NOVEL GLYCOPOLYPEPTIDE SYNTHESIS INDUCED

BY GAMETIC CELL

FUSION IN *CHLAMYDOMONAS REINHARDTII*

SHARON A. MINAMI and URSULA W. GOODENOUGH

From the Cell and Developmental Biology Department, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. Dr. Minami's present address is The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111.

ABSTRACT

Within the first hour of zygote maturation, *Chlamydomonas reinhardtii* cells stop synthesizing certain polypeptides that characterize the vegetative and gametic stages of the life cycle and initiate the synthesis of novel, zygote-specific polypeptides. At least six of these polypeptides are secreted into the medium, and fine-structural studies indicate that they represent components of the cell wall that is synthesized and secreted early in zygote development. We conclude that a new program of protein synthesis, and possibly also gene transcription, is initiated shortly after gametic cells fuse, a program that appears highly suited to cell-differentiation studies.

KEY WORDS *Chlamydomonas* · zygotes · secretion · cell differentiation

During its simple life cycle, the unicellular eukaryotic protist *Chlamydomonas reinhardtii* passes through three states of differentiation: the haploid vegetative state, the haploid gametic state, and the diploid zygotic state. Gametogenesis is triggered by nitrogen starvation of vegetative cells (27) and requires about 12 h (17, 22). Zygotes form when gametes of opposite mating type (mt^+ and mt^-) fuse to form quadriflagellated cells during the mating reaction, and early zygote development proceeds for about 24 h, during which time a thick zygote wall is elaborated (7).

We wished to learn whether either of these life-cycle transitions was suitable for studies of eukaryotic cell differentiation, our criterion for suitability being that the transition should involve the production of readily detected, novel polypeptides whose synthesis is specific for a particular state of differentiation. We therefore began by subjecting

cells at various stages of differentiation to 2-3-h pulses with [^{14}C]acetate, and analyzed the labeled polypeptides by slab-gel electrophoresis followed by autoradiography.

When such experiments were performed with cells undergoing gametogenesis (24), all of the major polypeptides synthesized by gametes were found to have apparent counterparts in the vegetative-cell samples. The synthesis of many vegetative-cell polypeptides was observed to abate or cease entirely during gametogenesis so that shifts occurred in the relative intensities of various bands on the gels, but no major polypeptides specific to the gametic state could be distinguished by a variety of approaches.

In contrast, we discovered that zygotic cell fusion in *C. reinhardtii* elicits the synthesis of a number of major zygotic polypeptides that are not evident in vegetative or gametic cells. Because the trigger for the onset of this "zygotic program" is gametic and probably nuclear fusion, the phenomenon is at least formally analogous to the trigger-

ing of protein synthesis by mammalian egg-sperm fusion (31), and an understanding of its mechanism may contribute toward an understanding of how fertilization initiates cell differentiation. We therefore conducted a detailed structural and gel-electrophoretic analysis of early zygote development in *C. reinhardtii*, the results of which are reported in the present paper.

MATERIALS AND METHODS

Strains and Culture Conditions

Mating types plus and minus of wild-type strain 137c were used. The cells used in all experiments were cloned and selected for high mating efficiency (8). Mating tests were performed as previously described (22). In each experiment reported here, 90-100% of the gametes formed quadriflagellated cells, the deviations from 100% mating probably arising largely from statistical errors in sampling and counting the cells.

Cells were grown synchronously under a regimen of 12 h of light and 12 h of darkness (17) in high salt medium (HSM) (29). Gametogenesis was induced by resuspension into nitrogen-free HSM (N-free HSM) as previously described (22). To obtain synchronous zygote formation, cultures of plus and minus gametes were mixed to give equal numbers of each mating type. Cell concentration was determined by counting with a hemacytometer.

The usual laboratory protocol for zygote maturation involves plating the cells 1-2 h after mating on 4% agar plates so that they do not aggregate; they are left in the light for 18-24 h, placed in darkness for 5 days, and then induced to germinate by transferring them to fresh plates and illuminating them (20). Inasmuch as our experiments involve leaving zygotes in liquid medium, we first ascertained that when such zygotes are subjected to the Levine-Ebersold protocol, they exhibit good germination (24). Zygotes older than 23 h were not analyzed because their germination is poor.

Electron Microscopy

Zygotes in their own medium were brought to a final concentration of 2% glutaraldehyde with a cold 4% solution of glutaraldehyde in 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7. The suspension was chilled and stored at 4°-6°C for at least 24 h. The cells were pelleted, resuspended in cold 1% OsO_4 for at least 4 h, and then dehydrated and embedded as previously described (16). Long fixation times prove essential for satisfactory preservation of zygotic cell structure.

Labeling Conditions

Sodium acetate uniformly labeled with ^{14}C was purchased from Amersham Corp., Arlington Heights, Ill.

(CFA-229). The [^{14}C]acetate was solubilized in 20% EtOH and filter-sterilized.

Cell cultures were labeled in 5-ml aliquots in sterile 2 cm × 19.5 cm test tubes equipped with bubblers. Labeling was done at 21° or 24 °C at a light intensity of 5,200 lux. Cell suspensions were bubbled with 5% CO_2 in air. At the end of the labeling period, uptake was stopped by diluting the cell suspension with 2 vol of ice-cold 20 mM Tris-HCl, 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.6 (Tris-Mg buffer). The cells were pelleted by centrifugation for 5 min at 5,000 rpm in a Sorvall SS-34 rotor (Sorvall, Ivan, Inc., Norwalk, Conn.). The pellet was rinsed with Tris-Mg buffer and then either frozen or immediately fractionated.

Fig. 1 shows the result of an experiment which compares the labeling patterns of zygotes pulse-labeled with either[^{14}C]acetate (A and C) or [^{3}H]arginine (B and D); details are given in the legend to the figure. Little difference is evident when the autofluorograms are compared, establishing that [^{14}C]acetate labels polypeptides, either via N-terminal acetylation (5) or by entering amino acid pools, in the same fashion as ^{3}H-amino acids (see also reference 1).

Preparation for Electrophoresis

Cells were resuspended into Tris-Mg buffer containing 1.6 mM phenylmethane sulfonyl fluoride (PMSF) and 5% EtOH at a concentration of 2-5 × 10^7 cells/ml. The suspension was placed in an ice bath and then sonicated for a total of $1\frac{1}{2}$ min with an MSE-Mullard Ultrasonic disintegrator. To completely break zygotes which were older than 4 h and had formed a pellicle, these cells were sonicated for a total of $3\frac{1}{2}$ min.

5 μl of a 5 mg/ml solution of ribonuclease I (Miles Laboratories, Elkhart, Ind.) in 1 mM HCl and 5 μl of a 1 mg/ml solution of deoxyribonuclease I (Worthington Biochemical Corp., Freehold N. J.) in 1 mM HCl were added to the sonicate. After 15 min the sonicate was centrifuged for 2 h at 40,000 rpm in a Beckman T40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) in a Beckman model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div.). The resulting pellet constitutes the pellet fraction, and the supernate constitutes the supernatant fraction. Each fraction was frozen if not prepared immediately for electrophoresis.

Before electrophoresis, each supernatant fraction was dialyzed against three changes of 50 mM NH_4HCO_3 at 4°C. After dialysis, the samples were lyophilized and then taken up in sample buffer (0.05 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate [SDS], 1% mercaptoethanol, 10% glycerol, 0.001% bromphenol blue) to a concentration of 1-2 μg/μl. The solubilized samples were boiled for 3 min.

Before electrophoresis, each pellet fraction was solubilized in sample buffer, boiled for 3 min, and then centrifuged for 10 min at 7,500 rpm in a Sorvall SS-34 rotor (Sorvall, Ivan, Inc.) to rid the fraction of starch and unsolubilized cell walls.
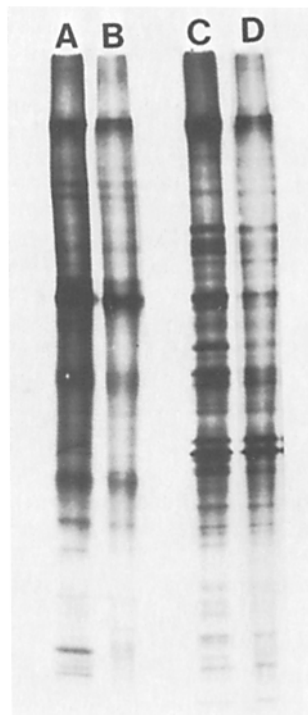
FIGURE 1 Autoradiograph comparing zygote polypeptides labeled with [³H]arginine and [¹⁴C]acetate. Synchronous 2¹/₂-h-old zygotes were labeled with either [¹⁴C]acetate (58 mCi/mmol) or [³H]arginine (11 Ci/mmol) at 20 µCi/ml for 15 min. The labeled cells were fractionated and the polypeptides were electrophoresed as described in Materials and Methods. The polypeptides were visualized by the autofluorography method of Bonner and Laskey (3), using Kodak RP Royal X-Omat film that had been preflashed (19). (A) and (B), pellet-fraction polypeptides labeled with [¹⁴C]acetate (A) and [³H]arginine (B). (C) and (D), supernatant-fraction polypeptides labeled with [¹⁴C]acetate (C) and [³H]arginine (D).

Protein Assay and Radioactivity Counting

The solubilized samples were assayed for protein by a modification (13) of the Lowry assay (21) using bovine serum albumin as a standard.

Radioactivity of the protein was determined by dissolving 5-µl aliquots in Aquasol (New England Nuclear, Boston, Mass.) and counting in a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). For autoradiography, samples were applied to gels to give equivalent counts in each slot. Identical autoradiograph patterns were in all cases obtained when samples were applied on the basis of protein concentration (data not shown), indicating that differences in electrophoretic mobilities for various polypeptides were not artifacts due to differential loading of gels.

Gel Electrophoresis

Gels were slabs of 0.75 mm × 16 cm × 22.8 cm dimensions; they were run at a constant current of 20 mA. The discontinuous gel system of Laemmli (18) was used with the modification that the running gel was a linear gradient of polyacrylamide from 7 to 15% or, in some cases, a linear gradient from 4 to 8%. The stacking gel was 5% polyacrylamide, or 3% in the second gel system. The gel apparatus was a modification of the vertical flat sheet apparatus of Reid and Bieleski (25). The following proteins were used as molecular weight standards: myosin (200,000), β-galactosidase (135,000), phosphorylase a (94,000), bovine serum albumin (68,000), γ-globulin large subunit (50,000), carbonic anhydrase (29,000), ribonuclease (13,500), myoglobin (17,800), and cytochrome c (12,400).

Staining Procedures

Gels were stained with Coomassie Blue and periodic acid-Schiff reagent as described by Fairbanks et al. (11).

Drying Gels and Autoradiography

Gels were dried onto Whatman 3 MM filter paper under vacuum over a steam bath. Kodak No-Screen x-ray film was exposed to the dried gel for autoradiography. Films were developed with Kodak KLX Developer and Rapid Fix.

RESULTS

General Features of Early Zygote Development

The diploid zygote of C. reinhardtii forms when gametes of opposite mating type are allowed to fuse. Fusion is complete within 5-10 min after gametes are mixed, and we routinely use strains that mate with near 100% efficiency. Therefore, a highly synchronized population of differentiating zygotes is readily obtained in the laboratory. The differentiation process can be subdivided into four stages, all of which occur normally in a nitrogen-containing medium and appear to proceed independently of the nutritional state of the environment. The motile quadriflagellated cell (QFC) stage (3 h) is followed by flagellar withdrawal and a secretory stage (~20 h), during which the thick zygote wall is produced; the ensuing maturation stage, during which zygotes prepare for meiosis, typically takes at least 2 days in the laboratory, and the final germination stage, during which meiosis and zygote-wall lysis occur, requires about 12 h. In this study, we have analyzed only the QFC and secretory stages of differentiation, which we denote as early zygote development.

Fine Structure of Developing Zygotes

Figs. 2–10 present electron micrographs of early zygotes which illustrate features relevant to our developmental analysis. Observations that parallel published reports (6, 7) are not presented, nor are observations relevant to the fate of the flagellar apparatus.

NUCLEAR FUSION: Fig. 2 shows a QFC fixed 45 min after mating was initiated, and the two nuclei are seen to have undergone a limited fusion. Such early nuclear fusion images are rare; in most cell sections from this sample, nuclei lie side by side but are separated by either a finger of chloroplast or an isthmus of cytoplasm. It appears, therefore, that nuclear fusion probably begins at about this time but that the initial sites of nucleoplasmic continuity are small in size and number. When a portion of the same QFC population is fixed 65 min after mating, fused nuclei are more often encountered, and by 95 min most of the nuclei appear to have fused to at least a limited extent. The channel connecting the two nuclei proceeds to widen so that, by 3 h, virtually every cell appears to contain a single nucleus at the cell anterior (Fig. 3).

ZYGOTE WALL DEPOSITION: Gametes break down their cell walls during the mating reaction (9) so that QFCs are totally devoid of cell walls (Fig. 2). The zygotes remain wallless until about 150 min after mating, at which time a few thin strands of wall material are found associated with the cell surface, particularly in regions where cells lie close together. By 180 min after mating, a distinct wall surrounds the entire cell (Fig. 3, arrow).

The zygote wall bears no structural resemblance to vegetative or gametic cell walls. A vegetative cell initiates wall synthesis with an amorphous mesh (16) which builds up to become an ordered, seven-layered structure (26). A zygote, in contrast, accumulates discrete fibers around its cell surface until a very thick wall is formed (Fig. 4). Moreover, adjacent cells contribute jointly to wall synthesis with the result that they stick to one another (Fig. 4). Cell-to-cell adhesions are first apparent by 150 min after mating; by 180 min, the cells are joined together as a fully adherent sheet which we denote a zygote pellicle.

Several types of fibers appear to be present in a mature zygote wall (Fig. 4): thick fibers (TF) lie next to the cell surface; a dense layer (DL) forms a continuous layer around each cell; and a meshwork of thin filaments (tf) fills the interstices between cells. Whether these represent different fiber types or different states of aggregation of a single type is not known.
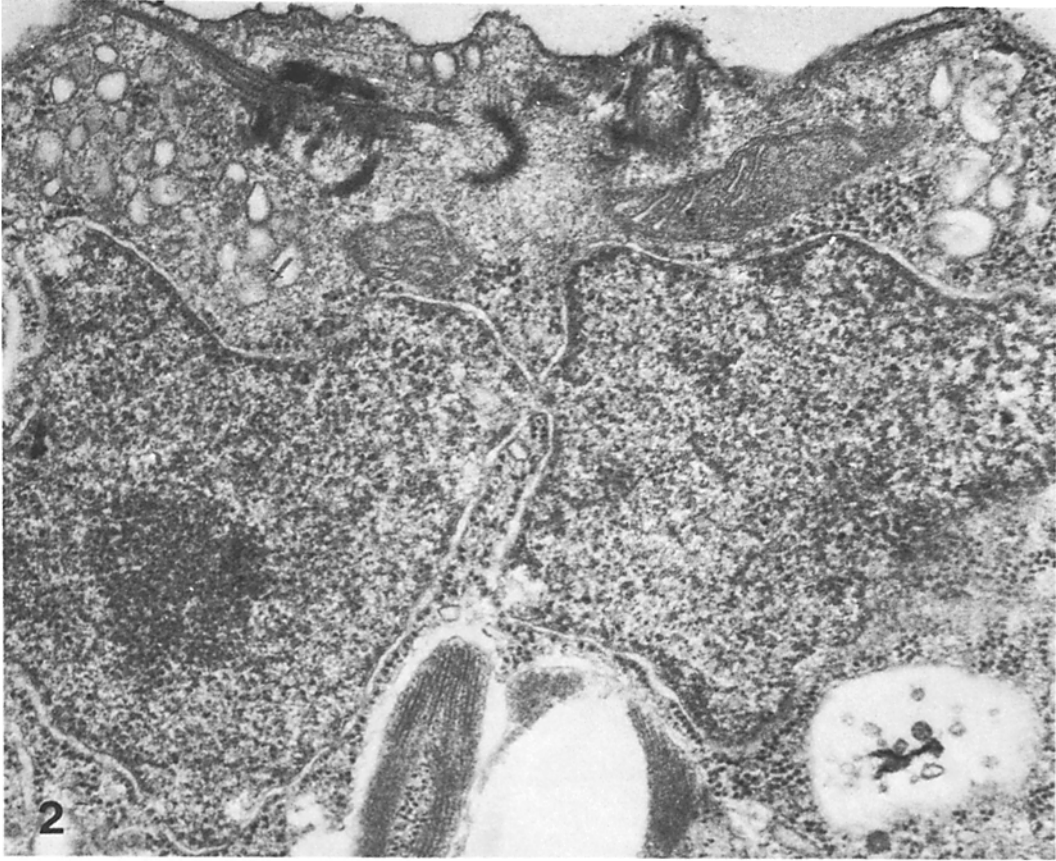
SECRETION OF ZYGOTE WALL FIBERS: The mode of synthesis and secretion of zygote cell-wall fibers is readily visualized in thin sections. Up to 125 min after mating, no fibers are present in vesicles associated with Golgi apparatus (Fig. 5), and indeed the Golgi regions are indistinguishable in morphology from those found in vegetative or gametic cells (16, 22). By 150 min after mating, however, fibrous material can be found in Golgi-associated vesicles of many cells (Fig. 6, arrowhead), and by 180 min after mating, fiber-containing vesicles are invariably found distal to Golgi regions (Figs. 3 and 7). Cell wall-fiber formation is thus initiated in a highly synchronous fashion in a differentiating zygotic culture.

That the fiber-filled vesicles originate from the Golgi apparatus is illustrated in Figs. 7 and 8, where fibrous material can be visualized in the distended ends of the more distal saccules (arrows). Fig. 9 illustrates a fiber-filled vesicle (arrow) that has moved next to the cell surface, apparently poised for secretion.

Once fiber formation in QFCs has begun, the production and secretion of the zygotic wall becomes a prominant cellular activity during the ensuing 20 h of zygote maturation. Rough endoplasmic reticulum proliferates from the nuclear

---

FIGURE 2   Zygote fixed 45 min after mixing mt^+ and mt^- gametes. A narrow interconnection has formed between the two nuclei. At the anterior end of the cell are the two basal apparatuses flanking the central, crescent-shaped mating structure which functioned during zygotic cell fusion (12, 15, 30). No cell wall material is present external to the cell. × 47,000.

FIGURE 3   Zygote fixed 180 min after mixing mt^+ and mt^- gametes. Extensive continuity now exists between the fusing nuclei. Arrowheads point to fiber-filled vesicles at the distal face of the Golgi apparatus; arrows point to similar fibers within the newly secreted zygotic cell wall. × 47,000.

2

3

envelope, and Golgi regions become larger and more numerous (Fig. 10). Indeed, zygotes during the wall-synthesis phase take on the fine structure of classical secretory cells.

Isolation of Zygote Wall Fibers

When a suspension of differentiating QFCs is washed to remove gametic cell-wall fragments and is then agitated on a rotary shaker, cell-to-cell aggregation is suppressed. If the cells are spun down after several hours and the medium is subjected to high-speed centrifugation, a white, extremely gelatinous pellet is obtained which, when examined by electron microscopy, proves to contain abundant zygote cell-wall fibers (Fig. 11). Shown for comparison in Fig. 12 is a pellet of gametic cell walls, which are seen to be very different in morphology.

Polypeptides Synthesized During the Course of Early Zygote Development

To analyze the overall patterns of polypeptide synthesis during the 24 h after zygotic cell fusion, cell samples were taken at various times from a differentiating culture and exposed to [^{14}C]acetate for 3 h. The samples were then washed, sonicated, and separated by centrifugation into a pellet fraction and a supernatant fraction, the latter containing cell material that fails to pellet after 2 h at 105,000 g. The polypeptides in each fraction were separated by electrophoresis, using SDS-polyacrylamide slab gels containing linear gradients of acrylamide from 7 to 15%; the radioactive polypeptides were visualized by autoradiography.

Fig. 13 shows a representative gel of the supernatant fraction. The polypeptides denoted by arrowheads on the right side of the figure show an increased synthesis during zygote development but have possible counterparts in the gamete sample and cannot therefore be designated as zygote-specific; the polypeptides labeled by arrowheads on the left side of the figure represent vegetative/gametic polypeptides whose synthesis is turned off as zygote development proceeds. The five polypeptides labeled by their estimated molecular weights[1] appear during zygote develop-
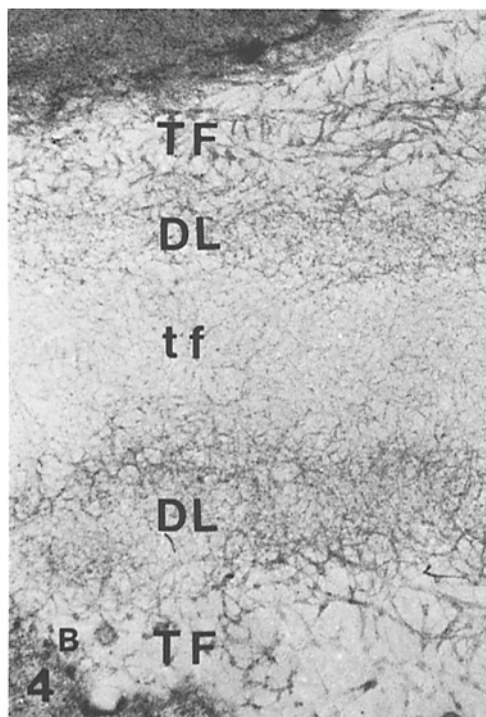

FIGURE 4 Mature pellicle produced by zygotes fixed 19 h after mating. A zone of thick fibers (*TF*) lies adjacent to each cell surface labeled *A* and *B*. This is overlain by a dense layer of fibers (*DL*), and a meshwork of very thin fibers (*tf*) fills the interstices to form a continuous matrix between all the zygotes in the pellicle. × 80,000.

ment and have no detectable counterparts in vegetative or gametic cells. Treatment of such gels with the periodic acid-Schiff (PAS) reagent, which stains carbohydrate, reveals that the 120K and 150K polypeptides are PAS-positive (not shown).

Parallel autoradiographs of pellet-fraction polypeptides (Fig. 14) reveal three major zygote-specific polypeptides, one of which has an apparent molecular weight of 120K. An intensely staining, narrow band is also seen in the >200K region of the gel. This narrow band is seen in Fig. 15 to be PAS-positive and to be absent from the gamete (G) sample, the glycopolypeptide bands in the gamete sample representing the major flagellar membrane protein (2, 32) and components of the gametic cell wall (24).

We should note here that the 7–15% gradient slab gels necessary to resolve the numerous pellet and supernatant polypeptides are not suitable for resolution of polypeptides in the >200K region; in a later section, we demonstrate, with 4–8%
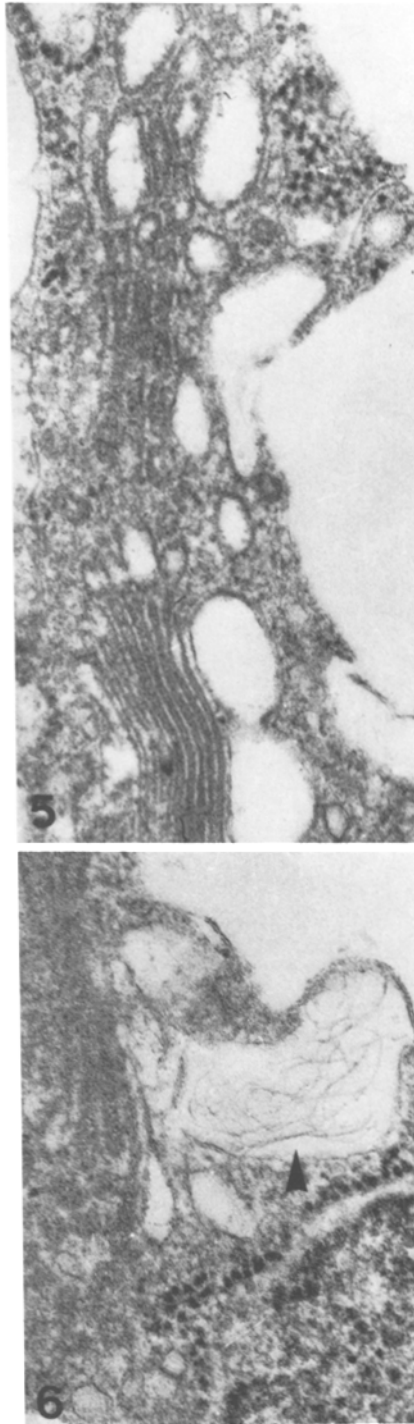
---

[1] For all slowly migrating glycopolypeptides described in this study, molecular weight assignments are estimates made for identification purposes; the anomalous migration of glycopolypeptides in SDS-polyacrylamide gels (4, 28) disallows accurate molecular weight estimates by this procedure.

FIGURE 5 Zygote fixed 120 min after mating. All Golgi-associated vesicles appear empty. × 66,000.

FIGURE 6 Zygote fixed 150 min after mating. Fibrous material (arrowhead) is present in a vesicle at the distal face of the Golgi apparatus. × 66,000.

gradient gels, that the narrow band evident in Figs. 14 and 15 can in fact be resolved into at least three bands. For present purposes, the distinctive appearance of this band in the 7–15% gels serves to identify it within the diffuse labeled material that often occupies this region.

Returning to Figs. 13 and 14, it is evident that most of the major turn-on and turn-off events in zygote polypeptide synthesis occur during the first three hours of differentiation, that is, during the QFC stage; the zygote-specific pattern established during this period is then simply amplified during the remaining hours of early zygote development. QFCs also prove to be more amenable to experimental analysis than mature cells, as it becomes increasingly difficult to break cells open, and subsequently to fractionate them into clean pellet and supernatant fractions, once the zygote wall has started to form. We therefore focused on the polypeptides synthesized by QFCs; we also shortened the duration of the [^{14}C]acetate pulse to 15 min in order to resolve more clearly the patterns of polypeptide synthesis during this stage.

Polypeptides Synthesized During the QFC Stage

Figs. 16 and 17 show, respectively, the supernatant and pellet fractions from cells subjected to brief [^{14}C]acetate pulses during the first 3½ of zygote development. When pulsed unmated gametes (sample G) are compared with cells labeled during the first 15 min after cells are mixed (sample 0), only one polypeptide (Fig. 16, arrowhead) is detectably different in its labeling. Major changes first appear in sample 30, which reveals polypeptides synthesized during a pulse from 30 to 45 min after the gametes are mixed. A new polypeptide with a molecular weight of 76K daltons is seen to appear in both the pellet and supernatant fractions, while one with a molecular weight of 120K daltons appears in the supernatant fraction alone. The supernatant fraction also shows significant quantitative shifts in band density; in sample 30, for example, bands in the bracketed region of the gel become very intense, and bands below this region become less intense. Because zygotic cell fusion is completed 5–15 min after gametes are mixed, the zygote-specific pattern of polypeptide synthesis can be said to initiate somewhere between 15 and 30 min after gametic fusion.

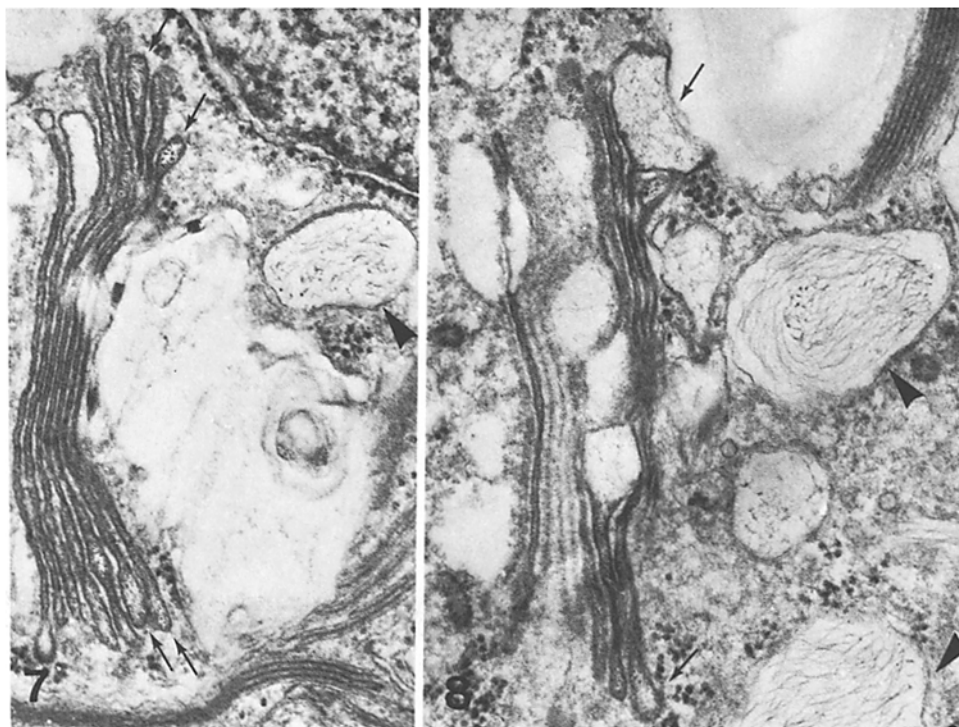Approximately 60 min after mating occurs, another novel polypeptide, with a molecular

Figure 7   Zygote fixed 10 h after mating. Fibers in distal Golgi sacs are indicated by arrows; fiber-filled vesicle is indicated by arrowhead. × 56,000.

Figure 8   Zygote fixed 13 h after mating. Distended Golgi sacs (arrows) and mature fiber-filled vesicles (arrowheads). Material in Golgi sacs appears fluffier than that in the vesicles, suggesting a maturation of fiber morphology, perhaps due to glycosylation, during passage through the Golgi cisternae. × 56,000.

weight of 150K daltons, is observed as a minor constituent of the supernatant fraction (sample 60 of Fig. 16). A polypeptide with similar molecular weight is evident in the pellet fraction 180 min after mating (Fig. 17). Inasmuch as the zygote wall, which forms by 180 min after mating (Fig. 3), is difficult to break by sonication, the 150K polypeptide may appear in the 180-min pellet because it becomes trapped in unbroken cells. Alternatively, the polypeptide may become associated with a pelletable structure at 180 min.

Finally, in the >200K region of the pellet-fraction gel (Fig. 17), a band with the narrow, intense appearance is prominent in samples 180 and 210 and detectable in sample 150. Poor resolution disallows a clear assessment of the polypeptides in this region of the supernatant-fraction gel (Fig. 16).

Polypeptides Secreted by QFCs

Inasmuch as a conspicuous activity of early zygotes is the elaboration of the zygotic cell wall (Figs. 3-10), and because cell-wall fibers are both synthesized and secreted by 3 h after cell fusion (Fig. 3), we next asked whether any of the novel polypeptides synthesized by QFCs are secreted into their medium. Synchronously formed zygotes were pulse-labeled as before, and the polypeptides in the lyophilized cell-free medium were examined.

Fig. 18 shows an autoradiograph of one of the resultant gels. The gametic medium (sample G) contains only a faint, >200K dalton band which is most likely the major flagellar membrane polypeptide (2, 32). Cells pulsed during mating reaction (sample 0) liberate a number of high molecular weight polypeptides into the medium, most of which can be assumed to derive from shed cell walls (15). Three novel polypeptides (150K, 120K, and 76K daltons) are first detectable in the medium in sample 30 and increase in amount for the next 1½ h of differentiation. They then
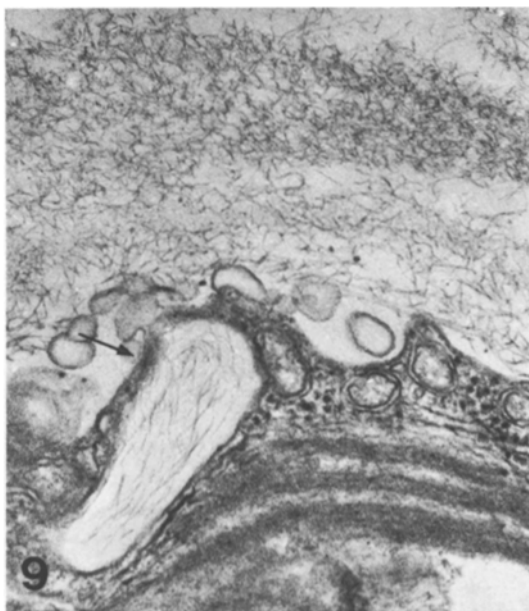
FIGURE 9 Zygote fixed 24 h after mating. Vesicle containing fibrous material is seen at cell surface, presumably poised for secretion. × 56,000.

decrease in abundance and are barely detectable in sample 210.

Resolution in the >200K region of Fig. 18 is poor, as noted earlier, but a very narrow band is evident in sample 60, becomes relatively prominent in samples 90 and 120, and decreases in intensity thereafter. To better resolve the polypeptides in this sector of the gel, pulse-labeled samples of cell-free medium were subjected to electrophoresis in 4–8% gradient slab gels. Fig. 19 shows an autoradiograph of one of the resultant gels. The cell-free medium is seen to contain three extremely slow migrating components, labeled A, B, and C; these are first distinguishable in the 60-min pulse, become prominent in samples 90–150, and abruptly abate in later samples. Levels of the 120K polypeptide are seen in Fig. 19 to parallel exactly those of the high molecular weight components.

The gels shown in Figs. 18 and 19, we should note, display samples from independent experiments. Zygote-specific secretion is seen to initiate in sample 30 and abate by sample 180 in the first experiment; in the second experiment, it initiates in sample 60 and abates in sample 210. In other words, the process consistently takes about 150 min, with the onset varying slightly from one experiment to the next. Such reproducibility attests to the high level of synchrony that characterizes this differentiation process.

Polypeptides Present in the Zygote Cell Wall

The zygote wall, which is designed to seal off the cell from its environment, has thus far proved resistant to every solubilization procedure that we and others have applied to it. By agitating differentiating zygotes and harvesting the medium, we were able to pellet a preparation of zygotic fibers (Fig. 11) which we attempted to solubilize by boiling for extended periods in SDS-mercaptoethanol solutions. Solubilization was clearly incomplete because a gummy residue remained, but, when the SDS-mercaptoethanol solution was applied to an SDS gel, a narrow, intensely PAS-positive band was prominent in the >200 K region.

DISCUSSION

Overview of Early Zygote Differentiation

This paper presents two independent analyses of early zygote development. By electron microscopy, limited nuclear fusion is shown to precede the onset of a major structural differentiation: rough ER proliferates, abundant fibers appear in Golgi-associated vesicles, and the fibers are secreted to form a thick extracellular wall. By gel electrophoresis, a major shift in patterns of polypeptide synthesis is shown to begin about the same time that nuclear fusion begins; novel polypeptides are shown to be secreted in soluble form for about 1½ h and then, about the time that wall deposition is visualized structurally, the secreted polypeptides can no longer be recovered in soluble form. Until some means is found to solubilize the zygote wall, or to prevent its solidification, the electron micrographs best serve to document the abundance of the zygotic gene products. It seems probable that this major synthetic activity of zygotes is accompanied by an association between rough ER and a similarly abundant zygote-specific class of mRNA. Experiments designed to isolate and characterize this mRNA in vitro appear imminently feasible.

Zygote Cell-Wall Fibers

Two observations suggest that the insoluble cell-wall fibers that form in the Golgi apparatus and
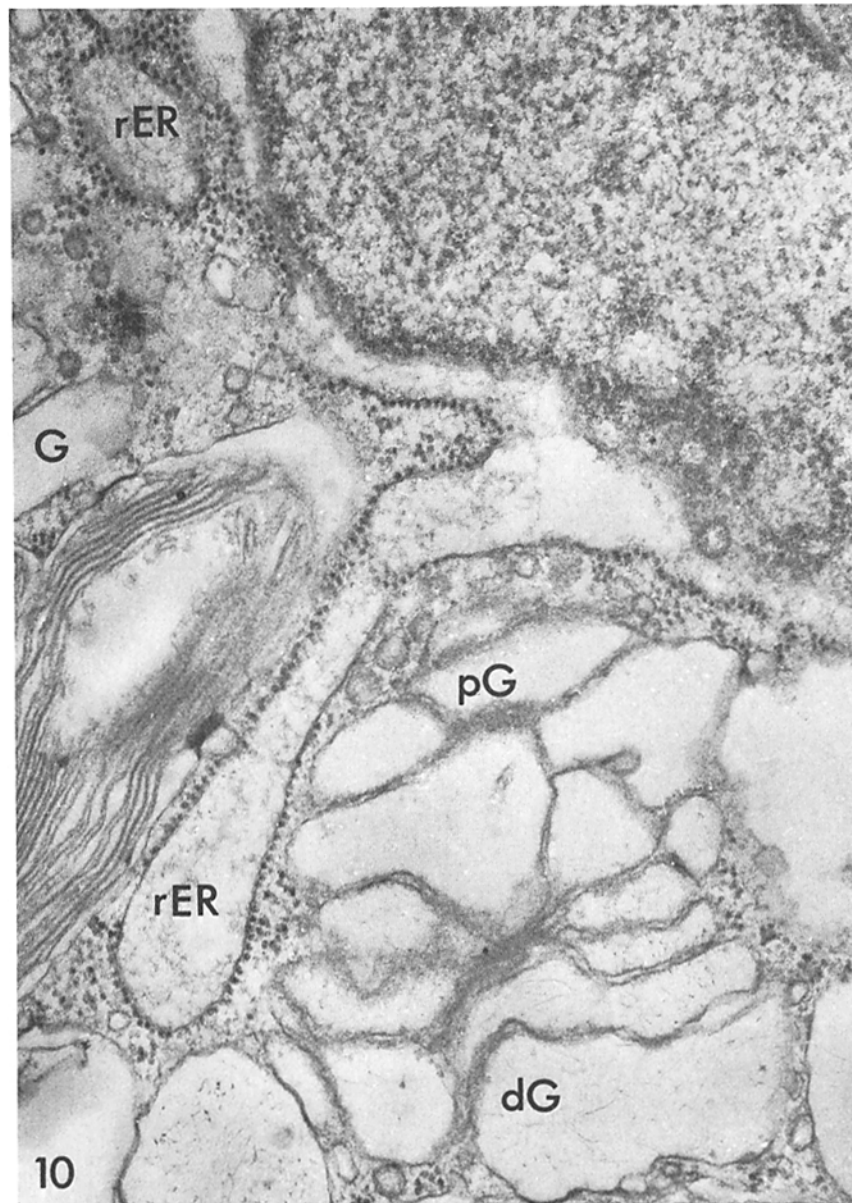
FIGURE 10 Zygote fixed 6.5 h after mating. Rough endoplasmic reticulum (*rER*) is dilated and filled with a flocculent material not evident in gametic or vegetative preparations. Golgi cisternae (*G*) are cut obliquely, showing the clear vesicles at the proximal face (*pG*) and the fiber-filled vesicles at the distal face (*dG*). × 56,000.

become part of the pellicle are composed, at least in part, of glycopolypeptides that form a narrow, slowly migrating band in 7–15% gradient SDS gels (Fig. 15). First, a band of this sort appears in the pellet fraction of broken zygotic cells (Fig. 17) at the same time (150–165 min after mating) that

fibers are first evident in Golgi vesicles (Fig. 6). Second, when secreted fibers are pelleted (Fig. 11) and exhaustively extracted, the solubilized material is found to form such a band when subjected to gel electrophoresis.

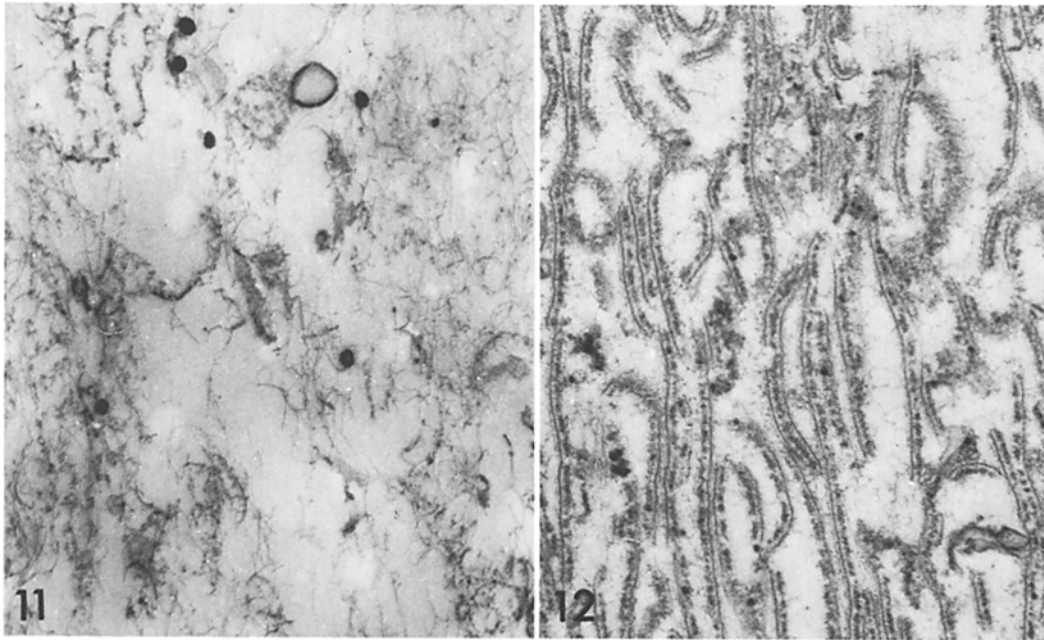A narrow band of this sort is likely to result if

FIGURE 11 Pelleted zygote cell wall fibers. × 48,000.

FIGURE 12 Pelleted gametic cell walls. × 48,000.

close associations form between (glyco)polypep-tides in a stacking gel and if these fail to "unstack" during the course of electrophoresis in the running gel (see also reference 14). By using 4-8% poly-acrylamide gels, we were able to resolve the narrow band into three components (Fig. 19). Because it is possible that still more bands may be resolved in this region if other gel systems are employed, we conclude that the zygotic cell-wall fibers are composed of at least three extremely slow migrating glycopolypeptide species that have no apparent counterparts in vegetative or gametic samples (Fig. 15, sample G).

In addition to the major morphological (Figs. 11 and 12) and electrophoretic differences be-tween the vegetative/gametic and zygotic cell-wall material reported here, the studies of Davies and Plaskitt (10) have shown that mutations affecting the production of vegetative/gametic polypeptides are without effect on the production of a zygote wall. Preliminary amino acid-composition studies, moreover, show that the pellicle material is rich in serine, threonine, and aspartic acid, whereas the vegetative/gametic cell wall is rich in hydroxypro-line (23). It thus seems probable that the zygotic wall contains novel gene products whose transla-tion is stimulated shortly after cell fusion. Whether control over this translation is exerted at the transcriptional or posttranscriptional level is not yet known.

Secreted Zygotic Glycopolypeptides

The cell-free medium of an unagitated zygotic culture comes to contain six major zygote-specific glycopolypeptides: the three species that normally migrate as a single intense band, plus the three glycopolypeptides with apparent molecular weights of 150K, 120K, and 76K. Novel glycopolypep-tides with equivalent apparent molecular weights are found in the pellet and supernatant fractions of broken zygotic cells (Figs. 13–17). Although it has not yet been proven, we shall assume for purposes of discussion that the intracellular and secreted polypeptides are equivalent molecular species, that is, that all six glycopolypeptides are first synthesized intracellularly and then secreted.

The relationship between synthesis and secre-tion is best studied with the 76K, 120K, and 150K species because of their satisfactory resolution in broken cell preparations. The 76K and 120K species, which are the most abundant, first appear intracellularly in cells pulsed 30–45 min after mating; the less abundant 150K species is not detectable until the 60–75-min pulse. All three
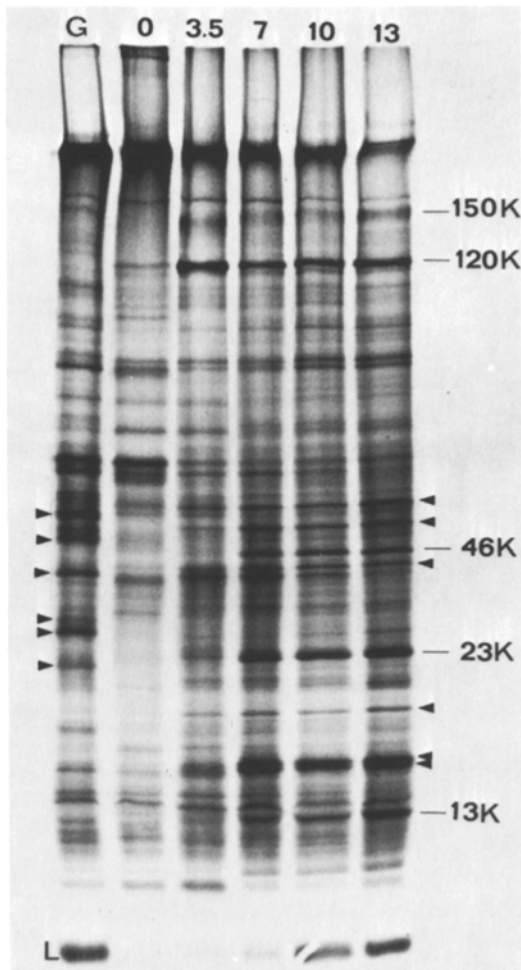
FIGURE 13 Supernatant-fraction polypeptides synthesized during zygote development. Synchronously grown plus gametes were mixed with an equal number of minus gametes to give a final cell concentration of 1.6 × 10⁷ gametes/ml. At 0, 3½, 7, and 10 h after the gametes were mixed, 5-ml aliquots of the cell culture were labeled with [¹⁴C]acetate (58 mCi/mmol) at 8 μCi/ml for 3 h. An aliquot was also labeled from hour 13 to hour 23. Unmated gametes (G) of minus mating type were labeled for 3 h as a control. The polypeptides were subjected to electrophoresis and autoradiography as described in Materials and Methods. The arrowheads on the right indicate bands that increase in density during zygote development; arrowheads on the left point out gametic bands that decrease in density during zygote development; the bands labeled with their molecular weights represent apparent zygotic-specific polypeptides. L indicates radioactive lipid. The numbers above each sample indicate the time in hours after the gametes were mixed at which the cells were labeled for the indicated periods.
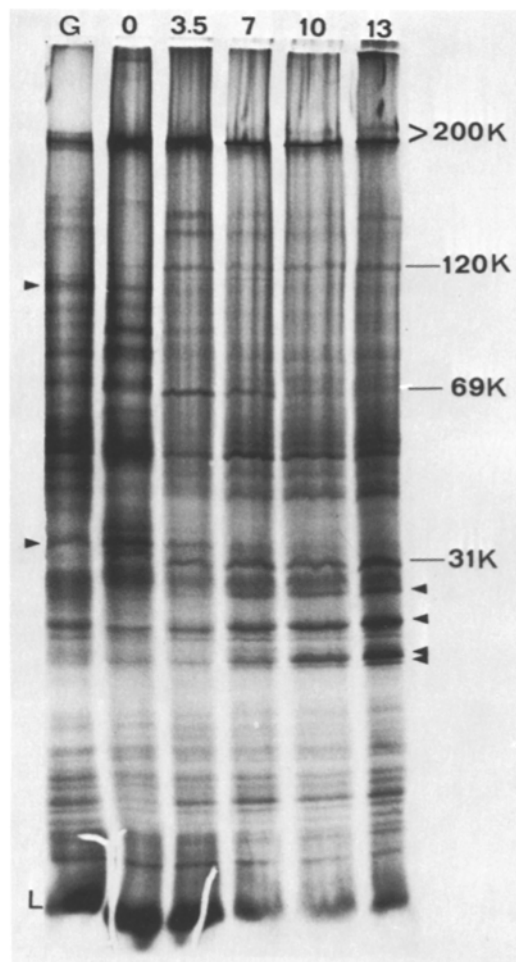
FIGURE 14 Autoradiograph of pellet-fraction polypeptides synthesized during zygote development. Zygotes in various stages of development and minus gametes (G) were labeled for 3 h with [¹⁴C]acetate as indicated for Fig. 13. The pellet-fraction polypeptides were electrophoresed and visualized by autoradiography as described in Materials and Methods. The bands labeled with their molecular weights represent zygote-specific polypeptides. Arrowheads indicate bands which increase and decrease in density during zygote development, as in Fig. 13. L indicates radioactive lipid. The numbers above the figure indicate the time in hours after the gametes were mixed at which the zygotes were labeled.

polypeptides also first appear in the medium during the 30–60-min period, so that they seem to be synthesized and secreted about the same time.

Although the 76K, 120K, and 150K polypeptides continue to be synthesized intracellularly throughout the first 24 h of zygote development,
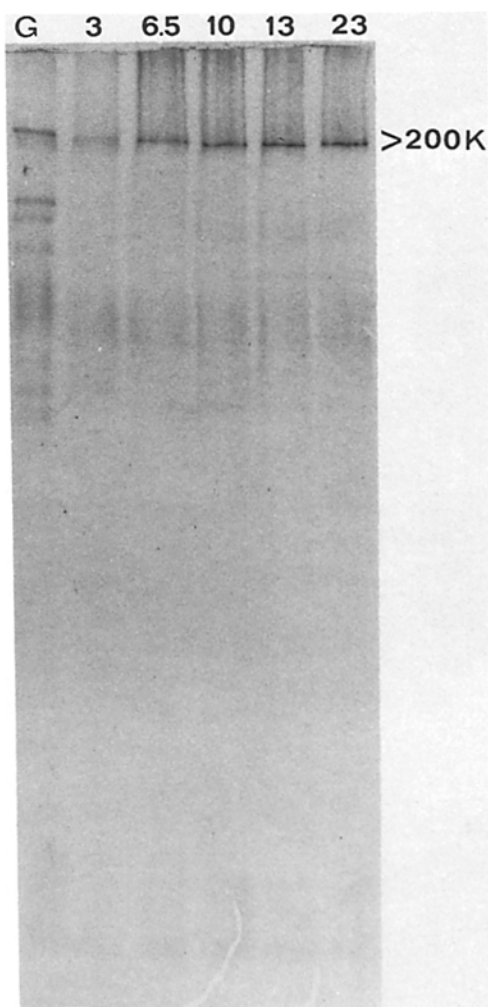
FIGURE 15 Zygote pellet-fraction polypeptides stained with periodic acid-Schiff reagent. Equal numbers of synchronously grown plus and minus gametes were mixed, and at various times in zygote development 5-ml samples were taken. The pellet-fraction polypeptides from the zygotes and unmated minus gametes (G) were subjected to electrophoresis, and the gels were stained with PAS reagent as described in Materials and Methods. Each sample on the gel contained 25 μg of protein. The numbers above the figure indicate the times in hours after the gametes were mixed at which the samples were taken. The 120K protein shown in Fig. 14, although PAS-positive, is not sufficiently abundant in the pellet fraction to be visible in this gel.
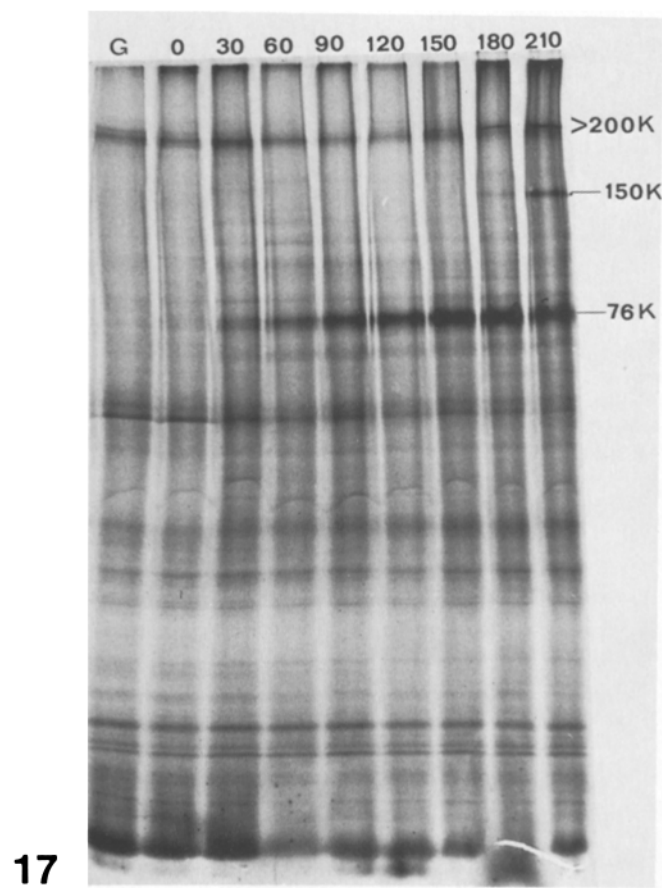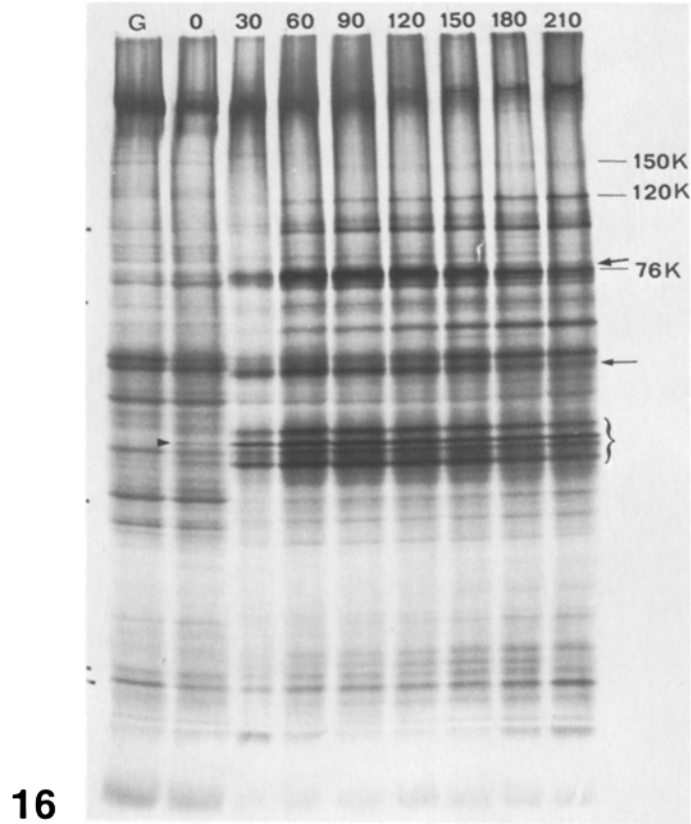
they decrease in abundance in the medium at 150 min and are undetectable in the medium by 3 h. Because the onset of this abatement coincides with the onset of zygote cell-wall formation, our

observations suggest the following sequence of events. (a) Pellicle-specific polypeptides are synthesized and secreted early in development but the initial products do not associate into an insoluble cell wall, perhaps because sufficient amounts of cell-wall material must first be present, or perhaps because a catalytic participant in cell-wall formation (e.g. a cross-linking reagent) is not elaborated until about 150 min after fusion. (b) Once cell-wall production is initiated, the secreted polypeptides associate with the forming wall and no longer appear in the cell-free medium. They can, however, be isolated from the medium of mature zygotes if the differentiating culture is agitated so that cell-wall deposition is disturbed.

Almost nothing is known about the role of 76K, 120K, and 150K glycopolypeptides in zygote wall formation, but it seems reasonable to propose that they either participate in fiber formation or else combine to serve as a matrix in which the fibrous components of the wall are embedded. In either case, because they are not extracted from the zygote-wall pellet by SDS-mercaptoethanol, they appear to be modified chemically in some fashion during wall deposition to account for their extreme insolubility.

Zygote Maturation as a Developmental System

Early zygote maturation in C. reinhardtii is shown in this study to be an excellent system for analyzing the turn off and turn on of polypeptide synthesis in a eukaryote. (a) The process occurs with a high degree of synchrony in genetically identical cells. (b) The process is triggered by a defined and readily manipulated stimulus, namely, gametic cell fusion, and proceeds under nonstarvation conditions. (c) Novel polypeptides, not detectable at any other stage in the organism's life cycle, begin to be synthesized within 25–40 min of the stimulus. (d) The novel polypeptides are not simply "bands on a gel" but can be ascribed a function, namely, to serve as components of the zygote cell wall. (e) Because the novel glycopolypeptides are secreted, they can be isolated and presumably purified from the QFC medium. (f) A rapid turn off of gametic patterns of polypeptide synthesis is coincidental with the turn on of zygotic patterns (Figs. 14 and 15), suggesting the existence of a major shift in the translational and, perhaps, transcriptional program of zygotic cells, a shift that should be ame-
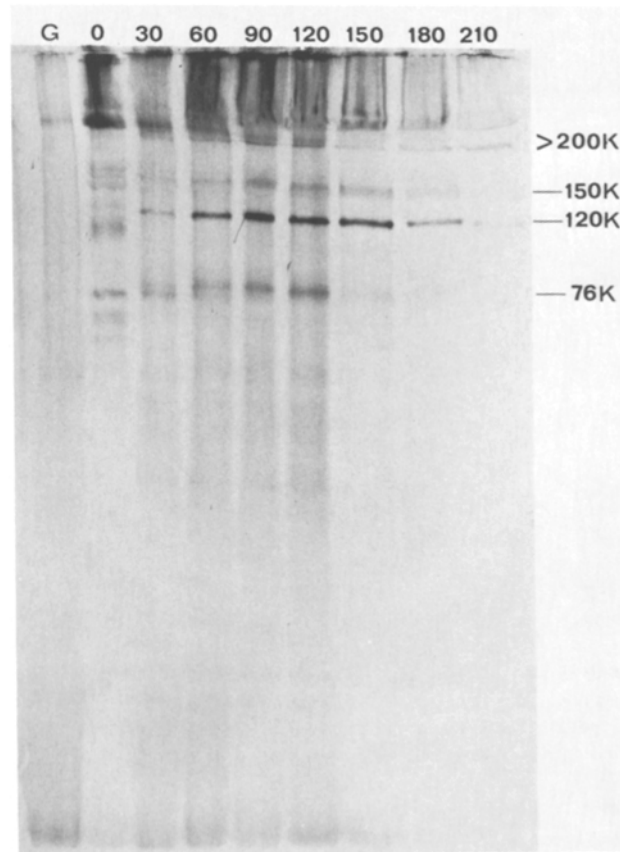
16

G 0 30 60 90 120 150 180 210

— 150K
— 120K
← 76K
←
}

17

G 0 30 60 90 120 150 180 210

>200K
— 150K
— 76K

FIGURE 18 Autoradiograph of polypeptides present in the cell-free medium of gamete and zygote cultures. 5-ml aliquots of gametes (G) and zygotes in early development were pulse-labeled for 15 min as described in Fig. 16. At the end of the labeling period, the cells were pelleted by centrifugation for 3 min at 3,000 rpm in a Sorvall SS-34 rotor (Sorvall, Ivan, Inc.). The resulting supernate was centrifuged for 5 min at 7,500 rpm. The supernate from the second centrifugation was mixed with 0.25 ml of 6 mg/ml PMSF in 95% EtOH and then dialyzed against two changes of 50 mM NH_4HCO_3. The dialyzed medium was lyophilized and then solubilized in Laemmli sample buffer at a concentration of about 1 mg/ml. The samples were then subjected to electrophoresis. The numbers above the figure indicate the time in minutes after the gametes were mixed at which the samples were labeled.

FIGURE 16 Autoradiograph of supernatant-fraction polypeptides synthesized during the QFC stage. Equal numbers of plus and minus gametes were mixed. Samples of the resulting zygote culture were taken at various times in the first 3½ h of zygote development and labeled with [14C]acetate at 16 μCi/ml for 15 min. Minus gametes (G) were labeled as a control. The labeled cells were pelleted by centrifugation, fractionated, and the supernatant-fraction polypeptides were subjected to electrophoresis as described in Materials and Methods. The numbers above the figure indicate the time in minutes after the gametes were mixed at which the samples were labeled. Bracketed polypeptides include those whose synthesis begins abruptly at 30 min. Arrowhead points to a band that appears during the first 15 min (Sample 0). Arrows point to two regions containing bands whose synthesis peaks at 120 min and then abates.

FIGURE 17 Autoradiograph of pellet-fraction polypeptides synthesized during early zygote development. Minus gametes (G) and zygotes in various stages of early development were labeled with [14C]acetate as described in Fig. 16. The pellet-fraction polypeptides of the labeled cells were subjected to electrophoresis as described in Materials and Methods. The numbers above the figure indicate the time in minutes after the gametes were mixed at which the samples were labeled.
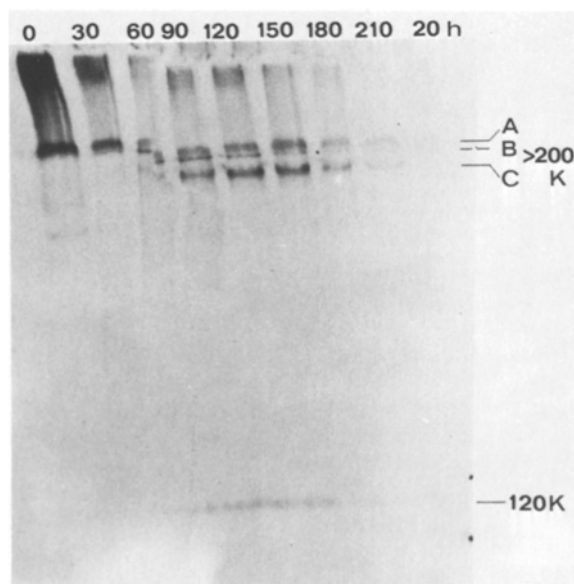
FIGURE 19   Gametes and zygotes were pulse-labeled with 40 μCi/ml of [³H]acetate (250 mCi/mmol) for 15 min. The polypeptides in the cell-free medium were prepared for electrophoresis as described in Fig. 18, and were then subjected to electrophoresis in a gel containing a 4–8% gradient of polyacrylamide. The labeled polypeptides were visualized by autofluorography (3, 19). The numbers above the figure indicate the time in minutes after the gametes were mixed at which the samples were labeled; the last sample was taken 20 h after mixing.

nable to genetic and molecular analysis. (g) Nuclear contact and limited nuclear fusion are shown to occur coincidentally with the onset of the zygote-specific program, namely, about 45 min after cell fusion. There is, therefore, at least a morphological basis for assuming early communication between plus and minus nuclei in the newly formed zygote, and for postulating that nuclear fusion, and not simply dikaryosis, triggers the ensuing developmental stages.

REFERENCES

1. BECK, D. P., and R. P. LEVINE. 1974. Synthesis of chloroplast membrane polypeptides during synchronous growth of Chlamydomonas reinhardtii. J. Cell Biol. 63:759-772.
2. BERGMAN, K., U. W. GOODENOUGH, D. A. GOODENOUGH, J. JAWITZ, and H. MARTIN. 1975. Gametic differentiation in Chlamydomonas reinhardtii. II. Flagellar membranes and the agglutination reaction. J. Cell Biol. 67:606-622.
3. BONNER, W. M., and R. A. LASKEY. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.
4. BRETSCHER, M. 1971. Major human erythrocyte glycoprotein spans the cell membrane. Nat. New Biol. 231:229-232.
5. BROWN, J. L., and W. K. ROBERT. 1976. Evidence that approximately eighty percent of the soluble proteins from Erlich ascites cells are N-Acetylated. J. Biol. Chem. 251:1009-1014.
6. CAVALIER-SMITH, T. 1970. Electron microscopic evidence for chloroplast fusion in zygotes of Chlamydomonas reinhardii. Nature (Lond.). 228:333-335.
7. CAVALIER-SMITH, T. 1976. Electron microscopy of zygospore formation in Chlamydomonas reinhardii. Protoplasma. 87:297-315.
8. CHIANG, K. S., J. R. KATES, R. F. JONES, and N. SUEOKA 1970. On the formation of a homogeneous zygotic population in Chlamydomonas reinhardtii. Dev. Biol. 22:655-669.
9. CLAES, H. 1971. Autolysis of the cell wall from gametes of Chalmydomonas reinhardii. Arch. Mikrobiol. 78:180-188.

10. DAVIES, D. R., and A. PLASKITT. 1971. Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardi. Genet. Res.* **17:**33-43.

11. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10:**2606-2617.

12. FRIEDMANN, I., A. L. COLWIN, and L. H. COLWIN. 1968. Fine structural aspects of fertilization in *Chlamydomonas reinhardi. J. Cell Sci.* **3:**115-128.

13. GEIGER, P. J., and S. P. BESSMAN. 1972. Protein determination by Lowry's method in the presence of sulfhydryl reagents. *Anal. Biochem.* **49:**467-473.

14. GLABE, C. G., and V. D. VACQUIER. 1977. Isolation and characterization of the vitelline layer of sea urchin eggs. *J. Cell Biol.* **77:**410-421.

15. GOODENOUGH, U. W., and R. WEISS. 1975. Gametic differentiation in *Chlamydomonas reinhardtii.* III. Cell wall lysis and microfilament-associated mating structure activation in wild-type and mutant strains. *J. Cell Biol.* **67:**623-637.

16. JOHNSON, U., and K. PORTER. 1968. Fine structure of cell division in *Chlamydomonas reinhardi.* Basal bodies and microtubules. *J. Cell Biol.* **38:**403-425.

17. KATES, J. R., and R. F. JONES. 1964. The control of gametic differentiation in liquid cultures of *Chlamydomonas reinhardi. J. Cell. Comp. Physiol.* **63:**157-164.

18. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature (Lond.).* **227:**680-685.

19. LASKEY, R. A., and A. D. MILLS. 1975. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56:**335-341.

20. LEVINE, R. P., and W. T. EBERSOLD. 1960. The genetics and cytology of *Chlamydomonas. Annu. Rev. Microbiol.* **14:**197-216.

21. LOWRY, O. W., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193:**265-275.

22. MARTIN, N. C., and U. W. GOODENOUGH. 1975. Gametic differentiation in *Chlamydomonas rein-*

hardii. I. Production of gametes and their fine structure. *J. Cell Biol.* **67:**587-605.

23. MILLER, D. H., I. S. MELLMAN, D. T. A. LAMPORT, and M. MILLER. 1974. The chemical composition of the cell wall of *Chlamydomonas gymnogama* and the concept of a plant cell wall protein. *J. Cell Biol.* **63:**420-429.

24. MINAMI, S. A. 1976. Protein synthesis during differentiation in *Chlamydomonas reinhardi.* Ph.D. Thesis, Harvard University, Cambridge, Massachusetts.

25. REID, M. S., and R. L. BIELESKI. 1968. A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. *Anal. Biochem.* **22:**374-381.

26. ROBERTS, K., M. GURNEY-SMITH, and G. J. HILLS. 1972. Structure, composition and morphogenesis of the cell wall of *Chlamydomonas reinhardi.* I. Ultrastructure and preliminary chemical analysis. *J. Ultrastruct. Res.* **40:**599-613.

27. SAGER, R., and S. GRANICK. 1954. Nutritional control of sexuality in *Chlamydomonas reinhardi. J. Gen. Physiol.* **37:**729-742.

28. SEGREST, J. P., R. L. JACKSON, E. P. ANDREWS, and V. T. MARCHESI. 1971. Human erythrocyte membrane glycoprotein: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **44:**390-395.

29. SUEOKA, N. 1960. Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. U. S. A.* **46:**83-91.

30. TRIEMER, R. E., and R. M. BROWN. 1975. Fertilization in *Chlamydomonas reinhardi,* with special reference to the structure, development, and fate of the choanoid body. *Protoplasma.* **85:**99-107.

31. VAN BLERKOM, J., and C. MANES. 1977. The molecular biology of preimplantation embryogenesis. *In:* Concepts in Early Mammalian Embryogenesis. M. J. Sherman, editor. The M.I.T. Press, Cambridge, Mass.

32. WITMAN, G. B., K. CARLSON, J. BERLINER, and J. L. ROSENBAUM. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* **54:**507-539.