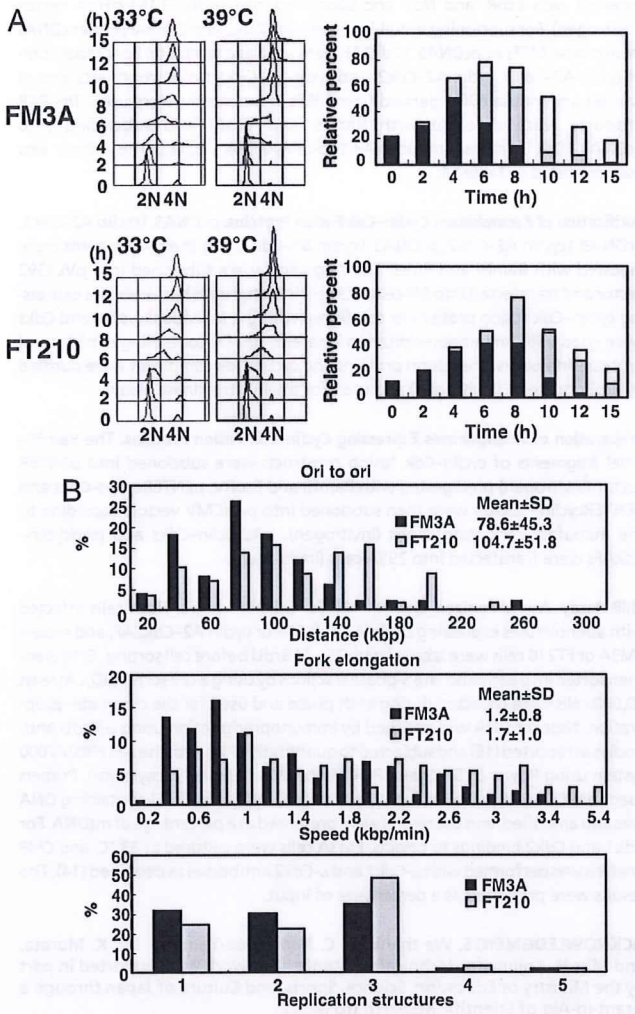

Fig. 4. Prolonged S phase in temperature-sensitive Cdk1 mutant FT210 cells. (*A*) FT210 and the parental FM3A cells were synchronized at M phase by nocodazole (0.5 μg/mL, 16 h) and then released at an either permissive (33 °C) or nonpermissive (39 °C) temperatures. Cells were then harvested 3 h after release (time 0) and at various times thereafter. Their replication sites were analyzed ($n > 300$) as in Fig. 3*B*. Data are means of at least 3 independent experiments. (*B*) Asynchronized FM3A and FT210 cells were shifted at 39 °C for 4 h. Cells were then harvested and subjected to molecular combing. Adjacent origins in replicon clusters (Ori to ori), fork elongation, and replication structure were determined ($n > 100$) as in Fig. 1*C*.

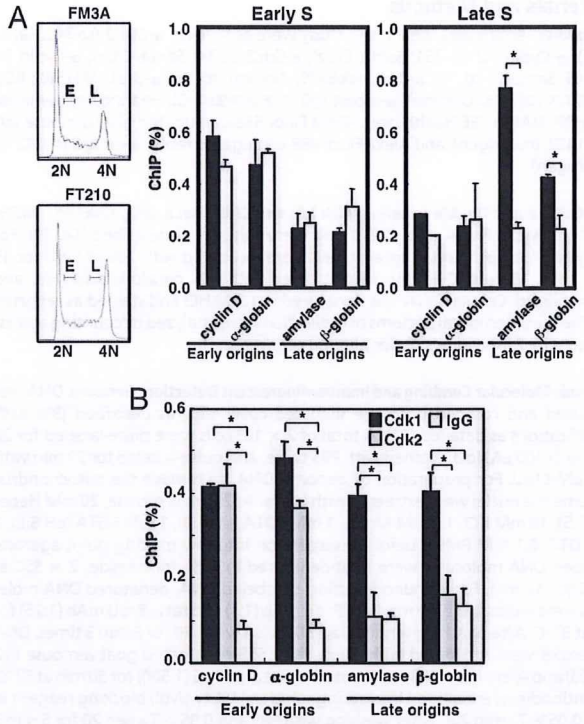

Fig. 5. Impaired late origin firing in temperature-sensitive Cdk1 mutant FT210 cells. (*A*) Asynchronous FM3A and FT210 cells were shifted at 39 °C for 4 h. Cells were pulse-labeled with BrdU (25 μM) for 1 h and sorted into early (E) or late (L) fractions. Replication firing at the indicated origins was analyzed by ChIP analysis as in Fig. 3*D*. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$). Filled bars indicate FM3A cells; empty bars indicate FT210 cells. (*B*) Asynchronous FM3A cells were cultured at 33 °C and harvested. Cell lysates were subjected to ChIP analysis as described in *Materials and Methods*. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$).

when replication structures were assessed by dynamic molecular combing technology (Fig. 3*C*). This idea was further supported by the observations that ectopic expression of cyclin A2–Cdk1AF and cyclin A2–Cdk2AF failed to induce DNA damage (Fig. 2 *B* and *C*). These findings present a clear contrast to the case with Chk1 depletion in which stability of replication forks during S phase was strikingly reduced. Therefore, Chk1 likely regulates the fork stability in a manner independent of cyclin–Cdk activities.

Cdk activities have both positive and negative roles during S phase, namely to initiate DNA synthesis and prevent rereplication. A quantitative model has proposed for explain the biphasic effects of Cdks (28). In addition to a quantitative model, the accessibility of Cdk to substrates could play a role in the regulation of the S-phase program. Studies in *Xenopus* and yeast systems suggested that Cdk1 specifically interacts with ORC and phosphorylates the components more efficiently than Cdk2 although this interaction is proposed to be involved in prevention of rereplication (23, 24). We found that Cdk1 could bind to both early and late origins but Cdk2 failed to bind to late origins (Fig. 5*B*). Thus, Cdk1 could potentially activate early origin firing. This notion is supported by the fact that Cdk1 could complement the Cdk2 function of S-phase initiation in Cdk2-depleted cells (26). However, because neither ectopic expression of cyclin A2–Cdk2AF nor cyclin A2–Cdk1AF resulted in the further enhancement of early origin activation (Fig. 3*D*), activation of endogenous cyclin A/E–Cdk2 at the S-phase onset appeared to be sufficient for early origin firing. Furthermore, given that the majority of endogenous Cdk1 and Cdk2 existed in soluble fractions (Fig. S8), the origin activation program appeared to be regulated not only by induction of Cdks and their binding to prereplicative complex components, but also by alternative ways such as complex formation with cyclins or regulation of inhibitory phosphorylation of Cdks. In this regard, it was very recently reported that Cdk1 started to form a complex with cyclin A2 after cyclin A2–Cdk2 complexes reached a plateau in mid S phase (21). Taken together, our results suggest that cyclin A2–Cdk1 may regulate origin firing program through both its specific accessibility to late origins and regulation of Cdk1 activity at late S phase.

In conclusion, the present results indicate that ATR/Chk1–cyclin A2–Cdk1 controls the activation of late replication origins and the density of active origins in mammals. Similar regulation was reported in a budding yeast system in which Clb5–Cdk1 was required for late origin firing (8). Taken together, these results suggest the existence of conserved mechanisms for the temporal program of origin activation among a number of eukaryotes.

Materials and Methods

Antibodies. Antibodies used in this study were as follows: α-CDK2 (sc-748; Santa Cruz), α-Cyclin A2 (sc-751; Santa Cruz), α-Cdc2 (sc-54; Santa Cruz), α-Cyclin B1 (sc-245; Santa Cruz), rat α-BrdU (ab6326; Abcam), mouse α-BrdU (347580; BD), α-γH2AX (05-636; Upstate), α-rabbit IgG HRP (NA934; GE Healthcare), α-mouse IgG HRP (NA931; GE Healthcare), Alexa Fluor 555-conjugated goat α-mouse IgG (A-21422; Invitrogen), and Alexa Fluor 488-conjugated rabbit α-rat IgG (A-21210; Invitrogen).

Cell Culture and Double Labeling with IdU and CldU. HeLa cells, Chk1$^{lox/-}$ MEFs, Chk1$^{del/-}$ MEFs, FM3A, and FT210 cells were cultured as described (14, 29). For analyses of origin firing programs, cells were incubated with 100 μM IdU for 15 min, then 100 μM CldU for 15 min, fixed with 4% paraformaldehyde, and permabilized. Cellular DNA was denatured in 1.5 M HCl and stained as reported (6). The spatiotemporal patterns of replication were analyzed by counting at least 300 cells by 2 individuals under blinded conditions.

Dynamic Molecular Combing and Immunofluorescent Detection. Genomic DNA was prepared and combed onto the silanated cover slips as described (30) with modifications as detailed (31). A total of 2×10^6 cells were pulse-labeled for 20 min with 100 μM IdU, washed with PBS twice, and pulse-labeled for 20 min with 100 μM CldU. For preparation of genomic DNA, to remove the mitochondrial genome the nuclei were extracted with buffer A [250 mM sucrose, 20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 6.8), 1 mM DTT, 0.1 mM PMSF] before resuspension into low-melting point agarose. Combed DNA molecules were heat-denatured in 50% formamide, 2 × SSC at 72 °C for 12 min. For immunodetection of labeled DNA, denatured DNA molecules were incubated with mouse α-BrdU mAb (1:5) and rat α-BrdU mAb (1:25) for 1 h at 37°C. After washing with PBS and 0.05% Tween 20 for 5 min 3 times, DNA molecules were incubated with Alexa Fluor 555-conjugated goat α-mouse IgG (1:500) and Alexa Fluor 488-conjugated rabbit α-rat IgG (1:500) for 30 min at 37°C. All antibodies were diluted in blocking solution [1% (wt/vol) blocking reagent in PBS, 0.05% Tween 20]. After washing with PBS and 0.05% Tween 20 for 5 min 3 times, coverslips were mounted in VECTASHIELD (Vector Laboratories). To estimate the extension of DNA molecules, coverslips were prepared with λ-DNA, and then the DNA molecules were stained with 6.7 mM YOYO-1 at 25°C for 1 h. YOYO-1-stained DNA molecules measured 21 ± 0.9 μm. As the virus genome is 48.5 kbp, the extension of DNA molecules is 2.32 ± 0.11 kbp/μm. DNA fibers were examined with a Zeiss Axioplan 2 MOT with a 63X Plan-APOCHROMAT (NA 1.4) objective lens, equipped with MicroMAX CCD camera (Princeton Instruments). Fluorescent signals were measured by using MetaMorph version 6.1 software (Universal Imaging).

Construction of Cyclin A2–Cdk1, Cyclin A2–Cdk2, and Cyclin B1–Cdk1 Fusion Vectors. For subcloning of full-length mouse Cdk1 and Cdk1AF, either cDNAs from mouse MEFs or pcDNA3.1Cdk1AF were used as a template. The PCR products were digested with EcoRI and NotI and subcloned into pcDNA3.1Myc/HisA vector (Invitrogen). For subcloning of full-length mouse Cdk2 and Cdk2AF, either cDNAs from mouse MEFs or pcDNA3.1Cdk2AF were used as a template. For preparation of cyclin A2–Cdk1, cyclin A2–Cdk2, and cyclin B1–Cdk1 fusion constructs, sets of primers and mouse cDNA derived from MEFs as a template were used. The PCR products were digested with BamHI and EcoRI and subcloned into pcDNA3.1Cdk1Myc/HisA or pcDNA3.1Cdk2Myc/HisA vectors. The primer sets used are listed in Table S1.

Purification of Recombinant Cyclin–Cdk Fusion Proteins. pcDNA3.1cyclin A2–Cdk1, pcDNA3.1cyclin A2–Cdk2, pcDNA3.1cyclin B1–Cdk1, and their AF mutants were digested with BamHI and PmeI. The fragments were subcloned into pVL1392 vector and transfected into Sf9 cells. Sf9 cells infected with baculoviruses expressing cyclin–Cdk fusion proteins or coinfected with the individual cyclins and Cdks were lysed with immunoprecipitation kinase buffer (7) containing a mixture of protease inhibitors. The fusion proteins and cyclin–Cdk complexes were purified by ProBond Resin (Invitrogen) and used for the in vitro kinase assay.

Preparation of Adenoviruses Expressing Cyclin–Cdk Fusion Proteins. The BamHI–PmeI fragments of cyclin–Cdk fusion constructs were subcloned into pENTER vector (Invitrogen) predigested with BamHI and EcoRV. pENTERcyclin–Cdks and pENTERcyclin–CdkAFs were then subcloned into pAdCMV vectors according to the manufacturer's instructions (Invitrogen). pAdcyclin–Cdks and pAdcyclin–CdkAFs were transfected into 293A cells (Invitrogen).

ChIP Assay. Asynchronized Chk1$^{lox/-}$ MEFs, Chk1$^{del/-}$ MEFs, HeLa cells infected with adenoviruses expressing cyclin A2–Cdk1AF or cyclin A2–Cdk2AF, and mouse FM3A or FT210 cells were labeled with 25 μM BrdU before cell sorting. Cells were then sorted into early and late S-phase fractions by using a cell sorter (BD). At least 60,000 cells were collected during each phase and used for the chromatin preparation. Nascent DNA was enriched by immunoprecipitation using α-BrdU antibodies as reported (16) and subjected to quantitative PCR with the ABI PRISM7000 system using Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used for PCR are listed in Table S1. As a control, mtDNA in BrdU-containing DNA was also amplified, and the results were presented as a percentage of mtDNA. For Cdk1 and Cdk2 bindings to origins, FM3A cells were cultured at 33 °C, and ChIP analysis was performed with α-Cdk1 and α-Cdk2 antibodies as described (14). The results were presented as a percentage of input.

ACKNOWLEDGMENTS. We thank Dr. C. Namikawa-Yamada, Mr. K. Murata, and Miss H. Kojima for technical assistance. This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan through a Grant-in-Aid of Scientific Research (to M.N.).

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