G1/S phase cyclin-dependent kinase overexpression perturbs early development and delays tissue-specific differentiation in *Xenopus*

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Accepted 5 February 2004

Development 131, 2577-2586
Published by The Company of Biologists 2004
doi:10.1242/dev.01121

Summary

Cell division and differentiation are largely incompatible but the molecular links between the two processes are poorly understood. Here, we overexpress G1/S phase cyclins and cyclin-dependent kinases in *Xenopus* embryos to determine their effect on early development and differentiation. Overexpression of cyclin E prior to the midblastula transition (MBT), with or without cdk2, results in a loss of nuclear DNA and subsequent apoptosis at early gastrula stages. By contrast, overexpressed cyclin A2 protein does not affect early development and, when stabilised by binding to cdk2, persists to tailbud stages. Overexpression of cyclin A2/cdk2 in post-MBT embryos results in increased proliferation specifically in the epidermis with concomitant disruption of skin architecture and delay in differentiation. Moreover, ectopic cyclin A2/cdk2 also inhibits differentiation of primary neurons but does not affect muscle. Thus, overexpression of a single G1/S phase cyclin/cdk pair disrupts the balance between division and differentiation in the early vertebrate embryo in a tissue-specific manner.

Key words: Cyclin, Cdk, Cell cycle, Differentiation, Epidermis

Introduction

The coordination of the cell cycle and differentiation is central to metazoan development but is poorly understood. Cyclins drive the cell cycle forward in conjunction with cyclin-dependent kinases. Much of what we know about cyclin activity comes from very detailed work using tissue culture cells. D-type cyclins, bound to cdk4/6 act early in G1, predominantly to phosphorylate Rb and allow G1 progression via effects on the E2F transcription factors. Cyclin E/cdk2 is then the main kinase complex needed for entry to S phase, after which point cyclin A/cdk2 becomes active. Finally, cdc2 bound to either an A- or B-type cyclin is needed for cells to enter mitosis. Under certain circumstances, overexpression of cyclin E or cyclin A alone is sufficient to drive cultured cells into or through the cell cycle (Connell-Crowley et al., 1998; Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995; Resnitzky et al., 1994; Resnitzky et al., 1995; Rosenberg et al., 1995).

Attempts have also been made to study the roles of different cyclins during development, and most success has been gained from taking a genetic approach in *Drosophila*. Overexpression of cyclin E in flies induces both undifferentiated and differentiating G1 cells to enter S phase in a variety of tissues (Li et al., 1999; Neufeld et al., 1998; Richardson et al., 1995). Ectopic overexpression of cyclin A also triggers entry into S phase, although this is limited by additional embryonic mechanisms that limit cyclin A activity, predominantly affecting protein stability (Sprenger et al., 1997). Work on tissue culture cells might imply that most, if not all, cyclins are important for cell cycle progression in all cells. However, for instance in *Drosophila* and *Xenopus*, expression patterns of various cyclins differ between tissues in ways that cannot always solely be explained by the proliferative index of the tissue concerned (Jones et al., 2000; Richardson et al., 1993; Vernon and Philpott, 2003a). In *Xenopus* embryos, our experimental system of choice, cell division is fairly uniform before gastrulation, but after this time proliferation adopts tissue- and region-specific patterns (Saka and Smith, 2001; Vernon and Philpott, 2003a). Methods of cell cycle regulation might differ between these distinct regions.

A number of G1/S phase cyclins have been identified in *Xenopus*. Although cyclin D has not been demonstrated to play an important role in early *Xenopus* embryogenesis (T. Hunt, personal communication) (see also Taieb et al., 1997; Taieb and Jessus, 1996), 3 cyclin E (Chevalier et al., 1996; Rempel et al., 1995) and 2 cyclin A homologues (Howe et al., 1995; Minshull et al., 1990) have been cloned. Interestingly, maternal cyclin E and cyclin A1 are degraded after the midblastula transition (MBT) or at the onset of gastrulation, respectively (Howe et al., 1995; Howe and Newport, 1996), and are replaced with zygotic transcripts. Cyclin A1, the pre-MBT form of cyclin A, complexes predominantly if not exclusively with cdc2, functions in mitosis and is fully degraded after MBT. It is then replaced by zygotic cyclin A2 (Howe et al., 1995). Cyclin A2 is found complexed with cdk2 and cdc2 soon after gastrulation but predominantly complexes with cdk2 from neurula stages onwards (Howe et al., 1995; Strausfeld et al., 1996). Thus, Xenopus cyclin A2 behaves more like a homologue of mammalian cyclin A than *Xenopus* cyclin A1 does, functioning

in S-phase entry (Strausfeld et al., 1996). Both cyclin E1 and cyclin A2 are detectable by in-situ hybridisation at least through to tailbud stages, although levels and sites of expression appear to differ somewhat at different developmental stages (Vernon and Philpott, 2003a).

Cell division and differentiation are generally thought to be mutually exclusive (e.g. Edlund and Jessell, 1999; Skapek et al., 1995; Zhang et al., 1999). While the effects of overexpression of cyclins and cdks have been studied in *Drosophila*, and some targeted expression has been attempted in mammals (Li et al., 1999; Miliani de Marval et al., 2001; Neufeld et al., 1998; Robles et al., 1996; Rodriguez-Puebla et al., 2000; Yamamoto et al., 2002), little work has been performed to investigate the short- and long-term effects of cyclin/cdk upregulation in multiple tissues in the vertebrate embryo in vivo. The aim of this study was to determine whether overexpression of a single cyclin/cdk pair alone was sufficient to promote proliferation in the early embryo, and to investigate whether this was incompatible with differentiation. Here, we demonstrate that, while overexpression of cyclin E results in apoptosis post-MBT, cyclin A2/cdk2 overexpression promotes proliferation in the early tailbud embryo. Moreover, cyclin A2/cdk2 RNA injection delays differentiation of epidermis and neurons but has no effect on differentiation of muscle. Thus, cyclin E and cyclin A2 levels can regulate cell cycle and differentiation in a stage- and tissue-specific manner.

Materials and methods

Embryos and injections

Xenopus laevis embryos obtained by hormone-induced laying were fertilised in vitro, de-jellied in 2% cysteine, pH 7.8-8.0, washed and incubated in 0.1×MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1984), fixed and stained for beta-galactosidase (Bgal) as described (Sive et al., 2000).

Xenopus cyclin E1, cyclin A2, and cdk2 (the latter two with or without myc-tag or HA-tag) were subcloned into pCS2+ and transcribed using the Ambion Message Machine kit. Typically, 1 ng of each RNA was injected (2 ng in Fig. 2G,H; 1.5 ng in Fig. 6). Where appropriate, 100 pg of Bgal was injected as a lineage tracer.

Kinase assays

Embryos were injected with the RNAs described and homogenised at stage 8, 9 or 23 (as labelled). H1 kinase assays were performed on cdk2 complexes immunoprecipitated from embryo extracts, as described in Philpott et al. (1997), approximately one embryo per lane.

In situ hybridisation, BrdU detection, antibody staining and histology

Whole-mount in-situ hybridisations were performed as described (Shimamura et al., 1994) with digoxigenin-labelled antisense RNA probes generated from epidermal keratin (EcoRI/SP6), neural-beta-tubulin (BamHI/T3) and muscle actin (HindIII/T7) clones. BrdU incorporation and detection was performed essentially as described in the Roche (Roche, Mannheim, Germany) instructions for the BrdU Labelling and detection kit 1 (1296 736). Embryos were injected with 1 nmol of BrdU 1 hour before MEMFA fixation. Anti-BrdU (1:100) was applied to 12 μm paraffin wax embedded sections overnight at 4°C, washed and detected with an anti-mouse Ig-rhodamine (1:200) for 1 hour at room temperature.

Whole-mount antibody staining was performed as described (Sive et al., 2000) using an anti-phosphohistone H3 antibody (TCS Biologicals, Buckingham, UK) at 1:1000 and detected with an alkaline phosphatase-conjugated secondary antibody using NBT/BCIP as substrates. Staining for skin differentiation (Fig. 6) and HA-tagged cyclin A2 (Fig. 4) was performed on embryos, fixed in 4% paraformaldehyde and sectioned by cryostat, using monoclonal antibody culture supernatant (Jones and Woodland, 1986) diluted 1:1 with PBS, or anti-HA at 1:250. For whole-mount staining of nuclei, embryos were fixed in MEMFA for 1-2 hours, bleached and stained with Hoechst at 10 μg/ml in PBS. Embryos were de-stained in PBS for at least 12 hours, then animal caps were cut, mounted under coverslips and viewed directly.

Western blotting was performed by standard methods and blots were probed with a monoclonal antibody to *Xenopus* cyclin A2, generously provided by Dr Tim Hunt.

Results

The aim of this study was to investigate the effect of global overexpression of G1/S phase cyclin-dependent kinase activity on cell proliferation and differentiation in the embryo. Initially, we overexpressed both E and A-type cyclins to determine whether these would promote cell division and whether differences existed between the two molecules.

To establish whether overexpression of G1/S phase cyclins was able to increase cdk2 kinase activity in embryonic blastomeres, RNAs encoding *Xenopus* cyclin A2 or cyclin E were injected into fertilised eggs with or without RNA encoding cdk2. At stage 8, after embryo homogenisation, cdk was immunoprecipitated and tested for its ability to phosphorylate histone H1 (Fig. 1). Injection of RNAs encoding the cyclin subunits alone did not result in a significant increase in cdk2 kinase activity, indicating that either the amount of cdk2 in the extract was limiting or that the newly-synthesised cyclin was unable to complex with or activate the endogenous cdk2 (Fig. 1, lanes 1, 2 and 6). In fact, we observed that overexpression of cdk2 alone led to a modest increase in cdk2 kinase activity (Fig.1, lane 3), indicating that the amount of cdk2 subunit in the early embryo was limiting and that there might have been uncomplexed cyclins present. Overexpression of cyclin E in particular was able to upregulate cdk2 kinase activity when exogenous cdk2 was introduced by RNA injection (Fig.1, lane 4), showing that active complexes can be formed from injected messages. In this system, neither cyclin A2 nor cyclin E overexpression resulted in appreciable activation of endogenous cdc2, as measured by immunoprecipitation assays (data not shown).

To investigate the effects of overexpression of cyclins and cdks on development, RNAs encoding *Xenopus* cyclin A2 and E1 were injected into one of two blastomeres at the two-cell stage (Fig. 2). At early stages pre-MBT, cyclin A2 at this level had very little effect on blastomere cleavage, either with or without co-injected cdk2 RNA (Fig. 2b,e), nor did injection of cdk2 alone or Bgal as a control (Fig. 2c,f). However, a subtle but reproducible slowing in the rate of blastomere cleavage was observed in embryos injected with cyclin E RNA by around stage 5, such that blastomeres around the site of injection were typically delayed by one to two cleavages compared with blastomeres in the uninjected side (Fig. 2a,d). When viewed again at midgastrula stage, embryos expressing cyclin A2, with or without cdk2, continued to develop essentially normally (Fig. 2B,E), although some modest slowing of cleavage was occasionally observed. By contrast, embryos injected with cyclin E or cyclin E/cdk2 showed extensive cell death on the

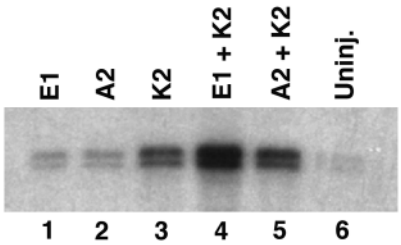

Fig. 1. Activation of H1 kinase activity by overexpression of cyclins and cdk2. RNA encoding cyclin E alone or with cdk2, cyclin A2 alone or with cdk2, or cdk2 alone were injected into fertilised eggs and allowed to develop until stage 8. Cdk2 was immunoprecipitated from injected embryos (lanes as labelled) and tested for its ability to phosphorylate histone H1.

injected side; cells dissociated from their neighbours and adopted a marbled appearance (Fig. 2A,D). The timing and morphology of these regions was entirely consistent with changes resulting from apoptosis, as documented previously in *Xenopus* embryos (Hensey and Gautier, 1997; Sible et al., 1997; Stack and Newport, 1997). Moreover, co-injection of the inhibitor of apoptosis Bcl-$_{XL}$ (Bellmeyer et al., 2003) prevented cell death, confirming that these cells were apoptotic (Fig. 2G,H).

Thus elevated levels of cyclin E, but not cyclin A2, induce apoptosis in the early embryo. This effect must be independent of the ability of cyclin E to globally raise cdk2 kinase activity because cyclin E injection alone, which does not significantly activate endogenous cdk2 kinase as measured in immunoprecipitation assays (Fig. 1, lane 1), is as efficient as cyclin E plus cdk2 at inducing apoptosis. One possible trigger for apoptosis is the disruption of the normal nuclear replication and division cycle. To investigate this, we cut animal caps from cyclin-injected embryos at stage 8.5 and stained them for DNA content (Fig. 3). Large areas of the animal pole where cyclin E message had been injected were devoid of any detectable nuclei (Fig. 3A, arrow). This lack of nuclei is consistent with elevated levels of cyclin E specifically blocking DNA replication, as has been demonstrated in egg extracts (Hua et al., 1997). This block would result in nuclear loss on subsequent cell division. However, even in the absence of DNA cell cleavage would continue, as embryos are able to sense this loss of nuclei only after the MBT when checkpoints kick in (Dasso and Newport, 1990) and when apoptotic pathways are activated in the early gastrula (Hensey and Gautier, 1997; Sible et al., 1997; Stack and Newport, 1997). Interestingly, overexpression of cyclin E resulted in loss of nuclei with or without co-injection of cdk2 (Fig. 3A,D). Therefore, the ability of cyclin E to induce nuclear loss in *Xenopus* embryos is independent of an effect on overall cdk2 kinase activity. Injections of RNA encoding cyclin A2 with or without cdk2, cdk2 alone or control Bgal had no effect on nuclear density within the dissected animal caps, as might be expected since these embryos develop essentially normally (Fig. 2B,E,C,F and see below).

The finding that cyclin E overexpression resulted in nuclear loss and apoptosis at MBT precluded us from further study to investigate the effect of its overexpression on later embryonic development. Instead we concentrated on studying the effect of cyclin A2 overexpression, which has no apparent adverse effects on the pre-MBT embryo.

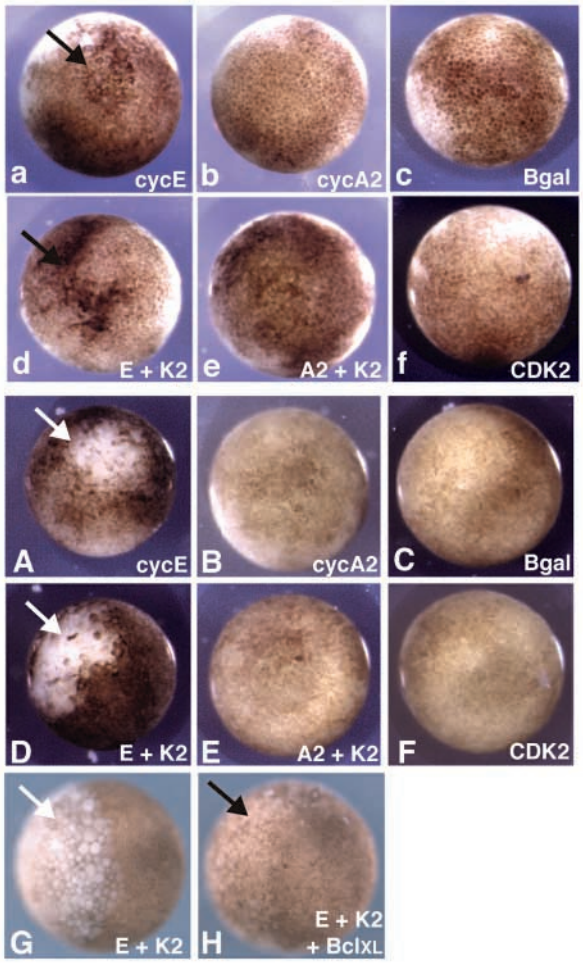

Fig. 2. Cyclin E but not cyclin A2 overexpression leads to apoptosis in the gastrula embryo. RNAs encoding cyclin E alone (a,A) or cyclin E with cdk2 (d,D,G), cyclin A2 alone (b,B) or cyclin A2 with cdk2 (e,E), cdk2 alone (f,F), Bgal as a control (c,C) or cyclin E with cdk2 and Bcl$_{XL}$ (H) were injected into one cell of a 2-cell embryo and allowed to develop to stage 9 (a-f) or stage 11 (A-H). Areas of slowed cell division are indicated by black arrows (a,d,H). Areas of apoptosis are indicated by white arrows (A,D,G).

We initiated experiments to determine the long-term effects of cyclin A2 overexpression on later stages of *Xenopus* development. As endogenous A-type cyclins are thought to be unstable (Funakoshi et al., 1999; Howe et al., 1995), we investigated whether cyclin A2 protein would be maintained at high levels as late as tailbud stages (stage 23) after injection of cyclin RNA into 2-cell embryos. Embryos injected with RNA encoding cyclin A2, cdk2, cyclin A2 and cdk2 together or Bgal as a control were allowed to develop until stage 23. Cdk2 was then immunoprecipitated and tested for kinase activity against histone H1 (Fig. 4A). Again, cyclin A2 alone was not able to significantly activate endogenous cdk2 even post-MBT (Fig. 4A, lane 3) but cyclin A2 co-injected with cdk2 RNA led to a substantial enhancement of cdk2 kinase activity (Fig. 4A, lane 4), even as late as stage 23 (tailbud stage).

Levels of cyclin A2 protein were measured by Western blotting (Fig. 4B). As development progressed, cyclin A2 protein accumulated in injected embryos (Fig. 4B, compare lanes 2 and 5). Furthermore, simultaneous overexpression of

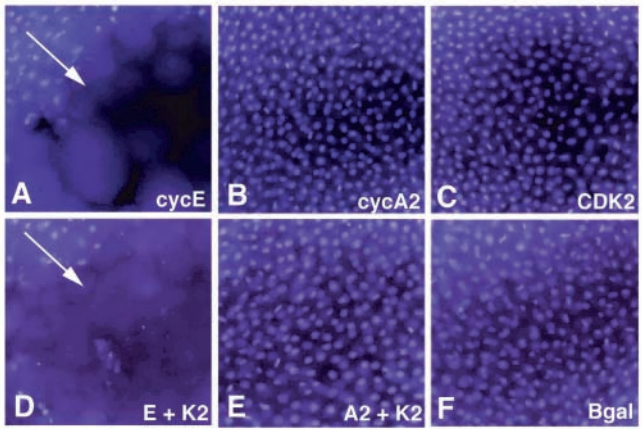

Fig. 3. Cyclin E overexpression results in nuclear loss. RNAs encoding cyclin E1 alone (A) or cyclin E1 with cdk2 (D), cyclin A2 alone (B) or cyclin A2 with cdk2 (E), cdk2 alone (C) or β-gal as a control (F) were injected into one cell of a 2-cell embryo and allowed to develop to stage 8.5. Embryos were fixed, depigmented and the DNA stained with Hoechst. Animal caps were dissected and viewed by fluorescence microscopy. White arrows indicate regions in which nuclei are absent.

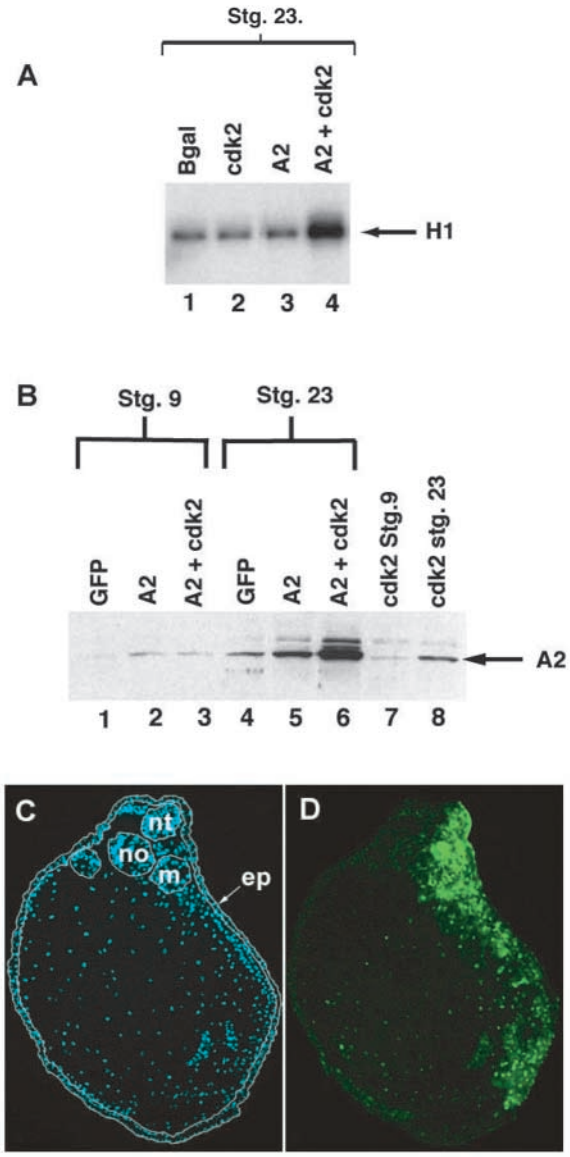

Fig. 4. Cyclin A2/cdk2 overexpression is sustained throughout early development. (A) Embryos were injected with RNA encoding cdk2, cyclin A2, cyclin A2 with cdk2, or Bgal as a control. Embryos were allowed to develop to stage 23, then immunoprecipitated cdk2 from injected embryos was tested for its ability to phosphorylate histone H1. (B) Embryos were injected with RNA encoding cyclin A2, cdk2, cyclin A2 with cdk2 or GFP as control and allowed to develop to stage 9 (lanes 1-3, 7) or stage 23 (lanes 4-6, 8). Extracts from embryos, injected as labelled, were western blotted to detect cyclin A2 protein levels. (C,D) Cyclin A2/cdk2-injected embryos were grown until tailbud stages (injected side to the right). Tagged cyclin A2 is stable in all tissues on the injected side (D), including neural tube (nt), notochord (no), muscle (m) and epidermis (ep); see Hoechst staining of the same section (C).

cdk2 substantially stabilised overexpressed cyclin A2 protein (Fig. 4B, lane 6). This effect might be analogous to stabilisation of cyclin E seen on binding to cdk2 in mammalian cells (Clurman et al., 1996). However, this stabilisation effect is not seen at stage 9, possibly because little injected cyclin A2 has accumulated at this time (Fig. 4B, compare lanes 2, 3 and 7) or due to regulated and abrupt degradation of A-type cyclins at the onset of gastrulation (Howe et al., 1995). To maintain cyclin A2-dependent kinase activity at high levels in older embryos that we studied in the following experiments, we co-injected cyclin A2 with cdk2 (cyclin A2/cdk2 RNA).

To investigate the stability of cyclin A2 protein in different tissues, we injected embryos with HA-cyclin A2/cdk2 RNA and allowed them to develop to stage 23. Embryos were sectioned and immunostained for expression of HA-tagged cyclinA2. We saw no overall difference in protein stability between epidermis, notochord, myotome or neural tube in the injected region (Fig. 4C,D). However, there was cell-to-cell variability, as expected from a protein that is degraded in a cell cycle-dependent manner. Cdk2 protein was also equally stable in all these tissue types (data not shown).

Next, to establish whether overexpression of cyclin A2 kinase is sufficient to force cells through the cell cycle, we injected cyclin A2/cdk2 RNA, along with Bgal as a lineage tracer, into one cell of a two-cell embryo. Embryos were allowed to develop to stages 16 and 19 when we investigated proliferation on the injected versus the uninjected side. We used the mitotic marker phosphorylated histone H3 to visualise proliferation and measured the number of cells in mitosis (Saka and Smith, 2001). To quantitate cellular proliferation, regions of equal size were drawn on the flank of the embryo on the injected versus the uninjected side and the number of phosphorylated histone H3-expressing cells were counted. On average, 46% more cells at stage 16 (n=20) and 63% more cells at stage 19 (n=12) (Fig. 5A,C) were undergoing mitosis on the skin of the injected side of the embryo compared with the uninjected side. By contrast, Bgal-injected control embryos showed no increase in number of phosphorylated histone H3-expressing cells on the injected side (data not shown). Therefore, overexpression of cyclin A2/cdk2 is able to increase cellular proliferation after MBT.

To determine its effect on S-phase entry in tissues

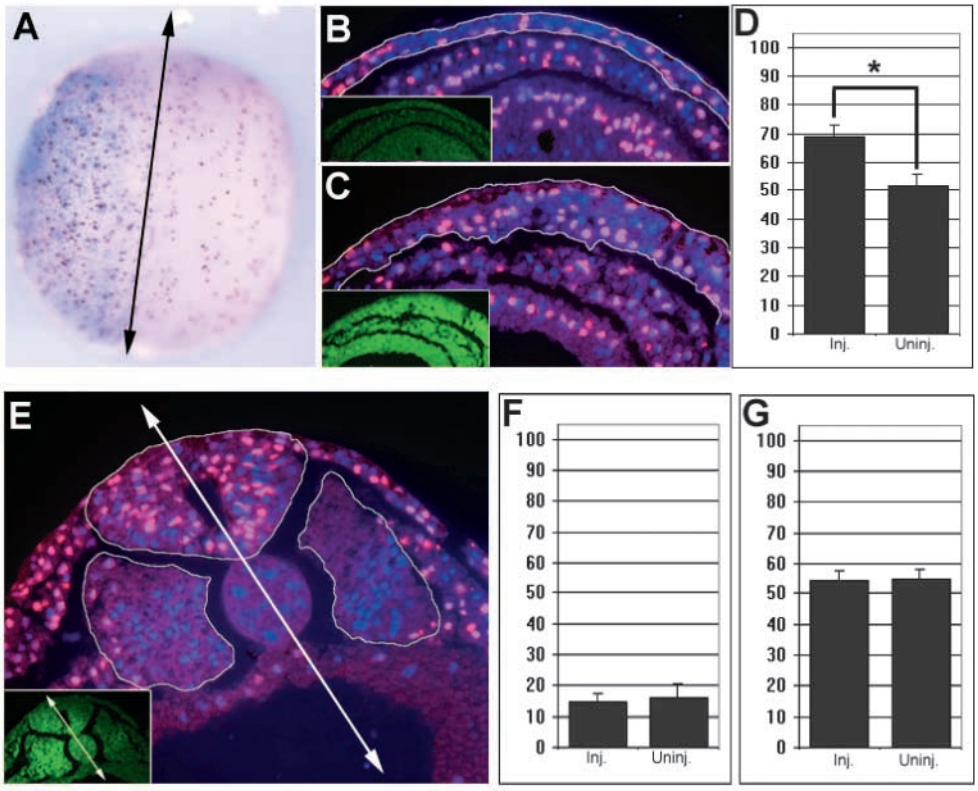

Fig. 5. Cyclin A2/cdk2 overexpression promotes cell proliferation in the embryonic epidermis. Embryos were injected into one cell at the 2-cell stage with cyclin A2 and cdk2 RNA along with Bgal (light blue). (A) Whole-mount immunostaining with antiphosphohistone H3 antibodies shows an increase in cell proliferation on the injected side (left). (B-G) Cyclin A2/cdk2-injected embryos were grown until tailbud stages and then allowed to incorporate BrdU for 1 hour before fixing (B,C,E; BrdU uptake) red; nuclei stained in blue with Hoechst dye; bottom left of each panel: β-gal auto-fluorescence. Epidermis overexpressing cyclin A2/cdk2 (C) is disorganised and shows increased BrdU incorporation compared with epidermis on the uninjected side (B). By contrast, cell proliferation does not differ in the neural tube and muscle between the injected side (left) and the uninjected side (right) (E). (D,F,G) Number of BrdU-positive cells expressed as a percentage of total number of nuclei (Hoechst-positive) were counted in regions of equal side on the injected and uninjected sides of the embryos. BrdU incorporation is increased in the epidermis on the injected side compared with the uninjected side of embryos (D). No significant difference in BrdU incorporation is seen for muscle (F) and neural tube (G).

throughout the embryo, cyclin A2/cdk2 RNA was injected into one cell at the 2-cell stage along with Bgal as a lineage tracer. Because the first cleavage in *Xenopus* embryos usually determines bilateral symmetry, RNAs are incorporated unilaterally into one side of the embryo, so injected and uninjected sides can be directly compared. At stage 23, bromodeoxyuridine (BrdU) was injected into the archenteron, and after 1 hour the embryos were fixed, sectioned and stained for BrdU incorporation. BrdU incorporation was enhanced in epidermis on the injected side (Fig. 5, compare C and B), where 69% of cells showed incorporation compared with 52% on the uninjected side ($P<0.05$). Moreover, while the uninjected sides of the embryos showed normal skin morphology, in which the epidermis is two cell layers thick and cells are flattened along the surface of the embryo (Fig. 5B), regions where cyclin A2 and cdk2 were most strongly overexpressed, as judged by a co-expressed Bgal marker, showed pronounced thickening. In these regions, the epidermis thickened up to five or six cell layers and cells often lost their flattened appearance (Fig. 5C). Whole-mount TUNEL staining did not reveal a significant increase in the number of apoptotic cells in the skin after cyclin A2/cdk2 overexpression at this level (data not shown). Thus, cyclin A2/cdk2 overexpression clearly promotes proliferation in the embryonic skin, a tissue in which cyclin A2 is usually expressed at a low level (Vernon and Philpott, 2003a).

To investigate whether cyclin A2/cdk2 overexpression would similarly promote proliferation in other early embryonic tissues, numbers of BrdU-labelled cells were counted in each half of the neural tube and myotome. There was no significant difference in the number of cells incorporating BrdU on the injected versus the uninjected side in these tissues (Fig. 5E,F,G): on average 15% BrdU-positive cells on the injected side versus 16% on the uninjected side for the muscle section and on average 54% BrdU-positive cells on both injected and uninjected sides for the neural tube. Nor was the myotomal tissue area visibly changed. Similarly, cyclin A2/cdk2 overexpression did not lead to proliferation in the notochord (Fig. 5E), which is postmitotic by this stage (Saka and Smith, 2001). These data indicate that in nerve and muscle at this stage any increased proliferative signal given by cyclin A2/cdk2 overexpression is overridden by intrinsic or extrinsic factors that control the rate of cell division. However, this is clearly not the case in the skin, in which cyclin A2/cdk2 overexpression alone can promote cell cycling.

While cyclin A2/cdk2 overexpression promotes ectopic cell division, we wanted to know whether these cells divide more rapidly, i.e. at a smaller size. To measure approximate cell size, we measured the average internuclear distance along the surface of either normal or thickened skin to produce a measurement of average cell diameter. In a typical experiment, internuclear distance was 26.3 μm±1.27 on the injected side versus 24.7 μm±1.22 on the uninjected side ($n=11$), demonstrating that there is not a significant difference in cell size in cyclin A2/cdk2-overexpressing regions and the control non-expressing regions. These data indicate that cyclin A2/cdk2 upregulation is not sufficient to allow cells to proliferate at a smaller than normal size.

Cells in the developing embryo are subject to many signals, extrinsic and intrinsic, which instruct cells to divide or to

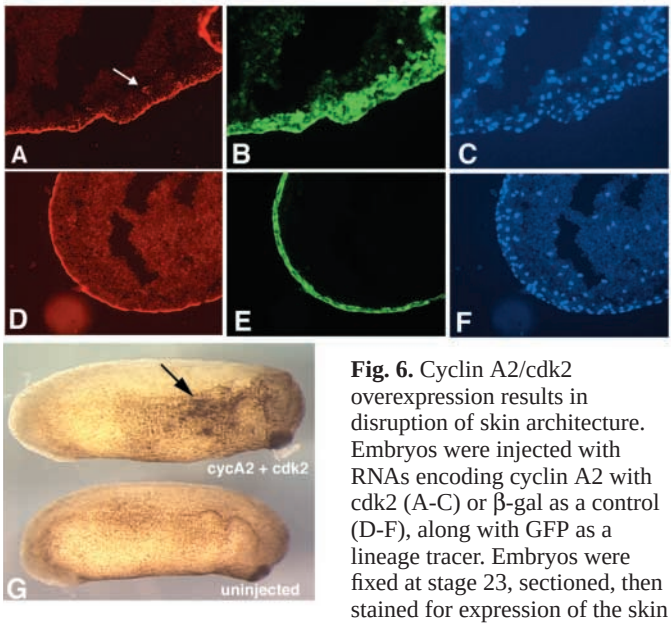

Fig. 6. Cyclin A2/cdk2 overexpression results in disruption of skin architecture. Embryos were injected with RNAs encoding cyclin A2 with cdk2 (A-C) or β-gal as a control (D-F), along with GFP as a lineage tracer. Embryos were fixed at stage 23, sectioned, then stained for expression of the skin marker, epi (A,D), confined to the epidermis in these sections. Region of epi expression deep within the thickened skin is indicated by a white arrow. GFP fluorescence (B,E). DNA is stained with Hoechst in blue (C,F). Alternatively, uninjected embryos or embryos injected with RNAs encoding cyclin A2/cdk2, as indicated, were photographed in whole-mount (G). A raised hyperpigmented lump, which often formed after cyclin A2/cdk2 injection, is indicated by the black arrow.

differentiate. While it has become increasingly apparent that cells must exit the cell cycle before terminal differentiation can occur, the ways in which cell cycle regulators and differentiation factors are coordinated is obscure. We wished to determine whether overexpression of cyclin A2/cdk2 alone was enough to overcome endogenous signals within the embryo promoting differentiation of the skin. First, we allowed injected embryos to grow up to tailbud stages and viewed them externally. We observed that overexpression of cyclin A2/cdk2, but not cyclin A2 alone or cdk2 alone, resulted in regions of hyperpigmentation in the injected region of the embryo, indicating that normal skin formation or architecture had been disrupted. This hyperpigmentation was often accompanied by raised regions of epidermis (‘lumps’, Fig. 6G, arrow). The penetrance of these phenotypes varied somewhat from batch to batch of embryos, but a typical experiment is shown in Table 1. However, we noted that even where hyperpigmentation and lumps were visible, skin integrity was always maintained, indicating that epidermal differentiation was occurring.

To investigate this further, we studied expression of 2F7.C7 (epi), a marker of differentiation of the surface layer of the skin. This marker first appears at the onset of neurulation in cells differentiating into the outer layer of the epidermis and becomes stronger as development progresses (Jones and Woodland, 1986). Embryos were injected in one cell at the two-cell stage with RNAs encoding cyclin A2 and cdk2 along with GFP as a tracer. Bgal RNA was injected along with GFP as a control in parallel embryos. Embryos were allowed to develop to stage 23, then sectioned and stained for the expression of epi (Fig. 6). Epi was strongly expressed in the outermost cell layer of the control-injected embryos (Fig. 6D).

Table 1. Effects of *cyclin A2* and *cdk2* injection

Injected?	Normal	Hyperpigmented	Hyperpigmented + lumps
Cyclin A2	45	2	0
cdk2	48	0	0
Cyclin A2 + cdk2	12	15	20
Uninjected	48	0	0

Table shows the number of embryos exhibiting regions of hyperpigmentation, with or without raised lumps following injection of cyclin A2 and cdk2, alone or in combination, as indicated.

Epi was also clearly seen in skin expressing cyclin A2/cdk2 at the same level as that seen in the control at this stage (Fig. 6A). However, while staining in control embryos was confined to the surface of the outermost layer of the epidermis, cyclin A2/cdk2-expressing embryos displayed staining deep in deep cell layers (Fig. 6A, arrow), indicating disrupted epidermal architecture. Hyperpigmentation might also be caused by this disrupted architecture, as pigment was also seen in deep skin layers in injected regions (data not shown). Thus, although cyclin A2/cdk2 overexpression causes inappropriate proliferation in the skin, overexpressing cells are still competent to respond to signals that promote terminal differentiation. Indeed, these extra cells differentiate even in their inappropriate position under the surface skin layer.

Thus, while overexpression of cyclinA2/cdk2 does not prevent epidermal differentiation, we wished to determine whether it was sufficient to delay differentiation in embryonic tissues. Hence, we injected cyclinA2/cdk2 RNA and allowed embryos to develop to neural plate and tailbud stages. Embryos at increasing developmental stages were stained by in-situ hybridisation for expression of: the skin marker epidermal keratin; the somatic muscle marker muscle actin; and neural beta tubulin, a marker of primary neurons, which are the first neurons to differentiate within the neural plate (Fig. 7). After cyclin A2/cdk2 overexpression, epidermal keratin expression was significantly delayed on the injected side of the embryo until stage 18 (Fig. 7A-C) while primary neurons failed to differentiate until tailbud stages (Fig. 7D-F). By contrast, expression of muscle actin (or the terminal muscle marker heavy chain myosin, data not shown) was not delayed by overexpression of cyclin A2/cdk2 (Fig. 7G-I). Thus, simple upregulation of a single cyclin/cdk pair is sufficient to overcome endogenous signals to differentiate, but only for a limited time and only in specific tissues.

Discussion

Much of what we know about the cell cycle has been derived from studies on single-celled organisms such as yeast, on tissue culture cells and on simplified cell cycles in amphibian eggs. None of these systems displays the complexity of regulation required in a multicellular organism to coordinate the complex patterns of cell division and differentiation that allow proper patterning of the early embryo. Where whole-organism studies have been attempted, perturbation of cyclin/cdk levels in *Drosophila* has led to significant disruption of cell division patterns in the tissues thus far studied (Duronio and O’Farrell, 1995; Jacobs et al., 2001; Knoblich et al., 1994; Li et al., 1999; Richardson et al., 1995; Sprenger et al., 1997). Targeted overexpression of D-type cyclins and cdk4 via transgenesis in

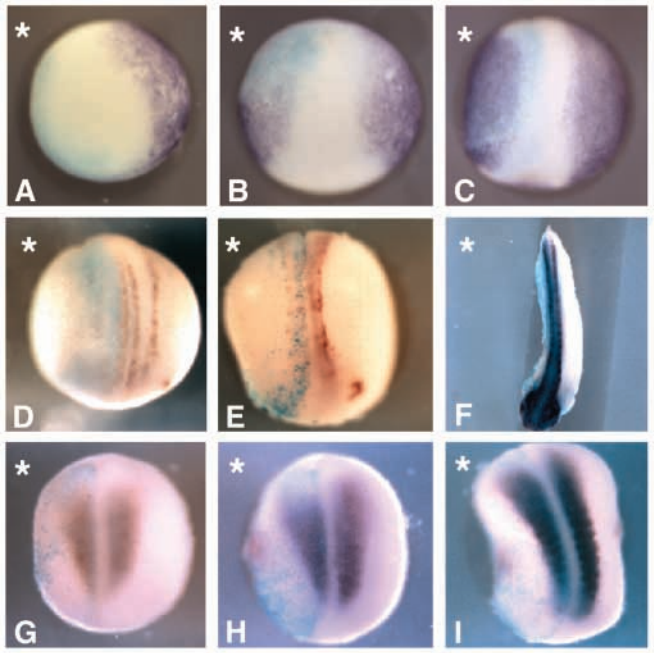

Fig. 7. Cyclin A2/cdk2 delays differentiation of epidermis and neurons. Whole-mount in-situ hybridisation analysis shows a delay in skin differentiation (A-C: epidermal keratin, stage 14, 16 and 18, respectively); and neural differentiation (D-F: neural β tubulin, stage 14, 19 and 25, respectively) in embryos injected with cyclin A2 and cdk2 RNA (injected side to the left, indicated by asterisks). Differentiation of the muscle is not affected (G-I: muscle actin, stage 13, 16 and 21, respectively).

mice has also been performed, largely in the epidermis and thymus (Miliani de Marval et al., 2001; Robles et al., 1996; Rodriguez-Puebla et al., 2000; Yamamoto et al., 2002). However, generalised overexpression of a cyclin/cdk pair in multiple tissues in a vertebrate embryo has not been attempted. To investigate the effects of a widespread increase in cyclin/cdk2 kinase activity in *Xenopus* embryos, we chose to overexpress E- and A-type cyclins.

In mammalian cell cycles, data that come largely from experiments in cultured cell systems suggest that accumulation of the cyclin, rather than the amount of cdk subunit, is rate-limiting for generation of cdk2-dependent H1 kinase activity and subsequent cell cycle progression (Connell-Crowley et al., 1998; Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995; Resnitzky et al., 1994; Resnitzky et al., 1995; Rosenberg et al., 1995). Strikingly in the pre-MBT embryo, this assumption seems not to hold true: overexpression of either cyclin E1 or cyclin A2 alone did not produce a significant increase in cdk2 kinase activity in embryo extracts (Fig. 1) (Strausfeld et al., 1996). While free cdk2 that is unable to complex with overexpressed cyclin E1 or A2 may exist in the embryo, overexpression of cdk2 alone by RNA injection also enhanced cdk2-dependent H1 kinase activity. This finding indicates that there may in fact be a pool of excess cyclins in pre-MBT embryos. Cyclin/cdk overexpression is unlikely to be titrating out cdk inhibitors (cdkis) at this stage, as the only described cdki in *Xenopus*, Xic1, is not expressed at significant levels prior to MBT (Shou and Dunphy, 1996; Su et al., 1995).

Microinjection of cyclin E RNA into *Xenopus* embryos resulted only in a mild slowing of cell cleavage pre-MBT. However, on closer inspection of dissected animal pole tissue, we determined that nuclei were largely absent from injected areas, even though cleavage had occurred. This observation is consistent with elevated levels of cyclin E blocking DNA replication, as has been demonstrated in egg extracts (Hua et al., 1997), which would result in nuclear loss on subsequent cell division. The fact that cleavage proceeds in the absence of nuclear DNA when cyclin E is overexpressed (Fig. 2) demonstrates the absence of active checkpoints in the embryo pre-MBT that monitor DNA replication and/or genome stability and chromosome segregation (Dasso and Newport, 1990). However, some slowing of cleavage does occur in the most highly overexpressing regions at this early stage (Fig. 2a,d, arrows), indicating either that nuclear events can be sensed to some extent or that cyclin E overexpression has a direct effect on cleavage events, possibly via an effect on centrosome duplication (Hinchcliffe et al., 1999) or on cdc2 activity. Apoptosis in these cyclin E-injected embryos, which apparently occurs in response to the absence of nuclear DNA, occurs at gastrula stages, when the apoptosis machinery becomes activated (Hensey and Gautier, 1997; Sible et al., 1997; Stack and Newport, 1997). Interestingly, overexpression of cyclin E in mammalian cells does not block DNA replication, nor does it result in obvious apoptosis (Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995; Resnitzky et al., 1994), but instead it promotes genetic instability (Spruck et al., 1999). Cyclin E is often highly expressed in tumours (Donnellan and Chetty, 1999), and the genetic instability caused could lead to loss of genes involved in apoptotic pathways in the outgrowing cells.

Importantly, we note that loss of nuclear DNA occurs after injection of cyclin E message alone, with or without additional cdk2. As overexpression of cyclin E alone does not lead to an overall increase in the level of immunoprecipitable cdk2 kinase activity (Fig. 1), nor will it activate cdc2 (data not shown), it is possible that increased kinase activity is not required to produce cyclin E-induced loss of nuclei. Alternatively, cyclin E overexpression might raise kinase activity only locally, which is not detected by overall cdk2 immunoprecipitation from lysed embryos, and this local activation might be sufficient to induce nuclear loss. Overexpression of cyclin A2 with or without cdk2 has no appreciable effect on either cell or nuclear division prior to MBT. This observation demonstrates that overexpression of cyclin E has specific effects that are not recapitulated by overexpression of cyclin A2, indicating distinct targets for these two molecules. This difference is surprising, given that endogenous cyclin A2 can compensate for the requirement of cyclin E for DNA replication in egg extracts (Jackson et al., 1995).

While the effect of cyclin E overexpression on nuclear maintenance in vivo is interesting and deserves further study, the focus of this work was to determine the effects of cyclin/cdk overexpression on development after the MBT. As overexpression of cyclin E induces apoptosis at gastrulation, we chose to concentrate on the effects of cyclin A2/cdk2 overexpression on later developmental processes.

Studies of phosphohistone H3 distribution in *Xenopus* demonstrated that cell proliferation is widespread in many areas of the neurula and tailbud stage embryo (Saka and Smith, 2001). We find that cyclin E1 and cyclin A2 messages have

overlapping distributions and are most highly expressed in the anterior neural tube (Vernon and Philpott, 2003a). While this distribution might be expected, since the neural tube is one of the most actively proliferating tissues at this time (Saka and Smith, 2001), it is surprising that expression is strikingly lower in other tissues that are still dividing at these stages, such as the embryonic epidermis (Saka and Smith, 2001; Vernon and Philpott, 2003a). Overexpression of cyclin A2/cdk2 alone is enough to promote enhanced proliferation in the embryo (Fig. 5). However, a dramatic increase in the proportion of proliferating cells occurs only in the epidermis. Strikingly, cyclin A2/cdk2 overexpression apparently cannot promote proliferation in the notochord or myotome, where cells normally exit the cell cycle at early to mid-neural plate stages (Fig. 5E,F) (Saka and Smith, 2001; Vernon and Philpott, 2003b), even though the proteins are stable in these tissues (Fig. 4C,D). Moreover, proliferation is not enhanced in the neural tube (Fig. 5G), where cyclin messages are normally abundant (Vernon and Philpott, 2003a). This indicates that in tailbud-stage embryos, of the tissues studied, cyclin A2/cdk2 overexpression is limiting only for proliferation in the skin, where G1/S phase cyclin message levels are usually low but where many cells are still proliferating (Vernon and Philpott, 2003a). This might reflect the fact that there are high levels of the cdk inhibitor Xic1 in the myotome, nervous system and notochord but only low levels in the epidermis (Hardcastle and Papalopulu, 2000; Ohnuma et al., 1999; Vernon et al., 2003; Vernon and Philpott, 2003b), and indicate that different tissues emphasise different methods of cell cycle regulation during embryogenesis.

Studies in *Drosophila* have shown that overexpression of cyclin E can result in cells dividing at a smaller size than usual, but the final area occupied by overexpressing cells is normal as extra cells are lost by apoptosis (Li et al., 1999). Interestingly, in some circumstances, while cyclin E overexpression accelerates passage through G1 phase, cell cycle length is not changed as there is a corresponding lengthening of S phase (Neufeld et al., 1998). By contrast, overexpression of E2F, a transcription factor required to upregulate a variety of S-phase progression factors as well as the M-phase String protein, accelerates the cell cycle. However, this accelerated proliferation correlates with decreased cell size, thus tissue volume is not affected (Neufeld et al., 1998). To determine whether cyclin A2/cdk2 overexpression caused cells to divide at a smaller than usual size, we measured the internuclear distance between cells overexpressing cyclin A2/cdk2 compared with uninjected cells in skin sections and found no significant difference. This is indicative of cell division occurring at normal cell size. Indeed, in cyclin A2/cdk2 overexpressing embryos, tissue expressing epidermal markers is more than the usual two cell layers thick (Fig. 6), again indicating that cells do not divide at a smaller size, but instead continue proliferating when they would normally have exited the cell cycle and so must occupy a greater than usual volume (Zuber et al., 1999). Therefore, cyclin A2/cdk2 overexpression appears to be insufficient to accelerate the overall cell cycle rate at the expense of cell growth in a vertebrate embryo, but instead it prolongs the period of proliferation.

In most systems, proliferation and differentiation are mutually exclusive. We wished to determine whether overexpression of cyclin A2/cdk2 delayed or prevented differentiation in the early embryo. Cyclin A2/cdk2 overexpression was able to significantly delay the appearance of epidermal keratin (Fig. 7A-C). However, differentiation did eventually occur (Fig. 6A; Fig. 7C), demonstrating that promotion of cell cycling in this way is sufficient to delay, but not to overcome, signals promoting epidermal differentiation. Mice have been generated in which D-type cyclins or cdk4 are specifically upregulated in epithelia, especially in the skin, and this results in hyperproliferation of the epidermis (Miliani de Marval et al., 2001; Robles et al., 1996; Rodriguez-Puebla et al., 2000; Yamamoto et al., 2002). Interestingly, under these circumstances differentiation appears to be largely normal. We note that D-type cyclins, which act in G1 phase of the cell cycle and earlier than cyclin E or cyclin A, are not detected in the epidermis of *Xenopus* embryos (Vernon and Philpott, 2003a).

The mammalian skin condition psoriasis results from hyperproliferation and thickening of the epidermis coupled to perturbed differentiation. Although epidermis structure differs significantly between species, we see a strikingly similar phenotype on overexpressing cyclin A/cdk2 in *Xenopus* embryos. Interestingly, psoriasis in humans is also accompanied by overexpression of cyclin A (Miracco et al., 2000), and our results suggest that this upregulation may help drive the condition. Moreover, a number of cancers, including those derived from skin, show elevated levels of A-type cyclins (Balasubramanian et al., 1998; Kim et al., 2002). Our results indicate that skin may be particularly susceptible to an increase in cyclin A-dependent kinase level. This overexpressing population of cells maintained in the cell cycle inappropriately might then be targets of further genetic ‘hits’, ultimately resulting in tumour formation. Indeed, a study looking at UV-induced skin cancers in mice showed that cyclin A overexpression occurs early on in the process of tumour formation (Kim et al., 2002).

At neural plate and tailbud stages, most of the cells destined to become neurons are still dividing and consequently express high levels of cyclin A2/cdk2 (Hartenstein, 1989; Saka and Smith, 2001; Vernon and Philpott, 2003a). However, a small subset of cells found in three stripes lateral to the midline exit the cell cycle early and differentiate into primary neurons (Hartenstein, 1989; Lamborghini, 1980). Overexpression of cyclin A2/cdk2 also delays differentiation of these cells (Fig. 7D-F). One can envisage several ways in which elevated cyclin A2/cdk2 could delay primary neuron differentiation. First, cyclin A2/cdk2 can promote cellular proliferation, preventing cell cycle exit, which is incompatible with differentiation. Second, we have recently shown that the cdki Xic1 is absolutely required for differentiation of primary neurons, and this activity extends beyond its ability to regulate the cell cycle (Vernon et al., 2003). While the exact mechanism of Xic1 action has yet to be elucidated, cyclin A2/cdk2 overexpression might inhibit primary neurogenesis by sequestering the required Xic1 protein. Interestingly, however, cyclin A2/cdk2 overexpression does not delay muscle differentiation, a process also shown to be Xic1-dependent, again showing that different tissues may rely on distinct molecular methods to regulate the balance between proliferation and differentiation. Third, the levels of the myogenic factor MyoD are regulated by ubiquitin-mediated proteolysis (e.g. Reynaud et al., 2000; Tintignac et al., 2000), which is, in turn, controlled by cell cycle kinases

and inhibitors. It is possible that proneural gene products are similarly regulated. We are currently investigating this possibility.

Our results demonstrate that overexpression of a single cyclin/cdk pair in a vertebrate can have substantial effects on the balance between division and differentiation in the early embryo, in a tissue-specific manner. Studies in experimentally tractable systems, such as those described here in *Xenopus* embryos, may prove very informative for understanding the role of cyclin/cdk upregulation in diseases of hyperproliferation, such as psoriasis and cancer, in which it might affect both cell proliferation and differentiation in a tissue-dependent manner.

We would like to thank: Tim Hunt and Elizabeth Jones for generously providing antibodies and constructs; Shinichi Ohnuma, Mike Zuber, Bill Harris and Ashok Venkitaraman for helpful discussions; and Doug Winton and Phil Jones for critical reading of the manuscript. This work was supported by Cancer Research UK (Grant No. SP2476/0101).

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