Organ renewal and cell divisions by differentiated cells in *Drosophila*

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Communicated by Gail R. Martin, University of California, San Francisco, CA, May 27, 2008 (received for review May 13, 2008)

For many organs, the processes of renewal and regeneration recruit stem cells to replace differentiated, postmitotic cells, but the capacity of an organ's differentiated cells to divide and contribute is uncertain. Most cells of the *Drosophila* adult are the descendants of dedicated precursors that divide and replace larval cells that are histolyzed during metamorphosis. We investigated the provenance of cells that reconstitute the second thoracic metamere of the tracheal system (Tr2). These cells contribute the precursors for Branchless(FGF)-dependent growth of the dorsal air sacs, the major tracheal organs of the adult fly. We found that, in contrast to the cells in other tracheal metameres that proceed through many cycles of endoreplication, the cells that constitute the Tr2 branches in young larvae do not. Like the cells in other tracheal metameres, these cells arrest mitotic cycling in the embryo and form differentiated, air-filled tracheal branches of the larva. We report here that they reinitiate cell divisions during the third instar (L3) to increase the Tr2 population by ≈10-fold with multipotent cells.

Branchless(FGF) | clonal analysis | *Drosophila* dorsal air sacs | tracheal morphogenesis | endoreplication

The mechanisms of organ renewal and neogenesis in animals are of both fundamental and clinical importance. Although the involvement of pluripotent stem cells has been established for many vertebrate and invertebrate organ systems, contributions may also come from dedicated pools of precursors or from differentiated cells that revert to a proliferative state. The cells of the imaginal discs of holometabolous insects such as *Drosophila* are examples of precursors that are dedicated to a particular epidermal fate and differentiate structures only after the larval epidermis has been eliminated at metamorphosis. These imaginal disc cells proliferate in an undifferentiated state throughout the foraging stages of larval development. There are also cases of cells that both express a differentiated state yet retain the capacity for growth. In the larval abdomen of *Drosophila*, larval cells occupy most of the epidermal surface and secrete most of the cuticle, but the imaginal histoblasts that will replace the larval cells at metamorphosis and make the adult abdominal cuticle occupy a small portion of the larval epidermis and secrete cuticle during the larval stages (1). Examples of differentiated cells that contribute to renewal or neogenesis in vertebrates include the insulin-producing beta cells in the mouse pancreas that may be a source of beta cells during adult life (2) and proliferating cardiomyocytes in regenerating the zebrafish heart (3).

We have been studying the development of the *Drosophila* dorsal air sacs, multilobed tracheal organs in the thorax of the adult fly that supply oxygen to the flight muscles. The primordium that generates the dorsal air sacs (the ASP) arises during the third larval instar (L3) from a transverse connective, one of the air-filled tracheal branches of the second tracheal metamere (Tr2) that associates with the wing imaginal disc (4, 5). The ASP forms as a tube in response to Branchless(FGF) [Bnl(FGF)] expressed by the wing imaginal disc. Its growth is characterized both by morphogenesis and by cell proliferation and migration (5, 6). The presence of dividing cells in the outgrowth makes it the only case of tubulogenesis in the fly that is characterized by both morphogenesis and cell division. It therefore represents a system in which a variety of cell biological and genetic mechanisms relevant to vertebrate organogenesis can be investigated. The research reported here examines the source of the dividing cells in the outgrowth and reports that they are descendants of the differentiated larval tracheal cells.

Results

Before ASP induction, the cell composition of Tr2 changes, for instance in the Tr2 dorsal trunk from 16–18 cells to ≈160, and in the Tr2 transverse connective from 6–8 cells to ≈64 (Fig. 1*A* and ref. 4). Incorporation of BrdU (4) and staining with α-phosphohistone H3 antibody (5) suggest that this transformation is effected largely or completely by cell divisions. Because cells in most other larval organs grow but do not divide (7, 8) and the program of widespread tracheal cell proliferation was unique to Tr2, we investigated the provenance of the mitotically active cells in Tr2 to learn whether, for example, they might be descendents of a distinct group of tracheoblasts, of mitotically active precursor cells, or of the larval tracheal cells themselves.

Cells that make up *Drosophila* larval organs, such as salivary glands and midgut, histolyze at metamorphosis in a process that coordinates their replacement with the mitotically active precursors of the adult organs (7, 9). Removal of the larval cells employs cell death and autophagy mechanisms (10). Studies of the abdominal tracheal system suggest that larval tracheal cells are also replaced to generate the pupal and adult tracheae (11, 12). To determine whether the larval cells of Tr2 have a similar fate, we probed at 8-h intervals through L3 for two markers of programmed cell death: activated Drice (13, 14) and TUNEL (15). Although both markers identified positive cells in the wing disc at all stages (Fig. 1 *B–K*), no positive tracheal cells were detected up to 48–50 h in L3 (Fig. 1 *E*, *F*, *J*, and *K*). Because cell death was not observed, the process that transforms the cell composition of Tr2 branches and the processes that remodel the midgut and salivary gland are apparently fundamentally different (7, 16).

The tracheal system grows as the larva grows (e.g., from L1 to 0–2 h L3, the length and diameter of the Tr2 dorsal trunk increase ≈2.5× and 5×, respectively), but growth is not accompanied by cell divisions. To document the programs of DNA synthesis in the larval trachea, we fed animals BrdU (17). Pulses of BrdU during L1, L2, and L3 revealed two distinct patterns of incorporation in the dorsal trunk and transverse connective. Most nuclei in the cells of Tr3 and the more posterior metameres labeled with anti-BrdU antibody after exposure to BrdU during L1 (data not shown) and L2 (Fig. 2*A*); labeling after exposure

Author contributions: A.G., L.L., and T.B.K. designed research; A.G. and L.L. performed research; A.G., L.L., and T.B.K. analyzed data; and A.G., L.L., and T.K. wrote the paper.

The authors declare no conflict of interest.

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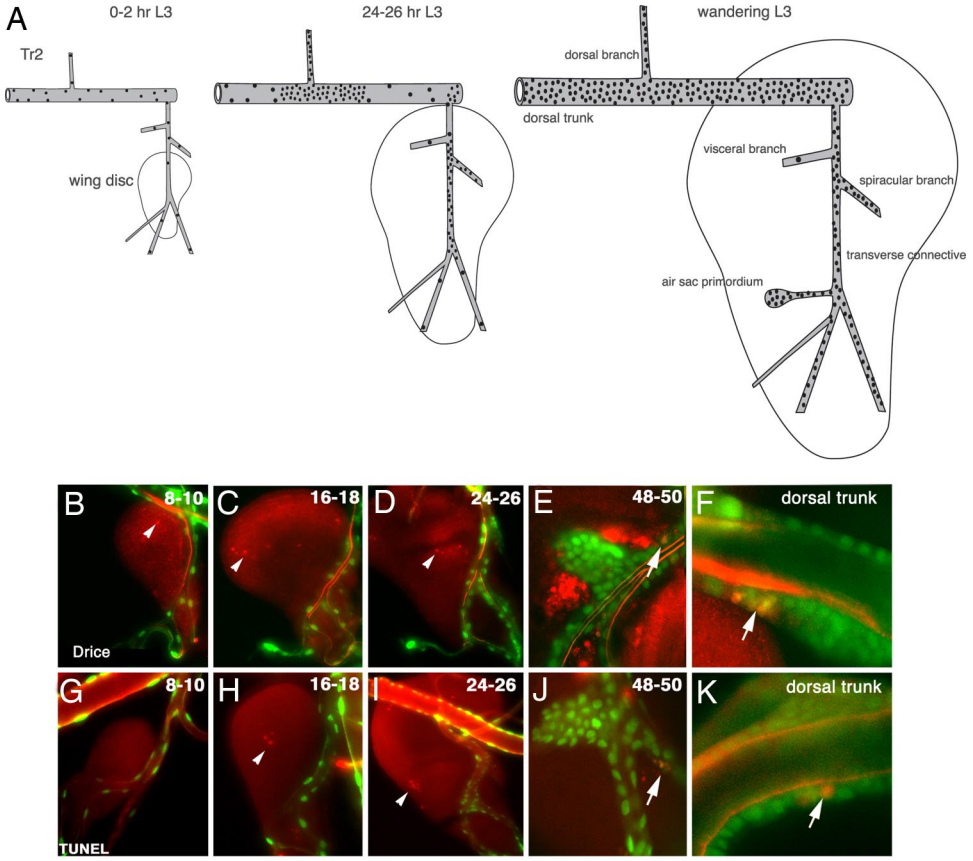

Fig. 1. Cell proliferation and programmed cell death in Tr2 trachea during L3. (*A*) Scaled drawings of the tracheal branches in Tr2 depicting the distribution of cells. (*B–K*) Wing discs and associated Tr2 tracheal branches. Anti-activated Drice (23) (*B–F*) and TUNEL (24) (*G–K*) staining identified cells undergoing programmed cell death in the wing disc (*B–D*, *H*, and *I*) at all time points tested (hours after L3 molt), but in the transverse connective (*B–E*, *G–J*; green nuclei) and dorsal trunk (*F* and *K*), signal was detected in only a few cells at 48–50 h. Arrowheads, stained cells in wing disc; arrows, stained tracheal cells.

during L3 (Fig. 2*B*) was less frequent (e.g., 2 of 15 preparations had labeled Tr3 dorsal trunk nuclei). BrdU incorporation was detected in most Tr2 nuclei by 16–18 h in L3 (Fig. 2*B*), when their numbers begin to increase (4); most nuclei in Tr2 did not label during L1, L2, or early L3 (Fig. 2*A*). These data indicate that tracheal cells in the metameres other than Tr2 undergo repeated endocycles of DNA synthesis as they grow during the larval instars. However, the absence of BrdU incorporation by most cells of Tr2 during L1, L2, and early L3 suggests that Tr2 cells do not undergo multiple cycles of endoreplication.

The tracheal branches of the larva are air-filled tubes whose apical, luminal surface is lined with a taenidial cuticle that provides structural rigidity. The taenidium has periodic ridges that can be discerned with bright-field microscopy. Fig. 2 *C* and *D* show portions of the Tr2 and Tr5 dorsal trunk that were dissected from a larva molting at the L2–L3 transition. These images show that cells of both metameres secrete taenidial cuticle, revealing that their differentiated character was indistinguishable.

To verify that the Tr2 tracheal cells do not increase their DNA content during L2, we measured nuclear DNA content in tracheal cells in L2 and L3 animals. The levels of Draq5 fluorescence, a cell-permeable DNA-binding dye, increased in Tr3 and Tr5 between L2 and L3, consistent with the incorporation of BrdU by these cells (Fig. 2*E*). However, we detected no increase in Draq5 levels in Tr2 nuclei (Fig. 2*E*). These measures of DNA content are consistent with the conclusion that although most tracheal cells endoreplicate through larval life, the cells of Tr2 tracheae do not. Moreover, robust staining with α-cyclin A antibody (data not shown) suggests that L2 Tr2 cells remain committed to a mitotic program (18).

Although centrioles are present in mitotically active cells, larval cells that endoreplicate appear to lack them (J. Raff, unpublished data). Given the contrasting patterns of BrdU incorporation and DNA content in cells of the larval trachea, we investigated the distribution of centrioles by examining two centriolar markers: DSas4-GFP (19) and GFP-PACT (20). During L2, almost all Tr2 cells contained fluorescent punctae (Fig. 2*F*), whereas most cells in Tr3 (data not shown) and Tr5 (Fig. 2*G*) did not. The distribution of Fzr-GFP was similar (data not shown). Late in L3, DSas4-GFP was detected in a distinctive and characteristic punctate pattern in the proliferating tracheal cells in Tr2 (Fig. 2*H*), but this pattern of GFP fluorescence was not detected in Tr3 (data not shown) or Tr5 cells (Fig. 2*I*). These observations confirmed that Tr2 cells are different from the cells in other metameres; we infer that Tr2 cells are not committed to a program of endoreplication but instead retain mitotic competence. We therefore tested directly whether the cells that constitute the Tr2 branches are the progenitors of the mitotically active cells that populate this metamere during L3.

We devised a lineage-tracing method to track single tracheal cells in real time (Fig. 3). This strategy is based on the expression of GFP after FLP-induced recombination (hsflp; actin 5C>CD2>Gal4; UAS nuclear-GFP). A brief heat shock during late embryogenesis induced GFP expression at a frequency low enough to mark single cells unambiguously. Importantly, this

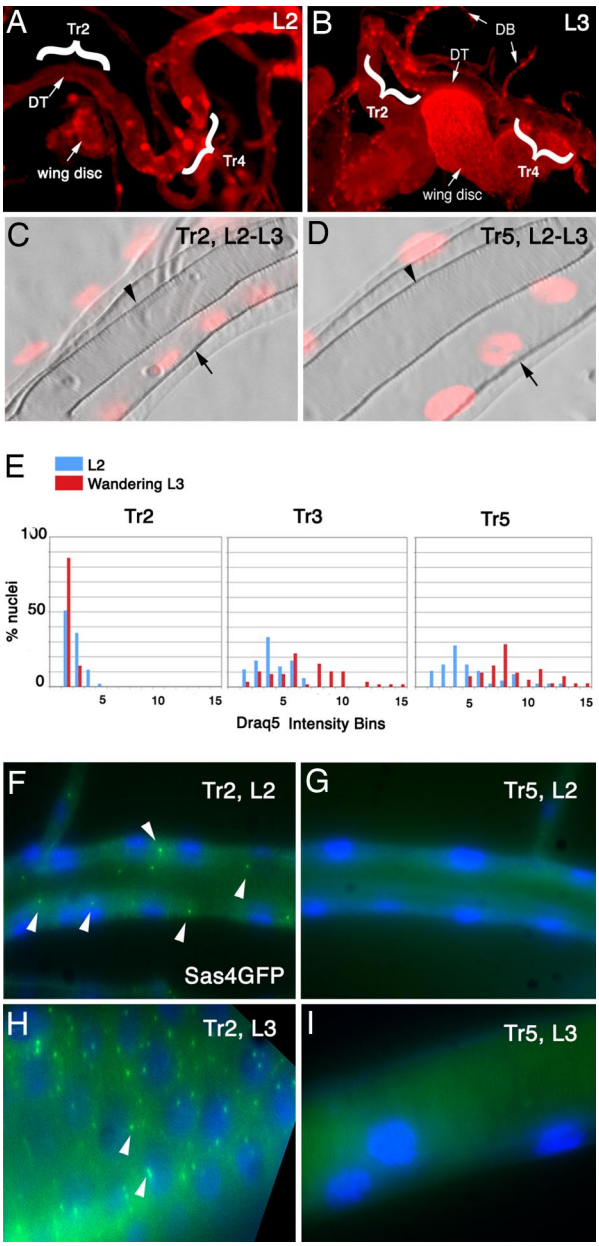

Fig. 2. DNA and centriole content of larval tracheal cells. (*A* and *B*) Cells that constitute Tr2 trachea did not incorporate BrdU during L2 (*A*) but did incorporate BrdU during L3 (*B*). Cells of the other tracheal metameres that are thought to undergo endoreplication through the larval instars incorporated BrdU in L2 (*A*), but incorporation during L3 was infrequent (*B*). Cells of the wing disc (arrows, *A* and *B*) incorporated BrdU at all stages that were examined. DT, dorsal trunk; DB, dorsal branch. (*C* and *D*) Bright-field images of portions of the dorsal trunk of Tr2 and Tr5 dissected from larva at the L2 to L3 molt. Striations of the taenidial cuticle can be seen in both the smaller L2 (arrowheads) and larger L3 (arrows) tubes. (*E*) Histograms showing DNA content of L2 (blue) and wandering L3 (red) nuclei in Tr2, Tr3, and Tr5. DNA content in Tr3 and Tr5 increased (*Middle* and *Right*) whereas the DNA content of cells in Tr2 did not (*Left*). The numbers of nuclei examined were 53 ($n = 7$ animals) for Tr2 L2 and 129 ($n = 11$ animals) for Tr2 L3, 51 ($n = 7$ animals) for Tr3 L2, 58 ($n = 8$ animals) for Tr3 L3, 47 ($n = 9$ animals) Tr5 L2, and 42 ($n = 6$ animals) for Tr5 L3. (*F–I*) Cells in Tr5 lacked Sas4-GFP, a marker for centrioles, both in L2 (*G*), and L3 (*I*) animals, whereas cells in Tr2 had Sas4-GFP-containing structures (arrowheads) in L2 and L3 (*F* and *H*).

regimen restricted recombination to a short interval in the late embryo because we found no evidence that additional GFP-expressing cells were induced after the pulse of FLP expression. L3 larvae (0–2 h) that had been heat shocked as embryos were screened without dissection, and animals with GFP-labeled cells in Tr2 were set aside. These animals were examined again after 24 and 48 h. Of the 36 clones we identified with individually marked Tr2 cells, 29 increased the number of GFP-expressing cells (from 2 to 8 cells) during the period of examination; seven were quiescent. An example of apparent clonal proliferation is shown in Fig. 3 *A–C*. In no case was GFP fluorescence lost, suggesting that none of the labeled cells was eliminated during this period. The persistence of these cells is consistent with the apparent absence of programmed cell death; their growth demonstrates that most of the postmitotic Tr2 tracheal cells restart a mitotic program in L3. Because most of the labeled cells increased in number, the fraction of cells in Tr2 with proliferative potential is high. We conclude that the postmitotic larval tracheal cells are the progenitors of the cells that proliferate and populate Tr2 tracheae during L3. Analysis of Tr2 clones induced at various times during embryonic and larval development indicates that the cell cycle is ≈10 h and that the increase in Tr2 cell number during L3 is driven entirely by larval Tr2 cell divisions (L.L. and T.B.K., unpublished data).

Discussion

This work offers several insights relevant to cell proliferation in differentiated organs undergoing repair or renewal. First, some of the cells that make up the larval tracheal system retain their capacity for proliferation. The ability of cells of the Tr2 metamere to restart their mitotic program revealed that expression of a fully differentiated state does not prohibit subsequent proliferation. For such cells, the term "terminal differentiation," with its implication that the cells lack growth potential, is inappropriate. Second, the process that reconstitutes the Tr2 metamere, increasing the number of constituent cells by ≈10-fold and entirely transforming its cellular composition, does so without compromising function. This metamere retains its multipartite structure [e.g., apical taenidial cuticle, cellular core, and basal lamina (A.G and T.B.K., unpublished data)] and functions as an air-filled conduit throughout the period of reconstitution. The term "dedifferentiation" may also therefore be inappropriate. Third, the larval cells of Tr2 show developmental plasticity, a property they manifest by contributing not only to the preexisting tracheal branches, but by also populating the ASP. In this context, they are multipotent. The dorsal air sacs that the ASP generates are arguably distinct organs, lacking the taenidial cuticle and radial symmetry that are characteristic of all tracheal branches (A.G. and T.B.K., unpublished data). Fourth, the ancestry of the cells that proliferate and renew the larval tracheal system is not the same in all of the metameres. Although proliferating cells that rebuild the tracheal network in Tr2 are derived from the cells that constitute Tr2 tracheal branches in the embryo, L1, L2, and early L3 larva, the polyploid state of the cells that constitute the other tracheal metameres makes it unlikely that these cells return to a mitotic program. Indeed, renewal of the tracheal branches in the abdominal metameres is reported to involve replacement of larval cells by a distinct population of imaginal tracheoblast precursors (12). Thus, within this single organ, the *Drosophila* tracheal system, mechanisms of remodeling vary. We have shown that the Bithorax Complex (BX-C) functions in the Tr3 metamere to block cell proliferation and tubulogenesis (4), establishing that such differences are regulated, in part, by the mechanisms that determine segmental pattern and identity.

The manner by which body forms change during the transformation of an egg into a sexually mature adult varies widely among different insect species. In hemimetabolous insects such as *Rhodnius* and *Schistocerca*, the passage to adulthood involves a series of intermediate nymphs that resemble the adult at all stages. Among holometabolous insects like *Drosophila*, however,

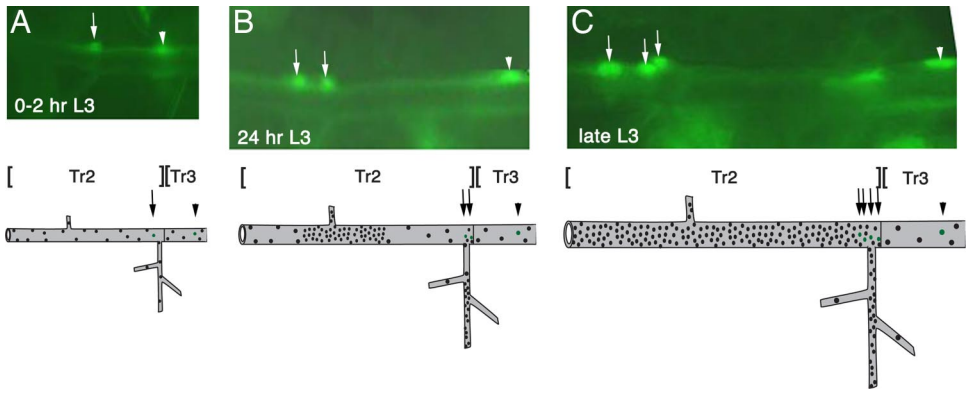

Fig. 3. Proliferation of the cells that constitute Tr2 during L3. (*A–C*) Lineage tracing of tracheal cells in a single larva imaged at three times during L3 reveals that a single larval cell in Tr2 (*A*, arrow) that had been marked during embryogenesis (see *Methods*) divided during L3 (*B* and *C*, arrows). A larval cell in Tr3 (*A–C*, arrowhead) that was also marked during embryogenesis did not divide during the same period. Distribution of cells and the marked cells are diagrammed below.

larval and pupal forms bear little resemblance to the adult. These differences in developmental sequence appear to correlate with different strategies for organ renewal and replacement.

Cells that constitute functional organs at the nymph stages of hemimetabolous insects can also proliferate and differentiate into multiple cell types at successive stages of development. Thus, they retain proliferative and multipotential capacity (their "embryonic" character) while serving functional roles. For example, in addition to proliferating and serving as a barrier at each larval stage, epidermal cells in *Rhodnius* can differentiate into distinct cell types (oenocytes, dermal gland cells, specialized sensory organs) at each molt (21). The imaginal discs of holometabolous insects exemplify the contrasting strategy: undifferentiated, pluripotent precursors of adult organs that do not contribute to larval function. This evolutionary adaptation that assigns the construction of an adult organ to a dedicated, separate class of progenitors is, in the case of the Tr2 tracheal cells, regulated by the BX-C. As noted above, the BX-C suppresses repopulation during L3 in the tracheal metameres posterior to Tr2 (4).

There are also examples of organs that use mechanisms of repopulation by both resident, differentiated cells and dedicated progenitors. In *Manduca* and *Tenebrio*, eyes and wings develop from imaginal discs, but in contrast to *Drosophila* whose imaginal discs form in the embryo, the *Manduca* and *Tenebrio* eye and wing discs form late in larval life. The epidermal cells in these insects that initiate imaginal disc development in late larval stages descend from cells that constitute the epidermis in young larvae (22). Our observations of the *Drosophila* tracheal system reveal that there are yet other ways that animals have combined distinct mechanisms for growth and replacement and that the capacity of differentiated cells to proliferate may be more common than has been generally appreciated.

Methods

Clonal Analysis. Embryos [*y w Hs-FLP/w*; *Act>CD2>GAL4/UAS-GFP*(nls)] were subjected to heat shock (15 min, 37°C) and a 54- to 56-h incubation at 23°C; larvae were examined 0–2 h after the L3 molt for GFP-positive nuclei in the Tr2 dorsal trunk, and animals with fluorescent nuclei were examined 24 and 48 h later.

DNA Quantitation. *btl*-GFP(nls)-expressing larvae were dissected in PBS, incubated with 5 μM Draq5 for 5 min (PBS), fixed in 5% paraformaldehyde (PBS) for 10 min, and rinsed in PBS + 0.3% Triton X-100. Trachea were flat-mounted in 50% (vol/vol) glycerol and compressed gently to expel air from the tubes to eliminate light scattering by air-filled tracheal tubes. Samples were imaged with a deconvolution imaging system (Intelligent Imaging Innovations). Outlines of tracheal nuclei were identified by nuclear-GFP fluorescence, and the integrated intensity of Draq5 fluorescence in the circumscribed region was quantified from image stacks by using Metamorph. The intensity bins are 50,000 units; intensities were corrected for background by subtracting an average intensity value of five selected regions devoid of nuclei from all pixels in the image.

Histology. TUNEL assays were with a Roche Diagnostics kit; Drice staining was according to ref. 14.

ACKNOWLEDGMENTS. We thank Jordan Raff (The Wellcome Trust/CRC Institute, Cambridge, U.K.) for fly lines, Bruce Hay (California Institute of Technology, Pasadena, CA) for α-activated Drice, and members of the Kornberg laboratory for helpful suggestions and assistance. L.L. thanks Markus Affolter, her Doktorvater, for his assistance, training, and support. The work was supported by National Science Foundation and National Heart, Lung, and Blood Institute/National Institutes of Health grants (to T.B.K.).

Note added in proof. Sato *et al*. (25) and Weaver and Krasnow (26) reach similar conclusions.

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