Supplementary material

Transcribed genes are localized according to chromosomal position within polarized *Drosophila* embryonic nuclei

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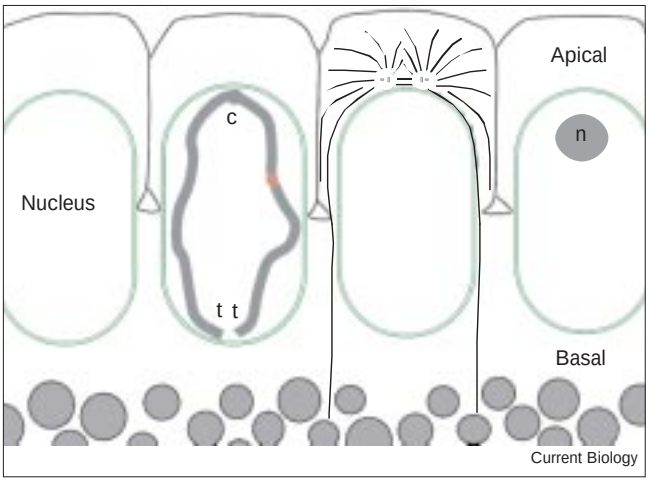
Supplementary background

Polarized cytology of the *Drosophila* embryo

The blastoderm embryo is a particularly suitable system for analyzing the spatial localization of genes and for testing the relationship between their transcription and their intranuclear distribution. The early embryo consists of a monolayer of approximately 6000 nuclei that are aligned in a polarized cortical cytoplasm [S1]. Blastoderm nuclei have a consistent apical–basal intranuclear polarity that mirrors that of the cytoplasm [S2,S3] (Figure S1). Chromosomes are organized with the centromeres tightly clustered within the apical hemisphere and the telomeres localizing within the basal hemisphere (Rabl conformation). Other parts of the chromosomes occupy intermediate positions in the apical–basal axis. Heterochromatin and rDNA genes are also localized at the apical pole of the nucleus, where the nucleolus is positioned. The interphase Rabl orientation of chromosomes is thought to result from the movement of chromosomes during the previous mitotic division, and has been observed in many tissue types including those of mammals [S4]. In contrast to their strict apical–basal polarity, there is no consistent radial positioning of chromosomes in *Drosophila* blastoderm nuclei. While the majority of loci are distributed at a random radial position, a small number of sites are positioned at the nuclear envelope, and other rare sites are excluded from the nuclear periphery [S5]. The random radial position of most loci is explained by the rapid stochastic movement of loci observed in living *Drosophila* embryos [S6,S7]. In contrast, the association of some sites with the nuclear periphery is due to their proximity to chromatin sites that are associated with the nuclear envelope [S5].

Figure S1



The polarized cytology of the *Drosophila* interphase 14, blastoderm embryo. The elongated nuclei are arranged along the periphery of the embryo, dividing the cortical cytoplasm into apical and basal compartments. Cell membrane furrows move down between the nuclei to cellularize the embryo. The polarized nuclei contain an apical nucleolus (n) and Rabl-oriented chromosomes with apical centromeres (c) and basal telomeres (t). The red circle represents a transcribed gene. A pair of centrosomes in the apical cytoplasm nucleates a basket of microtubules, which form an apical cap around the nucleus with a few microtubules trailing down into the yolk.

It remains unclear to what extent the intranuclear position of genes depends on their transcriptional state. The studies described above report the average position of chromosomal loci in a large number of nuclei throughout the entire embryo. Such studies would not necessarily reveal variations in the distribution of genes within nuclei in different regions of the embryo showing different expression levels. Therefore, we have examined the intranuclear position of transcribed genes within individual nuclei in different expression domains and compared the results with the distribution of chromosomal sites within the entire embryo.

Supplementary results

The apical–basal distribution of nascent transcript foci from chromosome arm 2L and the X chromosome

We analysed the apical–basal distribution of nascent transcript foci on the X chromosome and chromosome arm 2R (Figure S2a,b). The positions were determined by plotting all the data on to the outline of a single representative nucleus, in a similar manner to chromosome arm 3R, described in the main text (Figure 1c in the paper). Our results show that the position of each transcribed gene is restricted to a particular plane along the apical–basal nuclear axis as predicted by the cytological position of the gene and the apical–basal, Rab1 conformation of chromosomes.

The fluorescence intensity of nascent transcript foci is a measure of the relative amount of mRNA and level of transcription

To test whether the total level of fluorescence within *run* nascent transcript foci correlates with the level of transcription, we made use of the fact that *run* mRNA expression is known to vary in different regions of the embryo. Expression of *run*, an X-linked pair-rule gene, is detected in seven stripes and in two head patches during

Table S1

Intensity of fluorescence of nascent transcript foci in different expression domains of *run* and in paired or unpaired loci.

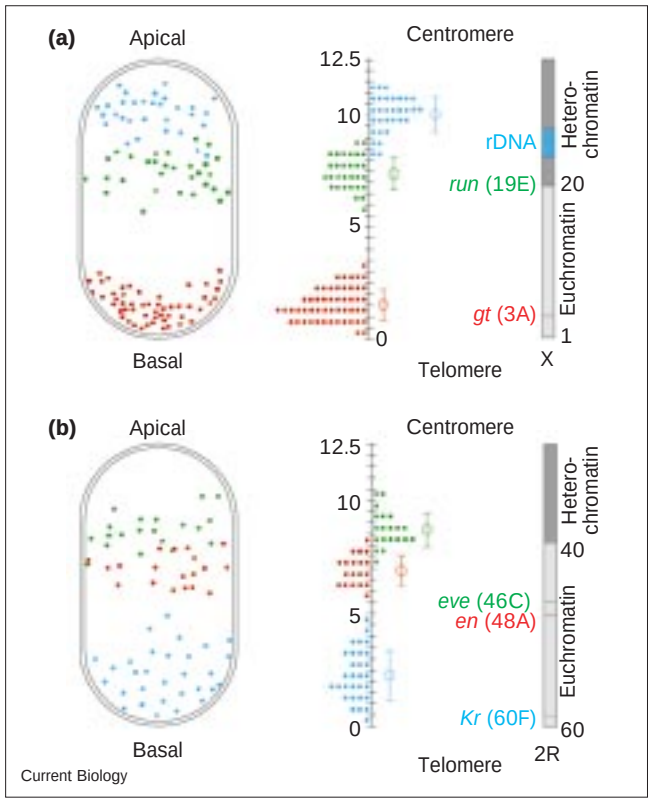
run expression stripe	Total intensity of nascent transcript foci
Representative male embryo:	
Stripe 1	648,771 ± 289,856 (*n* = 17)
Stripe 2	437,197 ± 104,496 (*n* = 10)
Head patch	173,606 ± 102,816 (*n* = 9)
Representative female embryo:	
Unpaired loci	891,446 ± 449,121 (*n* = 16)
Paired loci	1,994,649 ± 857,949 (*n* = 9)

Intensities of nascent transcript foci within nuclei in different expression domains of *run* in a single male embryo. In the second part of the table, paired and unpaired nascent transcript foci are compared within nuclei in the center of stripe 1 of a single female embryo. The data are representative of several embryos. Total integrated fluorescence intensities of nascent transcript foci are in arbitrary units ± the standard deviation, *n* = number of foci measured.

interphase 14 [S8]. We quantified the total fluorescence signal within nascent transcript foci of *run* in three dimensions (see Supplementary materials and methods). The *run* nascent transcript foci in the head patch were observed to have, on average, four times less fluorescent signal than nascent transcript foci in stripe 1, which was stronger than the other stripes in the same embryo, such as stripe 2 (Table S1 and Figure 3a–c in the paper). In addition, at earlier stages of *run* expression, we observed very faint nascent transcript foci within inter-stripe regions (data not shown). These results are in agreement with previous work describing the overall levels of *run* mRNA in different regions of the embryo [S8]. The *run* gene is first expressed over the majority of the middle of the embryo, followed by a gradual appearance of seven stripes, initially with lower expression in the inter-stripe regions. The head patch is expressed later and weaker than the seven stripes, and stripe 1 shows the strongest expression [S8].

We also quantified the fluorescence intensity of a number of different *run* nascent transcript foci within nuclei in the center of stripe 1 in female embryos. Such embryos have two copies of the gene, which are usually unpaired, but sometimes the homologues are paired, showing a single visible nascent transcript focus (Figure 3b). In contrast, all nuclei in male embryos show a single nascent transcript focus (Figure 3a). As expected, paired nascent transcript foci were found to contain twice the average intensity of nascent transcript foci in neighboring nuclei with unpaired homologues in female embryos (Table S1). Taken together, our results suggest that the total fluorescence intensity within a nascent transcript focus is a good measure of the relative transcriptional activity of a gene within different nuclei in the same embryo.

Figure S2



The apical–basal distribution of transcribed genes on the X chromosome and left arm of chromosome 2. Map of the intranuclear positions of six transcribed genes from two different chromosome arms. The observed nuclear positions are plotted onto an average nucleus, 12.5 µm in length (see Supplementary materials and methods). The cytological positions of the genes on metaphase chromosome arms are drawn to scale on the right of each panel (based on [S28]). In the center of each panel, a histogram is drawn from the distribution of the nascent transcript foci that lie within 0.5 µm intervals along the apical–basal axis of the nucleus. The average position is indicated by an open circle and the standard deviation by an error bar in each case. **(a)** The intranuclear position of two transcribed X-linked genes, *run* and *giant* (*gt*). The *gt* gene (red) is located relatively near the telomere of the X chromosome and is transcribed in the basal region of the nucleus whereas *run* (green) is located near the middle of the chromosome and is transcribed in the middle of the nucleus. Tandemly repeated rDNA genes (blue) are located in a more apical position in accordance with their more proximal cytological position. **(b)** The positions of three transcribed genes from chromosome arm 2R. *Kr* (blue) is located very near the telomere, and is transcribed in the basal region of the nucleus. In contrast, *en* (red) and *eve* (green) are situated nearer the middle of the chromosome arm and are transcribed in more apical regions of the nucleus in correspondence with their order on the chromosome.

The intensity of nascent transcript foci is independent of their close proximity to the nuclear envelope

Our results show that there is no relationship between the fluorescence intensity of nascent transcript foci and their distance from the nuclear periphery (see Figure S3, Figure 3c and Results and discussion section in the paper). We

Table S2

Intensity of fluorescence of nascent transcript foci at the periphery and in the interior of the nucleus.

Gene	Intensity at nuclear periphery	Intensity at nuclear interior
run	246,692 ± 87,442 (*n* = 8)	272,175 ± 137,304 (*n* = 7)
h	119,369 ± 46,450 (*n* = 35)	126,828 ± 58,905 (*n* = 31)
Ubx	600,795 ± 289,810 (*n* = 7)	588,316 ± 347,348 (*n* = 7)
X44N	1,442,708 ± 758,968 (*n* = 14)	1,501,704 ± 874,242 (*n* = 15)

A comparison of the intensities of unpaired nascent transcript foci within a single nucleus in which one focus is at the periphery and the other in the nuclear interior. For each gene the data were collected from a single embryo that is representative of other embryos studied. Total integrated fluorescence intensities of nascent transcript foci are in arbitrary units ± the standard deviation, *n* = number of foci measured.

also tested whether nascent transcript foci located near the nuclear periphery have a significantly different level of transcription from ones in the nuclear interior. We compared nascent transcript foci that were within 0.5 μm of the nuclear envelope with their twin nascent transcript foci that localize within the interior of the same nucleus in *run* stripe 1. The results show that the total fluorescence intensity of nascent transcript foci at the periphery and the interior of the nucleus are not significantly different. We obtained similar results for a number of different genes, including transgenes with unusually bright nascent transcript foci (Table S2). These results suggest that the level of transcription of a gene is not related in any way to whether a gene is in close proximity to the nuclear envelope.

Supplementary discussion

Hybridization foci represent nascent transcript accumulation at the site of transcription of a gene

To map the intranuclear position of transcribed genes, we used a fluorescent *in situ* hybridization method we have recently developed. Our data reveal localized intranuclear foci of intense fluorescent signal in the cases of all genes investigated. Previous work demonstrated that such foci contain nascent mRNA because, upon induction of transcription, 5′ sequences are detected before more 3′ sequences [S9]. In addition, the foci disappear on RNAse treatment and the number of foci depends on the number of genes, state of pairing and state of DNA replication [S10,S11]. By analyzing many more individual genes, we have obtained results that support this interpretation.

The number of foci depends on the number of chromatids, which changes after DNA replication in early cycle 14 and state of pairing between chromatids and sister chromatids, which varies between individual nuclei at the blastoderm stage [S12]. We have shown that the intranuclear position of the foci is correlated with their cytological position. In addition, for a given gene, the intensity of fluorescent signal within a focus is dependent on the expression level in that part of the embryo. Therefore, the bright fluorescent foci, we have observed, represent nascent mRNA colocalized with the gene from which they are transcribed.

Measurements of polymerase densities on nascent transcripts in early *Drosophila* embryos suggest that the genes we have studied are likely to contain approximately 10–30 nascent transcripts per locus [S13]. In any single nucleus, there are few other detectable fluorescent signals, suggesting that intermediates between the completion of transcription and processing and the export of mRNA through nuclear pore complexes are extremely rare. The cytoplasmic signal is restricted to 10–50 apical-localized small 'dots', which are clearly distinguishable from background, but each dot is an order of magnitude less bright than each nascent transcript focus.

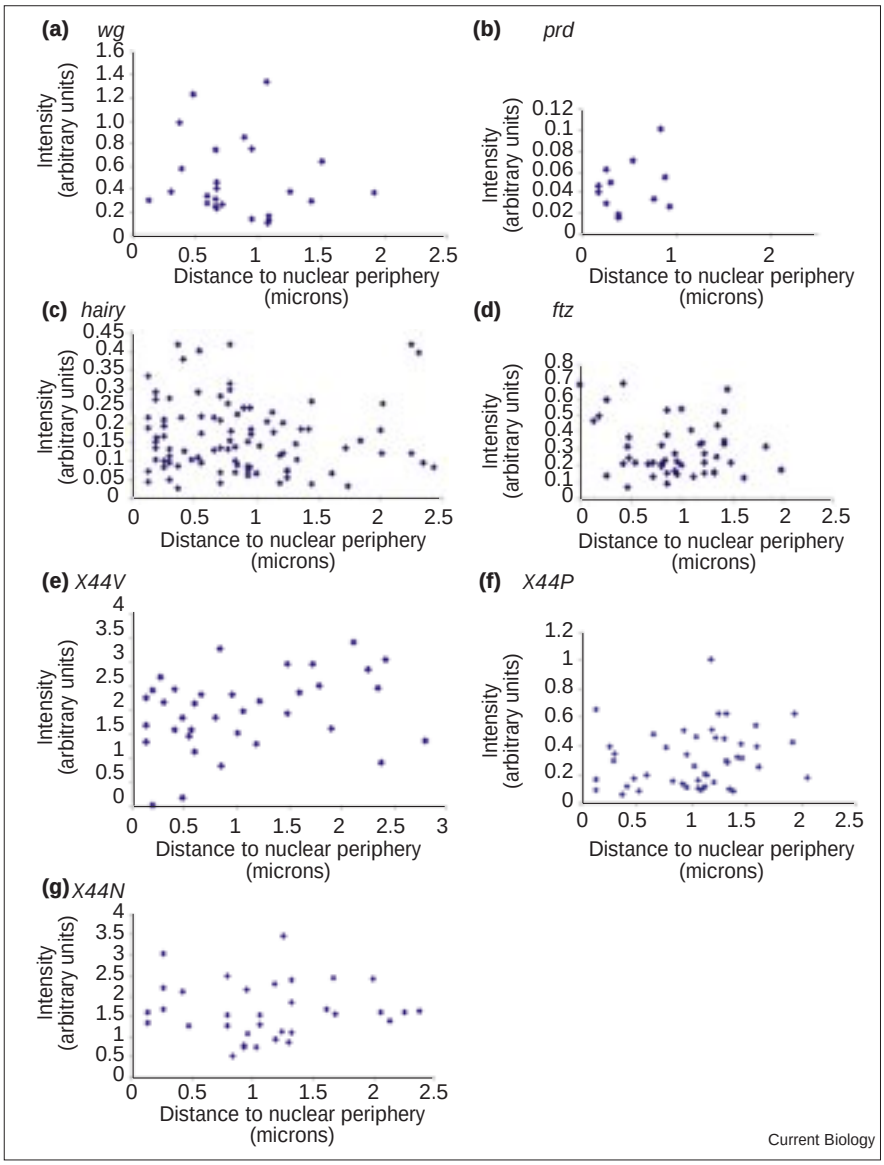
Export of transcripts requires long-range intranuclear transport of RNA

It has been suggested that transcribed genes are positioned at the nuclear envelope and this facilitates and perhaps causes directional export of mRNA [S3,S14,S15]. If such localization were to occur, it would bypass the need for long-range intranuclear transport of mRNA from the site of transcription to the nuclear periphery. At the stages examined, we found no evidence for preferential positioning of transcribed genes at or near the nuclear envelope. For all the sequences we investigated, most of the nascent transcript foci were localized to two foci at the site of the gene, a substantial distance from the nuclear periphery. The only exception was *prd*, which has been previously shown to be near a nuclear envelope attachment site [S5]. The scarcity of intranuclear RNA, outside the nascent transcripts, suggests that the movement of mRNA to the nuclear periphery and its export from the nucleus are very rapid in comparison to transcription and processing of RNA. Our results do not, however, allow us to distinguish between models for the movement of transcripts within the nucleus [S16]. While the movement of polyadenylated mRNA within the nucleus is probably diffusion-based [S17], the investigation of the mechanism of intranuclear movement of a single species of mRNA will probably require the ability to detect single mRNA molecules within living nuclei. It has recently become possible to detect single mRNA molecules in fixed material [S18] and mRNA decorated with the green fluorescent protein (GFP) in the cytoplasm of living cells [S19]. These techniques will, however, have to be substantially improved to allow detection *in vivo* of single mRNA molecules in transit from the nascent transcripts to the nuclear envelope.

We have also tested the possibility that the intranuclear location of transcribed genes affects their level of transcription by examining the intensity of the hybridization signal of individual transcribed loci. For example, transcription

Figure S3

The relationship between intensity of nascent transcript foci and their distance to the nuclear periphery for (a) *wg*, (b) *prd*, (c) *hairy*, (d) *ftz* and (e–g) three independent insertions of *X44*. Plots of the relative integrated nascent transcript intensity (in arbitrary units measured by the CCD camera) against their distance from the nuclear periphery. Each plot contains data collected from a single representative embryo. A visual inspection of the plots indicates that, in all cases, the intensity of the nascent transcripts and their distance from the nuclear periphery are not related. Regression analysis confirms this in all cases as the slopes of the regression curves are not significantly different from 0 (data not shown).



could be higher when a given gene is located at the nuclear periphery than when localized in the nuclear interior. In all cases examined, transcribed genes were found to have a relative level of fluorescence that was independent of their distance from the nuclear envelope. We conclude that the intranuclear position of a gene does not influence the level of transcription in the early *Drosophila* embryo.

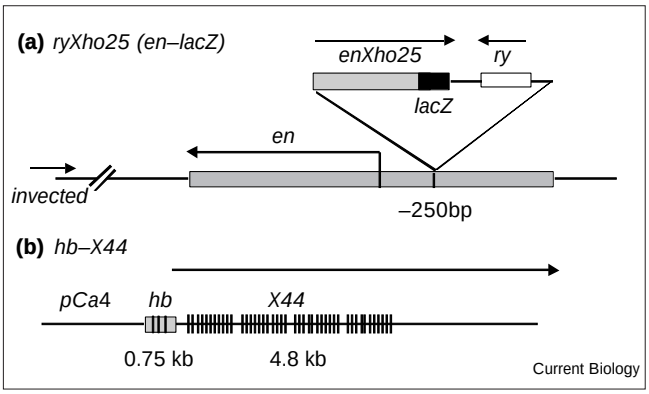
Although cytological location seems to be the most significant determinant of intranuclear position of genes in the embryo, additional factors might come into play in other biological settings. For example, heterochromatin can define a domain in the nucleus that is distinct from euchromatin, and there is strong evidence showing that some repressed genes are localized to heterochromatin. Furthermore, the same genes have very different localization sites when actively expressed [S20,S21]. It is possible that the introduction of substantial heterochromatin at later stages of development will mask the simplicity of the relationship we observe in the early embryo. Nevertheless, we conclude from our data that genes can be effectively transcribed without major relocalization within the nucleus.

Supplementary materials and methods

Drosophila *strains and construction of plasmids*

All fly stocks were raised on standard cornmeal-agar medium at 25°C. Oregon R was used as the wild-type strain. *Xho25* transgenic flies arose by a *P*-element-mediated insertion of an *engrailed–lacZ* fusion construct into the *en* locus by non-homologous, but targeted insertion [S22] (Figure S4a). *In situ* hybridization with probes against *lacZ* and

Figure S4



Maps of constructs lacking polyadenylation signals leading to very large nascent transcript foci. (**a**) Map of the *Xho25* (*en–lacZ*) transgene inserted 250 bp upstream of the transcription start site of the *en* gene, transcribed in the opposite direction and lacking polyadenylation and transcription termination sequences; and containing the *rosy* (*ry*) eye colour gene. The diagram is not drawn to scale and is based on [S22]. (**b**) Map of *X44*, a construct containing three *bicoid*-binding sites from a 0.75 kb fragment of the *hb* promoter region driving the expression of 44 tandem non-coding repeats and lacking polyadenylation and transcription termination sequences. The transgene was constructed in the pCaSpeR4 *P*-element transformation vector.

en upstream sequences, reveals expression in an *en*-like pattern, extension of transcription through the inserted *P*-element sequences and at least 20 kb upstream of the insert (M. Stark and P.H.O, unpublished data). Plasmid *pCahbX44* (*X44*) was constructed by fusing a 0.75 kb *Sal*I–*Xba*I restriction fragment of the *hb* promoter containing three *bicoid*-binding sites and the zygotic *hb* promoter upstream of 44 tandem copies of a non-coding sequence with no polyadenylation signal (Figure S4b). The fusion was inserted into the polylinker of the *P*-element transformation vector, pCaSpeR4 using *Kpn*I and *Bam*HI restriction sites. Plasmid *pBSX44* is an intermediate in the construction of *X44* which contains the 44 tandem repeats inserted into the *Bgl*II and *Bam*HI restriction sites of the bluescript plasmid vector. Further details of the cloning steps and sequences involved are available on request from the authors. After *P*-element-mediated germ-line transformation, 30 independent transgenic lines containing *X44* were recovered, representing insertions at different loci throughout the genome.

Collection and fixation of embryos

Embryos were collected from flies on yeasted grape-juice agar plates, then gathered in baskets and dechorionated in bleach. Embryos were fixed in a two-phase mixture of 37% formaldehyde and heptane for 5 min with gentle mixing [S23]. Fixed embryos were devitellinized in methanol, washed twice in methanol and stored in methanol at –20°C.

Hybridization and fluorescent detection

The visualization of nascent transcripts from individual genes has been possible for a decade, using dark histochemical stains [S10]. More recently, nascent transcripts have been detected using quantitative fluorescent methods, but these suffer from relatively poor resolution [S24] or a lack of sensitivity [S25]. It has been difficult to systematically study the intranuclear localization of nascent transcript foci using these methods. We have developed an alternative histochemical method of detecting mRNA, which is highly sensitive, and allows high-resolution fluorescent visualization of mRNA. The technique uses an enzymatic reaction to produce a highly reactive fluorescent product that covalently attaches to proteins near its site of production [S26]. We have applied this method to study the intranuclear localization of nascent transcripts by co-visualizing mRNA and the nuclear envelope. Antisense RNA probes were synthesized using T3 or T7 polymerases from plasmids containing cDNA sequences (unless stated) ranging in size from 1–3 kb. Plasmids for making probes were gifts from the following investigators (plasmid size in brackets): David Ish-Horowicz, *eve* (1 kb), *hairy* (1 kb), *prd* (3.5 kb), *wg* (2.3 kb); Elizabeth Gavis, *giant* (*gt*; 1.8 kb), *kni* (2 kb), *hkb* (1.7 kb); Bruce Edgar, *stg* (1.6 kb), *ftz* (2 kb), *hb* (2.4 kb), *Kr* (2.4 kb); Peter Gergen, *run* (2.4 kb); Ira Clark, *lacZ* (3 kb). Other plasmids originated in the authors' laboratories: *Ubx*, exon 1.2 (1.3 kb), *en* (2.3 kb), *pBSX44* (4.8 kb). *In situ* hybridization with fluorescent tyramide detection was performed as described previously [S26]. A detailed protocol is available on request. Briefly, embryos were rehydrated then post-fixed and rinsed five times in 1 × phosphate-buffered Tween20 (PBT: 1 × PBS + 0.1% Tween20) and prehybridized for at least 1 h at 70°C in hybridisation buffer (50% formamide, 5 × SSC, 100 μg/ml *Escherichia coli* tRNA, 50 μg/ml heparin and 0.1% Tween20 adjusted to pH 6.5 with HCl). Antisense RNA probes labeled with digoxygenin (DIG) were used at a concentration of 0.5 ng/ml and hybridized to embryos overnight at 70°C, then washed extensively at 70°C. Hybridized embryos were incubated in a horseradish peroxidase (HRP)-conjugated anti-DIG antibody (sheep anti-DIG-POD Fab fragment, Roche) and washed extensively in PBT. The HRP-coupled antibody was visualized by adding Cyanine-3 (Cy3) tyramides according to the manufacturer's instructions (TSA Direct, NEN Life Sciences). The reaction was allowed to proceed for 2–10 min before washing with PBT to remove unreacted tyramides. Following TSA staining, the nuclear envelope was labeled in the green fluorescent channel using AlexaFluor488-conjugated wheat germ agglutinin (AlexaWGA) from Molecular Probes. The rDNA was detected using a DIG-labeled DNA probe against externally transcribed sequences (ETS) of tandemly repeated ribosomal genes. A 0.7 kb probe was made by PCR from the Y22 plasmid (gift from Igor Dawid), hybridized in standard hybridization buffer containing 50% formamide at 45°C. The probe was detected with a rhodamine-coupled sheep anti-DIG antibody (Roche).

Three-dimensional fluorescence imaging and deconvolution

Embryos were mounted in Vectashield (Vector Laboratories) and three-dimensional image stacks (Z-series) were captured with a 12 bit PXL cooled CCD camera (Photometrics) on a Sedat/Agard widefield microscope (Applied Precision Inc.) based on an Olympus IX70 inverted microscope. An Olympus 100 ×/1.4NA oil immersion lens was used to view the embryos at high magnification. For imaging at the surface of the embryo, immersion oil with a refractive index (RI) of 1.520 was used, while oil with a higher refractive index (RI = 1.534) was used to correct spherical aberration when imaging sections from deeper in the embryo. DeltaVision software was used to control the movement of the motorized stage, and changing excitation and emission filters. Each image in a Z-series was obtained by moving the Z position by 0.2 μm increments. At every focal position, an image was captured for each fluorochrome, typically AlexaFluor488 (green) and Cy3 (red). Each Z-series from the same experiment was taken with an identical objective lens and camera settings (exposure, gain and degree of pixel binning) so that different images from the same sample could be compared. Out-of-focus light was reassigned to its original point source using Sedat/Agard three-dimensional constrained iterative deconvolution algorithms (DeltaVision software) after empirical determination of the point-spread function of each lens. The final images consist of a projection of the highest intensity signal from five consecutive 0.2 μm Z-sections, effectively providing an optical section 1 μm in thickness. Chromatic aberration was assessed and corrected, when necessary, using multicolor fluorescent 0.1 μm tetraspeck beads (Molecular Probes). In all cases, chromatic aberration resulted in a shift smaller than 0.4 μm between the wavelengths used [S27].

Data analysis

The fluorescence intensity within nascent transcript foci was measured using DeltaVision three-dimensional image analysis software. Two-dimensional polygons were found in deconvolved image stacks using

the appropriate threshold intensity to identify nascent transcript foci. Then three-dimensional objects were created from the two-dimensional polygons and the integrated fluorescence intensity, volume and position of each object were calculated. Identical threshold intensities were used to define nascent transcript foci within equivalent image stacks so that calculated intensity values were comparable between image stacks captured from the same sample of embryos.

Nascent transcript foci were usually identified as one or two very intense and highly localized fluorescent signals within nuclei. The number of foci was determined by viewing a series of focal planes through the entire nucleus. In some of the figures, nuclei appear to have only one nascent transcript focus, as the second focus is located in a different focal plane (for example, Figure 1b). Occasionally, the two sister chromatids are visible as closely localised twin spots (for example, in the male *run* embryos; Figure 3a). The embryonic expression patterns of all the genes studied here have been described previously, and we observed similar patterns of expression in all cases.

Nuclei were either imaged from the side (apical–basal view and longitudinal section) or surface (cross section) of several embryos at similar ages in interphase 14. The age of embryos was estimated from the progression of the cellularization membranes and the average length of nuclei [S12]. The positions of nascent transcript foci from many individual nuclei were plotted into an average nuclear outline. For the side views (Figures 1c and S2a,b), this was a nucleus 12.5 μm long, representing an embryo 30 min after the start of interphase 14. For cross sections (Figures 2b–m, 4b–i), a circle with a diameter of 6 μm was used as a standard nucleus. In a few nuclei which exhibited a markedly different size or shape from average, the position of nascent transcript foci was estimated with respect to the distance from the nuclear envelope and the position on the apical–basal axis by scaling the nucleus up or down in size to fit the average nuclear outline.

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