chain nitrogen (*39*). This suggests a mechanism for HK97 cross-linking, in which Glu^{363} increases the nucleophilicity of the Asn^{356} nitrogen, and a succinimide intermediate is followed by ligation, rather than cleavage. Although protein cross-links are not known to form by cyclic imide intermediates, peptides have been shown to ligate by this mechanism (*40*). Alternatively, formation of a Schiff-base intermediate involving Lys^{169} could be enhanced by deprotonation of the Lys^{169} ε-amino group by Glu^{363}.

The complexity of the HK97 capsid structure is striking, but represents only the final stage of maturation. Capsid assembly and maturation require a regulatory process of commensurate complexity to the final assembly product, and the goal is to understand this process in molecular detail. Prohead II, round in shape and extensively corrugated, with hexamers skewed into a dimer of trimers, is radically reorganized during maturation. Maturation involves an "ironing out" of these corrugations and an unskewing of the hexamers, producing the larger size, icosahedral shape, and smooth surface of Head II. Structures of three transitory intermediates between Prohead II and Head II, staging posts on the pathway of capsid reorganization, were recently determined by cryo-EM reconstruction (*10*). The stage is now set to understand HK97 morphogenesis in chemical detail.

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21. Domain A helices α5 and α6 from adjoining subunits pack together in an antiparallel arrangement. A cluster of hydrophobic residues form a pocket at the subunit interface, into which Trp^{309} from a neighboring subunit fits. Around annuli at the hexamer and pentamer axes, the subunit interactions are stabilized by symmetric hydrogen bond networks. The subunits form even more intricate associations around the quasi two- and threefold axes.
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23. The N-arm interacts with the E-loops from two different subunits at the quasi threefold axis. The face of the P domain three-stranded β sheet forms a concave pocket into which the knoblike end of a quasi threefold-related domain P fits. Arg^{194}, on the α2 helix knob, and Glu^{344}, in the pocket on strand βI, form a salt bridge, 3.5 Å from the putative catalytic Glu^{363} carboxylate.
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44. We thank J. Conway, N. Cheng, and A. Steven for providing the HK97 capsid cryo-EM reconstruction. Assistance of the Cornell High Energy Synchrotron Source (CHESS), Advanced Photon Source (APS), and Stanford Synchrotron Radiation Laboratory (SSRL) staffs is gratefully acknowledged. Supported by NIH grants AI40101 (J.E.J.) and GM47795 (R.W.H.). Coordinates for the crystal structure of the bacteriophage HK97 capsid have been deposited with the Protein Data Bank (accession no. 1FH6).

26 April 2000; accepted 28 July 2000

Activation of the DNA Replication Checkpoint Through RNA Synthesis by Primase

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When DNA replication is inhibited during the synthesis (S) phase of the cell cycle, a signaling pathway (checkpoint) is activated that serves to prevent mitosis from initiating before completion of replication. This replication checkpoint acts by down-regulating the activity of the mitotic inducer cdc2-cyclin B. Here, we report the relation between chromatin structure and induction of the replication checkpoint. Chromatin was competent to initiate a checkpoint response only after the DNA was unwound and DNA polymerase α had been loaded. Checkpoint induction did not require new DNA synthesis on the unwound template strand but did require RNA primer synthesis by primase. These findings identify the RNA portion of the primer as an important component of the signal that activates the replication checkpoint.

The DNA replication checkpoint prevents mitosis if DNA replication is either ongoing or blocked during S phase (*1*). Precisely which structural elements of replicating DNA serve to activate the checkpoint is currently not known. To address this issue, we have used *Xenopus* extracts, a biochemically tractable system, to define the replication structure(s) that activates the replication checkpoint.

Upon checkpoint induction, the Chk1 protein kinase is phosphorylated and activated (*2*). Activated Chk1 phosphorylates the Cdc25 protein phosphatase, resulting in negative regulation of Cdc25 and a subsequent delay on entrance into mitosis (*3*). In *Xenopus*, the Chk1 pathway functions in the replication checkpoint (*4*). Induction of the replication checkpoint in egg extracts induces Chk1 phosphorylation, and removal of Chk1 from extracts attenuates replication checkpoint control of mitosis.

Checkpoint-induced phosphorylation of

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Chk1 occurs in the nucleus and results in an easily observable mobility shift with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*2*, *4*). Thus, this Chk1 mobility shift can be used as a reliable assay for checkpoint induction. In *Xenopus* extracts, because nuclear transport of Chk1 is slow, only a small fraction of Chk1 is phosphorylated during checkpoint arrest. To overcome this, we used a fragment corresponding to the COOH-terminal 215 amino acids of Chk1 (Chk1 ΔKD) to assay activation of the replication checkpoint. Addition of aphidicolin to *Xenopus* extracts blocks DNA replication and activates the checkpoint. As a result of this activation, we found a large fraction of the Chk1 ΔKD added to the extract runs in a shifted position on SDS-PAGE (Fig. 1A). Treatment with calf intestine phosphatase reversed the mobility shift, implicating checkpoint-induced phosphorylation as the cause for Chk1 ΔKD shift (Fig. 1A). To eliminate the possibility that Chk1 ΔKD phosphorylation was not specific for checkpoint induction, we pre-incubated extract with sperm for 90 min to allow for a complete round of DNA replication before the addition of Chk1 ΔKD (Fig. 1A). Incubation was then continued for an additional 60 min. Under these conditions Chk1 ΔKD was not phosphorylated (Fig. 1A). If aphidicolin was included during the pre-incubation, then Chk1 ΔKD phosphorylation occurred, but phosphorylation did not occur if aphidicolin was added after the pre-incubation period (Fig. 1A). Moreover, treating the extract with caffeine, an inhibitor of the replication checkpoint, blocked phosphorylation of Chk1 ΔKD (Fig. 1A). Together, these data show that Chk1 ΔKD phosphorylation is a reliable assay for replication checkpoint induction in the *Xenopus* cell-free system.

To determine when chromatin first becomes competent to activate the replication checkpoint, we inhibited DNA replication at three discrete steps. The first step, assembly of the prereplication complex (pre-RC) onto chromatin, occurs during G_1 of the cell cycle and involves the sequential loading of Orc, Cdc6, and MCM proteins onto DNA (*5*). The second step, initiation, is catalyzed by the cdk2–cyclin E and cdc7-dbf4 protein kinases (*5*). During initiation, DNA is unwound and the separated strands are stabilized by replication protein A (RPA) association (*6*). After this step, DNA polymerase α (pol α) binds and synthesizes a RNA-DNA primer. The third step, elongation, involves proliferating cell nuclear antigen (PCNA)–dependent loading of DNA polymerase δ, and extension of the primers into nascent strands (*5*).

To determine if pre-RC assembly is required to generate the checkpoint signal, geminin (*7*), which inhibits pre-RC formation by blocking MCM binding to chromatin, was used. Addition of geminin to egg extract blocks DNA replication if added 10 min before the addition of sperm chromatin, but has no effect on DNA replication if added 10 min after the sperm chromatin (*7*) (Fig. 1B). The early addition of geminin, but not the late, prevented aphidicolin-induced Chk1 phosphorylation (Fig. 1C). Despite the replication block, early addition of geminin to cycling extracts did not delay mitosis, as assayed by nuclear envelope breakdown (Table 1). These data show that pre-RC formation is a prerequisite for activation of the replication checkpoint. The next step, initiation, was blocked through addition of the cdk2–cyclin E inhibitor $p27^{Kip}$ (*8*). Addition of $p27^{Kip}$ to extract blocked DNA replication (Fig. 1B) and prevented aphidicolin-induced phosphorylation of Chk1 (Fig. 1C). Therefore, chromatin only becomes competent to activate the replication checkpoint after the cdk2–cyclin E dependent step during initiation.

In *Xenopus* (*9*) and during SV40 DNA replication (*10*), the DNA unwinding step during initiation can be uncoupled from and is independent of primer synthesis. In these systems, aphidicolin-induced inhibition of primer synthesis, or removal of pol α, causes extensive unwinding of DNA following initiation. Under these conditions, long regions of single-stranded DNA (ssDNA) coated with the ssDNA-binding protein RPA are generated as the helicase moves away from the initiation site. To test if this RPA-bound, unwound DNA replication intermediate is sufficient to activate the checkpoint and to assay the direct involvement of pol α in checkpoint induction, pol α was removed

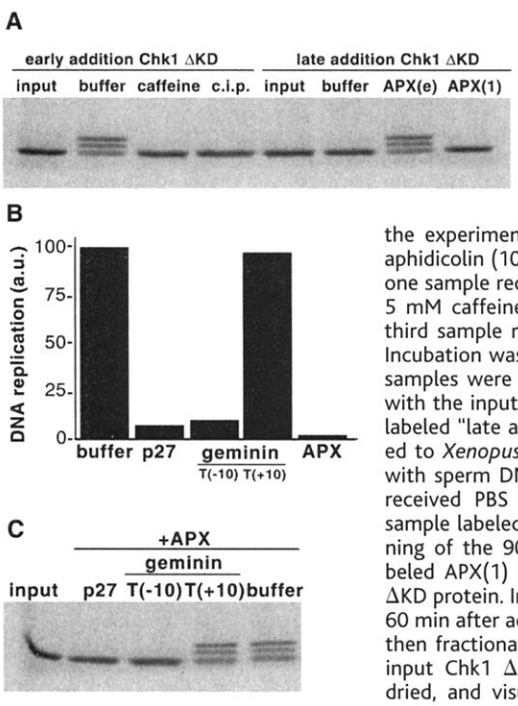

Fig. 1. DNA replication must pass through the pre-RC assembly and cdk2-dependent steps of initiation in order to trigger the replication checkpoint. (**A**) Chk1 ΔKD was assayed for phosphorylation in *Xenopus* egg extract. In the samples labeled "early addition," radiolabeled Chk1 ΔKD was added to *Xenopus* egg extract (*22*) at the beginning of the experiment, along with sperm DNA (2000/μl) and aphidicolin (100 μg/ml). The sample was split into three; one sample received buffer (buffer) and another received 5 mM caffeine (caffeine). After 50-min incubation, the third sample received calf intestine phosphatase (c.i.p.). Incubation was carried out for a total of 1 hour, and the samples were fractionated on SDS-PAGE and compared with the input Chk1 ΔKD protein (input). In the samples labeled "late addition," radiolabeled Chk1 ΔKD was added to *Xenopus* egg extract that had been pre-incubated with sperm DNA for 90 min. The sample labeled buffer received PBS along with the Chk1 ΔKD protein, the sample labeled APX(e) received aphidicolin at the beginning of the 90-min pre-incubation, and the sample labeled APX(1) received aphidicolin along with the Chk1 ΔKD protein. Incubation was carried out for an additional 60 min after addition of Chk1 ΔKD and the samples were then fractionated on SDS-PAGE and compared with the input Chk1 ΔKD protein (input). The gels were fixed, dried, and visualized with a PhosphorImager. (**B**) DNA replication analysis (*23*) in *Xenopus* egg extracts containing either buffer, Kip protein (p27) (200 nM), geminin protein (250 nM), or aphidicolin (100 μg/ml). Geminin was added either 10 min before (T_{-10}) or 10 min after (T_{+10}) the sperm DNA (2000/μl). Reactions, which also included [^{35}S]methionine-labeled Chk1 ΔKD (1/10 reaction volume), were terminated after 90 min. (**C**) Chk1 phosphorylation assays in extracts containing 100 μg/ml aphidicolin and either buffer, Kip protein (200 nM), or geminin protein (250 nM). Geminin was added either 10 min before (T_{-10}) or 10 min after (T_{+10}) the sperm DNA (2000/μl). Extracts contained [^{35}S]methionine-labeled Chk1 ΔKD at 1/10 reaction volume. Lane "input" refers to the input [^{35}S]methionine-labeled Chk1 ΔKD. Samples were harvested after 60 min.

Table 1. The effect of DNA replication inhibitors on entrance to mitosis in cycling extracts. Cycling extracts (*27*) were prepared and mixed with buffer [phosphate-buffered saline (PBS)], aphidicolin (APX) (100 μg/ml), recombinant geminin (250 nM), purified monoclonal antibody SJK-132 (1 mg/ml), or actinomycin D (Ac.D) (10 μg/ml). After 10 min on ice, the reactions were supplemented with sperm nuclei (2000/μl) and cycling was initiated by transferring the reactions to room temperature. Entrance to mitosis was taken as the time at which more than 50% of the nuclear envelopes had broken down [NEB (*27*)], which is indicated in minutes. For reactions where NEB did not occur during the 120-min time course of the experiment, a dash appears in the second column. The data are from a single experiment and are representative of three independent experiments.

Addition	T > 50% NEB
Buffer	60 min
APX	–
Geminin	55 min
SJK-132	–
Ac.D	55 min

from the extract by immunodepletion (*11*). After immunodepletion with antibodies to pol α or mock depletion with nonspecific antibodies, the extracts were incubated with sperm DNA for 60 min in the presence of aphidicolin. Both extracts contained similar amounts of RPA-bound ssDNA, as judged by the amount of RPA present in the isolated chromatin fractions shown in Fig. 2B. Only the mock-depleted extract contained detectable levels of chromatin-associated pol α. To determine if this difference was essential for induction of the replication checkpoint, we measured aphidicolin-induced Chk1 ΔKD phosphorylation in mock- and pol α–depleted extracts. As shown in Fig. 2B, the mock depleted extract exhibited Chk1 ΔKD phosphorylation but the pol α–depleted extract did not. Addition of human recombinant pol α to the pol α–depleted extract rescued Chk1 ΔKD phosphorylation and restored the replication checkpoint to the extract. This indicates that the checkpoint defect was due to the absence of pol α and not due to removal of unknown, coprecipitating factors. These data make two important points. First, because the pol α–depleted extract contained large amounts of unwound, RPA-coated ssDNA, this structure is, by itself, incapable of inducing the checkpoint. Second, the data show that pol α is required for induction of the replication checkpoint.

A requirement for pol α in the coupling of mitosis to the completion of S phase has been defined from genetic experiments in fission yeast (*12*, *13*). These experiments suggested that DNA synthesis by pol α is required to prevent mitosis during S phase (*13*). Aphidicolin inhibits DNA (*14*) but not RNA (*15*) synthesis by pol α. Our finding that removal of pol α prevents induction of the checkpoint, whereas aphidicolin induces the checkpoint, suggests that the DNA polymerase activity of pol α may not be required for the checkpoint response. To confirm this, we used the monoclonal antibody SJK-132 (*16*), a specific inhibitor of pol α that blocks DNA polymerase activity but not pol α RNA primase activity. Addition of SJK-132 to the extract abolished DNA replication (Fig. 2C), induced Chk1 ΔKD phosphorylation (Fig. 2D), and prevented mitosis in cycling extracts (Table 1). These findings demonstrate that SJK-132 is a potent activator of the replication checkpoint and strongly support the conclusion that activation of the checkpoint does not require the DNA polymerase activity of pol α.

Pol α, but not DNA synthesis, is required for replication checkpoint activation. This suggests that association of pol α with chromatin alone might be sufficient to induce the replication checkpoint. To test this notion, we examined the association of pol α and the replication factors RPA and PCNA with chromatin under conditions where the replication checkpoint had been induced. To do so, nuclei were formed in extracts containing either $p27^{Kip}$, aphidicolin (APX), or no exogenous additions (buffer). After a 60-min incubation, chromatin-associated proteins were examined by immunoblotting. Because PCNA requires synthesis of the complete RNA-DNA primer to bind to chromatin (*17*), and complete primer synthesis does not occur in the presence of either $p27^{Kip}$ or aphidicolin, PCNA was found only in the control sample (Fig. 3A). Chromatin isolated from the buffer-treated control sample contained moderate amounts of RPA and pol α, whereas no RPA or pol α bound to the chromatin in the $p27^{Kip}$ sample (Fig. 3A). This is consistent with reports showing that S phase cdks are required for initiation and unwinding of DNA (*9*). Surprisingly, a large increase in the amount of chromatin-associated pol α was observed when aphidicolin was added to the reaction (Fig. 3A). This result suggests that pol α, like RPA, was tightly associated with the ssDNA generated during unwinding. Despite this association, the lack of chromatin-associated PCNA in the aphidicolin sample demonstrates that pol α did not synthesize the DNA portion of the primer, which further supports the finding that DNA synthesis is not required for replication checkpoint induction.

To better understand the aphidicolin-dependent pol α–chromatin association during induction of the checkpoint, we performed a time-course experiment. The checkpoint was induced with aphidicolin and compared with a control reaction lacking aphidicolin. For 2 hours every 30 min, chromatin was isolated and probed for the presence of the replication factors pol α, Cdc45, and RPA. Cdc45 was examined because it forms complexes with pol α and has a proven role in the loading of pol α onto chromatin (*18*). In the control reaction, the association of pol α, Cdc45, and RPA with chromatin peaked at 60 min, and then decreased (Fig. 3B). This is consistent with these factors being loaded onto DNA and then displaced as replication is completed. By contrast, in aphidicolin-treated samples all three factors exhibited a gradual increase in chromatin association up to the 90-min time point (Fig. 3B). Initiation in the *Xenopus* egg extract system is synchronous and complete by 30 min. Therefore, when DNA synthesis is blocked, both Cdc45 and pol α continue to load onto the ssDNA generated as the helicase moves away from the site of initiation. As a result, Cdc45 and pol α accumulate on the DNA at much higher than normal levels.

In the presence of aphidicolin, the replication checkpoint is activated and pol α, Cdc45, and RPA are loaded onto DNA at high levels. These events also occur if the checkpoint is induced with SJK-132 instead of aphidicolin (*19*). We have shown that ssDNA and RPA alone are not sufficient to activate the replication checkpoint, whereas pol α appears to be necessary. Additionally, chromatin association of Cdc45 in the absence of pol α does not induce the checkpoint because Cdc45 binds to chromatin in the pol α–depleted extract (*19*). Together, these results suggest that activation of the replication checkpoint might be directly dependent on the physical association of pol α with ssDNA. To test this possibility, we took advantage of the observation that increasing the concentration of Cdc45 in *Xenopus* egg extracts increases the amount of chromatin-bound pol α (*18*). We reasoned that if loading of pol α onto chromatin was the rate-limiting event for induction of the checkpoint, and therefore Chk1 phosphorylation, then Chk1 phosphorylation might be influenced by the amount of Cdc45 present in the extract. To test this, we added recombinant Cdc45 to

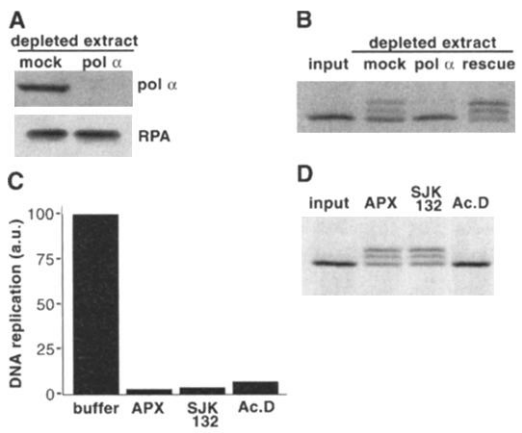

Fig. 2. Pol α, but not DNA primer synthesis, is required for induction of the replication checkpoint. **(A)** Egg extracts were depleted with either bead-bound antibodies to pol α (pol α–depleted) or nonspecific control antibodies (mock-depleted) and then were mixed with sperm DNA and aphidicolin for 60 min. The amount of chromatin-associated pol α (p70 subunit) and RPA (the 70-kD subunit) after incubation in the given extract was determined by isolation of the chromatin and immunoblotting. **(B)** Chk1 phosphorylation was assayed in mock-depleted extract, pol α–depleted extract, or pol α–depleted extract supplemented with 440 nM recombinant pol α (*28*) complex (rescue). **(C)** Egg extracts were supplemented with sperm DNA (2000/μl) and either PBS (buffer), aphidicolin (APX) (100 μg/ml), Mab SJK-132 (SJK-132) (1 mg/ml), or actinomycin D (Ac.D) (10 μg/ml). DNA replication was measured after 90-min incubation. **(D)** Chk1 ΔKD phosphorylation was assayed in extracts supplemented with sperm DNA (2000/μl) and either aphidicolin (100 μg/ml), Mab SJK-132 (1 mg/ml), or actinomycin D (10 μg/ml).

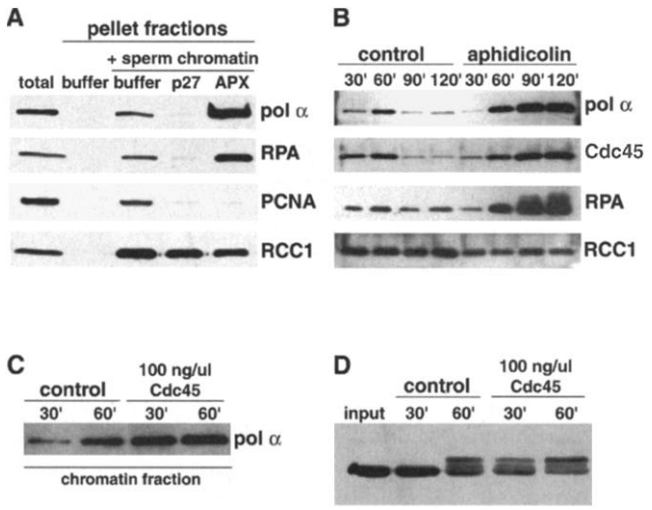

Fig. 3. Pol α–chromatin association controls Chk1 phosphorylation and induction of the replication checkpoint. (**A**) Immunoblot of either total extract (total) or pellet fractions from extracts supplemented with or without sperm chromatin. Extracts were additionally supplemented with buffer, Kip protein (200 nM), or aphidicolin (100 μg/ml). Extracts were incubated for 60 min before chromatin isolation. The given samples were immunoblotted with antibodies against the given DNA replication factor (*29*). The samples were also probed for RCC1, a protein not involved in DNA replication, which served as a loading control. (**B**) Extracts were supplemented with either buffer (control) or aphidicolin (100 μg/ml). At the given time (in minutes) an aliquot was removed and the chromatin extracted. Samples were analyzed as in (A). (**C**) Extracts were supplemented with either PBS (control) or recombinant *Xenopus* Cdc45 protein (*30*). After the given time (in minutes) of incubation chromatin was isolated and immunoblotted with antibodies to pol α. (**D**) Chk1ΔKD phosphorylation in the extracts described in (C). Recombinant Chk1ΔKD (*31*) was used instead of [^{35}S]methionine-labeled Chk1 ΔKD.

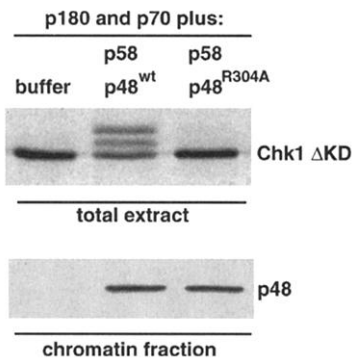

Fig. 4. Primase mutant defective in RNA synthesis binds to chromatin but does not induce Chk1 phopshorylation in response to aphidicolin. Pol α was depleted from extract and the extracts were then supplemented with aphidicolin (100 μg/ml), recombinant p180/p70 complex (500 nM), and either buffer or recombinant primase complexes (500 nM) containing wild-type (lane p58-p48^{wt}) or mutant (lane p58-p48^{R304A}) p48 protein. Recombinant proteins were prepared as described in (*32*). After a 60-min incubation with sperm DNA and [^{35}S]methionine-labeled Chk1 ΔKD, the supplemented extracts were assayed for Chk1 phosphorylation ("total extract"). Additionally, after the 60-min incubation, chromatin was purified from the supplemented extracts and examined, by immunoblotting, for the p48 primase subunit ("chromatin fraction") as described in (*23*).

aphidicolin-treated extract and measured both pol α–chromatin association and Chk1 phosphorylation relative to a sample that received no exogenous Cdc45. Addition of Cdc45 dramatically increased the rate at which pol α associated with DNA (Fig. 3C). By 30 min, pol α binding had become saturated in the Cdc45-treated sample. In contrast, the untreated sample required 60 min to achieve a level of pol α association that still lagged behind that seen at the 30-min time point in the Cdc45-treated sample. Therefore, under conditions of replication block, exogenous Cdc45 facilitated binding of pol α to chromatin. Exogenous Cdc45 also had a substantial effect on the kinetics of Chk1 phosphorylation. Chk1 phosphorylation was not detected at the 30-min time point in the control, but was detected at the 60-min time point (Fig. 3D). In contrast, addition of Cdc45 resulted in robust Chk1 phosphorylation after only 30 min (Fig. 3D). This result demonstrates a direct link between chromatin association of pol α and replication checkpoint activation.

Pol α is composed of four subunits, the p180 DNA polymerase subunit, the p48 and p58 primase heterodimer, and a p70 subunit of poorly characterized function. We have shown that pol α chromatin association, but not its DNA polymerase activity, is required for replication checkpoint induction. An explanation for this is that RNA primer synthesis, by primase, is the signal that activates the checkpoint. To test this, we used actinomycin D, which inhibits primase (*20*). Addition of actinomycin D to extract blocked DNA replication (Fig. 2C), as expected, but did not induce Chk1 phosphorylation (Fig. 2D) or delay mitosis in cycling extracts (Table 1). This suggests that primase activity is important for checkpoint activation.

To further explore the role of primase in triggering the replication checkpoint, pol α–depleted extract was prepared, supplemented with different recombinant pol α preparations, and assayed for replication checkpoint induction with the Chk1 phosphorylation assay. Pol α–depleted extract supplemented with the p180-p70 dimer alone did not phosphorylate Chk1, showing that primase is required for the checkpoint (Fig. 4). To see if RNA synthesis by primase was important, we analyzed a previously characterized mutation in human p48 [arginine 304 to alanine (p48^{R304A})] that is defective in RNA synthesis (*21*) for checkpoint activation. Supplementation of pol α–depleted extract with p180, p70, p58, and wild-type p48 induced Chk1 phosphorylation, whereas addition of a complex containing p48^{R304A} did not (Fig. 4). The p48^{R304A}-containing complex associated with chromatin to the same extent as the wild-type complex (Fig. 4). This demonstrates that pol α–chromatin association alone is not sufficient for checkpoint activation. Furthermore, because the p48^{R304A} protein was deficient for checkpoint activation, the data in Fig. 4 indicate that RNA primer synthesis by primase is the signal that triggers the replication checkpoint. The RNA portion of the primer is a transient structure that is produced throughout the course of S phase, as each Okazaki fragment on the lagging strand requires an RNA primer. Therefore, this structure would seem to be a logical activator of the checkpoint pathway that prevents entrance into mitosis until S phase has completed.

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11. Immunodepletions were performed using monoclonal antibody Mab SJK-132 [pol α, (*16*)] or nonspecific rabbit immunoglobulin G (mock). Purified antibody (100 μg) was applied to packed protein A–Sepharose beads (50 μl). After a 30-min incubation the beads were washed three times in egg lysis buffer (*22*) and were divided into three aliquots. *Xenopus* egg extract (100 μl) (*22*) was mixed with an aliquot of antibody beads and incubated for 1 hour at 4°C with rotation. The beads were pelleted and the supernatant was collected for incubation with a fresh aliquot of beads. A total of three incubations were performed before the extract was used for the given experiment.
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22. *Xenopus* egg cytosol and membrane fractions were prepared exactly as in (*23*) and frozen in small aliquots at −80°C. Reconstituted extracts were prepared by combining the cytosol and membrane fractions at a ratio of 10:1 (cytosol:membranes). Radiolabeled Chk1ΔKD (*24*) was produced by in vitro transcripition and translation in the presence of [^{35}S]methionine.
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28. The four-subunit human pol α complex was purified from coinfected Sf9 cells by immuno-affinity chromatography as described (*25*). Depletion of pol α resulted in diminished DNA replication relative to mock-depleted extracts [12% (±3%) of mock-depleted extract] and addition of recombinant pol α restored DNA replication in pol α–depleted extracts to 96% (±6%) the level measured in the mock-depleted extract.
29. Antibodies used for immunoblotting were against the p70 subunit of human pol α (gift of T. Wang, Stanford University), *Xenopus* RPA, human PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), *Xenopus* RCC1, and *Xenopus* Cdc45.
30. *Xenopus* Cdc45 protein (*18*) was expressed as a six-histidine tagged protein in insect Sf9 cells and was purified on nickel–nitrilotriacetic acid (nickel-NTA) agarose (*9*). Antibodies to the recombinant protein were produced (*9*) and used for immunoblotting.
31. The Chk1 ΔKD coding sequences were subcloned into a bacterial expression vector and expressed as a six-histidine tagged protein. Purified protein was added to extracts at 50 ng/μl. The protein was detected by immunoblotting with a T7-tag monoclonal antibody (Novagen, Madison, WI).
32. Recombinant p180 and p70 subunits were purified from coinfected Sf9 cells as described for the pol α complex (*25*). Primase proteins were produced through overexpression, in *Escherichia coli*, of a bicistronic expression vector [pETHis-Hp58-Hp48 (*26*)] encoding p58 and either wild-type or R304A p48 subunit. The p58/p48 complexes were purified on nickel-NTA agarose by virtue of a six-histadine tag fused to the p58 subunit.
33. We thank T. Wang for pol α antibodies, J. Walter for recombinant Cdc45, L. Kong for purified SJK-132, H.-P. Nasheuer for pETHis-Hp58-Hp48, and R. Kuchta for antibodies to p48 and for very helpful discussions. Supported by a postdoctoral fellowship from the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation (DRG-1460) to W.M.M., NIH grant RO1GM33523-16 to J.N., and NIH grant 52948 to E.F.

20 July 2000; accepted 7 August 2000

Patterning of the Zebrafish Retina by a Wave of Sonic Hedgehog Activity

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The *Drosophila* retina is patterned by a morphogenetic wave driven by the Hedgehog signaling protein. Hedgehog, secreted by the first neurons, induces neuronal differentiation and *hedgehog* expression in nearby uncommitted cells, thereby propagating the wave. Evidence is presented here that the zebrafish Hedgehog homolog, Sonic Hedgehog, is also expressed in the first retinal neurons, and that Sonic Hedgehog drives a wave of neurogenesis across the retina, strikingly similar to the wave in *Drosophila*. The conservation of this patterning mechanism is unexpected, given the highly divergent structures of vertebrate and invertebrate eyes, and supports a common evolutionary origin of the animal visual system.

The vertebrate neural retina develops from a layer of pseudostratified epithelium lining the inside of the optic cup, whereas the pigmented retina (RPE) develops from cells on the outside of the cup. The ganglion cell layer (GCL) forms part of the neural retina, and ganglion cells are the first neurons to be born in the retina. Neurogenesis proceeds in a wave from the central to the peripheral retina (*1*). Sonic Hedgehog (Shh) is expressed in the GCL and RPE and directs proliferation and differentiation of several late arising cell types, such as photoreceptors and glia (*2–5*). Here we show that at earlier stages, Hedgehog (Hh) signaling drives a wave of *shh* expression and neurogenesis across the GCL.

To investigate early functions of Shh in retinal neurogenesis, we constructed a zebrafish strain harboring a green fluorescent protein (GFP) transgene under the control of the Shh promoter (*6*). Two ShhGFP transformant lines faithfully recapitulate many aspects of *shh* RNA expression (*7*, *8*). In contrast to the observations of Stenkamp *et al.* (*5*), we detect zebrafish *shh* RNA and ShhGFP not only in the RPE, but also in the GCL (Fig. 1, A and E) (*8*), which is in agreement with the data from other vertebrates (*2–4*). ShhGFP and *shh* RNA expression is activated at 28 to 30 hours in a patch of cells ventral and nasal to the optic disc (Fig. 1B) (*8*). These cells are the first retinal ganglion cells (RGCs) to differentiate and express the RGC marker Zn5 (Fig. 1B) (*9*). ShhGFP and *shh* RNA expression then spreads from this point, together with Zn5 immunoreactivity, and fills the central retina by 52 hours (Fig. 1, A to E) (*8*). Only a subset of the RGCs express ShhGFP (Fig. 1I).

To determine whether *shh* expression might be regulated by Shh itself, we examined ShhGFP expression in *sonic you* (*syu*) mutants, in which the zebrafish *sonic hedgehog* gene is disrupted (*10*). In *syu* mutants, ShhGFP expression is initiated in the first RGCs, but then fails to spread further (Fig. 1, F to H). This is very similar to the *Drosophila* eye, where Hh signaling is required for the spread, but not the induction, of the first Hh-expressing neurons, which instead requires *decapentaplegic* signaling (*11*). In contrast, Zn5 immunoreactivity and RGC differentiation do spread in *syu* mutants, but this spread is retarded (Fig. 1, G and H), and the RGCs are disorganized and reduced in number (Fig. 1, I and J). The reduction of RGCs correlates well with the observation that the optic nerve is thinner in *syu* mutants (*7*). In addition, the layering of *syu* mutant eyes is not as pronounced as in wild-type eyes (Fig. 1, K and L). At 76 hours, there are many apoptotic cells in *syu* eyes (Fig. 1L) (*7*), but elevated cell death is not observed until after 50 hours (*7*), indicating that cell death is not responsible for the reduced ShhGFP expression, which is already evident well before this (Fig. 1G).

Since Shh is necessary for its own expression, we asked whether it might also be sufficient to induce itself. We injected a *shh* cDNA under the control of a heat shock–inducible promoter (*12*) into *syu* RNA null mutants (*13*) carrying ShhGFP. DNA injection into zebrafish embryos leads to mosaic expression of the transgene (*14*). We activated expression by heat shock at 28 hours and examined the effect on ShhGFP at 52 hours. Patches of cells expressing *shh* RNA were found to induce ShhGFP expression in the GCL (Fig. 2A), indicating that Shh is sufficient to activate its own expression. Consistent with this observation, wild-type cells transplanted into *syu* eyes are able nonautonomously to induce ShhGFP expression in mutant cells located in the vicinity (Fig. 2B). It is interesting that wild-type clones do not rescue ShhGFP expression if they do not

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