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Journal
Developmental Biology, 189(1)

ISSN
0012-1606

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Publication Date
1997-09-01

DOI
10.1006/dbio.1997.8665

DEVELOPMENTAL BIOLOGY **189,** 13–21 (1997)
ARTICLE NO. DB978665

Cell Cycle Length Affects Gene Expression and Pattern Formation in Limbs

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The relationship between growth and pattern specification during development remains elusive. Some molecules known to function as growth factors are also potent agents of pattern formation. This raises the possibility that growth factors could act in pattern formation via an effect on the cell cycle. We have tested the significance of the length of the cell cycle for gene expression and pattern formation in developing chick limb buds by locally slowing the cell cycle. When anterior cell cycles are lengthened by reversible inhibition of DNA replication or by other means, some genes characteristic of the posterior polarizing region are expressed, and digit duplication is observed. Conversely, when posterior cell cycles are slowed, expression of some posterior-specific genes is inhibited, but the pattern is normal. These results indicate that control of the length of the cell cycle could play a primary role in pattern formation by influencing the complement of genes expressed in a particular region of the embryo.

INTRODUCTION

Embryonic development is characterized by growth and pattern formation leading from a single cell to the structurally complex, multicellular organism. The mechanisms that function to integrate these two processes have been well studied during the development and regeneration of the vertebrate limb. From such studies it is clear that these processes are functionally related since alterations in the processes of pattern formation also affect growth (Bryant *et al.*, 1981; French *et al.*, 1976). This can be demonstrated directly in regenerating amphibian limbs where limb tissues can be manipulated surgically so as to alter pattern formation. Such manipulations predictably lead to either complete inhibition of growth and pattern formation on one extreme or the stimulation of threefold excess growth and pattern formation on the other (Gardiner and Bryant, 1998; Muneoka *et al.*, 1989). Other means of altering pattern formation, such as exposure to retinoic acid, also lead to linked changes in growth. Inhibition of pattern is linked to inhibition of growth; stimulation of supernumerary pattern is coupled to additional growth (see Bryant and Gardiner, 1992).

Several molecules have been identified as being important in the control of limb development (see Cohn and Tickle, 1996). Since some of these molecules (e.g., members of the BMP and FGF families and *shh*) play an active role in both growth and in pattern formation, it is possible that they could provide the functional linkage between the coordinated regulation of growth and gene expression leading to pattern formation. Although it is usual to view pattern formation as driving growth, the occurrence of bifunctional molecules raises the reciprocal possibility that changes in growth could precede alterations in gene expression and pattern formation. Pattern formation might begin with changes in the cell cycle which induce changes in gene expression leading to altered cell–cell interactions and further changes in gene expression and growth. By this view, rather than a linear cause and effect relationship between growth and pattern formation, the process could involve reciprocal interactions and feedback loops involving linked changes in the cell cycle and in gene expression.

In the experiments reported in this paper, we have tested the hypothesis that changes in cell cycle kinetics can alter gene expression and pattern formation. To do this, we transiently blocked the cell cycle of small regions of developing chick limb buds and assayed for changes in gene expression and pattern. We have observed that alterations in the cell cycle in different parts of the limb bud lead to altered patterns of expression of several genes known to be important in controlling limb pattern formation. In addition, we observed the paradoxical result that when anterior cell cycles were lengthened, despite the fact that growth was transiently inhibited, the eventual result was stimulation of extra growth and pattern.

0012-1606/97 $25.00

MATERIALS AND METHODS

Aphidicolin bead implantation. Beads (Amberlite, XAD-7, 200 μm in diameter) were soaked in an aphidicolin (Sigma) solution dissolved in DMSO (2^{-7} to 10 mg/ml) and were implanted in anterior, posterior, and extreme posterior regions of stage 20 wing buds as shown in Figs. 1b–1d, respectively. In some experiments, beads were soaked in a different mitotic inhibitor, nocodazole, at concentrations ranging from 0.1 to 0.5 mg/ml.

Analysis of BrdU incorporation. For BrdU incorporation, embryos were incubated *in ovo* with 100 μl of a 40 m*M* BrdU solution (5-bromo-2-deoxyuridine, Sigma; dissolved in H_2O) for 1 hr. Embryos were fixed in Carnoys (60% EtOH–30% chloroform–10% acetic acid). BrdU was detected using an anti-BrdU monoclonal antibody and an alkaline phosphatase-conjugated secondary antibody in whole mount preparations. At 7–9 days after bead implantation, some embryos were fixed and limb cartilage was stained with Victoria blue.

Analysis of cell death. Embryos were immersed in a solution of Nile blue (0.01 mg/ml in PBS) for 45 min at room temperature and then washed in PBS for 3–4 hr at room temperature.

In situ hybridization. Whole mount *in situ* hybridization was performed according to published methods (Wilkinson, 1992). Digoxigenin-labeled RNA probes were made from plasmids of *Bmp-2, Fgf-4, shh* (gifts from Juan Carlos Izpisua-Belmonte), *Hoxd-11,* and *Hoxd-13* (gifts from Denis Duboule). Proteinase K treatment was omitted in the cases of *Fgf-4* and in some cases of *Bmp-2* (Fig. 3E). Color reactions were performed with BCIP alone (Figs. 3A–3E) or with NBT/BCIP (all other cases).

RESULTS

We altered the length of the cell cycle in specific regions of developing chick limbs by implanting non-ionic macroreticular resin beads that had been soaked in different concentrations of aphidicolin, a specific inhibitor of DNA polymerase α (Ikegami *et al.*, 1978). Within 6 hr after bead implantation, a marked reduction in incorporation of BrdU was observed within a radius of 100–200 μm from the bead, regardless of bead location (Figs. 1b–1d). By 24 hr cells within the affected region showed evidence of renewed incorporation of BrdU, but at levels below that of comparable regions in control limbs (Figs. 1f and 1g). Limb buds examined 24 hr after treatment with aphidicolin ranged from normal size to slightly smaller and from normal shape to slightly irregular (Figs. 3 and 4), presumably as a direct consequence of aphidicolin-induced growth inhibition.

The effect of the transient increase in the length of G1 on final pattern was assessed in limbs fixed 1 week after aphidicolin exposure. When the bead was placed anteriorly beneath the AER, as in Fig. 1b, rather than the reduced patterns that might have been expected, paradoxically the major effect on pattern was the formation of an extra digit 2 on the anterior (Table 1; Figs. 1h and 1i). Neither limbs receiving beads with DMSO alone nor those soaked in a very low concentration of aphidicolin formed an extra digit.

To test further the hypothesis that alterations in cell cycle kinetics can influence pattern formation, we treated anterior cells with the mitotic inhibitor nocodazole, a drug that disrupts microtubules. This agent thus elongates the cell cycle, but by a different mechanism than aphidicolin. We also observed the induction of an extra digit 2 in nocodazole-treated limbs; however, the frequency of extra digit formation (17%, n = 12) was lower with nocodazole than with aphidicolin, and thus we used aphidicolin for experiments analyzing the effects of altering the cell cycle length on gene expression.

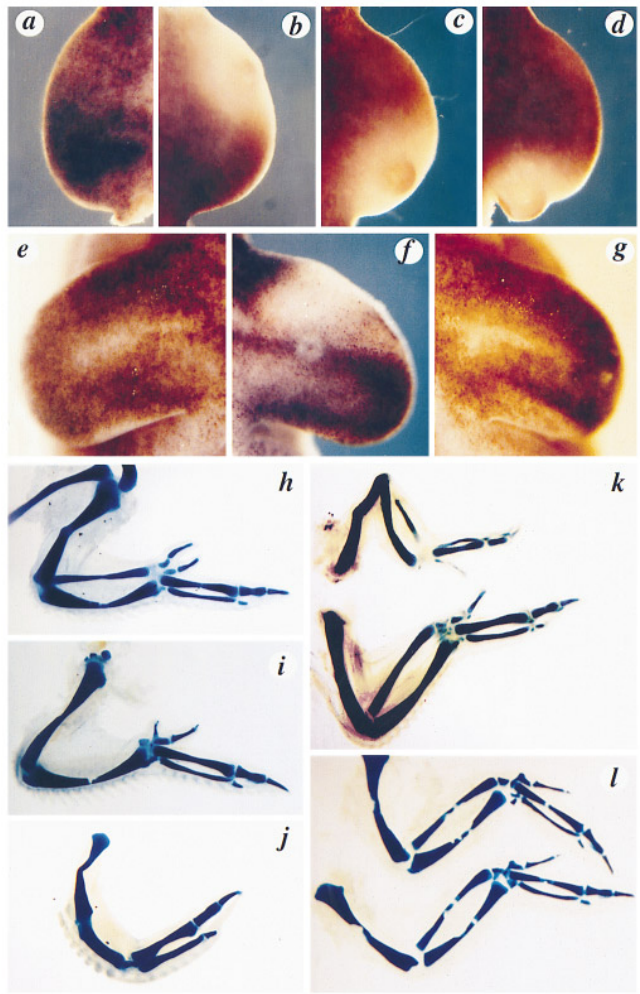

FIG. 1. BrdU incorporation (a–g) and the final limb pattern (h–l) in response to implants of aphidicolin beads in different positions. (a, e) Control limbs with BrdU incorporation 6 hr (a) and 24 hr (e) after a bead was implanted into the contralateral limb of a stage 20 embryo. (b–d) BrdU incorporation at 6 hr after aphidicolin bead implantation in anterior (b), posterior (c), and extreme posterior (d) positions and at 24 hr in anterior (f) and extreme posterior (g). Resultant cartilage patterns after aphidicolin bead implantation in anterior (h–j), in posterior (k), and in extreme posterior (l). Beads were soaked in a 0.5 mg/ml (h), 2 mg/ml (i, k, l), and 10 mg/ml (j) aphidicolin solution. (k) Experimental right limb of small size but normal pattern (top) and control left limb (bottom). (l) Experimental right limb of normal size and normal pattern (bottom) and control left limb (top).

TABLE 1

Dose-Dependent Effect of Anterior Aphidicolin Beads on Limb Pattern[a]

	Aphidicolin soaking concentration (mg/ml)								DMSO only
	10	2	1	2^{-1}	2^{-2}	2^{-3}	2^{-5}	2^{-7}	
Total number of limbs	10 (12)	19 (16)	14 (13)	12 (16)	8 (9)	14 (12)	13 (13)	13 (9)	17 (12)
Percentage of limbs with extra digits	30 (8)	90 (69)	79 (69)	58 (43)	12 (33)	28 (8)	23 (16)	0 (0)	0 (0)
Percentage of limbs with extra digits with cartilage	10 (8)	79 (44)	79 (46)	50 (25)	12 (11)	14 (8)	23 (8)	0 (0)	0 (0)
Percentage of limbs with extra digits without cartilage	20 (0)	11 (25)	0 (23)	8 (18)	0 (22)	14 (0)	0 (8)	0 (0)	0 (0)
Percentage of limbs with normal digits	20 (25)	10 (31)	7 (15)	25 (38)	88 (67)	72 (92)	77 (84)	100 (100)	100 (100)
Percentage of limbs with reduced digits	50 (67)	0 (0)	14 (15)	17 (19)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Note. Numbers in parentheses: beads left in.

[a] Beads removed at 1 day.

The pattern-duplication response to aphidicolin was dose-dependent and reproducible, with the maximal duplicating effect (~90%) observed when beads were soaked in 2 mg/ml aphidicolin and were removed 1 day after implantation (Table 1). Digit duplications were also obtained when beads were left in place, but at a lower frequency (~70%). In a fraction of the limbs with a duplicated digit, the extra digit failed to differentiate cartilage. Almost all limbs with an extra digit had a reduced or absent radius (86%; Fig. 1i). Digit reductions were occasionally observed at the duplicating doses and at higher frequency at higher doses (Fig. 1j).

The pattern-duplication response to aphidicolin was also position-specific. In contrast to the response of anterior limb bud cells, when an aphidicolin bead (2 mg/ml) was placed at the distal tip, the posterior (Fig. 1c), or extreme posterior (Fig. 1d), no extra digits formed, and the majority of limbs had a normal cartilage pattern (Table 2). With posterior beads, half of the limbs were smaller than normal (Fig. 1k). With extreme posterior beads, there was no effect on either the size or the pattern of the resultant limbs (Fig. 1l).

TABLE 2

Limb Pattern after Aphidicolin Beads at Apex, Posterior, and Extreme Posterior

	Apex	Posterior	Extreme posterior
Percentage of normal size, normal pattern	83	36	100
Percentage of small size, normal pattern	8	50	0
Percentage of missing elements	8[a]	14[b]	0
Number of samples	12	14	11

Note. Beads were soaked in 2 mg/ml aphidicolin.

[a] One limb, missing radius.

[b] One limb missing ulna, one limb missing ulna and digit 4.

We examined patterns of cell death to determine if there is a relationship between the pattern-duplication response and cell death associated with implantation of aphidicolin beads. We compared the patterns of cell death in 95 experimental limbs fixed 12 hr (data not shown) and 24 hr after bead implantation to the patterns of endogenous cell death in 95 normal limb buds. We observed that variable degrees of ectopic cell death are induced in response to aphidicolin bead implantation, ranging from ''none'' (Fig. 2b), ''minor'' (not illustrated), and ''moderate'' (Fig. 2d) to ''maximal'' (Fig. 2f). At the aphidicolin soaking concentrations that gave the highest frequencies of digit duplication (~90% at 2 mg/ml and ~70% at 1 mg/ml), we observed ectopic cell death in a lower percentage of cases: 63 and 53% of the limbs, respectively at 24 hr (Table 3). Of the limbs exhibiting ectopic cell death, most were in the ''minor–moderate'' category (58% at 2 mg/ml and 67% at 1 mg/ml; data not shown). Among limbs examined at 12 hr after treatment with the duplicating doses, the frequency of ectopic cell death was considerably lower (42% at 2 mg/ml and 11% at 1 mg/ml, data not shown), and the extent was limited to the ''minor'' category for all limbs.

To investigate the formation of the extra digit further, we examined changes in gene expression in response to aphidicolin treatment. A region of cells at the posterior edge of the limb bud simultaneously expresses several genes that are not expressed in anterior cells: *shh, Bmp-2, Hoxd-11,* and *Hoxd-13* (Francis, 1994; Izpisúa-Belmonte *et al.,* 1991; Nohno *et al.,* 1991; Riddle *et al.,* 1993; Yokouchi *et al.,* 1991). In addition, the AER overlying the posterior two-thirds of the bud expresses *Fgf-4* and *Bmp-2.* (Francis, 1994; Niswander and Martin, 1992; Suzuki *et al.,* 1992). We have found that the expression of these posterior genes is altered in a position-dependent manner when the cell cycle is lengthened by treatment with aphidicolin. When anterior cells are exposed to aphidicolin, expression of *Bmp-2* is induced in mesenchymal cells between the bead and the AER (Figs. 3a–3d) and in an anterior extension of the AER toward the bead (Fig. 3e) at both 12 and 24 hr after bead implanta-

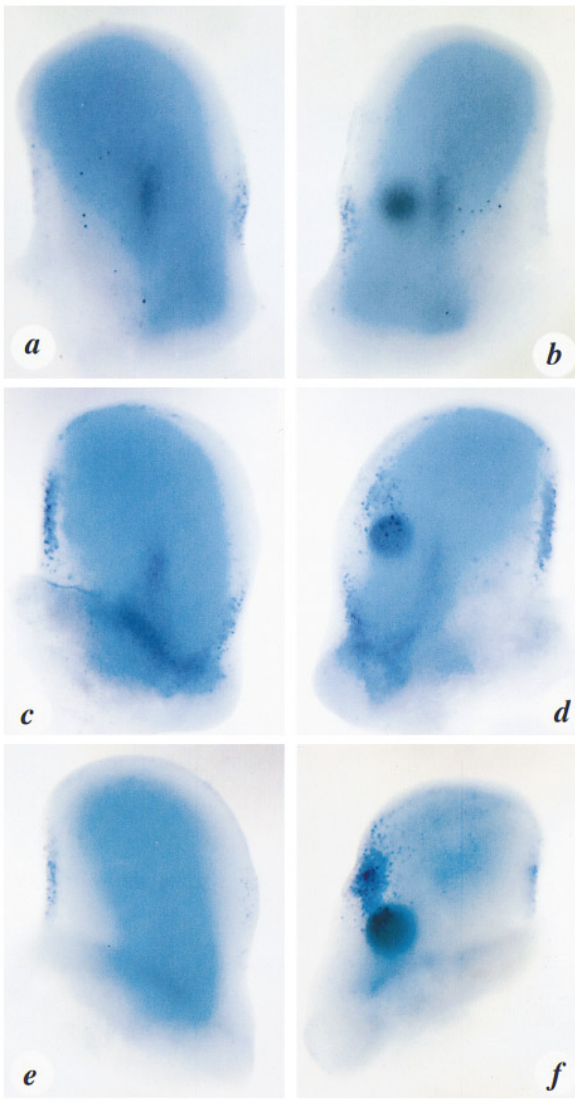

FIG. 2. Ectopic cell death induced in response to implantation of aphidicolin beads. Patterns of cell death in normally developing control limbs (a, c, e) compared to the contralateral limb that had been implanted 24 hr earlier with a bead soaked in aphidicolin at 2 mg/ml (b, d, f). Similar paired limbs were examined for limbs treated with lower concentrations of aphidicolin and/or shorter exposure times; however, in all cases, the frequency and the degree of cell death in those limbs were comparable or less than those in the limbs illustrated. The limbs are oriented with anterior to the right (a, c, e) or to the left (b, d, f). Regions of endogenous cell death are observed in the anterior and posterior regions. The degree of ectopic cell death ranged from ''no ectopic cell death'' (b), ''minor ectopic cell death'' (not illustrated), and ''moderate ectopic cell death'' (d) to ''maximal ectopic cell death'' (f).

tion. Anterior expansion of the AER is also accompanied by an extension of *Fgf-4* expression (Fig. 3j), beginning at 12 hr. Expansion of *Hoxd-11* expression in the mesenchyme between the bead and the normal expression domain was observed at both 12 hr and, more markedly, at 24 hr (Figs. 3f–3i). Neither *shh* nor *Hoxd-13* expression was detected in anterior cells (Figs. 3k–3m). In a recent study (Duprez *et al.*, 1996), BMP-2 producing cells implanted into the anterior of the limb bud induced the ectopic expression of *Hoxd-11* in the mesenchyme and *Fgf-4* in the anterior AER and resulted in the formation of an extra digit. Hence, in our study it is likely that ectopic expression of *Fgf-4* and *Hoxd-11* are downstream responses to the induction of ectopic *Bmp-2* expression in response to changes in the cell cycle.

When beads were implanted in the posterior or extreme posterior of the limb bud, where these genes are normally expressed, the cells responded to aphidicolin by showing decreased expression of some genes, whereas expression of others appeared unaffected. Expression of *shh* was either very reduced (posterior beads) or not detectable (extreme posterior beads) at 6, 12, 18 (not shown), and 24 hr after bead implantation (Figs. 4n–4v). By 36 hr, expression became detectable again in about half of the specimens, and by 48 hr, weak expression was observed that was equivalent to the weak expression in the contralateral limb at this stage. Extremely decreased expression of *Hoxd-13* was observed at 18 hr (not shown) and 24 hr (Figs. 4k and 4l) with posterior beads. Only slightly decreased expression of *Hoxd-13* was observed at 18 hr (not shown) and 24 hr (Figs. 4k and 4m) when beads were in extreme posterior positions. In contrast, expression of *Bmp-2* (Figs. 4a–4e), *Fgf-4* (Figs. 4f–4h), and *Hoxd-11* (Figs. 4i and 4j) were not significantly affected by altering cell cycle kinetics in posterior cells (Figs. 4b, 4d, 4g, and 4i) or extreme posterior cells (Figs. 4e, 4h, and 4j).

DISCUSSION

These studies show that local, reversible, alteration of the length of the cell cycle can lead to paradoxical changes in final limb pattern. This effect is independent of the type of agent used to slow the cell cycle, since aphidicolin, an inhibitor of DNA polymerase, as well as nocodazole, an inhibitor of microtubules, can both induce the formation of supernumerary pattern elements. This indicates that the mechanism by which these very different agents exert their effect on limb cells involves their common property of lengthening of the cell cycle.

To understand the changes in limb pattern induced by local slowing of the cell cycle, we examined the expression of several genes required for limb pattern formation. We have demonstrated that all four of the posterior-specific genes that are normally coexpressed in mesenchyme cells at the posterior margin are directly or indirectly sensitive to cell cycle length and respond by either ectopic expression or by inhibition when G1 is prolonged. In anterior mesenchyme cells, *Bmp-2* and *Hoxd-11* are induced in response to aphidicolin, and *Fgf-4* and *Bmp-2* are induced in the anteriorly extended AER. These results are consistent with the hypothesis that in anterior cells, the cell cycle is normally too short to permit the expression of either *Bmp-2* itself (and its downstream targets) or of a gene upstream of *Bmp-2*. A proposed link between cell cycle length and gene size is

TABLE 3

Relationship between Ectopic Cell Death and Ectopic Pattern Formation in Response to Aphidicolin Treatment (24 h after Bead Implantation)

Aphidicolin soaking concentration (mg/ml)	Bead position	Percentage of limbs with an extra digit	Percentage of limbs with ectopic cell death	No. limbs
2	Anterior	90	63	30
1	Anterior	79	53	15
2^{-3}	Anterior	28	0	20
2	Extreme posterior	0	100	7

discussed further below, but given that *Bmp-2* is a relatively small gene (Feng *et al.*, 1994), the second alternative is more likely.

The idea that expression of *Bmp-2* and its downstream targets is controlled by cell cycle length (directly or indirectly) is borne out by the behavior of the same set of genes in the posterior, where *Bmp-2, Hoxd-11,* and *Fgf-4* continue to be expressed normally after the cell cycle has been lengthened in response to aphidicolin. In contrast, expression of *shh* and *Hoxd-13* is inhibited when the cell cycle is lengthened in the posterior, and these genes are not induced in anterior cells by lengthening the cell cycle. This suggests that the cell cycle on the posterior margin, where expression of *shh, Hoxd-11, Hoxd-13,* and *Bmp-2* overlap, must be precisely controlled, providing a window that is appropriate for transcription of the upstream regulator of *Bmp-2* and as a consequence the downstream targets of *Bmp-2,* but not so long that *shh* and *Hoxd-13* are downregulated. Our results suggest that expression of at least several of the genes necessary for patterning the limb is controlled indirectly via control of the length of the cell cycle. Since some molecules with morphogenetic activity are also know to function as growth factors, it is possible that their effect on pattern formation is mediated via an effect on the cell cycle. Studies are in progress to analyze regional differences in the normal pattern of cell cycles in developing limbs at different stages.

Our studies have ruled out the possibility that the occurrence of extra digits in response to aphidicolin beads is directly related to the occurrence of ectopic cell death (Table 3). At 2 mg/ml aphidicolin soaking solution, nearly all experimental limbs with an anterior bead (90%) formed an extra digit; however, many (37%) did not exhibit increased levels of cell death. Conversely, at lower aphidicolin concentrations, no ectopic cell death was induced after anterior bead implantation, yet nearly a third of the limbs were induced to form an extra digit. Aphidicolin beads implanted into the extreme posterior induced ectopic cell death in all limbs, but had no effect on the limb pattern. Finally, results from previous studies indicate that it is unlikely that loss of anterior cells would lead to the formation of an extra digit. If as much as the anterior $\frac{1}{3}$ of the limb bud is removed or isolated by barriers (equivalent to a region of anterior cells in large excess in comparison to the ''maximal ectopic cell death'' that we observed), limbs form a normal distal pattern (Hinchliffe and Gumpel-Pinot, 1981; Summerbell, 1979). If the entire anterior half is removed, the resultant limbs are sometimes complete distally, but usually are missing digit 2. Extra digits are not induced as a result of loss of anterior cells.

We have demonstrated that a treatment that prolongs G1 mimics the effect of *shh* on *Bmp-2* expression (Laufer *et al.*, 1994). Hence in anterior cells, *Bmp-2* is expressed in the absence of *shh,* and in the posterior, *shh* is downregulated but *Bmp-2* continues to be expressed. This result raises the possibility that the effect (direct or indirect) of *shh* on target cells is to influence cell cycle kinetics, which in turn leads to the activation of *Bmp-2* and its downstream targets. Since *shh* interacts with *ptc,* a known tumor suppressor gene, such a mechanism is at least possible (Oro *et al.,* 1997). It remains to be determined why aphidicolin-induced *Bmp-2* expression in anterior cells, though capable of causing minor duplications and the expression of some posterior-specific genes, cannot substitute functionally for expression of *shh* in anterior cells. However, there are no major consequences of the absence of *shh* expression in posterior cells on the completeness of the limb pattern. With extreme posterior beads, where *shh* is undetectable for more than 24 hr, from stage 21 to stage 24, limbs have both normal morphology and size. This suggests either that in a posterior environment *Bmp-2* is capable of functionally replacing *shh* expression or that the role of *shh* in establishing the anterior–posterior limb pattern (Bitgood and McMahon, 1992; Riddle *et al.,* 1993) is restricted to very early stages of normal limb development and that continued expression is not required during later stages of limb bud outgrowth.

The formation of extra digits on the anterior suggests that cells exposed to aphidicolin acquire posterior properties and generate a weak, posterior polarizing signal (Duprez *et al.,* 1996). Interaction between these posteriorized cells and the normal anterior cells would secondarily lead to the observed digit duplication. Although there may be alternative mechanisms by which aphidicolin and nocodazole act to posteriorize limb bud cells, the common effect of these agents to reversibly block the cell cycle suggests that an increase in the length of the cell cycle is the most likely mechanism. Consistent with this conclusion, is the finding that when the AER is removed, a treatment that results in slowed growth of mesenchyme cells, anterior cells also acquire pos-

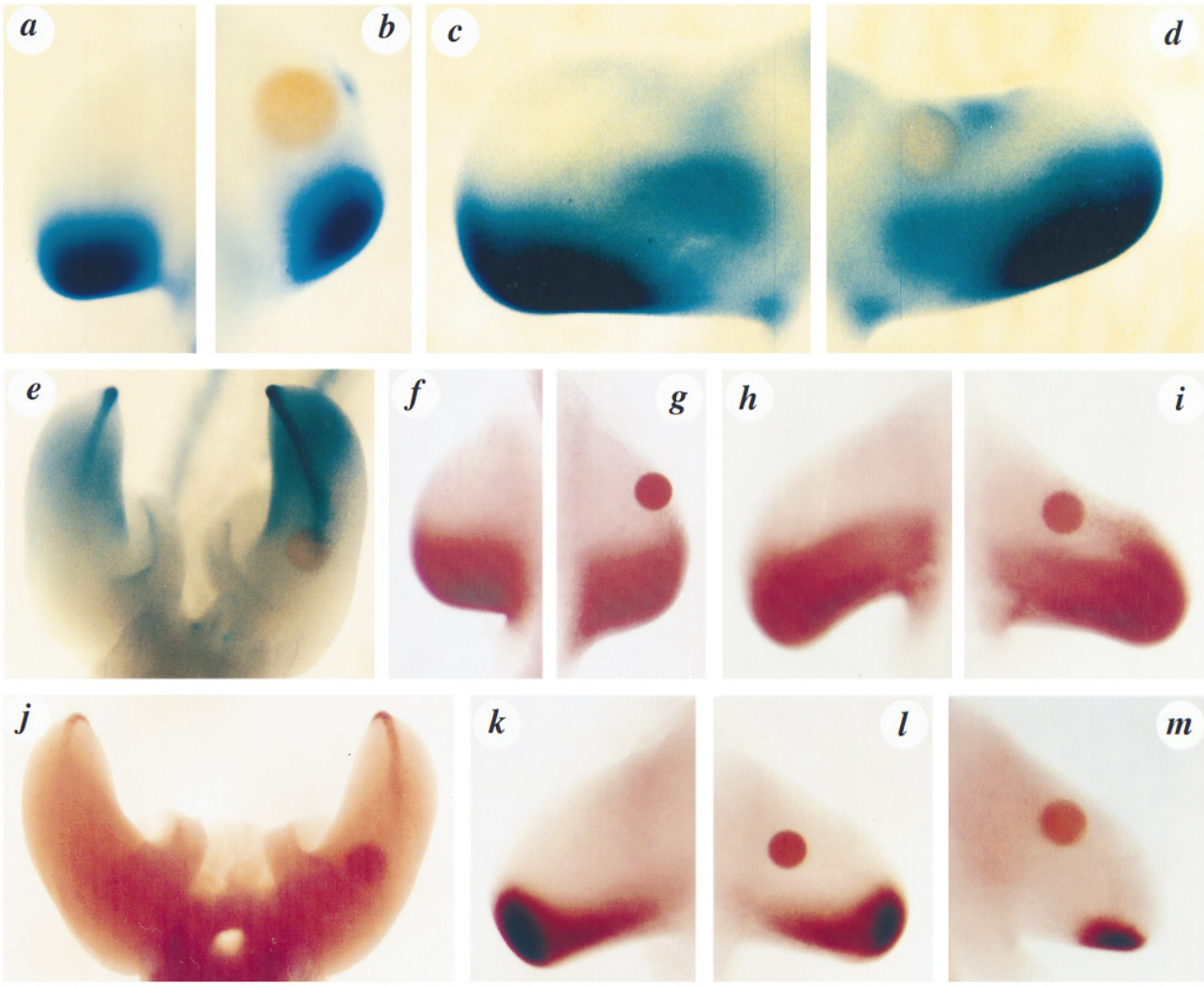

FIG. 3. Expression of genes after aphidicolin treatment of anterior limb bud cells. *Bmp-2* expression in experimental limbs at 12 hr (b) and 24 hr (d, e) after bead implantation and in control left limbs (a, 12 hr; c, 24 hr). (e) *Bmp-2* expression in bead-implanted limb (right) and control limb (left). *Hoxd-11* expression in experimental limbs at 12 hr (g) and 24 hr (i) after bead implantation and in control left limbs (f, 12 hr; h, 24 hr). (j) *Fgf-4* expression in an experimental limb 24 hr after bead implantation (right) and in a control limb (left). *Hoxd-13* expression in an experimental limb 24 hr after bead implantation (l) and in a control left limb (k). *Shh* expression in an experimental limb 24 hr after bead implantation (m). In all cases except b (10 mg/ml), beads were soaked in 2 mg/ml aphidicolin solution.

terior signaling activity (Anderson *et al.*, 1994). The fact that some teratogens cause preaxial polydactyly as well as reduced embryo size (Tanaka *et al.*, 1995) might be related to an effect on the cell cycle. If chemical exposure were to cause a transient lengthening of the cell cycle (growth inhibition resulting in smaller embryos), by analogy to our results, the consequences would be most pronounced in anterior limb cells where slowing of the cell cycle would lead to ectopic expression of some posterior genes, posteriorization of anterior cells, and induction of digit duplication.

It is well recognized that an inverse relationship exists between the cell cycle and differentiation. However, the idea that control of the length of the cell cycle could underlie developmental processes such as pattern formation, briefly explored earlier (Bryant *et al.*, 1993) has not previously been tested experimentally. Although the existence of cell populations in the limb bud with discrete cell cycle kinetics has not been extensively investigated (work in progress), there is evidence that such populations exist. Fate maps show that most of the distal part of the pattern originates from growth of a population of cells on the posterior side of the early chick limb bud (Summerbell and Honig, 1982). Since cells of the polarizing zone (ZPA) do not contribute significantly to the distal limb pattern (Wanek *et al.*, 1991), we predict the existence of a growth zone in the posterior half of the bud that is located posterior to the

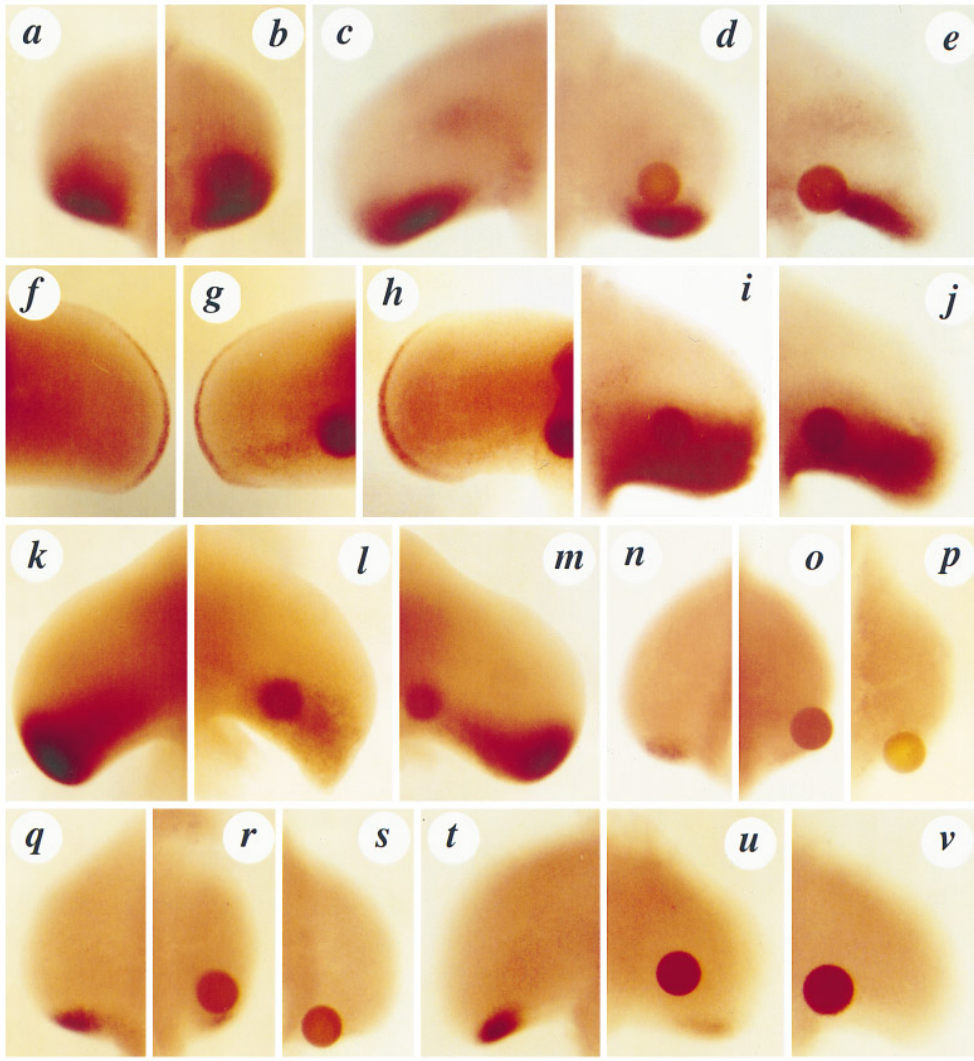

FIG. 4. Expression of genes after aphidicolin treatment of posterior and extreme posterior limb bud cells. *Bmp-2* expression in experimental limbs 12 hr (b) and 24 hr (d, e) after bead implantation in posterior (b, d) and in extreme posterior (e) positions and in control left limbs (a, 12 hr; c, 24 hr). *Fgf-4* expression in experimental limbs 24 hr after bead implantation in posterior (g) and in extreme posterior (h) positions and in a control limb (f). *Hoxd-11* expression in experimental limbs 24 hr after bead implantation in posterior (i) and extreme posterior (j) positions. *Hoxd-13* expression in experimental limbs 24 hr after bead implantation in posterior (l) and extreme posterior (m) positions and in a control limb (k). *Shh* expression in experimental limbs 6 hr (o, p), 12 hr (r, s), and 24 hr (u, v) after bead implantation in posterior (o, r, u) and in extreme posterior (p, s, v) positions and in control limbs (n, 6 hr; q, 12 hr; t, 24 hr). In all cases beads were soaked in 2 mg/ml aphidicolin solution.

midline, but anterior to the polarizing region. The existence of this predicted population can be visualized in limb buds exposed to BrdU and processed for whole mount immunohistochemistry (Fig. 1a). Given the complex expression patterns of the genes we have found to be sensitive to alterations in cell cycle length, we predict that more detailed analyses will reveal corresponding regions with unique cell cycle kinetics.

Although we have traditionally considered the control of growth to be a consequence of pattern formation, it is also possible that pattern formation is itself controlled by growth (Bryant *et al.*, 1993). According to such a view, pattern within a field is specified by gradients of critical transcription factors that are not set up by diffusion as in the early embryo (Lawrence and Struhl, 1996), but rather emerge as a result of underlying gradients in cell cycle lengths. One way in which a cell cycle length gradient could set up a gradient of a transcription factor is by dilution of transcripts, as has been suggested for segment specification in short germ band insects (Tautz and Sommer, 1995).

An alternative mechanism would involve cell cycle length-dependent accumulation of mRNA (Shermoen and

O'Farrell, 1991). In order for the length of the cell cycle to play a limiting role in accumulation of a gene product, the transcriptional unit would need to be large enough that the time required for complete transcription was of the same order of magnitude as the time available in the cell cycle for transcription. The restriction of mRNA accumulation by short cell cycles has been demonstrated for *Ubx* in *Drosophila* (Shermoen and O'Farrell, 1991) and for *dystrophin* in muscle cells (Tennyson *et al.*, 1995). For this mechanism to generate the spatial gradients needed for pattern formation, cells in an embryonic field would be cued to transcribe a gene with the appropriate characteristics, and the levels of mRNA accumulation would mirror variations in the length of the cycle in different parts of the field. Graded growth rates across a field could be established as a result of distance from a positional confrontation, as at a boundary, or as a result of distance from the source of a growth factor (or both), or perhaps as a direct result of the graded expression of a cell cycle length-dependent growth factor. Graded patterns of growth could provide the conditions needed to establish a graded distribution of an appropriate patterning gene (master regulatory gene) within the field. Downstream target genes (which could also include growth factor genes) would be activated at different threshold levels of patterning gene product, by analogy to the response of gap genes to maternal gradients of *bicoid* gene product in *Drosophila* (Lawrence and Struhl, 1996). Such a mechanism would establish a feedback control between cell growth and patterning gene expression resulting in dynamically stable organization in developing systems.

ACKNOWLEDGMENTS

We thank members of our lab and of the Developmental Biology Center for discussions and arguments about the issues raised in this paper. We also thank Juan Carlos Izpisúa-Belmonte for his generosity with expertise and reagents. This work was supported by the National Institutes of Health and in part by a Narishige Zoological Science Award.

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Received for publication April 10, 1997
Accepted June 12, 1997