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Integrin α subunit mRNAs are differentially expressed in early *Xenopus* embryos

Charles A. Whittaker and Douglas W. DeSimone*

Department of Anatomy and Cell Biology and the Molecular Biology Institute, University of Virginia, Box 439, Health Sciences Center, Charlottesville, VA 22908, USA

*Author for correspondence

SUMMARY

Adhesion of cells to extracellular matrix proteins is mediated, in large part, by transmembrane receptors of the integrin family. The identification of specific integrins expressed in early embryos is an important first step to understanding the roles of these receptors in developmental processes. We have used polymerase chain reaction methods and degenerate oligodeoxynucleotide primers to identify and clone *Xenopus* integrin α subunits from neurula-stage (stage 17) cDNA. Partial cDNAs encoding integrin subunits α_2, α_3, α_4, α_5, α_6 and an α_{IIb}-related subunit were cloned and used to investigate integrin mRNA expression in early embryos by RNase protection assay and whole-mount in situ hybridization methods. Considerable integrin diversity is apparent early in development with integrins α_2, α_3, α_4, α_5 and α_6 each expressed by the end of gastrulation. Both α_3 and α_5 are expressed as maternal mRNAs. Zygotic expression of α_2, α_3, α_4 and α_6 transcripts begins during gastrulation. Integrin α_5 is expressed at relatively high levels during cleavage, blastula and gastrula stages suggesting that it may represent the major integrin expressed in the early embryo. We demonstrated previously that integrin β_1 protein synthesis remains constant following induction of stage 8 animal cap cells with activin (Smith, J. C., Symes, K., Hynes, R. O. and DeSimone, D. W. (1990) *Development* 108, 289-298.). Here we report that integrin α_3, α_4 and α_6 mRNA levels increase following induction with 10 U/ml activin-A whereas α_5, β_1 and β_3 mRNA levels remain unchanged.

Whole-mount in situ hybridization reveals that α_3 mRNAs are expressed by cells of the involuting mesoderm in the dorsal lip region of early gastrulae. As gastrulation proceeds, α_3 expression is localized to a stripe of presumptive notochordal cells along the dorsal midline. In neurulae, α_3 mRNA is highly expressed in the notochord but becomes progressively more restricted to the caudalmost portion of this tissue as development proceeds from tailbud to tadpole stages. In addition, α_3 is expressed in the forebrain region of later stage embryos. These data suggest that integrin-mediated adhesion may be involved in the process of mesoderm involution at gastrulation and the organization of tissues during embryogenesis.

Key words: integrins, *Xenopus* embryo, amphibian embryo, gastrulation, neurulation, *Xenopus laevis*

INTRODUCTION

Cell adhesion and migration play important roles in the morphogenetic processes that help shape early embryos. Recent advances have led to the identification of multiple adhesive systems and families of adhesive molecules believed to mediate these processes (Hynes and Lander, 1992). The integrins are now recognized as an example of one such family made up of a large number of structurally related transmembrane receptors that participate in a variety of cell-cell and cell-extracellular matrix (ECM) interactions (for reviews see: Albelda and Buck, 1990; Ruoslahti, 1991; Hynes, 1992).

All integrins are heterodimers composed of structurally distinct α and β subunits. At least 8 different β subunits and 14 α subunits have been identified thus far. Although not all possible combinations are believed to occur, more than 20 distinct receptors have been reported (Hynes, 1992). Many of these receptors recognize the same ligand. For example, in the β_1 integrin subfamily alone there are at least four reported fibronectin receptors. Additional fibronectin receptor activity has also been attributed to the β_3 integrins and the $\alpha_V\beta_6$ and $\alpha_4\beta_7$ receptors. One possibility is that this high degree of integrin structural diversity and functional redundancy evolved to mediate the complex adhesive interactions involved in supporting the morphogenetic events that characterize embryonic development. Clearly, an important first step in our understanding of integrin function in the embryo is to identify those receptors expressed throughout development.

Primary attention has focussed on the expression and function of β_1 integrins in vertebrate embryos, in part

because this family includes receptors for a number of developmentally significant ECM proteins. Immunofluorescent localization studies reveal a near ubiquitous pattern of expression for β_1 integrins in chick (Krotoski et al., 1986; Duband et al., 1986) and amphibian embryos (Darribère et al., 1988; Smith et al., 1990; Gawantka et al., 1992; Howard et al., 1992). Attempts to determine the patterns of expression for specific α subunits during development have been limited by a lack of suitable reagents needed to undertake these investigations. Studies done in chick, however, suggest that $\alpha_5\beta_1$ is the predominant β_1 integrin expressed by day 4 of embryogenesis with levels of this receptor decreasing dramatically in late-stage embryos (Muschler and Horwitz, 1991). Bronner-Fraser et al. (1992) report that α_6 has a more limited distribution in the embryo and is expressed predominantly on neural and muscle tissues.

A number of studies address the importance of integrins in supporting embryonic cell migration and morphogenesis. In chick embryos, antibodies directed against β_1 integrins will perturb the migration of neural crest cells (Bronner-Fraser, 1986) and myoblasts (Jaffredo et al., 1988) and disrupt somite organization (Drake and Little, 1991). Similar reagents have been used to inhibit gastrulation in urodele (Darribère et al., 1988) and *Xenopus* embryos (Howard et al., 1992). Darribère et al. (1990) further showed that gastrulation and fibronectin matrix assembly can be perturbed by cytoplasmic injection of antibodies directed against the C terminus of the β_1 subunit. All of these studies demonstrate the overall importance of β_1 function but do not distinguish among the specific receptor types likely to be responsible (e.g., $\alpha_{1\text{-}8}\beta_1$). Integrins also play a number of important roles during *Drosophila* development (for review see Hortsch and Goodman, 1991). Originally identified as position-specific (PS) antigens by Wilcox and colleagues (1981, 1984), *Drosophila* integrins display dynamic patterns of expression in imaginal discs (Wilcox et al., 1981) and elsewhere in the embryo (e.g., Bogaert et al., 1987). One β (β_{PS3}) and two α (α_{PS1} and α_{PS2}) subunits have thus far been reported for *Drosophila*. The β_{PS3} subunit was demonstrated to be the product of the lethal myospheroid gene (MacKrell et al., 1988; Leptin et al., 1989). Studies also have shown the involvement of PS integrins in a variety of additional processes that include wing and eye development (Brower and Jaffe, 1989; Zusman et al., 1990) and sarcomere formation (Volk et al., 1990).

The most complete information concerning integrin structure and function comes from work done with human integrins, for which a large number of antibodies and cDNAs are now available. The majority of these reagents, however, are unsuitable for the study of integrin expression in non-human embryos and tissues. Previously, we reported the cDNA cloning of the integrin β_1 subunit from *Xenopus laevis* (DeSimone and Hynes, 1988). Although it is clear that β_1 integrins are expressed in *Xenopus* eggs and early embryos, very little is known about the α subunits that associate with β_1 during these times in development (Smith et al., 1990; DeSimone et al., 1991; Gawantka et al., 1992; Howard et al., 1992). The situation is likely to be complicated further by the recent identification of additional β subunits expressed in *Xenopus* embryos (Ransom, Hens and DeSimone, unpublished data). Furthermore, limited data are available concerning the expression and distribution of specific integrin receptors (i.e., αβ heterodimers) during embryogenesis in any vertebrate system. In the current study, we report the use of degenerate oligodeoxynucleotide primers and PCR methods to identify and clone several integrin α subunit cDNAs from *Xenopus*. These studies confirm that considerable diversity of integrin α subunit expression is present even at the earliest stages of embryonic development. At least three of these α subunits may be functioning as fibronectin receptors at gastrulation, consistent with the putative role of this extracellular matrix protein in supporting morphogenesis (Lee et al., 1984).

MATERIALS AND METHODS

Embryos

Adult wild-type and albino *Xenopus laevis* were obtained from Xenopus 1 (Ann Arbor, MI). Eggs were fertilized artificially (Newport and Kirschner, 1982), cultured in 0.1× modified Barths saline (MBS; Gurdon, 1977) and staged according to Nieuwkoop and Faber (1967).

Amplification of integrin α subunits by polymerase chain reaction

DNA template was purified from a *Xenopus* λgt10 neurula-stage (stage 17) cDNA library (Kintner and Melton, 1987) by CsCl step gradient centrifugation (Sambrook et al., 1989). PCR amplification was carried out using the following forward (A14F) and reverse (A2AR), degenerate oligodeoxynucleotide primers: A14F (5′-CGGAATTCGGIGA(G,A)CAG(A,C)TIG(C,G)I(G,T)CITA(C,T)TT(C,T)GG-3′) and A2AR (5′-CTCGTCGACG-GIGCI(C,G)CIA(C,T)IGCIA(C,T)(A,G)TCITI(A,G)(A,T)AICC(A,G)TC-3′). The sequence of these primers is identical to those described by Erle et al. (1991) with the exception of an *Acc*I site engineered onto the 5′ end of the A2AR primer. The amplification reaction consisted of 10 μg template DNA, 1 μg each of the forward and reverse primers, 2.5 units of Amplitaq (Cetus-Perkin-Elmer), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% (w/v) gelatin and 2.5 mM dNTPs. This reaction mix was subjected to 40 cycles of amplification (94˚C for 1 minute, 48˚C for 2 minutes, 72˚C for 3 minutes), followed by a final extension step of 7 minutes at 72˚C. Amplified cDNAs were subcloned into *Sma*I cut pBluescript S/K⁻(Stratagene) using standard techniques (Sambrook et al., 1989). Cloned amplification products were characterized by DNA sequencing on both strands using the dideoxy chain termination method (Sanger et al., 1977).

Synthesis of probes for RNAse protection assays

[α-^{32}P]rUTP-labelled probes were synthesized in vitro using appropriate RNA polymerases as described in DeSimone et al. (1992). Templates for the synthesis of antisense integrin α subunit transcripts were prepared by linearizing appropriate plasmids with *Eco*RI. Antisense transcripts prepared from the α_2, α_4, α_5, α_6 and α_{IIb} partial cDNAs are approximately 350 nucleotides (nt) in length, including 76 nt of plasmid sequence. Integrin α_3 template was prepared by digesting clone FF2E5 with *Xba*I to yield antisense transcripts of approximately 546 nt in length. FF2E5 is a subclone of a near full-length cDNA encoding *Xenopus* α_3 that was obtained previously by our laboratory and will be described elsewhere (Meng, Whittaker, Hens, Ransom and DeSimone, unpublished data). EF1-α template was prepared by digesting with *Alu*I, a *Pst*I-*Sac*I fragment of *Xenopus* EF1-α cloned into pSP65 (Krieg et al., 1989) to produce a transcript of approximately 90

nt in length. *Xenopus* brachyury template was prepared by linearizing with *Eco*RI, a *Bam*HI dropout subclone of pXbra (Smith et al., 1991) to yield a transcript of approximately 440 nt in length. Antisense integrin β_1 transcripts 580 nt in length were synthesized using *Eco*RI linearized pE1AH as template (DeSimone et al., 1991). The 530 nt antisense *Xenopus* integrin β_3 probe was synthesized from *Eco*RI linearized pXB3H1ΔH (Ransom, Hens and DeSimone, unpublished data).

RNA isolation and RNase protection assays

Total RNA was isolated from embryos exactly as described in DeSimone et al. (1992). Total RNA was extracted from *Xenopus* XTC cells (gift of Dr Igor Dawid, NIH) using the method of Chirgwin et al. (1979). 10 embryo equivalents of total RNA or 10 μg of XTC total RNA (except for α_4 assays where 40 μg was used) were hybridized to 50,000 or 100,000 cts/minute of purified probe per assay. Routinely, each hybridization also included 50,000 cts/minute of the EF1-α probe as an internal control for RNA levels. RNase protection assays were carried out as described previously (DeSimone et al., 1992).

For induction assays, animal caps were dissected from stage 8 embryos in 1× MBS. Uninduced animal caps were cultured in 1× MBS, 1% BSA (Sigma), and 10 μg/ml gentamycin until control embryos reached mid-gastrula (stage 11). Induced animal caps were cultured in the same way except for the addition of 10 units/ml activin-A (gift of Dr Jim Smith, NIMR, Mill Hill, London). Total RNA was then prepared from induced and uninduced explants. RNase protection assays were carried out as above with 20 μg of total RNA (35 μg used for α_4 assays).

Whole-mount in situ hybridizations

Whole-mount in situ hybridization was carried out using albino embryos as described by Harland (1991). Digoxigenin-rUTP-labelled (Boehringer Mannheim Biochemical) integrin α_3 probes were synthesized from *Eco*RI (antisense) or *Kpn*I (sense) linearized FF2ΔE (Meng, Whittaker, Hens, Ransom and DeSimone, unpublished data) cDNA templates. Embryos were photographed on a Zeiss Axiophot using bright-field and differential interference contrast optics.

RESULTS

Cloning of integrin α subunit cDNAs by homology PCR

Evidence suggests that integrins are likely to play important roles during early amphibian development (Darribère et al., 1988; 1990; Smith et al., 1990; Gawantka et al., 1992; Howard et al., 1992) but little is known about the identity of the specific αβ heterodimers involved. In order to identify integrin α subunits expressed in *Xenopus* embryos, we used degenerate oligodeoxynucleotides to amplify partial cDNAs by homology PCR. The nucleotide consensus sequences for these primers were derived from two highly conserved regions present in all known α subunits as identified by Erle et al. (1991). The location of the forward and reverse primers used in the PCR amplification are indicated in Fig. 1.

The major amplification product obtained from the stage 17 cDNA template was approximately 315 bp, which is within the size range predicted from published integrin α subunit sequences. Subcloning of the amplification product resulted in the characterization of 100 plasmids containing inserts. Of these, 30 were shown to encode putative integrin α subunits based on amino acid comparisons to known integrins (Fig. 2, Table 1). These sequences fall into six distinct categories, each of which shares a number of highly

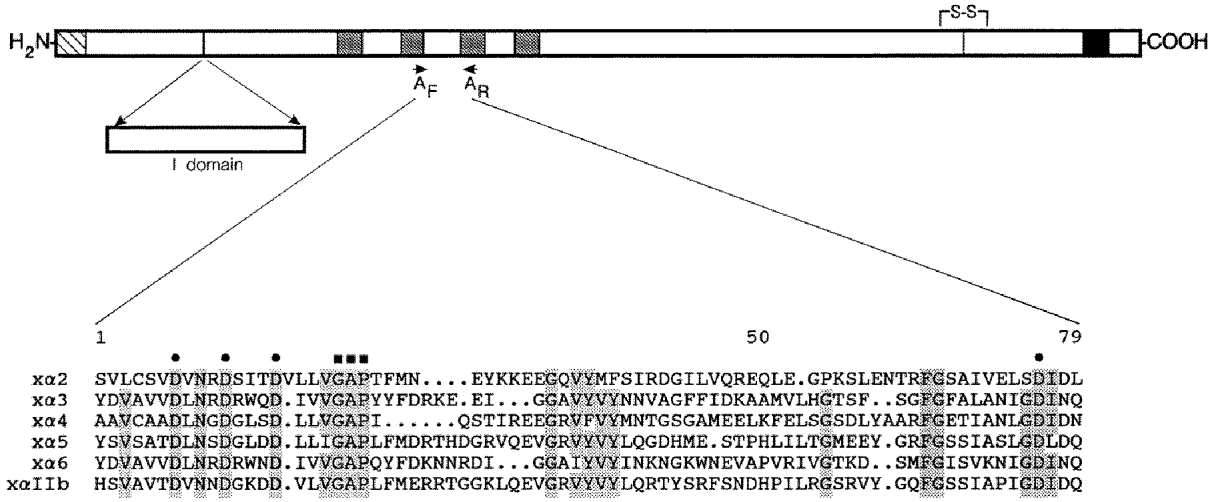


Fig. 1. Deduced amino acid sequences of *Xenopus* integrin α subunit partial cDNAs. Schematic illustrates the general structural features of a typical integrin α subunit. I domain box indicates the location of a 180 amino acid homologous segment present in the extracellular domains of some α subunits. Transmembrane domain (filled box), location of disulfide-bonded postranslational cleavage site present in some α subunits and the N-terminal signal sequence (hatched box) are also indicated. The degenerate oligodeoxynucleotide primers (see Materials and methods and Erle et al., 1991) used in the PCR amplification (A_F and A_R) are located in the highly conserved region of the α subunit cation-binding domains (shaded boxes). Small arrows delineate the amplified regions obtained by PCR of *Xenopus* neurula-stage cDNA. Deduced amino acid sequences of amplified regions (minus 5′ and 3′ oligo sequences) were aligned using the PILEUP program of the GCG sequence analysis software package (Devereux et al., 1984). Assignments of α subunit identities are based on pairwise comparisons with all known, published integrin α subunits sequences (see Fig. 2 and Table 1). Only 5- and 6-fold amino acid identities are highlighted. ●, conserved Asp residues of cation binding motifs; ■, location of Gly-Ala-Pro sequence conserved in all integrin α subunits.

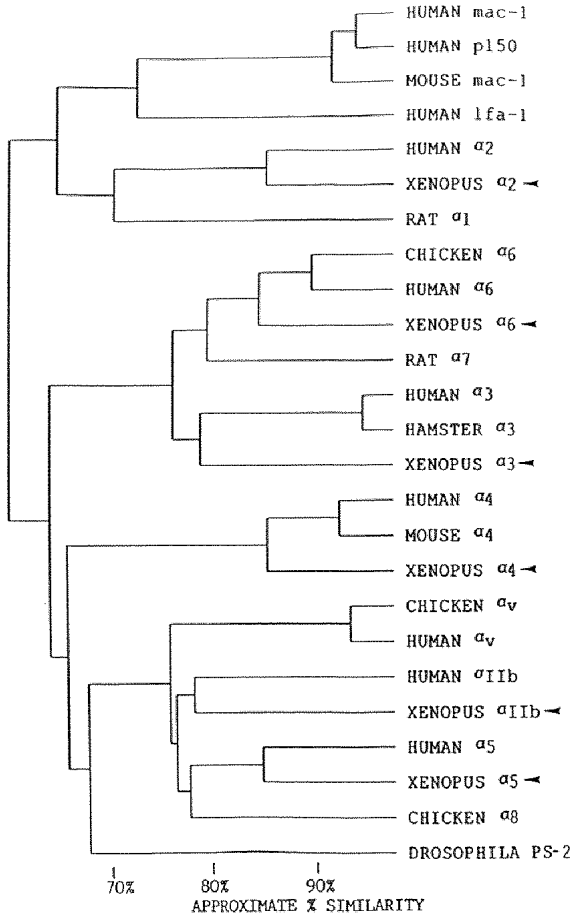


Fig. 2. Dendrogram of pairwise comparisons of *Xenopus* α subunit cDNAs with published integrin α subunit sequences. Deduced amino acid sequences of PCR-amplified *Xenopus* partial cDNAs were compared to the corresponding regions of published integrin α subunits using the PILEUP program of the GCG sequence analysis software package (Devereux et al., 1984). Progressive pairwise alignments result in the clustering of the most highly related pairs of sequences. Distance along horizontal axis is proportional to differences between clustered sequences and provides an approximate measure of amino acid similarity. Distance along the vertical axis has no significance (i.e., placement of clusters from top to bottom is in no particular order). The assignment of *Xenopus* α subunit identity is based on best % similarity score with known sequences (Table 1). The putative *Xenopus* α_2, α_3, α_4, α_5, α_6 and α_{IIb} subunits are indicated by the arrowheads. Sequence data for other α subunits: rat α_1 (Ignatius et al., 1990); human α_2 (Takada and Hemler, 1989); human α_3 (Tsuji et al., 1991; Takada et al., 1991); hamster α_3 (Tsuji et al., 1990); human α_4 (Takada et al., 1989); mouse α_4 (Neuhaus et al., 1991); human α_5 (Argraves et al., 1987); human α_6 (Tamura et al., 1990); chicken α_6, (deCurtis et al., 1991); rat α_7 (Song et al., 1992); chicken α_8 (Bossy et al., 1991); human mac-1 (Arnaout et al., 1988); mouse mac-1 (Pytela, 1988); human p150 (Corbi et al., 1990); human lfa-1 (Larson et al., 1989); human α_V (Suzuki et al., 1987); chicken α_V (Bossy and Reichardt, 1990); human α_{IIb} (Poncz et al., 1987); Drosophila PS-2 (Bogaert et al., 1987).

conserved elements including portions of 2 divalent cation-binding motifs and a 'Gly-Ala-Pro' (G-A-P) sequence conserved in all integrin α subunits. The amino acid sequences listed in Fig. 1 do not contain the flanking N- and C-terminal regions derived from the degenerate oligodeoxynucleotide primers because they are likely to differ from the actual *Xenopus* sequences.

Table 1. Percent similarity and identity of human and *Xenopus* α subunits

Subunit	% Similarity	% Identity
HUMAN α_2 : *Xenopus* α_2	84	68
HUMAN α_3 : *Xenopus* α_3	79	59
HUMAN α_4 : *Xenopus* α_4	86	76
HUMAN α_5 : *Xenopus* α_5	83	72
HUMAN α_6 : *Xenopus* α_6	89	71
HUMAN α_{IIb} : *Xenopus* α_{IIb}	74	58

The deduced amino acid sequences of each *Xenopus* cDNA were compared to all available integrin α subunits using the BESTFIT, LINEUP and PILEUP programs of the Genetics Computer Group (GCG) sequence analysis software package (Devereux, 1984). The dendrogram presented in Fig. 2 illustrates the relationship of each *Xenopus* partial amino acid sequence with the corresponding regions of various human, mouse, rat, hamster, chicken and *Drosophila* α subunit sequences. The *Xenopus* sequences fall between the range of 58-76% identity (74-89% similarity) with the human α_2, α_3, α_4, α_5, α_6 and α_{IIb} subunits (Table 1). We have assigned integrin subunit names to our partial cDNAs based on these identity scores.

Integrin α subunit mRNA expression during development

The results of the amplification experiments indicate that multiple integrins are expressed in neurula-stage embryos. To confirm and extend this observation, we investigated the temporal pattern of integrin expression throughout development using RNase protection assays. Each α subunit is expressed by the end of gastrulation in *Xenopus* (Fig. 3) with the exception of the putative α_{IIb} subunit, which is first detected at very low levels in neurulae. At this level of analysis, it appears that the expression of most α subunit mRNAs increases as development proceeds with the possible exception of α_5, which remains at relatively high but constant levels from egg through tadpole stages. Maternal α_3 transcripts are also detected in eggs and early blastulae but the levels of expression are considerably lower than that observed for the α_5 subunit.

Because of the possible importance of integrins to the process of gastrulation in *Xenopus*, we have analyzed the precise time course of integrin mRNA expression from the onset of zygotic transcription at the mid-blastula transition (MBT: Newport and Kirschner, 1982) through gastrulation (Fig. 4). RNase protection assays were undertaken with total RNAs from blastula (stage 8 and 9), early (stage 10), mid (stage 11) and late (stage 12) gastrula and early neural plate (stage 13)-stage embryos (staged according to Nieuwkoop and Faber, 1967; see also Keller, 1991). Both α_5 and β_1 mRNAs are expressed at similar levels throughout gastrulation with no obvious increase in expression noted during this period. Although α_3 mRNAs are also maternal, expression increases at stage 10 as a result of zygotic transcription. The α_4 and α_6 transcripts are first detected by stage 10 with α_2 expression apparent by stage 11. Zygotic expression of β_3 mRNA begins at the early

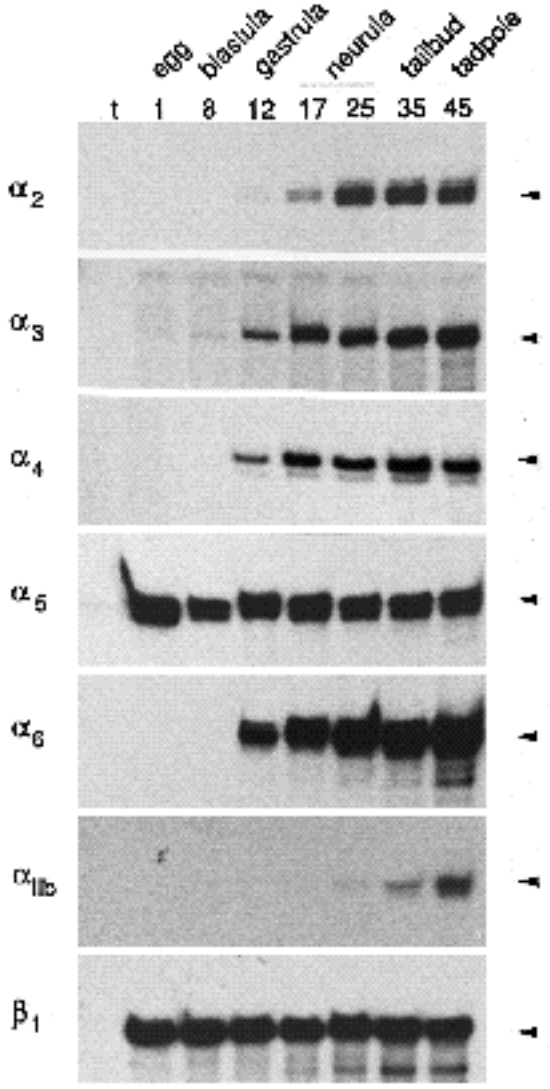


Fig. 3. Developmental time course of α subunit mRNA expression determined by RNase protection analysis.10 embryo equivalents of total RNA per lane were hybridized with ^{32}P-labelled antisense transcripts followed by digestion with RNase. Protected fragments are indicated by the arrowheads. The larger sized undigested probes run with each set of reactions are not shown. RNase protections of integrin β_1 and EF1-α (not shown) were included as controls for RNA loading at each stage. The α_3, α_5 and β_1 subunits are present in egg and cleavage-stage embryos as maternal mRNAs. Accumulation of α_2, α_4 and α_6 subunit mRNAs is first noted during gastrulation with α_{IIb} appearing during neurulation. The α_2, α_3, α_4, α_5, α_6 and β_1 autoradiograms were exposed for 2 days and the α_{IIb} for 11 days. Embryos staged according to Nieuwkoop and Faber, 1967. Approximate size of protected fragments indicated by arrowheads for α_2, α_4, α_5, α_6, and α_{IIb} is 245 nt. The α_3 protected fragment is about 460 nt and β_1 is 531 nt. t, tRNA control lane.

neural plate-stage (stage 13) but maternal transcripts for this subunit persist throughout gastrulation.

We were also interested in establishing which α and β subunits are expressed in *Xenopus* XTC cells (Pudney et al., 1973) because of the potential usefulness of this cell line for cellular and biochemical analyses of *Xenopus* integrins (Howard et al., 1992). All of our α and β subunit probes protect appropriately sized fragments in the XTC cell RNase protection assays (Fig. 4X). Relatively low levels of β_3, α_2 and α_4 mRNAs are detected in these cells whereas α_3, α_5, α_6 and β_1 transcripts are expressed at significantly higher levels. The EF1-α panels shown in Fig. 4 are included as controls for RNA loading (Krieg et al., 1989). The 90 nt EFI-a probe was routinely included in each assay (Figs 3, 4, data not shown) to confirm the presence of RNA and to normalize comparisons between different embryonic stages.

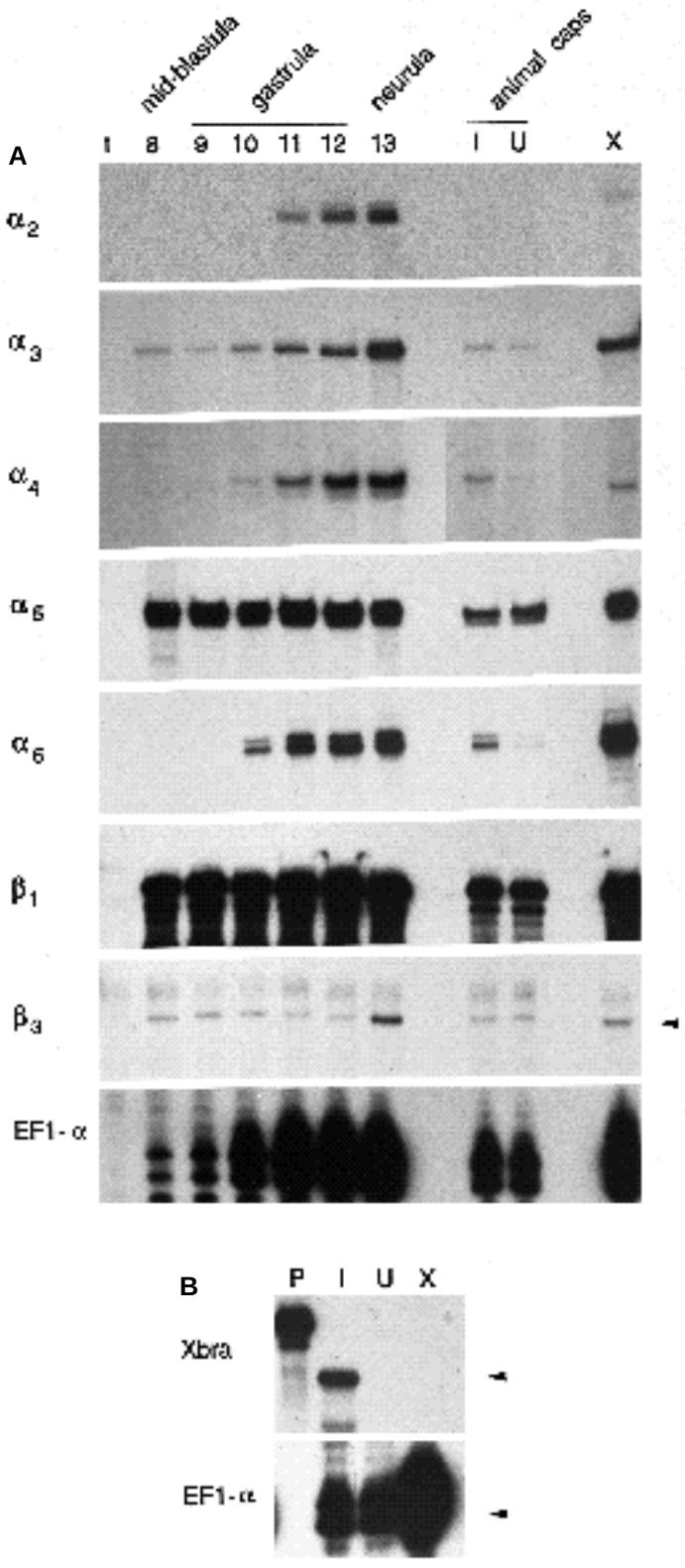
Integrin expression in response to induction with activin

In previous studies, we reported that *Xenopus* stage 8 animal cap cells rapidly adhere and spread on fibronectin-coated substrata when exposed to activin-A (Smith et al., 1990; DeSimone et al., 1991). This spreading can be inhibited in the presence of synthetic peptides containing the Arg-Gly-Asp sequence, thereby implicating integrins in this process. Metabolic and cell-surface-labelling studies indicate that little or no increase in β_1 integrin expression can be detected following activin induction (Smith et al., 1990) or during gastrulation (Howard et al., 1992; Gawantka et al., 1992). This leaves open the possibility that activin-A-induced changes in adhesive behavior may be caused by the expression of specific α subunits or other classes of β integrins. In order to test these possibilities, we have investigated integrin mRNA expression in response to induction with activin-A.

Animal cap tissue was isolated from stage 8 embryos and cultured in the presence of 10 Units/ml of activin-A. Under these conditions, both intact animal cap tissue and dispersed cells will adhere and spread on fibronectin (data not shown) as previously reported (Smith et al., 1990; DeSimone et al., 1991). Following exposure to activin-A, caps were cultured until the equivalent of early-mid gastrula stages (i.e., a mixture of stages 10.5-11) and total RNA isolated for use in RNase protection assays. Control caps were cultured in the absence of activin-A until the same developmental time point. Only caps exposed to activin-A displayed the gastrulation-like movements and morphology typical of induced tissues (Symes and Smith, 1987).

RNase protection assays demonstrate that the *Xenopus brachyury* gene *Xbra* was expressed in induced but not uninduced animal caps (Fig. 4B) as demonstrated previously by Smith et al. (1991). Fig. 4A (I,U) displays the results of typical integrin RNase protection assays utilizing the same batches of RNA used to obtain the *Xbra* results (Fig. 4B). The levels of both α_4 and α_6 subunit mRNAs are clearly increased in induced caps; however, 4-fold more RNA and longer exposure times were required in order to detect the α_4 transcripts. Similarly, a slight but reproducible increase in α_3 mRNA levels is noted under these conditions. Little or no change in the levels of expression for β_1, β_3, α_5 and EF1-α mRNAs are noted following induction with activin-A. No signal was detectable for α_2 mRNAs in animal caps in keeping with the observation that this mRNA is not expressed until late in gastrulation and then only at relatively low levels. Each assay included EF1-α antisense

probes to ensure that equivalent amounts of RNA were present in both the induced and uninduced samples. A representative example of the EF1-α pattern is included in Fig. 4A (taken from the α_5 assay) and in Fig. 4B for the *Xbra* assays.



Spatial expression of α_3 mRNAs

The RNase protection assays demonstrate that multiple integrin α subunit mRNAs are expressed in *Xenopus* embryos. One of our major interests is to elucidate spatial patterns of integrin expression during early development. Using the whole-mount in situ hybridization method of Harland (1991), we have investigated α_3 mRNA expression in gastrula, neurula and tailbud-stage embryos (Fig. 5).

A striking pattern of α_3 mRNA expression is noted in early *Xenopus* embryos. Initially, α_3 transcripts are localized to a group of individual cells at the dorsal side of the embryo in the region of the dorsal lip (Fig. 5A, arrow). When viewed from the side (Fig. 5B,D), these cells are identifiable as dorsal involuting mesoderm. The staining of this region is more intense in side-view photomicrographs because several layers of cells are imaged both above and below the plane of focus. The appearance of these stained cells coincides with the increase in α_3 mRNA synthesis noted at stages 10-11 (Fig. 4A). The anterior progression of the presumptive notochord is marked by the intense staining of α_3-positive cells (Fig. 5C,D). During neurulation, α_3 expression extends to the prechordal plate where relatively low levels of staining are detected (Fig. 5F). Faint staining of outer ectodermal cells is also observed at these stages.

Expression of α_3 mRNA decreases in the anterior portion of the notochord as development proceeds (Fig. 5G-J). By tailbud stages, α_3 mRNA localization in the notochord is limited to its most posterior aspect (Fig. 5H-J). Interestingly, α_3 transcripts are also localized anteriorly at the level of the prosencephalon in later stage embryos (Fig. 5H,I,K, arrowheads). In addition, low levels of α_3 mRNA are noted in the branchial arches (Fig. 5I,K).

Several control experiments were undertaken to confirm the specificity of the α_3 antisense probe. These include α_3 sense transcripts (Fig. 5L) and several other antisense probes that are expressed in distinctly different patterns (i.e., Xbra, β_1 and β_3 integrins; data not shown). Back-

Fig. 4. Integrin α subunit mRNA expression during gastrulation, mesoderm induction and in XTC cells. RNase protection analyses of total RNAs obtained from embryos, activin-A induced and uninduced animal caps, and the *Xenopus* XTC cell line. (A) Embryonic stages 8-13. 10 embryo equivalents of mRNA hybridized with ^{32}P-labelled antisense transcripts for *Xenopus* integrins α_2, α_3, α_4, α_5, α_6, β_1, β_3 and for EF1-α. Equal quantities (mass) of animal cap RNA (20 μg/lane, 35 μg in α_4 sample) were used in the activin-A-induced (I; 10 units/ml activin-A) and uninduced (U) samples. X, 10 μg total RNA was used for each XTC sample except for α_4 which contained 40 μg. EF1-α probe was included in each protection assay as a control to normalize for RNA loading (only 1 representative example of EF1-α, from the α_5 experiment, is shown). (B) Control for induction experiments shown in A. Brachyury is expressed in induced (I) but not in uninduced (U) animal caps. EF1-α mRNA levels are the same in both the induced and uninduced samples. No Xbra mRNA is detected in XTC cells (X). P, undigested Xbra probe. Sizes of protected fragments for α subunits and the β1 subunit are same as in Fig. 3. Approximate sizes of other protected fragments are indicated by arrowheads: β_3, 450 nt; Xbra, 410 nt; and EF1-α, 75 nt. Each assay was exposed to film for 48 hours with the exception of α_2 and α_4, which were exposed for 8 days.

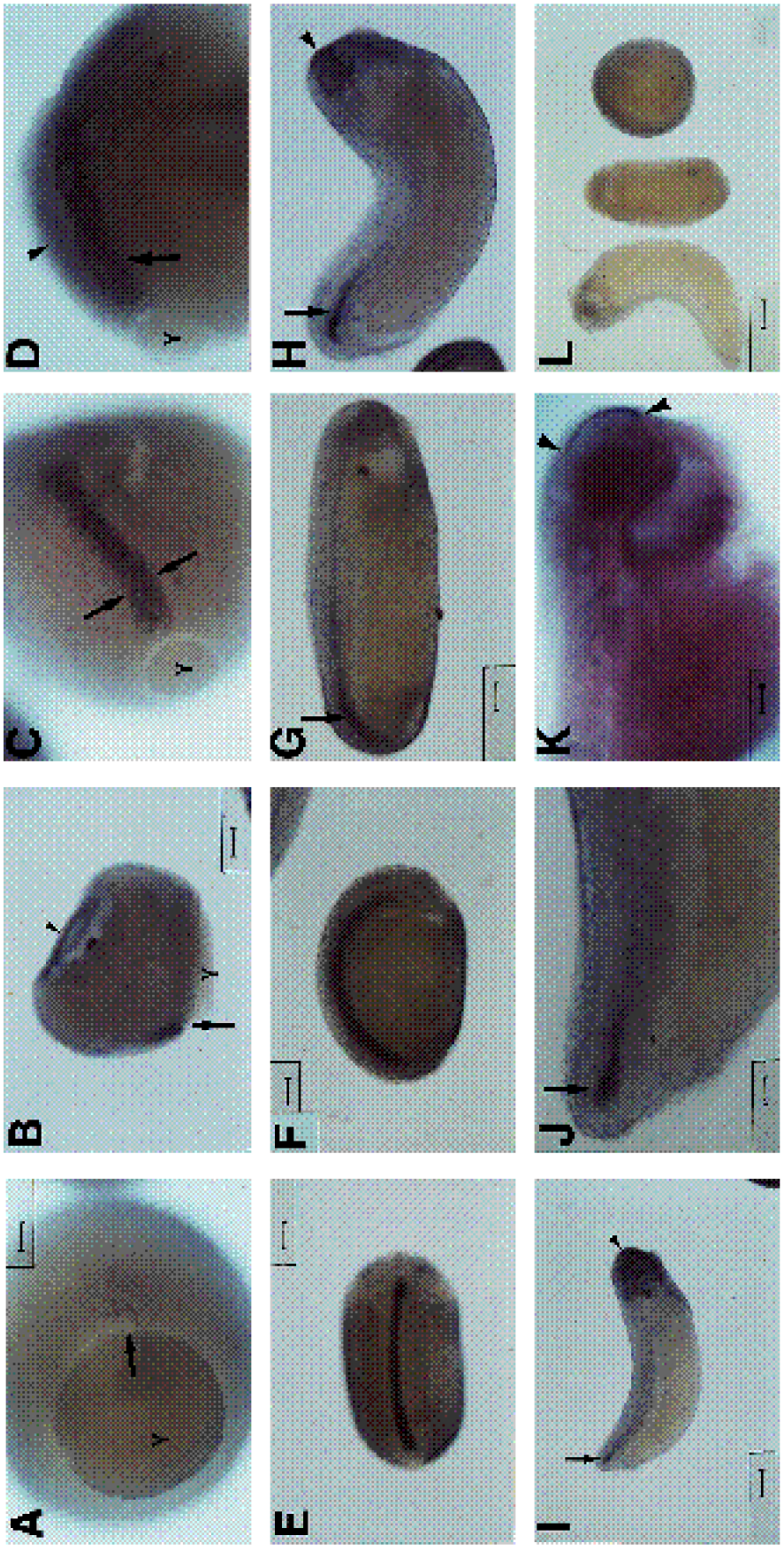


Fig. 5. Spatial expression of integrin α_3 mRNA. Whole-mount in situ hybridiztion was performed on albino *Xenopus* embryos using digoxigenin-labelled α_3 antisense (A-K) and sense (L) transcripts. Hybridization signal visualized as alkaline phosphatase chromogenic reaction product. Anterior end of embryos is oriented to the right in D-K. (A) Gastrula stage 10.5, vegetal pole view: staining of individual cells in dorsal lip region (arrow). (B) Stage 10.5, side view of dorsal involuting mesoderm (arrow). Arrowheads in B and D indicate faint background staining of outer ectodermal layer also observed with gastrula sense controls (L). Blastocoel roof in B is partially collapsed (arrowhead). (C) Dorsal and (D) side view of stage 12 embryo. Note intense staining of the presumptive notochord (arrows). (E) Dorsal and (F) side view of stage 20 embryo. Expression of α_3 observed along entire length of notochord. Anterior progression of staining extends to the prechordal plate (F). (G) Stage 24, side view. Expression of α_3 mRNA in the notochord (arrow) is decreased in the anterior portion. (H) Stage 28 and (I) stage 31 embryos, side views. Staining limited to caudal portion of notochord in the developing tailbud (I). Increased staining detected in the head with pronounced expression at the level of the forebrain (arrowheads). (J) Detail of a stage 31 tailbud. Note intense staining at the caudal tip of the notochord (arrow). (K) Stage 39, optical section of anterior end of embryo visualized by differential interference contrast microscopy. Arrowheads indicate borders of α_3 mRNA expression in the prosencephalon. (L) Representative tailbud, neurula and gastrula hybridized with α_3 sense transcript. Note faint background staining in ectoderm of gastrula and head of tailbud-stage embryo. Scale bar in A, same as in C,D,J,K, equals 100 μm. Scale bar in B, same as in E-H, equals 200 μm. Scale bars in I and L equal 400 μm. Y, yolk plug in A-D.

ground staining is noted in the blastocoel roof ectoderm and in the heads of tailbud and tadpole stages but this is easily distinguished from the more intense pattern of specific cellular staining obtained only with antisense probes. Our initial efforts with in situ hybridization focused on using PCR-generated partial cDNAs as templates for cRNA sense and antisense probes. In most cases, however, we have been unable to obtain adequate signals using these short (approximately 300 nt) transcripts. In contrast, the α_3 cRNA probes used in this study were generated from subclone FF2ΔE, which is a near full-length cDNA (approximately 4 kbp) obtained from a *Xenopus* stage 45 library (Meng, Whittaker, Hens, Ransom and DeSimone, unpublished data). Nucleotide sequence confirms that the PCR-generated α_3 cDNA (Fig. 1) is identical to a segment contained within FF2ΔE (data not shown).

DISCUSSION

Characterization of integrin α subunit cDNAs

This study reports the identification and cloning of multiple cDNAs encoding integrin α subunits from *Xenopus laevis*. These results provide some of the first evidence available for differential regulation of integrin α subunit expression in early vertebrate embryos.

Comparisons of the partial *Xenopus* and corresponding human α subunit deduced amino acid sequences reveal highest identity scores in the 58-76% range. This is lower than the 88-92% identities reported for several human and guinea pig sequences across the same region (Erle et al., 1991) and between human and other rodent sequences (Fig. 2). One obvious explanation for this difference is the evolutionary divergence of mammals and amphibia, which can account for the decreased relatedness of the *Xenopus* sequences. Our previous calculations showed that the *Xenopus* β_1 subunit shares 82-86% amino acid identity overall with other mammalian and avian β_1 sequences (DeSimone and Hynes, 1988). This suggests the possibility that at least some integrin α subunits diverged from one another earlier than have the β subunits. The larger number and relatively higher sequence diversity of α subunits may help account for the evolution of integrin functional variability with respect to ligand recognition (e.g., β_1 family). Correspondingly, β subunit sequence divergence may have been more constrained by the conservation of functional extracellular ligand-binding (D'Souza et al. 1988) and intracellular cytoskeletal-binding domains (Marcantonio and Hynes, 1988; Solowska et al., 1991; Reszka et al., 1992).

Another possibility is that the sequences reported here represent novel α subunits. It is improbable that this is the case given that different human α subunits share less than 45% amino acid identity overall (with the exception of the β_2 associated $\alpha_{M(mac\text{-}1)}$ and $\alpha_{X(p150)}$). These values are considerably lower than the sequence comparisons presented in Table 1 for human and *Xenopus* α subunits. Comparisons with chicken α subunit sequences provide a useful metric in this regard because humans are more distantly related to aves than to other mammals. For example, the chicken α_6 sequence is 76% identical to human α_6 across the region corresponding to the partial *Xenopus* α sequences (73% identical overall; de Curtis et al., 1991). This number is similar to that reported for the human versus *Xenopus* α_2, α_4, α_5 and α_6 subunits (i.e., 68-76% identity, Table 1). The *Xenopus* α_3 and α_{IIb} sequences are the least well conserved along this 75 amino acid stretch of sequence (58-59% identity, 74-79% similarity) and, conceivably, could represent other subunits. The putative αIIb is very similar to both the published α_V (57% identity) and α_8 sequences (57% identity, Fig. 2). The *Xenopus* cDNA that we have identified as α_3 is also similar to the reported sequence for rat α_7 (53% identity). The availability of *Xenopus* α subunit full-length cDNAs and additional nucleotide sequence will help to confirm the present assignments of α subunit identity.

Our inability to detect additional α subunits by homology PCR may be due to incompatability of the particular degenerate oligodeoxynucleotide primers used (Erle et al., 1991) or the absence of other α subunits at stage 17 (neurula). It is most likely that both situations are correct. With the exception of the putative α_{IIb} cDNA, each of the other cDNAs obtained by PCR encode α subunits known to associate with β_1 subunits, which we and others previously demonstrated are expressed in neurula-stage embryos (DeSimone and Hynes, 1988; Gawantka et al., 1992; Howard et al., 1992). Our studies confirm that β_3 is also expressed during neurulation (Fig. 4). At present, α_{IIb} is the only subunit that we have identified that is known to associate with β_3; however, α_{IIb} expression is very low at stage 17 (Fig. 3). It is likely, therefore, that other α subunits (e.g., α_V) are expressed coincident with β_3 in the early embryo but we have been unable to detect such subunit(s) using our primers.

Multiple integrin α subunits are sequentially expressed in early *Xenopus* embryos

The integrin α subunit mRNAs reported in this study originate at very early stages of development but each differs with regard to the timing and/or level of expression. The α_5 and β_1 mRNAs are both present as maternal transcripts with overall levels remaining constant throughout embryogenesis. This pattern of expression suggests that $\alpha_5\beta_1$ may be the major integrin present prior to gastrulation, coincident with the expression of fibronectin (Lee et al., 1984; DeSimone et al., 1992). One of the specific functions of the $\alpha_5\beta_1$ receptor in the early embryo, therefore, may be to participate in the assembly of fibronectin matrices. Fibronectin is synthesized at the mid-blastula stage and becomes incorporated into fibrils located along the inner surface of the blastocoel roof (Lee et al., 1984). Synthetic peptides containing the Arg-Gly-Asp cell-binding site are reported to disrupt the assembly of the fibronectin matrix in *Xenopus* (Yost, 1992) and *Pleurodeles* gastrulae (Boucaut et al., 1991) consistent with in vitro models of $\alpha_5\beta_1$-dependent fibril formation (McDonald et al., 1987; Fogerty et al., 1990). In addition to $\alpha_5\beta_1$, both $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrins are known to function as fibronectin receptors (Hynes, 1992). This apparent functional redundancy may be important for mediating multiple morphogenetic events that are fibronectin dependent.

Consistent with a general hypothesis of integrin involve-

ment in gastrulation is the timing of expression for these receptors. None of the integrin subunit mRNAs investigated thus far appear to be synthesized during the onset of zygotic transcription at the MBT. Rather, increased accumulation of most integrin transcripts occurs after the MBT coincident with the beginning of gastrulation. Of particular interest in this regard is the sequential pattern of expression for integrin α subunit mRNAs observed during this developmental time period (Fig. 4A). Both α_5 and α_3 transcripts are evident as maternal mRNAs with increased accumulation of α_3 noted as gastrulation commences at stage 10. Increases in α_4 and α_6 expression are also observed by stage 10 followed by the appearance of α_2 mRNAs at stage 11. A major objective of future studies will be to correlate the spatial patterns of integrin α subunit expression with the cellular rearrangements that characterize gastrulation movements in *Xenopus* (Keller, 1991).

Integrin α_3 is expressed in dorsal involuting mesoderm

The spatial pattern of α_3 mRNA expression is of particular interest because it is first detected in cells of the involuting marginal zone at the dorsal lip (Fig. 5A-D). Interestingly, α_3 expression is restricted to a tight band of cells in the dorsal midline of the embryo as gastrulation proceeds. Little or no α_3 is expressed by cells of the highly migratory 'head' mesoderm that involutes first or by the mesoderm that extends laterally on either side of the presumptive notochord. This spatiotemporal pattern of α_3 expression does not fit precisely with expectations for an integrin involved in supporting the migration of involuting mesodermal cells as gastrulation begins. Migratory mesodermal cells precede the involution of α_3-positive cells, which are actively converging toward the dorsal midline and extending along the anteroposterior axis (for a detailed description of these movements, see Keller 1991). In this capacity, it is possible that $\alpha_3\beta_1$ contributes to the process of convergence and extension. However, α_3 expression is limited to only a subset of those cells (i.e., prospective notochordal mesoderm) undergoing these morphogenetic movements during gastrulation. One alternative possibility is that $\alpha_3\beta_1$ is involved specifically in mediating adhesive activities required to organize and assemble the developing notochord.

The pattern of α_3 expression in the embryo is similar to that reported for *noggin*, which encodes a polypeptide with dorsalizing activity (Smith and Harland, 1992). Both *noggin* and α_3 are present at low levels as maternal mRNAs and are zygotically expressed by gastrulation. *Noggin* is expressed in the Spemann's organizer region at stage 9. We are unable to localize α_3 mRNAs until stage 10-10.5 but by this time α_3 is coexpressed with *noggin* in the dorsal involuting mesoderm. Both genes are highly expressed in the notochord as development proceeds. This pattern of notochord staining is also similar to that of the recently described *pintallavis* gene although, in this case, expression is observed in all three germ layers along the axial midline (Ruiz i Altaba and Jessel, 1992). Both *noggin* and *pintallavis* are expressed in the organizer region and are implicated in the induction and patterning of dorsal structures. It will be of interest, therefore, to determine whether these genes are involved in regulating the localized expression of integrins such as the α_3 subunit, which may in turn mediate subsequent morphogenetic events.

Integrin α subunit expression and mesoderm induction

Previous studies demonstrate that although fibronectins and β_1 integrins are likely to be involved in gastrulation, the onset of gastrulation-like movements in response to mesoderm induction is not triggered simply by an increase in the levels of expression of these proteins (Smith et al., 1990; DeSimone et al., 1991; Howard et al., 1992). The current results confirm that transcriptional activation of most integrin α and β subunits is unaffected following activin-A induction in the animal cap assay. Integrin α_3, α_4 and α_6 mRNA levels do increase in animal cap cells induced with activin-A at a concentration that causes the rapid adhesion and spreading of these cells on fibronectin (Smith et al., 1990). However, these mRNAs are not transcribed in whole embryos until stage 10 (Fig. 4). It is unlikely, therefore, that newly transcribed integrins can account for the increased adhesion to fibronectin that is noted in stage 8-9 animal cap cells shortly after induction with activin. Studies indicate that little change in the expression of β_1 integrins occurs at the cell surface during gastrulation or following mesoderm induction (Smith et al., 1990; Howard et al., 1992; Gawantka et al., 1992). In view of these results, it is likely that the mechanisms underlying this rapid change in adhesive behavior involve the functional activation of preexisting integrins at the cell surface. There is considerable evidence available from work in several systems that indicates that integrin function can be rapidly modulated through a variety of post-translational mechanisms (Hynes, 1992), some of which are likely to be operating during development.

The induction data, taken together with whole embryo expression studies (Fig. 4), suggest that integrins are not transcribed as 'rapid early response genes' following mesoderm induction but are possibly controlled by subsequent inductive events. This is in contrast to genes such as *Xbra* (Fig. 4B and Smith et al., 1991) and *pintallavis* (Ruiz i Altaba and Jessel, 1992) both of which are transcribed at the MBT and in animal caps shortly after induction with activin. The spatial localization of α_3 (Fig. 5) indicates that this subunit is likely to be regulated by inducers of dorsal mesoderm but α_3 levels increase little in response to activin induction. It is important to note, however, that the concentration of activin used in this study does not induce notochord tissue in these explants (Green et al., 1992). It is also possible that other factors such as members of the *wnt* gene family (Christian et al., 1992) or *noggin* (Smith and Harland, 1992) are required to sensitize cells to mesoderm inducers (Moon and Christian, 1992). The absence of these factors in animal caps might prevent the full range of dorsal patterning necessary to achieve the robust levels of α_3 expression observed in the dorsal involuting marginal zone of gastrulae.

The current study provides information on the patterns of expression for several integrin α and β subunit mRNAs in *Xenopus* embryos. It cannot yet be determined whether

these patterns of mRNA expression correlate with the synthesis of each α subunit or with the appearance of specific heterodimers at the cell surface. A complete analysis of integrin α subunit protein expression awaits the production of additional subunit-specific antibodies. We are currently using our PCR-generated partial cDNAs to clone full-length α and β subunit cDNAs in order to facilitate antibody production and functional studies.

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