed (Fig. 6, bottom panels). The kinetics of chromatin binding was slower than that for MCM proteins (Fig. 4B), consistent with the previous report that chromatin binding of Cdc45 depends upon both MCM and ORC proteins associated with chromatin. We occasionally detected three additional bands in anti-Cdc45 blots (indicated by an asterisk in the right margin of Fig. 6). Although the molecular identity is not yet clear, they do not seem to represent alternative or modified forms of Cdc45 proteins because (i) we see this band in cell samples in which no Cdc45 mRNA is detected (see time 0 in Figs. 6 and 7B, left lane; Cdc45 mRNA is below the detectable level in serum-starved cells, data not shown but see Fig. 5C), (ii) detection of this band is not reproducible (see Fig. 7B and 8D), and (iii) preincubation of the antibody with recombinant Cdc45 proteins abolished the Cdc45 band but had no effect on these three bands (data not shown). Thus, the transcription factor E2F or its downstream components are responsible for the loading of Cdc45 proteins onto chromatin as well as induction of its expression.

Requirement of Cdk2 Activity for Chromatin Binding of Cdc45 Proteins—Because the cyclin E/Cdk2 activity was required for E2F-1-mediated S phase induction (see Fig. 3), we next examined the role of Cdk activity in the induction and chromatin binding of MCM and Cdc45 proteins. To inhibit Cdk kinase activity in all cells at the same time, we treated the cells with a Cdk-specific chemical inhibitor, butyrolactone I (BLI) (37). BLI selectively inhibits the activities of Cdk2 and Cdk2, not affecting those of Cdk4 and other kinases. In G1 cells induced to express ectopic E2F-1, only marginal levels of Cdk4 and Cdk2 were activated. Thus, the main target of BLI in NIH-E2F cells stimulated with heavy metal is most likely to be Cdk2. NIH-E2F cells stimulated with heavy metal expressed as much endogenous mouse cyclin E proteins in the presence of BLI as its absence (Fig. 7A, top panel). Rb proteins migrated more slowly because of their hyperphosphorylation when stimulated with heavy metal (Fig. 2C), but treatment with BLI inhibited their phosphorylation (Fig. 7A, bottom panel). Therefore, BLI efficiently inhibited the activity of Cdk without affecting that of E2F under these conditions.

In BLI-treated cells, the expression of MCM4 and Cdc45 cDNA, we found an overlapping “E2F-binding site”-like sequence (Fig. 5A), with which E2F can specifically interact, as determined by an electrophoretic mobility shift assay (data not shown). The reporter construct containing this E2F-like sequence was activated in response to exogenously expressed E2F-1, -2, and -3, whereas the construct containing a modified E2F-like site failed to do so (Fig. 5B). As is often the case with most E2F-inducible genes, mutation of the E2F-binding site constitutively increased the basal promoter activity, suggesting that the Cdc45 promoter is negatively regulated through the E2F-binding site presumably by the E2F-Rb complexes. The Cdc45 promoter activity was dependent upon the cell cycle, low in G1 and high around G1/S (data not shown), in parallel with the endogenous Cdc45 mRNA expression. Mutation of the E2F site again abolished the responsiveness. Finally, overexpression of E2F-1 using a recombinant adenovirus vector induced endogenous Cdc45 mRNA in serum-starved REF52 cells (Fig. 5C). We thus conclude that the Cdc45 gene is one of the direct transcriptional targets of E2F.

We, next, examined in more detail the effect of E2F-1 induction on Cdc45 using NIH-E2F cells. In contrast to MCM proteins, Cdc45 proteins were not expressed in quiescent cells and were strongly induced to express by both serum and heavy metal stimulation (Fig. 6, top panels). The induction was delayed behind that of cyclin E but earlier than that of cyclin A (Fig. 2A). Consistent with the protein expression, an association of Cdc45 with chromatin was observed (Fig. 6, bottom panels). The latter represents the proteins tightly associated with chromatin, and these two forms can be easily separated by sequential extractions using detergents of different strength (differential extraction; see “Materials and Methods,” Ref. 14). Although MCM genes are reported to be the transcriptional targets of E2F, MCM4 and MCM7 proteins were expressed in serum-starved cells, and their total protein expression levels only marginally increased after serum and heavy metal stimulation (Fig. 4A, Whole). However, in quiescent cells, very few MCM proteins were found to be associated with chromatin and the amount of MCM proteins tightly bound to chromatin significantly increased after both stimulations (Fig. 4A, Pel.). In the time course experiment, we observed the cell cycle-dependent association of both MCM proteins with chromatin with a peak around G1/S transition (Fig. 4B). Comparing serum and heavy metal stimulations, we saw very little difference in the amount and induction kinetics of total and chromatin-bound proteins. Thus, ectopic E2F-1 expression induced the binding of MCM proteins onto chromatin (presumably in the vicinity of the origins of DNA replication) just as effectively as serum stimulation during G1.

E2F-1-mediated Expression and Chromatin Binding of Cdc45—Components of the DNA replication initiation complex other than ORC and MCM proteins include Cdc64. Cdc45 is not a component of the pre-replicative complex (pre-RC) but associates with pre-RC after activation of S phase-promoting Cdk (28, 29), which makes Cdc45 a good candidate for the targets of E2F-1 and cyclin E/Cdk2 kinase. After careful observation of the genomic DNA sequence adjacent to the 5′ of the human.
proteins was not significantly affected. However, the binding of Cdc45 to chromatin was markedly retarded. In contrast, MCM4 proteins (and MCM7 proteins as well, data not shown) efficiently bound to chromatin with or without BLI. Thus, Cdk2 activity is essential for the binding of Cdc45 to chromatin and expression of Cdc45 proteins and chromatin binding of MCM proteins were induced directly by E2F-1 or mediated by different molecules. These findings were consistent with results obtained in budding yeast and
text continues...
number of E2F-inducible genes. In certain systems, ectopic overexpression of cyclin E can induce the initiation of DNA synthesis without activating detectable levels of E2F (9), suggesting that cyclin E can substitute for E2F in S phase induction. In our system utilizing E2F-1-inducible mouse fibroblasts, the activation of the cyclin E-Cdk2 complex was required for E2F-1-mediated S phase induction and the complex played an important role in the chromatin binding of Cdc45. Therefore, we decided to investigate the precise effect of the ectopic expression of cyclin E in our mouse fibroblast system. In order to do this, we inserted the entire coding sequences of human cyclin E into a heavy metal-inducible expression vector, transfected them into NIH3T3 cells, and established several cell lines (NIH-cycE cells). In Fig. 8, we show the results obtained from one particular cell line, but basically similar results were observed in several independent cell lines. In quiescent cells, a small amount of human cyclin E was detected. This is presumably because of the leaky nature of the inducible promoter. Stimulation with heavy metal substantially increased the amount of human cyclin E (Fig. 8A) and activated the Cdk2 kinase (data not shown). Ectopic human cyclin E induced the expression of endogenous mouse cyclins E and A (Fig. 8A), suggesting the activation of endogenous E2F2 via phosphorylation of Rb proteins (Fig. 8, E and F, see below). Ectopic cyclin E enabled quiescent fibroblasts to initiate DNA replication (Fig. 8B). Although the efficiency was not as high as with serum, about 50% of the cells incorporated BrdUrd 16 h after stimulation and more cells did so at later time points. However, these cells did not progress through S phase as judged by flow cytometric analysis of the DNA content (Fig. 8C). This may sound paradoxical, but because we labeled the cells with BrdUrd only for a short time, BrdUrd positiveness does not necessarily guarantee that a certain amount of DNA synthesis has occurred, which is required for detection of S phase by flow cytometric analysis. At 16 h post-stimulation and even at later time points (up to 48 h), we did not detect the population of cells in S phase.

We next examined the effect of the ectopic expression of cyclin E on the action of MCM and Cdc45 proteins. As shown in Fig. 8D, the induction of Cdc45 and the chromatin binding of MCM4 and Cdc45 occurred with kinetics similar to those of serum stimulation. However, the maximum levels of induction and binding seemed low compared with those for serum stimulation. This slightly lower level of expression and chromatin loading might be sufficient for the initiation but not for the efficient progression of S phase.

Because ectopic human cyclin E induced expression of endogenous mouse cyclins E and A, chromatin binding of MCM proteins and expression of Cdc45 proteins, we investigated whether or not endogenous E2F is activated through phosphorylation of Rb proteins in these cells. First, Rb proteins were analyzed by a mobility shift assay in SDS-polyacrylamide gel electrophoresis (Fig. 8E). Cells stimulated with ZnSO4 contained slower migrating, hyperphosphorylated forms of Rb proteins. However, the amount of slower migrating Rb proteins was much less than in serum-stimulated cells. This is because serum induces cyclin D/Cdk4 activity in addition to cyclin E/Cdk2 activity, both of which are required for efficient phosphorylation and the total shift of Rb proteins (35). Next, E2F activity was measured using an E2F reporter construct (Fig. 8F). Although the mobility shift of Rb proteins was incomplete in ZnSO4-stimulated cells, similar levels of E2F activities were released by both stimulations.

Taken together, these findings indicate that the ectopic expression of cyclin E and the subsequent activation of Cdk2 induces expression of mouse cyclins and Cdc45 and chromatin binding of MCM proteins via phosphorylation of Rb proteins and activation of endogenous E2F. These events were probably sufficient to fire the origin of DNA replication, but not for the normal progression of S phase.

**DISCUSSION**

Transcription factor E2F plays an important role in the initiation of DNA synthesis but the details of its downstream
cascades largely remain to be investigated. The cyclin E gene, one of the most important E2F-target genes, creates a feedback loop with E2F, thereby increasing both activities near the G1/S transition (38). Although the precise role of cyclin E-Cdk2 complexes in E2F-mediated S phase induction remains to be elucidated, it is most likely that E2F and cyclin E-Cdk2 complex cooperate with each other to initiate S phase (39). However, recent reports that the overexpression of E2F or cyclin E alone can drive cells into S phase without activating the other suggest greater complexity of the signaling system in S phase induction (9, 10). In order to address these issues, we generated a cell system using mouse fibroblasts in which ectopic expression of E2F-1 can be extracellularly controlled by adding heavy metal to the culturing medium and analyzed the molecular mechanism of E2F-mediated S phase induction.

In this study, we showed that there are at least two separate signaling pathways downstream of E2F to initiate S phase, one involving the activity of the cyclin E-Cdk2 complex, the other not. The former requires the loading of Cdc45 onto chromatin, and the latter includes the expression of Cdc45 and the loading of MCM proteins onto chromatin. The chromatin binding of both MCM and Cdc45 proteins would be critical for DNA replication because the function of MCMs is required for the initiation of replication (11–13) and Cdc45 functions to facilitate loading of DNA polymerase onto chromatin (29). Thus, we conclude that the cooperation between E2F and cyclin E/Cdk2 kinase is required for the efficient initiation of DNA replication in a normal mammalian cell cycle.

Chromatin binding of MCMs requires ORCs and Cdc6 (15–17). The action of ORCs is constitutive during the cell cycle, while the expression and chromatin binding of Cdc6 is dependent upon the cell cycle, being expressed and activated before the onset of S phase. These facts make Cdc6 a good candidate for the E2F target gene. As a matter of fact, cell cycle-regulated expression of mammalian Cdc6 has been reported to be dependent on E2F (40–42). We attempted to address directly whether the E2F-1-induced MCM binding of chromatin observed in our system was mediated by endogenous Cdc6. Unfortunately, however, we do not have good antibodies that efficiently recognize mouse Cdc6 proteins, which precluded the experiments. As another approach, we tried to overexpress Cdc6 proteins in mouse fibroblasts to see whether we could detect the acceleration of S phase induction or the constitutive association of MCMs onto chromatin, but we failed to overexpress Cdc6 proteins in quiescent cells. This suggests the post-transcriptional and cell cycle-dependent regulation of Cdc6. Blow and co-workers (12, 43) have suggested that some unidentified cellular factors besides ORCs and Cdc6 are required for MCM binding of chromatin and the subsequent initiation of DNA replication. These unidentified factors might also be under the control of E2F transcription factors.

The substrate proteins for the cyclin E-Cdk2 complex essential for S phase induction remain to be determined. Substrate candidates include Rb proteins (35, 44), Cdc45, or its associated proteins (29), and NPAT (45). In our study, ectopic E2F induced the expression of cyclin E and the subsequent phosphorylation of Rb proteins without inducing cyclin D. Ectopic introduction of cyclin E, on the other hand, induced the expression of the E2F-inducible genes, presumably through Rb phosphorylation. Thus, our results are consistent with previous reports that Rb proteins are phosphorylated by the cyclin E-Cdk2 complex in vivo (44) and play an important role in creating a positive feedback loop between E2F and cyclin E around the G1/S boundary (38). The chromatin binding of Cdc45 is likely to be a critical step in the initiation of DNA replication. It is not clear whether Cdc45 itself or its associated protein is the substrate of cyclin E/Cdk2 kinase, but the Cdc45 binding of and the subsequent loading of DNA polymerase onto chromatin are dependent on the activity of Cdk2 (29). The expression of NPAT seems to be E2F-dependent and overexpression of NPAT can induce S phase. NPAT is one of the cyclin E/Cdk2 substrates and ectopic expression of cyclin E synergistically enhances the activity of NPAT in the S phase induction (45). These findings make NPAT an excellent candidate for both E2F and cyclin E/Cdk2 targets. Although the molecular action of NPAT remains to be elucidated, its effect on MCMs and Cdc45 should be determined.

It is obviously an important question why the introduction of E2F-1, but not cyclin E, enables quiescent cells to progress through S phase even though both are capable of inducing chromatin association of MCM and Cdc45 proteins and probably firing the origin of DNA replication. One interpretation is that the order of activation could be important. In this scenario, certain levels of E2F activity may need to be induced before the cyclin E-Cdk2 complex becomes activated. It is possible that a certain E2F-inducible gene product is inactivated through phosphorylation by cyclin E-Cdk2 complex and if the protein is phosphorylated shortly after synthesis, it is unable to function properly to promote DNA synthesis. Another possibility is that down-regulation of cyclin E/Cdk2 activity is important for cells to progress through S phase. Generally, degradation of cyclin is as important for cell cycle progression as induction of cyclin expression. If cyclin E/Cdk2 activity is maintained, S phase-specific checkpoint control mechanisms may be activated. In fact, a recent report indicates that constitutive overexpression of cyclin E, but not cyclin D1 or A, impairs S-phase progression and eventually results in chromosome instability (46).

On the induction of S phase, a number of experiments have been performed and a substantial amount of data has accumulated. Some of the conclusions obtained using similar but somewhat different systems are slightly different. This is understandable because the rate-limiting steps for the initiation of DNA synthesis may vary in different cell types and in different situations. The results obtained so far clearly indicate that both E2F and the cyclin E-Cdk2 complex play a pivotal role in governing the transition from G1 to S phase basically on all occasions. This is clearly different from the function of another G1 cyclin-Cdk complex, cyclin D-Cdk4, which plays an essential role in the somatic cell cycle in which G1 regulation controls the cell proliferation. Thus, the functions of E2F and cyclin E/Cdk2 kinase are more directly involved in the initiation of S phase and our E2F-inducible cell system would be a useful tool with which to investigate the molecular mechanism regulating DNA replication by these factors.

Acknowledgments—We thank Drs. S. Hiebert, M. Ohtsubo, C. J. Sherr, and H. Takisawa for plasmids.

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J. Biol. Chem. 2000, 275:6337-6345.
doi: 10.1074/jbc.275.9.6337

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