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Activation of a System for the Joining of Nonhomologous DNA Ends during *Xenopus* Egg Maturation

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Mature *Xenopus laevis* eggs provide an elementary reaction system of illegitimate recombination which efficiently joins nonhomologous DNA ends (P. Pfeiffer and W. Vielmetter, Nucleic Acids Res. 16:907–924, 1988). Here we show that stage VI oocytes, known to express a system for homologous recombination (D. Carroll, Proc. Natl. Acad. Sci. USA 80:6902–6906, 1983), are completely devoid of this joining system. Nonhomologous DNA end-to-end joining, however, attains full activity only at an extremely late stage of egg maturation. Cycloheximide inhibition patterns indicate that nonhomologous joining activity is regulated at the G_2 restriction point of the cell cycle. Implications of homologous and nonhomologous recombination activities during egg maturation are discussed.

Pathways of homologous as well as nonhomologous genetic recombination are ubiquitous in living cells and expressed at relative intensities that vary widely with cell type and state. Activities of homologous recombination may be transiently high in meiotic germ cells (42) but are maintained at levels several orders of magnitude below that of nonhomologous recombination in somatic cells of higher eucaryotes. This relation, first recognized and estimated during attempts of targeted gene transfer in mammalian cells (21, 34, 36, 38), is just inverse to the situation in procaryotes and yeasts (18, 39, 41).

The predominance of illegitimate DNA interactions in somatic cells of higher eucaryotes is reflected in the marked efficiency of nonhomologous end-to-end joining reactions between unrelated DNA molecules. Detailed studies in cultured CV1 monkey kidney cells (35, 37, 38, 45) and in extracts from fertilized *Xenopus laevis* eggs (33, 43) showed that pairs of DNA ends carrying nonhomologous protruding single strands are joined in any offered combination by strikingly similar patterns in both systems. In the *Xenopus* system, the joining mechanism appears to require only a limited number of functions, including fill-in DNA synthesis, ligation, and mismatch correction. In addition, an alignment function is indispensable to support the alignment of juxtaposed partner termini during repair reactions (43). Factors providing such an alignment function may be considered as proteins binding to DNA ends which have been suggested to play a key role in processes of DNA end joining in various other systems (12, 19, 23, 32). Thus, mechanisms of DNA end joining may be regarded as a widespread feature of the vertebrate domain. They have presumably evolved not only to eliminate highly lethal DNA double-strand breaks (DSBs) from duplex DNA but also to provide the fundamental mechanism for the rejoining of nonhomologous DNA elements in illegitimate recombination processes promoting chromosomal rearrangements (38).

While various aspects of DNA end joining have been fairly well studied (35, 38), little is known about its regulation within the cell cycle. This problem may be investigated in the egg maturation process of *X. laevis*, in which DNA metabolic activities are regulated at the G_2 restriction point of the cell cycle (22). In this well-studied model system, stage VI oocytes (10) are released by mitogenic stimulation from prophase I to undergo transition to metaphase II, where they again are arrested as mature eggs awaiting fertilization (8, 30). While fertilized *Xenopus* eggs were shown to be a rich source of joining activity (3, 24, 33, 40), an efficient machinery of homologous recombination has been described to be active in oocytes (4, 5). Of particular interest is how the activities of these two basically different recombinational systems are regulated.

Here we report experiments in which activities for nonhomologous DNA end joining are assayed in vivo by injection of suitable DNA substrates into *X. laevis* oocytes at various stages of maturation. We show that in contrast to other recombinational and DNA metabolic functions, nonhomologous joining is activated extremely late in the process of egg maturation. Possible implications for early embryogenesis are discussed.

MATERIALS AND METHODS

Preparation of substrate DNA. Linear nonhomologous DNA substrates (Fig. 1) were prepared from plasmids pSP65 (28) and pSP65 (Kpn) (33) as described previously (33).

Eggs, oocytes, and injection procedures. Fertilized *X. laevis* eggs were prepared as described previously (31). Stage VI oocytes (10) were sorted from collagenase (Boehringer)-digested ovaries (6) which had been dissected from females stimulated with 100 U of human chorionic gonadotropin 10 days before surgery. Oocyte nuclei were injected by either the centrifugation method (6) (experiment in Fig. 3) or the blind method (6) (experiments in Fig. 4 and 5). For each experimental value, at least 10 oocytes or eggs were injected with 0.1 ng of linear DNA substrate in 10 nl of 88 mM NaCl, resulting in a low DNA concentration of approximately 0.1 ng/0.1 μl in oocyte nuclei or 0.1 ng/μl in eggs to stimulate the intramolecular joining reaction. Injected cells were incubated in modified Barth's saline (13) at 18°C for the times indicated in the figure legends, frozen in liquid N_2, and stored at −70°C until further processing.

In vitro maturation of oocytes. For in vitro maturation, stage VI oocytes were checked for the absence of the white spot on the animal pole (indicative of germinal vesicle breakdown [GVBD]) and incubated in modified Barth's

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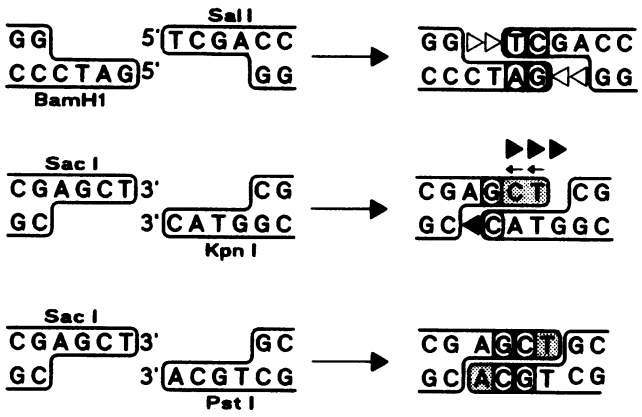

FIG. 1. Nonhomologous terminus configurations of the three DNA substrates used in this study and their presumed joining mechanisms (33). Pairs of protruding single-strand ends (PSS) with equal polarity (5'/5', 3'/3') overlap by a degree which is set by single fortuitously matching base pairs. The overlap register determines the patterns of fill-in DNA synthesis (*Bam/Sal*, 5'/5'), mismatch correction (*Sac/Kpn*, 3'/3'), and nick ligation (*Sac/Pst*, 3'/3'). Medallion-shaped letters, base matches; stippled letters, mismatched bases; white triangles, fill-in DNA synthesis of 5' PSS; black triangles, fill-in DNA synthesis of 3' PSS; small arrows, removal of noncomplementary PSS tails.

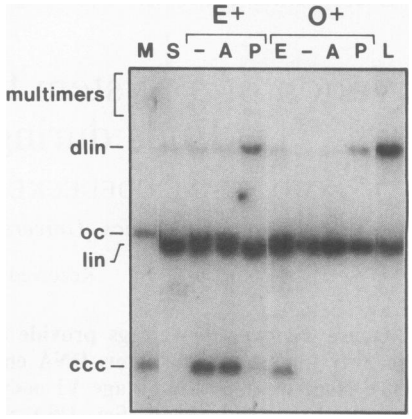

FIG. 2. In vitro joining assays in extracts from fertilized eggs (E) and oocytes (O). DNA substrate *Sac/Pst* (3'/3'; Fig. 1) was incubated with pure extracts (E+/−, O+/−), with extracts supplemented with 1 mM ATP (E+A, O+A) or 8% (wt/vol) PEG 8000 (E+P, O+P), and with a 1:1 mixture of oocyte and egg extract (O+E). Control lanes: M, circular 3-kb plasmid (oc, ccc); S, linear 3-kb substrate input (lin); L, T4 DNA ligase control. The absence of OC and CCC monomer confirms the nonhomologous terminus configuration of the DNA substrate used; formation of 6-kb linear dimers (dlin) indicates ligase activity.

saline containing 0.1 mM progesterone (Sigma). Addition of hormone was considered as $t=0$, and 1 h of maturation was defined as 1 h of incubation in progesterone-containing medium. After 2 to 3 h, 50% of the cells showed the white spot indicating successful induction of maturation (1, 13). This very early appearance of GVBD differs from values reported previously and may be explained by the high concentration of progesterone used and the stimulation of females with human chorionic gonadotropin prior to ovary excision. DNA substrate was injected into oocytes at various times after hormone addition and recovered after 1 h of incubation at 18°C.

Inhibition experiments with cycloheximide. Protein synthesis was inhibited by adding cycloheximide (Sigma) to a final concentration of 0.1 mg/ml to the oocyte medium at various times after progesterone-induced maturation. After 17 h of maturation, eggs were injected with DNA substrates and incubated for 1 h at 18°C.

Preparation of extracts from oocytes and fertilized eggs. Extracts from fertilized eggs and oocytes were prepared as described previously (11, 33). Extract joining assays, performed in total volumes of 10 μl containing 10 ng of DNA substrate and 8 μl of extract, were stopped after 90 min of incubation at 13°C by freezing in liquid N_2. Some joining assays were supplemented with 8% (wt/vol) polyethylene glycol (PEG) 8,000 (Sigma) or 1 mM ATP (pH 7) (Sigma).

Analysis of joining products. Reaction products were recovered from frozen samples as described previously (33). One microliter of extract sample corresponding to 1 ng of input DNA, or one oocyte or embryo equivalent corresponding to 0.1 ng of input DNA, was electrophoresed in 1% (wt/vol) agarose (Bethesda Research Laboratories) minigels in TA (40 mM Tris-acetate [pH 8], 2 mM EDTA) containing 1 μg of ethidium bromide per ml at 10 V/cm. Standard procedures were used for Southern transfer of gels on Hybond-N membranes (Amersham) and hybridization with pSP65 ^{32}P labelled by the random-priming method (Amersham) as the probe.

RESULTS

Assay system. The experiments reported here analyze the in vivo and in vitro capacity of oocytes and eggs from *X. laevis* to join linear DNA substrates with defined nonhomologous terminus configurations. The nonhomologous standard substrates used (Fig. 1) were prepared as described previously (33) by duplicate restriction cuts within the polylinker of plasmid pSP65 (28). Nonhomologous DNA end joining converts DNA substrate molecules into various mono- and multimeric reaction products which may be separated by gel electrophoresis (Fig. 2). Multimeric forms may arise by direct ligation of equal ends and are therefore useful indicators of ligation (Fig. 2, L). Only circular monomers are considered to be genuine joined reaction products which arise as covalently closed circular (CCC) monomers and as intermediate nicked or gapped open circular OC monomers. (Fig. 2, E+/−).

Comparison of joining activities in oocytes and eggs. Extracts from fertilized *X. laevis* eggs readily join nonhomologous DNA substrates (33) in vitro. This is exemplified in Fig. 2 for an extract-promoted joining reaction by the marked occurrence of CCC monomers (Fig. 2, E+/−). In contrast, extracts prepared from stage VI oocytes failed to generate any visible reaction products (Fig. 2, O+/−). Only if the local DNA concentration was increased by supplementing oocyte extracts with PEG 8000 (17), intermolecular ligation was stimulated to give rise to a dimer band (Fig. 2, O+P) which indicated the presence of DNA ligase activity. Thus, extracts from stage VI oocytes appear to be devoid of any activity for nonhomologous DNA end joining.

To rule out the possibility that oocyte extracts contain transferable inhibitors of nonhomologous DNA end joining, 1:1 mixtures with egg extract were prepared (Fig. 2, O+E). The result shows that joining activity dropped to approximately one-half of that of pure egg extract, indicating an effect of dilution rather than inhibition.

The in vitro results were verified in in vivo experiments by injection of nonhomologous DNA substrates into nuclei of

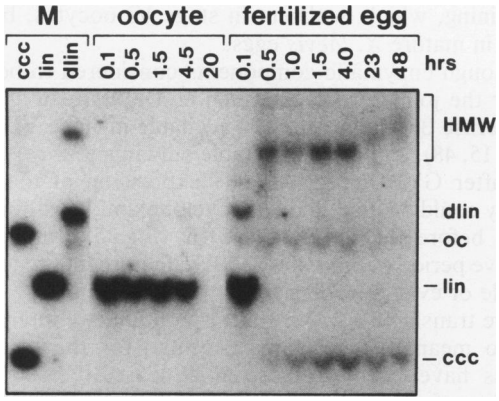

FIG. 3. Comparison of the in vivo joining reaction in stage VI oocytes and fertilized eggs. DNA substrate *Sac/Kpn* (3′/3′; Fig. 1) was injected into oocyte nuclei or animal poles of fertilized eggs and recovered after the incubation periods indicated above the lanes. Marker lanes (M): circular 3-kb plasmid (ccc, oc); linear 3-kb substrate input (lin); 6-kb linear dimer (dlin) (the additional bands in this lane are 6-kb CCC and OC forms resulting from a partial digest of a 6-kb plasmid). HMW, high-molecular-weight concatemers.

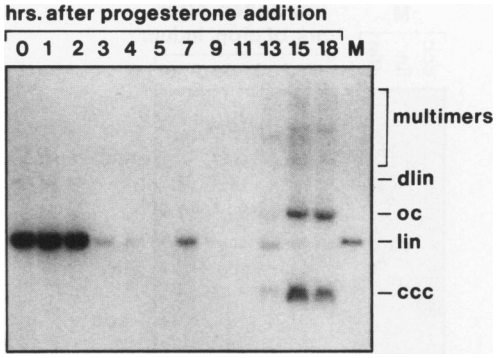

FIG. 4. Maturation kinetics of progesterone-stimulated stage VI oocytes. DNA substrate *Bam/Sal* (5′/5′; Fig. 1) was injected into oocytes at various stages of maturation and recovered after 1 h of incubation. Times indicated above the lanes represent the time of DNA recovery and thus the total time of incubation in hormone (= stage of maturation). The onset of GVBD appears at approximately 3 h after progesterone addition (lane 3). The increase of DNA concentration at 7 h is probably due to a variation during injection or DNA recovery and is not a reproducible finding. Abbreviations are as in Fig. 3.

stage VI oocytes or animal poles of fertilized eggs and DNA recovery after various times of incubation. The results of a typical experiment to define the kinetics of the joining reaction are shown in Fig. 3. As expected, injection of linear DNA substrates into fertilized eggs gave rise to OC monomers and linear dimers directly after injection (Fig. 3, 0.1). The joining reaction was completed at approximately 30 min postinjection, as indicated by the disappearance of linear input DNA and the occurrence of CCC monomers as well as high-molecular-weight multimers (Fig. 3, 0.5). The slight increase of CCC DNA at longer incubation times (Fig. 3, 1.0 and 1.5) was probably due to replication (25). Except for the multimers which were converted to even longer high-molecular-weight concatemers, this band pattern persisted during later embryonic stages (Fig. 3, 23 and 48).

In contrast to the joining reactions observed in fertilized eggs, DNA substrates injected into stage VI oocyte nuclei remained linear during the first hours postinjection (Fig.3, 0.1 to 4.5) but were completely degraded after longer incubation times (Fig. 3, 20). Since neither OC nor CCC monomers were seen at any time postinjection in stage VI oocytes (Fig. 3, 0.1 to 4.5), these cells lack factors necessary for nonhomologous DNA end joining, which is consistent with previous findings (7, 26, 29, 47). These results taken together indicate that nonhomologous DNA end joining in fertilized eggs is a feature of their differentiated state and presumably of physiological importance.

Detection of joining activity during egg maturation. Stage VI oocytes are arrested in prophase of meiosis I. Incubation of stage VI oocytes in progesterone-containing medium induces maturation in vitro, leading to completion of meiosis I and fragmentation of the female pronucleus, the so-called germinal vesicle. Successful induction of maturation may be monitored by the appearance of a white spot on the animal pole of the oocyte indicating GVBD. Thereafter, cells pass meiosis II and are arrested in metaphase II, the physiological state of the mature fertilizable egg (1).

To determine at which time during maturation joining activity appears, stage VI oocytes were induced with progesterone at $t = 0$ as described in Materials and Methods; nonhomologous DNA substrates were injected at various times thereafter and recovered after 1 h of incubation. The occurrence of joining activity in the course of oocyte maturation is displayed in the maturation kinetics presented in Fig. 4. The amount of linear input DNA remained constant for the first 2 h of maturation (Fig. 4, 0 to 2), while a significant loss of substrate DNA was observed approximately at the onset of GVBD (Fig. 4, 3). Presumably, fragmentation of the nuclear compartment causes an increased accessibility of unprotected DNA to cytoplasmic exonucleases (46). Joining activity was detectable for the first time after 13 h of maturation (Fig. 4, 13) and came to a maximum between 15 and 18 h (Fig. 4, 15 and 18). This time corresponds to the period when the physiological state of the mature fertilizable egg is reached. Occurrence of abortive cleavage furrows on the animal pole after microinjection, which mimics fertilization (13, 15), confirmed the completion of maturation.

In contrast to linear DNA, circular plasmid DNA, although prone to nucleolytic degradation in the oocyte cytoplasm, exhibits a remarkable stability in the oocyte nucleus which is possibly due to unknown nuclear factors (46, 47). These factors may be released into the cytoplasm upon GVBD and thus considered to be responsible for the known stability of circular DNA within the whole cell lumen of the mature egg (46). This may explain why we did not observe any significant losses of circular DNA injected into the cell lumen of maturing oocytes after GVBD and mature eggs in our control experiments (data not shown). Therefore, the failure to detect joining activities during maturation is unlikely to be an artifact caused by nucleolytic degradation of joined CCC products. On the contrary, DNA end joining, once activated in the mature egg, is able to rescue a substantial portion of linear DNA from exonucleolytic degradation by converting it into stable inert circles.

The results in Fig. 4 show that the joining system becomes active only in the final phase of egg maturation. It remains unknown whether the activity of the system is switched on by the activation of preexisting precursor proteins or by the de novo synthesis of essential components of the system.

Kinetics of cycloheximide inhibition during egg maturation. The question of whether protein synthesis is required for the

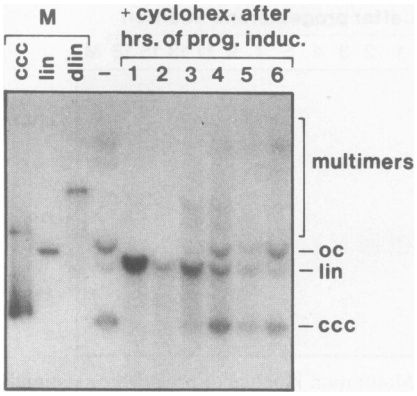

FIG. 5. Cycloheximide inhibition. For inhibition of protein synthesis, cycloheximide was added to oocytes at various times after progesterone induction as indicated above the lanes. DNA substrate *Bam/Sal* (5′/5′; Fig. 1) was injected into stage VI oocytes after 17 h of maturation (when DNA end-joining activity is maximal; Fig. 4) in the presence or absence (= positive control without added cycloheximide [−]) of the drug and recovered after 1 h of incubation. Abbreviations are as in Fig. 3.

activation of the joining system was addressed by inhibiting translation with cycloheximide. Cells blocked with the drug at various stages of maturation were injected with linear DNA substrate at the stage of maximal joining activity (17 h after hormone induction; compare Fig. 4, 15 and 18) and recovered after 1 h of incubation. The results of a typical inhibition experiment are presented in Fig. 5. As expected, in vitro-matured oocytes whose translation had not been inhibited showed full joining activity (Fig. 5, −). When cycloheximide was added within the first 2 to 3 h of maturation, before the onset of GVBD, joining activity was completely abolished (Fig. 5, 1 to 3). After this critical time, cycloheximide did not further affect the expression of the joining activity (Fig. 5, 4 to 6).

This pattern of inhibition implies that the protein components required for a functioning joining system are all available at GVBD, 3 h after maturation induction (Fig. 5, 3). Joining activity, however, is not detected until 10 h later (Fig. 4, 13). If the activation of the joining system were due to de novo synthesis of one or more components, joining activity would be expected to appear directly after GVBD. However, the delay of 10 h points to the involvement of time-consuming and therefore regulatory processes. In *X. laevis*, protein synthesis is required for the initiation of maturation and for progression to meiosis II (22, 44). Translation of factors regulating the activity of maturation-promoting factor, which initiates egg maturation, has been shown to occur in this cycloheximide-sensitive period (9, 20, 22, 30, 44). It may be therefore considered that all protein components required for the assembly of an active joining system are already stockpiled in stage VI oocytes but that their activities are regulated as a function of the meiotic cell cycle of egg maturation.

DISCUSSION

During egg maturation, amphibian oocytes, arrested in prophase I, differentiate into mature fertilizable metaphase II-arrested eggs with new, distinct properties (14). Here, we have shown that a system promoting nonhomologous DNA end joining, which is absent in stage VI oocytes, becomes active in mature *X. laevis* eggs.

Although enzymatic components considered to be essential for the joining reaction, such as DNA ligase and DNA polymerase β (43), are already available in stage VI oocytes (2, 10, 15, 48), joining is detectable only in mature eggs about 10 h after GVBD (Fig. 4). The expression of the joining activity could be inhibited with cycloheximide within a short period before the onset of GVBD (Fig. 5). In this drug-sensitive period of time, the decisive factors that regulate the cascade of events leading to the completion of egg maturation are translated (22, 30). In this context, we interpret our data to mean that functions essential for the end-joining process have been synthesized at least 10 h before the detection of actual joining activity. Thus, activation might depend on the translation of cell cycle-regulating factors and is therefore completed considerably later. It is reasonable to assume that the activation of the joining system is regulated by posttranslational modification of one or more key functions. The alignment function may be considered a possible candidate (43). In addition, preexisting basal levels of enzymatic factors involved in the joining mechanism, e.g., DNA ligase might also be stimulated in the course of maturation. Since microinjection mimics fertilization (14, 16), our experiments cannot strictly distinguish whether or not the joining activity requires a fertilization stimulus as a final activation step.

The extremely late activation of the joining system suggests that it can be dispensed within oocytes but exerts its biological function during embryogenesis and is conveyed to the adult cell to maintain there its known high level (36). In contrast, prophase I-arrested stage VI oocytes exhibit high activities of nonconservative homologous recombination (4, 5). This activity was considered to be involved in DSB repair or resolution of amplified ribosomal DNA or, less likely, as a remnant of meiotic recombination activities (27). Since it has not yet been shown whether this homologous recombination system is also active in mature eggs or whether it is replaced by nonhomologous DNA end joining, we cannot exclude the possibility that both recombinational systems coexist in mature eggs. If so, they would compete for free DNA ends as their common substrates. Both activities could be involved in the elimination of DSBs arising during the extremely rapid replication cycles (38) of the cleavage divisions in the early embryo (14). Since the more precise homologous recombination process requires extensive sequence homologies for DSB repair, it might be able to affect only DSBs that occur in close proximity of sequence repeats. The less precise process of nonhomologous DNA end joining, however, might develop its full efficiency because it can dispense with time-consuming homology searches. In this context, it might be used mainly to facilitate the joining of spatially close DNA ends. Since the ratio of nonessential to essential DNA is high in vertebrate genomes, it has been proposed that minor alterations at junctions introduced by small nucleotide deletions or insertions would not be fatal for the cell (38).

It remains to be shown whether the well-timed and regulated activation of nonhomologous recombination is a general feature initiated by the first meiotic recombination wave during germ cell maturation. The process of egg maturation in *X. laevis* and its already known regulatory switches might be used as a useful model system to further explore the problem of the regulation of recombination during the cell cycle.

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