Activation of transcription in *Drosophila* embryos is a gradual process mediated by the nucleocytoplasmic ratio

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We have observed that zygotic transcription does not initiate at a single point in *Drosophila* embryos. Rather, a gene initiates transcription in a few nuclei of a fraction of embryos. During succeeding cycles, the frequency of transcribing embryos, and of nuclei transcribing in those embryos, gradually increases. For the *fushi tarazu* (*ftz*) gene, the timing of this process is regulated by the concentration of the maternally loaded, repressing transcription factor *tramtrack* (*ttk*). Altering the dose of Ttk protein in embryos shifts the activation of *ftz* transcription either forward or backward during development but does not effect *Krüppel* (*Kr*) activation. We have observed that the transcription of several genes, including *ftz*, is triggered in embryos at a critical nuclear density; therefore, we suggest that titration of transcription factors like *ttk* by the nucleocytoplasmic ratio triggers zygotic transcription in *Drosophila*.

[*Key Words*: *Drosophila* embryogenesis; transcriptional activation; Mid-Blastula Transition; titration of maternal repressor]

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The early stages of development in many animals are characterized by an absence of transcription. The activation of zygotic transcription starts at different developmental stages in different animals. For example, in *Drosophila* embryos, transcription first becomes detectable around the tenth nuclear division or "nuclear cycle" as the embryo is completing the process of nuclear migration (Zalokar 1976; Anderson and Lengyel 1979; Knipple et al. 1985; Weir and Kornberg 1985; Edgar and Schubiger 1986; Erickson and Cline 1993).

Based on these studies, it has been generally assumed that zygotic transcription is initiated in an embryo at a specific stage during development. However, a limitation of these studies is that the assay methods available to detect transcription were either insensitive or did not allow precise staging of embryos. Tautz and Pfeifle (1989) have developed a sensitive whole-mount in situ hybridization technique. Using this technique the sites of gene transcription appear as "nuclear dots," two spheres of staining within nuclei (Shermoen and O'Farrell 1991). For several genes we have demonstrated that transcription is gradually activated. When genes first begin to transcribe during cycles 8, 9, or 10, nuclear dots are observed only in a few nuclei in a small fraction of embryos. During each succeeding nuclear cycle the frequency of nuclei showing nuclear dots gradually increases and more embryos show transcribing nuclei. Finally, after several nuclear cycles, most nuclei in all embryos show nuclear dots.

Before cycle 8, nuclei are migrating from the center of the embryo out to the cortex and the embryo is transcriptionally silent. However, between cycles 8 and 10, the nuclei reach the cortex, complete nuclear migration, and transcription begins. One of several developmental events might trigger the initiation of transcription at this point, including the position of the nuclei (position), the age of the embryo (time), the lengthening of the cell cycle, or the embryo reaching a crucial nuclear density (nucleocytoplasmic ratio) (Edgar and Schubiger 1986; Kimelman et al. 1987; Newport and Kirschner 1982a,b; Brown et al. 1991; Yasuda et al. 1991). To distinguish between these alternatives, we have assayed the effect of a series of experimental and genetic manipulations that alter the timing and/or pattern of nuclear migration on the initiation of transcription of the representative genes *fushi tarazu* (*ftz*) and *Krüppel* (*Kr*). These studies have indicated that a critical nuclear density triggers transcription of *ftz* and *Kr*.

In *Drosophila*, as well as in *Xenopus*, activation of transcription at a specific nuclear density has been interpreted in terms of titration of a maternally loaded, DNA-binding transcriptional repressor by nuclei; at a critical threshold the repressor is titrated out (Newport and Kirschner 1982a,b). A good candidate for a transcriptional repressor of *ftz* transcription is the *tramtrack* (*ttk*) gene. *ttk* is a maternally loaded, alternatively spliced zinc finger transcription factor whose 69-kD form binds

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to the *ftz* promoter and enhancer (Harrison and Travers 1990; Brown et al. 1991; Read and Manley 1992). Ectopic expression of the 69-kD Ttk protein in cellular blastoderm embryos represses *ftz* but not *Kr* expression, whereas deletions of *ttk*-binding sites from a *ftz–lacZ* promoter construct causes premature *ftz* expression (Brown et al. 1991; Read et al. 1992). We have tested the *ttk* titration hypothesis in vivo by increasing and reducing the dose of maternal Ttk protein supplied to embryos. We have found that maternally loaded *ttk* is a repressor of *ftz* but not *Kr* transcription and that the dose of Ttk protein in the early embryo regulates the timing of initiation of *ftz* transcription.

Results

Using the sensitive, digoxigenin, nonradioactive in situ hybridization technique developed by Tautz and Pfeifle (1989), O'Farrell and colleagues demonstrated that small spheres of intense staining termed nuclear dots are detected within nuclei with a *string* gene probe (O'Farrell et al. 1989). Based on experiments with the *Ultrabithorax* gene, Shermoen and O'Farrell (1991) proposed that transcription dots correspond to the sites of transcription of genes within a nucleus. Our observations also indicated that nuclear dots represent sites of transcription. RNasing embryos before hybridization abolished all hybridization signal, including nuclear dots (data not shown). Furthermore, varying the gene copy number in cycle-14 embryos caused corresponding alterations in the number of nuclear dots observed (Shermoen and O'Farrell 1991; Pignoni et al. 1992). For example, from crosses between flies heterozygous for *ftz*, *even-skipped* (*eve*) or *Antennapedia* (*Antp*) deletions, we scored embryos having zero, one, or two dots per nucleus. In one experiment embryos from *Df(2R) eve*$^{1.27}$/*CyO* stock and a wild-type stock were fixed during cycle 14 and probed with an *eve* probe. In wild-type embryos, two dots were observed in 10/10 embryos, whereas in progeny from *Df(2R)eve*$^{1.27}$/*CyO* parents, no dots were observed in 19, one dot was observed in 30, and two dots were observed in 17 embryos.

When does transcription begin?

Using the appearance of nuclear dots as the marker for the activation of transcription of genes, we determined the onset of transcription of genes expressed during early *Drosophila* development. We fixed 60–120± 5 min after egg deposition (AED) or 120–210± 5 min AED wild type embryos and hybridized them with 13 gene probes. We defined the first nuclear cycle during which hybridization was detected (Table 1; Fig. 1A,B). In every case, nuclear dots were the first hybridization signal observed. The developmental stage at which these genes were activated fell into two categories: four late expressed genes activated transcription during cycle 14 (which is after the onset of zygotic transcription and were therefore not characterized further), and nine early expressed genes that first initiated transcription during either nuclear cycles 8, 9, or 10. Transcription of these genes was observed earlier than reported previously for many of them (Knipple et al. 1985; Rothe et al. 1992; Erickson and Cline 1993).

Table 1. *Activation of transcription of early and late expressed genes*

Class	Gene	Cycle first detected
Gap	*Krüppel*	10
	tailless	10
	knirps	9
Pair–rule	*fushi tarazu*	9
	even-skipped	9
Dorsal–ventral	*snail*	8
	zerknüllt	9
Cellularization	*nullo*	9
Sex determining	*sis A*	8
Homeotic	*Antennapedia*	14
?[a]	*Toll*	14
Cell cycle	*string*	14
Segment polarity	*engrailed*	14

[a]Maternally loaded *Toll* is involved in forming the dorsal–ventral axis. The function of the zygotically transcribed *Toll* is unknown.

Nuclear cycles 1–10 each last 9 min. These early cycles are biphasic, consisting of alternating S and M phases with no detectable gap phase. S phase occupies the entire interphase, and DNA replication has been proposed to be incompatible with transcription. We observed that when these early genes begin to transcribe during cycles 8, 9, or 10, they are transcribed in a cell cycle phase-specific hybridization pattern (Fig. 1). No hybridization signal was visible in interphase and telophase nuclei. In prophase nuclei we detected nuclear dots, but during metaphase and anaphase, the nuclear dots disappeared and a diffuse halo of RNA around or between the chromosomes appeared. In each case, only a small percentage of nuclei within a small fraction of embryos showed nuclear dots, and in most cases only one nuclear dot was observed in each nucleus. We refer to prophase, metaphase, and anaphase as the transcription phases to signify the phases of the early cell cycle in which RNA can be detected in nuclei. In all further experiments we only assay embryos fixed during the transcription phases. Starting when the cell cycle lengthens during cycle 12, nuclear dots were first observed in late interphase as well as prophase, and hybridization signal became noticeable in the cytoplasm. In the next section, we quantify the activation of *ftz* and *Kr* transcription in greater detail.

Transcription is gradually activated

We first detected *ftz* nuclear dots in embryos fixed during nuclear cycle 9 (Table 1). We determined whether or not all embryos initiate *ftz* transcription during the same nuclear cycle by calculating the fraction of embryos in the transcription phases during each nuclear cycle that

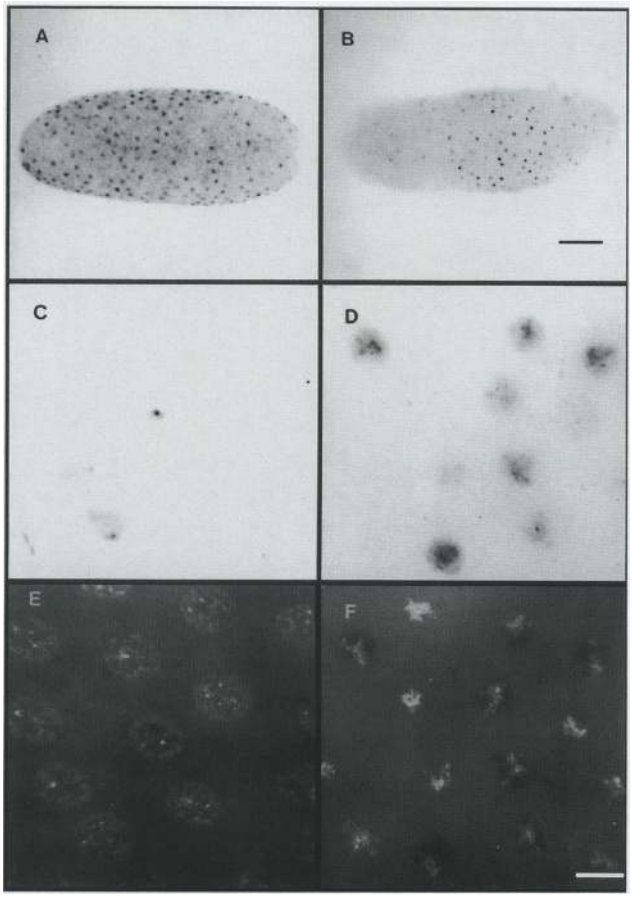

Figure 1. Early *ftz* and *Kr* transcription is nuclear and has a cell-cycle specific pattern. (*A,B*) Bright-field images of wild-type embryos in nuclear cycle 10, probed with *ftz* (*A*) or *Kr* (*B*) probes. Note the punctate nuclear expression of *ftz* in some but not all nuclei. *ftz* is expressed across the entire embryo. *Kr* is expressed in a domain between 12% and 65% egg length. Scale bar, 50 μm. The embryos are oriented with anterior poles to the *left*. (*C,E*) Bright-field (*C*) and DAPI (*E*) images of a field of nuclei in prophase of cycle 11 from a wild-type embryo, probed with a *ftz* probe. Note the nuclear dots present in some but not all the nuclei. (*D,F*) Bright-field (*D*) and DAPI (*F*) images of a field of nuclei in metaphase of cycle 11 wild-type embryo probed with a *ftz* probe. Note the halos of stain present in some nuclei. Scale bar, 10 μm.

showed *ftz* hybridization signal. At cycle 9, only 13 of 28 embryos showed hybridization (Fig. 2A). By cycle 10, 20 of 24 embryos showed hybridization, and by cycle 12, every embryo had *ftz* hybridization. These results indicated that embryos initiated *ftz* transcription during different cycles and suggested that *ftz* transcription activates gradually rather than abruptly.

We determined whether individual nuclei in an embryo also activated *ftz* transcription gradually by calculating the frequency of nuclei that showed *ftz* hybridization in each of the embryos scored in Figure 2A. For each embryo, we scored a minimum of 50 nuclei. As documented in Figure 2B, the frequency of transcribing nuclei within an embryo varied. For example, during cycle 10, four embryos showed no *ftz* hybridization in any nuclei, whereas one embryo showed hybridization in ~70% of its nuclei. This clearly indicates that not all nuclei within an embryo initiate *ftz* transcription during the same nuclear cycle.

We also observed that on average more nuclei transcribed *ftz* during each cycle after 9. We calculated the average frequency of *ftz* nuclear transcription in all of the embryos we scored in Figure 2, A and B, during cycles 8–12 and plotted this result in Figure 2C. During each cycle the frequency of nuclei that showed *ftz* hybridization increased. At cycle 9 only 3% of nuclei transcribed *ftz*, whereas by cycle 12 this increased to ~70%. These observations clearly indicate that *ftz* transcription is gradually activated.

We also characterized the activation of *Kr*. *Kr* nuclear dots were first detected during cycle 10, one cycle later than *ftz*. *Kr* transcription was concentrated in a band encompassing the central and posterior half of the embryo (Fig. 1B). For example, the *Kr* expression domain (defined by the farthest extent of transcribing nuclei) was observed between 12% and 65% egg length in cycle 10 but was restricted to 24% and 65% egg length by cycle 12. In this domain *Kr* also showed gradual activation of transcription. As seen in Figure 2D, less than half of the embryos showed *Kr* transcription during cycle 10, whereas by cycle 12 all embryos transcribed. There was extensive variation in the frequencies of transcribing nuclei within the *Kr* expression domain from embryo to embryo during cycles 10–12 (Fig. 2E), and the average frequency of nuclear transcription increased during cycles 10–12 (Fig. 2F). The gradual activation of *ftz* and *Kr* transcription was also observed among the other seven early expressed genes (data not shown), and for each of these genes, a high frequency of transcription was observed during cycle 12.

These observations indicate that the activation of transcription is a gradual process initiated at several developmental stages. Each individual nucleus makes an independent stochastic decision whether or not to begin transcribing a gene. Next, we address how the activation of transcription is regulated.

ttk *is a repressor of* ftz *but not* Kr *transcription*

It has been proposed that the maternally loaded 69-kD protein form of Ttk is a repressor of *ftz* but not *Kr* transcription (Read et al. 1992). We altered maternal Ttk protein levels in embryos and determined the effects on the activation of *ftz* and *Kr* transcription. Read et al. (1992) transformed flies with a construct in which a *ttk* cDNA encoding the 69-kD form of Ttk was fused to a heat shock promoter. We increased the amount of maternal Ttk protein loaded into embryos by keeping mothers homozygous for the heat shock-inducible 69-kD *ttk* expression construct at 29°C during oogenesis (hereafter termed *hs 69-2* embryos). We collected *hs69-2* and wild-type embryos from mothers incubated at 29°C and then fixed them at 90 ± 5 min AED. From staged cycle 9 embryos the amount of Ttk protein was determined on

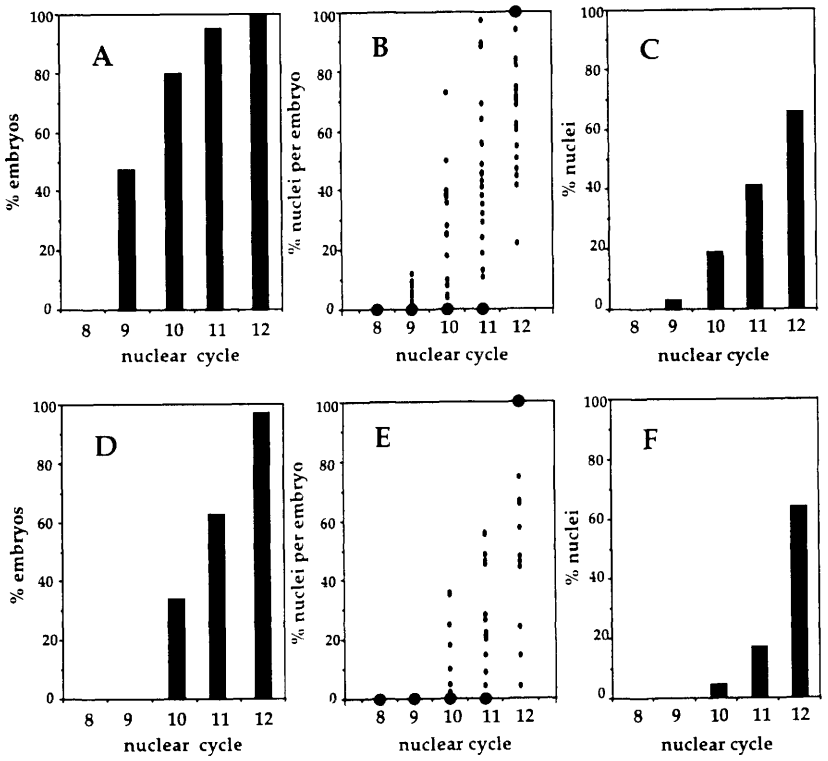

Figure 2. Gradual activation of *ftz* (*A–C*) and *Kr* transcription (*D–F*). (*A,D*) Frequency of embryos that show *ftz* (*A*) or *Kr* (*D*) hybridization between nuclear cycles 8 and 12. On the *x*-axis nuclear cycles are plotted; on the *y*-axis percentage of wild-type embryos in the transcription phases of a specific cell cycle that show one or more transcribing nucleus are plotted. A minimum of 20 embryos per nuclear cycle was scored for each time point. (*B,E*). Scatter plot presenting the frequency of *ftz* (*B*) or *Kr* (*E*) hybridization for each embryo scored in *A* or *D*. On the *x*-axis nuclear cycles are plotted; on the *y*-axis percentage of nuclei per embryo are denoted. Each embryo is represented by a dot; larger dots represent multiple data points with the same value. For each embryo a minimum of 50 nuclei were scored. (*C,F*) Plot of the average frequency of transcription of embryos in the different nuclear cycles scored in *B* or *E*. On the *x*-axis nuclear cycles are plotted; on the *y*-axis % nuclei denotes the percentage of 1000 nuclei scored from a minimum of 20 embryos all in the transcription phases that are transcribing *ftz* or *Kr* during each specified nuclear cycle. Each value represents a minimum of 1000 nuclei scored from a minimum of 20 embryos.

quantitative Western blots. *hs 69-2* embryos fixed during cycle 9 had 1.32 times (1.32±0.066 Ttk protein; $n=6$ trials) as much protein as wild-type controls (1.00±0.058 Ttk protein; $n=6$ trials). Antibody staining of *hs 69-2* embryos fixed during cycles 9–12 indicated that Ttk protein was evenly distributed in the embryo (data not shown). We tested whether the increased levels of Ttk protein in *hs 69-2* embryos affected initiation of *ftz* during nuclear cycles 9–12. None of the *hs 69-2* embryos (0/21) fixed during nuclear cycle 9 showed *ftz* hybridization, whereas 11 of 21 of the wild-type embryos fixed during the same cycle showed *ftz* hybridization. We also observed a lower frequency of nuclei transcribing *ftz* during cycles 9–12 (Fig. 3A). *ftz* patterning was not affected by the delayed initiation and lower frequency of transcription in *hs 69-2* embryos. Nor was this lowered expression of *ftz* deleterious, as wild-type and *hs 69-2* embryos incubated at 29°C hatched at identical rates (79.2% for wild type and *hs 69-2*; $n=120$ for both). These results provide the first in vivo demonstration that maternal *ttk* is a dose-dependent repressor of *ftz* transcription and that altering the concentration of a maternally loaded repressor can effect the temporal pattern of activation of a gene.

We also determined whether mothers heterozygous for the amorphic *ttk* allele *1e11* (hereafter termed *1e11* embryos) had reduced levels of Ttk protein in embryos. We collected *ttk 1e11* and wild-type embryos fixed at 90±5

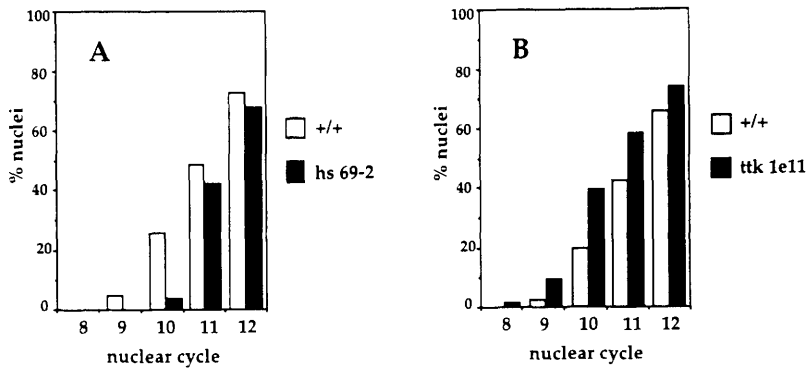

Figure 3. Dose of maternal *ttk* regulates the activation of *ftz* transcription. Comparison of the average frequency of *ftz* nuclear transcription in wild-type and *hs 69-2* (*A*) or wild-type and *ttk 1e11* (*B*) embryos during nuclear cycles 8–12. On the *x*-axis, nuclear cycles are plotted; along the *y*-axis, % nuclei is plotted as the percentage of 1000 nuclei that are transcribing *ftz* during each specified nuclear cycle scored from a minimum of 20 wild-type or *hs 69-2* (*A*) or wild-type and *ttk 1e11/TM3* embryos (*B*). The differences in frequency of transcription observed in *A* are only statistically significant during cycles 9 and 10 ($P<0.05$, Mann-Whitney test). The differences in frequency observed in *B* are statistically significant for cycles 8–11 ($P<0.05$, Mann-Whitney test).

min AED and processed them for quantitative Western blots. *1e11* embryos fixed during cycle 9 had <5% (*1e11*; <0.05 Ttk protein; $n=6$ trials) protein as compared to wild-type controls (1.00±0.079 Ttk protein; $n=6$ trials); however, *1e11* embryos developed normally. We determined whether reduced Ttk protein was correlated with premature *ftz* transcription. *1e11* embryos (10/60) fixed in the transcription phases of cycle 8 showed *ftz* hybridization, whereas none (0/71) of the wild-type embryos fixed during cycle 8 showed hybridization. Also, *ttk 1e11* embryos showed a significantly higher frequency of *ftz*-transcribing nuclei than control embryos during cycles 8–11 (Fig. 3B).

We also assayed the effects of altering the dose of *ttk* in the *1e11* and *hs 69-2* embryos on the time of initiation and pattern of gradual activation of *Kr* expression. No significant differences were observed (data not shown). The observation that *ttk* is not a repressor of *Kr* transcription confirms previous studies suggesting that *ttk* represses pair–rule genes but not gap genes (Read et al. 1992).

When early expressed genes first initiate the process of gradual activation of transcription during cycles 8–10, the nuclei are completing or have just completed nuclear migration to the cortex. This observation suggests that either the position of the nuclei, the time AED, the cell cycle length, or global or local nuclear density could act as a developmental trigger for the gradual activation of transcription (Newport and Kirschner 1982a,b; Edgar and Schubiger 1986; Brown et al. 1991; Yasuda et al. 1991). In the following sections, we have developed three experimental and genetic manipulations that alter the timing and/or pattern of nuclear migration to determine which developmental event triggers *ftz* and *Kr* transcription.

Delaying nuclear migration by early UV irradiation delays the onset of ftz *and* Kr *transcription*

Yasuda et al. (1991) observed that early UV irradiation (EUV) of the anterior poles of wild-type embryos during cycles 3 and 4 blocked the replication of some of the nuclei and delayed nuclear migration. The completion of nuclear migration in embryos was recognized by the formation of pole cells and somatic buds in cycle 10. Using time-lapse video we observed an average delay of 16 min in the formation of pole cells, and somatic buds in EUV ($n=38$) versus control ($n=48$) embryos (Fig. 4A,B). We observed one or two extra premigratory nuclear divisions in EUV embryos accounting for the 16-min delay in nuclear migration. The lengthening of the cell cycle, which normally starts in cycle 10 in control embryos, was delayed by one or more cycles in EUV embryos consistent with the delay in migration. Nuclear counts of fixed material indicated that whereas pole cell formation is delayed in EUV embryos versus controls, they occurred at a similar nuclear density. Eight of the 10 embryos analyzed by time-lapse video also had five instead of four blastoderm divisions (Fig. 4B). However, despite these deviations, EUV embryos hatched at a rate of 44% of control embryos.

Does delaying nuclear migration also delay the onset of *ftz* and *Kr* transcription? We fixed control and EUV embryos at 95±5 min AED, and after hybridization, grouped them into three classes: the premigratory stage (no pole buds, corresponding to wild-type cycle 8 or younger), pole bud stage (wild-type cycle 9), and the pole cell stage (wild-type cycles 10 and 11). At this time point 75.6% of the control embryos ($n=111$) had reached either the pole bud or pole cell stage, whereas only 44% of the EUV ($n=120$) embryos reached these stages (Table 2), indicating that nuclear migration was delayed in the EUV embryos versus control. However, *ftz* transcription was first detectable only in embryos at the pole bud stage in both control (20%) and EUV (14%) embryos despite the delay in nuclear migration in EUV embryos. This delay in *ftz* transcription argues that the initiation of *ftz* transcription is not triggered by a time-dependent mechanism. Consistent with this hypothesis, 29% of the control embryos showed hybridization versus only 14% of the EUV embryos ($P<0.001$ X^2 analysis) (Fig. 5B).

We fixed EUV and control embryos at 120±5 min AED and probed them with a *Kr* probe. We observed that 34% of the control embryos ($n=80$) showed *Kr* hybridization signal versus only 7% of the EUV embryos ($n=118$), indicating once again that delaying nuclear migration in EUV embryos correlated with a delay in initiation of *Kr* transcription ($P<0.01$ X^2). Although these experiments demonstrate that *ftz* and *Kr* transcription were not activated by a time-dependent mechanism, they do not differentiate between position dependent and nuclear density dependent activation of transcription.

Delaying the onset of transcription delays ftz *and* Kr *transcriptional patterning*

The spatial patterns of expression of *ftz* and *Kr* progress over time from an initial pattern of gene expression through several intermediate expression patterns until the mature pattern is expressed during cycle 14 (Weir and Kornberg 1985). We distinguished five different patterns of *ftz* expression (Fig. 5A). We determined whether or not delaying the onset of *ftz* and *Kr* transcription caused corresponding delays in the progression of their transcriptional patterning. Control and EUV embryos were fixed at 95, 120, 145, 170, and 220±5 min AED. Figure 5 shows that at each time point the *ftz* transcription pattern of the EUV embryonic population has a higher percentage of embryos with a less advanced *ftz* pattern than the control. This indicates that delaying the onset of *ftz* transcription also significantly delays *ftz* patterning (Fig. 5 legend). Similarly, in EUV and control embryos probed with a *Kr* probe, the control population has a higher percentage of embryos with more developed *Kr* transcriptional patterns and the differences are also significant (data not shown). These results indicate that the progression of *ftz* and *Kr* patterning takes a defined amount of time. Therefore, delaying the initiation of the process by delaying the activation of transcription causes corresponding delays in later patterning. This result confirms the earlier proposal of Yasuda et al. (1991).

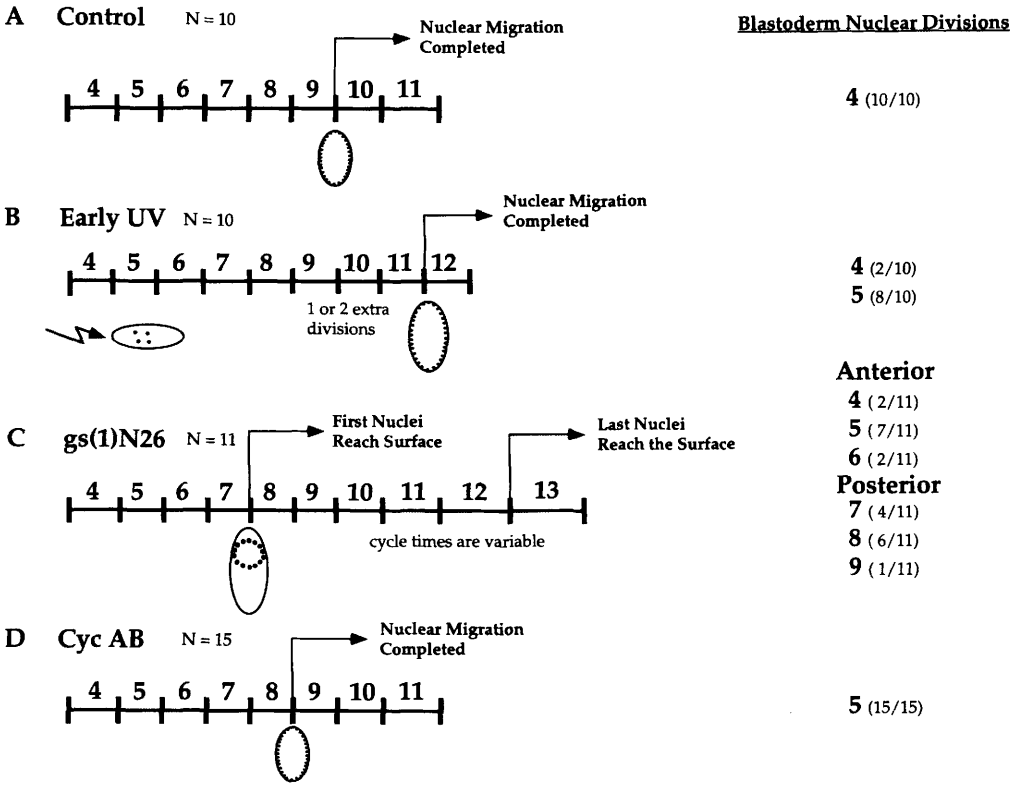

Figure 4. Experimental manipulations alter the time of nuclear migration. A summary of the effect different experimental manipulations have on the time and pattern of nuclear migration as observed by time-lapse video microscopic analysis on living embryos. The time lines summarize the nuclear cycles in which nuclear migration is completed. The numbers above the time lines (N=) indicate the number of embryos analyzed. The column Blastoderm Nuclear Divisions details the number of nuclear divisions that take place between the first nuclear cycle during which nuclei reach the cortex and gastrulation. The number of cases is summarized to the *right*. (*A*) Wild-type control embryos. (*B*) EUV-irradiated wild-type embryos irradiated at their anterior poles at 30±5 min AED. (*C*) *gs(1)N26* embryos. (*D*) *Cyc AB* embryos.

The onset of ftz and Kr transcription is dependent on nuclear density

The previous observations indicated that transcription was not activated by a time-dependent signal. However, in migration-delayed embryos, transcription of nuclei occurred when the nuclei were out at the cortex (position) at normal density (nucleocytoplasmic ratio). To determine whether or not transcription was triggered by nuclear density or nuclear position, we took advantage of the phenotype produced by embryos from mothers heterozygous for null alleles of *Cyclin A* and *B* (hereafter termed *Cyc AB* embryos). We fixed both wild-type and *Cyc AB* embryos at the pole bud stage. We found that pole bud stage wild-type embryos had 4.1 nuclei per 4900 μm^2 ($n = 20$), whereas *Cyc AB* embryos had only 2.1 nuclei ($n = 21$). Using time-lapse video microscopy, we found that *Cyc AB* embryos ($n = 15$) completed nuclear migration one cycle earlier than wild type, forming pole buds during cycle 8 and pole cells and somatic buds during cycle 9 (Fig. 4D) (Edgar et al. 1994). To compensate for the early migration to the cortex, *Cyc AB* embryos also underwent an extra blastoderm division so that they cellularized with normal density. No significant difference was observed between the lengths of the premigratory cell cycles in *Cyc AB* and wild-type embryos. We observed that *Cyc AB* embryos continued to develop and hatched.

We fixed control and *Cyc AB* embryos between 60 and 120 min AED and then probed with either a *ftz* or *Kr*

Table 2. *Onset of* ftz *transcription is delayed in EUV embryos*

Stage[a]	Transcribing[b] EUV	Transcribing[b] control	Percent embryos[c] EUV (n = 120)	Percent embryos[c] control (n = 111)
Premigratory	–	–	55.8	24.3
Pole buds	+	+	26.6	36.9
Pole cells	+	+	17.4	38.7

[a]EUV and control embryos were fixed at 95 ± 5 min AED and probed with a *ftz* probe. They were then categorized according to their developmental stage.
[b]The developmental stage at which *ftz* transcription is detectable is denoted by a + under the transcribing category.
[c]The percentage of embryos in each developmental stage from both control and EUV embryos.

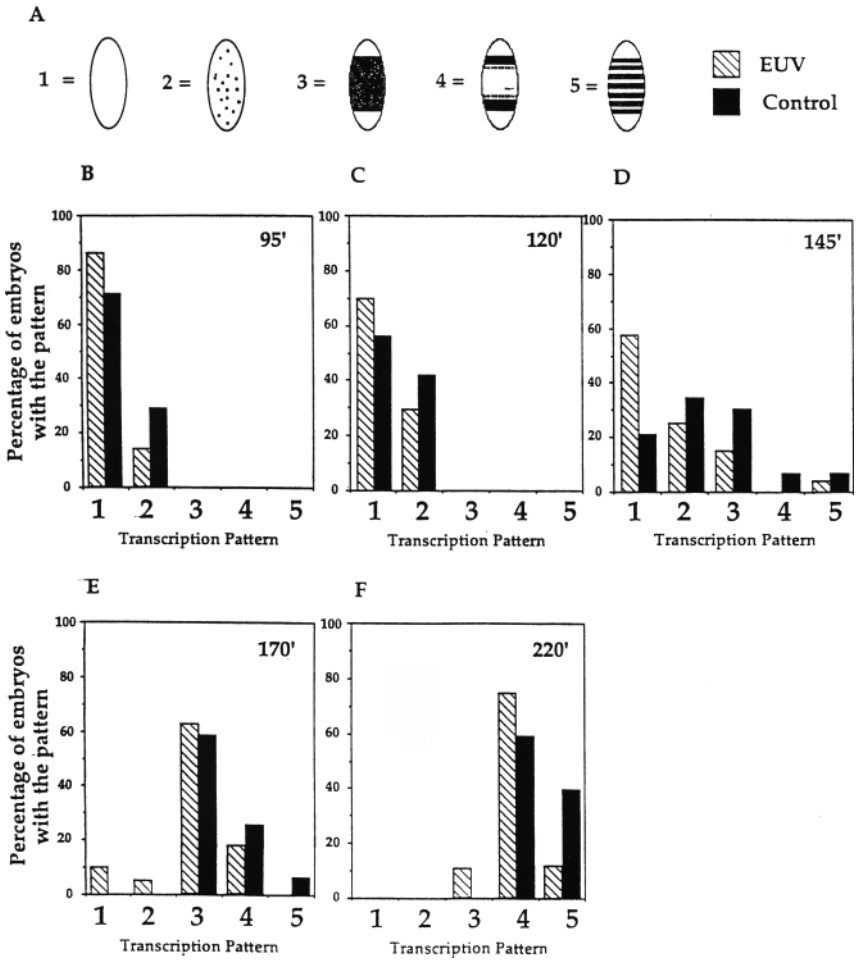

Figure 5. Delaying the onset of *ftz* transcription delays the resolution of the mature *ftz* pattern. (*A*) *ftz* transcription before gastrulation was divided into five patterns. Its time of expression was determined by nuclear counts of fixed material. (1) No hybridization; (2) nuclear pattern of expression (nuclear cycles 9–12); (3) one band pattern of expression (nuclear cycles 12,13); (4) 2 or more but <7 stripes of expression (nuclear cycle 14); (5) the mature 7-striped pattern of expression (nuclear cycle 14). Each graph represents the distribution of embryos expressing the different *ftz* patterns from control (solid bars) and EUV-treated (hatched columns) embryos. At each time point a minimum of 50 control and experimental embryos were scored. Embryos fixed at 95±5 min (*B*), 120±5 min (*C*), 145±5 min (*D*), 170±5 min (*E*), 220±5 min (*F*). At each time point the differences between EUV and control embryos were statistically significant ($P<0.043$ X^2).

probe. As seen in Table 3, *ftz* transcription was first detected in cycle 9 in both *Cyc AB* and wild-type embryos and correlated with embryos having a nuclear density of ~4 nuclei per 4900 μm^2. The initiation of *ftz* transcription did not correlate with position of the nuclei out at the cortex, as *Cyc AB* embryos reached the pole bud stage one cycle earlier than wild type. Similarly, initiation of *Kr* transcription correlated with reaching a global nuclear density of ~8 nuclei per 4900 μm^2 rather than when nuclei completed migration and formed pole cells. These results indicate that the activation of *ftz* and *Kr* transcription correlates with a critical nuclear density.

Table 3. *Nuclear density regulates the activation of* ftz *and* Kr *transcription*

Cycle[a]	Type	Stage[b]	Density[c]	*ftz*[d]	*Kr*[d]
8	+	premigratory	2.1		
	Cyc AB	pole bud	2.1		
9	+	pole bud	4.1	+	
	Cyc AB	pole cell	4.3	+	
10	+	pole cell	8.0	+	+
	Cyc AB	pole cell	7.6	+	+

Wild-type (+) or *Cyc AB* embryos were collected and fixed between 60 and 120 min AED and then probed with either a *ftz* or *Kr* probe.
[a]For each cycle, a minimum of 20 embryos fixed during the transcription phases were analyzed.
[b]The *Cyc AB* embryos formed pole buds and pole cells one cycle earlier than wild type.
[c]*Cyc AB* embryos complete nuclear migration and reach the cortex one cycle earlier than wild type and do so at a lower nuclear density (average number of nuclei per 4900 μm^2).
[d]For each cycle, the entire population of embryos was scanned for the presence of nuclear dots or halos. If any one of the embryos had initiated *ftz* or *Kr* transcription this was denoted by a +.

In gs(1)N26 *embryos, nuclear density varies. Is activation of* ftz *and* Kr *transcription dependent on local nuclear density?*

Observations from the previous experiment indicated that transcription was activated at a critical nuclear density. However, there is precedent for the local nucleocytoplasmic ratio (nuclear density of a distinct group of nuclei) rather than the global nucleocytoplasmic ratio regulating diverse processes including cell division, nuclear migration, maternal RNA breakdown, and transcription (Edgar et al. 1986; Yasuda et al. 1991). To determine whether the global or local nucleocytoplasmic ratio triggered transcription, we studied embryos from homozygous *gs(1)N26* mothers in which nuclei migrate to the cortex unevenly, arriving both earlier and later than normal (Niki 1984; Hatanaka and Okada 1991).

We compared the pattern of nuclear migration in wild-type and *gs(1)N26* embryos. In wild-type embryos, initial nuclear divisions were internal and anterior. During succeeding nuclear divisions, the nuclei migrated both posteriorly and anteriorly within the embryo. During cycles 7–10, the nuclei migrated out to the cortex of the egg (Fig. 4A). In *gs(1)N26* embryos, nuclei divided properly but failed to migrate along the anterior–posterior axis. They remained located anteriorly and defined a sphere that expanded with each division. Thus, nuclei reached the cortex unevenly arriving both earlier anteriorly and later posteriorly than normal (Fig. 6A,B). Time-lapse video microscopic observation of *gs(1)N26* embryos ($n=11$), as well as nuclear counts of fixed embryos, indicated that nuclei first reached the cortex during nuclear cycle 7 or 8 in an anterior band (Figs. 4C and 6B), and by cycle 9, nuclei reached the anterior pole. By cycle 12 or 13, nuclear migration was completed when nuclei reached the posterior pole (Figs. 4C and 7). Because of the abnormal migration, the nuclear density of the nuclei at the cortex in the anterior was higher than that of the nuclei still migrating posteriorly. As a consequence of this uneven nuclear density, the anterior nuclei underwent <14 nuclear divisions and the posterior >14 divisions to form a properly spaced blastoderm (Fig. 4C). Despite this striking alteration in the pattern of migration, 85% ($n=140$) of embryos laid by *gs(1)N26* homozygous mothers hatched.

We characterized the pattern of *ftz* hybridization in wild-type and *gs(1)N26* embryos fixed at 70±5 min AED. This time corresponds to nuclear cycles 7 and 8. In wild type, no nucleus had reached the cortex, but in *gs(1)N26* embryos, nuclei did (Fig. 6). No wild-type embryos showed *ftz* hybridization ($n=102$). Twelve percent of *gs(1)N26* embryos ($n=49$) showed *ftz* hybridization. However, *ftz* hybridization was detected only in nuclei at the surface or occasionally in yolk nuclei directly underneath nuclei that had migrated to the cortex (Fig. 7), whereas internal migrating nuclei, which were at a lower nuclear density, did not transcribe. The lack of hybridization signal in internal migrating nuclei is not likely to be caused by problems with probe penetration, as nuclear dots could be detected easily in yolk nuclei at the center of wild-type embryos.

We have found that the local density of the transcribing nuclei at the cortex is 4.5 nuclei per 4900 μm^2 ($n=17$), which is similar to the nuclear density of transcribing cycle 9 wild-type embryo (4.1 nuclei per 4900 μm^2). As can be seen in Figure 6B, the nuclear density of vitellophages (nuclei that never migrate to the cortex) is highest underneath the nuclei that have reached the cortex. We only observed transcription in these vitellophages. Thus, we conclude that transcription initiation depends solely on the local rather than the global nucleocytoplasmic ratio. We determined the effect of the uneven transcriptional activation in *gs(1)N26* embryos on the progression of *ftz* expression patterns in *gs(1)N26* embryos. We fixed control and *gs(1)N26* embryos at 70, 95, 120, 145, and 170±5 min AED and have characterized the *ftz* expression patterns along the anterior–posterior axes of the embryos using the classes of *ftz* expression patterns introduced in Figure 5A. Many of the *gs(1)N26* embryos showed mosaic patterning with more than one expression pattern observed within the same embryo (Fig. 7). This was never observed in control embryos ($n>130$ for each time point) (Fig. 7A–F). The youngest transcriptional pattern was always observed in the posterior half of the embryo, consistent with the delayed transcriptional activation in the posterior of *gs(1)N26* embryos. For example, at 145 min AED, 54 of 75 embryos were mosaic and a variety of mosaic patterns were observed; however, the most common pattern was one in which the embryo was beginning to form stripes

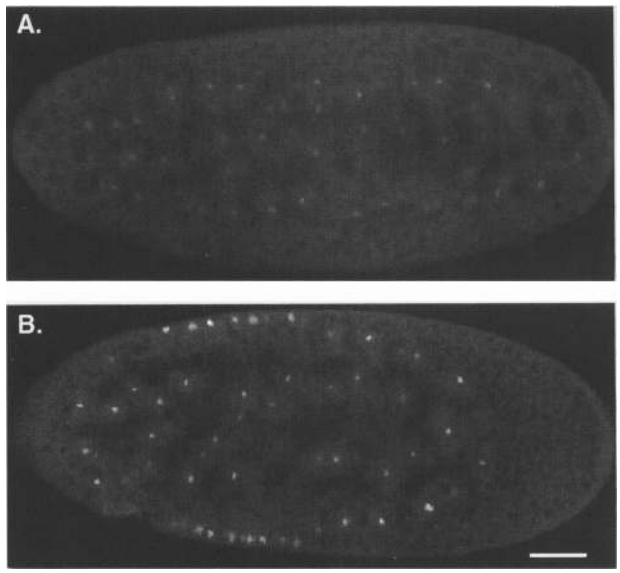

Figure 6. A comparison of patterns of nuclear migration in wild-type vs. *gs(1)N26* embryos. Distribution of nuclei as observed by confocal microscopy of a wild-type (*A*) and a *gs(1)N26* embryo (*B*) both fixed during nuclear cycle 8. The nuclei in the *gs(1)N26* embryos have reached the cortex (note the high density of nuclei at the cortex), although all the nuclei are still internal in the wild-type embryo. Scale bar, 50 μm.

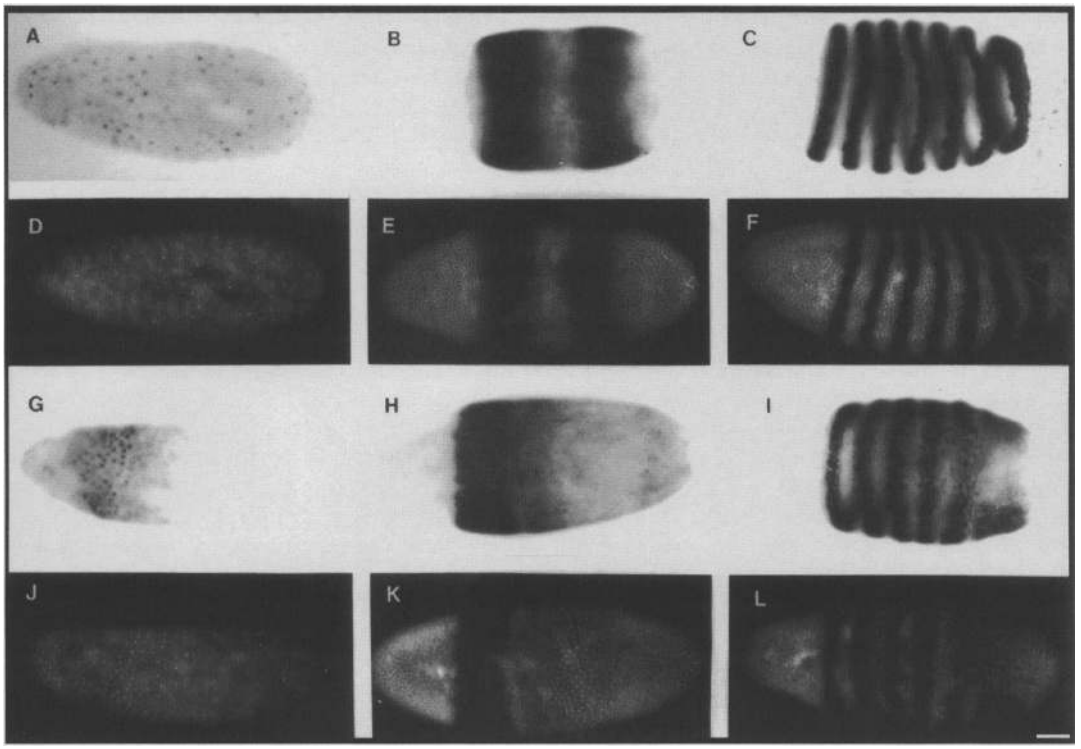

Figure 7. Mosaic *ftz* expression in *gs(1)N26* embryos. (*A–F*) Wild-type embryos fixed at 95, 145, and 170±5 min, respectively, and hybridized with a *ftz* probe and visualized with bright-field optics (*A–C*) or DAPI counterstaining (*D–F*). (*G–L*) *gs(1)N26* embryos fixed at 95, 145, and 170±5 min, respectively, and hybridized with a *ftz* probe and visualized with bright-field optics (*G–I*) or DAPI counterstaining (*J–L*). The *gs(1)N26* embryos have more than one transcriptional pattern within the same embryo. Scale bar, 50 μm.

of expression in the anterior part of the embryo while in the posterior uniform expression was observed (Fig. 7H,K).

The observations on mosaic *ftz* patterning in *gs(1)N26* embryos further support the idea that the *ftz* patterning process is a time-dependent process initiated at the activation of zygotic transcription.

Discussion

The process of transcriptional activation has usually been interpreted in terms of the concept of the mid-blastula transition (MBT)—a defined stage during development when a variety of developmental events, including the activation of transcription, are coordinately initiated throughout an embryo (for review, see Yasuda and Schubiger 1992). Our observations suggest a different view of how transcriptional activation is regulated. We have observed that transcription of early expressed genes does not begin during one nuclear cycle. Instead, the onset of transcription of these genes is first detected at multiple points in development during cycles 8, 9, or 10. Furthermore the activation of transcription for these genes occurs gradually, with individual nuclei activating transcription during different cycles. These observations indicate that each individual copy of a gene makes a stochastic decision whether or not to begin transcription of a particular gene. Our observations support a modified view of the MBT in which it consists of a series of coordinated independent processes, rather than a single event (Yasuda and Schubiger 1992).

To understand how transcription is activated in *Drosophila* embryos when nuclei reach the cortex, we have devised a series of experimental and genetic perturbations of nuclear migration and assayed their effects on the activation of *ftz* and *Kr* transcription. These experiments demonstrate that reaching a critical local nucleocytoplasmic ratio triggers transcription of *ftz* and *Kr*. There is precedent for the local nucleocytoplasmic ratio regulating developmental processes, including cell division, nuclear migration, maternal RNA breakdown, and transcription (Edgar et al. 1986; Yasuda et al. 1991). Because the local nucleocytoplasmic ratio coordinates diverse processes, we speculate that it may be involved in temporally coordinating many of the early events of *Drosophila* embryogenesis as well as providing the early embryo with some plasticity to regulate an otherwise hard-wired developmental program.

Control of transcription by the nucleocytoplasmic ratio has been interpreted in terms of titration of a maternally loaded repressor by DNA. Consistent with this proposal, we have demonstrated that altering the concentration of maternal Ttk protein in embryos alters the time of initiation and pattern of activation of *ftz* but not *Kr* transcription, as suggested previously by ectopic expression and reporter construct studies (Read et al. 1992).

We also demonstrated for the first time in vivo that the concentration of a maternal transcription factor can temporally regulate the activation of a gene. Our observation that Ttk is not a repressor of *Kr* transcription predicts that other Ttk-like maternally loaded repressors are present in the early embryo, each repressing a different set of genes.

Based on the prediction that other Ttk-like repressors are present in embryos and that the transcriptional activation of genes is regulated by the nucleocytoplasmic ratio, we suggest a working model: Transcription of genes in the early, precycle 8 embryos is actively repressed by maternally loaded transcription factors. As nuclei near the cortex during cycles 8, 9, and 10, the local nucleocytoplasmic ratio reaches a threshold level where titration of the Ttk-like repressors is sufficient to enable low-level transcription of a given gene in a few nuclei. As the nucleocytoplasmic ratio increases during succeeding cycles, titration decreases the effective concentration of the repressors and transcriptional frequency gradually increases. This proposal is mechanistically similar to the patterning of early zygotic genes where the interaction of different maternal and zygotically produced transcriptional repressors defines the mature pattern of expression of a gene (Gaul and Jäckle 1989).

Although the simplicity of this model is appealing, in reality the process of transcriptional activation is likely to be more complex. Other potential transcriptional regulators such as histones or histone variants, components of chromatin, or elements of the basic transcriptional apparatus could be titrated by the nucleocytoplasmic ratio to trigger transcription as has been suggested in *Xenopus* (Prioleau et al. 1994). Furthermore, our model does not address the observation that maternally loaded activating transcription factors like *ftz F1*, *bicoid* (*bcd*), and *Caudal* are also likely to be important for transcriptional activation (Dearolf et al. 1989; Lavorgogna et al. 1991; Ueda et al. 1991). Nor does it explain our observations that there is considerable variability in frequency of *ftz* or *Kr* transcription between individual embryos fixed during the same nuclear cycle. Finally, our model does not consider the observation of Edgar et al. (1986) that the length of the cell cycle regulates the amount of transcription product a gene makes. It is likely that transcriptional activation is a complex, multifactorial process regulated at multiple levels.

Implications of the gradual and variable nature of transcriptional activation

A commonly held tenet is that temporal control of gene expression is regulated by when the genes initiate transcription. For example, it has long been assumed that gap genes like *Kr* would begin to transcribe before pair–rule genes like *ftz*, because *Kr* is upstream of *ftz* in the segmentation hierarchy. However, we have observed that *ftz*, not *Kr*, is transcribed first, contradicting the hypothesis.

We suggest that the initial activation of zygotic transcription in embryos is a generic noninformational event, with genes initiating transcription at a low and variable level. During this period neither *ftz* nor *Kr* mRNA is observed in the cytoplasm and no Ftz or Kr protein is detectable (Carroll et al. 1988; Gaul and Jäckle 1989). It is only later, during cycle 12, when the cell cycle first lengthens significantly, that transcription of *ftz* and *Kr* reaches a high level, becomes detectable in interphase, accumulates in the cytoplasm, and the Kr protein becomes detectable (Gaul and Jäckle 1989). The absence of *ftz* and *Kr* mRNA from the cytoplasm during cycles 9–11 might be attributalble to the extreme rapidity of the early cell cycles, which may not allow sufficient time to completely transcribe and process mRNAs of early expressed genes during one cell cycle (Shermoen and O'Farrell 1991; Rothe et al. 1992).

We suggest that tight temporal and spatial control of early gene transcription is not necessary for the proper development of the animal. Supporting this hypothesis, we can alter the frequency of *ftz* transcribing nuclei from 4% to 40% in a population of cycle 10 embryos by manipulating Ttk protein level; however, this has no effect on the development of the animal or the formation of the mature *ftz* pattern (Fig. 3). This early generic transcription has also been observed in other organisms such as *Caenorhabditis elegans* and *Xenopus* (for review, see Yasuda and Schubiger 1992). This weak transcriptional activity precedes the large increase in transcription associated with the MBT. So what is the signicance of the early generic transcription? We suggest that transcription is an essential prerequisite before a gene can begin to pattern. Let us use an analogy. Before one can drive a car it is essential to first turn on the engine. The time between activating the engine and putting the car in drive is irrelevant, but the car will not drive unless the engine is engaged.

Materials and methods

Fly collections

Embryo collections followed a standard procedure. Except as noted, all flies were raised and embryos collected at 25°C. Embryos were collected from 3- to 7-day old females after 1 hr precollection on fresh food. Embryos were collected on 2% agar plates supplemented with acetic acid and yeast. The first three collections were 30, 15, and 15 min long and were discarded to remove overaged embryos. Experimental embryos were then collected for 10 or 60 min as denoted in the text and fixed at the appropriate time. The *Sevelen* strain was used as the wild-type stock. For the experiments in which we altered the numbers of copies of *eve*, *ftz*, and Antp genes, we collected embryos from $Df(2R)eve^{1.27}/CyO$, $Df(3R)NS^{+R17}/TM6$, or $X/Dp(y;3)Antp^{+}$ stocks.

To examine embryos with reduced concentrations of maternal *ttk*, embryos were collected from *ttk 1e11/TM3* mothers that are heterozygous for the *ttk* null allele *ttk 1e11* (Xiong and Montell 1993). To examine embryos with increased levels of maternal *ttk*, embryos were collected from mothers homozygous for the *hs 69-2 P* element insertion that contains a heat shock-inducible *ttk* gene encoding the 69-kD Ttk protein isoform (Read et al. 1992). To induce the gene, *hs 69-2* and *Sevelen* control flies were kept at 29°C for 3 days, longer than the time of oogenesis, before collecting the embryos.

For the *gs(1)N26* experiments, a *gs(1)N26/FM7* stock was used (Niki 1984; Hatanaka and Okada 1991). Homozygous *gs(1)N26* females were crossed to *gs(1)N26/Y* males at 25°C (the restrictive temperature for the temperature-sensitive *gs(1)N26* mutation).

For the *Cyc AB* experiments, $CycA^{neo114}/TM3$ and $CycB^{Df(2R)59AD}/CyO$ flies were crossed to generate $CycB^{Df(2R)59AD}/+$; $CycA^{neo114}/+$ females (Edgar et al. 1994). Embryos with premature migration were collected from $Cyc\ B^{Df(2R)59AD}/+$; $Cyc\ A^{neo114}/+$ females crossed to *Sevelen* males.

In situ hybridization

In general, plasmids containing full-length cDNAs were employed to make probes for these studies although the *Antp*, *ftz*, and *tailless* (*tll*) probes were not full length. Digoxigenin-labeled DNA probes were prepared using the procedure of C. Oh and B. Edgar (unpubl.). Probes were made according to the procedure of Tautz and Pfeifle (1989), with the following modifications: The random hexamer concentration was raised to a final concentration of 5 mg/ml in the probe-making reaction, and the reaction was incubated overnight at 15°C, then incubated at 22°C for 4 hr.

In these experiments, we used the in situ hybridization protocol of Tautz and Pfeifle (1989), with the following modifications: Hybridization of probe occurred in 20 μl of hybridization solution containing 1.5 mg/ml of sheared salmon sperm DNA and no tRNA. Hybridization washes were performed at 50°C. All hybridized embryos were counterstained with 1 mg/ml of DAPI and were mounted in Water Mounting Media (Gurr).

α-Amanitin injection

α-Amanitin was injected at 0.5 mg/ml into embryos to block transcription following the procedure of Edgar and Schubiger (1986). In situ hybridization then followed the standard protocol.

Scoring embryonic transcription

Hybridization signal was scored using bright-field optics. Tech Pan (Kodak) film was used for all photography. Photographs were then scanned using a HSD Scan-X scanner and assembled into figures using the program Adobe Photoshop v. 3.5 and a Power Mac 6100. Figures were then printed on a Tektronix Phaser IISDX Dye Sublimation printer.

Embryo observation and staging

Embryos were staged by nuclear cycle and cell cycle phase according to Foe and Alberts (1983). Cell cycle phase was determined by direct observation of the DAPI-stained embryos. Living embryos were observed with phase-contrast optics. Time-lapse video microscopy was performed according to Baker et al. (1993) using differential interference contrast (DIC) optics and the following components: an Mti 65 video camera, a Koyo black and white monitor, and a Gyyr time-lapse video recorder.

EUV

Irradiations were performed according to the procedure of Yasuda et al. (1991), with the modification that irradiation took place at 30±5 min AED. In these experiments, control and irradiated embryos were incubated at room temperature (22°C) as opposed to 25°C.

Quantification of Ttk protein levels in embryos

Quantification of Ttk protein levels in embryos was performed as in Edgar et al. (1994). Embryos were collected from mothers homozygous for the heat shock-inducible 69-kD *ttk* expression construct or wild-type mothers incubated at 29°C for 3 days (*hs 69-2* experiments) or from *ttk 1e11/TM3* and wild-type mothers incubated at 25°C (*1e11* experiments). They were then fixed at 90±5 min AED. Embryos fixed during cycle 9 were pooled. In each experiment, protein was prepared from a minimum of 50 embryos of each genotype. An amount of protein equivalent to 10 embryos was loaded onto an acrylamide gel. Ttk protein was detected on Western blots using a rat anti-Ttk 69-kD antibody (1:400) (Read et al. 1992) and a peroxidase-conjugated goat anti-rat antibody (Amersham; 1:300) using the ECL chemiluminescent kit (Amersham). To normalize for protein loading, the blot was stained with a rabbit anti-CDC 2 antibody (1:400) and a peroxidase-conjugated goat anti-rabbit antibody (Amersham; 1:300) using the ECL kit. The intensity of bands on the Western blot was determined by scanning the images into a Power Macintosh 7100 with an HSD Scan-X scanner and analyzing the band intensity using the program NIH Image. The amount of Ttk protein in each lane was normalized for protein loading using the relative intensity of the CDC 2 signal. In each case quantification of the amount of protein in embryos during cycle 9 was based on six separate trials.

Confocal microscopy

Embryos were stained with a rhodamine-conjugated mouse antihistone antibody at 1:500, as described in Baker et al. (1993) and visualized on a Bio-Rad(600) Confocal microscope.

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Activation of transcription in Drosophila embryos is a gradual process mediated by the nucleocytoplasmic ratio.

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