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Conditional Transgenic System for Mouse Aurora A Kinase: Degradation by the Ubiquitin Proteasome Pathway Controls the Level of the Transgenic Protein

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Aurora A is a mitotic kinase that localizes to centrosomes. Expression of this protein is normally limited to the mitotic stage (G_2-M) of the cell cycle, whereas human cancer cells frequently exhibit overexpression of Aurora A protein regardless of the cell cycle stage. In the present study, Aurora A transgenic mouse lines were generated with a new conditional expression system (cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter-Z-enhanced green fluorescent protein) in order to analyze the function of this protein. Although transcripts for Aurora A were elevated in multiple organs of the transgenic mice, the corresponding protein was not detected in extracts analyzed by immunoblotting. The treatment of transgenic-derived embryonic fibroblasts (MEF) with proteasome inhibitors markedly increased the protein level of transgenic Aurora A, indicating that the transgenic Aurora A protein is readily degraded in normal mouse tissues. Under the exponential growth conditions of MEF cells, transgenic Aurora A was detected within the mitotic stage of the cell cycle and localized to centrosomes. In contrast, the marker of the transgenic promoter (enhanced green fluorescent protein) was continuously expressed throughout the cell cycle, indicating the constitutive transcription of transgenic mRNA. These results indicate that transgenic Aurora A is protected from degradation within G_2-M but is immediately degraded after translation in the G_1-S stage of the cell cycle. The findings obtained with this transgenic model and derived cells support that the transition from protection to degradation by the ubiquitin proteasome system at the end of mitosis is an important step in controlling the level of Aurora A protein during the cell cycle.

The Aurora A protein belongs to a family of serine/threonine kinases that also include Aurora B and Aurora C. The three kinases have a relatively conserved C-terminal catalytic domain but differ with regard to length and sequence in the N-terminal domain (3). Each member of this kinase family exhibits a specific pattern of localization and function (7). Earlier genetic studies in *Drosophila melanogaster* revealed that Aurora A has a critical role in chromosomal and centrosome separation (11, 12, 14). Aurora A localizes to the centrosome and also to the bipolar mitotic spindle poles (7). Localization studies by electron microscopy revealed that this kinase is associated with the filamentous structure at the surface of the centrosome, which is known as the pericentriolar material (29).

Expression of Aurora A protein is highly dependent on the stage of the cell cycle (3, 22). In accord with a role in mitotic progression, slight increases of Aurora A message and protein occur during the end of S phase and are maximum at the G_2-M phase (32). The increased mRNA of Aurora A around G_2-M was confirmed with a reporter assay for the promoter region, and the putative transcriptional element responsible for cell cycle dependency was identified (32). Phosphorylation sites in Aurora A protein are important for its activation. Kinase activity requires phosphorylation of a threonine residue (Thr288 in human Aurora A) in the activation loop of the C-terminal catalytic domain (19). TPX2 (target protein for *Xenopus* kinesin-like protein 2) binds to Aurora A and is considered to be important for autophosphorylation at this site and protection of the kinase from phosphatase activity (9, 35).

The collective findings from several laboratories indicate that Aurora A can function as an oncogene (2, 8, 25, 39). As supporting evidence of this notion, the Aurora A gene has been mapped to the 20q13 chromosome, which is a region frequently amplified in many human cancers (27). Amplification of this region has been reported in 12% of primary breast tumors and in 40% of breast cancer cell lines (39). Amplification of the 20q13 region also occurs at a frequency of 52% in colorectal tumors (3). In addition, most (94%) of the primary invasive mammary carcinomas analyzed for Aurora A immunoreactivity were positive (33). In line with these clinical data, exogenous overexpression of Aurora A in Rat1 fibroblasts causes transformation accompanied by centrosome amplification and chromosome instability (2). Furthermore, the Rat1 cells that expressed a constitutively active mutant of Aurora A formed subcutaneous tumors when inoculated into nude mice (2). The expression of human Aurora A in human MCF10A breast cancer cells and mouse primary embryonic fibroblasts also led to centrosome amplification and genomic instability (1, 39). On the basis of these observations, the Aurora A protein is considered important to maintain the accuracy of chromosome separation, and de-

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fects in its function might result in genomic instability and cancer progression (6, 20, 23).

In the present study, we developed a mouse conditional transgenic system (cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter-Z-enhanced green fluorescent protein [CAG-Z-EGFP]) to express Aurora A protein. Although Aurora A mRNA was efficiently expressed in the transgenic mouse tissues, the corresponding protein was shown to be degraded by the ubiquitin proteasome pathway under quiescent conditions. We also showed that the transgenic Aurora A protein is expressed during mitosis and localizes to the centrosome under the exponential growth conditions of embryonic fibroblasts derived from transgenic mice. These studies clearly demonstrate the importance of degradation in regulating the level of Aurora A protein during the cell cycle.

MATERIALS AND METHODS

Plasmid construct and transgenic mouse production. The cDNA fragment of mouse Aurora A (GenBank no. U69106) was obtained by reverse transcription-PCR (RT-PCR) with total RNA of mouse embryonic tissue at developmental stage of 13 days (Clontech). The hemagglutinin (HA) protein tag sequence was introduced at the amino terminus of Aurora A protein by the PCR. For the efficient translation of transgenic protein, the Kozak sequence was introduced just upstream of the start codon of the HA protein tag. The amplified cDNA fragment was cloned into the EcoRV site of pBlueScript SK+ (Stratagene) by the TA-cloning method (21). The sequence accuracy of the coding region of mouse Aurora A cDNA was confirmed in the clones selected. The selected cDNA was subcloned into the PstI site of the CAG-Z-EGFP transgenic cassette in the correct orientation by the TA-cloning method. Primer sequences are available from us on request.

The details of the method used to create the basic transgenic vector (CAG-Z-EGFP) are described elsewhere (Fukuda et al., unpublished data). The transgenic plasmid (CAG-Z-EGFP-Aurora A) was digested with SwaI to obtain the linear transgenic construct. The linearized transgenic construct was diluted (to 2 ng/μl) with 0.1× Tris-EDTA buffer and injected into the pronucleus of mouse eggs (FVB strain, 0.5 day) with a glass capillary. The transgenic founders were initially screened by the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of tail tissue. Mouse mammary tumor virus (MMTV)-Cre mouse line A was purchased from the Jackson Laboratory (Bar Harbor, ME). The genotypes of MMTV-Cre mice were determined by a PCR amplification specific to Cre recombinase protein. All mouse experiments were performed in accordance with National Institute of Environmental Health Sciences guidelines covering the humane care and use of animals in research.

Preparation of Cre-expressing BM25.8 competent bacteria and plasmid. The BM25.8 *Escherichia coli* strain was obtained from Clontech and used for the preparation of competent bacteria that expresses the Cre recombinase protein. The competent BM25.8 *E. coli* was prepared by Hanahan's method (31). The transgenic plasmid (CAG-Z-EGFP-Aurora A) was introduced into BM25.8 for Cre-mediated recombination. All of the plasmids were recovered with the QIAprep system (QIAGEN).

The detection of transiently expressed Aurora A protein in COS7 cells. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (50 U/ml), at 37°C and 5% CO_2. Cells were maintained in a rapid growth phase prior to use in experiments. The cells were transfected with 2 μg of the appropriate plasmids by the lipofection method as suggested by the manufacturer (Fugene 6; Roche).

The transiently expressed Aurora A protein was visualized by fluorescence confocal microscopy. First, transfected cells were fixed with neutralized 4% paraformaldehyde solution and then exposed to methanol for 1 min at −20°C. Next, the cells were treated with primary antibodies to HA protein tag (sc-7392, 1/100 dilution; Santa Cruz) and rabbit anti-gamma tubulin (PRB-433C, 1/500 dilution; Covance) in 5% normal goat serum-phosphate-buffered saline (PBS). Alexa 350-labeled goat anti-mouse immunoglobulin G (IgG) and Alexa 568-labeled goat anti-rabbit IgG (Molecular Probe) were used for detection at a 1:200 dilution in 5% normal goat serum-PBS. DNA was stained with SYTOX Green (Molecular Probe). The results were obtained with a Zeiss LSM510 confocal microscope system.

X-gal staining, Western blots, and Northern blots. Tissues were stained by X-gal to determine expression of the transgene (10). For Western blotting, whole tissue and cultured cells were lysed in HIPS buffer (28). Protein was subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore). After blocking with 1% nonfat dry milk-TBST (Tris-buffered saline and 0.1% Tween 20), the membrane was initially probed with antibody to HA protein tag (1/200 dilution; Santa Cruz), Aurora A (kindly provided from Peter Donovan, Kimmel Cancer Center, Thomas Jefferson University; 1/1,000 dilution), EGFP (Clontech; clone JL-8, 1/2,000 dilution), cyclin B1 (Santa Cruz; SC-245, 1/1,000 dilution), and actin (Santa Cruz; C-11, 1/2,000 dilution). The polymer-immunocomplex method was used for the detection of rabbit primary antibody (13). Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz; 1/5,000 dilution) or donkey anti-goat (Santa Cruz; 1/5,000 dilution) antiserum, and immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

For the Northern blot analysis, total RNA was extracted by the guanidine phenol chloroform method using TRIZOL (Invitrogen). Five micrograms of total RNA were applied to formaldehyde denatured gels for Northern blots and then transferred to a Nylon membrane. The membrane was hybridized at 65°C with the random hexamer labeled probe in hybridization buffer (Clontech). Full-length mouse Aurora A cDNA that was obtained by RT-PCR and the NotI-EcoRI fragment of EGFP-1 plasmid (Clontech) were used for the probe preparation. After overnight hybridization, the blots were washed once with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and three times with 2× SSC–0.1% SDS buffer at 65°C. The hybridization signal was detected by X-ray film (Hyperfilm MP; Amersham Biosciences) and an intensifying screen at 80C°.

Establishment of mouse embryonic fibroblasts derived from wild-type and Aurora A transgenic embryos. Mouse embryonic fibroblasts (MEFs) were obtained from both wild-type and transgenic embryos at developmental stage of 13.5 day. The genotypes of the embryos were detected by EGFP fluorescence and were confirmed by the PCR amplification to be specific to the EGFP sequence. Two embryos for each genotype (wild-type and recombined Aurora A transgenic embryos) were homogenized with a stainless steel mesh (0.39 mm; Thomas Scientific). Cells were cultured in DMEM with 10% fetal calf serum, penicillin, streptomycin, and 50 μM 2-mercaptoethanol. The MEF cells were frozen as stocks at the second passage and were used for the subsequent studies at the third passage.

Treatment of primary embryonic fibroblasts with proteasome inhibitors. All proteasome inhibitors were initially dissolved in dimethyl sulfoxide (DMSO; 25 mM stock solution) and then diluted to 25 μM with culture medium to treat the MEFs. *N*-acetyl-Leu-Leu-norleucinal (LLnL, MG101), MG132, clasto-lactacystin β-lactone (cLL), and *N*-acetyl-Leu-Leu-methional (ALLM) were obtained from Sigma Chemical Co. MEFs were treated with the inhibitors for 7 h prior to analysis of the cells for transgene products.

The immunohistochemical detection of endogenous and transgenic Aurora A protein during the cell cycle of MEFs. For these experiments, transgenic and wild-type MEF cells were grown to approximately 70% confluence on tissue culture slides (Beckton Dickinson). The cells were fixed in ice-cold methanol for 5 min and blocked with normal goat serum (5%) in PBS. For the detection of endogenous Aurora A protein in MEF cells, mouse monoclonal antibody for IAK-1 (Transduction Laboratory) was used at a 1:800 dilution in PBS with 5% normal goat serum. Alexa 488-labeled goat anti-mouse IgG (Molecular Probes) was used for the detection. The transgenic Aurora A protein was detected with a rat monoclonal antibody to the HA protein tag (Roche). After the incubation of primary antibody at a 1:800 dilution, the signal was detected by Alexa 564-labeled goat anti-rat IgG (Molecular Probes). The fluorescence signal of EGFP was detected after fixation in 4% paraformaldehyde solution. Fluorescent signals were evaluated with a Zeiss LSM510 laser confocal microscope.

Cell cycle synchronization of MEF cells with nocodazole treatment. Transgenic and wild-type MEF cells were grown to about 70% confluence and treated with vehicle or nocodazole (Sigma; 100 μg/ml in dimethyl sulfoxide for stock solution) at 100 ng/ml for 8 h to increase the percentage of the cells in the mitotic stage of cell cycle. The cells in mitotic stage were collected by mitotic shake-off (34) and then collected by centrifugation. Cell pellets were lysed in HIPS buffer (28) and subjected to SDS–10% PAGE.

RESULTS

Strategy and design for the conditional Aurora A transgenic mouse. Overexpression of Aurora A is reported to induce

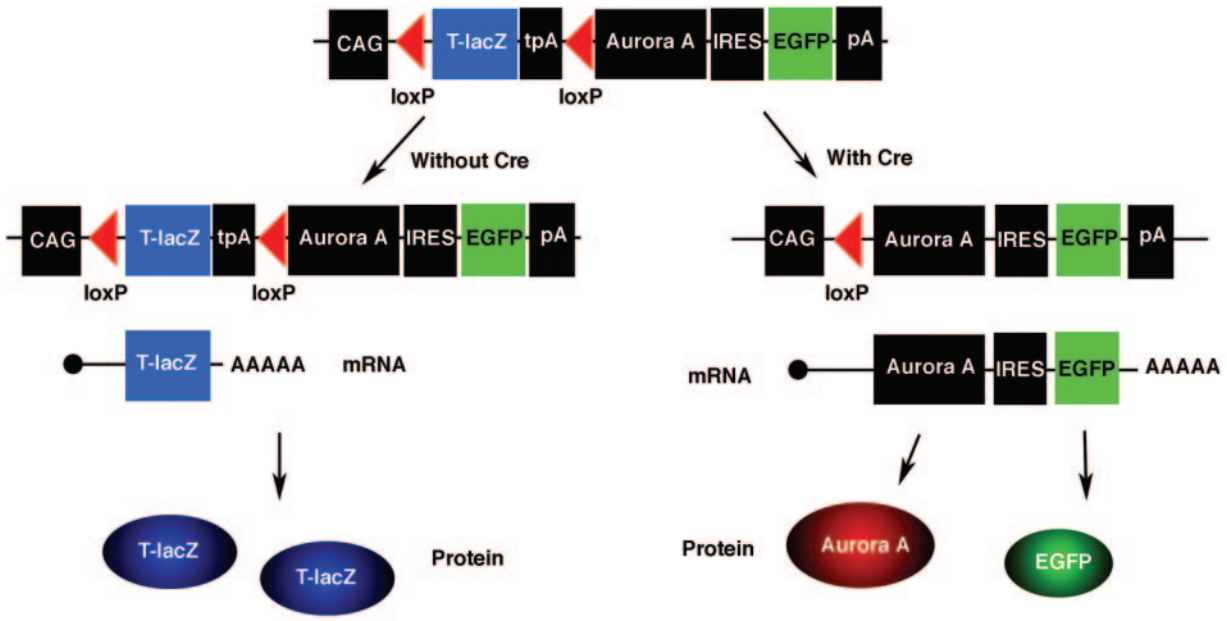

FIG. 1. Strategy for the conditional transgenic CAG-EGFP system. Top, structure of conditional mouse Aurora A transgene using the CAG-Z-EGFP system. CAG, cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter; T-lacZ, beta galactosidase with nuclear localization signal; tpA, triple polyadenylation signal; IRES, encephalomyocarditis virus internal ribosome entry site; EGFP, enhanced green fluorescent protein; pA, polyadenylation signal. Left, T-lacZ will be transcribed from the transgenic cassette. Right, Cre recombinase will excise the DNA sequence flanked by *loxP* sequences. Ribosomes can access the bicistronic mRNA either at the 5′ end to translate the mouse Aurora A or at the IRES to translate the EGFP reporter gene. Parallel protein translation between Aurora A and EGFP proteins should be observed.

abnormalities in cell division in vitro; hence, we selected a conditional transgenic system (CAG-Z-EGFP; Fig. 1) to prevent the possible embryonic lethality of founder animals. The configuration of this transgenic cassette was chosen so that mouse HA-tagged mouse Aurora A and EGFP would be expressed in the transgenic mouse only in the presence of Cre recombinase. We predicted that transcription of mRNA would stop in the middle of the transgenic cassette due to the polyadenylation of mRNA caused by triple poly(A) (Fig. 1, left). T-*lacZ* should show blue X-gal staining in mouse tissues that express the transgenic cassette. If Cre recombinase is present in the transgenic tissue, the DNA fragment flanked by *loxP* will be excised (Fig. 1, right), resulting in the transcription of Aurora A and EGFP. Due to the internal ribosome entry site (IRES) sequence in the middle of the transgene, ribosomes can access to bicistronic transgenic mRNA for translation either at the 5′ end or at the IRES sequence. The access of ribosomes to the bicistronic RNA enables the parallel translation of both Aurora A and EGFP, making the EGFP fluorescent protein a reporter for HA-tagged mouse Aurora A protein.

In vitro validation of the CAG-Z-EGFP system and concurrent expression of Aurora A protein in cultured cells. We first verified whether the recombination of CAG-Z-EGFP and the concurrent expression of transgenic Aurora A would work properly under in vitro conditions. The Aurora A transgenic plasmid was introduced into Cre expressing bacteria (BM25.8) to detect the recombination. The actual restriction map obtained from the recombined construct in Cre expressing bacteria was in good agreement with the size of the predicted map (Fig. 2A and B). The recombined plasmid in Cre expressing bacteria was introduced into COS7 cells by lipofection in order to detect HA-tagged Aurora A protein. The HA positive protein (Aurora A) was detected at the centrosome and in the cytoplasm of COS7 cells under transient conditions (Fig. 2C, upper left). Aurora A protein was observed regardless of the cell cycle stage; for example, the protein was detectable in both resting cells as well as mitotic cells (data not shown). The extracts of COS7 cells gave an appropriate band at 45 kDa when analyzed by Western blotting with the HA antibody (Fig. 2D). From these data, we conclude that the CAG-Z-EGFP system works properly under in vitro conditions.

Establishment and characterization of transgenic mouse founders for Aurora A expression. We successfully obtained five conditional transgenic founders by pronuclear injection to mouse embryos. The two lines showing the strongest LacZ activity were selected for further analysis. All tissues except mammary gland and uterus showed the blue X-gal staining (Fig. 3A, tail tissue), indicating ubiquitous expression of the transgenic cassette. Female conditional Aurora A transgenic mice were mated with male MMTV-Cre transgenic mice which have a wide tissue distribution for the expression of Cre recombinase, e.g., mammary gland, skin, liver, and lung (36). While the double transgenic mouse tissues showed strong EGFP fluorescence, tissues of the Cre transgenic mouse showed minimal fluorescence that can be considered as background (Fig. 3B and C). Interestingly, the intensity of EGFP was different among individual mice even though they had the same genotype at 6 weeks of age (Fig. 3B, middle). X-gal staining was detected in tails that showed weak EGFP fluores-

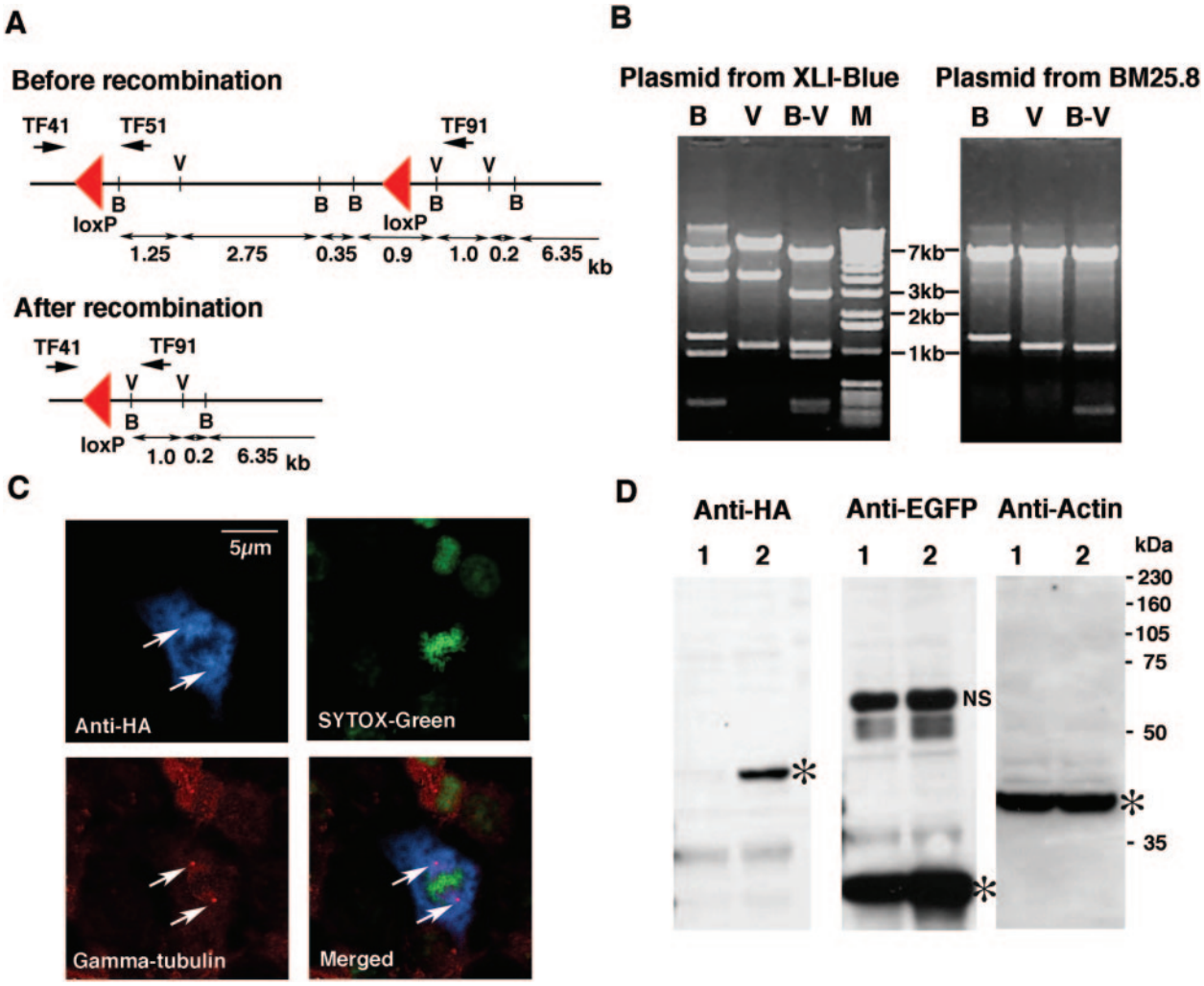

FIG. 2. Cre-mediated recombination of the transgenic cassette in Cre-expressing bacteria and the transient expression of Aurora A protein in COS7 cells. (A) The predicted restriction plasmid map for the conditional Aurora A transgenic mouse. B and V indicate the positions for BamHI and EcoRV. The red triangles indicate *loxP* sequences. The size of each DNA fragment is indicated below. The predicted plasmid maps before and after the recombination are shown. (B) The restriction pattern of DNA fragments of Aurora A transgenic plasmid after the introduction to normal bacteria (XL-1 Blue) or Cre-expressing bacteria (BM25.8). Enzymes used for the digestions are indicated at the top of each lane; BamHI (B), EcoRV (V), and double digestion for BamHI and EcoRV (B-V). Molecular weight markers (M) are expressed in kilobases. Note that the actual restriction pattern of the fragment obtained from BM25.8 matched with that predicted in the map (after recombination in panel A). (C) Transient expression of HA-tagged mouse Aurora A protein in COS7 cells transfected by recombined transgenic constructs. The HA-tagged mouse Aurora A protein is visualized with the antibodies to HA and Alexa 350 (upper left, blue panel). Arrows indicate the position of the centrosome. Centrosomes are visualized by the antibody to gamma-tubulin and Alexa 546 (lower left, red panel). DNA is visualized by the SYTOX-Green (upper right, green panel). The overlap of staining is shown in the merged picture at the lower right. Note that HA antibody shows blue staining at the position of centrosome and cytoplasm. Bar, 5 μm. (D) The Western blot detection of Aurora A protein in the lysate of COS7 cells transfected by the recombined transgenic cassette. Results obtained with the HA, EGFP, and actin antibodies are shown in each column. The specific band obtained with each antibody is indicated by an asterisk. Lane 1, cells transfected by recombined blank transgenic plasmid; lane 2, cells transfected with recombined Aurora A transgenic plasmid. Note that the specific band at 45 kDa with anti-HA was detected only in lane 2 but not in lane 1. The nonspecific band(s) is indicated by NS.

cence (Fig. 3B, bottom). In addition, the intensity of EGFP fluorescence in tail tissues increased with age of the mouse (data not shown). These data led us to assume that recombination within the transgenic cassette increases with age of the mouse. The recombination of the transgenic cassette in Aurora A/MMTV-Cre double transgenic tissues was detected by PCR amplification (Fig. 3D). These data indicate that the CAG-Z-EGFP conditional transgenic system works properly in vivo.

Detection of Aurora A mRNA but not the corresponding protein in mice. The presence of the EGFP protein in multiple mouse tissues indicates that transgenic Aurora A should also be produced in mouse tissues. The Aurora A/MMTV-Cre double transgenic mice did not show any abnormalities during mouse development. The EGFP protein was detected by immunoblotting lysates of brain, liver, lung, and tail from double transgenics (Aurora A/MMTV-Cre); however, these same ly-

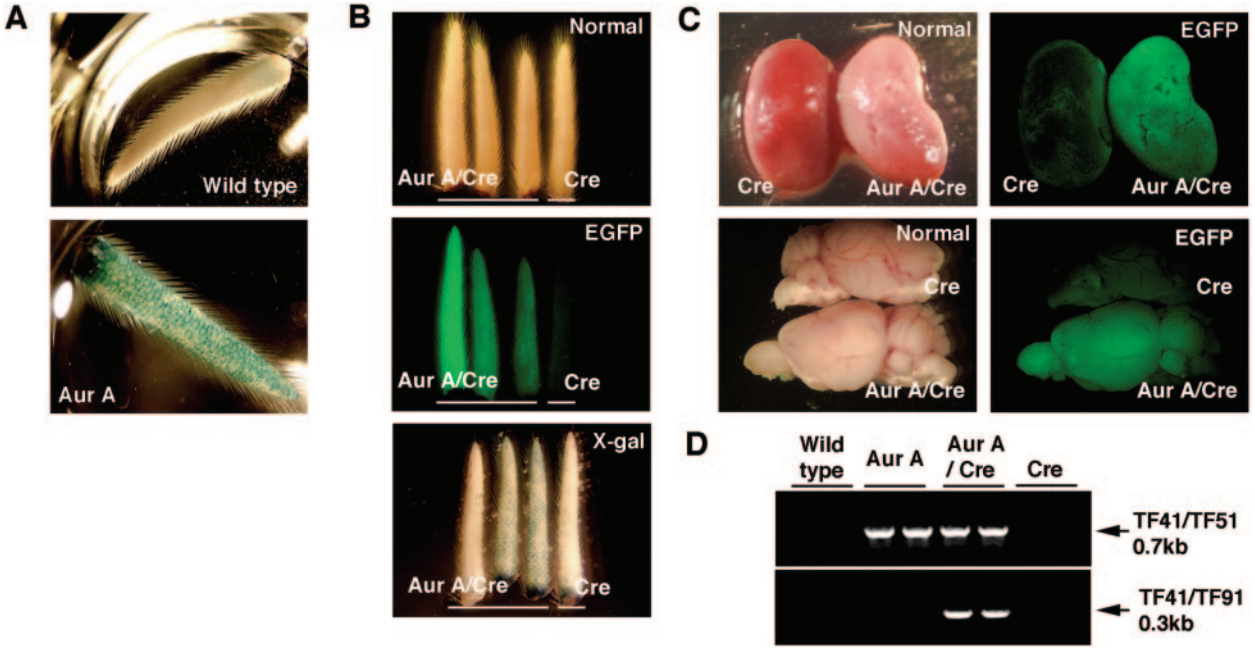

FIG. 3. LacZ and EGFP detection in the tissues of double transgenic mice (Aurora A/MMTV-Cre). (A) LacZ expression in the tail of a wild-type mouse (upper panel, wild type) and the tail of an Aurora A transgenic mouse (lower panel, Aur A) as detected by X-gal staining. (B) EGFP and LacZ detection in the tail tissue of the Aurora A/MMTV-Cre double transgenic mice and MMTV-Cre mouse. Upper panel, macroscopic observation of tails. Middle panel, EGFP fluorescence detection in double (Aur A/Cre) and single transgenic tails (Cre). Aur A/Cre indicates the Aurora A and MMTV-Cre double transgenic mice. Cre indicates the MMTV-Cre transgenic mouse. The EGFP fluorescence was detected in double transgenic mice (Aur A/Cre) but not in MMTV-Cre mouse (Cre). The EGFP fluorescence was activated upon Cre-mediated excision of floxed T-lacZ and poly(A) sequence. Note the difference in the intensity of EGFP fluorescence among the three tails from double transgenic mice. Lower panel, LacZ expression detected by X-gal staining. Note *lacZ* expression is detected in the tail tissues of two double transgenic mice that showed weak fluorescence in the middle panel. (C) The detection of EGFP fluorescence in the kidney and brain of double transgenic mouse. Upper panels, macroscopic observation of brain and kidney. Lower panel, EGFP fluorescence intensity in the tissues of double transgenic mice (Aur A/Cre) is much higher than that of MMTV-Cre mouse (Cre). (D) PCR detection of Cre-*loxP* recombination in the tail tissues of the Aurora A/MMTV-Cre double transgenic mice. The genomic DNA obtained from tails of wild-type, Aurora A transgenic mice, Aurora A/MMTV-Cre double transgenic mice, and MMTV-Cre transgenic mice were subjected to PCR analysis. The corresponding annealing positions of each PCR primer within the transgenic cassette are listed in Fig. 2A. TF41/TF51 is the specific primer set that allows amplification of the unrecombined transgenic allele. TF41/TF91 are the primers that allow the specific amplification of the recombined allele. Note that only the Aurora A/MMTV-Cre double transgenic shows the recombined specific amplification due to the Cre-mediated excision of floxed T-lacZ and poly(A) sequence.

sates from single transgenic mice (MMTV-Cre) were negative (Fig. 4A, middle panels). Surprisingly, the transgenic Aurora A protein was not detectable with an antibody to HA tag regardless of the mouse genotype (Fig. 4A, top panels). Likewise, transgenic Aurora A protein was not detected when a polyclonal antibody to native Aurora A was used. Transfected COS7 cells, on the other hand, readily showed Aurora A protein by immunoblotting with antibodies for HA and endogenous Aurora A (Fig. 4A, top panels), indicating that antibody and method of detection are valid.

To address whether the transgenic mRNA is properly transcribed in these tissues, total RNA was subjected to Northern blotting (Fig. 4B). For accurate signal evaluation, we selected one MMTV-Cre mouse and two Aurora A and MMTV-Cre double transgenic mice that had different EGFP fluorescence intensities. The band detected by the EGFP probe showed good correlation with the intensity observed by fluorescence microscopy (Fig. 4B, middle). The Aurora A probe showed an identical expression pattern to that of the EGFP probe (Fig. 4B, top and middle), indicating that these probes detect the same bicistronic mRNA. Furthermore, these two probes detected bands at the predicted size (5.5 kb), which is larger than that of native Aurora A mRNA (2.5 kb) (15, 16), indicating these signals are derived from the transgenic mRNA. Thus, these data show that the transgenic Aurora A mRNA is expressed in mouse tissues after Cre-dependent recombination, although the corresponding protein is not detected.

Expression of transgenic Aurora A protein in MEF cells treated with proteasome inhibitors. Since the treatment of HeLa cells with proteasome inhibitors was reported to increase the protein level of Aurora A (17), it is likely that transgenic Aurora A is undetectable due to degradation by the ubiquitin proteasome system, even if the transgenic mRNA is efficiently transcribed. To test this notion, we evaluated the effects of proteasome inhibitors on mouse embryonic fibroblasts (MEF) from transgenic and nontransgenic animals. Since Cre recombinase expression starts in the later stages of embryogenesis, the MEF cells obtained from double transgenics (Aurora A/MMTV-Cre) will be composed of a mixed population of cells, recombined and nonrecombined. To increase the percentage of recombined MEF cells, a germ line-recombined Aurora A transgenic allele was created. Since the MMTV-Cre

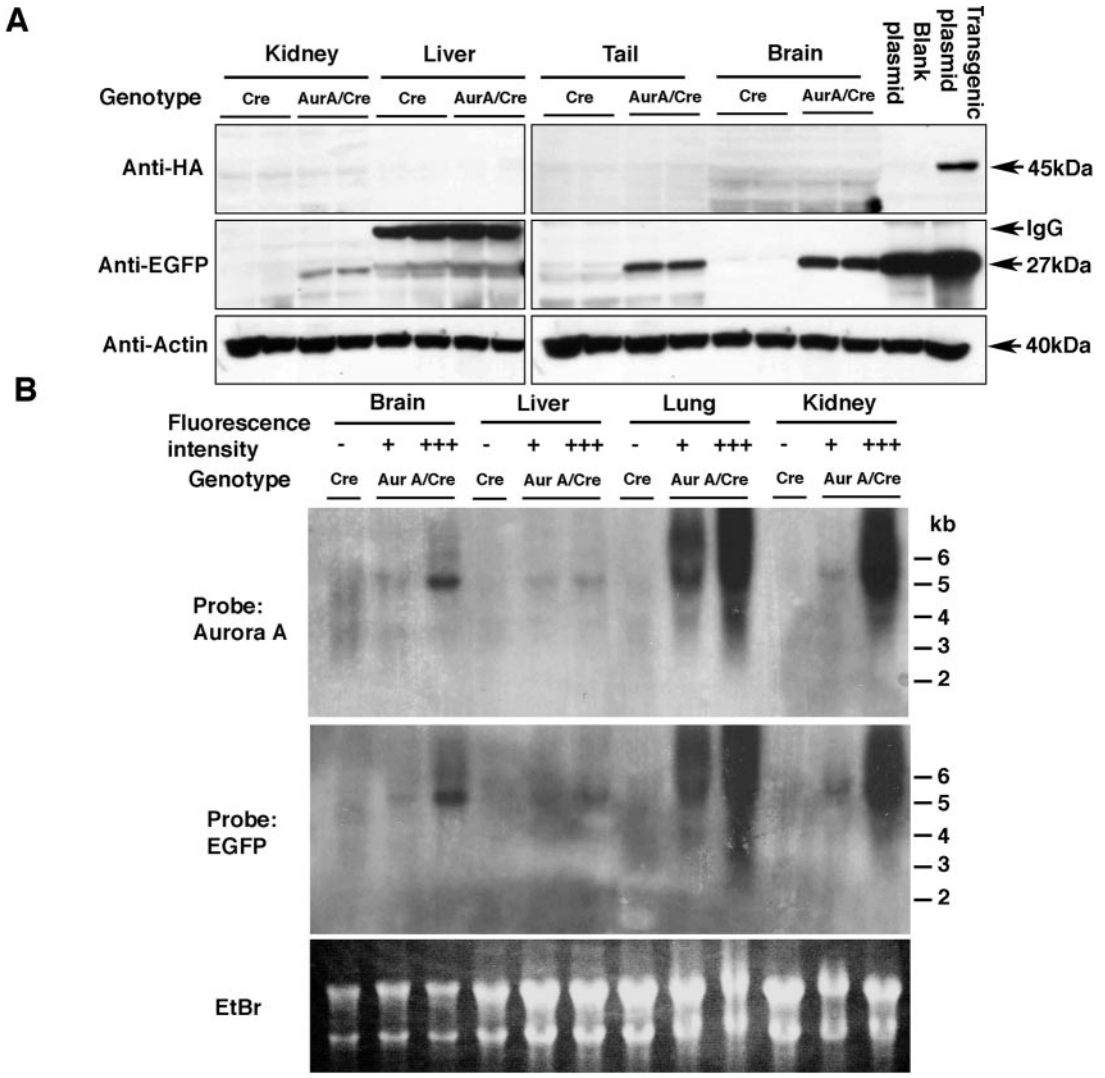

FIG. 4. Detection of transgenic Aurora A protein and mRNA in Aurora A, MMTV-Cre double transgenic, and MMTV-Cre mice. (A) The detection of HA-tagged Aurora A, EGFP, and actin in tissues of double transgenic (Aur A/Cre) and MMTV-Cre mice (Cre) by Western blotting. Results obtained from two mice for each genotype are shown in this picture. Protein from COS7 cells transfected with recombined blank vector and recombined transgenic vector was analyzed by immunoblotting as a negative and positive control (transient expression). Note that EGFP expression is observed only in double transgenics but not in MMTV-Cre mice. Note there is no difference in the column of HA between double transgenic (Aur A/Cre) and MMTV-Cre mice (Cre). The IgG band (around 55 kDa) was detected due to the binding of secondary antibody in liver sample. (B) Detection of transgenic mRNA by Northern blot. The results from two double transgenic mice (Aur A/Cre) and one MMTV-Cre mouse (Cre) are shown. The double transgenic mice show different EGFP fluorescence intensities under the microscope even though these two mice are the same genotype (see the fluorescence intensity). Total RNA from brain, liver, lung, and kidney are analyzed. Top, the results obtained with the Aurora A probe. Middle, the results obtained with the EGFP probe. Bottom, ethidium bromide staining of total RNA. Note that the signal derived from the Aurora A probe showed the same expression pattern with that derived from the EGFP probe.

mouse expresses Cre protein during oogenesis, transgenic mice that have the recombined allele at the germ line level can be obtained from mating these mice (Fig. 5A). Mouse embryos that have germ line recombined transgenic Aurora A showed increased EGFP fluorescence when compared with wild-type mouse embryos (Fig. 5B). The lysates from untreated (no proteasome inhibitors) wild-type or transgenic MEF cells did not reveal HA immunoreactivity by Western blotting (Fig. 6A, top panel). However, after treatment with the proteasome inhibitor MG132, the transgenic MEF cells showed a significant increase in transgenic Aurora A protein (Fig. 6A, top panel). In addition, this significant increase of transgenic Aurora A protein in MEF cells was also detected with an antibody to native Aurora A (Fig. 6A, second from the top). Based on the morphology of MEF cells, there was no obvious cell toxicity of the MEF cells caused by the MG132 treatment. Treatment with vehicle or MG132 did not change the level of EGFP protein. This would be anticipated since the EGFP is not processed by the ubiquitin proteasome pathway. It is known that MG132 can also activate heat shock proteins and JNK-1 (24), in addition to having an inhibitory effect on the 26S proteasome. Therefore, other proteasome inhibitors, LLnL and cLL, which do not activate either heat shock proteins or JNK-1, were used for the treatment of MEF. cLL is reported

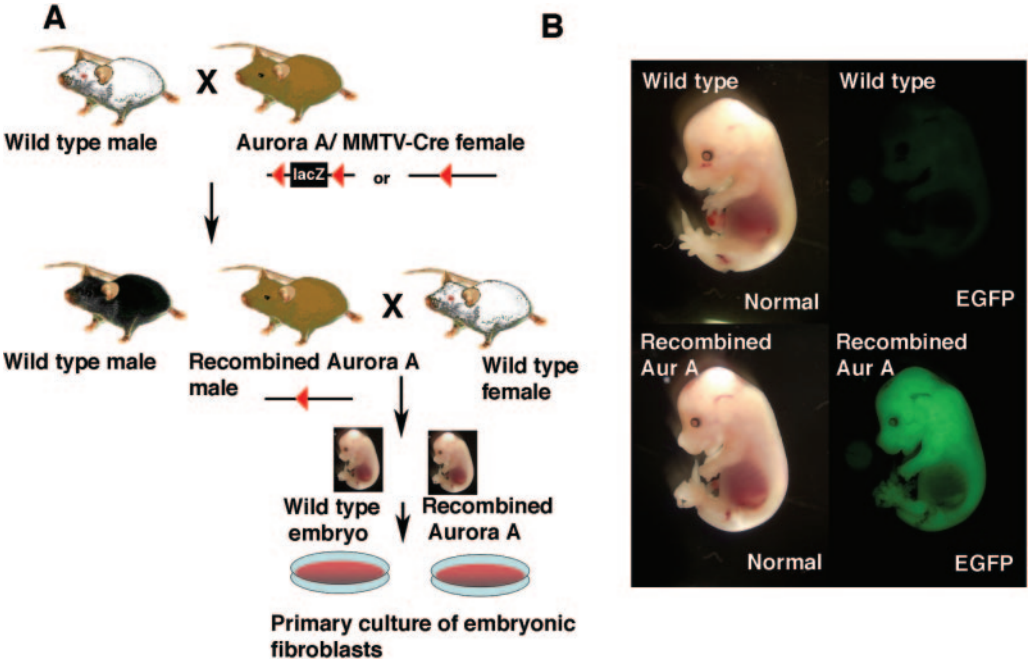

FIG. 5. Establishment of mouse embryonic fibroblasts (MEF) containing the recombined Aurora A transgenic cassette. (A) Schematic representation to show how MEF cells that have the recombined Aurora A transgenic cassette were obtained. A wild-type male was bred with a double transgenic female (Aurora A and MMTV-Cre). Due to the Cre expression during oogenesis, a male mouse was obtained that has a recombined Aurora A transgenic cassette at the germ line level. As a result of mating between a recombined Aurora A transgenic male and wild-type females, mouse embryos were obtained that have the recombined Aurora A transgenic cassette throughout the organs. MEF cells were established from the primary culture of embryonic tissues. (B) Detection of the EGFP reporter protein in the mouse transgenic embryo that has the recombined allele. Note that EGFP expression is observed in the recombined Aurora A transgenic mouse embryo.

to be a specific proteasome inhibitor with trypsin-like inhibitory activity (17). As with MG132, treatment with LLnL and cLL also increased expression of transgenic Aurora A (Fig. 6B and C). Since treatment with ALLM, a specific inhibitor of calpain, did not affect the level of Aurora A (Fig. 6D), inhibition of this protease was ruled out as the cause of increased Aurora A protein levels. Thus, interfering with proteasome activity leads to an increase in transgenic Aurora A protein.

Mitotic expression of transgenic Aurora A protein in MEF cells: degradation by the proteasome limits Aurora A expression to G_2-M. It has been reported that the levels of both Aurora A mRNA and protein are rapidly decreased at the end of the mitotic phase during the cell cycle (16). However, it is not clear what step is responsible for the decrease of Aurora A protein, decreased mRNA transcription or increased protein degradation. Since our transgenic cassette is driven by an artificial promoter (CAG promoter), transcription of the transgenic cassette should be independent of the cell cycle stage. If the transgenic Aurora A protein has a G_2-M-dependent pattern while the corresponding mRNA is constitutively expressed throughout the cell cycle, protein degradation of Aurora A should be the dominant step controlling the level of Aurora A protein rather than transcription.

To test this notion, the level of Aurora A protein is detected by immunostaining during the cell cycle of both wild-type and transgenic MEF cells. Although an increased endogenous Aurora A protein level in G_2-M was reported in COS7, HeLa, and NIH 3T3 cells (16, 17), the pattern of Aurora A protein expression has not been reported for cycling MEF cells. To verify the cell cycle dependency of Aurora A in MEF, an antibody to endogenous Aurora A was used to detect this protein in wild-type MEF cells. Aurora A was essentially undetectable in the resting stage of the cell cycle (Fig. 7A). The Aurora A was detected at prometaphase (data not shown). During metaphase and anaphase, the level of Aurora A protein increased and was evident at each end of the bipolar spindles, indicating localization of Aurora A to the centrosome and spindle microtubules (Fig. 7B and C). During cytokinesis, Aurora A was still associated with the centrosomes. This cell cycle dependency and intracellular localization of Aurora A in MEF cells are in agreement with that previously reported in NIH 3T3 cells (16). Normal mouse IgG did not show any staining, which demonstrates specificity of detection (Fig. 7K). The transgenic MEF cells were then analyzed for the transgenic Aurora A protein with an antibody to HA tag. The pattern and intracellular localization of transgenic Aurora A were in accord with that of native Aurora A protein (Fig. 7E to H). Again, transgenic Aurora A was essentially undetectable in cells at the resting stage. Wild-type MEF cells did not show any staining, indicating the specificity of the HA antibody (Fig. 7L). In transgenic MEF cells, the EGFP protein is cotranslated with transgenic Aurora A from the same mRNA. Since EGFP is not degraded by the ubiquitin proteasome pathway, the protein level of EGFP can be used as a reporter for transcriptional activity of the transgenic cassette. The EGFP protein was detected continuously during the cell cycle in the transgenic MEF cells (Fig. 7I). Wild-type MEF cells did not exhibit EGFP fluorescence, indicating detection specificity for the EGFP protein (Fig. 7J).

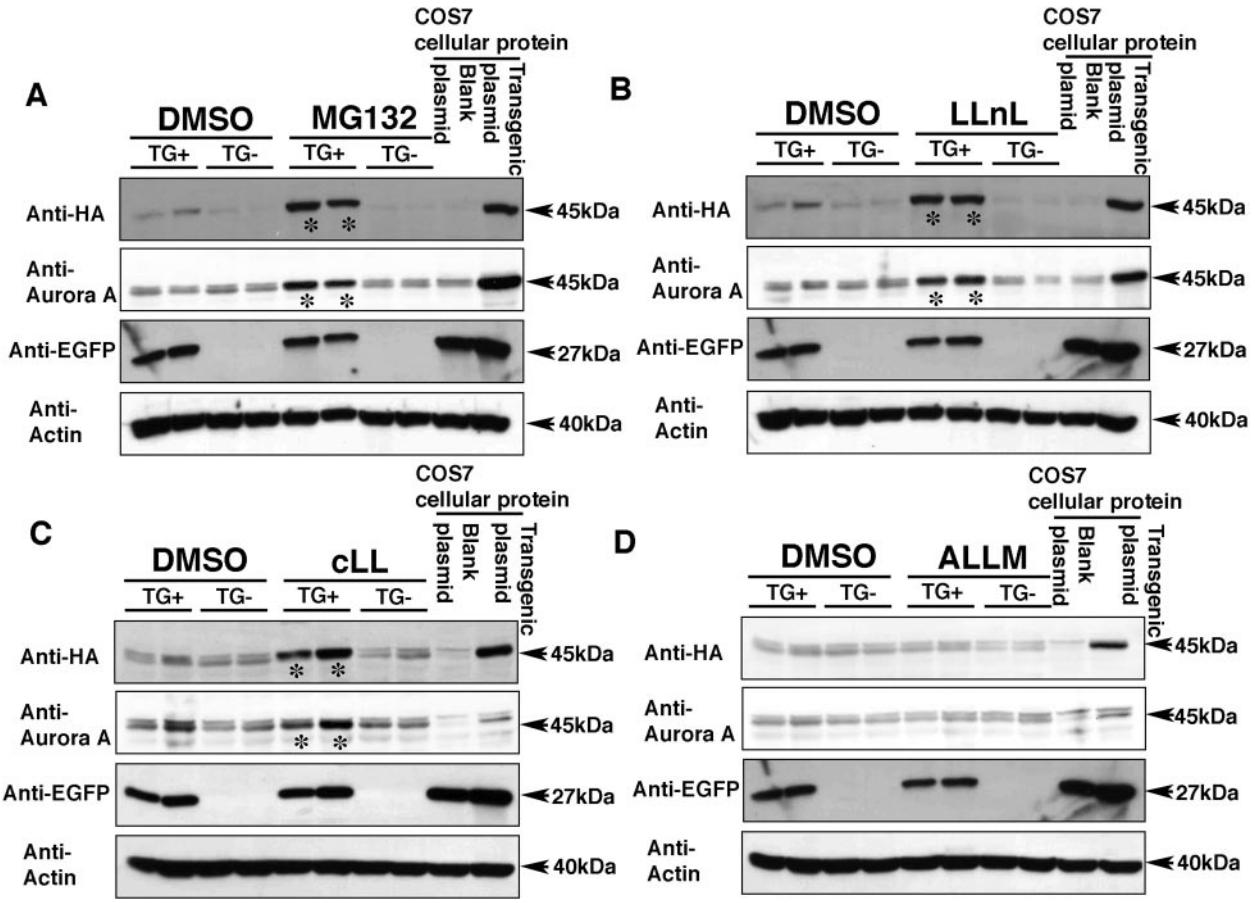

FIG. 6. Expression of transgenic Aurora A in MEF cells treated with proteasome inhibitors. Transgenic and wild-type MEF cells were treated with several proteasome inhibitors. Lysates were analyzed by immunoblotting for HA, Aurora A, EGFP, and actin. The results obtained following treatment with (A) MG132, (B) LLnL, (C) cLL, and (D) ALLM are shown. Lysates from COS7 cells transfected with the recombined transgenic vector and empty vector were used as positive and negative controls, respectively (transient expression). Two independent samples were tested for each genotype. Note that an increase in Aurora A transgenic protein was detected in transgenic MEF cells treated with MG132, LLnL, and cLL but not in MEF cells treated with vehicle (dimethylsulfoxide). EGFP expression in MEF cells derived from the recombined transgenic showed no difference whether treated with or without inhibitors.

Although immunostaining is a useful method to determine the intracellular localization of a protein, this method is at best semiquantitative. To evaluate the level of transgenic Aurora A during the cell cycle, wild-type and transgenic MEF cells were synchronized by nocodazole treatment and cell lysates analyzed by immunoblotting. Since nocodazole is toxic to MEF cells at a high concentration (1 μg/ml), the mitotic cells were collected by the shake-off method after treatment at a lower concentration (100 ng/ml) (see Materials and Methods). As expected, the protein level of cyclin B increased after the M-phase synchronization with nocodazole (Fig. 8) (19). The transgenic MEF cells in the mitotic stage showed an increased level of transgenic Aurora A protein when detected with the HA antibody, which is in agreement with our immunofluorescence data (Fig. 8). Accordingly, the total amount of Aurora A protein (endogenous plus transgenic Aurora A), as measured with an antibody to the native protein, was increased in the transgenic cells when compared to that of wild-type MEF cells. In contrast, the level of the EGFP protein was fairly constant with and without cell synchronization. These observations confirm that maximal expression of the transgenic Aurora A occurs during the mitotic stage of the cell cycle. We propose that the transgenic Aurora A protein is translated and then rapidly degraded outside of G_2-M.

DISCUSSION

In this study, we established Aurora A transgenic mouse lines as an effort to obtain overexpression of Aurora A protein with a new conditional transgenic system (CAG-Z-EGFP). The expression of Aurora A was regulated by the recombination within the transgene mediated with the Cre protein and its target sequences (*loxP*). The CAG-Z-EGFP system has two marker genes, *lacZ* and EGFP, in order to follow the activated/silent status of the transgenic cassette. Even when the transgenic Aurora A protein was undetectable in our animals, the EGFP marker was still observed in mouse tissues. The cumulative findings from our experiments showed that the design of the CAG-Z-EGFP enabled us to create a transgenic mouse that expresses both mouse Aurora A and markers in multiple organs. Furthermore, the cellular localization of the transgenic Aurora A protein in transgenic-derived embryonic fibroblasts

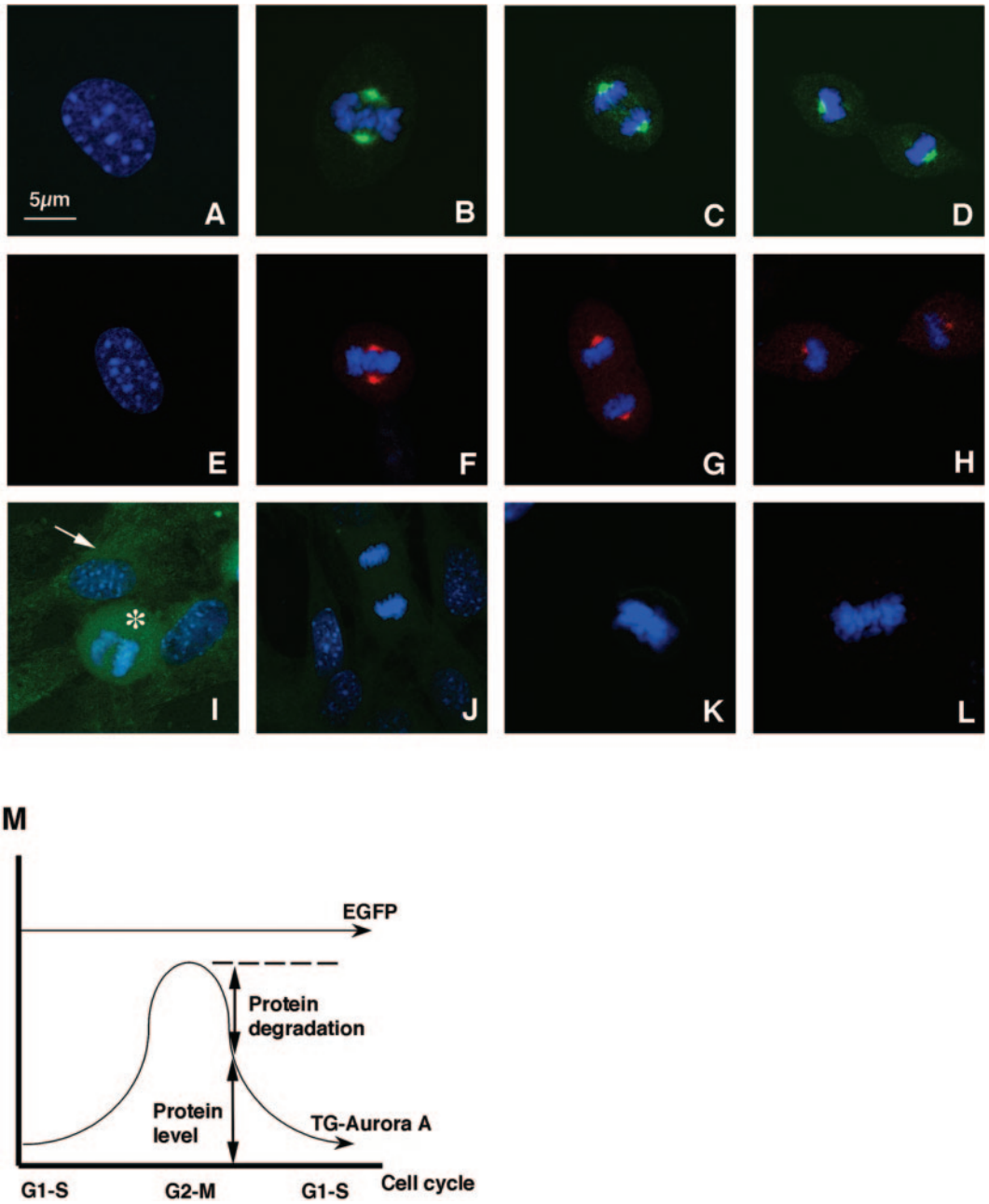

FIG. 7. Detection of G_2-M-dependent expression of native and transgenic Aurora A protein in wild-type and transgenic MEF cells. (A to D) Detection of native Aurora A protein at the following stages of cell cycle: (A) interphase, (B) metaphase, (C) late anaphase, and (D) cytokinesis. Native Aurora A is visualized by green fluorescence (Alexa 488). The chromosome is visualized by blue fluorescence (4*N*,6*N*-diamidino-2-phenylindole). (E to H) Detection of transgenic Aurora A protein in transgenic-derived MEF cells at the following stages of cell cycle: (E) interphase, (F) metaphase, (G) late anaphase, and (H) cytokinesis. Transgenic Aurora A protein is visualized by red fluorescence (Alexa 546). (I) The detection of EGFP reporter protein in cycling MEF cells. The mitotic stage of cell division is denoted by an asterisk. Note that the fluorescence intensity of the EGFP marker protein during cell division appears similar to that observed when the cells are in interphase (arrow). (J) Detection of EGFP in wild-type MEF cells. (K) Control staining of native Aurora A (IgG control). (L) Control staining of HA antibody in wild-type MEF cells. (M) Model for the cell cycle dependent expression of transgenic Aurora A protein. The longitudinal axis represents the level of protein expression. The horizontal axis indicates the stage of the cell cycle. The transgenic promoter is active throughout the cell cycle (as represented by EGFP). Aurora A is protected from the degradation during the G_2-M of the cell cycle but is degraded during G_1-S of the cell cycle.

(MEF) was equivalent to that of endogenous Aurora A protein.

The treatment of transgenic-derived MEF cells with proteasome inhibitors increased the level of transgenic Aurora A protein. The same inhibitor profile was shown for the degradation of native human Aurora A in HeLa cells (17). This indicates that the transgenic Aurora A protein is processed by a pathway that is similar to that used for the degradation of

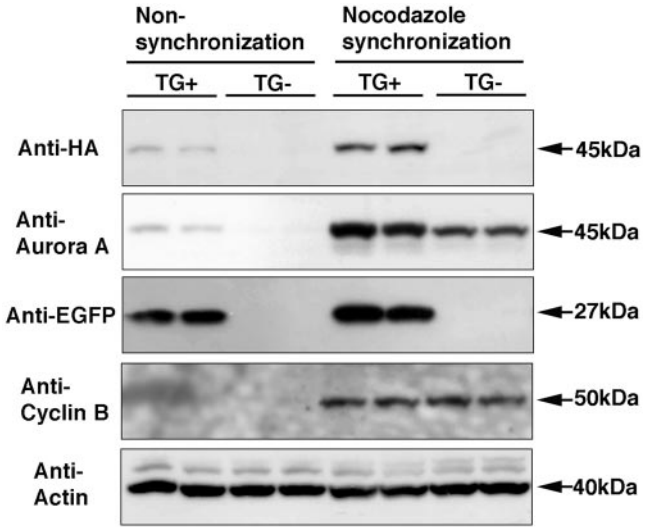

FIG. 8. Changes in protein level of native and transgenic Aurora A in nocodazole-synchronized MEF cells. Wild-type and transgenic MEF cells were treated with vehicle (dimethyl sulfoxide) or nocodazole. Lysates were analyzed by immunoblotting for HA, Aurora A, EGFP, cyclin B, and actin. Two independent samples were tested for each genotype. A comparable increase in cyclin B was observed in transgenic (TG+) and wild-type (TG−) cells exposed to nocodazole. Note that the signal of transgenic Aurora A (anti-HA) increased in the TG+ cells after the nocodazole treatment and that the level of total Aurora A in these cells was at least twofold greater than that of wild-type cells. By contrast, a strong signal for EGFP was detected in the transgenic cells with and without nocodazole treatment.

endogenous Aurora A protein. Furthermore, the recombinant human Aurora A protein was reported to be degraded by the proteasome pathway in *Xenopus* egg extracts (4, 19). The latter studies with *Xenopus* egg extracts showed that degradation of Aurora A is regulated by ubiquitination which is mediated by the anaphase promoting complex/cyclosome (APC/C) and its activator, Cdh1 (19). However, HeLa cells may involve possible abnormalities in the ubiquitin proteasome pathway, since this cell was established from malignant cancer tissue. The protein level of Aurora A in these cells is still relatively high in the absence of proteasome inhibitors (5). The present study is the first report that Aurora A protein is unstable and rapidly degraded in normal animal tissues.

The analysis of MEF cells under exponential growth conditions allowed us to detect the level of transgenic Aurora A protein during the mitotic stage of the cell cycle, even though this protein was essentially undetectable in mouse tissues. Transgenic Aurora A protein is detected during G_2-M and localizes to the centrosome in transgenic-derived MEF cells. Studies with HeLa and NIH 3T3 cells have also shown this same pattern and localization; namely, that Aurora A is detected and localizes at the centrosome during the early stage of mitosis and disappears at the mitotic exit (2, 16, 22). Although the level of Aurora A mRNA as well as Aurora A protein was reported to decrease at the mitotic exit (2, 16, 32), it was not clear which is the limiting step in determining the level of the Aurora A protein outside of G_2-M, the decrease in the mRNA or the protein degradation. The constitutive expression of EGFP in our transgenic MEF indicates our transgenic promoter (CAG promoter) transcribes mRNA regardless of the cell cycle stage, whereas the transcriptional activity of the endogenous Aurora A promoter is limited mostly to G_2-M. The constitutive expression of transgenic mRNA during cell quiescence was suggested by the intense signal of transgene detected in Northern blots (most of the cells are under the resting stage in the adult mouse tissues). On the other hand, transgenic Aurora A protein was undetectable during cell quiescence.

The cumulative findings from earlier studies have led to a model for cell cycle-dependent expression/degradation of Aurora A that is shown in Fig. 7M. Our data from both the transgenic animals and derived cell lines support this model. The expression of transgenic Aurora A mRNA is invariant during the cell cycle (along with constant EGFP expression) (see Fig. 7M). After translation, Aurora A protein is degraded by the ubiquitin proteasome pathway in G_1-S but is protected from degradation during G_2-M. As a consequence of its degradation in G_1-S and its protection in G_2-M (with no change at the mRNA level), the level of protein mimics a waveform during the cell cycle. The previous reports are consistent with our conclusion that the transition from protection to degradation is a critical step for the level of Aurora A protein during the cell cycle. Aurora A degradation after the G_2-M phase release from nocodazole block was inhibited by proteasome inhibitor(s) (17), and the treatment of cycling cells with proteasome inhibitors results in accumulation of the ubiquitinated form of Aurora A (17, 37). However, it is not clear in these experiments whether the accumulation of Aurora A protein by inhibitors is due to a direct effect on proteasome degradation or an indirect effect resulting from blockage of cell cycle progression. In our work, comparison of the stability of the transgenic Aurora A protein and the EGFP marker protein, which is not affected by proteasome inhibitors, clearly show that the transition from protection to degradation of Aurora A at the end of mitotic stage is an important step to control the level of Aurora A protein.

In our study, the transient expression in COS7 cells showed the level of Aurora A protein changed little regardless of the cell cycle stage. Furthermore, Aurora A protein is detected in the cytoplasm of COS7 cells as well as in the centrosomes. This expression pattern and intracellular localization that resulted from transient expression were much different from that observed under stable conditions. The abundant protein production observed under transient conditions might overwhelm the degradation capacity of the proteasome pathway. Immunoblot studies support the notion that the amount of Aurora A in extracts of transiently transfected cells is greater than that in extracts of stably transfected cells. A previous study established NIH 3T3 cell lines that stably express *Xenopus* Aurora A. In their study, mRNA of introduced *Xenopus* Aurora A was readily detected by RT-PCR, but the level of corresponding Aurora A protein was too low to be reliably detected on immunoblots (19). Their difficulty in detecting the target protein in stable cell lines might be explained by the proteasome degradation of Aurora A. Our results have a direct relevance for experiments that use an expression system of Aurora A, both under transient and stable conditions.

During the preparation of the manuscript, Zhang et al. described a conditional transgenic mouse that expresses human Aurora A in mammary epithelial tissue (38). The expression of

human Aurora A was regulated by Cre recombinase driven by a whey acidic protein promoter. The stable expression of human Aurora A was reported to induce abnormal cell division (binuclear cells) in the mouse mammary gland, resulting in hyperplasia and increased incidence of apoptosis. In our study, an obvious phenotype that corresponded to the expression of transgene was not observed up to an age of one year. The outcome in our transgenic mice might be explained by differences in susceptibility to degradation between human and mouse Aurora A proteins. Although the kinase domains of human and mouse Aurora A exhibit a high degree of identity (95.5% in amino acids 124 to 371), the N-terminal region is not well conserved (38.5% in amino acids 1 to 123). A difference in amino acid sequences in the A and D boxes of human and mouse Aurora A was reported to be important for the rate of proteasome degradation (5, 19). A species difference in the rate of protein degradation of Aurora A between human and *Xenopus* has been reported (5). Human Aurora A may be more resistant to protein degradation in mouse tissue than mouse Aurora A protein. Although the nonhomologous expression systems (e.g., human Aurora A into mouse cell, *Xenopus* Aurora A into mouse cell) (2, 20) have been used to detect the oncogenic activity of Aurora A protein, it may be important to utilize a homologous transgenic system (mouse Aurora A protein in mouse tissue) to achieve an accurate functional analysis of Aurora A. Although the transgenic animals of the present study did not develop tumors, these animals might be more sensitive than wild types to carcinogenic stimuli.

A recent study by Crane et al. showed that addition of N-terminal epitope tags to Aurora A protein can reduce the efficiency of degradation by APC/C in vitro (5). This was particularly so when the large green fluorescent protein (GFP) was used as an N-terminal tag for human Aurora A in their in vitro system (5). In contrast, addition of the smaller 6-amino-acid AU1 tag only slightly inhibited degradation of Aurora A when compared to the untagged protein. Even though our findings suggest that the transgenic mouse Aurora A was readily degraded in whole animals and derived fibroblasts, it is possible that N-terminal HA tag used in the present study reduced the rate of protein degradation.

The cell synchronization experiments in the present study revealed that total amount of Aurora A protein within G_2-M (transgenic plus endogenous Aurora A) in transgenic MEF cells is significantly higher than that of wild-type cells. In contrast, the protein level of Aurora A in transgenic MEF cells was almost identical with that of wild-type cells in other stages of the cell cycle. In the cancer-derived cell lines, abnormal expression of Aurora A occurs regardless of the cell cycle stage (18). It might be necessary to increase the protein level of Aurora A outside of G_2-M to mimic the phenotype in cancers. In this regard, two groups have indicated that the level of Aurora A protein in tumors does not always correlate with amplification of the Aurora A gene (26, 30). An abnormality in the degradation pathway for Aurora A could result in accumulation of Aurora A protein without genomic amplification. Thus, it is important to find out what kinds of genetic alterations are responsible for the increased protein level of Aurora A outside of G_2-M to address the significance of overexpression of Aurora A during carcinogenesis.

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