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Identification of novel genes specifically expressed in *Chlamydomonas reinhardtii* zygotes

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Abstract

The maturation of zygotes formed by the fusion of two gametes is the essential part of the diploid phase of the *Chlamydomonas reinhardtii* sexual life cycle and results in mature zygotes competent to germinate. To understand the molecular mechanisms underlying zygote maturation and the attainment of competence for germination we isolated genomic clones representing three different genes that are specifically expressed in *Chlamydomonas reinhardtii* zygotes. Accumulation of the RNAs started more than 24 h after mating, setting these genes apart from genes expressed in young zygotes [9]. Upon light-induced germination of zygotes, the mRNAs disappeared. The patterns of RNA accumulation and disappearance were gene-specific and suggested a function of these genes in maturation and/or in initial steps of germination.

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* undergoes a sexual life cycle which includes defined stages of differentiation. Vegetative cells differentiate into gametes and gametes of opposite mating type fuse to form a diploid zygote. The zygotes – which represent the resting form of *Chlamydomonas* – germinate after a maturation period (i.e. undergo meiosis and release haploid spores). Both gametogenesis and zygote germination are controlled by environmental conditions. Under laboratory conditions gametogenesis is induced by nitrogen starvation [23] and light [26]. For the maturation period, the newly formed zygotes are transferred to agar plates and incubated for about 20 h in the light and at least for 4 days in the dark [17]. Mature zygotes can subsequently be induced to germinate by transferring them into the light.

Vegetative cells, gametes and zygotes represent distinct cell types displaying unique gene expression programs as shown by comparison of their *in vivo* protein patterns as well as their content in *in vitro* translatable RNA [20, 26, U. Treier and C.F. Beck, unpublished]. Ferris and Goodenough [9] identified genes expressed in zygotes immediately after gamete fusion. Woessner and Goodenough [29] showed that one of these genes codes for a zygote-specific cell wall protein. Our interest is focused on zygote maturation and zygote germination. It was not known

whether later stages of zygote maturation display specific gene expression programs and whether mRNAs controlling germination are synthesized during maturation.

We recently developed two prerequisites for the analysis of zygote maturation and germination at the molecular level [28]: first, procedures for the generation of mature zygotes in large quantities, and second, a method for the quantitative breakage of these thick-walled cells. Using single-stranded cDNAs generated from poly(A)$^+$ RNA of mature zygotes and vegetative cells we differentially screened a genomic DNA library and isolated zygote-specific clones which represent protein-encoding genes (*zym*). The clones were used to study the *zym*-RNA accumulation patterns during the sexual life cycle of *C. reinhardtii*. These RNAs were not detectable in vegetative cells. The accumulation of the *zym*-RNAs was found to be restricted to the zygote maturation phase; during germination, the RNAs disappeared.

Materials and methods

Culture conditions

C. reinhardtii vegetative cells, strains 137c mt+ and 137c mt−, were grown in TAP medium [11] with aeration at 23 °C under continuous illumination (30 μmol m^{-2} s^{-1}). The cells were transferred to nitrogen-free TAP medium (TAP-N) to induce gametes.

Generation of zygotes

Equal numbers of gametes were mixed for mating 18–20 h after induction of gametogenesis. Zygote maturation and induction of germination were performed as described previously [28]. For germination experiments zygotes after harvest were suspended in TAP medium to a density of about 1×10^9 cells per ml. By sonication for 60 s at a power of 30 W using a Branson model B 12 sonifier equipped with a microtip the zygote pellicles were disrupted into individual zygotes and clusters of 2–30 zygotes. These zygotes after centrifugation were suspended in TAP medium to a density of $1–2 \times 10^6$ cells per ml and incubated with shaking (120 rpm) in the light (30 μmol m^{-2} s^{-1}). For 'liquid maturation' experiments zygote pellicles were not transferred to agar plates, but 200 ml aliquots of mating mixtures were incubated in 500 ml beakers without shaking for 20 h in the light and then in the dark.

Determination of germination efficiency

Zygote suspensions were sampled at different time points after induction of germination. The cells were fixed with glutaraldehyde (0.2% final concentration) and microscopically scored for zygotes (Z), dyads and tetrads (DT), and flagellated cells (S). We assumed that the flagellated cells represent spores and that each zygote released 4 spores after meiosis. The germination efficiency was calculated using the following equation:

$$\% \text{ germination} = 100 \times (DT + S/4)/(Z + DT + S/4)$$

RNA isolation

Mechanical breakage of the zygotes and RNA isolation from vegetative cells, gametes, and zygotes were performed as described previously [28]. Poly(A)$^+$ RNA was prepared from total RNA by oligo(dT) cellulose affinity chromatography [18].

Hybridizations

Radiolabelled DNA representing the cloned sequences was generated by the random oligolabelling technique [8]. Radiolabelled cDNA was synthesized in a reaction mixture (50 μl) containing 50 mM Tris hydrochloride pH 8.3, 75 mM KCl, 30 mM $MgCl_2$, 10 mM dithiothrei-

tol, 0.1 μg/μl BSA, 0.1–1 μg heat-denatured poly(A)$^+$ RNA, 4 μM each of dATP, dCTP, dTTP, 40 μg/ml oligo $(dT)_{15}$, 0.05 μg/μl actinomycin D, 150 μCi of α^{32}P-dCTP (specific activity 3,000 Ci per mmol; Amersham Buchler, Braunschweig, FRG), 200 U of M-MLV reverse transcriptase (BRL) and incubated for 60 min at 37 °C. The template RNA was subsequently degraded by alkaline hydrolysis. Plaque hybridizations were made as described [27] but at 50 °C. Washes were in 2 × SSC, 1% SDS at 50 °C for 30 min and 0.2 × SSC, 1% SDS at 60 °C for 30 min. Nylon membranes (GeneScreen; Dupont/NEN Research Products, Boston, MA) were used for DNA gel blot analyses. Prehybridizations and hybridizations were performed in 5 × SSC, 50 mM Tris hydrochloride pH 7.5, 0.1% sodium pyrophosphate, 10 × Denhardt's solution [7], 1% SDS, 10% dextran sulfate, 50% formamide, 150 μg of sheared, denatured salmon sperm DNA at 50 °C. Washes were in 0.3 M NaCl, 60 mM Tris hydrochloride pH 8, 2 mM EDTA, 1% SDS at 55–75 °C for 60 min. RNA gel blot analyses were performed as described previously [27] at 60 °C. The membranes were washed in 0.1 × SSC, 1% SDS at 65 °C for 30 min. For RNA dot hybridizations the RNA was applied to nylon membranes according to Boll *et al.* [2]. Hybridizations were performed as described for the RNA blot hybridizations. All filters were wrapped in plastic wrap and exposed to Fuji X-ray film RX at − 70 °C using intensifying screens (Titan 2HS, Siemens).

Quantification of hybridization signals

The relative intensities of the hybridizations signals were quantified by elution of the silver grains from developed X-ray films and measurement of the absorbance of the eluate as described [24].

Library screening

A library of genomic *C. reinhardtii* DNA (kindly provided by M. Goldschmidt-Clermont) in phage lambda EMBL 3 was screened with single-stranded radiolabelled cDNA generated from poly(A)$^+$ RNA of zygotes and of vegetative cells. Preparation of filter replicas, phage lysis and DNA fixation were as described [1]. Clones selected after the primary screening were assayed in a second screening according to the plaque dot method [22]. Confirmed zygote-specific clones were plaque-purified. Phage DNA was isolated, digested with restriction endonucleases, and the fragments that hybridized to the cDNA reverse transcribed from zygote poly(A)$^+$ RNA were cloned in pUC18.

Hybrid selection

Binding of plasmid DNA to nitrocellulose filters and hybrid selection were performed as described [19], with the following modifications: hybridizations were performed for 4–5 h at 50 °C with 100–200 μg of total RNA isolated from zygotes. The nitrocellulose filter pieces were removed directly from the boiling eluate.

In vitro *translation and SDS-PAGE*

The hybrid-selected RNAs were translated *in vitro* in a nuclease-treated rabbit reticulocyte lysate (kindly provided by K. Hilse, University of Freiburg, Freiburg, FRG) [21] or in a wheat germ extract (kindly provided by H. Kössel, University of Freiburg, Freiburg, FRG) [5] using ^{35}S-methionine (specific activity 800 Ci/mmol; Amersham Buchler, Braunschweig, FRG). Proteins were separated on 13.5% SDS-polyacrylamide gels or 7.5–20% gradient SDS-polyacrylamide gels using a discontinuous buffer system [15]. Gels were prepared for fluorography as described [3].

Results

Isolation of clones containing sequences specifically expressed in zygotes

Poly(A)$^+$ RNA isolated from vegetative cells and from mature zygotes were reverse-transcribed to produce single-stranded ^{32}P-labelled cDNA. These cDNAs were used to screen approximately 35000 phages from a lambda EMBL 3 genomic library [10]. 15 clones were isolated that hybridized to the zygote-specific cDNA probe and not to the cDNA generated from RNA of vegetative cells. DNA gel blot analyses using the DNAs of these clones digested with different restriction enzymes revealed that each clone contained only a single *Sal* I or *Sal* I/*Eco* RI fragment hybridizing to the cDNA probe. These fragments were subcloned in plasmid vectors. On the basis of shared restriction fragments and cross-hybridization studies (data not shown) the clones were grouped into three classes named zymA, zymB, and zymC. One clone of each class (zymA-9, zymB-14 and zymC-16) was used for further analyses. Clone zymA-9 contained a 4 kb *Sal* I fragment, zymB-14 a 3.4 kb *Sal* I/*Eco* RI fragment, and zymC-16 a *Sal* I fragment of 4.6 kb. The *Sal* I site of clone zymB-14 and one of the *Sal* I sites of clone zymC-16 were from the ends of the lambda clones.

Assay for transcripts and gene copy number

To confirm that the cloned DNA fragments represent transcribed sequences and to verify the zygote specificity of their expression, they were used to probe gel blots of total RNA isolated from vegetative cells, gametes and mature zygotes. Figure 1 shows that each cloned DNA hybridized to a RNA species of a single size class: zymA-9 and zymB-14 both to RNA species of about 0.55 kb, zymC-16 to a RNA of about 1.6 kb. Each RNA was present in the poly(A)$^+$ and not in the poly(A)$^-$ fraction of an oligo(dT) cellulose chromatography (data not shown). The clones zymB-14 and zymC-16 hybridized to RNA present only in zygotes. RNA hybridizing to zymA-9 was present in zygotes and also at a low level in gametes (Fig. 1).

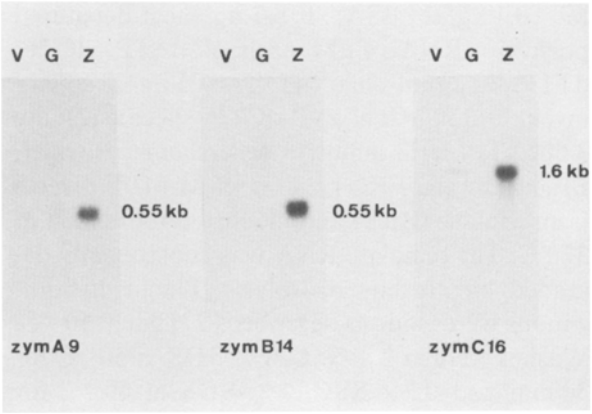

Fig. 1. Gel blot analysis of RNA from different stages of the sexual life cycle using ^{32}P-labelled zym DNAs as probes. 5 μg of total RNA isolated from vegetative cells (V), gametes (G), and mature zygote (Z) were separated by electrophoresis on formaldehyde-containing agarose gels, transferred onto nylon membranes and hybridized under high stringency conditions with DNA of clones zymA-9, zymB-14, and zymC-16.

In order to determine genomic organization and the repetition frequencies of the zym homologous sequences DNA gel blot analyses were performed with nuclear *C. reinhardtii* DNA digested with various endonucleases. In addition five- and single-copy equivalents – assuming a genome complexity of 100000 kb [13] – of the cloned DNAs were probed. As shown in Fig. 2, the three zym clones hybridized to genomic fragments of different sizes, indicating that they are not directly adjacent in the *C. reinhardtii* genome. Colinearity of the cloned DNA fragments with genomic DNA was demonstrated for zymB-14 and zymC-16 for *Eco* RI-*Pst* I and *Sal* I-*Pst* I subfragments, respectively, comprising approximately two thirds of the original clones (data not shown). The intensities of the hybridization signals indicated that the zym-homologous genomic sequences may be present as single copies per haploid genome.

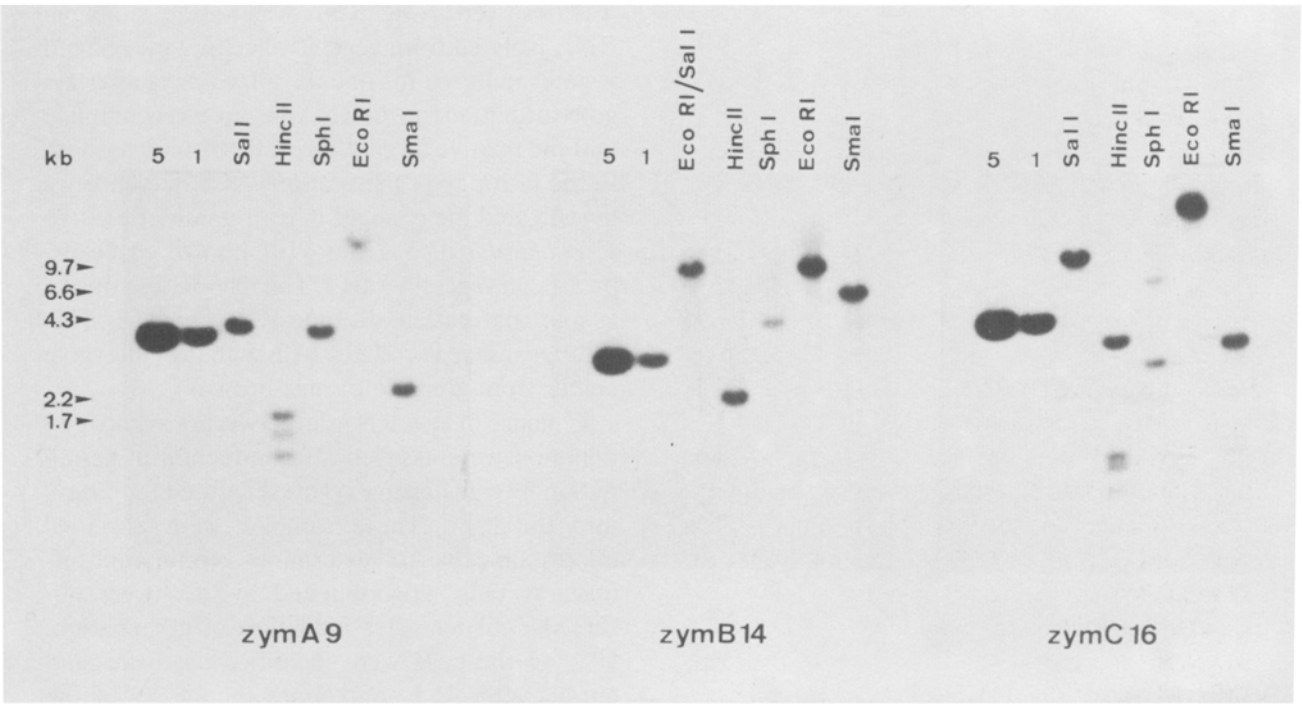

Fig. 2. Gel blot analysis of *C. reinhardtii* nuclear DNA using ^{32}P-labelled zym DNAs as probes. Nuclear *C. reinhardtii* DNA was digested with the restriction endonucleases indicated, separated on agarose gels, transferred to nylon membranes, and hybridized with the DNA of clones zymA-9, zymB-14, and zymC-16. Five- and single-copy equivalents of the cloned fragments were run in lanes labelled 5 and 1, respectively.

zym sequences encode proteins

Hybrid-select translations were used to assay whether the three cloned DNA fragments represent protein encoding genes (or parts thereof). RNA selected by hybridization with zymC-16 DNA was translated in a rabbit reticulocyte lysate. RNA species hybrid-selected with DNA of clones zymA-9 and zymB-14 were translated in wheat germ extract because co-migration of endogenous rabbit globin with the *in vitro* translation products was expected. The radio-labelled translation products were analyzed by SDS-PAGE (Fig. 3). RNA that hybridized to zymA-9 DNA and RNA that hybridize to zymB-14 DNA each directed the translation of a protein of a molecular weight of about 18000 (Fig. 3B). The RNA selected by clone zymC-16 was translated into two proteins of molecular weights of about 41000 and 42000 (Fig. 3A). These two products may represent distinct proteins, or, alternatively, one was the product of incomplete translation or post-translational processing. The actual molecular weights of the translation products were consistent with those predicted from the RNA sizes (Fig. 1). We conclude from these data that the three zygote-specific clones represent unique *C. reinhardtii* genes.

Changes in zym-RNA abundance during the sexual life cycle

To analyze *zym* gene expression we followed *zym*-RNA abundance patterns throughout the sexual life cycle of *C. reinhardtii*. Zygote maturation and germination were assessed in greater detail. For analysis of the changes in RNA levels during zygote maturation, zygote pellicles 6 h after mating were transferred to agar plates. Total

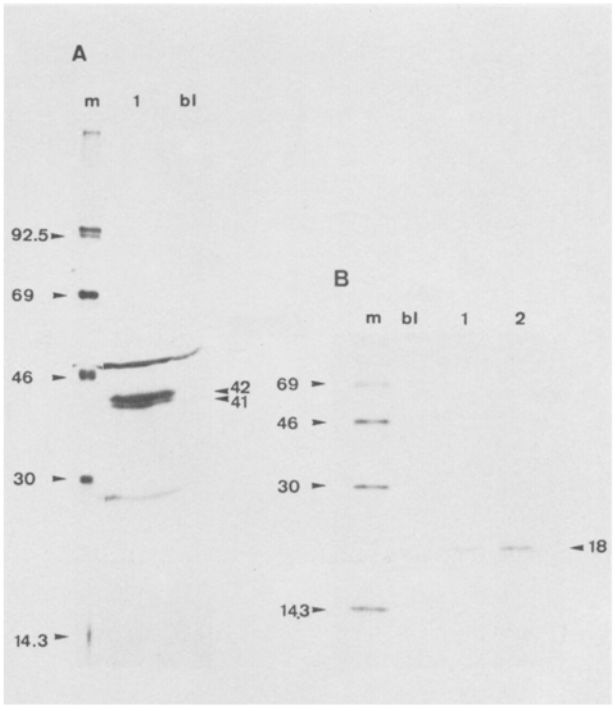

Fig. 3. SDS-PAGE of products from hybrid-select translation. A. Zygotic mRNA isolated by hybrid selection with immobilized DNA of clone zymC-16 was translated *in vitro* in a rabbit reticulocyte lysate (lane 1). B. Zygotic mRNA species isolated by hybrid selection with immobilized DNA of clones zymA-9 (lane 1) and zymB-14 (lane 2) were translated *in vitro* in a wheat germ extract. In lanes labeled bl, no additional RNA was added. The molecular weight standards (in thousands) are indicated.

RNA was isolated from zygotes that were harvested 1, 2, 3, and 5 days after mating. After a 5-day maturation zygotes were able to germinate at a high rate. In addition, RNA was isolated from vegetative cells and mature gametes of the same cultures used for generation of the zygotes. Figure 4A shows that during zygote maturation the *zym*-RNAs accumulated following gene-specific temporal patterns. *zymA*-RNA was not detectable in vegetative cells, but was present in mature gametes at similar levels as in zygotes one day after mating. During maturation the RNA level increased, reaching a maximum 5 days after mating. The *zymB* RNA was detectable only one day after mating. From the first to the third day of maturation a 10-fold increase of *zymB*-RNA was observed. *zymC* RNA was not detectable in RNA isolated from vegetative cells, gametes and zygotes matured for one day. Two days after zygote formation *zymC* RNA began to accumulate and the relative level of *zymC* RNA increased up to the fifth day of maturation. As a reference we investigated the changes in expression of the two *C. reinhardtii rbcS* genes [10] known to be expressed in vegetative cells. The *rbcS* RNAs during zygote maturation disappeared (Fig. 4A). This disappearance correlates with a change in zygote colour from green to orange-brown.

Changes in *zym* RNA levels during zygote germination were assayed after induction of germination by transferring zygotes matured for 5 days into the light. These samples were observed microscopically to determine germination efficiency. Only ungerminated zygotes were observed until 6 h after induction of germination, 50% of the cells were identified as dyads and tetrads after 15 h, and more than 82% of the zygotes had germinated after 24 h. As shown in Fig. 4B, the *zym* RNAs disappeared during zygote germination. The *zymA* RNA decreased gradually during a 24-h period. The decrease of *zymB* RNA was slightly accelerated as compared to that of the *zymA* RNA. The most drastic decline was observed for the *zymC* RNA. This RNA species was reduced to 30% within the first 3 h of germination and was detectable only in very low amounts after 6 h. *zymC* RNA thus disappeared before any changes in sub-cellular structures became evident and before zygotes entered meiosis which is initiated about 10 h after the zygotes were introduced to the light [16]. The RNA levels of *zymA* and *zymB* decreased in temporal correlation with the decrease in ungerminated zygotes, albeit with different kinetics. In contrast to the decrease in *zym* RNA levels, the *rbcS* RNAs accumulated during zygote germination. They were detectable 6 h after induction of germination and then increased continuously to the level observed in vegetative cells. The kinetics of *rbcS* RNA accumulation coincide with greening of the cell suspension and the onset of photosynthetic activity [12]. The accumulation of the *zym* RNAs appears to be restricted to the zygotic stage of the

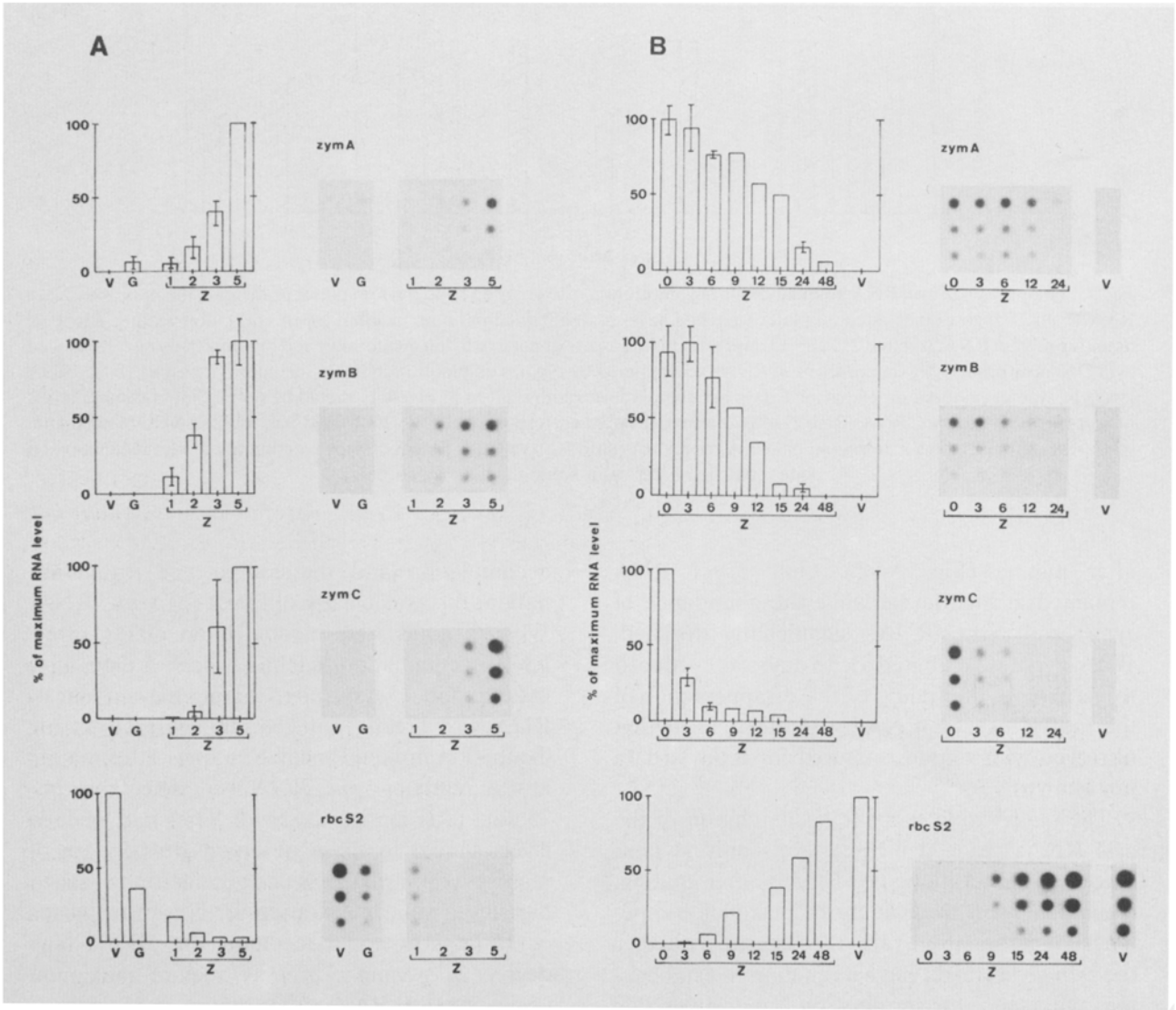

Fig. 4. Changes in abundance of *zym* and *rbcS* RNAs during zygote maturation and germination. A. Zygote maturation: 2 μg, 1 μg and 0.5 μg of total RNA isolated from vegetative cells (V), mature gametes (G) and zygotes (Z) harvested 1, 2, 3, and 5 days after mating were applied to nylon membranes and hybridized to the ^{32}P-labelled zym and rbcS [10] DNAs as probes. B. Zygote germination: 2 μg, 1 μg and 0.5 μg of total RNA isolated from vegetative cells (V) and zygotes (Z) harvested at different time points (h) after induction of germination were applied to nylon membranes and hybridized.

C. reinhardtii sexual life cycle. The *zym* genes are thus the first *C. reinhardtii* genes identified that are specifically expressed in maturing zygotes.

Effect of zygote maturation conditions on zym RNA abundance

Chlamydomonas zygotes retain their ability to sporulate when incubated for several years [25]. To investigate the effect of extended incubation of zygotes on *zym* RNA levels, we compared the *zym* RNA levels from zygotes harvested 5 and 15 days

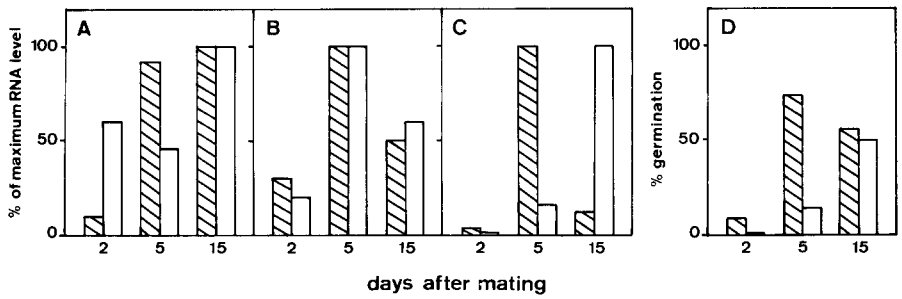

Fig. 5. Comparison of *zym* RNA abundance during maturation of zygotes up to 15 days on plates or in liquid medium. A–C. *zym* RNA levels of zygotes incubated on plates (hatched bars) or maintained in liquid medium (open bars) after mating. Identical amounts of total RNA, isolated 2, 5 and 15 days after mating, were applied to nylon membranes and hybridized using ^{32}P-labelled zym DNAs as probes. Maximum levels of RNAs accumulated by zygotes on plates or in liquid medium were set as 100%. RNA levels of zygotes matured on plates for 5 days are comparable to those given in Fig. 4. It should be noted that maximum levels of *zymA*, *zymB*, and *zymC* RNAs from zygotes matured in liquid correspond to 100%, 200% and 50%, respectively, of maximum RNA levels from zygotes matured on plates. A, zymA; B, zymB; C, zymC. D. Extent of zygote germination after incubation on plates (hatched bars) or in liquid medium (open bars).

after mating (Fig. 5A–C). Only *zymA* RNA remained at high levels while the abundance of *zymB* and *zymC* RNAs significantly declined. When zygotes incubated for 15 days were induced to germinate, the timing of the disappearance of the individual *zym* RNAs was identical to that observed with zygotes matured for 5 days (data not shown).

The observation that the accumulation of the *zym* RNAs reached highest levels only at later stages of maturation, i.e. 5 days after mating, coincident with the gaining of maximal competence for germination, raised the question whether the *zym* genes might have a function in establishing competence for germination. To evaluate this hypothesis we compared *zym* RNA accumulation patterns of zygotes that were transferred to solid support after mating to zygotes that were maintained in liquid medium. Continued incubation of freshly mated cells in liquid medium is known to result in zygotes that differ morphologically from plate matured zygotes and exhibit only poor germination [4]. Fig. 5A–C shows the relative *zym* RNA levels of zygotes matured on plates or in liquid medium harvested at various time after mating. Also, zygotes matured on plates or in liquid medium were assessed for germination (Fig. 5D). While a correlation was not evident between the patterns of *zymA* and *zymB* RNA accumulation and the competence for germination, this was clearly different for *zymC* RNA. When zygotes were incubated on plates, *zymC* RNA accumulation reached a peak 5 days after mating, and 15 days after mating the amount of RNA was greatly reduced. In contrast, during incubation in liquid medium 5 days after mating low amounts of *zymC* RNA were detectable, but 15 days after mating the *zymC* RNA had reached levels similar to those observed after 5 days of maturation on plates. Since germination reached high levels after maturation for 5 days on plates or 15 days in liquid medium (Fig. 5D), a high degree of germination may require maximum levels of *zymC* RNA either before or at the time when germination is induced.

Discussion

By differential screening of a genomic library of *C. reinhardtii* DNA we identified 3 zygote-specific clones. The genomic sequences homologous to the cloned DNAs were present as single copies per haploid genome (Fig. 2). Hybrid-select translations revealed that the clones selected RNA species from total RNA that were translatable *in vitro*. These data suggest that the cloned DNAs represent 3 different *zym* genes or fragments

thereof. The zygote specificity of these genes was confirmed by RNA gel blot analyses. Each gene hybridized to a single transcript present only in zygotes, not in vegetative cells and, with the exception of *zymA*, neither in gametes. Two genes (*zymA* and *zymB*) hybridized to transcripts of 0.55 kb, the third (*zymC*) to a transcript of 1.6 kb.

The levels of the individual *zym* RNAs were analyzed at different stages of the sexual life cycle of *C. reinhardtii*. The RNA abundance patterns for each gene showed an increase during zygote maturation and a decrease after induction of germination (Fig. 4). The temporal patterns though were gene-specific. The *zym* genes thus represent novel types of *C. reinhardtii* genes exhibiting zygote-specific expression. The virtual absence of these transcripts in vegetative cells and gametes and the high level of accumulation during zygote maturation argues for an activation of transcription of these genes 1 to 3 days after mating. The decrease in *zym* RNA levels upon induction of germination may be accounted for by the shutoff of transcription and degradation of the RNAs. The decrease of *zymC* RNA levels (Fig. 4B) is of particular interest since light is the essential environmental signal required for induction of germination. The reduction in *zymC* RNA level may thus be considered a very early step in a program of differentiation which ultimately results in vegetative cells. Disappearance of mRNAs during early stages of zygote germination were also documented by *in vitro* translation experiments using zygote RNA (U. Treier and C.F. Beck, unpublished).

Genes specifically expressed in zygotes after gamete fusion have been identified [9]. These genes, one of which was shown to encode a zygote cell wall protein [29], are not expressed in mature zygotes (data not shown). In contrast, expression of *zymB* and *zymC* was not detectable after cell fusion and in early stages of zygote maturation; *zymA* RNA was present in low amounts (Fig. 4A and data not shown). This indicates that early and late stages of zygote maturation exhibit different gene expression patterns. Since the zygote wall is already formed when *zym* RNAs start to accumulate, a function of these genes in zygote wall formation appears unlikely. Although the functions of the proteins encoded by the *zym* genes remain to be elucidated, the kinetics of RNA accumulation and disappearance are suggestive of their function in zygote maturation and/or initial steps in germination. The accumulation of *zymC* RNA, albeit transient, correlates with the zygotes' competence to germinate (Fig. 5) and the product of the *zymC* gene may possibly have a function in the attainment of competence for germination or in the initiation of germination itself. In contrast to *zymB* and *zymC* RNA levels, the *zymA* RNA levels remained high when zygotes were incubated for prolonged periods after maturation (Fig. 5A). With a system for the generation of stable transformants at hand [6, 14], an approach to the analysis of the function of the *zym* genes is available. Irrespective of the function of these genes, they are useful tools as markers to follow zygote maturation and germination at a molecular level and thus will facilitate the analysis of this stage of the sexual life cycle of *C. reinhardtii*.

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