Dynamics of the Dorsal morphogen gradient

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The dorsoventral (DV) patterning of the Drosophila embryo depends on the nuclear localization gradient of Dorsal (Dl), a protein related to the mammalian NF-κB transcription factors. Current understanding of how the DI gradient works has been derived from studies of its transcriptional interpretation, but the gradient itself has not been quantified. In particular, it is not known whether the DI gradient is stable or dynamic during the DV patterning of the embryo. To address this question, we developed a mathematical model of the DI gradient and constrained its parameters by experimental data. Based on our computational analysis, we predict that the DI gradient is dynamic and, to a first approximation, can be described as a concentration profile with increasing amplitude and constant shape. These time-dependent properties of the DI gradient are different from those of the Bicoid and MAPK phosphorylation gradients, which pattern the anterior and terminal regions of the embryo. Specifically, the gradient of the nuclear levels of Bicoid is stable, whereas the pattern of MAPK phosphorylation changes in both shape and amplitude. We attribute these striking differences in the dynamics of maternal morphogen gradients to the differences in the initial conditions and chemistries of the anterior, DV, and terminal systems.

computational modeling | Drosophila | systems biology | parameter estimation

A tissue patterned by morphogen gradients can change its transcriptional state, grow, or deform either in response to the gradients or independently of them (1–3). When these changes are much slower than the dynamics of the gradient, a tissue responds to a stable signal. Transcriptional interpretation of such signals can rely on differences in the expression thresholds of target genes with respect to the spatially distributed repressors or activators (2, 4). A different strategy for signal interpretation is required when the formation of positional information becomes intertwined with the dynamics of the patterned system (2, 5). Here, we suggest that the dorsoventral (DV) patterning of the Drosophila embryo operates in this regime.

The DV patterning of the Drosophila embryo depends on the nuclear localization gradient of Dorsal (DI), a protein related to the NF-κB family of transcription factors (6–10). Transcriptional interpretation of the DI gradient depends on the differences in the affinities of the DI binding sites in the DI-target genes and several gene expression and signaling cascades initiated by DI (6, 11, 12). A ventral-to-dorsal occupancy gradient of the Toll cell surface receptor provides the activating signal for the DV patterning (13). In the absence of this signal, DI is sequestered in the cytoplasm, in complex with an inhibitory protein I-κB, called Cactus (Cact) in Drosophila. In response to Toll signaling, the DI–Cact complex dissociates, Cact is degraded, and DI enters the nucleus to control gene expression. In the current model of DV patterning, positional information is established by the spatial pattern of Toll occupancy (13, 14).

The DI gradient forms during the last five nuclear divisions of a syncytial blastoderm, a single cell with multiple nuclei (15). Because nuclei can be viewed as competing with Cact for DI, an increase in the number of nuclei can influence the DI gradient, but whether or not this happens is currently unknown. DI undergoes rapid nucleocytoplasmic shuttling with a nuclear residence time of several minutes (16). Nuclei change in volume and undergo five synchronous divisions (15, 17). To explore how these processes contribute to the formation of the DI gradient, we formulated a mathematical model that accounts for the nuclear import and export of DI, its interaction with Cact, and the dynamics of nuclear density and volumes in the syncytial blastoderm. Based on the computational analysis of this model and a number of our model-based experiments, we argue that the DI gradient is dynamic and, to a first approximation, can be described as a spatial pattern with constant shape and increasing amplitude.

Results

At the outset of this work, the only quantitative information about the spatial distribution of nuclear DI could be found in the study by Zinzen et al. (18), who had characterized the DV pattern of nuclear DI at a single time point. The domains of the DI-target genes begin to form several nuclear cycles before cellularization, and it is important to determine whether these genes respond to a constant or time-dependent signal. This question has been prompted by recent studies of the anterior-posterior (AP), DV, and terminal systems. First, the nuclear levels of Bcd, a morphogen that specifies the anterior structure of the embryo, are stable throughout the last five syncytial nuclear divisions. Bcd undergoes nucleocytoplasmic shuttling on the scale of several minutes. After mitosis, the nuclear levels of Bcd drop to zero, but are then rapidly reestablished to the premitosis level. Thus, with the exception of a rapid transient associated with nuclear divisions, a particular point in the embryo is exposed to a constant level of nuclear Bcd, which is distributed in a spatial pattern with constant shape and amplitude. In contrast, the pattern of phosphorylated ERK/MAPK (dpERK), which specifies the terminal regions of the embryo, changes in both shape and amplitude. Over the five last nuclear divisions in the syncytial blastoderm, the nuclear levels of dpERK increase at the poles and decrease in the midbody of the embryo.

We asked whether the spatial pattern of nuclear localization of DI is stable, similar to the pattern of Bcd, or dynamic, similar to the pattern of dpERK. Answering this question requires quantitative characterization of the nuclear levels of DI along the DV axis and at multiple time points. Here, this goal is achieved using a combination of quantitative imaging and mathematical modeling of the biophysical processes associated with the DV patterning of the embryo. Our experimental and computational results reveal that the DV pattern of nuclear DI behaves differently from both the Bcd and dpERK gradients. Similar to Bcd, the shape of the DV pattern of nuclear DI has approximately constant shape, but the amplitude of this pattern increases with time.

To visualize the DV distribution of nuclear DI, we used a transgenic line where one endogenous copy of DI was replaced by


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a $dl$-gfp transgene (see Materials and Methods). Several control experiments were done to test whether the DI gradient, quantified from the GFP signal in this line is close to the DI gradient obtained by quantifying the wild-type $\alpha$-DI antibody stainings. First, fixed embryos were stained with $\alpha$-DI and $\alpha$-GFP antibodies and the fluorescent intensities of the nuclear $\alpha$-DI and $\alpha$-GFP stainings were compared with each other (Fig. 1A–D). As shown in Fig. 1D, there is a strong linear correlation between the intensities of the $\alpha$-DI and $\alpha$-GFP antibody stainings. Thus, except for the differences in the background levels, the DV pattern of nuclear DI obtained on the basis of the GFP staining is proportional to the pattern that is based on the $\alpha$-DI antibody staining. Because the $\alpha$-DI antibody detects both endogenous and GFP-tagged DI, whereas the $\alpha$-GFP antibody detects only GFP-tagged DI, the linear correlation suggests that the GFP tag does not significantly interfere with the normal processes of DI transport and interactions. As an additional control experiment, we used the $\alpha$-DI antibody and stained the wild-type embryos together with embryos with one wild-type and one GFP-tagged copy of $dl$ (Fig. 1E). Comparison of the nuclear DI gradients in the wild-type and transgenic embryos (Fig. 1F) indicates that they are very close to each other. Thus, the line with one wild-type and one GFP-tagged $dl$ can be used to monitor the dynamics of the DI gradient in live imaging experiments.

To follow the dynamics of the DV pattern of nuclear DI, we used the “end on” imaging technique (19), where embryos are mounted with their AP axis perpendicular to the horizontal surface, enabling the imaging along the DV axis of the embryo. The space–time plot of nuclear DI extracted from a live-imaging experiment with $>$130 time points between cycles 11 and 14 is shown in Fig. 2A. The gradient of nuclear DI is very dynamic. Concentration of nuclear DI increases during interphase, which is followed by a drop to a low value during mitosis and a subsequent increase during the next cycle (Fig. 2A and B).

One of the main sources of variability in the end-on imaging of the DI gradient is introduced by the movement of nuclei in and out of the focal plane, both during interphase and between different nuclear division cycles. The inherent dynamics of the arrangement of the nuclei induces large variability in the profiles of the DI gradients along the DV axis. This effect, which is particularly significant during the earlier nuclear division cycles, makes the quantitative analysis of the DI gradients during these cycles extremely challenging. At the same time, the arrangement of nuclei is much more regular during cycle 14, when they are tightly packed under the plasma membrane. Thus, we use cycle-14 data from four separate live imaging experiments to extract the DV pattern of nuclear DI at a fixed time point: $\sim$15 min into cycle 14 (Fig. 2C). To characterize the dynamics of the DV pattern of nuclear DI at the previous time points, we use the data in Fig. 2C as a quantitative constraint for the mathematical model that accounts for the dynamics of DI/Cact interactions and nuclear divisions.

The objective of our model is to characterize the dynamics of the DV pattern of nuclear DI during the last five syncytial cell cycles. We model the syncytium as a periodic arrangement of cuboidal compartments, each of which contains a single spherical nucleus and an associated cytoplasmic region (Fig. 3A). DI, Cact, and DI–Cact complex diffuse rapidly within each of the compartments and undergo slow exchange with the neighboring compartments. The kinetic part of our model is a subset of reactions included in the more detailed models of the mammalian NF-κB system (Fig. 3B) (20). The association of DI and Cact is modeled as a second-order reaction with a spatially uniform rate constant. The dissociation of the DI–Cact complex is modeled as a first-order process with a rate constant that depends on the DV position, reflecting a DV pattern of Toll activation. We assume that this pattern remains constant during the entire patterning process. The rates of the nuclear import and export of DI depend on the surface area of the nucleus. Finally, we assume free Cact is produced at a constant rate and degraded in a first-order reaction.

Within each nuclear cycle nuclear radius increases (17). As a consequence of this change, nuclear and cytoplasmic concentrations are affected by both the chemical processes and volume changes. At specific time intervals that correspond to the detailed measurements of Foe and Alberts (15), nuclei divide. During
mitoses, the content of each nucleus is instantaneously redistributed in the corresponding cytoplasmic region. After mitosis, the nuclear envelope reforms, and the number of cytoplasmic compartments doubles. The model for doubling of compartments is shown in Fig. 3C; more details can be found in SI Appendix (Fig. S1).

The dynamics of the model depend on nine dimensionless parameters that characterize the spatial pattern of Toll activation, the rates of nuclear import and export of DI, the relative amounts of total DI and Cact, the rates at which the species move between the adjacent cytoplasmic compartments, and the formation and degradation rates of the DI–Cact complex (see SI Appendix, Table S1). Given these parameters, numerical solution of the model predicts the spatial patterns of DI, Cact, and DI–Cact complex during nuclear cycles 10–14. Although the exact values of model parameters are largely unknown, they can be constrained by the available experimental data. Specifically, it is known that the DI levels are mainly nuclear/cytoplasmic at the most ventral/dorsal points, respectively and that the levels of Cact at the dorsal side of the embryo are higher than those at the ventral side (2–4, 14). Based on the results of the live imaging studies, we required that the ventral-most level of nuclear DI increases monotonically within the interphase of cycle 14 and that this level reaches 90% of its final value in <15 min (16). Finally, we used the spatial pattern of nuclear DI at the nuclear cycle 14 (Fig. 2C), based on the quantification of the GFP autofluorescence from our live imaging experiments. This information does not define the parameters of our model uniquely. Rather, it constrains them to a “cloud,” or ensemble, in the

nine-dimensional space of parameters. Each point in this ensemble, i.e., a particular parameter vector, can be used to predict the properties of the DV system that cannot be readily measured experimentally. In particular, we are interested in the dynamics of nuclear DI levels at all times from nuclear cycle 10 to cycle 14 and along the entire DV axis.

Thus, we start with a relatively small amount of experimental data on the DV pattern of nuclear DI at a specific time point during the cycle 14, identify an ensemble of parameter vectors that satisfy these constraints, and then use this ensemble to predict the DI gradient at all times. A similar approach has been successfully used to make model-based statistical predictions about the dynamics of other cell signaling systems (21). To identify parameter sets that satisfy the experimentally based constraints for our model, we used a stochastic evolutionary optimization technique (22). The DI gradient dynamics predicted for one particular parameter vector is presented in Fig. 4A that shows a surface plot that represents the dynamics of nuclear DI at all times and all positions along the DV axis. Fig. 4B shows a comparison of the nuclear DI gradients reached at the end of all nuclear cycles, and Fig. 4C shows the dynamics of the nuclear DI level at the ventral-most point. After obtaining a large set of acceptable parameters (~40,000), we used the resulting ensemble as the basis for statistical analysis of the DI gradients predicted by the model.

As a first step in the statistical analysis of model predictions, we used the identified ensemble to calculate a distribution function for the ratio of the amplitudes of the DI gradients at the end of nuclear cycles 14 and 10 (A14/A10; Fig. 5A). We chose this metric based on the previous analysis of the gradients of dpERK, which patterns the terminal regions of the embryo, and the Bcd gradient, which patterns the AP axis (17, 23). For the nuclear Bcd gradient, which is stable during cycles 10 and 14, A14/A10 ~ 1. For the dpERK gradient, however, A14/A10 > 1, which reflects a pattern that is amplified at the poles. The distribution function for the ratio of the maxima of the spatial patterns of nuclear DI at cycles 14 and 10 has a single peak at ~1.5 (Fig. 5A). Upon further examination, we found that approximately two-thirds of the parameter sets predict a continuous increase in the ventral levels of nuclear DI at the end of each cycle (from cycle 10 to 14; Fig. 5A Upper Inset). The remaining parameter sets predict multiple combinations of increase and decrease in nuclear DI level from one cycle to another (Fig. 5Aii Lower Inset).

To test whether the amplitudes of the DI gradients change monotonically between subsequent nuclear cycles, embryos expressing the histone–GFP transgene, which marks the nuclei, were stained using the α-DI antibody. In this case, we used lateral images of embryos and located the peak of the gradient in each image (see Materials and Methods). Using a previously developed image processing approach (23), we assigned each embryo to one of the four temporal classes, corresponding to the nuclear cycles 11, 12, 13, and 14. For each group, we measured the amplitude of the gradient at the ventral-most point in the embryo (Fig. 5B). We analyzed the resulting dataset using a linear regression model and found a strong correlation (P < 0.001) between the age of the embryo (i.e., the nuclear division cycle) and the amplitude of the gradient (Fig. 5B). Based on these results, we conclude that the amplitude of the DI gradient is an increasing function of the number of nuclei. Based on this, we restricted the further statistical analysis to those members of the identified ensemble of parameter sets that satisfied this additional constraint (~67% of the parameter vectors in the original ensemble).

Focusing on these remaining two-thirds of parameter sets, we examined how the shape of the DI gradient changes from one nuclear cycle to another. We constructed a distribution function for the change in the half-width of the gradients from cycles 10 to 14 (Fig. 5C). Strikingly, we found that this distribution function has a peak at 0.05 and corresponds to the half-width change of at most 15%, which implies that the shape of the gradient is only weakly

Fig. 3. Mathematical modeling of reaction and transport processes in the syncytium. (A) (i) Schematic of the DV cross-section of the embryo. (ii) Model of the syncytium, with compartments arranged in a spatially periodically manner. (B) Reaction and transport processes within a single compartment of the syncytium. (C) Model for division of syncytiat compartments. The cuboids divide in a way such that the height (w) of compartment remains the same while the length (l) and width (b) reduce by a factor of \( \sqrt{2} \). Thus, the volume of the compartment halves, but the surface area shared by the two compartments reduces by \( \sqrt{2} \).
affected by nuclear divisions. We have also confirmed that the same is true for other nuclear cycles. In fact, the DI gradients at the end of the five nuclear cycles can be collapsed into one shape by simple rescaling (Fig. 5D). To a first approximation, the DV distribution of nuclear DI can be viewed as a pattern with constant shape and increasing amplitude. In addition to characterizing how the amplitude of the gradient changes from one nuclear cycle to another, we found that all points along the DV axes are exposed to a monotonically increasing level of DI within each nuclear cycle. Thus, based on the statistical analysis of the gradients predicted by the model, we conclude that the nuclear levels of DI are monotonically increasing in time during the interphase of syncytial nuclear divisions; after mitosis, they are reestablished, reaching a value that is higher than in the previous cycle.

Discussion

The DV patterning of the Drosophila embryo by the DI gradient is arguably the best-studied morphogenetic patterning event (6, 11, 24, 25). Multiple genes controlled by DI were identified, and sequence-specific patterns of their transcriptional regulation have become progressively understood. At the same time, the spatial distribution of DI and its dynamics have not been directly characterized. Both of these pieces of information are essential for quantitative understanding of the DV patterning. For instance, the relative arrangement of the expression domains of the DI target genes has been interpreted within the framework of thermodynamic models (18, 25). A key assumption of such models is that the input “seen” by the regulatory regions of the DI target genes is stable, but whether or not this is the case is unknown. Given the fact that the DI undergoes nucleocytoplasmic shuttling in a medium with increasing number of nuclei, the answer to the question about the stability of the nuclear levels of DI is far from obvious. Here, we answer this question based on the imaging experiments with fixed and live embryos, mathematical modeling, and computational analysis.

Each of these approaches has its relative advantages and limitations. Experiments with fixed embryos have limited temporal resolution and cannot follow the dynamics of the gradient within the same embryo. We have used imaging of fixed embryos to test the applicability of the DI-GFP transgene and characterize how the ventral-most levels of nuclear DI change from one nuclear cycle to the next. In contrast to experiments with fixed embryos, live imaging provides a dynamic view of the DI pattern as a function of time. At the same time, high-resolution images are typically collected from a single optical section, and quantitative analysis of the DI dynamics is compounded by the dynamics of nuclear rearrangements. We have used live imaging to quantify the spatial pattern of DI at a specific time point when the arrangement of nuclei is stable. This information provides one of the inputs for our mathematical model, which is clearly an approximation of processes in the real embryo. We assumed that the spatial pattern of Toll occupancy and the total amount of DI in the embryo remain constant and used a simple geometric model to describe the syncytial embryo. As more data become available, our model can be extended to describe these additional effects. For example, including the DI-dependent synthesis of Cact can explain why the shape of the DI gradient is dynamic during the DV patterning in other insects (26, 27). Nevertheless, by capturing what we believe are the essential features of the DI gradient and its interaction with the dynamics of the syncytium, our model provides a basis for more complex mathematical models that are essential for understanding the DV patterning in the wild-type and mutant backgrounds and exploring the evolvability of the DV patterning system (24, 28).

Based on the computational analysis of this model, we argue that the DI morphogen is distributed in a dynamic pattern with increasing amplitude and constant shape. These dynamics are different from the dynamics of Bcd and MAPK phosphorylation gradients.
which are formed in the same medium and at the same time as the DI gradient (29). Specifically, neither the shape nor the amplitude of the nuclear levels of Bcd are affected by changes in the number of nuclei. The two-peaked pattern of dpERK, however, changes in both shape and amplitude.

What is the origin of these striking differences between the dynamics of the three gradients? The terminal and DV gradients are initiated only when nuclei have reached the plasma membrane and respond to signals from the activated cell surface receptors. At the same time, the functional Bcd gradient is already established at this stage (30). Thus, the stability of the nuclear Bcd gradient during the last five nuclear divisions can be due to the fact that this gradient starts to form earlier and is not affected by nuclei, which sample only a small fraction of total Bcd at any given point along the AP axis (17, 31). Another key factor is the difference in the “chemistries” of Bcd, DI, and dpERK molecules. We have previously proposed that Bcd is not degraded on the time scale relevant for the formation of the gradient (31). In contrast, the molecules that pattern the DV and terminal regions are reactive: DI interacts with Cact and dpERK is dephosphorylated. Both of these processes can be affected by changes in nuclear density. Why, then, does the same change in nuclear density lead to different changes in the DI and dpERK gradients?

We attribute the fact that amplification of the dpERK levels is restricted to the poles, whereas the nuclear DI levels increase everywhere, to the different spatial extents of the sources that activate the DV and terminal systems. Our recent experiments suggest that the occupancy of the Torso receptor, which activates MAPK, is sharply localized to the poles and that the formation of the dpERK gradient relies on the diffusion of activated MAPK to the midbody regions (23, 32). By decreasing the distance to which the dpERK molecule can diffuse before being trapped or dephosphorylated, an increase in the nuclear density can prevent the lateral transport of dpERK to the midbody regions and amplify its level at the poles. However, we argue that a significant fraction of nuclei along the DV axis are exposed to appreciable levels of Toll signaling. As a consequence, lateral movement of the intracellular signaling components is less important for the DV patterning than for patterning of the terminal system, which agrees with the imaging experiments that revealed a slow DI exchange between the adjacent cytoplasmic regions (16).

Understanding how a dynamic DI gradient specifies multiple gene expression boundaries along the DV axis requires quantitative studies of the dynamics of other patterning signals in the early embryo (18, 33–37). As an example, we discuss the expression of genes that are expressed in the ventral and lateral regions of the embryo. Consider the regulation of sog, a gene repressed in the
prospective mesoderm and expressed in more lateral regions. It is possible that the ventral boundary of this pattern is stabilized by the feed-forward loop that is induced by DI and relies on the positive autoregulation of Twist, a high threshold target of DI (18). If this network operates in a bistable regime, then its output, and hence the ventral boundary of Twist, can be stable even with increasing levels of DI (28). However, it is not clear how the joint regulation of sog by both DI and Zelda, a maternally provided activator of early zygotic transcription (38, 39), we speculate that the dorsal boundary of the sog pattern is stabilized by the combined effect of the temporally increasing levels of spatially distributed DI and decreasing levels of spatially uniform Zelda. Thus, increasing levels of nuclear DI can be important for the dynamic expression of DI-target genes. Direct tests of this prediction can rely on the combined experimental, modeling, and computational strategy described in this work.

Materials and Methods

**Fly Lines.** Dorsal mutants dl1 and dl6 were obtained from the Bloomington Stock Center at the Indiana University and crossed with the dl-GFP/MTM3 line. Live imaging was performed with embryos of the genotype dl1/+;dlp[w−→dl-GFP]+.

**Antibody Staining and Live Imaging.** Embryos were dechorionated in bleach for 1 min and then washed in 0.7% NaCl containing 0.05% Triton X-100. They were then treated with heptane for 30 s and fixed in a 1:1 mixture of heptane/4% paraformaldehyde in PBS for 20 min at room temperature. After fresh heptane was added, embryos were devitellinized in equal volumes of methanol and stored in methanol at −20 °C. For immunostaining, embryos were rehydrated for 10 min in PBS with 0.3% Triton X-100 (PBT) and blocked for 1 h with 2% BSA in PBT at room temperature; this was followed by a 2-h incubation with the primary antibody. The primary antibodies used were mouse anti-Dorsal 7A4 (Developmental Studies Hybridoma Bank at the University of Iowa) at 1:50 and rabbit anti-GFP (Molecular Probes) at 1:1,500. Embryos were washed in PBT and subsequently stained with fluorescently coupled secondary antibodies (Molecular Probes). For end-on imaging, embryos were mounted on their posterior pole and imaged as described (19).

Embryos were imaged at ~70 μm from the posterior pole, at a position where the diameter of the embryo was ~140–150 μm. Control embryos for secondary background, wild-type embryos, and dl1/dl-GFP dorsal embryos were immunostained on the same day and imaged under the same settings of the confocal microscope. For quantification, small regions of interest were drawn in the nuclei marked by Hoechst dye (Molecular Probes) using the software Image J. Average fluorescence intensity with corresponding positions of nuclei was obtained for anti-DI and anti-GFP. The gradients were normalized between zero and one by subtracting the minimum intensity measurement within a nucleus and then dividing the entire gradient by the maximum intensity.

Live imaging was performed with dl heterozygous embryos expressing GFP-dorsal (dl1/+;dlp[w−→dl-GFP]) to match the wild-type dl copy number. Flies were allowed to lay embryos for 1 h at 25 °C on grape juice agar plates. Embryos were dechorionated and mounted on their posterior pole in Lab Tek chambers coated with silane (40), immersed in PBS, and imaged on an LSM 510 confocal microscope. Imaging was performed at ~70 μm from the posterior pole. Quantification was performed with ImageJ.

For lateral imaging of fixed embryos, they were dechorionated for 1 min in 100% bleach, fixed for 20 min by gentle shaking a nutator, and devitellinized by vigorous 1-min shaking in a mixture of heptane and methanol. Next, embryos were quickly rehydrated and transferred to the blocking and antibody steps of the protocol. All further processing was done with 0.02% PBS-Triton X-100 as the diluting solution. DI antibody (Developmental Hybridoma Bank) was used in 1:100 dilution, and goat anti-mouse Alexa Fluor 546 antibody (Invitrogen; 1:500 diluting solution). Dl antibody (Developmental Hybridoma Bank) was used for 10 min in PBS with 0.3% Triton X-100 (PBT) and blocked for 1 h with 2% BSA in PBT at room temperature; this was followed by a 2-h incubation with the primary antibody. The primary antibodies used were mouse anti-Dorsal 7A4 (Developmental Studies Hybridoma Bank at the University of Iowa) at 1:50 and rabbit anti-GFP (Molecular Probes) at 1:1,500. Embryos were washed in PBT and subsequently stained with fluorescently coupled secondary antibodies (Molecular Probes). For end-on imaging, embryos were mounted on their posterior pole and imaged as described (19).

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