Transcriptional regulation of \textit{string} (\textit{cdc25}): a link between developmental programming and the cell cycle

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SUMMARY

During postblastoderm embryogenesis in \textit{Drosophila}, cell cycles progress in an invariant spatiotemporal pattern. Most of these cycles are differentially timed by bursts of transcription of \textit{string} (\textit{cdc25}), a gene encoding a phosphatase that triggers mitosis by activating the Cdc2 kinase. An analysis of \textit{string} expression in 36 pattern-formation mutants shows that known patterning genes act locally to influence \textit{string} transcription. Embryonic expression of \textit{string} gene fragments shows that the complete pattern of \textit{string} transcription requires extensive \textit{cis}-acting regulatory sequences (>15.3 kb), but that smaller segments of this regulatory region can drive proper temporal expression in defined spatial domains. We infer that \textit{string} upstream sequences integrate many local signals to direct \textit{string}'s transcriptional program. Finally, we show that the spatiotemporal progression of \textit{string} transcription is largely unaffected in mutant embryos specifically arrested in G\textsubscript{2} of cycles 14, 15, or 16, or G\textsubscript{1} of cycle 17. Thus, there is a regulatory hierarchy in which developmental inputs, not cell cycle inputs, control the timing of \textit{string} transcription and hence cell cycle progression.

Key words: \textit{string}, \textit{cdc25}, \textit{Drosophila}, pattern formation

INTRODUCTION

From the past 15 years of genetic and molecular studies of \textit{Drosophila}, a molecular description of the initial stages of embryonic pattern formation has emerged (see Lawrence, 1992; Bate and Martinez-Arias, 1993, for reviews). A cascade of genetic interactions directs the expression of numerous transcription factors in localized patterns in the blastoderm stage embryo and these transcription factors direct subsequent morphogenesis in the regions where they are expressed. But how do localized transcription factors direct the events of morphogenesis? Neither the molecules nor the mechanisms involved are known. We have addressed this question by examining the mechanisms directing one important aspect of morphogenesis, namely embryonic cell proliferation.

In \textit{Drosophila}, mechanisms of cell cycle control change as development progresses. The first 13 embryonic cell cycles are rapid and synchronous, and are driven by a maternally installed mechanism that does not require zygotic gene expression (Foe et al., 1993; Edgar et al., 1994). In cycle 14, decay of a maternal message that encodes the Cdc2 activator, String (\textit{Cdc25}), leads to a transient arrest of the cell cycle in G\textsubscript{2} (Edgar and O'Farrell, 1989; Edgar et al., 1994). The next three cell cycles are controlled at the G\textsubscript{2}/M transition by the availability of zygotically produced String (Edgar and O'Farrell, 1990). In cycle 17 many of the embryonic cells arrest in an extended G\textsubscript{1} (or G\textsubscript{0}) phase (Edgar and O'Farrell, 1990) due to the down-regulation of a G\textsubscript{1} cyclin, cyclin E, which is required for DNA synthesis (Knoblich et al., 1994). Here, we focus on the three G\textsubscript{2}-regulated cell cycles that occur between removal of maternal \textit{string} function in cycle 14 and the introduction of this new rate-limiting step in cycle 17. These cell cycles progress according to an intricate spatiotemporal pattern that is essentially invariant and appears to be under genetic control (Fig. 1; Hartenstein and Campos-Ortega, 1985; Foe, 1989; Foe and Odell, 1989; Arora and Nusslein-Volhard, 1992). Three observations indicate that the pattern of these cycles is directed by modulated expression of \textit{string}. First, loss-of-function \textit{string} mutants are blocked in interphase 14 (Edgar and O'Farrell, 1989); second, ectopic \textit{string} expression is sufficient to drive ectopic cell divisions during cycles 14, 15 and 16 (Edgar and O'Farrell, 1990); and third, \textit{string} is normally expressed in a dynamic spatiotemporal pattern that is identical to the mitotic pattern, but precedes it (Edgar and O'Farrell, 1989; Fig. 2).

This experimental evidence is further supported by biochemical characterization of \textit{string} and its influence on cell cycle regulators. \textit{string} encodes a conserved Cdc25 type tyrosine phosphatase that triggers mitosis by dephosphorylating and activating the mitotic kinase, Cdc2 (Edgar and O'Farrell, 1989; Kumagai and Dunphy, 1991; Gautier et al., 1991; Edgar et al., 1994). Active Cdc2 phosphorylates many substrates in the cell, directly catalyzing mitotic events such as chromatin condensation, nuclear envelope breakdown and spindle formation (Nigg, 1993). Other factors required for cell proliferation, including Cdc2 itself, its cyclin cofactors and factors required for DNA replication, are expressed in excess in the embryo during cycles 14-16 and are not rate-limiting for cell cycle progression (Lehner and O'Farrell, 1989; 1990a, b;
serve as the interface between the pattern-formation gene network and cell cycle control. These sequences integrate numerous position-specific signals to generate the complex spatiotemporal program of *string* transcription and this in turn executes the pattern of embryonic cell divisions.

**MATERIALS AND METHODS**

### Analysis of string expression and cell division patterns

In situ hybridization was done with digoxigenin-labeled DNA probes (Boehringer Mannheim) essentially as described by Tautz and Pfeifle (1989) and in Ashburner (1989). Our major modification was that the template used for the *string* DNA probe was size-reduced to less than 200 bp to improve the signal and reduce background staining. Size reduction of a gel-purified 2.3 kb fragment of the *string* cDNA was accomplished by digestion with a cocktail of six restriction enzymes, and digoxigenin labeling by the random primer reaction was done at 22°C for 16 hours. *string* protein expression patterns were visualized using an affinity-purified rabbit polyclonal antibody (AP4; Edgar et al., 1994), preabsorbed goat anti-rabbit-biotin secondary antibodies (Jackson Labs) and streptavidin-HRP (Chemicon) according to standard protocols (Ashburner, 1989). In vivo labeling with BrdU was used to assay cell cycle patterns according to Bodmer et al. (1989) and Edgar and O’Farrell (1990).

### Mutants

Pattern-formation mutants were obtained in large part from the Mid-America Drosophila Stock Center. For most lines, mutant embryos were identified by characteristic defects in morphology and *string* expression. In some cases, lines carrying a *lacZ* marked balancer chromosome were stained for both *string* and *lacZ* RNA, and mutant embryos were identified by the absence of *lacZ* expression. *string*, *cyclinA* and *cyclinA*, *cyclinB* double-mutant embryos (*stg* */stg* */stg* ) and *string*, *cyclinA* neotic and *cyclinB* double-mutant embryos (*stg* */stg* */stg* ) were identified by their lack of mitotic figures and abnormally low cell densities for morphological stage. *cyclinE* deficient embryos were generated by the cross: *Df(cycE)GW1*/*CyO* *wg-lacZ* × *Df(cycE)GW3*/*CyO* *wg-lacZ*. Transheterozygosity for these two deficiencies deletes only the *cyclinE* gene (Knoblich et al., 1994; R. Duronio, personal communication). Transheterozygotes were identified by the absence of *wg-lacZ* expression from the balancer. In addition, we analysed *string* expression in *cycE* / *cycE* / *wg-lacZ* mutant embryos derived from *l(2)DA4B5* pr en *w* */w* bw/CyO *fit-lacZ* parents (see Knoblich et al., 1994). These homozygous embryos were identified by the absence of *fit-lacZ* expression from the balancer, with similar results.

### Cloning and transformation

A 10.5 kb *string* transgene was isolated from a phage lambda clone (HF2A), cloned into the Carnegie 20 transformation vector and three independent lines with integrations on chromosome 2 were generated by standard P-element-mediated germline transformation (Spradling, 1986). Expression patterns and rescue of this fragment were assessed in embryos homozygous for a transcription-null allele at the *string* locus (genotype *P[(w+)stg15.3]* / *stg15.3*). The 31.3 kb transgene was isolated from a genomic cosmid library (NotBamNot-CoSpeR provided by J. W. Tamkun) and two independent transformant lines, one on chromosome 2 and one on chromosome 3, were isolated by P-mediated transformation. Expression patterns and rescue were assessed in the genotypes *P[(w+)stg31.3]* / *stg31.3* and *P[(w+)stg31.3]* / *stg31.3* / *P[(w+)stg31.3]*. The 15.3 kb transgene was isolated as a *SalI* fragment from the 31.3 kb clone, inserted into CaSpeR and three independent lines on the X chromosome were generated. Expression patterns and rescue were assessed in the genotypes *P[(w+)stg15.3]* / *P[(w+)stg15.3]* / *stg31.3* / *stg31.3*.
P-element excision deletions

We generated 225 w- revertants by excision of P[w+ AA53] (Fig. 4). This insertion is homozygous viable and has only minor effects on string expression patterns, but is 90% lethal as homozygous alleles of string (stgAA1 and stgB). Eight stg- mutants (stgAR1-8) were then identified among these w- revertants by their failure to complement the null allele stgAA1. Lesions at the string locus in these mutants were analyzed by genomic Southern blotting using probes covering the P element and much of the string genomic region (−28.6 kb to +4.5 kb). Probes were generally the EcoRI fragments shown in Fig. 4 and genomic DNA was likewise restricted with EcoRI. To unambiguously map the deletions in stgAR2 and stgAR5, genomic DNA was extracted from about 0.5 ml of homozygous mutant embryos. These embryos were generated by collecting eggs from the cross allowing the eggs to age for 36 hours and then collecting the unhatched embryos. Heterozygous and wild-type larvae (from hatched eggs) were effectively removed from the egg collection plates by placing yeast paste, into which larvae crawl, at the edge of the plate and replacing it three times. This procedure allowed the analysis of purely mutant DNA, greatly simplifying mapping of the deletions. Southern blots were done using digoxigenin-labeled DNA probes, Hybond-N membranes (Amersham) and Lumiphos chemiluminescent detection, according to the manufacturer (Boehringer Mannheim).

RESULTS

The dynamic program of string transcription

The embryonic expression pattern of string mRNA, assayed by in situ hybridization, is shown in Fig. 2. Although the pattern is too intricate to be described exhaustively here, several aspects are noteworthy. After the degradation of maternal string transcripts during early interphase 14 (Fig. 2A,B), expression occurs in a sequence of brief pulses that are timed differently in different regions of the embryo. The first wave of pulses precedes and matches the cycle 14 mitotic domains (MDs) mapped by Foe (1989; compare Figs 1 and 2C-G, M-Q). The order of string mRNA appearance generally corresponds to the order of mitoses, but shows deviations in some tissues. For instance, expression in MD10 (the mesoderm) precedes expression in MD2 (part of the head) by at least 5 minutes, even though mitosis in MD10 follows mitosis in MD 2 by 10 minutes (compare Fig. 2M and N). However, accumulation of transcripts proceeds more slowly in MD10 than in MD2, suggesting that, in addition to the time of initiation of string transcription, string RNA level is an important parameter in timing mitosis. The three postblastoderm cell cycles (14, 15 and 16) are regulated in three distinct, though related, spatial patterns. Consistent with its role in governing these cycles, string RNA accumulates prior to each division in patterns that anticipate the division patterns (Fig. 2).

During cycles 14 and 15 string transcription in most MDs ceases immediately following mitosis and does not resume until just prior to the next mitosis, 40-100 minutes later. In contrast, string mRNA is not rapidly down-regulated following mitosis 16. Instead, high levels of RNA persist in virtually all external tissues for about 2 hours after mitosis (stages 11-13; Fig. 2K,U,V). Expression is then extinguished in the epidermis (which has ceased proliferation) and is maintained only in proliferating cells of the peripheral and central nervous systems (Fig. 2L,W,X). string RNA expression in proliferating neuroblasts of the CNS would appear to be periodic, since not all of the neuroblasts express string at any given time. As neuroblasts cease proliferation they too extinguish string RNA expression (not shown).

string transcription is controlled by pattern-formation genes

The cycle 14 mitotic domains coincide precisely with features of the blastoderm fate map (Foe, 1989) and embryos with mutations in the pattern-formation genes that generate this fate map have characteristic defects in mitotic patterns (Foe and Odell, 1989; Rushlow and Arora, 1990; Arora and Nusslein-Volhard, 1992). Since mitotic patterns are executed by regulated transcription of string and since many of the pattern-formation genes encode position-specific transcription factors, we have proposed that string transcription is controlled, directly or indirectly, by pattern-formation gene products (Edgar and O’Farrell, 1989; O’Farrell et al., 1989).

To investigate this possible connection, we visualized string mRNA and protein patterns in a representative collection of pattern-formation mutants. Almost without exception, these mutations alter string expression in the regions where they affect cell fate (see Fig. 3 and Table 1 for summary data). In some mutants (such as twi, sna and btd), string expression is cleanly deleted in a specific domain that corresponds, spatially and temporally, to the normal expression of the mutant gene. This is consistent with direct regulation. In other mutants (such as bcd, hb and Kr), string expression patterns are not deleted, but are globally distorted. This suggests indirect, combinatorial, or concentration-dependent regulation. The earliest onset of abnormal expression in the mutant embryos is also informative. For instance, the pair-rule periodicity of string expression in MD11 (Fig. 2N; lateral epidermis) is not significantly affected in pair-rule mutants, but is altered in gap mutants (Fig. 3). Pair-rule mutants first show defects in string expression in late cycle 14, in the segmentally reiterated patterns of MDs 16, 17 and 21. Likewise, segment-polarity genes, homeotic genes and genes involved in neural patterning do not have significant effects until late cycle 14, in the intricately patterned ventral neurogenic region (MDs 16, 17, 21, 25, N and M). Such observations indicate that string is a sophisticated pattern integrator that responds to genes at all levels of the pattern-formation network.

Extensive regulatory sequences at string contain separable position-specific elements (PSEs)

To assess the size of the string gene, we isolated large fragments of the locus, introduced these into flies via P-element-mediated germline transformation, and tested their ability to rescue mutations of string. Fragments of approximate sizes 10.5 kb, 15.3 kb and 31.3 kb that included the complete 3.4 kb string transcription unit were tested (Fig. 4). Each of these fragments provided string function, as assessed by restoration of some cell division to string null mutants (stg-6, stgAR2 and stgAA1). Nevertheless, none of these transgenes restored cell division to all mitotic domains, nor viability to string null mutants, and thus none appears to include all the essential regulatory sequences.

The patterns of string RNA expressed by these string transgenes were assessed following crosses to introduce each transgene into embryos homozygous for transcription-null alleles of stg (stgAA1 or stgAR2). The largest transgene (stg-31.3
The dynamic expression of *string* mRNA in wild-type embryos. Embryos were in situ hybridized with a digoxigenin-labeled *string* cDNA probe, which is detected immunohistochemically giving a purplish stain (see methods). The embryos were also stained for DNA with Hoescht 33258 and photographed with simultaneous bright-field and UV illumination to highlight nuclei (light blue) and morphology. A sequence of increasing ages is shown from ventral (A-L) and lateral (M-X) views. Approximate stages (upper right corners) and age in min AED at 25°C (lower right corners) are noted in each panel. Stages and timing are according to Campos-Ortega and Hartenstein (1985); times noted are not terribly accurate: ±15 minutes to stage 10 and at least ±30 minutes thereafter.

(A) Maternal expression in a cycle 12 embryo; (B) lack of expression during early interphase 14 and residual maternal RNA in the pole cells; (C) cycle 14 expression in MDs 1-10; (D) expression in MDs 1-17; (E) expression in MDs 1-21; (F) cycle 14 expression in MD N and MD 25, cycle 15 expression in the head and lateral ectoderm; (G) cycle 14 expression in MD M, and cycle 15 expression in the head and lateral epidermis; (H) further cycle 14 expression in MD M, and cycle 15 expression in the head and lateral epidermis; (I) cycle 15 (?) expression in the ventral neurogenic region; note lack of expression in lateral epidermis; (J) cycle 16 expression in the tracheal placodes, cycle 15 (?) expression in the VNr; (K) cycle 16 expression in the epidermis, lack of expression in the salivary placodes; (L) post cycle 16 expression in the CNS, PNS and brain.
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Fig. 2. (M) very early cycle 14 expression in MDs 10 and 4; (N) cycle 14 expression in MDs 1-11; (O) expression in MDs 1-18; (P) expression in MDs 10-21; (Q) cycle 14 expression in MD N, MD 25 and early cycle 15 expression in the lateral epidermis; (R) cycle 15 expression in the head and the lateral epidermis; (S) cycle 15 (?) expression in the VNr; note lack of expression in lateral epidermis; (T) cycle 16 expression in the tracheal placodes, expression also in the VNr and head; (U) early cycle 16 expression in the epidermis; (V) expression in the epidermis continuing during G1 of cycle 17; (W) partial loss of expression in the epidermis, continued expression in the brain, CNS and PNS, and dorsal vessel; (X) expression in the brain and CNS.
occurs in the tracheal placodes, many neuroblasts in the head and the ventral nerve cord, and in a few external cells of the head (Fig. 5D-F). Expression in the epidermis, which is strong and persistent from the endogenous gene after mitosis 16 (Fig. 2K,V), is not observed from stg-31.3 kb (Fig. 5F). During later embryogenesis, the 31.3 kb transgene continues to be expressed in many of the cells that normally continue dividing, namely neuroblasts in the peripheral nervous system, the ventral nerve cord and the brain (Fig. 5G). Analysis of String protein, mitotic figures and BrdU incorporation patterns in the transgenic, stg− mutants confirmed that protein expression, mitoses and S phases occur in all regions exhibiting string mRNA expression (not shown).

Removal of 16 kb of 3′ sequences from the 31.3 kb transgene, producing the stg-15.3 kb transgene (Fig. 4), did not alter the expression patterns described above in any detectable way. While it remains possible that these 3′ sequences contain redundant transcriptional elements, or that there are regulatory sequences lying more than 16 kb 3′ of the gene, we suggest that few, if any, string regulatory sequences lie 3′ to +4.5 kb (region E, Fig. 4).

In contrast, removal of 4.8 kb of 5′ sequences, producing the stg-10.5 kb transgene, greatly restricted expression. stg-10.5 kb expression is limited to cycles 14 and 15 and occurs only in the ventral neurogenic region (MDs 16, 17, 21, N; Fig. 6A). We detected no strong expression in any of the other MDs that are driven by stg-31.3 kb or stg-15.3 kb. Thus, we infer that the position-specific elements (PSEs) responsible for expression in these other MDs reside between −5 kb and −9.8 kb (region B, Fig. 4). We could not assess expression in the mesoderm (MD 10) due to interference from a mesodermal enhancer of a rosy gene that marked the stg-10.5 kb transgene (but see below).

Deletion mutations in string further localize the PSEs

P[w+ AA53] is a 12 kb transposon inserted 2.1 kb upstream of the string transcription start site (Fig. 4). It is homozygous viable and retains substantially normal spatial patterns of string expression, though it is semilethal in trans to string null alleles. Since P[w+ AA53] lies between many of the string PSEs and the basal string promoter, correct spacing of these PSEs relative to the promotor is evidently not crucial for their function.

As an alternative method for dissecting the string regulatory region, we generated deletion mutations in vivo by excision of the P[w+ AA53] transposon. One of these (stgAR2) is deleted for the entire transposon and downstream sequences, including the string transcribed region. Not surprisingly, this mutant produces no string transcripts. A more pertinent mutation, stgAR5, deletes all of P[w+ AA53] and sequences extending upstream to at least −28.6 kb. This deletion eliminates string expression in almost all tissues: cycle 14 expression is maintained only in the mesoderm (MD 10) and invaginated regions of the head (MDs 8, 15), which lie next to the mesoderm and form the anterior midgut (Fig. 7B,C). During later stages, we noted weak, uniform expression throughout the embryo and scattered high level expression in a few randomly located cells. In vivo labeling with BrdU confirmed that the mesoderm underwent two postblastoderm cell cycles in stgAR5/stgAR5 mutant embryos (Fig. 7D). In addition, scattered internal cells,
perhaps mesodermal, incorporated BrdU at later stages, as did a few cells in the lateral epidermis. These latter cell cycles appeared to be driven by very low levels of *string* expression and may not reflect normal patterning. Since the 31.3 and 15.3 kb transgenes also exhibited mesodermal expression, we conclude that a mesoderm PSE is located between −2.1 kb and +4.5 kb (Fig. 4, region D).

In summary, our experiments define four *string* PSEs that drive transcription in distinct sets of cells. These regulatory regions are denoted A-D in Fig. 4 and their inferred functions are listed in Table 2. Region D contains a mesoderm PSE, region C contains an early acting ventral neuroectoderm PSE, region B contains a PSE that acts in a number of cell types in the head, the nervous system and the trachea, and region E appears to be beyond the boundary of the *string* locus. By elimination, we assign all unidentified PSEs to region A, upstream of tested sequences. Nevertheless, it is possible that we have missed PSEs within the tested region due to weak expression or disruption by breakpoints. We also note that, since we assessed expression in embryos that lacked cell proliferation in certain tissues, the late-stage expression patterns we saw may have had some abnormalities. However, the PSE locations we infer from these studies correlate well with the locations being mapped by an independent method, using *string-lacZ* reporter gene fusions (B. A. E. and D. A. L. unpublished).

**The cell cycle influences deactivation, but not activation, of *string* transcription**

In *Drosophila*, progress of the developmental program continues quite normally even after the cell cycle is blocked (see Hartenstein and Posakony, 1990; Gould et al., 1990). Since *string* transcription is evidently driven by developmental regulators, we expected that progression of the *string* transcription program would be cell cycle independent. Nevertheless, the coincidence of *string* transcriptional shut off with passage through mitoses 14 and 15 suggests that the cell cycle does have a role in orchestrating *string* expression patterns (see above). To test the influence of cell cycle progression on *string* transcription, we studied embryos arrested by different cell cycle mutations. Maternal supplies of the various cell cycle regulators decay with different kinetics and thus mutations in these regulators arrest the cell cycle at different developmental stages. *string* mutants arrest in G2 of cycle 14 (Edgar and O’Farrell, 1989), *cyclin A, cyclin B* double-mutants arrest in G2 of cycle 15 (Knoblich and Lehner, 1993), *cyclin A* single mutants arrest in G2 of cycle 16 (Lehner and O’Farrell, 1989) and *cyclin E* mutants arrest in G1 of cycle 17 (Knoblich et al., 1994).

In embryos arrested in G2 of cycle 14 by *stg*7B, an EMS-induced allele that produces an inactive protein but has no deficit in RNA expression, *string* transcription commences in a normal sequence of mitotic domain patterns, but is not extin-

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**Table 1. Effects of pattern gene mutations on *string* expression**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Tissue specification</th>
<th>Earliest noted effects on <em>string</em> expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X bcd+</td>
<td>A/P axis</td>
<td>Expansion of all head MDs, contraction of all abdomen MDs.</td>
</tr>
<tr>
<td>bcdE1</td>
<td>A/P axis</td>
<td>Contraction of all head MDs, duplication of MD4 in anterior.</td>
</tr>
<tr>
<td>dll</td>
<td>D/V axis</td>
<td>Homogeneous pattern along D/V axis.</td>
</tr>
<tr>
<td>torXR1</td>
<td>Maternal: embryo poles</td>
<td>Deletion/contraction of head and tail MDs 1,3,4.</td>
</tr>
<tr>
<td>hsp25</td>
<td>Gap: head and thorax</td>
<td>Anteriorward compression of head MDs 1,2,3,5, fusion of anterior two sections of MD11 w/MD6.</td>
</tr>
<tr>
<td>Kr256</td>
<td>Gap: thorax and abdomen</td>
<td>Compression and fusion of head and thorax MDs 2,5,6,11.</td>
</tr>
<tr>
<td>kni5H</td>
<td>Gap: abdomen</td>
<td>MD11 reduced from 5 to 3 repeated units.</td>
</tr>
<tr>
<td>gt37</td>
<td>Gap: head/abdomen</td>
<td>Morphological distortions in head and abdomen at early germ-band extension, <em>string</em> MDs accordingly distorted.</td>
</tr>
<tr>
<td>tll</td>
<td>Gap: head/tail</td>
<td>Global distortions of head MDs, deletion of tail MD4.</td>
</tr>
<tr>
<td>dpg48</td>
<td>Gap: head/abdomen</td>
<td>Dorsal fusion of MD1 and of MD5, MD11 weak, delayed, or absent.</td>
</tr>
<tr>
<td>zen7</td>
<td>Dorsolateral</td>
<td>Fusion/expansion of MD1 across dorsal midline.</td>
</tr>
<tr>
<td>twi1096</td>
<td>Ventral mesoderm</td>
<td>Deletion of MD10 (the mesoderm).</td>
</tr>
<tr>
<td>snm1605</td>
<td>Ventral mesoderm</td>
<td>Ventral fusion of MD2 and MD4, MD10 (mesoderm) deleted, MD14 (mesectoderm) expanded to cover ventral midline.</td>
</tr>
<tr>
<td>ems7D99</td>
<td>Head/tail pattern</td>
<td>Narrowing or deletion of MD2.</td>
</tr>
<tr>
<td>btkA1</td>
<td>Head pattern</td>
<td>Deletion of MD2, fusion of MD8 with MD14.</td>
</tr>
<tr>
<td>Sce7F28</td>
<td>Head pattern</td>
<td>Defects in MD21 and MD N in labial and T1 segments.</td>
</tr>
<tr>
<td>b7M</td>
<td>Pair rule: segmentation</td>
<td>Pair-rule fusions in MD16, 17, 21, N, 25.</td>
</tr>
<tr>
<td>run3E96</td>
<td>Pair rule: segmentation</td>
<td>Pair-rule fusions in MD16, 17, 21, N, 25.</td>
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<tr>
<td>eva1D19</td>
<td>Pair rule: segmentation</td>
<td>Pair-rule fusions in MD21 MDN, and MD25.</td>
</tr>
<tr>
<td>ftcVal2</td>
<td>Pair rule: segmentation</td>
<td>Pair-rule fusions in MD21 MDN, and MD25.</td>
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<tr>
<td>enhC41</td>
<td>Segment polarity</td>
<td>Segmental defects in mitosis 15 and mitosis 16.</td>
</tr>
<tr>
<td>wgcX41</td>
<td>Segment polarity</td>
<td>Segmental defects in mitosis 15 and mitosis 16.</td>
</tr>
<tr>
<td>nkdE7</td>
<td>Segment polarity</td>
<td>Segmental defects in mitosis 15 and mitosis 16.</td>
</tr>
<tr>
<td>sc587</td>
<td>Ventral ectoderm</td>
<td>Disorganization of MD 21, N, M.</td>
</tr>
<tr>
<td>da2116</td>
<td>Proneural</td>
<td>Reduction in number of expressing cells in MD 21, N, M, reduction in CNS and PNS thereafter.</td>
</tr>
<tr>
<td>N08</td>
<td>Neurogenic</td>
<td>Reduction in MD M expression, disorganized expression in VNr (Ventral Neurogenic region) thereafter.</td>
</tr>
<tr>
<td>E/Spl1</td>
<td>Neurogenic</td>
<td>Reduction in number of expressing cells in MD 21, N, M, reduction in CNS expression and elimination of PNS expression thereafter.</td>
</tr>
<tr>
<td>nesX90</td>
<td>Neurogenic</td>
<td>Overexpression in VNr beginning in cycle 15.</td>
</tr>
</tbody>
</table>

Mutants with no noted defects in cycle 14: *sim*99, otdDM1, *fkh*200-17, *Dfd R*, *spi A14*, *sal 11A55*, *Antp RW10*, *BX-C Df(P9),Acts 58*. 

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mitoses 14, 15 and 16 (Fig. 8A,B). This persistent expression could be extinguished by delivering active String from an inducible HSP70-string fusion gene and triggering mitosis (not shown, see Edgar and O’Farrell, 1990). Thus, string activity, or its consequence (Cdc2 activation and mitosis), contributes to the shut-off of string transcription in the ectoderm at the close of cycle 14.

While mitosis extinguishes string transcription in many of the cycle 14 expression domains, it is not uniformly essential. Many cells in the heads of arrested stg7B embryos extinguish transcription normally, at the time corresponding to interphase of cycle 15 (Fig. 8A; arrow), and additional cells shut-off expression at the time of cycle 16 (Fig. 8B; arrows). Moreover, the inactivation of transcription during the interval corresponding to interphase 17 is normal in stg7B embryos: inactivation starts in the dorsal epidermis, spreads to cells of the presumed PNS and finally encompasses the ventral nerve cord (Fig. 8C,D). At very late stages (after stage 16), stg7B mutant embryos maintain expression in only a few cells of the rudimentary brain, just as do wild-type embryos (compare Fig. 2X to Fig. 8D). Thus the developmental programing of later transcriptional activations and inactivations continues even though the timing of the initial shut-off is disturbed.

Mitosis plays a minor role in extinguishing string transcription after cycle 14. In cycle 15-arrested, cyclin A, cyclin B mutants, we noted prolonged expression of cycle 15 string RNA patterns in some parts of the lateral epidermis and the ventral neurogenic region during stages 9-11. However, most cells shut off their cycle 15 expression patterns with near normal timing (Fig. 8E; arrows), and essentially all cells activate and inactivate cycle 16 expression patterns correctly (Fig. 8F,G). In embryos arrested in G2 of cycle 16 by cyclin A mutations, the shut off of string expression after arrest is also essentially normal, as is continued expression in the brain and CNS (not shown). Likewise, embryos arrested in G1 of cycle 17 by mutations in the cyclin E gene (Knoblich et al., 1994) show continued string expression after the cell cycle arrest. This is limited to neuroblasts of the peripheral nervous system,

Fig. 4. A physical map of string locus. The 50 kb chromosome walk of the string locus is shown to scale (above), with position 0 at the transcription start site of string and the positions of restriction enzyme cleavage sites (E, EcoRI; S, SalI). Transcribed regions inferred from northern blot experiments (bold lines) are indicated above the restriction map. The string intron is stippled. The transcription unit downstream of string, pathless (ptl), appears to be involved in neural development (E. Giniger, personal communication) and the upstream transcription unit remains unidentified. Below the restriction map, we show the position of the transposon P[w+ AA53] and the two deletion mutants (AR5, AR2) generated by its excision. The presence of all sequences except for those downstream of +4.5 kb (striped arrows) were tested by Southern blotting. The DNA remaining in the mutants is indicated by solid arrows and the deleted DNA is not shown. Below this, the extent of the 10.5 kb, 15.3 kb and 31.3 kb gene fragments tested as transgenes is indicated. The endpoints of the deletions and transgenese divide the string locus into five regions (A-E) that were tested for regulatory function. We find that regions A-D contain distinct position-specific elements (PSEs), whereas region E appears to be unrelated to string function. The inferred activities of these regions are listed in Table 2.

Table 2. Position-specific elements of the string locus

<table>
<thead>
<tr>
<th>DNA REGION (Fig. 4)</th>
<th>A*</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>Lateral epidermis</td>
<td>Head</td>
<td>Ventral neurogenic</td>
<td>Mesoderm</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>(MD14,16, 7, 11, 19)</td>
<td>(MD14,15,1, 2, 3, 20) 23, 24</td>
<td>(MD14,16, 17, 21, N)</td>
<td>(MD14,15 10)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>(MD14,16, 7, 11, 19)</td>
<td>(MD14,15,1, 2, 3, 20) 23, 24</td>
<td>(MD14,16, 17, 21, N)</td>
<td>(MD14,15 10)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>Mesectoderm</td>
<td>Ventral neuroblasts</td>
<td>Tracheal placodes</td>
<td>Anterior midgut</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>(MD14,14)</td>
<td>(delaminated, M15,16)</td>
<td>(M15,16)</td>
<td>(MD17,15)</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>Tail</td>
<td>Tracheal placodes</td>
<td>(M15,16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>(MD14,12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Head</td>
<td>PNS</td>
<td>Ventral neurogenic</td>
<td>Ventral nerve cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(MD14, 5, 9)</td>
<td>(7-9 hrs AED?)</td>
<td>(MD14, 25, M)</td>
<td>(8-12 hrs AED)</td>
</tr>
</tbody>
</table>

*Domains listed in region A were inferred by negative deduction only; that is, by their lack of expression from the 31.3 kb, 15.3 kb, 10.5 kb transgenese or in the stg4AE5 mutant.
Transcriptional regulation of string brain and ventral nerve cord, where proliferation continues after cycle 17 in wild-type embryos. We did note, however, that fewer neuroblasts express string in these late-stage cyclin E mutant embryos than in wild-type embryos (Fig. 8H). We conclude that, while mitosis or an associated S phase event may contribute to the abruptness of the shut-off of string expression, this effect is rather slight except in some of the cycle 14 mitotic domains. Importantly, there seems to be little influence of the cell cycle on the periodic activation of string transcription.
Since the isolation of a large collection of pattern mutants in the early 1980s (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984), studies in Drosophila have revealed the mechanisms that specify positional information during early embryogenesis (see Lawrence, 1992; Bate and Martinez-Arias, 1993, for reviews). Despite this, our understanding of how positional information is translated into cell behaviors, and thus into morphogenesis, remains rudimentary. Here we address how embryonic positional information is used to orchestrate one fundamental aspect of morphogenesis, namely patterns of cell proliferation. During much of Drosophila embryogenesis cell proliferation is regulated by the expression of string, which encodes a conserved Cdc25-type phosphatase that activates Cdc2 (Edgar and O’Farrell, 1989; Millar and Russell 1992; Edgar et al., 1994). string expression is controlled at the transcriptional level and we show here that it is patterned by positional information supplied by a large set of genes that determine many aspects of cell fate (see also Foe and Odell, 1989; Rushlow and Arora, 1990; Arora and Nusslein-Volhard, 1992). Extensive (>15.3 kb) regulatory sequences of string integrate this positional information to generate the complex patterns of string transcription that execute the mitotic program.

Spatial regulation of string

The regulatory DNA of string contains separable position-specific elements (PSEs) that drive transcription in specified regions of the embryo at specified times (Fig. 4; Table 2). We have defined four such PSEs, but these are large, and in some cases multifunctional, and we expect that each one is a conglomeration of smaller, more specific PSEs. Like the control regions of several pattern-formation genes that have been studied in detail, string’s control region appears to be a patchwork of elements that can function independently and which, when summed, generate the overall expression pattern (see Small and Levine, 1991; Pankratz and Jäckle, 1993; for reviews).

Perturbations of string expression in mutant backgrounds indicate that string’s expression program depends on known patterning genes, many of which encode position-specific regulators of transcription (Fig. 3; Table 1). For example, twist and snail mutants fail to express string specifically in the mesoderm (MD10) and buttonhead mutants fail to express string specifically in a single stripe in the head (MD2). Since twist, snail and buttonhead encode transcription factors that are expressed specifically in MD10 (twi and snl) and MD2 (btd), they may be direct effectors of string transcription in these domains (see Thissel et al., 1987, 1988; Boulay et al., 1987; Wimmer et al., 1993). Our mapping indicates that the MD10 and MD2 PSEs reside in regions B and D of Fig. 4, and thus we might expect to find binding sites for these factors in these regions.

Although these examples suggest simple regulation, complex regulatory relationships are indicated as well. For example, string expression in MD11 (dorsal ectoderm) shows a transient pair-rule periodicity (Fig. 2N), but this pattern is not significantly affected by mutations in pair-rule genes. Rather, MD11 expression is affected by mutations in an earlier-acting set of genes, the gap genes (Fig. 3; Table 1). Thus string expression in MD11 is probably regulated by combinations of gap-gene products, in a concentration-dependent manner, in the same fashion that pair-rule genes are regulated (Small and Levine, 1991; Small et al., 1992; Pankratz and Jäckle, 1993). Expression of string in MD14 (the mesectoderm) provides another example of independent regulation of related patterns. Although string expression in MD14 is precisely coincident with expression of single-minded, a transcription factor involved in mesectoderm specification (Fig. 2E; Nambu et al., 1991), string expression is unaffected by single-minded mutations. We presume that string and single-minded are regulated independently, in parallel, by similar mechanisms.

The PSE driving string expression in MD14 most likely responds, like single-minded, to combinations of broadly dis-
Transcriptional regulation of *string* 3141

...ributed dorsoventral pattern gene products to produce its highly restricted expression pattern (see Kasai et al., 1992).

Patterning genes that encode cell signaling molecules presumably influence *string* transcription by altering activity or expression of a transcription factor. For example, *dpp*, which encodes a TGF-β type signaling molecule, is known to regulate the spatial patterns of *twist* and *zen*, two transcription factors shown to alter *string* expression. Similarly, neurogenic genes such as *Notch* are likely to exert their influence on *string* by alterations in the activity of the helix-loop-helix proteins encoded by the *achaetescute* complex.

Given the complexities of integrating positional information, it seems likely that different PSEs that share the same spatio-temporal response have a common evolutionary origin. Perhaps the *string* PSEs arose as regulators of other genes and were spliced into *string* piecemeal during evolution. In this regard, it is interesting to consider that *string* homologues appear to regulate G2/M transitions in eukaryotic cells ranging from yeast to humans and where it has been studied, this regulation occurs at the transcriptional level (Moreno et al., 1990; Sadhu et al., 1990; Kakizuka et al., 1992). Thus, like its catalytic function, *string*’s mode of responding to positional information through complex transcriptional control may be evolutionarily conserved.

**Temporal regulation of *string***

Studies of the cell cycle in cleavage-stage frog and marine invertebrate embryos led to the suggestion that cell cycle timing is governed by an autonomous cell cycle oscillator. While this may be an accurate characterization of the relentless progress of some early embryonic divisions, ultimately, embryogenesis requires the coordination of cell proliferation with other aspects of morphogenesis (see Edgar and O’Farrell, 1990). Although this coordination might be achieved through modulation of the rate of an autonomous oscillator, the *Drosophila* embryo appears to follow a different alternative. Its cell cycle oscillator is interrupted by loss of one oscillator component, String, after mitosis 13 (Edgar et al., 1994). Subsequently, developmental regulators determine the timing of *string* transcription and thus control progression of the cycle until removal of a second oscillator component, cyclin E, leads to a G1 arrest in cycle 17, or later (Knoblich et al., 1994).

The constellation of stage- and position-specific transcription factors that regulate *string* control multiple aspects of cell fate and are not specialized exclusively for control of the cell cycle. For this reason, we were not surprised to find that *string* transcription continues to be periodically activated and inactivated according to normal spatial patterns even after the embryonic cell cycle is arrested. The independence of *string*’s transcription program from cell cycle progression is most clearly documented by our finding that arrest in cycles 15, 16 or 17, (achieved by the various *cyclin* mutations) causes little perturbation in the dynamics of *string* transcription following arrest (Fig. 8). Thus, like a number of DNA synthesis genes expressed at the G1 to S transition, *string* is not actually a ‘cell

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**Fig. 8.** Expression of *string* mRNA in cell cycle arrest mutants. (A-D) A progression of *string* mRNA expression pattern in stg2B mutants, which arrest in G2 of cycle 14. Red numbers refer to stages and purple numbers are minutes AED. Both are estimates based on morphology. In stg2B mutants many cells show persistent expression through periods when they would normally shut off *string* periodically, but some cells do extinguish expression at the appropriate times (yellow arrows). Following germ band shortening at stage 12, *string* is shut off in the epidermis as in wild-type embryos (C,D). (E-G) *string* mRNA in *cyclin A*, *cyclin B* double mutant embryos, which arrest in G2 of cycle 15. In this case, expression patterns are nearly normal. After a cycle 15 expression, epidermal cells turn off (yellow arrows) and then turn on again to express cycle 16 patterns (treacheal placodes in E and the rest of the epidermis in F). At later stages, CNS cells express *string*, as in wild type (G; compare with Fig. 2L). (H) Expression of *string* protein in a *cyclin E* mutant, arrested in G1 of cycle 17. As in G, expression occurs in CNS neuroblasts despite the cell cycle arrest.
cycle-regulated’ gene in vivo (see Knoblich et al., 1994; Duronio and O’Farrell, 1994).

Although developmental cues are evidently the primary regulators of string expression, we did find that cell cycle progression plays a role in the shut-off of transcription in some cell types at some stages. This influence is most significant in cycle 14. If embryos are arrested in cycle 14 (by a string mutation), a major subset of ectodermal cells fails to deactivate string transcription on schedule (Fig. 8). However, in other regions of the embryo and, at other stages, the timely inactivation of string transcription does not require passage through mitosis. This complexity might be attributed to variation in the stability of the different factors that presumably activate string transcription in different cells at different stages. The transcription complexes that activate string in the ectoderm in cycle 14 may require mitotic phosphorylation or DNA replication to be disrupted and inactivated on schedule. Perhaps because of this, the reprogramming of string transcription in the cycle 14 ectoderm appears to require mitosis. In contrast, later reprogramming events may involve distinct transcriptional complexes that are less stable and do not require passage through the cell cycle for their inactivation. Such complexes might be inactivated by newly expressed repressors, or simply by the cessation of expression of some of their components.

Developmental regulation of effector genes

Finally, we would like to offer a perspective somewhat different than that emphasized in many investigations of the molecular basis of developmental patterning. We suggest that the distinctions between different body structures such as wings and legs are largely organizational, and are not likely to be understood in terms of the induction of distinct tissue-specific gene products. Studies of string may provide a paradigm for another type of developmental control, in which the upstream regulatory regions of a variety of ‘housekeeping’ genes control the spatial and temporal programming of cellular events (such as cell division, cell adhesion or cytoskeletal changes) that have profound, specific effects on tissue organization and structure (see Costa et al., 1994).

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