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RNase sensitivity of an anterior morphogenetic determinant in an insect egg (*Smittia* sp., Chironomidae, Diptera)

(developmental biology/homeotic mutations/ribonucleoprotein particles/microinjection/spatial pattern formation)

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ABSTRACT **In chironomid midges, the development of head and thorax in the embryo requires the function of cytoplasmic determinants localized near the anterior pole of the egg. Experimental inactivation of these determinants causes a dramatic switch in the developmental program of the embryo. Instead of the normal segment pattern, the aberrant pattern "double abdomen" is formed. Head, thorax, and anterior abdominal segments are then replaced by an additional set of posterior abdominal segments joined in mirror image symmetry to the original abdomen. Such double abdomens have been produced, with a maximum yield of 29%, by application of RNase (ribonuclease I, ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) to the anterior pole region of the egg. This was achieved by microinjection or puncturing the eggs during submersion in RNase. Control experiments with inactive RNase S fragments reliably proved that double abdomen formation resulted from RNase activity. Neither application of other enzymes to the anterior pole region nor application of RNase to other egg regions produced double abdomens in significant yields. The effects of RNase concentration and stage of development were determined. The data from these and earlier experiments are compatible with the idea that stored cytoplasmic RNP particles act as anterior determinants. Similarities to genetically caused switches in developmental pathways (homeotic mutations) are discussed.**

The emergence of spatial patterns from apparently homogeneous layers of cells is a most fascinating feature of early embryogenesis. The elucidation of the underlying mechanisms is a major challenge in contemporary developmental biology. In animal eggs, cytoplasmic determinants are apparently prelocalized in a spatial arrangement so as to initiate channeling of the embryonic cells into different developmental pathways (1). For a causal analysis of these processes, test systems allowing manipulation of pattern formation in a specific and controlled manner are most valuable (2). In eggs of chironomid midges, the development of the longitudinal body segment pattern can be switched into aberrant pathways reproducibly and with high yields (3, 4). In the aberrant pattern "double abdomen," the head, thorax, and anterior abdominal segments are replaced by an additional set of posterior abdominal segments joined with reverted polarity to the original abdomen (Fig. 1a). The double abdomens appear symmetrical in their external and internal structures except that germ cells are only found in the posterior abdomen (5).

In earlier experiments with a terrestrial chironomid of the genus *Smittia*, double abdomens were produced by UV irradiation of the anterior pole region. The egg components which had to be irradiated in order to cause double abdomen formation were characterized as follows. The effective targets are extranuclear; they are apparently prelocalized during oogenesis. A multiplicity of targets is radially distributed about the longitudinal egg axis. The efficiency of double abdomen induction decreases as the irradiated area is shifted from the anterior pole towards the middle, as well as from peripheral to central regions of the egg (4). The effective targets are contained in the clear cytoplasm without association to mitochondria or endoplasmic reticulum (6). A detailed action spectrum for double abdomen induction suggests that the targets consist of a nucleic acid–protein complex (7). The involvement of a nucleic acid moiety is also indicated by the photoreversibility, i.e., the reduction of the double abdomen yield by visible light after UV (7).

To make a further test of the nature of the anterior determinants in the egg of *Smittia*, we set out to manipulate the developmental program by applying different enzymes to the anterior pole region of the egg. We report here that the aberrant pattern "double abdomen" can be generated by RNase (ribonuclease I, ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) action.

MATERIALS AND METHODS

Eggs. Eggs were obtained from the laboratory strain of *Smittia* sp. used in earlier studies. The parthenogenetic female lays about 80 eggs in a gelatinous matrix which can be torn apart to release the eggs. All eggs in a cluster are at the same stage of development which can be determined by morphological criteria as described previously (8). Stages referred to in this communication are: P_0 (no pole cells), P_2 (2 pole cells), P_4 (4 pole cells), M_1 (migration of cleavage nuclei into periplasm), M_2 (some cleavage nuclei have reached periplasm), *PB* (preblastoderm), and *Bl* (blastoderm).

Puncturing. Eggs were punctured (1 hole per egg) with needles mounted on a micromanipulator (Fonbrune type). Needles with solid tips of 1–2 μm diameter were pulled from 1 mm glass capillaries and used without further sharpening. Eggs were held in the tip of a capillary connected by plastic tubing to a syringe with a screw-driven plunger, all filled with paraffin oil to facilitate holding and prompt release of the eggs with a minimum of pressure. Puncturing was accomplished gently so that extraovates at the anterior pole were formed only rarely (less than 1%). Such eggs were discarded since extraovate formation is often followed by the development of double abdomens without further treatment (9).

During and immediately after puncture, eggs were submerged in RNase or other enzyme solutions (Fig. 2). Groups of five to six eggs were punctured within 2 min and allowed to remain in the enzyme solution for another 2 min. The time of incubation in the enzyme solution thus ranged from 2 min to 4 min. Thereafter, the eggs were rinsed and incubated in boiled and filtered tap water. This work was done in an air conditioned room at 20°. Eggs were then allowed to develop under water in small glass dishes at 20° in the dark.

Injection. The injection technique was similar to the puncturing procedure described, with the following modifications. Hollow glass needles with an outer diameter of 3 μm were ground under an angle of 20–30 degrees on a steel wheel

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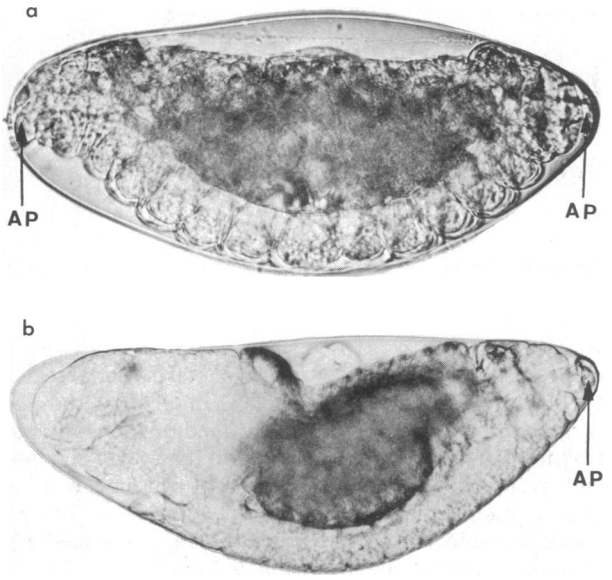

FIG. 1. (a) *Smittia* embryo showing the aberrant segment pattern of double abdomen. Anal papillae (*AP*) can be seen at both ends. Each abdomen consists of the seven terminal posterior segments, fused in mirror image symmetry near the egg equator. The aberrant pattern developed after injection of RNase at the anterior pole. (b) *Smittia* embryo with normal segment pattern, which developed after application of RNase to the posterior pole.

with diamond paste and then cleaned with acetone, ethanol, and distilled water. For loading with enzyme and subsequent injection, the needles were connected to a syringe system. During injection, eggs were submerged in paraffin oil.

Enzymes. RNase A from bovine pancreas was obtained from both Sigma (Munich) and Serva (Heidelberg). RNase S, RNase S protein, RNase S peptide, and oxidized RNase A were supplied by Sigma. RNase S is a subtilisin-modified enzyme (10). Peptide bond 20 as numbered from the NH_2-terminal is hydrolyzed, a change which has virtually no effect on RNase activity. RNase S can be split by trichloroacetic acid fractionation into S-peptide (20 amino acids) and S-protein. Both fragments are enzymatically inactive except for some residual activity in the S-protein. RNase activity is largely regained upon mixing S-protein and S-peptide at an equimolar ratio. Oxidized RNase is prepared from RNase A with performic acid (10). Phosphodiesterases from snake venom and calf spleen, phosphodiesterase I, oligonucleolate 5′-nucleotidohydrolase, EC 3.1.4.1, and alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] (from *Escherichia coli*), and DNase I from bovine pancreas (deoxyribonuclease I, deoxyribonucleate 5′-oligonucleotidohydrolase, EC 3.1.4.5) were purchased from Boehringer (Mannheim), Pronase P 1 from Serva, and trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) from Sigma.

Classification of Results. Two days after puncturing or injection, the embryos could be unequivocally classified as normal larvae (*NL*), double abdomens (*DA*), or undifferentiated eggs (*ud*). The double abdomens then showed, besides their typical outline (Fig. 1a), a proctodaeum and anal papillae at each end. Embryos with normal segment patterns but dwarfed or defective heads were scored as normal larvae.

RESULTS

Preliminary Experiments: Variability of Results. Enzymes can be introduced into *Smittia* eggs either by injection or by puncturing during submersion in enzyme solutions. The latter

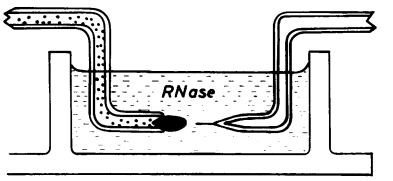

FIG. 2. Diagram shows how *Smittia* eggs were punctured during submersion in RNase. Eggs were held in a capillary filled with paraffin oil. Length of egg 0.2 mm, diameter of glass needle at the tip 1–2 μm.

method is much less laborious. In an earlier study (11), this technique had been developed for application of ethidium bromide and other agents. From these experiments we knew that eggs punctured at stages M_1 to *PB* in aqueous solutions survived well and produced no extraovates.

Since the results of our earlier UV experiments suggested that ribonucleoprotein particles act as anterior determinants in *Smittia* eggs, we began by applying RNase. After puncturing eggs at the anterior pole during submersion in RNase, double abdomens were indeed produced. As a preliminary result it emerged that the aberrant segment pattern would preferentially develop after puncturing at stages M_1 or M_2 with RNase concentrations between 0.5 and 1.0 μg/ml. RNase A from Serva and Sigma gave similar results. It also turned out that, for effective double abdomen production, RNase solutions had to be prepared with distilled water rather than buffer. The enzyme molecules are then apparently carried by the water osmotically drawn into the egg.

Despite our efforts to standardize the experimental procedure, the results remained variable. With a given set of controlled experimental parameters, the double abdomen yield varied between 5 and 70% in the most extreme cases. While the RNase activity (with transfer RNA as substrate) was fairly constant, the mechanical resistance of the egg shell (chorion) to the penetration of the glass needle varied between eggs from different clusters. This may partly be ascribed to slight differences in egg size and shape which influence the mechanical stress inflicted upon the egg by the holder capillary. Uncontrolled differences between individual glass needles also appeared to contribute to the variability of the results. Eggs from the same cluster were therefore punctured with one needle under the different controlled conditions to be tested.

Proof that Double Abdomens Result from RNase Activity. The double abdomen yield clearly depended upon the RNase concentration (see below, Fig. 4). Puncturing and incubation in water did not produce double abdomens (Table 1). Oxidized RNase A as well as RNase A denatured by boiling for 1 hr were completely ineffective. Perhaps the most convincing evidence that the double abdomens are caused by RNase activity is provided by the results of the experiments with RNase S, and its components RNase S protein and RNase S peptide. They were used in equimolar concentrations (RNase S, 1 μg/ml: S-protein, 0.84 μg/ml: S-peptide, 0.16 μg/ml). While RNase S produced 40% double abdomens in the surviving eggs, the S-peptide was inactive and the S-protein almost inactive even if the concentration was doubled (Table 1). A mixture of equal volumes of S-protein (1.68 μg/ml) and S-peptide (0.32 μg/ml), resulting in nearly 1 μg/ml of the reconstituted enzyme, produced 36% double abdomens in the surviving eggs. These results, together with those of the other controls are regarded as reliable proof that the double abdomens produced in the experiments reported here result from the activity of the RNase introduced into the eggs.

Application of Other Enzymes. Eggs at stages P_0 through

Table 1. Puncture of *Smittia* eggs during submersion in RNase

Agent	Concentration (μg/ml)	Batches	Total eggs	No. of normal larvae	No. of undifferentiated eggs	Double abdomens		
						No.	% of total eggs*	% in surviving eggs†
RNase A	0.8	19	382	95	203	84	22	47
RNase A denatured	0.5–1.0	23	476	436	40	—	0	0
RNase A oxidized	10.0	4	93	93	—	—	0	0
H_2O		8	158	151	7	—	0	0
RNase S	1.0	9	189	76	63	50	26	40
S-Peptide	0.16	9	205	195	10	—	0	0
S-Protein	0.84	4	81	77	3	1	1	1
	1.68	7	170	151	15	4	2	3
S-Peptide plus S-protein	0.16 + 0.84	8	184	69	77	38	21	36

Eggs at stages M_1 or M_2 (5.5–7.5 hr after deposition) were punctured at the anterior pole during submersion in RNase (Fig. 2).
* Percentage of double abdomens from total of eggs.
† Percentage of double abdomens from surviving eggs, i.e., total minus undifferentiated eggs.

M_2 were also punctured in other enzyme solutions. Phosphodiesterases from *Crotalus* venom and calf spleen (2–10 μg/ml), alkaline phosphatase (2 μg/ml) added to the phosphodiesterases, DNase I (100 μg/ml), trypsin (10–100 μg/ml), chymotrypsin (10–100 μg/ml), and Pronase P (0.01–2 mg/ml). None of these treatments resulted in double abdomen formation. Defective and dwarfed heads were found but the frequency of undifferentiated eggs was not significantly increased.

We cannot exclude the possibility that some of these enzymes penetrated into the punctured eggs less efficiently than RNase. To avoid this difficulty, enzymes were injected into *Smittia* eggs. The injected volume was about 36 pl, as determined by liquid scintillation counting after injection of isotopes with known radioactivity per volume. The injected volume corresponded to about 4% of the egg volume. RNase A, at a concentration of 5 μg/ml, produced double abdomens in 14 from a total of 79 injected eggs. Injection of RNase in lower concentrations hardly produced any double abdomens. The other injected enzymes (Table 2) did not result in double abdomen formation except one case when a double abdomen was found after injecting a mixture of phosphodiesterase from calf spleen and alkaline phosphatase. We cannot exclude, however, that our enzyme preparation had some endonuclease activity.

From the comparison of the double abdomen yields resulting from injection of and puncturing in RNase, injection appears less effective. This could be ascribed to a leakage of fluid from the injected eggs which was observed in some cases. The bulk of the injected enzyme might also become located in an egg region where the RNase targets are less sensitive and/or less concentrated than in the most anterior egg region which is primarily exposed to RNase after puncture at the anterior pole. This interpretation is supported by the observation that methylene blue, after injection at the anterior pole, became located near but not just beneath the anterior pole where the maximum efficiency of double abdomen induction by UV was found (4, 6).

Application of RNase to Different Egg Regions. To test the influence of RNase on egg regions other than the anterior pole, eggs at stage M_1 or M_2 were punctured in RNase solution (0.5 μg/ml) as described above, but the punctured site was either at the posterior pole or near the egg equator. A total of 98 eggs from four clusters was punctured at the posterior pole. While

Table 2. Injection of enzymes into *Smittia* eggs

Agent	Concentration (μg/ml)	Batches	Total eggs	No. of normal larvae	No. of undifferentiated eggs	Double abdomens*		
						No.	% of total eggs	% in surviving eggs
RNase A	5	6	79	50	15	14	18	22
PDE (snake venom) plus alkaline phosphatase	50 + 50	3	43	43	—	—	0	0
PDE (calf spleen) plus alkaline phosphatase	50 + 50	3	29	26	2	1	3	4
DNase I	10	2	30	28	2	—	0	0
	50	2	31	30	1	—	0	0
	100	2	40	39	1	—	0	0
Pronase P	100	3	45	39	6	—	0	0

Eggs at stages M_1 or M_2 (5.5–7.5 hr after deposition) were injected at the anterior pole with 36 pl (about 4% of egg volume) of enzyme solution. PDE = phosphodiesterase.
* See footnotes to Table 1.

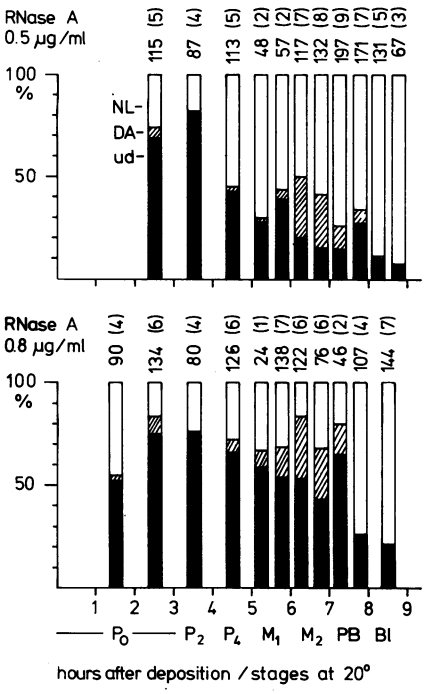

FIG. 3. Influence of the stage of development (abscissa) on the results of puncturing *Smittia* eggs in RNase. The percentages of normal larvae (*NL*), double abdomens (*DA*), and undifferentiated eggs (*ud*) are represented by the white, hatched, and black parts of the columns. Figures on top of each column indicate the number of eggs analyzed and, in parentheses, the number of clusters from which the eggs were derived. The RNase concentration was 0.5 µg/ml (top) or 0.8 µg/ml (bottom). *PB* refers to preblastoderm; *Bl* refers to blastoderm.

64 eggs developed apparently normally (see Fig. 1b), 24 eggs remained undifferentiated. Only one double abdomen was found, and this was also exceptional because it carried at least one incomplete segment in the anterior abdomen. Defective or dwarfed head structures and/or incomplete dorsal closure were observed in nine embryos. These signs of deficiency however, are probably nonspecific since they were also observed after UV irradiation, restricted oxygen supply, or even in untreated control eggs. Eggs were also punctured dorsally, ventrally, or laterally near the egg equator. From a total of 111 eggs (five clusters), 60 developed apparently normal, eight showed defective or dwarfed head structures and/or incomplete dorsal closure, and 43 remained undifferentiated. Obviously, RNase must be applied to the anterior pole region in order to produce double abdomens with a reasonable frequency.

Influence of the Stage of Development. Eggs were punctured at the anterior pole during submersion in RNase A at different stages of development. As shown in Fig. 3, the yield of double abdomens was generally below 5% during early stages (P_0 to P_4). At stage P_2 (3.5 hr after deposition), only one double abdomen was found in 167 eggs. After this lag period, the double abdomen yield increased markedly at about 6 hr after deposition (stage M_1/M_2). A maximum yield of about 30% was found after puncturing between 6 and 7 hr after deposition (stage M_2). The frequency of double abdomens decreased again around 7.5 hr after deposition (stage *PB*), and no double abdomens were found in 211 eggs punctured 8.5 hr after deposition (early blastoderm stage). This stage dependence of double abdomen production was consistently observed in two independent series of experiments with RNase concentrations of 0.5 and 0.8 µg/ml, respectively.

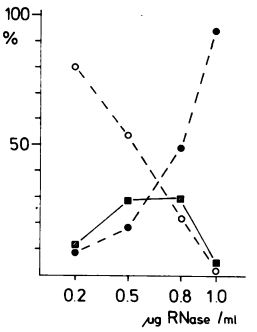

FIG. 4. Influence of the RNase concentration on the results of puncturing *Smittia* eggs during submersion in the enzyme. With increasing RNase concentration (abscissa), the percentage of normal larvae (○) decreased, while the frequency of undifferentiated eggs (●) increased. The maximum yield of double abdomens (⧈) was 28% and 29% at concentrations of 0.5 and 0.8 µg/ml, respectively. Eggs were punctured at the anterior pole between 6 and 7 hr after deposition. Mean number of eggs (clusters) checked at each concentration was 172 (9).

Effect of RNase Concentration. To determine the effect of RNase concentration over a wider range, we punctured eggs between 6 and 7 hr after deposition. As shown in Fig. 4, the influence of the enzyme concentration was rather dramatic. At 0.2 µg/ml, 91% of the punctured eggs survived, and 80% (87% of the survivors) produced normal larvae. With 0.5 and 0.8 µg of RNase per ml, the double abdomen yield was nearly equal (28 and 29%), but with the higher concentration, the frequency of undifferentiated results increased from 18 to 49% at the expense of normal larvae. At 1.0 µg/ml, as much as 94% of the eggs remained undifferentiated, while the surviving eight eggs yielded five double abdomens.

DISCUSSION

The experiments reported here show that introduction of RNase into the anterior pole region of *Smittia* eggs causes the formation of the aberrant pattern "double abdomen." The control experiments, in particular those with RNase S fragments, are considered as reliable proof that double abdomens are caused by RNase activity and are not due to any unknown contaminants or other uncontrolled effects of the puncturing or injection procedure. It may come as a surprise that inactivation of an RNA moiety in the egg causes a switch in the morphogenetic pathway of the embryo and not just defects or gaps in the segment pattern. The same switch, however, can be produced by UV irradiation of the anterior pole region and other unrelated types of experimental interference (15). Double abdomens are also found in the *bicaudal* mutant syndrome of *Drosophila melanogaster* (12). (Since the maternal genotype is the controlling factor in this mutant, the formation of the abnormal segment pattern must be ascribed to a defective oogenetic condition.) It is hard to conceive how mutagenesis and several unrelated types of experimental interference could *de novo* generate specific determinants for the formation of an abdominal end. It is much more likely that the different methods have in common the *displacement or inactivation* of crucial egg components necessary for head and thorax formation. This implies that the conditions which result in the formation of an abdomen exist not only in the posterior but potentially also in the anterior egg half. The formation of double abdomens instead of normal embryos can thus be visualized as a switch from one developmental pathway to another, triggered by the functional elimination of a crucial component. Similar bistable control systems are thought to operate in the determination and

transdetermination (13), and in the genetic control of compartmentalization of imaginal discs in *Drosophila* (14).

It may also appear surprising that RNase can inactivate egg components necessary for the formation of the normal segment pattern without killing the egg entirely. This is indeed what occurred when the RNase concentration was raised slightly over the optimum range of 0.5–0.8 μg/ml (Fig. 4). This sublethal range is apparently sufficient to inactivate crucial components near the point of influx into the egg. These components required for head and thorax formation may be especially RNase sensitive, at least around stage M_2. As the RNase diffuses further into the egg, the enzyme may be diluted and/or degraded to concentrations which can be tolerated by the egg.

The data obtained so far are compatible with the idea that the effective targets for RNase in these experiments are identical with the effective targets for UV induction of double abdomens (see above). This identity is suggested by two lines of evidence. First, ribonucleoprotein (RNP) particles appear to be the most likely candidates for the effective UV targets as judged from the action spectrum (7) and the localization of the targets in the clear cytoplasm not in association with mitochondria (6, 11). We have also found that photoreactivation after UV inactivation of *Smittia* eggs is correlated with splitting of uridine dimers, and that the photoreactivable sector after inactivation at different wavelengths is correlated to the amount of uridine dimers produced in the eggs by UV of that wavelength (H. Jäckle and K. Kalthoff, unpublished results). Second, the sensitive periods for double abdomen induction by UV and RNase both extend from egg deposition to blastoderm formation, with maximum yields at stages M_1 and M_2 in both cases (ref. 4, and Fig. 3 of this communication). However, the double abdomen yields after UV irradiation at stages P_0 through P_4 are almost as high as the maximum yields at stages M_1 and M_2, whereas the double abdomen yields after application of RNase at the early stages are very poor. This difference could be explained by the assumptions that the putative RNP particles act as effective targets of both UV and RNase, and that the conformation of the RNP particles changes around stage M_1 so as to render them more accessible to RNase action, whereas the sensitivity to UV is rather constant.

It is believed that "masked" messenger RNA or "informosomes" exist as RNP particles in which protein components serve to inhibit both the premature translation (16, 17) and RNase digestion of the RNA moiety prior to translation (18). Within this conceptual framework, the results of our experiments suggest that mRNA is present in the anterior pole region of *Smittia* eggs, and that its inactivation by either RNase or UV causes double abdomen formation. This speculation implies that the message is not translated until about stage M_1, because photoreverting treatment, after UV irradiation at stage P_2, can be delayed until stages M_1 or M_2 without much detriment to its efficiency (11), a result which suggests that the effective targets are inactive from egg deposition until at least stage M_1 or M_2.

The gross change in the morphogenetic program of the *Smittia* embryo apparently caused by function or dysfunction of stored RNP particles, is probably not as unique as it may appear. Homeotic mutations which switch morphogenetic pathways from, e.g., antenna to leg or haltere to wing in *Drosophila*, may be regarded as related phenomena. Such switches in morphogenetic pathways are apparently caused by dysfunction of single genes (19). As such genes might be transcribed into RNA, and our data suggest that stored RNP particles act as anterior determinants in *Smittia* eggs, the molecular mechanisms underlying these phenomena may be similar. A theoretical model for pattern specification during early embryogenesis that includes gene regulation by prelocalized RNA has been presented by Davidson and Britten (20).

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