Novel Splicing Variant of Mouse Orc1 Is Deficient in Nuclear Translocation and Resistant for Proteasome-mediated Degradation*

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DNA replication is controlled by the stepwise assembly of the pre-replicative complex and the replication apparatus. Loading of the origin recognition complex (ORC) onto the chromatin is a prerequisite for the assembly of the pre-replicative complex. To define the physiological functions of the mammalian ORC, we cloned ORC subunit cDNAs from mouse NIH3T3 cells and found novel variant forms of Orc1, Orc2, and Orc3 each derived from alternative RNA splicing. The variant form of Orc1, Orc1B, lacks 35 amino acid residues in exon 5; the variant of Orc2, Orc2B, lacks 48 amino acid residues in exon 2. In the Orc3 variant, Orc3B, only 1 amino acid residue is deleted in exon 15. Reverse transcription-PCR analysis showed that the full-length Orc1–3 subunits, Orc1A, Orc2A, and Orc3A, as well as Orc2B and Orc3B, were widely expressed in various mouse cell lines and mouse tissues. In contrast, Orc1B was only expressed in the thymus and at an early embryonic stage. Overexpression of these Orc subunits in cultured cells revealed that Orc1A, Orc2A, Orc3A, Orc2B, and Orc3B are localized in the nucleus, whereas Orc1B remains exclusively in the cytoplasm. Moreover, fusion of the 35 amino acids spliced fragment from mOrc1A with β-galactosidase resulted in its translocation into the nucleus. When Orc1B is expressed transiently, its degradation occurs in a proteasome-dependent manner, whereas Orc1A is rapidly degraded by the ubiquitin-proteasome pathway. Taken together, we conclude that mouse Orc1, Orc2, and Orc3 each exist in two alternative-splicing variants and that naturally occurring Orc1B lacks a functional domain that is essential for nuclear translocation and proteasome-dependent degradation.

Eukaryotic DNA replication is strictly controlled during the cell cycle thus ensuring that cells enter S phase only once per cell cycle. A considerable body of evidence from both yeast genetics and biochemical analysis using Xenopus egg extracts has shown that the initiation of replication requires the stepwise assembly of protein complexes on chromatin to form a pre-replicative complex (pre-RC). The pre-RC consists of the origin recognition complex (ORC), the minichromosome maintenance protein complex, as well as the Cdc6 and Cdt1 proteins. After activation of the pre-RC by phosphorylation through cyclin-dependent kinases and the Dbf4-dependent Cdc7 kinase, Cdc45, Mcm10, and the GINS (Go-Ichi-Ni-San; 5-1-2-3 in Japanese) complex associate with the pre-RC to form the pre-initiation complex. After Cdc45-mediated recruitment of DNA polymerases to the pre-initiation complex, DNA polymerase α initiates DNA synthesis. To maintain genome integrity and the correct ploidy, each step in the formation of the pre-RC, pre-initiation complex, and replication apparatus is tightly controlled by multiple checkpoint mechanisms (1–4).

As the first step in pre-RC assembly, the ORC binds to pre-replicative chromatin throughout the genome. In Saccharomyces cerevisiae, DNA binding sites for the ORC, consisting of an autonomously replicating sequence (ARS) consensus sequence and a B element located within regions of several hundred base pairs, were shown to act as replication origins and were defined as an ARS. Characterization of ARS binding proteins in S. cerevisiae has led to the identification of replication initiation factors as ORC (5). The S. cerevisiae ORC is composed of six subunits and is stably associated with the ARS throughout cell cycle (6), where it serves as a landing pad for the pre-RC components Cdc6, Cdt1, and Mcm2–7. ARS-dependent formation of the pre-RC is solely responsible for ORC binding in S. cerevisiae and Schizosaccharomyces pombe (5, 7). In addition, the S. cerevisiae ORC has additional functions within the nucleus, including transcription silencing, heterochromatin formation, and nucleosome positioning (8–10).

Because most of the pre-RC components identified in S. cerevisiae have been found in other eukaryotes, including human, it is believed that the mechanisms controlling the initiation of replication are conserved in all eukaryotes (1). However, the origin sequence remains to be elucidated, particularly in metazoan cells. Although several sequences have been characterized as metazoan origins of replication, most studies have suggested that initiation of DNA synthesis in metazoans is triggered at broad zones within discrete chromosomal loci (11, 12). Recent findings, that homologues of the S. cerevisiae ORC exist throughout most eukaryotes, imply that its further characterization will shed light on the initiation of DNA replication in metazoan cells.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB190255, AB190256, and AB190257.

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1 The abbreviations used are: pre-RC, pre-replicative complex; ORC, origin recognition complex; ARS, autonomously replicating sequence; NLS, nuclear localization signal; DMEM, Dulbecco's modified Eagle's medium; CDK, cyclin-dependent kinase; HA, hemagglutinin A; RT, reverse transcription; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen.
Splicing Variant of Mouse Orc1 Is Deficient in Nuclear Translocation.

Studies of the metazoan Orc proteins have revealed that the ORC complex of higher eukaryotes is unexpectedly different from the yeast ORC. First, mammalian Orc1 and Orc6 are loosely associated with the other ORC subunits, Orc2–5 (13, 14). Second, mammalian Orc2–5 associate with chromatin at late telophase and remain bound during subsequent cell cycle progression, whereas Orc1 binds chromatin during the G1 to S phases and gets selectively released from chromatin during the S to M phase transition (15, 16). Third, chromatin-bound Orc1 is modified during S phase by ubiquitination, which results in its degradation by a proteasome-dependent pathway during S phase (15, 17).

Thus, the metazoan ORC might have evolved to control replication at different levels, as compared with S. cerevisiae, and may play additional roles in DNA replication of higher eukaryotes. To understand the molecular mechanisms of DNA replication in metazoan cells, it is essential to characterize the function of each subunit of the ORC complex in the replication process, especially the mechanism by which the ORC complex is loaded onto chromatin and the interplay between the DNA replication and ORC and other replication components of the pre-RC at the onset of replication.

To understand the physiological function of the ORC complex in higher eukaryotes, we characterized the role of Orc1, Orc2, and Orc3 in mouse cell lines. By cloning cDNAs of mouse Orc1, Orc2, and Orc3, we found that an alternative splicing variant exists for each protein. Using a naturally occurring truncated mOrc1 protein, we characterized a domain crucial for nuclear translocation and degradation.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of Mouse Orc1, Orc2, and Orc3 Homologues—cDNAs for mouse Orc1, Orc2, and Orc3 were amplified by PCR using sequence-specific primers and a mouse NIH3T3 cell line cDNA library (Clontech, Palo Alto, CA). The following primers were used: mOrc1, 5'-GG-GGTACGGATCCATGCTCTTCACTCTACAAG-3' and 5'-CCGGTA-CCAAATGTGCTCTTCCTTAGGACC-3'; mOrc2, 5'-GGGAATTCG-GATCAGAGAGTGAGACTCGTATAA-3' and 5'-GGCTCGTCGACGGTACTGAC-3'. cDNAs from mice were amplified with the following primers: 5'-GG-GAGTAGTATGTTATGTCAC-3' and primer 5'-AGCGAATA-CCTAACCCTACTCTAGCA-3'. cDNAs for mOrc2A and mOrc2B were specifically amplified with the forward primer g, 5'-CTGGTCAAGGCCTAAACAGCA-3' and one of the reverse primers: mOrc2A primer h, 5'-CTGACCTTCTCTCTGACTGTTGTC-3' or mOrc2B primer i, 5'-CTGACCTTCTCTCTGACTGTTGTC-3'. A PCR fragment from the 3'-untranslated region of the mOrc1, mOrc2, and mOrc3 cDNAs was amplified using the reverse primer (primer b in Fig. 2A) 5'-GAAGATCCTGAGTTAGATGCTTGCT-3' and one of the forward primers for mOrc1a and b: mOrc1a primer a, 5'-GACTCCGACCTCTCTCCTTT-3'; mOrc1a primer c, 5'-GTTTATCTAGAAGCCTAAACAGCA-3'; mOrc1b primer d, 5'-GGTCTGACGCAAGCTTCTGCTGCT-3'; mOrc2A primer e, 5'-GGGGATCCATGAGCACTCTGCAGTTAAA-3' and mOrc2B primer f, 5'-GGGGATCCATGAGCACTCTGCAGTTAAA-3'. PCR products for mOrc1A and mOrc1B were sequenced on both DNA strands.

Southern Hybridization—PCR products of mOrc1A/mOrc1B and mOrc2A/mOrc2B were detected by Southern hybridization using RT-PCR samples of NIH3T3 or Swiss3T3 cells. PCR products were separated by 2% agarose gel electrophoresis and detected by staining with SYBR Green (Molecular Probes, Eugene, OR).

Cell Cycle Analysis—Swiss3T3 cells were synchronized by serum starvation using DMEM containing 0.2% fetal bovine serum for 72 h and then released by changing the medium to DMEM containing 15% fetal bovine serum. Cells were harvested at the following periods: 0, 4, 8, 16, 24, and 48 h. Synchronized growth was monitored with a Laser Scanning Cytometer (LSC2, Olympus, Tokyo, Japan) with propidium iodide, according to the manufacturer's instructions.

Southern Hybridization—PCR products of mOrc1A/mOrc1B and mOrc2A/mOrc2B were detected by Southern hybridization using RT-PCR samples of NIH3T3 or Swiss3T3 cells, respectively, as probes. Each probe was labeled using the Random Primer DNA Labeling Kit version 2 (TaKaRa, Otsu, Japan). The DNAs were transferred to a Hybond-N+ membrane (Amersham Biosciences) overnight and then fixed on the membrane by heating for 2 h at 80 °C. Hybridization was performed for 2 h at 65 °C with gentle agitation using Rapid-hyb buffer (Amersham Biosciences). The blots were washed extensively, and signals were detected by using a BAS3000 phosphorimaging device (Fuji Film, Tokyo, Japan) with exposure to x-ray film (BioMax, Eastman Kodak).

Antipeptide Antibody Preparation—Rabbit polyclonal antibodies were raised against synthetic peptides corresponding to the mOrc1A internal region (C-KKSSCSDLSLDYQTSKRAAPF) and the boundary region sequences in exon 5 of mOrc1B (C-KKSSCSDLSLDYQTSKRAAPF) (see Fig. 3A). Immunization and affinity purification by anti-peptide-conjugated columns were performed as described previously (21).

**Indirect Immunofluorescence**—Cells were fixed and permeabilized as described before (22). The slides were then blocked with PBS containing 5% fetal bovine serum (blocking buffer) for 30 min at room temperature, incubated with anti-T7 antibody (Novagen, EMD Biosciences, San Diego, CA) for 1–2 h at room temperature, washed three times with PBS, and counterstained with a mixture of the following antibodies: anti-calreticulin (Stressgen Biotechnologies, Victoria, BC, Canada), MitoTracker (Molecular Probes), and anti-β-galactosidase (5′-Prime, Inc., Boulder, CO).

Preparation of Cell Extracts and Western Blot Analysis—After transfection, COS-1 cells were washed with PBS, scraped from the plates in ice-cold PBS, centrifuged for 5 min, resuspended in cytoskeletal buffer (10 mM Tris, 1 mM piperazineethanesulfonic acid, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoro-
mOrc1B, mOrc2B, and mOrc3B are shown in 0.02% NaN₃) for 12 h at 4 °C, the membranes were washed three times (Tropix, Applied Biosystems, Foster City, CA), 0.1% Tween 20, and are shown in at the alternatively spliced sites are shown for mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B. Intron-exon junctions are indicated with lowercase letters. The sequences referring to splicing junctions are underlined. Deleted nucleotide and amino acid sequences in mOrc1B, mOrc2B, and mOrc3B are shown in italics. Specific amino acid repeats found in mOrc1A are depicted by bold letters.

A
mOrc1A
mOrc1B
mOrc2A
mOrc2B
mOrc3A
mOrc3B

B
introns
1
4
5

C

D

Fig. 1. A schematic representation of two isoforms of mouse Orc1, Orc2, and Orc3. A, exon organization of mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B. Intron-exon junctions are indicated with arrows, and coding sequences are shown as boxes. The positions of amino acid sequences eliminated by alternative splicing are shown under the figures. B–D, the partial genomic sequences of intron-exon boundaries at the alternatively spliced sites are shown for mOrc1 (B), mOrc2 (C), and mOrc3 (D). Exon sequences are shown in capital letters, intron sequences are shown in lowercase letters. The sequences referring to splicing junctions are underlined. Deleted nucleotide and amino acid sequences in mOrc1B, mOrc2B, and mOrc3B are shown in italics. Specific amino acid repeats found in mOrc1A are depicted by bold letters.

RESULTS
cDNA Cloning of Variant Forms of Mouse Orc1, Orc2, and Orc3 Derived from Alternative Splicing—To identify the cDNAs coding for mouse Orc1, Orc2, and Orc3, we performed RT-PCR analysis using cDNAs from several embryonic stages as well as multiple mouse tissues and cultured cells. Specific primers for

foryl fluoride, 1 mM dithiothreitol) containing Protease Inhibitor mixture (Roche Applied Science) and 0.1% Triton X-100 for 30 min at 4 °C, and subjected to low-speed centrifugation (3,000 rpm for 5 min). The supernatant (S1) was collected, and the insoluble chromatin fraction was digested for 30 min at 25 °C with 1,000 units/ml of RNase-free DNase I (Roche Applied Science) in cytoskeleton buffer containing 0.1% Triton X-100. The solubilized fraction was collected by low-speed centrifugation (S2). The insoluble fraction was further extracted with cytoskeleton buffer containing 0.1% Triton X-100 and 0.3 M KCl for 30 min at 4 °C and separated by high-speed centrifugation (S3 and P).

For Western analysis, samples were separated by electrophoresis using a 9% SDS-polyacrylamide gel and transferred electrophoretically onto 0.45-μm polyvinylidene difluoride membranes (Millipore, Billerica, MA). After incubation of the membranes with primary antibodies in Blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% I-block (Tropix, Applied Biosystems, Foster City, CA), 0.1% Tween 20, and 0.02% NaN₃) for 12 h at 4 °C, the membranes were washed three times with blocking buffer, then incubated at 1 h at room temperature with alkaline phosphatase-conjugated secondary antibodies (Sigma) in Blocking buffer. Blots were developed using the enhanced chemiluminescent reagent CDP-Star reagent (Tropix), following the manufacturer’s instructions. To inhibit the proteases, transfected COS-1 cells were treated with 20 μM Me₂SO or MG132 (Calbiochem) for 10 h.

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mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B were designed as shown in Fig. 2A, and specific products were detected by agarose gel electrophoresis followed by SYBR Green staining and/or Southern hybridization. A single transcript of the predicted size was observed when cDNA of each Orc subunit was used as a template (Fig. 2B). When primers a and b were used for mOrc1, PCR fragments corresponding to mOrc1A and mOrc1B were amplified simultaneously as 505- and 400-bp fragments, respectively (Fig. 2B, lanes 4–6). Alternatively spliced exons are shown by a shaded box.)

Fig. 2. Expression of mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B mRNA. A, schematic strategy used for amplification of mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B. Alternatively spliced exons are shown by a shaded box. B, PCR using mOrc1A (lanes 4–6) or mOrc1B (lanes 7–9) cDNA (0.5 pg) as a template demonstrates primer specificity for the two types of cDNA and provides size controls for specific amplification products. Lanes 1–3 show the negative controls without template DNA. The analogous PCR products are shown for mOrc2A/mOrc2B (lanes 10–12) and mOrc3A/mOrc3B (lanes 13–18). C, RT-PCR analysis is shown for mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B in mouse early developmental stages and multiple tissues. E7, E11, E15, and E17 represent embryonic days 7, 11, 15, and 17, respectively. After amplification of cDNA using the transcript specific primer pairs, PCR products were electrophoretically separated on a 2% agarose gel and detected by Southern blot analysis (mOrc1 and mOrc2) or SYBR Green staining (mOrc3 and β-actin). β-Actin was used as a loading control. D, RT-PCR detects mOrc1A/mOrc1B, mOrc2A/mOrc2B, and mOrc3A/mOrc3B transcripts in mouse cell cultures. Swiss3T3 cells were serum-starved and then stimulated for cell growth by serum addition. Cells were harvested at the indicated times after addition of serum, and cDNA was prepared directly from the cells using the Cells-to-cDNA kit at each time point. The number of PCR cycles is indicated in parentheses. Cell growth was monitored by laser scanning cytometry, as indicated in the upper panel. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a loading control.

As shown in Fig. 2C, PCR products for mOrc1A, mOrc2A, mOrc3A, mOrc2B, and mOrc3B were detectable in embryos as well as in most tissues, including heart, brain, muscle, kidney, spleen, testis, and small intestine. Interestingly, in contrast to mOrc3A and mOrc3B, mOrc1 and mOrc2, and variants, were scarcely detected in the liver under our conditions (lane 8), suggesting that Orc3 may have a specific function for homeostasis in mouse liver. As for mOrc2B, specific signals were found in most of the tissues. However, the ratio of mOrc2B to mOrc2A was not constant; for example, mOrc2B expression in lung, spleen, and small intestine was lower than in other tissues. Furthermore, mOrc3A and mOrc3B were scarcely detected in the lung and the thymus. Although the PCR product for mOrc1A was clearly detected throughout the developmental stages E7 to E17, mOrc1B was only detected in embryonic stage 17. In addition, a PCR product for mOrc1B was only detected in the thymus. Taken together, these results strongly suggested that mOrc1B was substantially expressed in specific environments and at certain time points during development.
A previous report for human Orc1 showed that Orc1 expression associated with the nuclear matrix (16, 17). To determine the subcellular distribution of mOrc subunits, including the novel variants, mOrc proteins transiently overexpressed in COS-1 cells were fractionated by simple extraction of the cells and harvested after a 72-h incubation. Western blotting with anti-Xpress was used to detect mOrc1 isoforms, and with anti-T7 to mOrc2 or mOrc3 isoforms. Western blot analysis using anti-Mcm7, anti-PCNA, and anti-lamin B antibodies are shown as fractionation controls.

Using this anti-mOrc1B antibody, we analyzed FM3A or thymus tissue extracts. In both extracts, a 97-kDa polypeptide band corresponding to the mOrc1A protein was detected (Fig. 3B, lanes 5 and 6, and at higher magnification in Fig. 3C, lanes 1 and 2). Moreover, a 93-kDa band, which corresponds to the mOrc1B protein, was specifically detected in thymus whole cell extracts from Swiss Webster mice (Fig. 3B, lane 5, or Fig. 3C, lane 2). When the anti-mOrc1A antibody was used for Western analysis using recombinant mOrc1A or mOrc1B protein, a single 97-kDa band was detected (data not shown), indicating that this antibody specifically detects mOrc1A, but not mOrc1B. Note that the band marked with an asterisk (in Fig. 3C, lane 3) is a nonspecific signal detected by the anti-mOrc1A antibody. Thus, using anti-peptide antibodies, we confirmed that the novel variant form of mOrc1, mOrc1B, can be detected in thymus tissues.

Subcellular Distribution of Ectopically Expressed mOrc1A and mOrc1B Analyzed by Cellular Fractionation and Western Analysis—A previous report for human Orc1 showed that Orc1 is localized primarily in the insoluble nucleosome-resistant fraction associated with the nuclear matrix (16, 17). To determine the subcellular distribution of mOrc subunits, including the novel variants, mOrc proteins transiently overexpressed in COS-1 cells were fractionated by simple extraction of the cells using detergent, nuclease, and increasing salt concentrations, as shown in Fig. 4A. We found that transiently overexpressed mOrc1B was mainly extracted in the S1, S2, and S3 fractions, as well as the pellet, but was not detected in the S4 fraction. However, mOrc1A was detected in S4, the 2M salt-resistant chromatin bound fraction (Fig. 4B). In contrast, no differences in fractionation were observed between mOrc2A and mOrc2B.
or between mOrc3A and mOrc3B, and these proteins fractionated in the S1–S3 fractions and the pellet, but not S4 (Fig. 4, C and D). Human and hamster Orc1 have been recovered in the S3 fraction, which contains chromatin-binding proteins that are tightly associated with nucleosome-resistant matrix proteins (16, 28).

**Ectopically Expressed mOrc1A and mOrc1B Localized in the Nucleus and Cytoplasm, Respectively**—To examine the subcellular localization of the splicing variants, cDNAs were transiently transfected into mammalian cultured cells, and expressed proteins were visualized by indirect immunofluorescence. When T7-tagged mOrc1A was ectopically expressed in COS-1 cells, proteins were exclusively localized in the nucleus (Fig. 5A), as reported previously for human Orc1 (25, 29). Moreover, after extraction with 0.5% Triton X-100, mOrc1A proteins predominantly co-localized with heterochromatin regions, which specifically stained with Hoechst 33258 and anti-HP1β (heterochromatin protein 1β) (Fig. 5B). In sharp contrast, when mOrc1B was expressed in the COS-1 cells, mOrc1B was localized to small punctate structures in the cytoplasm (Fig. 5A). These results indicated that the splicing variant mOrc1B was deficient in nuclear translocation due to its lack of the 35-amino acid fragment in exon 5. Endogenous Orc4 or Mcm4 did not co-localize with overexpressed mOrc1B protein, in double staining of transfected cells (data not shown). These results suggest that ectopically expressed mOrc1B might not associate with endogenous components of the pre-RC in COS-1 or NIH3T3 cells. In addition, cytoplasmic mOrc1B did not co-localize with the endoplasmic reticulum marker calreticulin or the mitochondrial marker MitoTracker (Fig. 5, C and D). T7-tagged mOrc2A, mOrc2B, mOrc3A, and mOrc3B were transfected into COS-1 cells and their subcellular localization was analyzed. All these subunits were predominantly localized in the nucleus, some of them forming fine dot-like structures (Fig. 5A). Apparent differences in the subcellular distribution of the two splicing variants of mOrc2 and mOrc3 were not detected.

**Identification of the Nuclear Localization Signal for mOrc1A**—Nuclear proteins are transported into the nucleus by a specific import system composed of importin-α, -β, and GTP-dependent Ran (30). Classic nuclear translocation is solely dependent on a defined amino acid motif called a nuclear localization signal (NLS). The NLS is not only essential for nuclear translocation, but also sufficient for the entry of cytoplasmic proteins, such as ectopically expressed bacterial β-galactosidase, into the nucleus when fused to the NLS sequence. Because mOrc1B was exclusively expressed in the cytoplasm while mOrc1A was localized to the nucleus, we hypothesized that the amino acid region eliminated in mOrc1B functions as an NLS. Thus far, an NLS has not been identified in Orc1, because common search algorithms do not recognize a functional NLS in this protein (31). To define the NLS in mOrc1A, we constructed a recombinant plasmid that encoded a fusion protein of the mOrc1A-specific 35 amino acids and β-galactosidase, into the nucleus when fused to the NLS sequence. Because mOrc1B was exclusively expressed in the cytoplasm while mOrc1A was localized to the nucleus, we hypothesized that the amino acid region eliminated in mOrc1B functions as an NLS. Thus far, an NLS has not been identified in Orc1, because common search algorithms do not recognize a functional NLS in this protein (31). To define the NLS in mOrc1A, we constructed a recombinant plasmid that encoded a fusion protein of the mOrc1A-specific 35 amino acids and β-galactosidase, as well as positive and negative controls as shown in Fig. 6A. The expression plasmids were transfected into COS-1 cells, and the expressed proteins were visualized by indirect immunofluorescence using an anti-β-galactosidase antibody. The fusion protein containing the spliced fragment was exclusively localized in the nucleus while wild type β-galactosidase was localized in the cytoplasm (Fig. 6C). That is, nuclear translocation-deficient bacterial β-galactosidase protein was transported into the nucleus due to its fusion with the mOrc1A fragment.

Next, to identify the exact amino acid sequences responsible for nuclear translocation of β-galactosidase, several basic lysine and arginine residues were replaced with neutral asparagine and glutamine residues and the proteins were expressed in COS-1 cells (Fig. 6B). Whereas mutant I, mOrc1246–280KK(252–253)NN, was transported into the nucleus as well as the wild-type mOrc1246–280β-galactosidase, mutant II, mOrc1246–280KRR(266–268)NNQ, was localized to the cytoplasm and mutant III, mOrc1246–280KK(279–280)NN, was localized to both the nucleus and the cytoplasm (Fig. 6D). It is
possible that the NLS mutations made in the truncated form of mOrc1A fused to β-galactosidase protein behave differently in the context of full-length mOrc1A protein. Therefore, we constructed the NLS mutant of the full-length mOrc1A protein (mOrc1A:KRR(266–268)NQQ), and examined its subcellular distribution. We confirmed that this mutant (Fig. 7A) was localized to the cytoplasm (Fig. 7D, see below). Taken together, we concluded that amino acid residues 266–280 are not only essential, but also sufficient, for nuclear translocation of mOrc1A, and, moreover, the loss of this NLS by alternative splicing in mOrc1B, results in its localization to the cytoplasm.

*mOrc1 Stability Is Regulated by the Proteasome*—Recently several lines of evidence have indicated that metazoan Orc1 is an unstable protein and is modified by polyubiquitination and degraded through a proteasome-dependent pathway during cell cycle progression (15, 17, 28, 32). To verify that mOrc1A and mOrc1B are modified and degraded by the proteasome-dependent pathway, transiently transfected COS-1 cells were treated with the proteasome-specific inhibitor MG132. Positive and negative controls for the inhibitor treatment were monitored using anti-p27 and anti-PCNA antibodies, respectively. Ectopically expressed proteins were detected by Western blot analysis (Fig. 7B). Treatment of cells with MG132 increased the p27 protein level severalfold but did not affect that of PCNA, indicating that proteasome-dependent protein degradation can be specifically examined in this system using 20 μM MG132.

Treatment of MG132 increased protein levels of mOrc1A significantly, whereas the level of mOrc1B remained unchanged (Fig. 7, B and C). To address the question of whether degradation is dependent on nuclear localization, we used an mOrc1A construct in which the functional NLS is mutated (mOrc1A:KRR(266–268)NQQ), and examined the effect of MG132 treatment on its protein stability (Fig. 7A). This mutant was localized to the cytoplasm, and the signal intensity of this protein increased after treatment with MG132 (Fig. 7, B–D). These results indicate that mOrc1A is degraded by the ubiquitin-mediated proteasome pathway. In contrast, mOrc1B, also localized to the cytoplasm, is not affected by MG132 treatment and remains stably expressed in the cells. Thus, the stabilities of these proteins are independent of their subcellular localization.

Because mOrc1B is resistant to proteasome-dependent degradation, we attempted to identify the essential sequences required for degradation in mOrc1A. With the aid of amino acid alignment of Orc1 sequences from various organisms (Fig. 8A), we noticed that mOrc1B lacks a putative CDK2-dependent phosphorylation site ((S/T)PXY(R/K)) in the NLS. This phosphorylation site is conserved in vertebrates from *Xenopus* to humans but not in *Drosophila* (Fig. 8A). It was reported that human Orc1 (hOrc1p) is phosphorylated by cyclin A/CDK2 at multiple sites, most of them located in the N-terminal half of the protein, and gets degraded due to its interaction with the F box protein Skp2, a substrate recognition component of the Skp1-cullin 1-F box ubiquitin-ligase complex (17). The N-terminal domain of hOrc1p, which contains most of the phosphorylation sites and overlaps with one of the Skp2-interacting domains, thus functions as a regulatory element for hOrc1p stability. Another report demonstrates that phosphorylation of hOrc1p is carried out by cyclin A/CDK and regulates its translocation into the cytoplasm via the Crm1-mediated export (29). To test whether the consensus phosphorylation site conserved in vertebrates, but lacking in mOrc1B, is important for degradation or nuclear transport, we analyzed mutant forms of mOrc1A in which the conserved phosphorylation site Ser-276 was substituted to an Ala, Asp, or Glu residue (Fig. 7A). Inter-

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**Fig. 6. Localization of a chimera consisting of the spliced fragment from mOrc1A fused to β-galactosidase.** A, schematic representation of the fusion constructs and summary of localization. Wild-type (wt) β-galactosidase protein localized in the cytoplasm. p54t-β-gal, a positive representation in which β-galactosidase is fused to the NLS of DNA primase subunit p54 (RIRKKLR) (19), localized to the nucleus. The mOrc1A:KRR(266–268)NQQ, a chimera construct of β-galactosidase fused with the mOrc1 alternatively spliced fragment, amino acids 246–280 (PNTBWSKKSS(DS)LDYQKTSKRRAAFSETPSPPKK), is also localized in the nucleus. B, schematic representation of the mutant constructs. Three clusters of basic residues (KK252–253, KRR266–268, and KK279–280) located within the spliced region were substituted with neutral amino acid residues resulting in NN, NQQ, and NN substitutions, respectively. The percentages of cells ectopically expressing proteins in the nucleus, the cytoplasm, or both nucleus and cytoplasm (nuc + cyto) are shown. C, subcellular distribution of the constructed proteins by indirect immunofluorescence analysis. Each construct was transfected into COS-1 cells. All proteins were detected 48 h after transfection by indirect immunofluorescence analysis using a polyclonal anti-β-galactosidase antibody and an Alexa-488 secondary antibody. Nuclei were counterstained with Hoechst 33258. D, immunofluorescence localization of mutant proteins fused with the β-galactosidase gene. Mutant I (KK252–253NN) is localized in the nucleus, mutant II (KRR266–286NNQQ) in the cytoplasm, and mutant III (KK279–280NN) in both nucleus and cytoplasm.
Interestingly, the signal intensity of the phosphorylation-deficient mutant (S276A) was unchanged in the presence or absence of MG132, whereas in those mutants that mimicked phosphorylation (S276D and S276E), the signals were increased similar to mOrc1A (Fig. 7, B and C). In addition, these mutants were localized to the nucleus, at levels comparable to that of the wild-type mOrc1A (Fig. 7D). Therefore, these results indicate that the conserved phosphorylation site, localized in the sequence that mOrc1B lacks, is important for degradation of vertebrate Orc1.

**DISCUSSION**

In this study, we identified novel alternative splicing variants of mouse Orc1, Orc2, and Orc3 genes. This alternative splicing results in the elimination of 35 amino acids in exon 5 of the Orc1 gene, 48 amino acids in exon 2 of the Orc2 gene, and 1 amino acid in exon 15 of the Orc3 gene. As a result, three novel protein isoforms, Orc1B, Orc2B, and Orc3B, consisting of 805, 528, and 714 amino acid residues, respectively, are produced. Each one of these Orc genes exists as one allele located at a specific locus on the mouse genome.
The expression of the splicing variants was confirmed by RT-PCR analysis. Thereby, we found that mOrc1B was expressed specifically in the thymus and in late embryonic stages, whereas the other Orc subunits and variants were widely expressed throughout different mouse cell lines and tissues as well as at different time points during development. Characterization of transiently overexpressed Orc subunits revealed that only mOrc1B was localized to the cytoplasm and was resistant to the ubiquitin-mediated proteasomal pathway, whereas all of the other Orc subunits were exclusively detected in the nucleus. Taken together, these results suggest that additional variants of Orc subunits are involved in ORC complex formation and may indicate that there are various cellular functions of these proteins that are not fully understood. Moreover, truncated Orc1B revealed functional domains for nuclear translocation and recognition by the ubiquitin-mediated proteasomal pathway, suggesting that mouse Orc1 may be controlled by unique mechanisms not found in other eukaryotes.

In S. cerevisiae, ORC serves as the landing pad for the pre-RC. In yeast cells, the assembly of Cdc6, Cdt1, and Mcm proteins is dependent on the prior binding of ORC to the ARS. The S. cerevisiae ORC stably associates with the ARS throughout the cell cycle, and its interaction with other replication factors changes dynamically during cell cycle progression. In contrast, recent reports suggested that the counterparts of Orc subunits in metazoans exhibit different association profiles, especially Orc1. Human, hamster, and Drosophila Orc1 loosely associate with Orc2–5 subcomplexes (2, 15, 33), and their expression from G1 to S phase is dependent on the transcription factor E2F. The timing of chromatin binding of Orc1 is different from that of Orc2–5. Orc2–5 associates with chromatin at late telophase, whereas Orc1 binds to chromatin at G1 to S phase, with a peak at the G1/S transition. Moreover, chromatin-bound Orc1 is degraded during the S to M phase in human, but not in hamster cells (15, 17, 28). In the case of hamster cells, Orc1 seems to be mono-ubiquitinated in S phase and phosphorylated in M phase, but the roles of these modifications remains unclear (28, 32). These findings in metazoan systems imply that Orc1 is the limiting step for the assembly of the pre-RC before its conversion to the pre-initiation complex rather than a landing pad for other initiation factors of the pre-RC. Therefore, the characterization of Orc1 transcription and translation, its translocation into the nucleus, and its association with other Orc subunits are important features for the elucidation of the physiological functions of the mammalian ORC.

We identified a mouse Orc1 splicing variant form that is deficient in nuclear translocation, suggesting a novel regulatory mechanism for controlling Orc1 activity in the cell. It is tempting to speculate that nuclear translocation controls Orc1 function. If Orc1 loses its nuclear localization signal, Orc1 remains in the cytoplasm thereby preventing the onset of replication. Thus, we hypothesize that alternative splicing may act as an accurate and transient brake to cell cycle progression without the need for post-translational modifications, degradation, or transcriptional regulation.

We further found that mOrc1B is detectable in embryonic and thymus tissues where the overall developmental program is closely linked to cellular differentiation. In particular, many kinds of alternative splicing variants play specific roles in the immune and nervous system (34). It has been reported that very short splicing variants of human interleukin-4 (IL-4) exist in the thymus (35). In the IL-42-2 protein, exon two was skipped resulting in the deletion of 16 amino acids and a conformational change from β-sheet to loop structure. This splicing form might function as a co-stimulator for T-cell proliferation. Moreover, the DNA-binding protein SATB1 is expressed specifically in the thymus and selectively binds to DNA in nuclear matrix/scaffold-associated regions (36). SATB1 has a cage-like "network" localization circumscribing heterochromatin (37). mOrc1A is also associated with heterochromatin (see Fig. 5B), whereas mOrc1B is localized in the cytoplasm exclusively and is specifically expressed in the thymus. Thus, mOrc1B appears to be a cell type-specific protein that might play a crucial role during thymocyte differentiation.

The stability of human Orc1 is regulated by a proteasome-dependent pathway, whereas Drosophila Orc1 is shown to be regulated by anaphase-promoting complex-dependent degradation with its N-terminal domain being responsible for degradation and nuclear translocation (33). The relationship between phosphorylation and degradation remains to be elucidated. Our studies showed that mOrc1B is localized stably in the cytoplasm (Fig. 5A) due to the lack of important domains for degradation, phosphorylation, and nuclear localization (Fig. 5B). Furthermore, a conserved phosphorylation site is essential for degradation. Thus, Orc1 degradation may be regulated by its phosphorylation at this specific site. Taken together, these findings are consistent with the previous characterization of hOrc1p (17) and further identify a critical residue for phosphorylation and degradation.

Alternative splicing increases the diversity of protein functions. Because the human genome project revealed that only 22,000 genes are involved in the human genome, the heterogeneity and complexity of higher eukaryotic systems has been considered to depend on alternative splicing. Indeed, a recent bioinformatics analysis showed that 40–60% of transcribed genes contain alternate splice variants (34). Alternative splicing occurs by adding or deleting functional domains thereby leading to additional protein functions. Expressed sequence tag sequences automatically predict alternative splicing events, however, the biological significance of alternative spliced variants must be examined by individual characterization of the respective proteins. Moreover, although the expressed sequence tag data base accumulates exponentially, many alternative splicing events are very rare and occur only in a specific tissue, at a specific time in development and/or under certain physiological conditions. These splicing forms will probably not be well represented in the expressed sequence tag data collection. In fact, human and mouse homologues for Orc1B and Orc2B have not yet been found in the NCBI data base. Concerning splicing variants of ORC subcomplexes in other eukaryotes, it was reported that human Orc5 has a splicing variant, HsOrc5Tp (38). The alternative spliced form of HsOrc5 was expressed in a wide range of human tissues and malignant cells, however, in mouse cell lines, we have not detected the truncated Orc5T homologue thus far (data not shown).

In this study, although we characterized the subcellular distribution and expression in various tissues of each variant of Orc1, Orc2, and Orc3, apparent differences between mOrc2A and mOrc2B or mOrc3A and mOrc3B were not detected. Amino acid sequences flanked by the splicing junction have not shown the typical properties for functional domains. As Orc2 has been reported to associate with various replication factors, including Mcm10, Cdt1, and Orc3 (14, 23, 39–41), we speculate that mOrc2B and mOrc3B splicing variants might also be involved in protein-protein interactions or complex formation. Thus, biochemical and physiological characterization of individual splicing variant is an essential step in further post-genomic analysis.

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