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An extracellular matrix response element in the promoter of the LpS1 genes of the sea urchin *Lytechinus pictus*

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ABSTRACT

The extracellular matrix (ECM) has been shown to play an important role in development and tissue-specific gene expression, yet the mechanism by which genes receive signals from the ECM is poorly understood. The aboral ectoderm-specific LpS1-α and -β genes of *Lytechinus pictus*, members of the Spec gene family, provide an excellent model system to study ECM-mediated gene regulation. Disruption of the ECM by preventing collagen deposition using the lathrytic agent β-aminopropionitrile (BAPN) inhibits LpS1 gene transcription. LpS1 transcription resumes after removal of BAPN and subsequent collagen reformation. Using a chloramphenicol acetyltransferase (CAT) reporter gene assay, we show that a 125 bp region of the LpS1-β promoter from –108 to +17 contains an ECM response element (ECM RE). Insertion of the 125 bp region into the promoter of the metallothionein gene of *L.pictus*, a gene unaffected by ECM disruption, caused the fused promoter to become ECM dependent. As with the endogenous LpS1 genes, CAT activity directed by the fused LpS1-β promoter resumed in embryos recovered from ECM disruption. A mutation in a *cis*-acting element called the proximal G-string, which lies in the 125 bp region, caused CAT activity levels in ECM-disrupted embryos to equal that of the wild-type LpS1-β promoter in ECM-intact embryos. These results suggest that the intact ECM normally transmits signals to inhibit repressor activity at the proximal G-string in aboral ectoderm cells. Consistent with these results were our findings which showed that in addition to expression in the aboral ectoderm, the proximal G-string mutation caused expression of the CAT gene in oral ectoderm cells. These studies suggested that the proximal G-string serves as a binding site for negative regulation of the LpS1 genes in oral ectoderm during development. We also examined *trans*-acting factors binding the proximal G-string following ECM disruption. Band shift gels revealed a predominant set of slower migrating nuclear proteins from ECM-disrupted embryos which bound the proximal G-string. This work suggested that ECM disruption initiates signaling that induces a repressor to bind the ECM RE and/or modifies ECM RE binding proteins, which in turn represses LpS1 gene activity.

INTRODUCTION

Growing evidence shows that the extracellular matrix (ECM) plays a vital role in cell differentiation and gene regulation. The ECM helps govern the differentiation of mouse mammary tissue, hepatocytes, keratinocytes and other murine cell types (1) and rat Sertoli cells change from a squamous to a columnar morphology when in contact with a basement membrane (2). Several genes have been identified in these tissues *in vitro*, in which the ECM also participates in the regulation of transcription. Expression of the mouse β-casein gene in mammary epithelial cells is increased in the presence of prolactin only when cultured on an ECM preparation (3), the whey acidic protein (WAP) gene in mouse mammary epithelial cells requires an intact ECM for expression (4) and the albumin gene in mouse hepatocytes is activated by the ECM (5–9). The ECM can act as a negative influence on transcription, as in the case of the involucrin gene in keratinocytes (10–12) and the transforming growth factor-β1 (TGF-β1) gene in epithelial cells (13).

An *in vivo* model system that has been studied with regard to the role of the ECM in development and differentiation is the sea urchin embryo. Disruption of any of several ECM components arrests development just prior to gastrulation and spiculogenesis at the mesenchyme blastula stage. Inhibition of collagen deposition in the embryo by treatment with proline analogs or the lathrytic agent β-aminopropionitrile (BAPN), which specifically inhibits the collagen crosslinking enzyme lysyl oxidase, arrests development in the sea urchin embryo (14) and inhibits transcription of the LpS1 genes in *Lytechinus pictus* (15). The effects of the inhibitory agents are reversible and removal of the agents allows normal development and LpS1 transcription to resume.

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The studies described in this paper examine the mechanism by which the tissue-specific LpS1 genes are regulated by the ECM. The two LpS1 (*L.pictus* Spec 1) genes (α and β) encode proteins that belong to the calmodulin gene superfamily of calcium binding proteins and are expressed only in aboral ectoderm cells (16,17). Nuclear run-on assays showed that disruption and redeposition of collagen in the *L.pictus* embryo caused LpS1 transcription to be turned off and on respectively (15). These results suggested that an ECM signaling pathway regulates LpS1 transcription via a *cis*-acting ECM response element (ECM RE). We tested this hypothesis by identifying a 125 bp region on the LpS1-β promoter that responds to ECM disruption and recovery. Evidence is presented showing that a *cis*-acting element called the proximal G-string (17,18) in the 125 bp region is an (the) ECM RE and that it is involved in spatial regulation of the LpS1 genes during development. This is one of the few ECM REs identified and the first in a developmental system.

MATERIALS AND METHODS

Embryo cultures

Lytechinus pictus were obtained from Marinus Inc. (Long Beach, CA). Gamete collection and embryo culturing were performed as described (19). The BAPN treatment and recovery experiments with *L.pictus* embryo cultures were done as previously described (19).

DNA constructs

The constructs used for these studies are shown in Figure 1. The –762 LpS1–CAT, –511 LpS1–CAT and –108 LpS1–CAT constructs (17,18) were gifts from Dr W.H.Klein (M.D.Anderson Cancer Center, University of Texas, Houston, TX). The –143 LpS1–CAT construct was made by digesting the –762 LpS1–CAT construct DNA with *Stu*I and *Xho*I, making the *Xho*I end blunt-ended and religating the DNA strands. The proximal G-string in the –762 LpS1–CAT construct was converted to a *Bgl*II restriction site (5′-AGATCT-3′) by *in vitro* mutagenesis (18) and was a gift from Dr W.H.Klein. The –4000 LpMT1–CAT construct (20) was a gift from Dr B.Brandhorst (Simon Fraser University, Burnaby, BC, Canada). The –4000 LpMT1/LpS1–CAT construct was generated by inserting the –108 to +17 *Xho*I–*Sal*I DNA fragment of LpS1 into compatible sites of the –4000 LpMT1–CAT construct.

CAT assays

Chloramphenicol acetyltransferase (CAT) assays and CAT DNA determination assays were performed as described (21). Some of the CAT DNA determination assays shown in Figure 5 were carried out using PCR with CAT gene-specific primers 5′-GTCAGTTGCTCAATGTACC-3′ and 5′-CACCGTAACACGCCACATC-3′ (Gibco BRL, Gaithersburg, MD) to generate a 307 bp amplified DNA product. Carrier *L.pictus* genomic DNA and the CAT constructs were digested with the same restriction enzyme, which was usually *Xho*I.

Band shift assays

Crude nuclear protein extracts were isolated from *Lytechinus* embryos as described (21). Protein binding was assayed using double-stranded oligonucleotides (Genosys, Woodlands, TX) representing the wild-type proximal G-string (5′-CCGGGATCATTATCTTCGCATGGGGGGCGTGGTCTGTGTTGGT-3′) and a mutated proximal G-string (5′-CCGGGATCATTATCTTCGCATAGATCTCGTGGTCTGTGTTGGT-3′) (the proximal G-string region is underlined and only the sense strand sequences are shown). Competition band shift assays were carried out using double-stranded oligonucleotides representing the proximal G-string (5′-TCGCATGGGGGGCGTGGT-3′), the distal G-string (5′-TCGGAGCCCCCCTATGTT-3′) and the USF (5′-TCATTTCACGTGATTGAG-3′) binding sites (the G-string and USF sites are underlined and only the sense strand sequences are shown). DNA was radiolabeled at the 5′-end with [γ-^{32}P]ATP using T4 polynucleotide kinase or by fill-in reactions with [α-^{32}P]dCTP using the Klenow fragment. Specific activity was ~1×10^4 c.p.m./ng DNA. The band shift assays were performed as described (22).

Whole-mount *in situ* hybridization

The procedure is a modification of the protocol of Harkey *et al.* (23). All procedures were carried out in flexible 96-well microtiter plates under a stereomicroscope. Riboprobes were made with digoxigenin-UTP using SP6 RNA polymerase. Sense and antisense RNA probes were transcribed *in vitro* from the *Sac*I-linearized p64 and *Hin*dIII-linearized p65 CAT DNA constructs (24). Collected embryos injected with the wild-type and mutated proximal G-string –762 LpS1–CAT constructs were fixed overnight at 4°C in 2.5% glutaraldehyde, 0.14 M NaCl, 0.2 M phosphate buffer (PB) and dehydrated in successive 20, 35, 50 and 70% ethanol baths. The embryos were rehydrated in successive 50, 35 and 10% ethanol baths and two washes in PBST (0.2 M PB, 0.5 M NaCl and 0.1% Tween-20). Embryos were incubated in PBST containing 20 μg/ml proteinase K for 5 min at room temperature. The protease reaction was stopped with PBST containing 2 mg/ml glycine. The embryos were washed twice in PBST, fixed in PBST with 4% paraformaldehyde for 30 min on ice and rinsed twice with PBST. Hybridization buffer (1× HB: 50% formamide, 10% polyethylene glycol, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris, 500 μg/ml yeast tRNA, 2× Denhardt's solution and 0.1% Tween-20) was added sequentially to the embryos at 0.25, 0.5, 0.75 and 1× solutions. The embryos were incubated in 1× HB for 1–4 h at 50°C. An equal volume of 1× HB containing the riboprobe (0.6 ng/ml) was added, the wells were sealed with parafilm and the embryos were incubated at 50°C overnight. Following hybridization, the HB was diluted by adding several drops of PBST. The embryos were washed twice in PBST for 20 min at 50°C, three times in 0.5× SSC for 30 min at 60°C and once in PBST at room temperature. The embryos were incubated in PBST with 4% ovine serum for 30 min at room temperature and further incubated with the same buffer containing a 1:500 dilution of anti-digoxigenin antibody for 4 h to overnight at room temperature. The embryos were washed twice in PBST, once in AP (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$), pH 8.0, and once in AP, pH 9.5. The embryos were stained according to the instructions accompanying the digoxigenin color kit (BioRad, Hercules, CA) for 1 h in AP, pH 9.5. The reaction was stopped with PBST containing 1 mM EDTA. The embryos were dehydrated in successive 35, 50, 70, 90 and 100% ethanol baths and suspended in Terpineol for mounting on microscope slides.

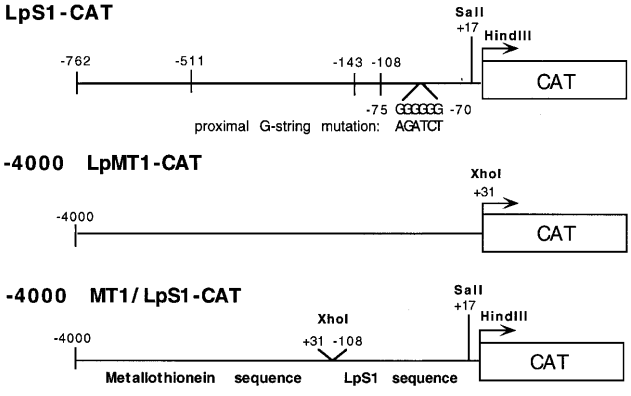

Figure 1. A diagram of the DNA constructs used in this study.

RESULTS

Delineation of the ECM RE to a 125 bp region on the LpS1 promoter

Nuclear run-on studies suggested that signals transmitted by the ECM were mediated via *cis*-acting elements on the LpS1-β promoter (15). We set out to identify the putative ECM RE and assumed that it resided within 762 bp of 5′ upstream promoter region, because it was shown that this region conferred proper temporal and spatial regulation on the LpS1-β gene (17). The putative ECM RE was delineated to a relatively smaller region on the LpS1-β promoter using a CAT assay system. The strategy was to delete different regions from the promoter of the –762 LpS1–CAT construct and test them in untreated control and BAPN-treated (ECM-disrupted) embryos. The rationale was that: (i) deletion of an ECM RE that bound a positive regulator would cause CAT activity in the control embryos to drop to that of the ECM-disrupted embryos; (ii) deletion of an ECM RE that bound a repressor would cause CAT activity in ECM-disrupted embryos to rise to control levels; (iii) the ECM RE would be delineated to a relatively smaller region for easier manipulation and identification if the smaller LpS1-β promoters continued to be inactivated by ECM disruption. Four constructs with LpS1-β promoter lengths of –762, –511, –143 and –108 fused to the CAT gene (Fig. 1) were injected into *L.pictus* eggs. The eggs were fertilized and untreated and BAPN-treated embryos were cultured until control embryos reached the pluteus larva stage. CAT activity levels from lysates of ECM-disrupted embryos fell to ~20% of control levels (Fig. 2). These results suggested that the ECM RE had not been deleted at –108 and was contained in a 125 bp region from –108 to +17 of the LpS1-β promoter. BAPN added directly to the nuclear run-on assays (15) or to the CAT assays (C.A.Seid and C.R.Tomlinson, unpublished data) had no effect. In all cases BAPN-treated embryos were arrested in development at the mesenchyme blastula stage. Because the LpS1-α and LpS1-β genes have identical 5′ flanking upstream DNA sequences for 326 bp (17), we concluded that the same ECM RE was used by both LpS1 genes. We also concluded that the distal G-string (–726 to –721) and USF binding site (–531 to –526) were not required for the ECM response because they lie well upstream of the 125 bp region.

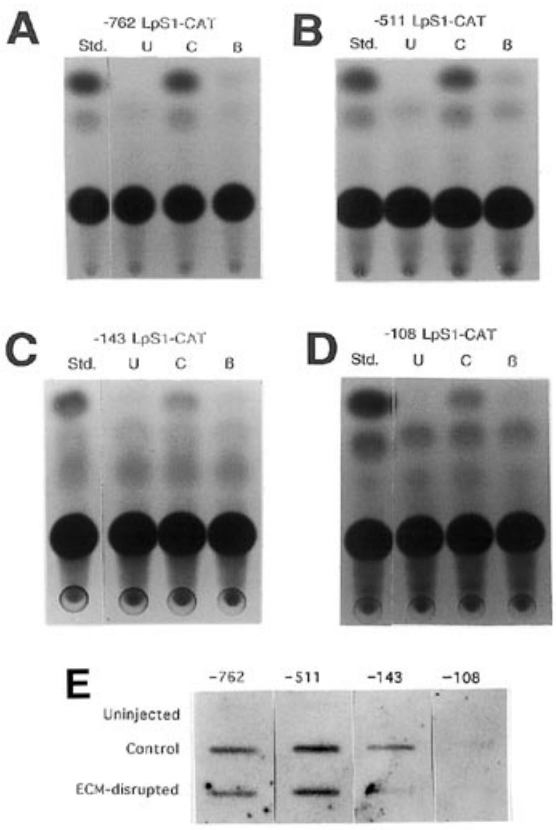

Figure 2. A region from –108 to +17 of the LpS1 promoter responds to the ECM. A series of LpS1–CAT constructs representing the regions –762 (**A**), –511 (**B**), –143 (**C**) and –108 (**D**) to +17 of the LpS1-β promoter was injected into *L.pictus* eggs. The eggs were fertilized and cultured for 48 h to the pluteus stage in the absence of BAPN as untreated control embryos (C) or in the presence of 100 μg/ml BAPN (β). Approximately 200 embryos were collected and assayed for CAT activity. Uninjected embryos (U) were used as background controls. The standard (Std.) was 0.01 U bacterial CAT. Relative amounts of CAT DNA are shown on the blot in (**E**).

Table 1. A mutation at the proximal G-string allows LpS1 promoter-directed CAT expression in the oral ectoderm

	Number of scored embryos[a]					
	Wild type –762LpS1–CAT			Mutant pG –762LpS1–CAT		
	A	O	A+O	A	O	A+O
Experiment 1	12	0	2	3	2	13
Experiment 2	20	0	2	1	1	6
Experiment 3	12	0	1	2	3	10
Experiment 4	18	0	3	1	1	22
Total	62 (89%)	0 (0%)	8 (11%)	7 (11%)	7 (11%)	51 (78%)

[a]Positive staining for CAT expression in: A, aboral ectoderm; O, oral ectoderm; A+O, aboral ectoderm plus oral ectoderm.

The 125 bp region from the LpS1 gene responds to ECM disruption in a heterologous promoter

In order to confirm that an ECM RE resided in the 125 bp region of the LpS1 promoter, the 125 bp fragment was inserted into a

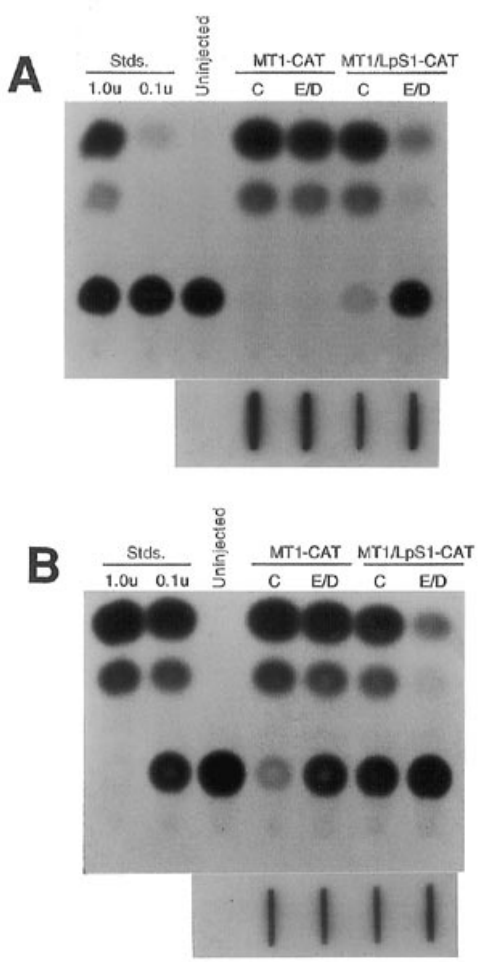

Figure 3. The region on the LpS1 promoter from –108 to +17 causes the metallothionein promoter to become ECM dependent. Duplicate experiments (**A** and **B**) were carried out in which a *Xho*I–*Hin*dIII DNA fragment containing a region on the LpS1 gene from –108 to +17 was inserted at the junction between the metallothionein promoter and CAT gene of the –4000 MT1–CAT construct (+31 on the metallothionein gene) to create the –4000 MT1/LpS1–CAT construct (see Fig. 1). CAT activities were determined from *L.pictus* embryos cultured for 48 h to the pluteus stage in the absence or presence of 100 µg/ml BAPN as untreated controls (C) or ECM-disrupted (E/D) embryos respectively. An equal number of uninjected embryos were used as background controls and the standards (Stds.) were 1.0 and 0.1 U bacterial CAT. DNA was isolated from equal volumes of uninjected and injected embryo lysates, spotted onto nitrocellulose and probed with CAT DNA. Relative amounts of CAT DNA are shown on the blot directly below the corresponding CAT assays.

promoter that is unaffected by ECM disruption. The RNA accumulation levels of many genes in the sea urchin embryo appear to be independent of the ECM, including the metallothionein gene of *Lytechinus*, in which nuclear run-on assays showed that collagen disruption had little or no effect on metallothionein gene transcription (15). A –4000 MT1–CAT construct (Fig. 1) containing the 5′ regulatory region of the metallothionein gene (20) was used in the assays. As duplicate experiments in Figure 3 and Table 1 show, the –4000 MT1–CAT construct was unaffected by ECM disruption. There was little or no difference in the CAT activities between untreated control embryos, which reached the pluteus larva stage, and ECM-disrupted embryos, which arrested at the mesenchyme blastula stage. However, insertion of the 125 bp region from the LpS1-β promoter into the –4000 MT1–CAT construct (now called –4000 MT1/LpS1–CAT; see Fig. 1) caused CAT activity regulated by the fusion promoter to drop dramatically in ECM-disrupted embryos with respect to untreated control embryos. The relative amount of injected CAT DNA in each batch of embryos was approximately the same, as shown in the DNA slot blot directly below the CAT assays. The control experiments in Figure 3 showed that the intact exogenous metallothionein promoter is ECM independent, like the endogenous promoter, and that insertion of the 125 bp did not interfere with promoter activity in ECM-intact embryos. Figure 3 further shows that the inserted 125 bp region caused the near abolition of CAT activity in ECM-disrupted embryo. These data demonstrate that an ECM RE is contained in the 125 bp region of the LpS1 promoter.

The LpS1 ECM RE confers ECM dependence and recovery

ECM-disrupted sea urchin embryos remain arrested indefinitely at the mesenchyme blastula stage. Transfer of the ECM-disrupted embryos to fresh seawater allows development (14) and LpS1 transcription to resume (15,19). If the 125 bp DNA fragment contains the ECM RE, then the –4000 MT1/LpS1–CAT construct should resume CAT activity in embryos transferred from BAPN-treated seawater to fresh seawater (Fig. 4A and B). Eggs were injected with the –4000 MT1/LpS1–CAT construct, fertilized and cultured for 36 h as untreated controls to the pluteus stage (lanes labeled C), for 36 h as BAPN-treated and arrested at the mesenchyme blastula stage (lanes labeled E/D), for 24 h as BAPN-treated and then 12 h in fresh sea water for recovery (lanes labeled E/R) and for 14 h as an untreated control to the mesenchyme blastula stage (lanes labeled MB). CAT activity dropped to 6.5% in ECM-disrupted/mesenchyme blastula stage-arrested embryos but resumed in the recovered embryos to 68.5% of control levels (Fig. 4C). The drop in CAT activity in ECM-disrupted/mesenchyme blastula-arrested embryos is not merely a stage-specific phenomenon, because CAT activity in untreated control mesenchyme blastula stage embryos was 78% of that of the ECM-disrupted embryos. The results shown in Figure 4 indicate that a complete ECM RE is contained in the 125 bp region because the fragment confers ECM dependence and recovery.

The proximal G-string binds an ECM-regulated repressor

The proximal G-string is composed of six contiguous guanine deoxynucleotides located at –70 to –75 and is only one of two identified *cis*-acting elements in the ECM-responsive, 125 bp region of the LpS1-β promoter (18,25) and thus was a candidate element for an ECM RE. The wild-type and mutated proximal G-string –762 LpS1–CAT constructs (Fig. 1) were tested for CAT activity in *L.pictus* embryos (Fig. 5). This same mutation in DNA fragments representing the proximal G-string region did not compete for binding (16,26) nor bind proteins (25) in electrophoretic mobility shift assays. Embryos injected with the wild-type construct produced CAT activity 10-fold (Fig. 5A) and nearly 40-fold (Fig. 5C) greater in the untreated embryos relative to ECM-disrupted embryos. However, CAT activity levels driven by the mutated proximal G-string LpS1 promoter in ECM-disrupted embryos were equal to or greater than the CAT activity levels produced by the wild-type LpS1 promoter in the untreated control group. These

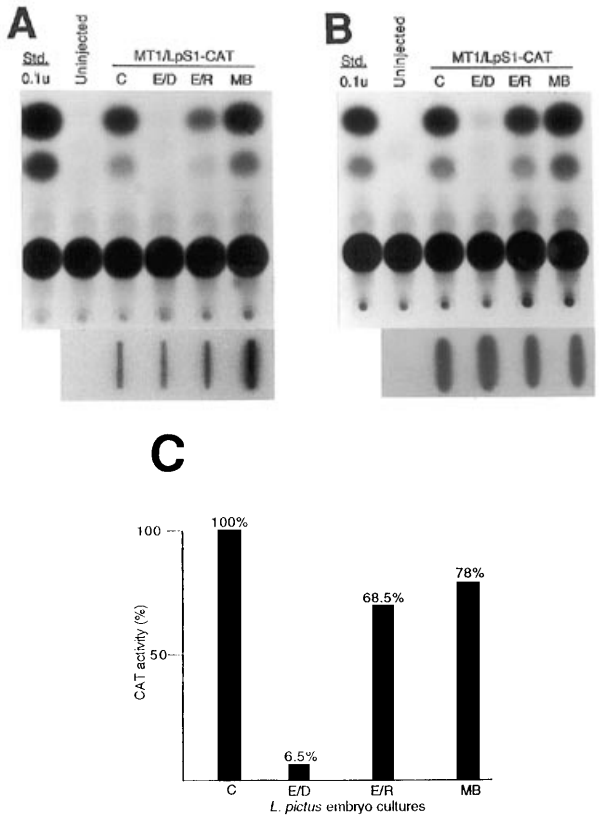

Figure 4. CAT activity resumes for the –4000 MT1/LpS1–CAT construct in BAPN-treated embryos allowed to recover in fresh seawater. Duplicate experiments (**A** and **B**) were carried out in which *L.pictus* eggs were injected with the –4000 MT1/LpS1–CAT construct and fertilized. Approximately 200 embryos were cultured for 36 h as untreated controls to the pluteus stage (C), for 36 h in 100 µg/ml BAPN and arrested at the mesenchyme blastula stage as ECM-disrupted embryos (E/D), for 24 h in 100 µg/ml BAPN and arrested at the mesenchyme blastula stage and for 12 h in fresh seawater as recovered embryos to early pluteus stage (E/R) and ~400 (A) or 250 (B) embryos for 12 h to the mesenchyme blastula stage (MB). Background controls (uninjected) and the CAT standards (Stds.) are as in Figure 3. Relative CAT DNA amounts were determined as described in Figure 3 and are shown on the blot directly below the corresponding CAT assays. The averages of the relative CAT activity levels shown in (A) and (B) are presented in (**C**).

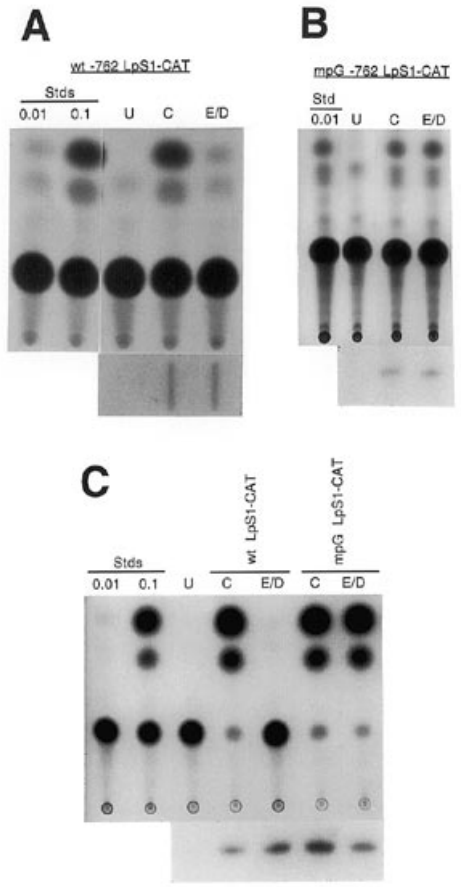

Figure 5. The proximal G-string acts as an ECM RE in the LpS1 promoter. Wild-type (wt) and mutated proximal G-string (mpG) –762 LpS1–CAT constructs were injected into *L.pictus* eggs, which were fertilized and cultured for 48 h as untreated control (C) and ECM-disrupted (E/D) embryos (100 µg/ml BAPN). The wild-type and mutated proximal G-string constructs were injected into two different egg batches in (**A**) and (**B**) and in the same egg batch in (**C**). Uninjected embryos (U) served as background controls. CAT standards (Stds) are as in Figure 3. Relative CAT DNA amounts are shown directly below the corresponding CAT assays.

results indicate that the proximal G-string is a *cis*-acting ECM RE for ECM-regulated repressor activity.

ECM disruption alters DNA binding activities on the ECM RE

Identification of the proximal G-string as the ECM RE allowed examination of the DNA binding properties of the ECM RE binding factor(s). Xiang *et al.* (18) demonstrated by band shift assays that two different factors bound the proximal G-string. One of the factors was specific to ectoderm cells and showed a slower rate of migration on band shift assays. The other factor was specific to endoderm and/or mesoderm cells and showed a relatively faster rate of migration on the same band shift gels. Similar band shift assays were carried out to examine how ECM disruption might affect protein binding to the ECM RE (Fig. 6). Nuclear extracts were isolated from untreated control embryos cultured to the pluteus larva stage, ECM-disrupted embryos arrested at the mesenchyme blastula stage and ECM-recovered embryos which had developed to the pluteus larva stage. These nuclear extracts were incubated with a 43mer oligonucleotide representing the proximal G-string region of the LpS1 promoter. Figure 6A shows the results of triplicate experiments using nuclear protein preparations from three different batches of embryos. In each preparation the proteins isolated from the ECM-disrupted embryos showed two predominant, slower migrating protein–DNA complexes (indicated by the arrows). The slower DNA–protein complexes that are predominant in the ECM-disrupted lanes are also visible in the control and recovered lanes, but are considerably less intense. The predominant bands suggested that ECM disruption either caused different proteins to bind the proximal G-string or caused modification of the original G-string binding factor(s). Based on earlier work (18), the rate of migration indicated that it was the ectodermal rather than the endodermal/mesodermal factor that was affected by ECM disruption. This conclusion was confirmed by the results in Figure 6B, which were in agreement with those of Xiang *et al.*

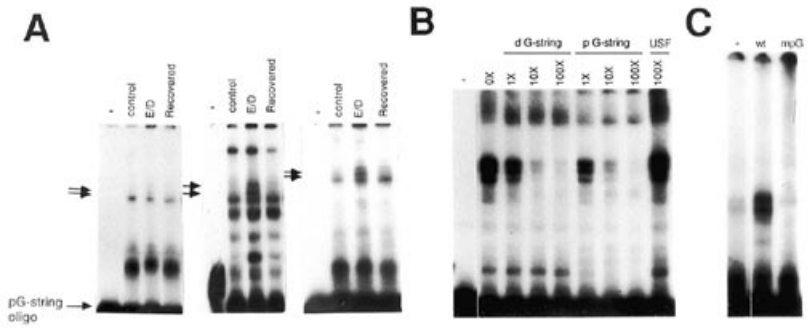

Figure 6. The DNA binding activity of the proximal G-string binding factor(s) is altered by ECM disruption. Triplicate band shift assays (**A**) were carried out with the proximal G-string oligonucleotide incubated with three different preparations of nuclear extracts from untreated 48 h control, ECM-disrupted (E/D) and ECM-recovered *L.pictus* embryos. The arrows denote the position of the more prominent bands produced by the nuclear extracts from ECM-disrupted embryos. A competition band shift assay (**B**) was carried out with the proximal G-string oligonucleotide incubated with nuclear extracts from ECM-disrupted *L.pictus* embryos. The indicated relative amounts of unlabeled distal G-string (dG-string), proximal G-string (pG-string) and USF oligonucleotides were incubated with the nuclear extracts for 20 min prior to incubation with the radiolabeled proximal G-string oligonucleotide. A band shift assay (**C**) was carried with radiolabeled wild-type (wt) and mutated (mpG) proximal G-string oligonucleotides incubated with nuclear extracts from ECM-disrupted *L.pictus* embryos. The radiolabeled oligonucleotide alone (–) is shown in the left lane of each gel.

(18) and which show that the proximal G-string oligonucleotide competes for both the ectodermal and the endodermal/mesodermal factors, while the distal G-string oligonucleotide competes only for the ectodermal factor. Figure 6B also shows that protein binding to the proximal G-string, including the proteins affected by ECM disruption, is specific because an oligonucleotide representing the USF binding site does not compete. Also in agreement with earlier studies (18,25), the same altered sequence at the proximal G-string site inhibits protein binding (Fig. 6C).

The proximal G-string is involved in spatial regulation of the LpS1 genes

Evidence that the proximal G-string bound a repressor in ECM-disrupted embryos suggested that it may have a role in normal development as a *cis*-acting element involved in spatial regulation of the aboral ectoderm-specific LpS1 genes. To test this hypothesis, *L.pictus* eggs were injected with wild-type and mutated proximal G-string –762 LpS1–CAT constructs, fertilized and cultured to the desired developmental stage. The embryos were fixed and prepared for whole-mount *in situ* hybridization using an antisense RNA probe to detect expressed CAT transcripts (Fig. 7 and Table 1). Embryos injected with the wild-type construct expressed CAT RNA appropriately only in the aboral ectoderm of gastrula and pluteus larva stage embryos in 89% of the embryos scored (Fig. 7B and C). Embryos injected with the mutated proximal G-string construct showed staining for CAT gene expression in both the aboral ectoderm and oral ectoderm in 78% of the scored embryos (Fig. 7D–F). Little staining above background levels was observed in non-ectoderm cells. These results suggest that the proximal G-string normally binds an oral ectoderm-specific repressor which when inhibited from binding by a mutation at the proximal G-string allows LpS1 promoter-directed transcription in oral ectoderm cells to proceed.

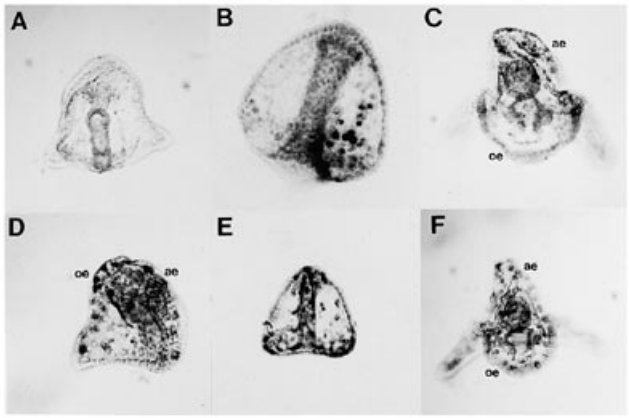

Figure 7. A mutation in the proximal G-string allows LpS1 promoter-directed transcription in oral ectoderm cells. Eggs of *L.pictus* were injected with the wild-type –762 LpS1–CAT (**A**–**C**) or mutated proximal G-string –762 LpS1–CAT (**D**–**F**) DNA constructs, fertilized and cultured to the indicated developmental stage. Fixed embryos at the gastrula (B and E), prism (A and D) and pluteus (C and F) larval stages were incubated with control sense RNA (A) or antisense RNA (B–F) representing the CAT gene and prepared for whole-mount *in situ* hybridization. ae, aboral ectoderm; oe, oral ectoderm.

DISCUSSION

We have identified an ECM RE in the LpS1 promoter. First, by a series of deletions a 125 bp fragment of the LpS1 promoter from –108 to +17 was shown to respond to collagen disruption. CAT activity in ECM-disrupted embryos with a deleted promoter was ~20% of control embryos. Second, insertion of the 125 bp fragment into the metallothionein promoter (the –4000 MT1/LpS1–CAT construct) caused the fused promoter to become ECM dependent, with CAT activity levels in embryos treated with BAPN falling to 23% of CAT activity levels in untreated control embryos. Third, as with the endogenous LpS1 gene, the fused metallothionein–LpS1 promoter resumed activity in ECM-recovered embryos. Fourth, a mutation in the 125 bp region at the proximal G-string demonstrated that this element is an ECM RE which binds an ECM-regulated repressor. By these experiments we identified the first ECM RE in an *in vivo* system.

There have been three proposed mechanisms by which the ECM may exert transcriptional regulation. First, positive *trans*-acting factors may require an ECM for transcriptional induction, which is the means by which the β-casein (27) and albumin (8,9) genes are regulated. Second, the ECM can signal repressor binding or activity, which is the means by which the mammary

TGF-β1 gene appears to be regulated (13). Third, the absence of an ECM may allow a transcriptional repressor to bind and inhibit transcription, which may be the way in which the mouse WAP gene is inactivated during early mammary gland development (1). Our data indicate that the LpS1 genes are regulated by an ECM signaling process resembling the third mechanism and support a model in which an intact ECM is required for LpS1 gene activity.

Several *cis*- and *trans*-acting elements have been identified and analyzed in the LpS1 promoter (17,18,22,28,29). Early CAT assays suggested that the proximal G-string bound a positive factor required for transcription (18). However, the role of the proximal G-string has been revised in the light of recent findings which showed instead that it was possibly the 5′ portion of a *cis*-acting domain that includes the region immediately downstream (25). In agreement with our findings (Fig. 5B and C), it was shown that the proximal G-string mutation in the –108 LpS1–CAT construct had no effect on LpS1 promoter-directed CAT activity in ECM-intact (control) embryos. However, a mutation at a site immediately downstream of the proximal G-string reduced CAT activity by 50% and mutations at both the proximal G-string and downstream sites reduced CAT activity to ~25% (25). These results suggest that these two sites form a binding domain in which the proximal G-string, while not required for binding, may contribute to binding of a positive factor in aboral ectoderm cells.

Our results suggest an additional role for the proximal G-string, in which it acts as a binding site for a repressor. This conclusion is based on results that showed that a proximal G-string mutation which inhibits protein binding caused the LpS1 promoter to overcome transcriptional inactivation caused by ECM disruption. The results suggest that ECM disruption initiates signals that repress LpS1 transcription in aboral ectoderm cells by modifying the existing proximal G-string binding proteins or by inducing new protein binding. Consistent with these results were: (i) band shift assays that showed that ECM disruption caused altered banding patterns; (ii) our findings that showed that in addition to expression in the aboral ectoderm, proximal G-string mutation caused inappropriate expression of the CAT gene in the oral ectoderm of normal gastrula and larva stage *L.pictus* embryos. Together the results suggest that the repressor resides in both aboral and oral ectoderm cells. We propose a model in which during normal development the repressor binds the proximal G-string site in oral ectoderm to repress LpS1 gene transcription, while in the aboral ectoderm it is inhibited from binding, perhaps by signals from the ECM. Disruption of the ECM would halt ECM signaling to allow repressor binding in the aboral ectoderm. All the evidence suggests that the proximal G-string may serve a dual role as a binding site for positive regulation in aboral ectoderm cells and for negative regulation in oral ectoderm cells.

The G-string was first described as an element in mouse collagen genes that bound the repressor IF1 (30,31), a zinc finger protein now called c-Krox (32). The LpS1 proximal G-string element was shown to interact with two proteins, one specific to the ectoderm of larva stage embryos and the other specific to the endoderm/mesoderm. The ectoderm-specific factor was shown to have DNA binding properties similar to c-Krox (18). A G-string binding factor of 59.5 kDa, called suGF1, has been purified from the sea urchin *Parechinus angulosus*, in which it was shown to bind the LpS1 proximal G-string sequence with high affinity (26). An implied role for suGF1 was regulation of gene expression by altering chromatin structure (33). The sequence and structure of suGF1 are not known. A G-string binding protein called SpGCF1 was recently cloned from *Strongylocentrotus purpuratus* and was shown to act as a positive transcriptional regulator for the CyIIIa and Endo16 genes (34,35). SpGCF1 was also shown to bind the LpS1 proximal G-string (25), but SpGCF1 is not a zinc finger protein nor similar to c-Krox.

The ECM may also play a role as a temporal activator of LpS1 and, in the case of *Lytechinus*, regulate primarily tissue-specific genes. The LpS1 genes are activated at the late cleavage/early blastula stage (36) and most of the components that comprise the basal and apical ECM are laid down in the embryo by this time (37). The sea urchin genes identified thus far that appear to be affected by ECM disruption are all tissue-specific genes, including, in addition to the aboral ectoderm-specific LpS1 genes, the gut-specific Endo1 (14), alkaline phosphatase (38) and LvN1.2 (39) genes and the primary mesenchyme cell-specific gene SM30 (40). Known tissue-specific genes that are exceptions are the primary mesenchyme cell-specific CyIIa and SM50 genes (15,38) and the aboral ectoderm-specific genes Spec1, Spec2a and CyIIIa (15), all of *S.purpuratus*, which suggests the possibility that differentiation in this species may be less dependent on the ECM than in *Lytechinus*.

A major question that arises from this work is the mechanism by which the ECM transmits signals to the LpS1 gene. Previous work in our laboratory suggested that platelet-derived growth factor (PDGF)-like and epidermal growth factor (EGF)-like signaling pathways are involved in regulating LpS1 transcription (19). In that work we showed that mammalian PDGF and TGF-α rescued ECM-disrupted/mesenchyme blastula-arrested embryos such that development and LpS1 transcription resumed. Those results and other work in our laboratory (41,42) have given rise to the hypothesis that signals originate from the ECM via endogenous growth factors that interact with and require the ECM for signaling activity (43). Disruption of the ECM may disrupt ligand–receptor binding to interrupt the downstream PDGF-like and EGF-like growth factor signaling pathways, causing development to arrest and LpS1 transcription to cease. As noted earlier, many tissue-specific genes in *Lytechinus* are ECM dependent. Thus, differentiation may in part be regulated by qualitative and/or quantitative differences in the composition of the ECM to provide a binding substrate for different growth factors in regulation of different tissue-specific genes. Tests of this hypothesis will require identification of additional signaling components and *cis*-acting elements and identification of an ECM RE in the LpS1 genes may serve well for this purpose.

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