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Drosophila mode of metamerization in the embryogenesis of the lepidopteran insect *Manduca sexta*

(segmentation/hunchback/Krüppel/runt/wingless)

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ABSTRACT **Insect embryos have been classified as intermediate- and short-germ embryos, in which posterior segments are thought to be generated sequentially from an uncommitted growth zone, or as long-germ embryos, such as *Drosophila melanogaster*, which develop primordia for all segments simultaneously. In *Drosophila* the coordinated activities among a three-tiered cascade of zygotic segmentation genes subdivide the embryo into progressively smaller units along the anterior–posterior axis. The mode of pattern specification in lepidopteran embryos has not been determined, although on morphological grounds they have been characterized as intermediate-germ insects. We have cloned orthologues of *Drosophila* segmentation genes from the tobacco hawkmoth *Manduca sexta* and have found that the blastoderm expression patterns of these genes show a molecular prepatterning typical of *Drosophila*. Thus, successive segment formation in *Manduca* embryos may not be due to sequential addition but rather may be the consequence of a lateral compression of the embryo proceeding in an anterior-to-posterior progression. These data challenge the view that the classification of insect development according to morphological criteria can serve as a reliable indicator of the molecular mechanisms underlying segmentation.**

Embryos of lower insects develop from a small portion of the blastoderm and are called "short-germ embryos." In contrast, "long-germ embryos" of higher insects such as *Drosophila melanogaster* are derived from cells covering the entire blastoderm (1). *Drosophila* genetics has revealed three classes of zygotic segmentation genes: gap, pair rule, and segment polarity (2–4). Most of these genes encode transcription factors that produce local gradients along the blastoderm, partitioning the embryo synchronously into segmental units earlier than the germ band is formed (5, 6). In short-germ embryos such as *Schistocerca americana* (7) and possibly *Tribolium castaneum* (8, 9), segmentation is asynchronous and visible segment formation is preceded by the sequential expression of stripes of the segment polarity gene engrailed (*en*) in the posterior growth zone. Lepidopteran insects have been judged by morphological criteria to undergo an intermediate-germ type development (10, 11) and it has been suggested that posterior segmentation proceeds according to the mode observed in short-germ embryos, i.e., that abdominal segments are generated by cellular proliferation from an uncommitted growth zone (11).

To analyze pattern formation in a lepidopteran embryo, we have utilized PCR (12) to clone sequences from the tobacco hawkmoth *Manduca sexta* that correspond to the *Drosophila* gap genes hunchback (*hb*) (13) and Krüppel (*Kr*) (14), the pair rule gene runt (*run*) (15), and the segment polarity gene wingless (*wg*) (16).* By examining the expression of these genes in *Manduca* embryos by whole-mount *in situ* hybridization (17), we show that a molecular prepattern is already evident at the cellular blastoderm that is analogous to the *Drosophila* prepattern. Thus, the formation of abdominal segments during *Manduca* embryogenesis involves preestablished metameric units characteristic of long-germ development rather than the sequential addition of posterior segments as is seen with short-germ embryos.

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MATERIALS AND METHODS

Cloning of Segmentation Gene Orthologues from *Manduca sexta*. Fragments were isolated from *Manduca sexta* genomic DNA by PCR (18, 19) and cloned into plasmid vectors (20, 21). *Manduca sexta* genes will carry the prefix *Ms* herein. Degenerate primers for *Kr* have been described (22). For *Mshb*, the primers used were (all in 5′–3′ orientation) proximal AARCACCAYYTNGARTAYCA and distal CGGATATCTCGCCRCACATRTTRCA, where R is G or A, Y is T or C, and N is G, A, T, or C (nt 5524–5543 and 6910–6886 of the *Drosophila hb* sequence in ref. 13). For *Msrun*, the primers used were proximal TGCCAATTGCYTTCAARGTIRTIGC and distal GCTCCTAGGCTCRCKKGGDCCATC, where K is G or T and D is G, A, or T (nt 671–695 and 951–928 of the *Drosophila run* sequence in ref. 15). For *Mswg*, the primers used were proximal AGACTCGAGTGYAARTGYCAYGG and distal GACTGCGCARCACCARTGGAA (nt 1105–1127 and 1776–1756 of the *Drosophila wg* sequence in ref. 16). Multiple independent clones were sequenced for each gene (23). Alignment of *hb* sequences was facilitated by the program CLUSTAL4 (24).

Embryo Collection and Whole-Mount *in Situ* Hybridization. *Manduca sexta* was raised under standard conditions (25). Eggs were treated for 5 min in 50% (vol/vol) Chlorox, rinsed briefly in 0.1% Triton X-100 and distilled water, and fixed in 4% (vol/vol) formaldehyde in phosphate-buffered saline for 2–5 h on a low-speed rotating shaker (26). Embryos were dissected free from the chorion and yolk, fixed an additional 6 h to overnight at 4°C without shaking, and then stored at −20°C in methanol with 5% 0.5 M EGTA (pH 8.0). Single-stranded DNA probes were labeled with digoxigenin (Boehringer Mannheim) by PCR (27) and whole-mount *in situ* hybridizations were performed (17) with minor modifications (28).

RESULTS

Early *Manduca sexta* Embryogenesis. Embryonic development of *Manduca sexta* takes ≈100 h at 25°C (29). The early stages of *Manduca* development have been described (30, 31). Briefly, the cellular blastoderm forms ≈8 h after oviposition. The embryo continues to develop from a portion of the blastoderm cells and the remaining cells give rise to the

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z30278–Z30281).

extraembryonic serosa. The germ anlage appears initially as a broad sheet of morphologically homogeneous cells that detaches from the serosa and sinks into the yolk mass (Fig. 1*A*). About 10 h after oviposition, a cleft forms along the anterior midline and the embryo starts to compress laterally to form the two head lobes (Fig. 1 *B* and *C*). Gastrulation proceeds by the invagination of cells along the ventral midline, starting in the presumptive gnathal–thoracic position (Fig. 1*D*). Ventral furrow formation extends primarily toward the posterior. This is accompanied by a constriction of the embryo laterally, proceeding in an anterior-to-posterior progression as well (Fig. 1*E*). As the ventral furrow reaches its final posterior position, segmentation is first visible in the gnathal–thoracic region, followed by the sequential appearance of abdominal segments (Fig. 1*F*). By the time the segmented germ band is complete, differentiation among the gnathal, thoracic, and abdominal segments has begun (Fig. 1*G*). This observed sequence of morphological events is consistent with the previous classification of lepidopteran embryos as intermediate-germ embryos (10, 11).

Isolation of *Manduca sexta* Segmentation Genes. *hb* and *Kr* are members of the gap class of segmentation genes and both encode zinc-finger-type transcription factors (13, 14). *hb* is expressed maternally and zygotically and plays a critical role in integrating the functions of the anterior and posterior maternal systems (13, 32). *Kr* is expressed zygotically and its activity is required for the establishment of thoracic and anterior abdominal segments of the embryo (33). *run* is a pair-rule gene, is expressed in a series of equally spaced stripes covering the trunk anlagen in the blastoderm embryo (34), and encodes a transcription factor sharing a conserved motif with the human AML1 protein (35, 36). Expression of the segment polarity gene *wg* is restricted to a distinct band of cells in each segment anlage (37). *wg* is a member of the Wnt gene family (38) and encodes a secreted glycoprotein believed to be a crucial component of a signal transduction pathway instructing the fate of responding cells (39). We have amplified sequences from *Manduca sexta* genomic DNA that are homologous to these *Drosophila* genes and have designated these clones *Mshb*, *MsKr*, *Msrun*, and *Mswg*. The protein sequences encoded by the *Manduca* fragments are compared to the orthologous genes from *Drosophila* and other insect species in Fig. 2.

Gap Gene Expression in *Manduca sexta* Embryos. *Mshb* and *MsKr* are already expressed in the *Manduca sexta* germ anlage when it separates from the extraembryonic blastoderm cells. *Mshb* expression forms a broad stripe in the anterior of the blastoderm (Fig. 3*A*) that fades in a graded manner toward posterior. Whether these transcripts are both maternally and zygotically expressed is not yet known. At the midblastoderm stage, *Mshb* expression can also be detected in the posterior most region of the embryo (Fig. 3*B*). Therefore, the expression patterns of *hb* in *Drosophila* and the *Manduca* orthologue *Mshb* correspond roughly in space and time (13). This pattern is also shared by the *hb* orthologue in *Tribolium* (40). In the *Drosophila* blastoderm, *Kr* is expressed in a broad band posteriorly adjacent to the *hb* expression domain, while *Kr* in *Tribolium* forms a cap covering the posterior most region of the germ anlage (8). This early *Tribolium* expression appears to be confined to the growth zone where sequential abdominal segmentation occurs, but as the embryo elongates, *Kr* expression gradually occupies a more central domain (8). In *Manduca*, *MsKr* expression is initially seen as a broad band in the central region of the blastoderm embryo (Fig. 3*C*). The transcripts do not remain evenly distributed, for in midblastoderm there appears to be less transcript in the center than at the borders of this domain (Fig. 3*D*). Thus, although the sequential appearance of abdominal segments in *Manduca* resembles the short-germ type segmentation process in *Tribolium*, the spatial and temporal

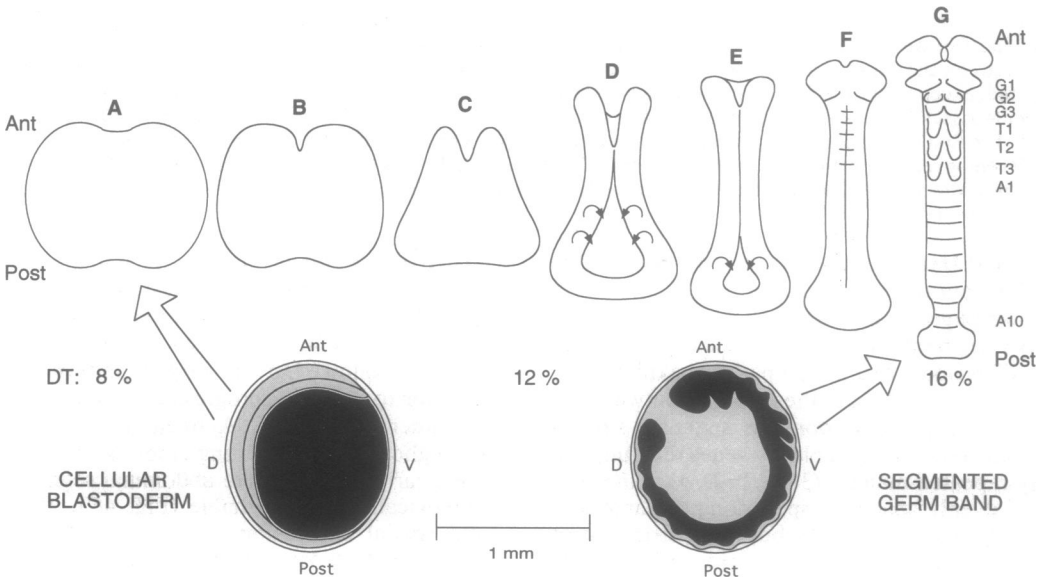

FIG. 1. Diagrammatic representation of early *Manduca sexta* embryogenesis. Percent development time (DT; 0% DT signifying oviposition and 100% DT representing hatching) is approximate; at 25°C 1% DT is roughly equivalent to 1 h (29). Ventral views of embryos removed from the egg are illustrated with anterior (Ant) and posterior (Post) labeled. The stages depicted curve dorsally at all margins. The positions of 8% DT and 16% DT embryos within the chorion are shown below with ventral (V) to the right and dorsal (D) to the left. Lightly and darkly shaded areas represent yolk and embryo, respectively. (*A*) The germ anlage is first recognized at cellular blastoderm as a layer of cells that is distinct from the adjacent serosa. Note that the germ anlage does not extend the entire length of the anterior–posterior or dorsal–ventral axes. (*B*) A cleft develops anteriorly, separating the two presumptive head lobes. (*C*) Gastrulation is initiated at ≈12% DT and coincides with the onset of embryo elongation. (*D*) Invagination of the embryo along a ventral midline furrow commences in the gnathal–thoracic region (26) and then extends anteriorly and most prominently posteriorly (signified by arrows). (*E*) Elongation of the embryo continues and there is a reduction in the width of the embryo in an anterior-to-posterior progression. (*F*) Segmentation first becomes evident in the gnathal–thoracic region as gastrulation nears completion in the posterior of the embryo (30, 31), followed by the appearance of segments in the abdominal region. (*G*) By 16% DT, the gnathal (G1–G3), thoracic (T1–T3), and abdominal (A1–A10) segmental primordia are clearly distinguishable.

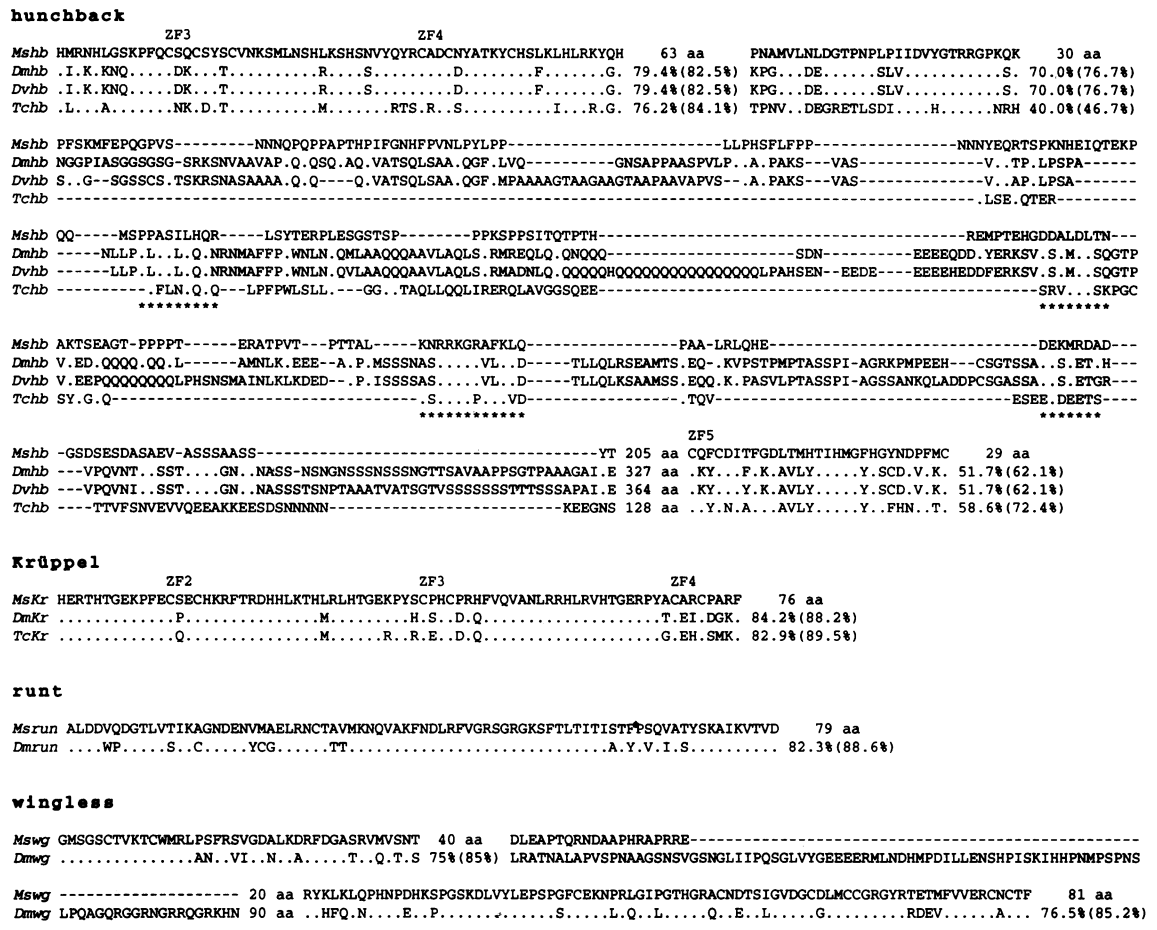

FIG. 2. Comparisons of protein sequences between orthologous developmental genes from *Manduca sexta* and *Drosophila melanogaster*. Dots denote identities; dashes denote insertions/deletions. The conceptual translation of the cloned *Manduca sexta (Ms)* sequence is aligned with the *Drosophila melanogaster (Dm)* and, when known, the *Tribolium castaneum (Tc)* sequence (*Tchb* and *TcKr* are from refs. 40 and 22, respectively), plus the *Drosophila virilis (Dv)* sequence for *hb* (41). Percentages signify identical amino acids and the percentage of conserved amino acids (42) between the *Manduca* sequence and the corresponding sequence is in parenthesis. Only sequences amplified between the primers are presented, except when the final amino acid encoded by a primer is unambiguous. The *Drosophila hb* sequence extends from codons 287 to 735 and encompasses 63 aa of the first finger domain, an adjacent 30-aa conserved box, an intervening 327 aa, and 29 aa of the second finger domain (13). The first Cys residues of zinc fingers (ZF) within *hb* and *Kr* are identified. Four short conserved motifs within the otherwise nonconserved intervening region are underscored by asterisks. The *Drosophila Kr* sequence extends from codons 240 to 315 (14), the *Drosophila run* sequence is from codons 148 to 226 (15), and the *Drosophila wg* sequence is from codons 237 to 447 (16). The cloned *Manduca* fragments do not contain intron sequences.

patterns of *Mshb* and *MsKr* expression are as observed in the long-germ development of *Drosophila*.

***Msrun* and *Mswg* Expression in *Manduca sexta* Embryos.** A pivotal step in the segmentation gene cascade of *Drosophila* is the formation of periodic expression patterns exhibited by pair rule genes (3, 4). Pair rule activities mediate between the gap genes that have expression domains spanning several segment anlagen and the segment polarity genes that function in a segmentally repeated manner (3–5). In *Drosophila, run* is expressed in 7 evenly spaced stripes along the anterior–posterior axis of the blastoderm (34), but these stripes arise asynchronously (43). The seven stripes correspond to alternating metameric units and are later processed to a metameric expression pattern of 14 stripes (43) similar to segment polarity genes such as *wg*, which is expressed at the onset of gastrulation in *Drosophila* (37, 44). *Msrun* is expressed in a series of 8 stripes along the anterior–posterior axis of the *Manduca sexta* blastoderm (Fig. 4 *A–C*). The additional eighth stripe in *Manduca* as compared to *Drosophila* is consistent with the formation of 10 abdominal segments in the *Manduca* embryo. It appears that *Msrun* transcripts are initially more abundant in the 4 anterior stripes (Fig. 4*A*). By late blastoderm, *Msrun* is also expressed in the head lobes (Fig. 4*C*), comparable to *Drosophila* (43). Transcription of *Mswg* (Fig. 4 *D–F*) is first detected just prior to gastrulation. The way in which the expression pattern emerges closely resembles *Drosophila wg* expression (37, 44). *Mswg* transcripts are initially observed in an anterior and posterior domain, followed by a striped pattern proceeding in an anterior-to-posterior progression, the first stripe being most prominent (Fig. 4*D*). A metameric pattern can be seen throughout the gastrulating embryo (Fig. 4*E*) and 16 stripes are clearly visible before abdominal segments become morphologically distinguishable (Fig. 4*F*). Thus, the temporal and spatial expression patterns of *Msrun* and *Mswg* are analogous to those observed for *run* and *wg* in *Drosophila*.

DISCUSSION

The finding of conserved patterns of pair rule and segment polarity gene expression in *Drosophila* and *Manduca sexta* embryos is different from what has been observed in the embryos of *Schistocerca* and *Tribolium*. In *Tribolium* the pair rule gene hairy orthologue is first expressed in two stripes in the central region of the blastoderm and posterior stripes are successively expressed during gastrulation in a region that may correspond to the uncommitted growth zone where abdominal segments form, suggesting a conserved pair rule

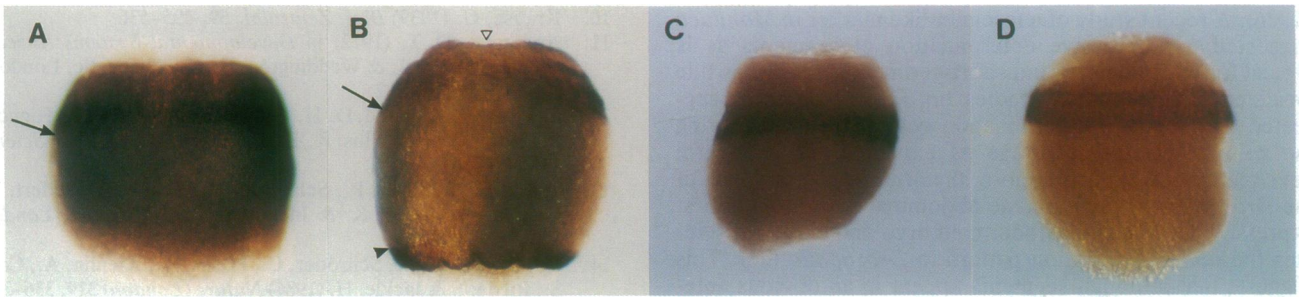

FIG. 3. Expression of *Mshb* and *MsKr* in *Manduca sexta* blastoderm embryos. Ventral aspects are shown, with anterior to the top. Approximate embryo size can be judged from Fig. 1. (*A*) *Mshb* staining in an anterior domain at early blastoderm; the posterior limit is indicated by an arrow. (*B*) *Mshb* expression at midblastoderm stage. A cleft in the anterior margin between the incipient head lobes has formed (open triangle). In addition to the anterior domain, *Mshb* is now also detected in a posterior domain (the anterior limit is marked by an arrowhead) similar to *hb* in *Drosophila* (13). (*C* and *D*) *MsKr* staining in early (*C*) and mid (*D*)-blastoderm embryos equivalent to those in *A* and *B*, respectively. Comparable to *Kr* expression in *Drosophila* (33), a band is evident across the embryo posterior to the *Mshb* anterior domain. In *D* staining has also arisen at the anterior margins of the presumptive head lobes but is not visible due to embryo curvature.

function (8, 9). In contrast, the *Schistocerca* orthologue of the pair rule gene even-skipped apparently has no conserved role in the segmentation process (45). In both insects, a distinct stripe of *en* expression appears in the posterior region of the germ band before the formation of each abdominal segment (7, 8). Therefore, although *Tribolium* and *Manduca* have both been described as undergoing intermediate-germ type development due to the presumed addition of some abdominal segments by cellular proliferation (9, 46), the divergent expression of *Kr*, pair rule, and segment polarity genes indicates that these two insects do not share the same pattern and timing of posterior segmental fate determination.

The position of lepidopterans within the three categories of insect germ band development has been a matter of debate (1,

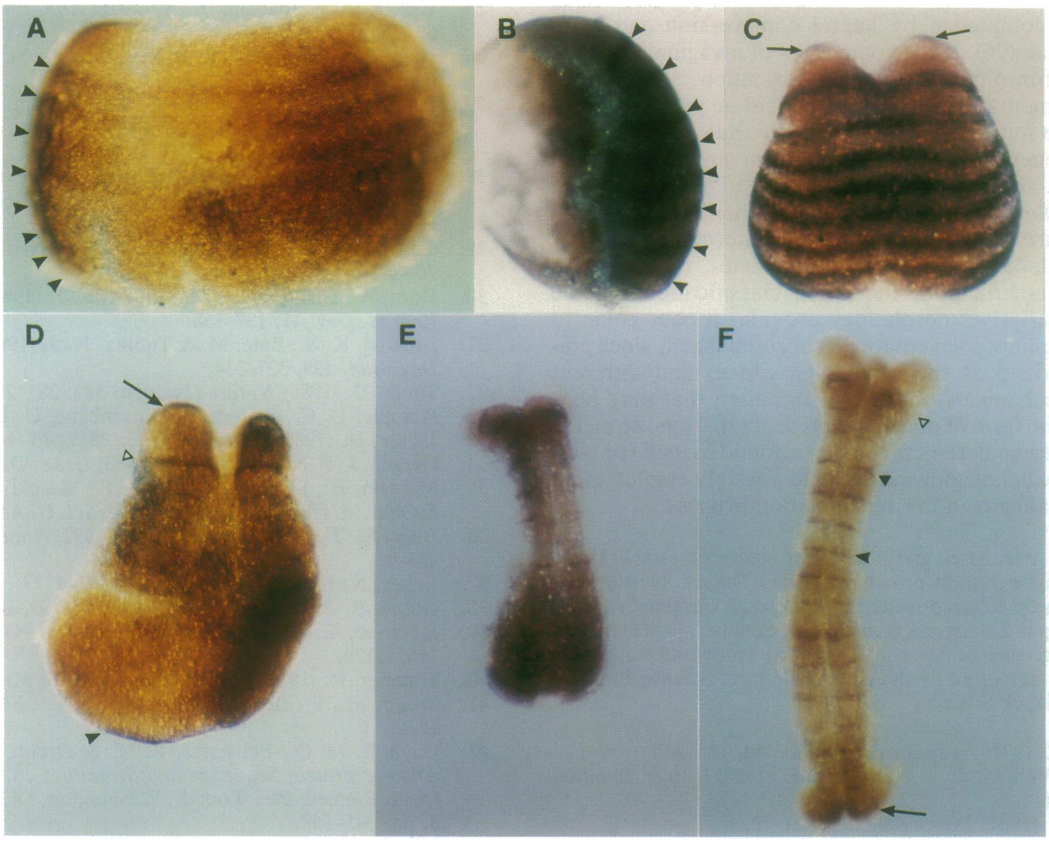

FIG. 4. Pattern and timing of *Msrun* and *Mswg* expression in *Manduca sexta* embryos. Ventral aspects are shown, except for *B*, with anterior to the top. Approximate embryo size can be judged from Fig. 1. (*A*) *Msrun* staining in an early blastoderm embryo. Eight stripes (arrowheads) are seen along the anterior–posterior axis, although the anterior-most four stripes are more prominent than the posterior four (the last being out of the focal plain and not visible). (*B*) In a lateral view of a midblastoderm embryo, the pair rule pattern of *Msrun* expression is evident (arrowheads). (*C*) In the late blastoderm embryo, *Msrun* is expressed in two head spots (arrows), in addition to the pair rule pattern. (*D*) *Mswg* staining at the start of gastrulation resembles the *wg* pattern in *Drosophila* (37, 44), with an anterior domain (arrow), a posterior domain (arrowhead), and a striped pattern, starting with a strong anterior stripe (open triangle), appearing in an anterior-to-posterior progression. (*E*) At mid-gastrulation, a striped pattern of *Mswg* expression can be detected throughout the embryo. (*F*) *Mswg* expression in an embryo after gastrulation is complete, but before posterior segmentation is evident. Sixteen stripes corresponding to 3 gnathal (G1 marked by an open triangle), 3 thoracic (T1 marked by a solid triangle), and 10 abdominal (A1 marked by an arrowhead) segment primordia are seen, in addition to the persistence of the posterior domain (arrow) and patches of expression in the head lobes not visible here.

11, 26). A recent study of a fasciclin-like marker in *Manduca* suggested a progressive determination of segments as is typical for intermediate- and short-germ embryos (46), while localized UV-irradiation studies on *Tineola* embryos suggested that segment determination occurs before or during the cellular blastoderm stage as expected for long-germ development (47). Furthermore, the *Manduca* orthologue of the *Drosophila* homeotic gene abdominal-A (*abd-A*) is first expressed in the late blastoderm embryo and closely resembles the *abd-A* expression pattern in *Drosophila* (26). This suggests that although segments appear in an obvious anterior-to-posterior progression, specification of the abdominal segments in *Manduca* occurs before the embryo has undergone gastrulation. These findings are in agreement with our results demonstrating that, in molecular terms, the metameric organization is laid down already at the cellular blastoderm stage. Therefore, the occurrence of a germ anlage that occupies only a portion of the anterior–posterior axis of the cellular blastoderm as is seen in *Manduca* does not preclude the generation of a molecular prepattern for all segments found later in the extended germ band.

How is it then that segments appear sequentially during *Manduca* germ band formation, while they appear synchronously in *Drosophila*? One major difference between *Manduca* and *Drosophila* development is that mesoderm involution occurs simultaneously along the entire anterior–posterior axis of the gastrulating *Drosophila* embryo (48), whereas it occurs in an anterior-to-posterior progression in *Manduca*, accompanied by lateral compression and germ band elongation (26). If the completion of invagination is the prerequisite for physical segment formation, the delayed posterior segmentation in *Manduca* could be just the consequence of the initial asynchrony, rather than be due to the sequential generation of segments from an uncommitted growth zone. These results demonstrate that blastoderm embryo size and the order in which segments become distinguishable are not reliable criteria for determining whether an insect undergoes a short-, intermediate-, or long-germ mode of metamerization. Indeed, a classification scheme based on descriptive morphology provides a poor guide to understanding how segmentation is accomplished, since embryos categorized as the same type can exhibit different molecular mechanisms of pattern formation that may be a reflection of the form of oogenesis present (9). Thus, so called short- and intermediate-germ embryos found associated with meroistic or panoistic oogenesis could possibly employ quite distinct mechanisms in the segmentation process.

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