the notochord, (ii) induced by the notochord at a distance (implying that the inducer is diffusible), or (iii) secondarily induced by the notoplate by means of a propagating (homeogenetic) induction (*22, 23*). The last possibility has been called into question recently, since evidence for homeogenetic induction in *Xenopus* has not been obtained (*6*). All three of the above possibilities could explain the areas of expression extending away from the notochord in our recombinants (Fig. 4). However, none of the three possibilities explains the rectangular shape of the stage 14 patch of expression of En-2 in the normal embryo (*9*). It is possible that a gradient within the neuroectoderm in the anterior-posterior direction is responsible for controlling the shape of the patch by modulating the response to the inducer. Thus, signals passing through the plane of the neurectoderm may not be essential for initiating *En-2* expression, but they might be important for fine-tuning the area of *En-2* expression in normal embryos. Alternatively, dorsal mesoderm may induce other region-specific genes in the neuroectoderm and local interactions between these gene products may sharpen and further elaborate anterior-posterior pattern in the neural plate, a situation similar to the establishment of anterior-posterior pattern in *Drosophila* (*24, 25*).

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Specification of Jaw Muscle Identity in Zebrafish: Correlation with *engrailed*-Homeoprotein Expression

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Molecules that determine the specific features of individual muscles in vertebrates are unknown. Antibody labeling studies described here revealed a molecular difference among muscles in the zebrafish head, in that two functionally related jaw muscles (the levator arcus palatini and the dilator operculi), and not other head muscles, expressed *engrailed*-homeodomain proteins. Expression began in mesoderm-derived muscle-precursor cells in the paraxial mesenchyme and continued during muscle morphogenesis and differentiation. Growth cones of trigeminal motoneurons that innervate these muscles associated with the precursors within a few hours of the time they began to express *engrailed*. It is proposed that the *engrailed* proteins in these cells establish muscle identity and neuromuscular target recognition.

EACH MUSCLE HAS A CHARACTERISTIC location, relationship to supporting structures, and association with selectively innervating motoneurons. These features are signatures of individual muscle "identities," but molecules that specify muscle identities are unknown (*1–3*). Homeodomain proteins and other DNA-binding proteins are likely candidates, since they specify cell fates and pattern formation in invertebrates, and accumulating evidence suggests that they have similar functions in vertebrates, particularly within the central nervous system (*4*). Here we report the expression of *engrailed*-type homeodomain proteins (Eng) in cells that develop to form two specific jaw muscles in the zebrafish.

We used the monoclonal antibody 4D9 that was originally produced against *invected*, one of the two Eng proteins in *Drosophila*, and that also recognizes Eng in diverse animals (*5–8*). Homeobox gene expression patterns are generally conserved among vertebrates. For example, homologs of the murine *engrailed*-type gene *En-2* (*9, 10*) are expressed as a stripe in the neural tube at the border of the midbrain and hindbrain of other tetrapods (*6*) and of zebrafish (*8*).

The time course of Eng expression during zebrafish jaw muscle development is shown in Fig. 1. The first cranial expression outside of the central nervous system was observed in a scattered group of about 15 mesenchymal cells, caudal to the eyes and lateral to the hindbrain, at 24 to 26 h (*11*). The number of labeled cells increased to approximately 30 to 40 on each side of the head at 28 h, at which stage the cells are only loosely associated (*12*). By 36 h the Eng-positive cells moved rostrally, aggregated, and subsequently compacted to form a single cluster located in the mandibular arch by 48 h. At 72 h (when many embryos have hatched), Eng was expressed in two jaw muscles that develop at the same location as the earlier labeled mesenchyme. These muscles are the levator arcus palatini (LAP) and the dilator operculi (DO). LAP and DO are closely apposed and cooperate functionally (*13*). Eng was expressed in the nuclei of every cell in these two muscles, and expression persisted for at least 3 weeks while the number of labeled nuclei increased to a few thousand. Other head and gill arch muscles expressed little or no Eng (*14*). Expression is transient; we could find no muscles in adult fish (at 5 months) that expressed Eng.

The head mesenchymal cells that express Eng might originate from either paraxial mesoderm or the neural crest: although some (possibly all) head muscles in the zebrafish and other vertebrates derive from mesoderm (*15, 16*), the earliest Eng expression is in neuroectoderm (*5*), from which the neural crest originates. Thus, the mesenchyme that expresses Eng might be derived from either or both sources (*17*). We used lineage tracing to clarify the origins of LAP and DO, and showed directly that the muscle precursor cells were already in the head paraxial mesenchyme at a stage before neural

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crest migration began (Fig. 2). In the early gastrula, cells that would give rise to LAP and DO were present in the marginal region of the blastoderm, the region containing progenitors of other head muscles, axial muscle in the trunk and tail, and other mesodermal derivatives, but not neural crest. The cells that would later form the jaw muscles involuted during gastrulation and migrated as part of the hypoblast to the head paraxial mesenchyme. The position of the cells in the early fate map and the subsequent pattern of cell movement were characteristic of mesoderm rather than ectoderm (*16*). Therefore, in zebrafish, at least some jaw muscles and some or all of the cells that express Eng in the cranial paraxial mesenchyme are mesodermal (*17*). We obtained no evidence for Eng expression by neural crest–derived cells.

Jaw muscles, including LAP and DO, are innervated by the trigeminal (fifth cranial) motor nerve. We double-stained embryos for Eng and early developing neurons and found that the first trigeminal growth cones entered the periphery by 28 h, where they contacted the paraxial mesenchyme containing the Eng-positive muscle precursor cells (Fig. 3). The axons remained associated with the precursor cells at least through 36 h. Thus, the neurons appeared to interact with their eventual targets at about the time Eng expression was first detected, and the association persisted (*18*). The motoneurons themselves are located in two brain segments where neighboring subsets of cells also express Eng.

Early contacts and subsequent comigration of axonal growth cones and the precursors of their eventual targets are also seen during development of the midbody lateral line in zebrafish. The leading growth cones of the sensory nerve that innervates the lateral line migrate together with the precursors of their eventual targets, the hair cells of the neuromasts (*19*). Thus, association of axonal growth cones and early target cells may be a common mechanism for growth cone navigation and nerve-target interaction in both motor and sensory systems.

We have described a molecular difference between muscles during their development. All of the cells comprising two jaw muscles express Eng, while cells in other head muscles do not. This difference, the onset of expression in the muscle precursors, and the maintenance of expression in the differentiated muscles, are all consistent with the hypothesis that Eng is involved in determining the identity of these two muscles. Further, since LAP and DO differ not only from other head muscles, but also from one another, Eng may specify their identities only in combination with other transcriptional regulators. Such a mechanism, well documented for insect body segments, has also been proposed for establishing the identity of vertebrate hindbrain segments (*4, 20*).

Muscles are recognized specifically by the neurons that innervate them. Eng in LAP and DO precursors may regulate neurotrophic or -tropic factors (*21*), or cell surface molecules, or both, that may control the neuronal association. Since the expression of Eng is specific to these muscles, it can be used in experiments to identify the target

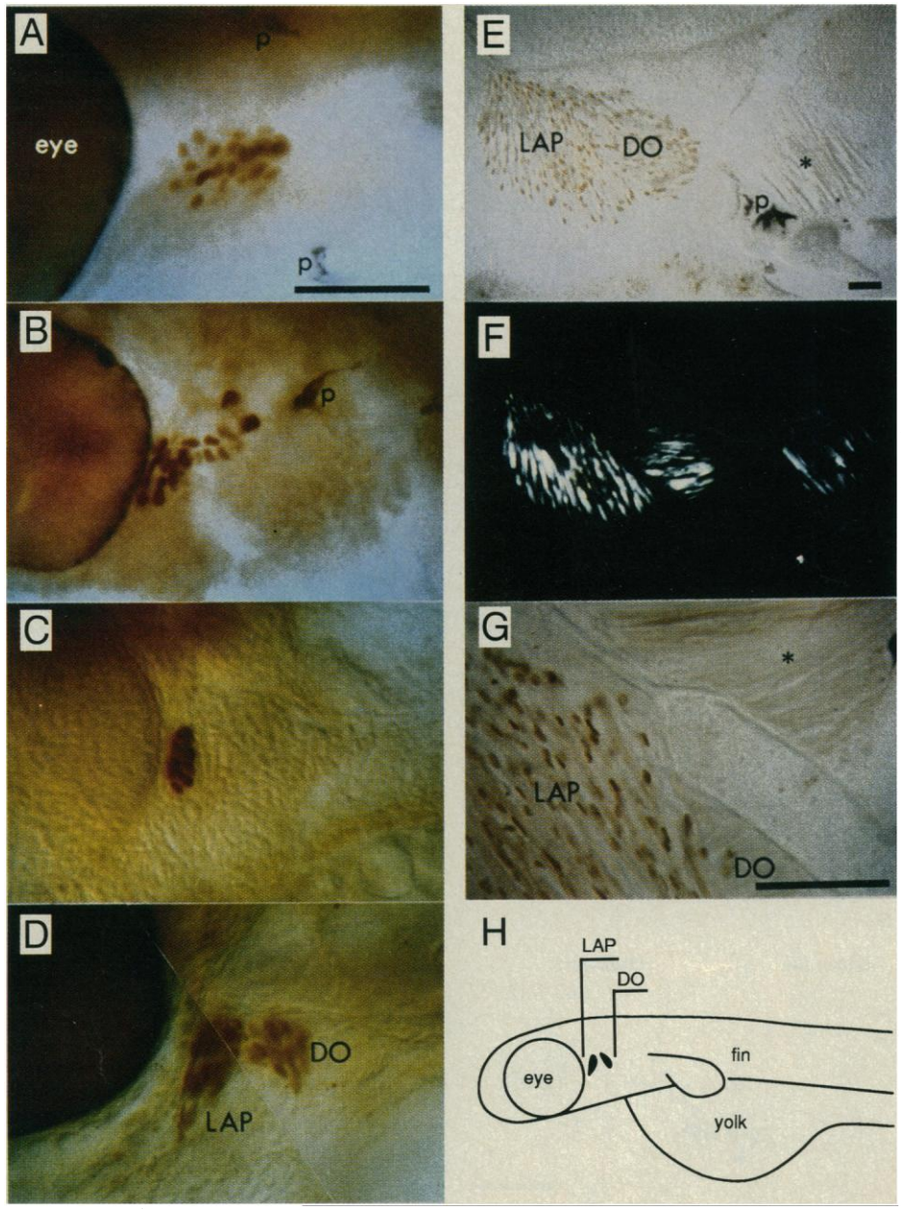

Fig. 1. Specific jaw muscles express Eng during development. (**A**) At 28 h, loosely clustered mesenchyme expresses Eng (p, pigment cells that are visible but are not labeled by the 4D9 antibody). (**B**) At 30 h, labeled cells have moved anteriorly, relative to the eye. (**C**) At 48 h, labeled mesenchyme is compacted into a single aggregate. (**D**) At 72 h, two jaw muscles (LAP and DO) are labeled. (**E**) The same muscles at 3 weeks (advanced larva), in sagittal section. Note that only two of the three muscles in the field express Eng. [Asterisks in (E) and (G) indicate head muscles that do not express Eng.] (**F**) Same section as (E) photographed with polarized light to show muscle birefringence. (**G**) Transverse section, 3 weeks, photographed at higher magnification to show Eng expression localized to nuclei of differentiated myofibers. (**H**) Schematic representation of the position of Eng-expressing muscles at 72 h. The muscles are identified by their connections with bones and the operculum at 3 weeks, as described for larval *Amia* (*2*) and the adult perch (*13*). For (C) and (D), we used golden (*gol1-1/gol-1*) mutants (*23*) that have less pigmentation than wild-type embryos. (A) to (D), whole mount; (E) to (G), 20-μm sections. (A), (B), (F), (G), bright field; (C to E), Nomarski optics. Whole-mount or section immunostaining was done with peroxidase-antiperoxidase complex and visualized by diaminobenzidine (*24*). Bars represent 50 μm.

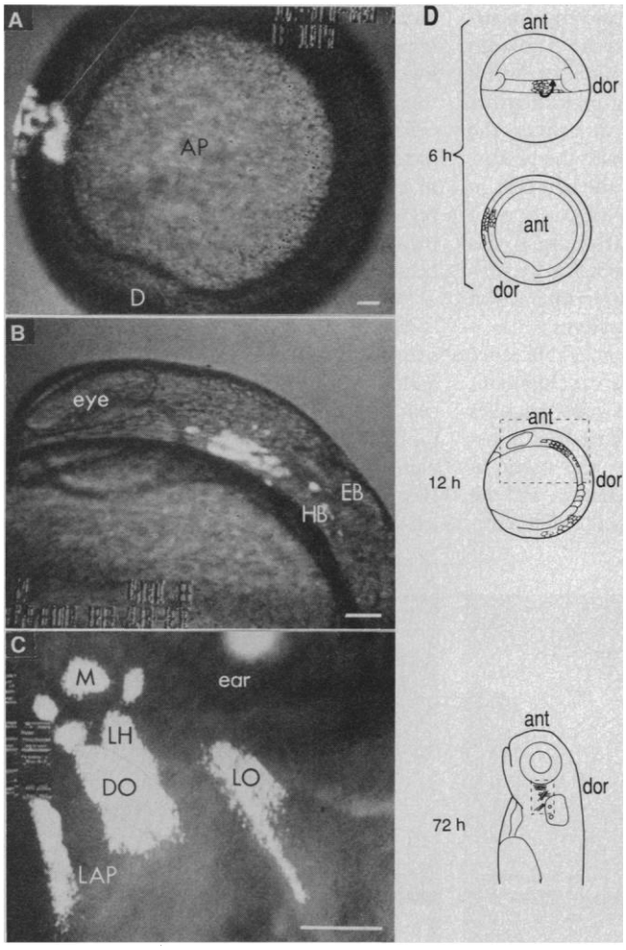

Fig. 2. Jaw muscles originate in paraxial mesoderm. Cells labeled with rhodamine-dextran and giving rise to LAP and DO are shown at three successive stages in the same embryo, after injection of their precursor blastomere at the 1000-cell stage (3 h). Fluorescent and Nomarski images were combined by computer (*16*). (**A**) Marginal cells in the gastrula (6 h), 3 hours after the cell was injected. The view is from the animal pole (AP) and shows labeled cells in the lateral germ ring (at the circumference of the gastrula in this view), which is formed by a layer of deep, involuted cells. The outermost cells are enveloping layer (EVL) cells, which do not involute but differentiate into periderm. Here we focus only on the deep cells, the innermost group of which gives rise to the mesoderm. The germ ring is thickened at the dorsal midline, marked D. (**B**) Paraxial mesoderm labeled on the left side of the developing head (12 h). At this stage, a visible boundary separates the hypoblast (HB, prospective mesoderm and endoderm) and epiblast (EB, prospective ectoderm) (*16*). The labeled cells lie within the hypoblast and caudal to the eye. (**C**) Differentiated muscle fibers in LAP and DO (72 h). In this experiment, the microinjected mesodermal cell also contributed to the levator hyomandibulae (LH) and levator operculi (LO) muscles located more caudally, and to some dorsal mesenchyme (M). Bars represent 50 μm. (**D**) Summary diagram of the same animal shown in (A) through (C). Lateral and animal pole views at 6 h show involution movements (arrow) of mesodermal precursors. Future anterior (ant) and dorsal (dor) sides are already established. The precursors converged into the head mesoderm by 12 h, separating from the more posterior EVL cells, and differentiated as head muscles at 72 h.

cells and to examine the details of their interactions with the motoneurons. The hypothesis we have proposed here also may be tested more directly by mutational analysis or molecular genetic manipulation (*22*) to alter Eng expression during development and by searching for other specific proteins and mRNAs that may be involved in the control of muscle identity.

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8. An *engrailed*-type homeobox gene in zebrafish was cloned that corresponds to the murine *En-2* gene [A. Fjose, H. G. Eiken, P. R. Njølstad, A. Molven, I. Hordvik, *FEBS Lett.* **231**, 355 (1988); P. R. Njølstad and A. Fjose, *Biochem. Biophys. Res. Commun.* **157**, 426 (1988)]. Other cells in zebrafish express Eng earlier (*5*) and during the same stages we describe here. A detailed description of the 4D9

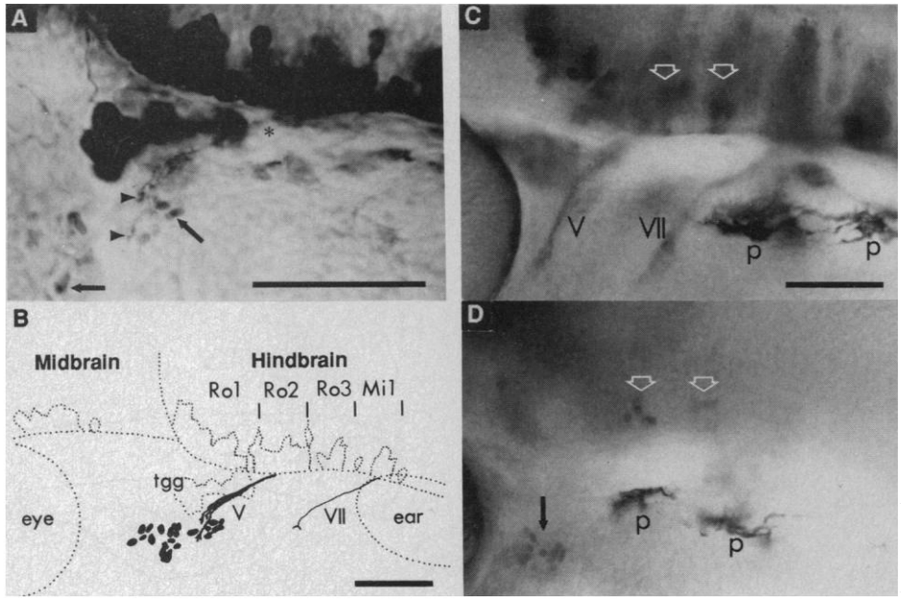

Fig. 3. The growth cones of trigeminal motor axons specifically associate with Eng-expressing mesenchyme. (**A**) Double-staining of a whole-mounted embryo at 28 h, with 4D9 and zn-1, in left-side view. The monoclonal antibody zn-1 recognizes a cytoplasmic antigen in most early developing neurons (*24, 25*). Some growth cones (arrowheads) are near to and in the same plane of focus as Eng-expressing mesenchyme (filled arrows). The trigeminal nerve can be traced deeper into the tissue, to its origin in the hindbrain, as shown here in computer-combined images from seven different focal planes. The nerve (asterisk) originates from the second hindbrain segment and passes through the caudal part of the trigeminal sensory ganglion, which is visible as a cluster of large zn-1–positive cells just above the cell nuclei expressing Eng. Segmental clusters of hindbrain cells are also labeled with zn-1 (*25*). (**B**) Camera lucida drawing of the same preparation shown in (A). The hindbrain segments are named as in (*25*). (**C**) Whole-mounted embryo (36 h) labeled with zn-1, left-side view. The open arrows are centered on the Ro2 and Ro3 segments. (**D**) Whole-mounted embryo (36 h) labeled with 4D9, left-side view, at the same magnification and orientation as (C). Filled arrow indicates the labeled jaw muscle precursors in the periphery, located at the same position where the trigeminal axons terminate in (C). The open arrows show clusters of brain cells in the Ro2 and Ro3 segments that are labeled by 4D9. The clusters are present exclusively in the first three hindbrain segments and are different from the Eng-positive cells previously described (*5*) in the prominent stripe at the hindbrain-midbrain boundary. For double staining, 4D9 and zn-1 were used together as primary antibodies. The photographs were taken with Nomarski optics. (V, trigeminal motor axons; VII, facial motor axons; p, pigment cells; tgg, trigeminal ganglion. Bars represent 50 μm.)

labeling pattern in zebrafish is in preparation (K. Hatta *et al.*, in preparation). Recent molecular studies have shown that there are two *engrailed* homeobox genes in zebrafish (M. Westerfield, personal communication). It is likely that 4D9 reacts with proteins encoded by both of them, and whether one or both of these genes is expressed in the jaw muscles is currently unknown.
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12. This labeling pattern is similar to that previously described for the cranial mesenchyme of mouse (*En-1*) (*10*) and chicken (*6*) embryos, although the eventual fates of those cells were not characterized.
13. The two muscles cooperate to enlarge the buccal cavity and to cause the mouth to protrude (the mechanical advantage of DO is increased by LAP action; DO also enlarges the opercular cavity [R. Chiasson, *Laboratory Anatomy of the Perch* (Brown, Dubuque, IA, ed. 3, 1980)].
14. Sometimes we saw a faintly stained mesenchymal aggregate just proximal to the LAP and DO precursors. We did not further examine the origin and fate of these cells, but, because they appear similar to the LAP and DO precursor cells, it is possible that their fate is to form jaw muscles proximal to LAP and DO. Expression disappears by 72 h.
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17. In the chicken, mesenchyme derived from both cranial neural crest and mesoderm appears to express Eng [(*6*); C. A. Gardner and K. F. Barald, personal communication].
18. A similar association, between trigeminal axons and a patch of mesenchyme that accumulates laminin, has been reported in the chicken, where the cells were thought to be muscle precursors [S. A. Moody *et al.*, *J. Comp. Neurol.* **283**, 38 (1989)].
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cdc2 Gene Expression at the G_1 to S Transition in Human T Lymphocytes

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The product of the *cdc2* gene, designated $p34^{cdc2}$, is a serine-threonine protein kinase that controls entry of eukaryotic cells into mitosis. Freshly isolated human T lymphocytes (G_0 phase) were found to have very low amounts of $p34^{cdc2}$ and *cdc2* messenger RNA. Expression of *cdc2* increased 18 to 24 hours after exposure of T cells to phytohemagglutinin, coincident with the G_1 to S transition. Antisense oligodeoxynucleotides could reduce the increase in *cdc2* expression and inhibited DNA synthesis, but had no effect on several early and mid-G_1 events, including blastogenesis and expression of interleukin-2 receptors, transferrin receptors, c-*myb*, and c-*myc*. Induction of *cdc2* required prior induction of c-*myb* and c-*myc*. These results suggest that *cdc2* induction is part of an orderly sequence of events that occurs at the G_1 to S transition in T cells.

THE SERINE-THREONINE PROTEIN kinase $p34^{cdc2}$ plays a key role in the regulation of the eukaryotic cell cycle. Originally identified as the product of the *cdc2* gene of *Schizosaccharomyces pombe* and the *CDC28* gene of *Saccharomyces cerevisiae*, $p34^{cdc2}$ is now known to be the catalytic subunit of the mitosis-regulating protein kinase complex known as maturation promoting factor (MPF) or growth-associated H1 kinase (*1*). In growing HeLa cells, the amount of $p34^{cdc2}$ remains constant throughout the cell cycle, but $p34^{cdc2}$ H1 kinase activity increases dramatically as cells progress from G_1 to mitosis (*2*). The regulation of $p34^{cdc2}$ function is incompletely understood but is believed to be modulated by associated proteins, such as $p13^{suc1}$ and cyclins. Also, the phosphorylation of $p34^{cdc2}$ fluctuates as a function of the cell cycle (*2–4*) and full activation of $p34^{cdc2}$ in mitotic metaphase requires that residues in the adenosine triphosphate (ATP) binding site be dephosphorylated (*2, 5*).

In fission yeast, *cdc2* is required both at the G_2 to M (G_2-M) transition and in G_1 at "Start" (*6*). The requirement, if any, for $p34^{cdc2}$ in the G_1 phase of the cell cycle in higher eukaryotes has been difficult to assess, partly because of the lack of suitable cell cycle control mutants, although a potential role for $p34^{cdc2}$ at the G_1-S transition has been postulated (*7*). Microinjection of antibodies to $p34^{cdc2}$ into serum-stimulated rat fibroblasts caused cells to arrest in G_2 but had no effect on DNA replication (*8*). However, the function of $p34^{cdc2}$ may be different in G_1 than G_2, and $p34^{cdc2}$ could be less susceptible to antibody neutralization in G_1 and S because of a difference in intracellular localization or associated proteins.

Early events in cell cycle control have been studied in hematopoietic cells because these cells spontaneously arrest in G_0 during the process of differentiation. T and B lymphocytes can be induced to reenter the cell cycle in response to specific antigen or mitogenic lectins, whereas myeloid cells are terminally differentiated and remain in G_0-G_1 (*9*). To investigate the role of $p34^{cdc2}$ in G_1 cell cycle control in human cells, we have examined the expression of *cdc2* in T cells after lectin stimulation and have used antisense oligodeoxynucleotides to modulate *cdc2* expression.

The DNA content of freshly isolated blood T cells was analyzed by flow cytometry after staining with propidium iodide. As described previously (*10*), all T cells were found to be in G_0, but in response to the mitogenic lectin phytohemagglutinin (PHA) they could be synchronously induced to undergo blastogenesis (the morphological changes of the G_0-G_1 transition) (after 8 to 12 hours), begin DNA synthesis (after 18 to 24 hours), and initiate mitosis (after 24 to 48 hours). The expression of *cdc2* mRNA (*11*) was evaluated by Northern blotting (Fig. 1A) and reverse PCR (polymerase chain reaction) (Fig. 1B) in T cells for up to 96 hours after stimulation. Before PHA stimulation, *cdc2* mRNA was undetectable, and it first became detectable at 15 to 18 hours by reverse PCR and at ~24 hours by Northern blot. The expression of $p34^{cdc2}$ was simultaneously evaluated by immunoblotting with a monospecific polyclonal antibody to a human $p34^{cdc2}$ fragment (*12*).

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