when they are most needed, and "conditional laggards" who lag farthest when they are most needed (*28*).

Our analysis has revealed unexpected diversity in lion behavior. Some females cooperate unconditionally and others only cooperate when most needed. Both strategies ensure the long-term rewards of protecting essential companions and a stable territory. However, other females opt for additional short-term benefits by lagging behind pride mates during territorial disputes, and some females lag farthest when their help is most needed. Although leaders recognize laggards and behave more cautiously in their presence, they continue to lead the response. In a broader interpretation of cooperative behavior, leaders and laggards may be analogous to "producers" and "scroungers" in foraging groups (*29*) or "bold" and "shy" individuals in other contexts (*30*). As laggards avoid the costs of fighting ("producing"), their rewards are clearly frequency-dependent, and they exploit their pride's corporate territoriality if enough of their companions cooperate (*22*). Under these conditions, laggards may coexist with leaders in a mixed evolutionarily stable strategy (*31*). This study suggests that cooperative groups can include a great variety of behavioral strategies. Most theory on the evolution of cooperation has focused on two-person games and has revealed extraordinary levels of complexity (*32*). Individual behavior in contests between larger groups may prove to be even more complex.

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12. Between 6 and 15 of these playbacks were conducted to each pride over a 24-month period and only in the absence of adult males; all responses were videotaped. Roars from extra-pride females were played from 200 m with a Panasonic SV-250 digital audio tape recorder, an ADS p120 amplifier, and a Klipsch Hersey speaker hidden in long grass or bushes. All playbacks were conducted between 0.5 and 1.0 hours before dusk; recordings were between 20 and 55 s in duration and were played at 116 dB at 1 m. Females clearly distinguish between the roars of one and three intruders when played from a single loudspeaker (*7*). Use of particular recordings was randomized with respect to the identity and composition of each experimental group, and individual responses were independent of the number of times an individual had heard a recording (*7, 8*).
13. All females were between 3 and 14 years old. Each individual's mean rank did not depend on age [degrees of freedom ($F_{1,32}$) = 1.31] or body size (estimated from chest girths, n = 14, $F_{1,12}$ = 0.83). To ensure that lagging was not caused by poor health, playbacks excluded all animals that were either emaciated or injured.
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15. In 14 cases of known maternity, the daughter's mean rank was not significantly correlated with her mother's (r = 0.19, t = 0.66).
16. Five of 12 laggards had at least one sister that consistently approached the speaker in the front half of the group.
17. Although we lack direct observations of the hunting behavior of most of these females, one laggard consistently failed to participate in group hunts, whereas another was an active hunter.
18. Juvenile females (6 to 42 months) also differ in their preferred position relative to other juveniles (n = 38, $0.001 > P > 0.0001$), and juvenile behavior predicts adult behavior (10 of 10 juvenile laggards continued to lag as adults). Juveniles are not considered here because they all tended to stay behind the adults when approaching the speaker.
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28. The two conditional strategies appear to result entirely from the behavior of the laggards. Although each pride responded more cautiously when the odds were less favorable [also see (*7*)], the lag time between leaders (as measured by the lag between the first- and second-arriving females) did not vary in any systematic manner.
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Endoreduplication in Maize Endosperm: Involvement of M Phase–Promoting Factor Inhibition and Induction of S Phase–Related Kinases

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Endoreduplication is an endonuclear chromosome duplication that occurs in the absence of mitosis and in *Zea mays* (L.) is required for endosperm development. Induction of DNA synthesis during early stages of endosperm development is maintained by increasing the amount and activity of S phase–related protein kinases, which was demonstrated here by their ability to interact with human E2F or with the adenovirus E1A proteins. In addition it was shown that endoreduplicated endosperm cells contain an inhibitor that suppresses the activity of the M phase–promoting factor (MPF). These results demonstrate that in maize endosperm, endoreduplication proceeds as a result of two events, inhibition of MPF and induction of S phase–related protein kinases.

A common method by which polyploidy occurs is through chromosome endoreduplication. This is an endonuclear duplication of the genome occurring in the absence of mitosis, and it leads to the production of chromosomes with 2^n chromatids (*1*). This process is common in tissues with high metabolic activity, such as the silk glands of dipterans and the developing endosperm of seeds, but it can also occur in tumor tissues (*1*). Endoreduplication appears to be an essential process for the development of *Zea mays* (L.) endosperm (*2*). One would predict that endoreduplication proceeds from two events: inhibition of mitosis and the constitutive induction of DNA synthesis. As the mechanism controlling endoreduplication is unknown, we have begun to characterize the factors regulating this process in maize endosperm.

We established the temporal expression of endoreduplication in maize endosperm

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by flow cytometry. These results were consistent with previous descriptions (*2*), showing that the process initiates 10 to 12 days after pollination (DAP) (Fig. 1). The size of the nucleus increased considerably during endoreduplication (Fig. 1A), with DNA content ranging from 3C to 96C (where C represents the DNA content of the haploid genome) (Fig. 1B). Endoreduplication was detected until 28 DAP, at which time the percentage of 3C nuclei was 15% (Fig. 1B).

To investigate the mechanism by which mitosis is inhibited, we analyzed the amount and activity of the M phase–promoting factor (MPF) ($p34^{cdc2}$/cyclin B complex) by monitoring the in vitro phosphorylation of histone H1 (*3*). The H1 kinase activity (Fig. 2A) eluted from a column of $p13^{suc1}$ conjugated to agarose beads increased during the early stages of endosperm development and peaked at 10 to 12 DAP, after which it declined. This is consistent with the reduction in mitotic activity that occurs at the initiation of endoreduplication. The amount of $p34^{cdc2}$ was determined by immunoblot analysis with a specific polyclonal antibody ($p34^{cdc2}$Zm) raised to the $COOH_2$-terminal portion of a functional maize $p34^{cdc2}$ protein (*4*). Two closely related (96% identical) *cdc2* complementary DNAs (cDNAs) (*cdc2ZmA* and *cdc2ZmB*) were previously isolated from maize that encode proteins that are 64% identical to human $p34^{cdc2}$ and 63% identical to *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* $p34^{cdc2}$ proteins (*4*), and *cdc2ZmA* was shown to complement a *cdc28* mutant of *S. cerevisiae* (*4*). The amount of $p34^{cdc2}$ in endosperm was high between 10 and 12 DAP and was only slightly reduced by 14 and 16 DAP (Fig. 2B). Further analysis showed that the amount of $p34^{cdc2}$ remained unchanged to 28 DAP (*5*). This result suggests the reduction in histone H1 kinase activity at 16 DAP could result from $p34^{cdc2}$ phosphorylation, lack of mitotic cyclins, or activation of CDC2 kinase inhibitors (*6*, *7*).

To determine if inhibition of the MPF is active or passive, we performed experiments in which extracts from 10-DAP endosperms were combined with varying amounts of 15-DAP extract. These mixtures were preincubated for different periods, after which samples were tested for H1 kinase activity. The 10-DAP extract displayed a high level of kinase activity, and very low activity was detected in the 15-DAP extract (Fig. 2C). Kinase activity decreased in a concentration-dependent manner when 10-DAP extract was mixed with that from 15-DAP (Fig. 2C). Bovine serum albumin (BSA) had no effect on histone H1 kinase activity. We analyzed the proteins after the preincubation period by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with $p34^{cdc2}$Zm antibody and found no evidence for degradation (*5*), indicating that an active inhibitor of the MPF is produced in endoreduplicated cells.

The fission yeast Wee1 kinase negatively regulates entry into M phase by phosphorylation of $p34^{cdc2}$ in a dose-dependent manner (*6*, *8*). This reaction causes changes in the mobility of $p34^{cdc2}$ as determined by SDS-PAGE (*9*). The maize endosperm $p34^{cdc2}$ resolved into two polypeptides between 33 and 35 kD (Fig. 2D); however, we could not detect changes in mobility of either protein during endosperm development. Whether these two polypeptides represent different forms of the same protein or two different but closely related proteins is unknown.

Cell cycle regulators are highly conserved among eukaryotes (*10*). We therefore used heterologous proteins that interact with mammalian S phase kinases to investigate the induction of DNA synthesis during endoreduplication. Several kinases implicated in the onset of S phase in mammalian cells appear to interact with cellular and viral proteins, such as the human E2F and adenovirus E1A proteins (*11*). Consequently, we tested the glutathione-S-transferase (GST) fusion proteins GST–E2F-1, GST-E1A, and GST-E1AΔCR2 (*12*) for their ability to interact with maize protein kinase complexes.

We mixed these three fusion proteins, or GST alone, with 8- or 16-DAP endosperm extracts and analyzed the complexed proteins by immunoblot analysis for a $p34^{cdc2}$-related protein. A maize CDC2-related protein was recovered after association with the GST fusion proteins, and the amount of this p34 was higher at 16 DAP than at 8 DAP (Fig. 3A). Histone H1 kinase activity recovered from 16-DAP extract with either fusion protein was higher than from 8-DAP extract (Fig. 3B). Low enzyme activity was detected in precipitates directed by the GST protein alone, suggesting that the maize protein kinase complexes interacted specifically with the E2F-1 and E1A proteins. The higher level of protein kinase activity recovered from the $p13^{suc1}$-agarose

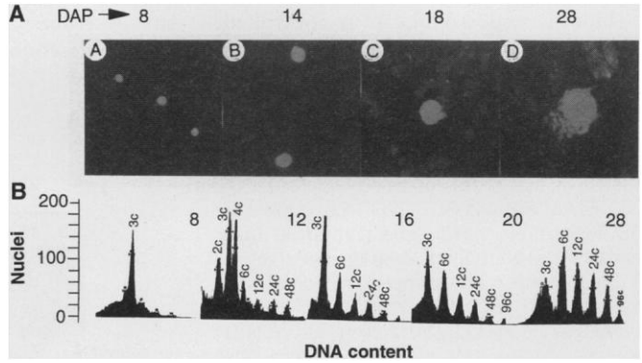

Fig. 1. Endoreduplication during maize endosperm development. (**A**) Increase in nuclear size during endosperm development [indicated by days after pollination (DAP)]. Stained nuclei were photographed at the same magnification (×40) with a Zeiss photomicroscope. (**B**) Flow cytometry analysis of DNA content of endosperm nuclei during development (numbers at the top right indicate DAP) in which DNA content (indicated as C values) is plotted against the number of nuclei. Methods are described in (*19*).

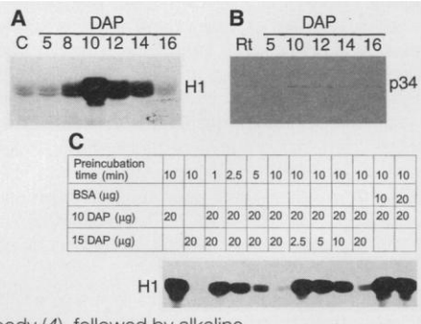

Fig. 2. Histone H1 kinase activity is reduced during endoreduplication by an active inhibitor. (**A**) In vitro histone H1 phosphorylation by kinase eluted with $p13^{suc1}$-agarose from developing endosperm. Methods are described in (*20*). C is a control with no endosperm extract. (**B**) The amount of the maize $p34^{cdc2}$ homolog during endosperm development. Analysis was performed by protein immunoblotting, with each lane containing 20 μg of total protein extract from the developmental stages indicated. Proteins were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose (Schleicher & Schuell). The membrane was treated with affinity-purified $p34^{cdc2}$Zm antibody (*4*), followed by alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G. Rt indicates extract from the root tips of 5-day-old maize seedlings used as a control. (**C**) Inhibition of histone H1 kinase activity by extracts of 15-DAP endosperm. Extracts from 10-DAP endosperms were preincubated at room temperature with various amounts of 15-DAP extract for different periods (as indicated) in kinase buffer [50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 0.2 mM adenosine triphosphate]. The reactions were stopped by quick freezing in liquid nitrogen, passed through a $p13^{suc1}$-agarose column, and subjected to histone H1 assay. BSA (Pierce) was used as a control. (**D**) The maize $p34^{cdc2}$ resolves into two polypeptides. Total protein extract (50 μg) from 10 or 15 DAP was separated by 15% SDS-PAGE and analyzed by immunoblotting with $p34^{cdc2}$Zm antibody.

column at 8 DAP compared with 16 DAP implies that the kinases associated with E1A and E2F are distinct. To verify the difference between them, the enzyme complex eluted from the GST-Suc1 column was reloaded onto GST-Suc1 and GST-E1A columns. Kinase activity was re-recovered with GST-Suc1, but not with GST-E1A (Fig. 4). This suggests that different cyclin-dependent kinase (CDK) complexes are involved in cell cycle regulation in maize: an M phase kinase that can interact with $p13^{suc1}$, and an S phase kinase that can interact with the human transcription factor E2F-1 and the adenovirus E1A proteins. Because the p34 components of M and S phase kinases have similar molecular masses and cross-react with a specific antibody for maize CDC2, it appears the CDK complexes share the same or very closely related catalytic subunits and are probably regulated by different cyclins. The fact that a maize kinase is able to interact with mammalian and viral proteins implies the existence of a human E2F homolog and other mammalian-like cell cycle regulators in maize endosperm.

How is endoreduplication controlled? In plants and mammals endoreduplication was induced after treatment with protein kinase inhibitors (*13*). Yeast cells carrying the *cdc2* or *cdc13* mutations that lead to MPF inactivation were found to undergo extra rounds of DNA synthesis (*14*). Consistent with these observations, our results demonstrate that endoreduplication in maize endosperm requires on the one hand the inactivation of the mitotic $p34^{cdc2}$/cyclin B kinase and on the other hand the induction of S phase–related kinases. Our results support a hypothesis that couples DNA replication to the cell cycle by inactivation of the M phase and induction of the S phase kinases (*15*).

In a variety of eukaryotic cells endoreduplication was induced by various reagents that either inhibit mitosis or cause abnormal alteration in cell surfaces and the cytoskeleton (*16*). On the basis of its wave-like pattern of development (*2*), induction of endoreduplication in maize endosperm might involve a signaling pathway that responds to as yet unknown physiological stimuli. Unraveling the factors regulating this process may lead to strategies that make it possible to increase the productivity of storage tissues and to potentially control cell proliferation.

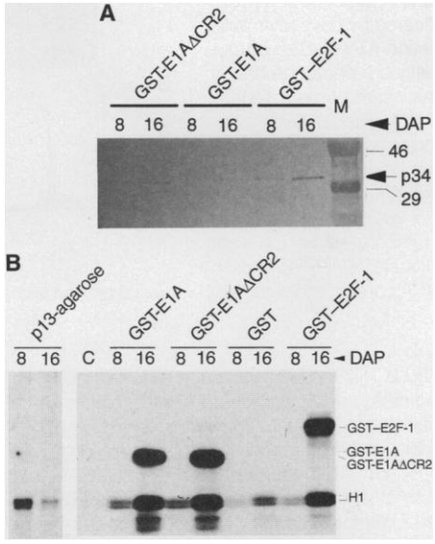

Fig. 3. The amount and activity of H1 kinase that binds to GST-E1A or GST-E2F increase during endoreduplication. (**A**) Immunoblot analysis of $p34^{cdc2}$ in precipitates obtained with GST-E1A, GST-E1AΔCR2, and GST–E2F-1 fusion proteins. GST fusion proteins (*12*), either purified (30 μg) or as bacterial extract (1 mg), were incubated with 8- or 16-DAP endosperm extracts (1 mg) on a rocker platform at 4°C for 2 hours in NETN buffer. Complexes were purified by incubation with glutathione-agarose beads for an additional 1 hour, after which the beads were washed five times with NETN and the complexes eluted and dialyzed as described (*12*). Half of the eluted proteins were analyzed by immunoblotting with $p34^{cdc2}$Zm antibody. Note that in GST-E1A precipitates, $p34^{cdc2}$ is evident as a faint band and was poorly reproduced in the figure. M shows size markers (in kilodaltons) of prestained proteins. (**B**) Analysis of histone H1 kinase activity in GST fusion protein precipitates. Bound proteins (equivalent to 0.5 mg of total protein) were analyzed for histone H1 kinase activity (*21*). Precipitates obtained with the GST protein alone were used as a negative control. The position of histone H1 and phosphorylated GST–E2F-1, GST-E1A, and GST-E1AΔCR2 proteins are indicated on the right. Histone H1 kinase activity in $p13^{suc1}$-agarose precipitates was added for comparison.

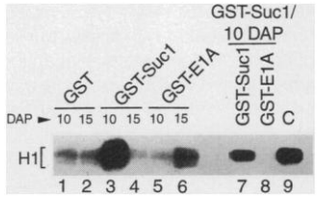

Fig. 4. The kinase complex that binds to $p13^{suc1}$-agarose is different from that binding to E1A protein. GST, GST-Suc1, and GST-E1A proteins were cross-linked to glutathione-Sepharose (*22*), mixed with 2 mg of total protein extract from 10- or 15-DAP endosperm, and the precipitates (equivalent to 0.5 mg of total protein extract) were analyzed for H1 kinase activity (lanes 1 to 6). The kinase complex recovered from 10-DAP extract by GST-Suc1 was eluted with 0.8% deoxycholate, dialyzed against NETN buffer, and reloaded onto either GST-Suc1 (lane 7) or GST-E1A (lane 8) and tested for H1 kinase activity. As a control (C), a GST-E1A column was also loaded with 1 mg of 15-DAP endosperm extract. H1 on the left indicates phosphorylated histone H1.

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19. Maize (Pool 33 QPM) plants were field grown during 1993. Developing ears were collected, quick frozen with liquid nitrogen, and stored at −80°C. Nuclei were prepared by chopping the kernels in modified Honda buffer (*17*). The homogenate was passed through three layers of 100-μm nylon mesh; nuclei were stained with propidium iodide and subjected to flow cytometry with an Epics Elite Model 2362 (Coulter).
20. Degermed kernels were homogenized with ice-cold NETN buffer (20 mM tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing aprotinin (10 μg/ml), 5 mM NaF, 1 mM sodium orthovanadate, and 1 mM dithiothreitol (DTT). Homogenates were cleared by centrifugation at 15,000*g* for 30 min, aliquoted, and stored at −80°C. Protein concentration was determined with Coomassie Plus (Pierce). Protein kinase complexes were purified by affinity with $p13^{suc1}$-agarose (Oncogene) according to the manufacturer's protocol, except that the last wash was with EB buffer [80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and pepstatin (10 μg/ml)] (*18*). The beads (equivalent to 100 μg of protein extract) were used directly for histone H1 kinase assay (*18*).
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