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Digital nature of the immediate-early transcriptional response

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SUMMARY

Stimulation of transcription by extracellular signals is a major component of a cell's decision making. Yet the quantitative relationship between signal and acute transcriptional response is unclear. One view is that transcription is directly graded with inducer concentration. In an alternative model, the response occurs only above a threshold inducer concentration. Standard methods for monitoring transcription lack continuous information from individual cells or mask immediate-early transcription by measuring downstream protein expression. We have therefore used a technique for directly monitoring nascent RNA in living cells, to quantify the direct transcriptional response to an extracellular signal in real time, in single cells. At increasing doses of inducer, increasing numbers of cells displayed a transcriptional response. However, over the same range of doses, the change in cell response strength, measured as the length, frequency and intensity of transcriptional pulses, was small, with considerable variation between cells. These data support a model in which cells have different sensitivities to developmental inducer and respond in a digital manner above individual stimulus thresholds. Biased digital responses may be necessary for certain forms of developmental specification. Limiting bias in responsiveness is required to reduce noise in positional signalling.

KEY WORDS: *Dictyostelium*, Immediate-early response, Live cell imaging, Transcription

INTRODUCTION

The transcriptional response to extracellular signals is integral to cellular choices of proliferation, stress response and differentiation. Cells must assess signal strength to respond appropriately. However, the acute transcriptional response, the quantitative relationship between inducer and RNA polymerase activity at a gene, is unclear. One model is that response strength directly reflects strength of stimulus. With more signal, more receptors are bound and more intracellular signalling components are activated, leading to a greater probability of transcriptional firing. This view is supported by studies on the responsiveness of embryonic tissue to inducers, which imply graded responses (Ashe and Briscoe, 2006; Green et al., 1992; Gurdon et al., 1999; Lander, 2007). An alternative model, a digital or all-or-none response (Hazzalin and Mahadevan, 2002; Hume, 2000), is one in which cells respond only above a threshold signal concentration. This model was suggested by studies of prokaryotic and viral gene expression (Ko et al., 1990; Novick and Weiner, 1957; Ozbudak et al., 2004).

Earlier studies measured protein or bulk RNA synthesis, hours or days after initial transcriptional responses. These delays allow adaptation to responses and extensive feedback (Becskei et al., 2001; Vilar et al., 2003). Protein-based measures are time-averaged and include combined fluctuations in multiple steps of gene expression. Standard RNA-based measures rely on population-averaged measurements of RNA from disrupted cells. These techniques do not distinguish events occurring in individual cells nor do they follow cells over time, therefore cannot discriminate between digital and graded models of signal-induced transcription. With this in mind, we have directly visualised the dose-response behaviour of a signal-induced gene in individual living cells. Our data indicate the immediate-early transcriptional response is predominantly digital.

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MATERIALS AND METHODS

Molecular biology

A *Bam*HI fragment containing 24 MS2 repeats upstream of a blasticidin selection cassette (Chubb et al., 2006b) was inserted between promoter (–905 to +60) and 5′ (+45 to +1040) fragments of the *ecmA* gene, just downstream of the ATG. The targeting fragment was released by *Hin*dIII/*Not*I digestion, transformed into *Dictyostelium* AX2 cells and selected with 10 μg/ml blasticidin S. Correct recombinants were then transformed with an MS2-GFP expression vector (Chubb et al., 2006b). To visualise DimB, cells were transformed with a DimB-GFP expression vector (from Yoko Yamada, University of Dundee, UK).

Stimulation experiments

Cells were maintained in HL5 media supplemented with 20 μg/ml G418. G418 was removed 1 day before development, which was initiated as described (Chubb et al., 2006a). For reproducible developmental timing, cells were incubated in humidified chambers at 6°C for 16 hours, then 22°C for 5 hours, taking cells to the equivalent of 10 hours of normal development (parental cells, which normally aggregate at 6 hours, aggregated 1 hour after return to 22°C). Before 10 hours, cells did not respond to stimuli. From 11 hours, 40% of cells displayed MS2 RNA spots (see Fig. S1B in the supplementary material), obscuring stimulation. Mounds were harvested in 1 ml KK2/10 mM EDTA, diluted 10-fold then disaggregated by passing through a 20G needle (BD) 15 times. The resulting cell suspension was plated (200 μl per well) into 8-well dishes (Nunc 155411). After loading onto the microscope, a 3D stack was captured before addition of 200 μl DIF (Biomol) and cAMP (Sigma) in KK2/EDTA (at the indicated concentrations).

Imaging

Cells were viewed on a previously described inverted fluorescence microscope using protocols optimised for long-term high-resolution 3D imaging of photosensitive samples (Muramoto and Chubb, 2008). Three dimensional stacks (43 slices) were captured at multiple positions every 3.5 minutes with 250 nm *z*-steps and 50 msecond exposures. Cell fields

were selected without prior fluorescence exposure, using bright-field illumination. Proportions of cells expressing *ecmA*-MS2 RNA were measured by scoring each cell 'on' or 'off', as described previously (Chubb et al., 2006b). Pulse length and intensity measurements were taken from complete pulses. Images were deconvolved using Volocity (15 iterations, refractive index of 1.518, NA of 1.4, emission 508 nm) for intensity measurements. Intensity values were measured from 5×5×5 voxel cubes centred on the brightest pixels. Intensity was defined as mean intensity per voxel within cubes, averaged over each time point within a pulse, after background subtraction. Background intensities were calculated from similar cubes applied to cell bodies, averaged over two measurements. To account for day variation in background, values were scaled to mean background intensities for the day.

RESULTS AND DISCUSSION

To visualise signal-induced transcription, we used a fluorescent live-cell RNA reporter to measure nascent RNA production in single cells (Bertrand et al., 1998; Chubb et al., 2006b; Golding et al., 2005). MS2 RNA stem loops have a high affinity, sequence-specific

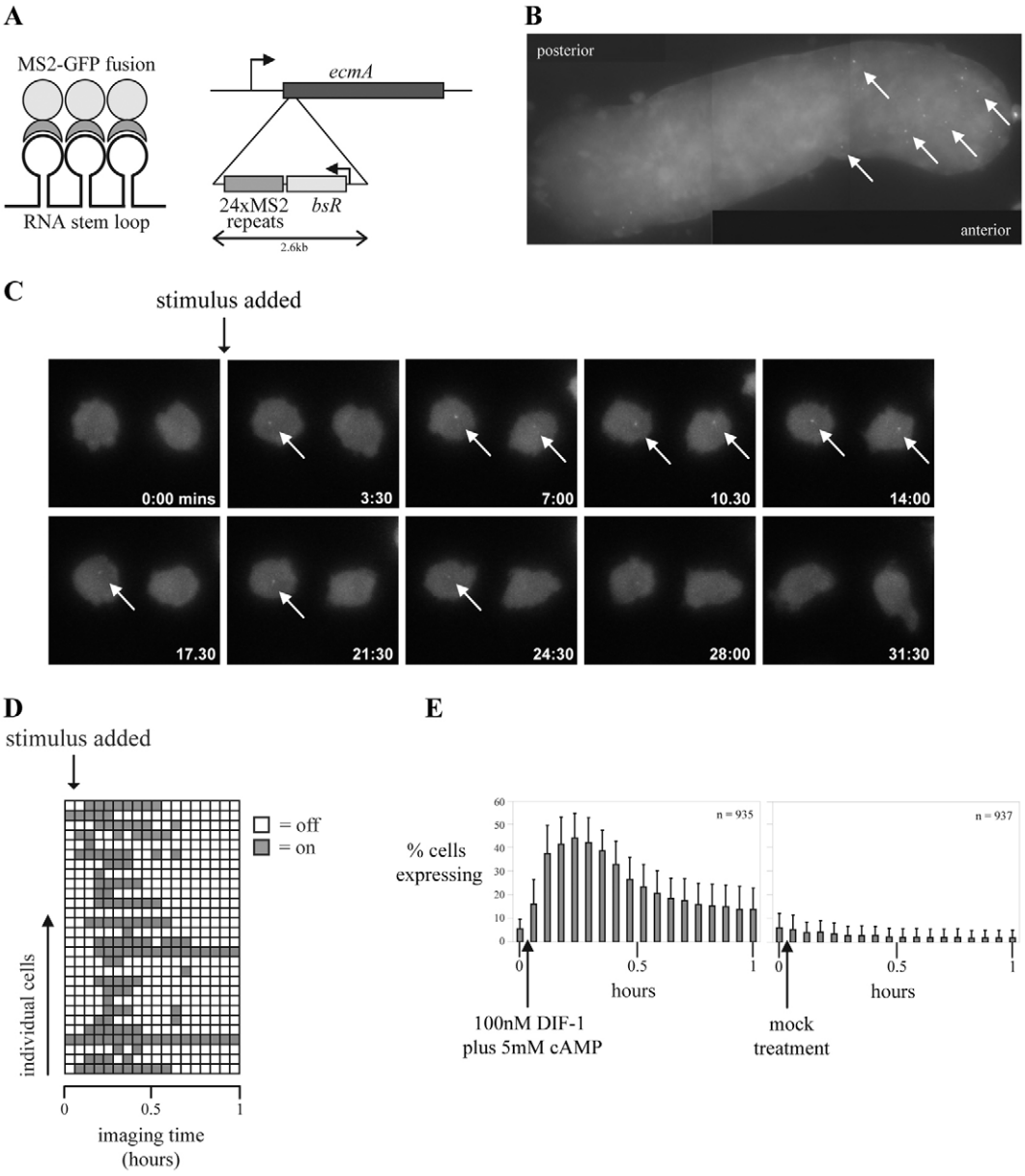

Fig. 1. Visualising immediate-early transcription in living cells. (**A**) Detecting transcription in living cells. MS2 protein is fused to GFP to allow the detection of MS2 RNA repeats. Twenty-four MS2 repeats were inserted into the *ecmA* gene (insertion was confirmed by Southern blot, see Fig. S1A in the supplementary material). (**B**) Spatial distribution of transcription in a live multicellular organism. Spots reflect nascent *ecmA*-MS2 transcripts (arrows) in the anterior (prospective stalk cells) of the slug stage (16 hours of development). (**C**) Visualising signal-induced transcription. After the initial image was taken, 100 nM DIF/5 mM cAMP was added to cells. The left cell had a nascent RNA spot for seven frames; the right cell for three frames (arrows). Images are projections of 3D stacks. (**D**) Variability of the transcriptional response to a uniform signal. Cells were scored as 'on' (transcribing *ecmA*) when a spot was visible. Each horizontal record represents one cell. Typical responses are shown after stimulation with 100 nM DIF/5 mM cAMP. (**E**) The dynamics of recruitment of transcribing cells after stimulation. The stimulus was added after first time point. The left graph shows stimulation with 100 nM DIF/5 mM cAMP; the right graph shows mock stimulation (buffer). A total of 935 (stimulated) and 937 (mock) cells were captured from 14 experiments.

interaction with the MS2 coat protein. A fusion of GFP to the MS2 protein directs GFP to stem loops. Twenty-four MS2 stem loop repeats were integrated into the developmentally induced gene *ecmA* by homologous recombination in *Dictyostelium* cells (Fig. 1A; see also Fig. S1A in the supplementary material). Upon transcription, MS2 loops are incorporated into newly synthesised RNA and are detected by the MS2-GFP fusion. MS2-GFP recruitment allows nascent RNA to be detected as a single spot at the site of transcription (Fig. 1B,C).

Dictyostelium cells adopt a combination of two fates during development, stalk and spore. Stalk fate is triggered by the extracellular signals DIF and cAMP. (Williams, 2006). Transcription of *ecmA* is an immediate-early response to these signals. The gene is induced rapidly in a cycloheximide-resistant fashion, implying no intermediate gene expression (Verkerke-van Wijk et al., 1998), and the gene can be disrupted with minimal developmental effect (Morrison et al., 1994). MS2-tagged *ecmA* showed correct spatial and temporal induction. We observed a high frequency of transcription spots at the anterior of the multicellular slug structure (Fig. 1B), where stalk precursors reside. Strong induction of MS2 transcription was observed in multicellular structures after 10 hours of development, which is consistent with wild-type *ecmA* expression (see Fig. S1B in the supplementary material). Estimates of the RNA load at transcription sites, by comparison with a previously characterised gene, suggest most spots have 10 or fewer RNAs, with a few outliers containing 20-30 RNAs (see Fig. S1C in the supplementary material).

We defined the dynamics of the immediate-early transcriptional response in single cells. Ten-hour developed cells were stimulated with 100 nM DIF and 5 mM cAMP. Stills from a movie of these experiments are shown in Fig. 1C (see Movies 1 and 2 in the supplementary material). Cells were stimulated after the first frame (t=0). The left cell responded rapidly, with a spot visible by 3.5 minutes, which persisted for more than 20 minutes. The right cell displayed a slower induction of transcription, with a spot appearing after 7 minutes that disappeared 10 minutes later. We collected images from 935 stimulated cells over 14 experimental days. The pattern of the transcriptional response in representative expressing

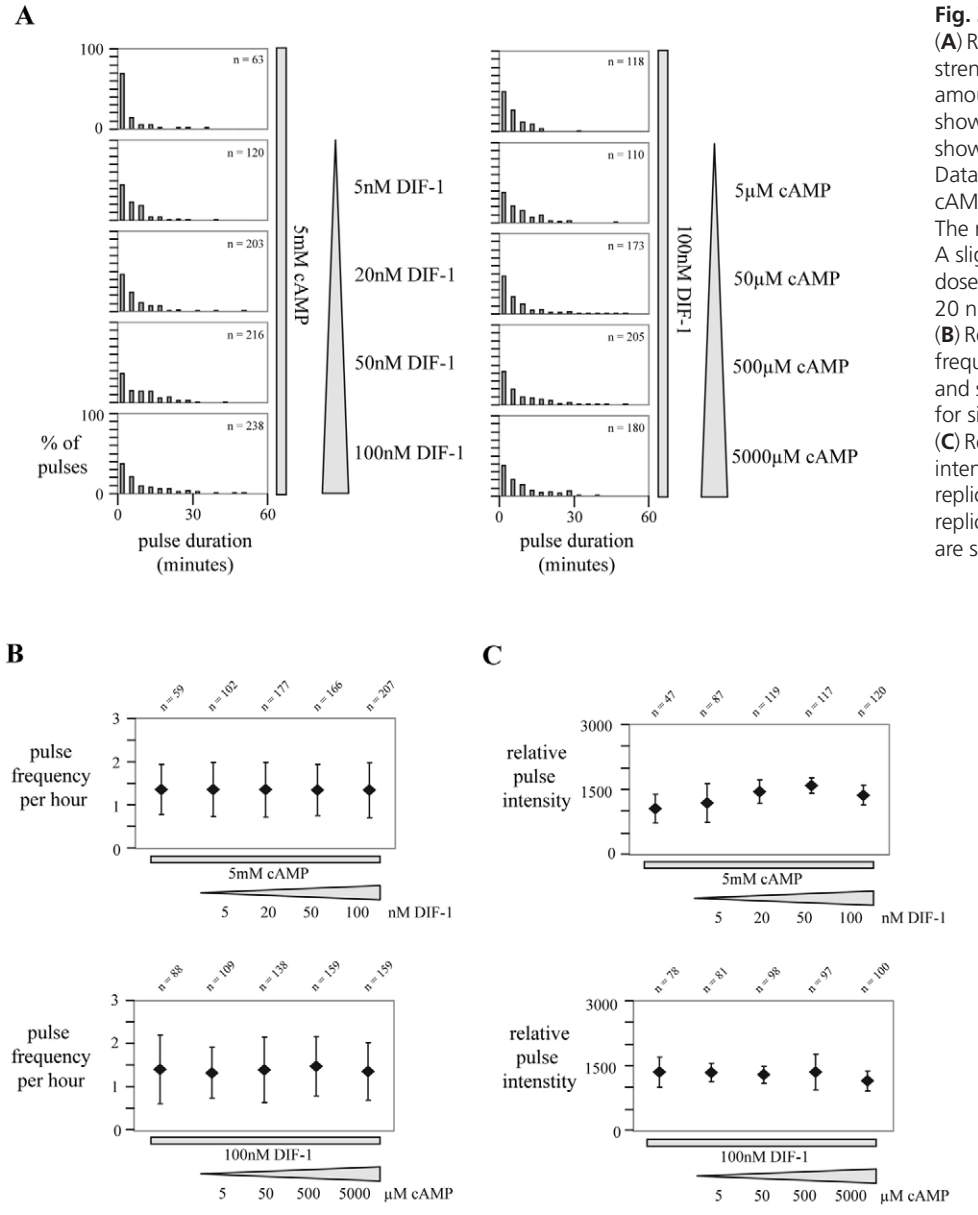

Fig. 2. Minimal grading of transcription. (**A**) Relationship of pulse duration to signal strength. Pulse duration (the continuous amount of time that a cell displays a spot) is shown on the horizontal axis. Vertical axes show percentage of pulses of a given length. Data shown for DIF (left, six replicates) and cAMP (right, five replicates) dose responses. The numbers of pulses analysed are indicated. A slight increase in pulse length at high DIF doses was observed (P=0.039; paired t-test, 20 nM versus 100 nM, 5 degrees of freedom). (**B**) Relationship of stimulus strength to pulse frequency. Mean pulse frequency (pulses/hour) and s.d. are shown for different signal doses for six DIF and five cAMP experiments. (**C**) Relationship of stimulus strength to pulse intensity. Top, DIF dose response (six replicates); bottom, cAMP dose response (five replicates). Means and s.e.m. from replicates are shown; cell numbers are indicated.

cells showed high variability of response (Fig. 1D), even under these conditions of strong uniform stimulation, which are normally sufficient to induce all cells in the culture to terminally differentiate into stalk cells (Thompson and Kay, 2000). The gene pulsed on for only one frame in some cells, whereas it was expressed strongly for nearly the entire hour of capture in others. Some cells showed multiple pulses of transcription. Expression was not detected in many cells. The proportion of cells expressing during the hour following stimulation is plotted in Fig. 1E. Induction of expression occurred within 3.5 minutes of signal addition, and number of responding cells peaked 15 minutes after stimulation, when over 40% of cells had detectable RNA spots. The proportion of cells responding then diminished, although the distribution had a long tail, with cells still pulsing 1 hour after stimulation. The cumulative proportion of the population expressing in the 1-hour period was 70%. Similar movies of 937 mock-treated cells showed basal transcription in a few cells, but no stimulation (Fig. 1E). The decay of response to stimulus was not a consequence of cAMP degradation, as the response trajectory was similar when the cAMP was substituted by a non-degradable cAMP analogue (Soede et al., 1996) (see Fig. S1D in the supplementary material).

To investigate the relationship between signal dose and transcriptional response, we induced cells at different doses of the stimuli, DIF and cAMP. One signal was held constant, and the other varied. Experiments at different doses were carried out in parallel, sampling from the same starting cell population, using multi-well imaging dishes.

To address whether immediate-early transcriptional responses are graded, we measured the duration and frequency of transcriptional pulses and the intensity of RNA spots in expressing cells. Transcriptional pulses displayed considerable variability in duration, frequency and intensity (Fig. 2). Durations of pulses measured from DIF dose-responses from six independent experiments are shown in Fig. 2A. The horizontal axis represents pulse duration; vertical axes represent the incidence of pulses of specific durations. Shorter pulses were more common, but some lasted for tens of minutes. The distributions in Fig. 2A are shifted slightly to the right at higher doses of DIF. When mean pulse lengths were extracted from these data, the mean for 100 nM DIF (10.9 minutes) was higher than the mean for 20 nM DIF (8.2 minutes; P=0.039). A stimulus range of 20-fold caused a 33% increase in mean pulse length. Considering the large variances of pulse length distributions (Fig. 2A), this effect indicates very shallow grading of the transcriptional response. No change in pulse duration was observed over a 1000-fold cAMP dose range (Fig. 2A).

Pulse frequency was independent of signal dose. At all signal doses, there was large diversity in frequency between cells. Some cells pulsed only once after stimulation, others showed three or more pulses. Mean pulse frequency (pulses per hour) at maximal stimulation by DIF or cAMP did not differ from the pulse frequencies of expressing cells at the lowest doses of these signals (Fig. 2B). Furthermore, frequencies at the highest doses were similar to frequencies for the few cells expressing without signal.

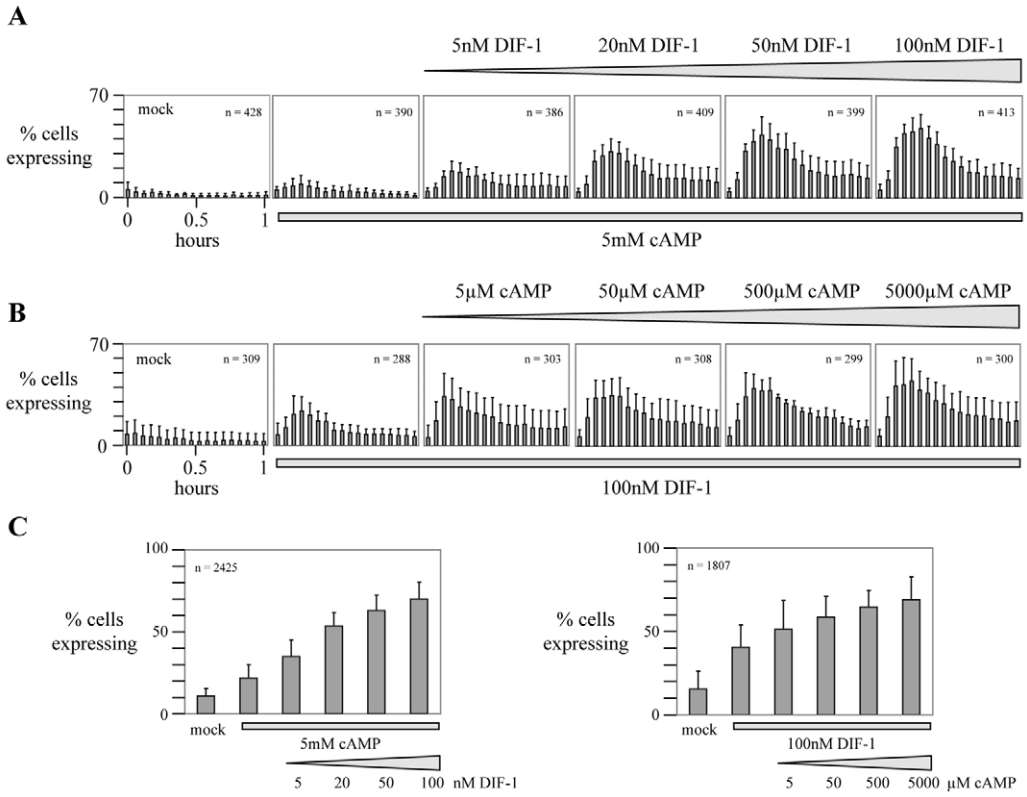

Fig. 3. Probability of expression increases at higher signal concentrations. (**A**) More cells showed a transcriptional response with increasing DIF concentrations. Indicated DIF concentration was added together with 5 mM cAMP. More than 380 cells were recorded per dose over six replicates (s.d. is shown). Signals were added after the first frame. (**B**) cAMP dose response. Indicated cAMP concentration was added to cells with 100 nM DIF. More than 280 cells were imaged per dose over five replicates. (**C**) Cumulative transcriptional response. Data in A and B represented as the total number of cells expressing in the hour after stimulation; DIF (left) and cAMP (right) dose-responses with s.d. indicated.

Pulse intensity was also independent of signal dose. Spot intensity reflects nascent RNA production (Kumaran and Spector, 2008; Zenklusen et al., 2008). Natural variation of spot intensity in the population was considerable, illustrated by within-replicate standard deviations of a similar magnitude to the mean intensity values. The slight increase in mean intensity between 5 nM and 50 nM DIF visible in Fig. 2C was not significant (P=0.16, paired t-test, 5 degrees of freedom). No intensity changes were observed over a 1000-fold range of cAMP.

To address to what extent cells are induced to express at different stimulus strengths, we measured how changes in signal dose affect the proportion of cells showing transcriptional responses. Fig. 3A shows responses to increasing doses of DIF. In the first panel is the basal response. A slight stimulation was observed with cAMP alone. In the next four panels, effects of increasing DIF dose are shown. Over a 20-fold range of DIF concentrations, a 2.5-fold increase in the peak number of responding cells was observed. This change in the proportion of responding cells at higher doses of DIF was also reflected in the cumulative proportion responding over the entire hour after stimulation (Fig. 3C). Fig. 3B shows the responses of cells to a 1000-fold range of cAMP. DIF alone induced considerable expression, with the 1000-fold increase of cAMP causing a 1.3-fold increase in proportion responding. There was no evidence for specific response thresholds for either signal. Bias in response was not caused by bias in nuclear recruitment of DimB, a transcription factor required for *ecmA* transcription (Huang et al., 2006; Zhukovskaya et al., 2006); DimB-GFP was rapidly recruited into all nuclei during stimulation (see Fig. S2A in the supplementary material). Nor was there an obvious cell cycle bias in responding cells, as assessed using the marker RFP-PCNA (Muramoto and Chubb, 2008). At 10 hours of development, all cells had the diffuse nuclear PCNA distribution of G2. Differences in cycles of developing cells could only be detected at 11 hours (see Fig. S2B in the supplementary material), when a wave of S-phase (as indicated by PCNA foci) was observed. Biases may not have a single cause (Raj and van Oudenaarden, 2008).

Our data indicate that immediate-early transcriptional responses to uniform developmental signalling are highly variable between cells. No uniform response thresholds were identified. Over a 20-fold range of DIF, we detected a 2.5-fold increase in number of cells responding. The frequency and intensity of pulsing was unchanged by increasing signal dose. Increased pulse durations were observed, implying a grading of the intracellular response to stimulus; however, this grading was small given the large variability in pulse length. A 1000-fold range of cAMP caused an increase in the number of cells responding, but no change in pulse length, intensity or frequency. These data favour a view where individual cells have different sensitivities to inducer, with cells transcribing only when a signal exceeds individual cell thresholds and with relative insensitivity to doses above the threshold. All-or-none behaviour implies an amplification of the signal solely by components of the immediate-early response. Amplification does not require intermediate or secondary gene expression, as is inferred for the *lac* operon (Vilar et al., 2003).

What are the implications of digital and biased responses for developmental patterning? *Dictyostelium* cells are motile and resolve scattered, stochastic induction events by sorting (MacWilliams et al., 2006; Thompson et al., 2004). With a global inducer and cell motility diminishing the effects of microenvironment, biases are necessary for stable differentiation. The existence of response biases was predicted by earlier models, in which heterogeneity in the threshold is necessary for the stability of patterning systems based on negative feedback and cell sorting (Blaschke et al., 1986). Heterogeneity in threshold is not unique to *Dictyostelium*. Models of lateral inhibition require initial biases between cells (Fior and Henrique, 2008). Embryonic and haematopoietic stem cell differentiation is biased by fluctuations in pluripotency regulators (Chambers et al., 2007; Chang et al., 2008; Hayashi et al., 2008; Kalmar et al., 2009), and stochastic patterning selects cells destined to form the apical ectodermal ridge of vertebrate limb buds (Altabef et al., 1997).

Digital responses are not inconsistent with positional information. Positional models involving multiple thresholds only require distinct signalling thresholds for additional genes. There may be graded transcriptional responses in other immediate-early embryonic contexts, which remains to be determined. However, cells responding to positional information require strategies to remove heavily biased responses. Biases present a problem in the conversion of shallow signalling gradients into sharp boundaries. Feedback mechanisms could sharpen rough specification (Arias and Hayward, 2006). However, for cells to detect only a 10% difference in signal (Lander, 2007) there would be little for feedback to work upon, with biases as strong as we report. Model systems for instructive signalling in animal development often display potential noise-reducing features. These include cell cycle synchronisation, low motility so responding cells have similar environmental and lineage histories, and limited early zygotic transcription, which prevents the accumulation of gene expression differences. The syncitial development of flies extends this theme (Gregor et al., 2007). With no boundaries between cells, cytoplasmic mixing should equalise biases. All these features potentially generate a more uniform template for signalling.

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Competing interests statement
The authors declare no competing financial interests.

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