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A G-string positive *cis*-regulatory element in the LpS1 promoter binds two distinct nuclear factors distributed non-uniformly in *Lytechinus pictus* embryos

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Summary

The LpS1α and β genes of *Lytechinus pictus* are activated at the late cleavage stage of embryogenesis, with LpS1 mRNAs accumulating only in lineages contributing to aboral ectoderm. We had shown previously that 762 bp of 5′ flanking DNA from the LpS1β gene was sufficient for proper temporal and aboral ectoderm specific expression. In the present study, we identified a strong positive *cis*-regulatory element at −70 bp to −75 bp in the LpS1β promoter with the sequence $(G)_6$ and a similar, more distal *cis*-element at −721 bp to −726 bp. The proximal 'G-string' element interacted with two nuclear factors, one specific to ectoderm and one to endoderm/mesoderm nuclear extracts, whereas the distal G-string element interacted only with the ectoderm factor. The ectoderm and endoderm/mesoderm G-string factors were distinct based on their migratory behavior in electrophoretic mobility shift assays, binding site specificities, salt optima and EDTA sensitivity. The proximal G-string element shared homology with a binding site for the mammalian transcription factor IF1, a protein that binds to negative *cis*-regulatory elements in the mouse α1(I) and α2(I) collagen gene promoters. Competition experiments using wild-type and mutant oligonucleotides indicated that the ectoderm G-string factor and IF1 have similar recognition sites. Partially purified IF1 specifically bound to an oligonucleotide containing the proximal G-string of LpS1β. From our results, we suggest that the ectoderm G-string factor, a member of the G-rich DNA-binding protein family, activates the LpS1 gene in aboral ectoderm cells by binding to the LpS1 promoter at the proximal G-string site.

Key words: sea urchin embryos, G-rich *cis*-regulatory elements, sea urchin Spec/LpS1 genes, transcription factor IF1.

Introduction

Proteins that regulate transcription play key roles in early developmental events (eg. Melton, 1991 and references therein). These proteins can be activated in restricted areas of an embryo by a variety of mechanisms, resulting in the downstream activation or repression of target genes (eg. Gilmore, 1990; Jones, 1990; Driever and Nüsslein-Volhard, 1988). Recent investigations on sea urchin gene expression have implicated a variety of transcription factors as being important in the activation of cell lineage-specific genes following the establishment of the founder cell lineages in the cleaving embryo (Davidson, 1989; Zhao *et al.* 1991; Calzone *et al.* 1991; Höög *et al.* 1991; Venuti *et al.* 1991; Kozlowski *et al.* 1991). How such transcription factors themselves are regulated to achieve the subsequent regionalized response is unknown. However, because of the brief period between fertilization and cell lineage-specific gene activation, it is likely that some of the transcription factors involved in activating these early downstream genes are represented in the unfertilized egg in an inactive state or as untranslated maternal mRNA.

To address questions regarding cell lineage-specific gene activation, we have used a family of genes whose expression is restricted to aboral ectoderm lineages. In *Lytechinus pictus*, two closely related genes, LpS1α and LpS1β, are activated at the end of cleavage; the LpS1 messages accumulate only in aboral ectoderm cells (Xiang *et al.* 1988, 1991; Tomlinson and Klein, 1990). These genes are related to the *Strongylocentrotus purpuratus* Spec1 and Spec2 genes, which encode intracellular calcium-binding proteins and serve as markers for the differentiation of the aboral ectoderm (Klein *et al.* 1990, 1991). In an earlier study, we analyzed 5′ flanking DNA from the LpS1β gene and showed that 762 bp of upstream DNA was sufficient for

proper temporal and spatial expression of reporter genes in a sea urchin embryo expression system (Xiang *et al.* 1991). In this report, we continue our analysis of the LpS1β gene promoter. We show that a guanosine-rich, or 'G-string', motif located 70 to 75 base pairs upstream of the LpS1β transcriptional start site is a major positive control element for this gene. The G-string element binds two distinct nuclear factors, one specific to ectoderm cells and one to endoderm/mesoderm cells. Our results suggest that the ectoderm factor is a positive regulatory protein likely to activate the LpS1β gene in aboral ectoderm cells during embryogenesis. The ectoderm factor has many properties in common with IF1, a transcriptional repressor that binds to a G-string motif on the mouse α1(I) and α2(I) collagen promoters (Karsenty and de Crombrugghe, 1990, 1991).

Materials and methods

Plasmid constructs

The −511 LpS1β–CAT and −108 LpS1β–CAT plasmid constructs were generated from the Bluescript vector (Stratagene) and pSV_0·CAT (Gorman *et al.* 1982) as follows: A 1635-bp *Hin*dIII–*Bam*HI fragment containing the CAT reporter gene and SV40 polyadenylation site was isolated from pSV_0·CAT and cloned into the *Hin*dIII and *Bam*HI sites of Bluescript II SK(+) to create $pCAT_0$. A 5′ deletion containing LpS1β 5′ flanking regions from +17 bp to −511 bp (Xiang *et al.* 1991) was then inserted in a forward orientation into the *Sal*I site of $pCAT_0$ to generate −511 LpS1β–CAT. The −108 LpS1β–CAT construct was subsequently produced by digestion of −511 LpS1β–CAT with *Mlu*I (a single site at −108 bp) and *Kpn*I (a single site in $pCAT_0$).

Site-directed mutagenesis

A mutant plasmid containing an altered G-string motif was created following manufacturer's instructions supplied with the T7-GEN™ *in vitro* mutagenesis kit (USB) based on the method of Vandeyar *et al.* (1988). Single-stranded DNA was rescued from −511 LpS1β–CAT using an M13K07 helper phage (Promega, Biotec.). A G-string mutant oligonucleotide, GS(MT), containing LpS1β sequence from −57 to −88 bp with the GGGGGG motif mutated to an AT-rich *Bgl*II site, AGATCT, was phosphorylated and annealed to single-stranded −511 LpS1β–CAT DNA at a molar ratio of 5:1. Complementary DNA strands were synthesized with T7 DNA polymerase using 5-methyl-dCTP, and ligated with T4 DNA ligase. The unmethylated parental DNA strands were subsequently cut by *Msp*I and digested with exonuclease III. The circular, methylated mutant DNA strands were used to transform *Escherichia coli* SDM cells. Isolated mutant −511 LpS1β–CAT plasmids were confirmed by *Bgl*II digestion and DNA sequencing.

Microinjection of sea urchin zygotes and CAT assays

Microinjection of sea urchin zygotes and CAT assays were performed as described previously (McMahon *et al.* 1985; Gan *et al.* 1990*a*; Xiang *et al.* 1991). Each *L. pictus* zygote was injected with about 2000 molecules of linearized plasmid plus *L. pictus* sperm carrier DNA at a molar ratio of 1:5. After microinjection, *L. pictus* embryos were cultured at 18°C and allowed to develop to the desired stage. $pCAT_0$ and −511 LpS1β–CAT were linearized with *Xho*I and *Kpn*I. The −108 LpS1β–CAT construct was released from −511 LpS1β–CAT by digestion with *Mlu*I and *Kpn*I and was gel purified before injection.

Microinjected embryos were harvested at mesenchyme blastula, gastrula or prism/pluteus stage and mixed with approximately 1500 uninjected embryos. Half of the embryos were assayed for CAT activity, and the other half were used to measure the plasmid DNA levels (Gan *et al.* 1990*a*).

Preparation of sea urchin nuclear extracts

Sea urchin nuclei and nuclear extracts were isolated and prepared as described previously (Morris *et al.* 1986; Tomlinson and Klein, 1990; Tomlinson *et al.* 1990; Kozlowski *et al.* 1991). To prepare *L. pictus* blastula stage nuclear extracts, we isolated nuclei from whole blastulae homogenates using sucrose gradient ultracentrifugation as previously described (Morris *et al.* 1986). To prepare *L. pictus* ectoderm and endoderm/mesoderm nuclear extracts, pluteus stage embryos were fractionated into ectoderm and endoderm/mesoderm according to McClay (1986). Nuclei isolated from these two fractions were monitored and quantified by measuring DNA content and directly counting the nuclei. The nuclear extracts prepared from these fractionated nuclei were stored in aliquots at −70°C in a buffer containing 15% glycerol, 25 mM Hepes (pH 7.8), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (phenylmethylsulfonyl fluoride).

Electrophoretic mobility shift analysis

Electrophoretic mobility shift analyses were performed as described by Tomlinson *et al.* (1990) with the following modifications: DNA probes were generated by 5′-end labelling with [γ^{32}P] ATP and T4 polynucleotide kinase. Approximately 5×10^4 cts min^{-1} of radiolabelled DNA (1–5 fmoles per reaction) were used for each DNA–protein binding reaction. Most reactions were performed at 4°C for 15 min in a final volume of 20 μl and contained 12 mM Hepes (pH 7.8), 100 mM KCl, 0.12 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 100 $\mu g\,ml^{-1}$ poly(dI-dC) with 1–3 μg of nuclear extracts. Competition reactions were performed by addition of the desired amount of DNA fragment or oligonucleotide. In the analyses to determine the effects of KCl and EDTA concentrations on DNA–protein interactions, the KCl concentration was varied from 0 to 0.4 M and EDTA concentration from 0 to 4 mM. Reaction mixtures were electrophoresed in 5% nondenaturing polyacrylamide gels and reaction complexes visualized by autoradiography. Densitometry was used to quantitate signal intensity. To measure the relative affinity constants and number of nuclear molecules per embryo for the G-string factors, the data obtained from electrophoretic mobility shift experiments were plotted according to Calzone *et al.* (1988). Values of Kr (defined below) and number of nuclear molecules per embryo (Po) were obtained directly from these plots as described in Calzone *et al.* (1988).

Electrophoretic mobility shift assays using an IF1 oligonucleotide (Table 1) were performed as previously described (Karsenty and de Crombrugghe, 1990, 1991). One μl of DNA affinity purified IF1 was incubated with 10^4 cts min^{-1} (5 fmoles) of end-labelled oligonucleotide in a final volume of 10 μl. All binding reactions contained 12 mM Hepes (pH 7.9), 12% glycerol, 0.2 M KCl, 0.1 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 1 $\mu g\,ml^{-1}$ pepstatin, 1 $\mu g\,ml^{-1}$ leupeptin and were electrophoresed on a 5% nondenaturing polyacrylamide gel.

Methylation interference assay

A 5′ end labelled *Eco*RI–*Hin*cII fragment containing the

LpS1β upstream sequence from +17 to −762 bp was digested with *Dde*I (at −683 bp) to generate the *Eco*RI–*Dde*I fragment *c* with only the coding strand labelled at its 5′ end (Fig. 1A). The 5′ end-labelled fragment *c* was then cut with *Taq*I (at −745 bp) to create a fragment with only the non-coding strand labelled at its 5′ end. Approximately 1×10^7 cts min^{-1} of these probes were used for methylation interference assays (Siebenlist and Gilbert, 1990). The probes were partially methylated by dimethyl sulfate as described by Maxam and Gilbert (1980). DNA–protein binding reactions were performed with *L. pictus* blastula nuclear extracts and approximately 1×10^6 cts min^{-1} of methylated probes. The DNA–protein complexes and free probes were eluted from the polyacrylamide gel and cleaved with piperidine at 90–95°C. 1×10^4–1×10^5 cts min^{-1} of purfied DNA–protein complexes and the same or half the amount of free probes were used in cleavage reactions. The cleavage products were then separated on an 8% DNA sequencing gel and visualized by autoradiography.

Purification of IF1

A complete purification procedure for IF1 will be presented elsewhere (M.M. and G.K., in preparation). Briefly, nuclear extracts (12 ml) from 7 g (wet weight) of HeLa cells were prepared as previously described (Dignam *et al.* 1983) except that PMSF (0.5 mM), pepstatin 5 $\mu g\,ml^{-1}$ and leupeptin 5 $\mu g\,ml^{-1}$ were added to each of the buffers. The protein extracts were suspended in buffer A (25 mM Hepes, pH 7.9, 20% glycerol, 0.1% NP40, 0.5 mM EDTA). After dialysis for three hours against buffer A with 0.1 M NaCl, the extracts were applied to a 5 ml phosphocellulose column. After washing with buffer A with 0.1 M NaCl, IF1 activity, as measured by electrophoretic mobility shift analysis using the IF1 oligonucleotide, was eluted with buffer A with 0.5 M NaCl. Active fractions were pooled, dialyzed against buffer A with 0.1 M NaCl, then mixed for 15 min with poly(dI-dC) (20 $\mu g\,ml^{-1}$) and loaded onto a 1 ml DNA affinity column containing the concatemer of the IF1 oligonucleotide. The DNA affinity column was prepared as previously described (Kadonaga and Tjian, 1986). The purification was approximately 800-fold with respect to total protein in the HeLa nuclear extracts.

Results

A G-string-positive cis-regulatory element in the LpS1β gene promoter

In a previous study, we microinjected LpS1β promoter–reporter gene constructs into *L. pictus* eggs and monitored promoter activity in the injected embryos using CAT and *lac Z* reporter genes for quantitative and spatial analysis, respectively (Fig. 1; Xiang *et al.* 1991). Constructs containing 762 bp of upstream DNA plus 17 bp of untranslated leader sequence resulted in strong CAT activity and appropriate aboral ectoderm expression in greater than 90% of the injected embryos. Constructs with 511 bp or 368 bp of 5′ flanking DNA had one third and half the CAT activity, respectively, and both constructs displayed a significant loss of aboral ectoderm restricted expression (Fig. 1B; Xiang *et al.* 1991). Further deletion to 108 bp of upstream DNA caused no additional loss of CAT activity (Fig. 1B, C). Thus, while some activity was lost in constructs lacking the region between −762 bp and −511 bp, the major quantitative elements regulating

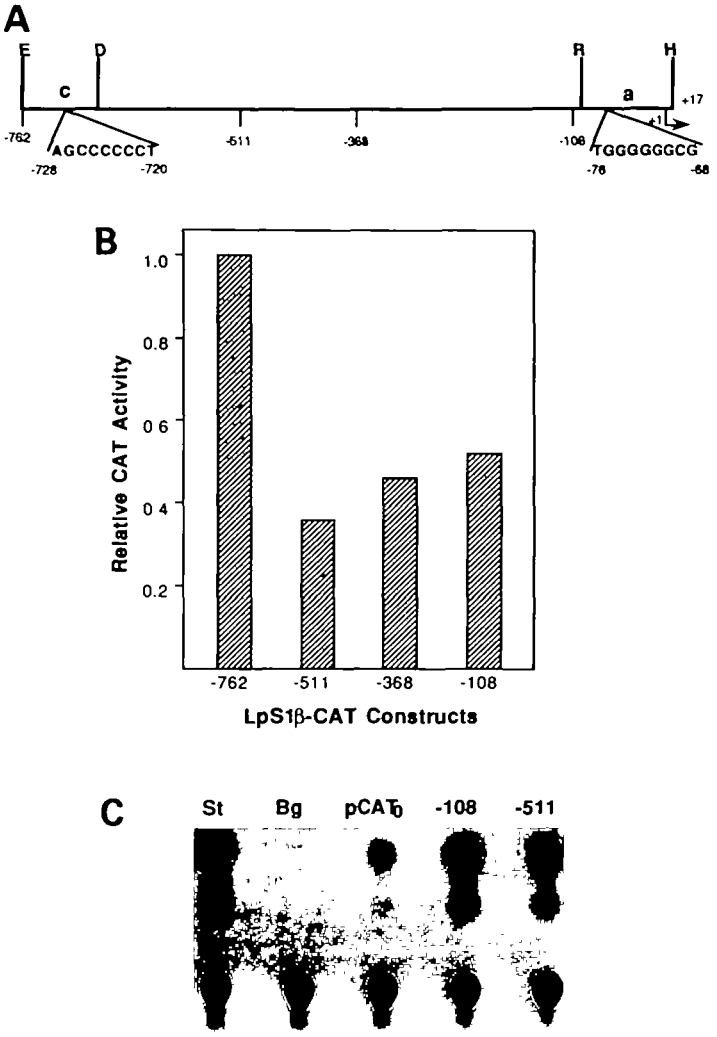

Fig. 1. Comparison of the promoter activities of the LpS1β 5′ deletion constructs. (A) Schematic illustration of the LpS1β promoter region. The arrow indicates the transcription initiation site, +1. Fragment *a* is a 120-bp *Hinc*II–*Rsa*I fragment containing the LpS1β sequence from +17 to −103 bp and fragment *c* represents the 80-bp *Dde*I–*Eco*RI fragment from −683 to −762 bp. Also indicated are the GC-rich sequence motifs in fragment a between −68 and −76 bp, and in fragment *c* between −720 and −728 bp. Relative positions of the LpS1β deletions −511, −368 and −108 used in a previous report (Xiang *et al.* 1991) and in this report are marked. D, *Dde*I; E, *Eco*RI; H, *Hinc*II; R, *Rsa*I. (B) Diagram of the relative CAT activities of LpS1β–CAT constructs. (C) Comparison of CAT activity between −108 LpS1β–CAT and −511 LpS1β–CAT. The CAT assay was performed with 320 mesenchyme blastula stage embryos for each plasmid. Bg, uninjected background embryos; St, uninjected embryos plus 0.1 unit of bacterial CAT enzyme.

LpS1β gene expression apparently were between −108 bp and +17 bp.

To investigate the LpS1β promoter further, we monitored DNA–nuclear protein interactions *in vitro* by mixing DNA fragments generated from +17 bp to −762 bp of the LpS1β gene with nuclear extracts from

Table 1. *DNA sequences that inhibit fragment* a *binding to the ectoderm G-string factor*

Oligonucleotide or fragment	Sequence[3]
a, G-string	...T C T T C G C A T **G*G G*G*G*G*C** G T G G T C T G...
c[1]	...C A A A A C A T A G G G G G G C T C C G A A T T ..
IF1	...T T G C G G G A G G G **G*G*G*G** C G C T G G G T G G A C...
G_3CG_3	...T C T T C G C A T G G G C G G G G T G G T C T G...
PolydG-dC[2]	...G G G G G G G G G G G G G G G G G G G G G G G G G G G...

[1] The sequence within fragment *c* is complementary and inverted with respect to the orientation shown in Fig. 1A.
[2] Poly(dG-dC) was a much weaker competitor than the other oligonucleotides or fragments listed in the Table.
[3] Bold type indicates the homology between the G-string and IF1 binding sites encompassing the G-rich region. Astericks indicate the bases that have been changed to generate the mutant G-string and IF1 oligonucleotides. In the case of the mutant G-string oligonucleotide, the $(G)_6$ was changed to AGATCT. In the case of the mutant IF1 oligonucleotide, the $(G)_3$ was changed to $(A)_3$. Neither mutated sequence has significant affinity for the ectoderm G-string factor or for partially purified IF1.

L. pictus embryos and performing electrophoretic mobility shift assays. Two fragments, '*a*' and '*c*', corresponding to +17 to −103 bp and −683 to −762 bp, respectively, displayed three similar closely associated protein–DNA complexes that appeared identical based on competition experiments (see Fig. 4). Examination of the sequences within these fragments revealed the presence of $(G)_6C$ in the coding strand of fragment *a* and in the non-coding strand of fragment *c* (Fig. 1A, Table 1; the complete sequence of these fragments is shown in Xiang *et al.* 1991). No other significant homologies were present in these two fragments (Xiang *et al.* 1991). Fragment *c* was used to determine whether the G-strings within these fragments interacted with nuclear proteins. Methylation interference analysis with blastula stage nuclear extracts and radiolabelled fragment *c* showed that at least one protein made strong contact with four Gs and weak contact with two Gs in the G_6-string of the non-coding strand of fragment *c* but with no other guanines in the coding or non-coding strands (Fig. 2). The G-string in fragment *c* (and by inference in fragment *a*) was thus a binding site for a nuclear protein.

These results implicated the G-strings in fragments *a* and *c* as potential *cis*-regulatory elements. Because 108 bp of 5′ flanking LpS1β DNA was sufficient for high levels of CAT activity, we focused on the element contained within fragment *a* at −70 bp to −75 bp to define more precisely the properties of the G-string regions (Fig. 1A). Using the −511 LpS1β–CAT construct, the G-string was replaced with a *Bgl*II site (AGATCT) and both wild-type and mutant constructs were injected into *L. pictus* eggs. Mesenchyme blastula, gastrula and prism stage embryos were analyzed for CAT activity with the same result. A representative example of mesenchyme blastula stage embryos is shown in Fig. 3. In this experiment, the mutant construct had approximately 1/30 the activity of the wild-type when an equivalent amount of plasmid DNA was present in the injected embryos (data not shown). To insure that the CAT gene had not been inactivated inadvertently during mutagenesis, the mutated fragment was excised from the original vector and re-ligated into a second pSV_o·CAT derivative. Activity from this construct also remained very low compared to the wild-type construct (data not shown).

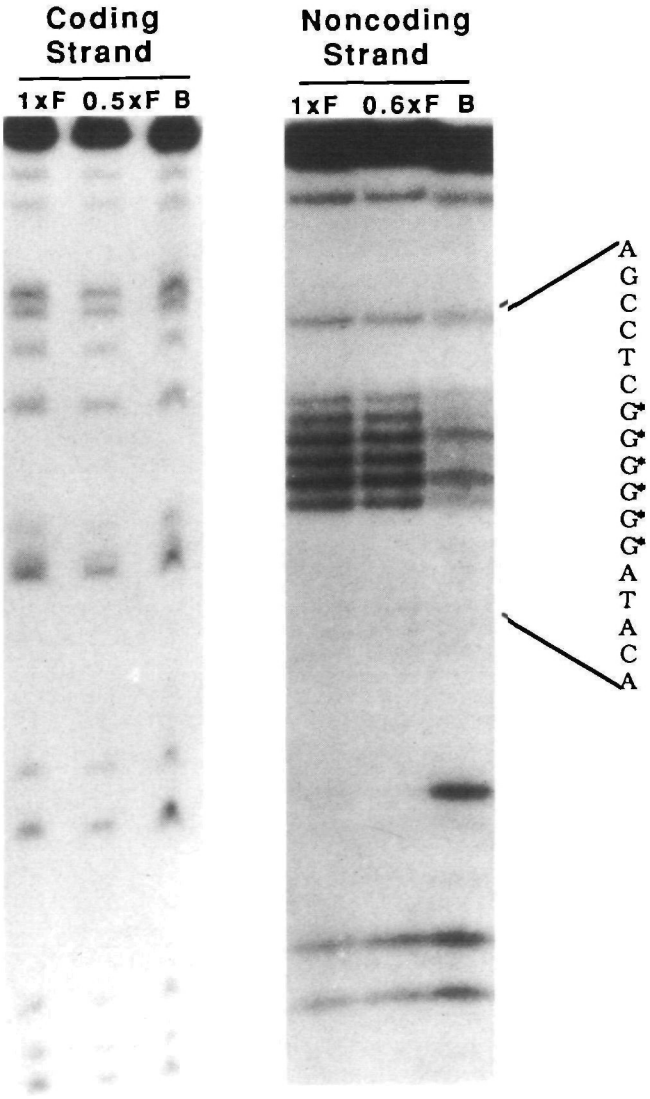

Fig. 2. Determination of the contact sites of fragment *c* with blastula stage nuclear extracts. The methylation interference assay was performed with radiolabelled fragment *c* coding and noncoding strands. Shown on the right is the DNA sequence surrounding the contacted guanine residues which are indicated by asterisks. F, free DNA probe; B, bound DNA probe.

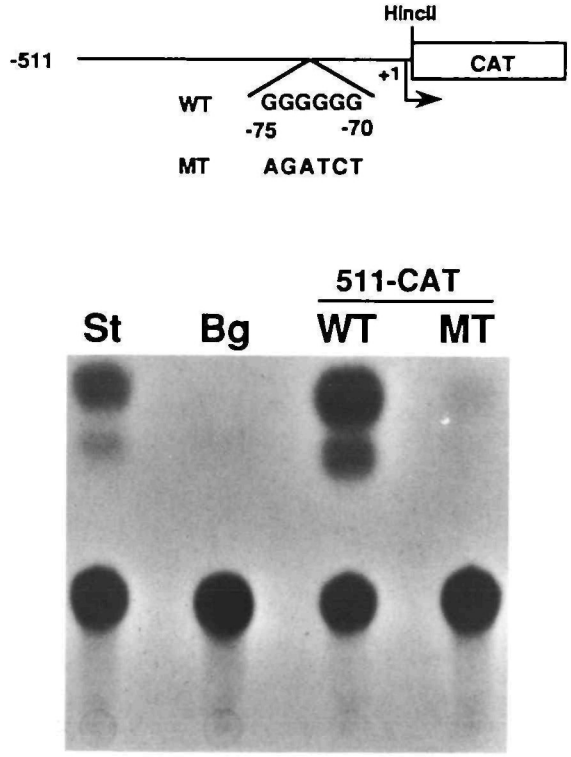

Fig. 3. Effect of a G-string site mutation on the CAT activity of the −511 LpS1β–CAT construct. To create the mutant construct (MT), the GGGGGG motif in the wild-type construct (WT) was mutated to an AT-rich *Bgl*II site, AGATCT. Both wild-type and mutant plasmids were injected into *L. pictus* eggs and CAT activity assayed with 340 mesenchyme blastula stage embryos for each plasmid. Bg, uninjected embryos; St, background embryos plus 0.1 unit of bacterial CAT.

These *in vivo* expression experiments identified the G-string at −70 bp to −75 bp as a major positive element in the LpS1β promoter. It was also possible that the removal of the G-string in fragment *c* was responsible for the three-fold drop in promoter activity observed between the −762 and −511 LpS1β–CAT constructs, although this has yet to be shown directly.

Two distinct factors bind the G-string elements

To examine the tissue distribution of proteins binding to the G-string regions, *L. pictus* plutei were fractionated into ectoderm or endoderm/mesoderm. Nuclear extracts prepared from these fractions were used in electrophoretic mobility shift experiments with fragment *a* as probe. Fig. 4 shows that both ectoderm and endoderm/mesoderm fractions contained nuclear proteins that bound fragment *a*. Ectoderm nuclear extracts formed three closely associated complexes with fragment *a*, and the formation of these complexes was effectively inhibited by either fragment *a* or fragment *c* but not by an unrelated oligonucleotide (CTB oligonucleotide) containing a USF binding site (Fig. 4A, lanes 1–5). Nuclear extracts from the endoderm/mesoderm fraction formed a different set of complexes with faster mobility (Fig. 4A, lane 6). Several lines of evidence supported the hypothesis that the factor present in the endoderm/mesoderm nuclear extracts was different from that in the ectoderm extracts. First, the binding of the endoderm/mesoderm factor to fragment *a* was inhibited by a 200-fold molar excess of fragment *a* but not by the same molar excess of fragment *c* (Fig. 4A, lanes 8, 9), whereas the binding of the ectoderm factor to fragment *a* was inhibited by the same molar excess of either fragment *a* or fragment *c* (Fig. 4A, lanes 4, 5). Second, the salt requirement for binding fragment *a* was different for the ectoderm *versus* endoderm/mesoderm factor. The ectoderm factor showed a salt optimum with a peak at 0.2 M KCl (Fig. 5A, B). In contrast, the endoderm/mesoderm factor showed a broader salt curve with optimal binding at 0.1 M KCl (Fig. 5A, B). Third, increasing concentrations of EDTA abolished the binding of the endoderm/mesoderm factor to fragment *a* but did not affect the binding of the ectoderm factor to the same fragment (Fig. 6A, B). Fig. 6B shows results when an *L. pictus* blastula stage nuclear extract was incubated with fragment *a* in different concentrations of EDTA; in these unfractionated blastula extracts both sets of complexes were observed (Fig. 6B, lane 2). However, 0.5 mM EDTA abolished the faster migrating (endoderm/mesoderm) complexes but had no effect on the slower (ectoderm) complexes (Fig. 6B, lanes 2–6).

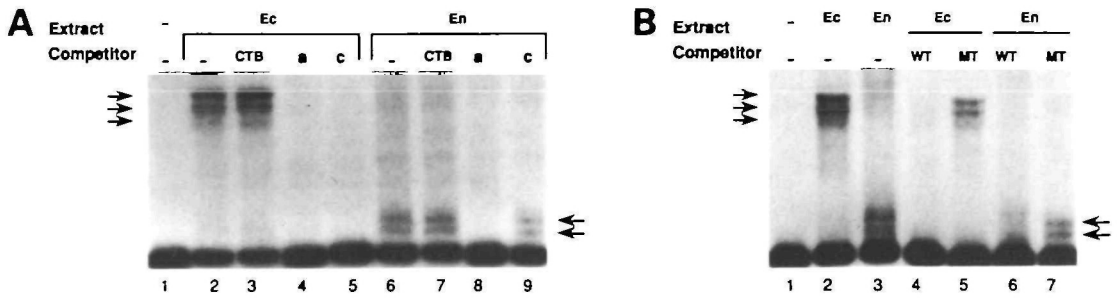

Fig. 4. Demonstration of two tissue-specific G-string factors in *L. pictus* embryos. Fragment *a* was used as the probe in panels A and B for electrophoretic mobility shift analysis with equal amounts of ectoderm (Ec) and endoderm/mesoderm (En) nuclear extracts prepared from *L. pictus* pluteus stage embryos. A 200-fold molar excess of CTB oligonucleotide, fragment *a* or fragment *c* were used for competition in A. A 150-fold molar excess of either wild-type (WT) or mutant (MT) G-string oligonucleotides were used for competition in B. Arrows indicate DNA–protein complexes.

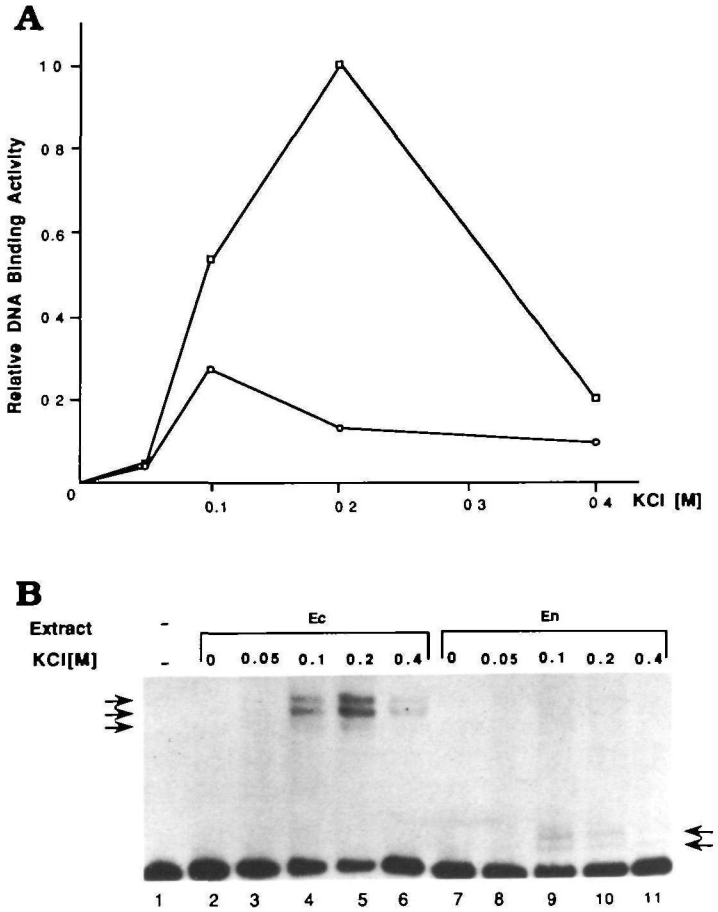

Fig. 5. Differential salt requirement of the G-string factors for optimal DNA-binding. Radiolabelled fragment a was utilized for electrophoretic mobility shift analysis with nuclear extracts from ectoderm (Ec) and endoderm/mesoderm (En) with KCl concentrations ranging from 0–0.4 M (B). Relative intensities of these complexes are shown in A. The open squares represent DNA-binding activity of the ectoderm G-string factor and the open circles represent DNA-binding activity of the endoderm/mesoderm G-string factor.

These experiments implied a metal co-factor was required for endoderm/mesoderm factor binding to DNA. Addition of Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, or Co^{2+} did not reverse the inability to bind (data not shown), suggesting that the endoderm/mesoderm factor may have been irreversibly denatured when its metal co-factor was removed. Finally, the endoderm/mesoderm factor did not appear to be a degradation product of the ectoderm factor since preincubation of whole embryo extracts for 30 min at 37° prior to the incubation with fragment *a* did not change the mobility of either set of complexes in an electrophoretic mobility shift assay (data not shown).

The ectoderm and endoderm/mesoderm factors also displayed different avidity for a 24-bp GC-rich oligonucleotide containing a $(G)_7$-string capable of binding the mammalian factor IF1 (Karsenty and de Crombrugghe, 1990, 1991). In Fig. 7, the formation of the ectoderm factor–fragment *a* complexes was strongly inhibited by either a 24 bp oligonucleotide containing the G-string region of fragment *a* (defined as the G-string oligonucleotide) or by the IF1 oligonucleotide, but not by mutant oligonucleotides lacking the G-strings (Fig. 7A, B, data not shown). In a parallel experiment, however, the endoderm/mesoderm complexes, while efficiently inhibited by the G-string oligonucleotide at molar ratios of 50:1, were inhibited only two-fold by the IF1 oligonucleotide at molar ratios of 200:1 (Fig. 7C, D).

While these results demonstrated the difference between the ectoderm and endoderm/mesoderm factors, both factors bound fragment *a* at the G-string region. This is shown in Fig. 4B where the 24-bp oligonucleotide containing the G-string region of fragment *a* effectively inhibited binding to fragment *a* of both ectoderm and endoderm/mesoderm factors (Fig. 4B, lanes 2, 3, 4, 6), while a 31-bp oligonucleotide that had the G-string replaced with a *Bgl*II site did not inhibit binding (Fig. 4B, lanes 5, 7). Of course the ectoderm and endoderm/mesoderm factors may have different binding affinities for the G-string region of fragment *a*. To address this issue, we estimated the relative affinity constants in a manner similar to that used by Calzone *et al.* (1988) to estimate equilibrium constants and number of nuclear molecules for factors binding to the sea urchin CyIIIa actin gene promoter. In this treatment, a parameter, Kr, is defined as the ratio of the equilibrium constant for the complex between a given factor and its specific site to that for the complex between the factor and the nonspecific competitor

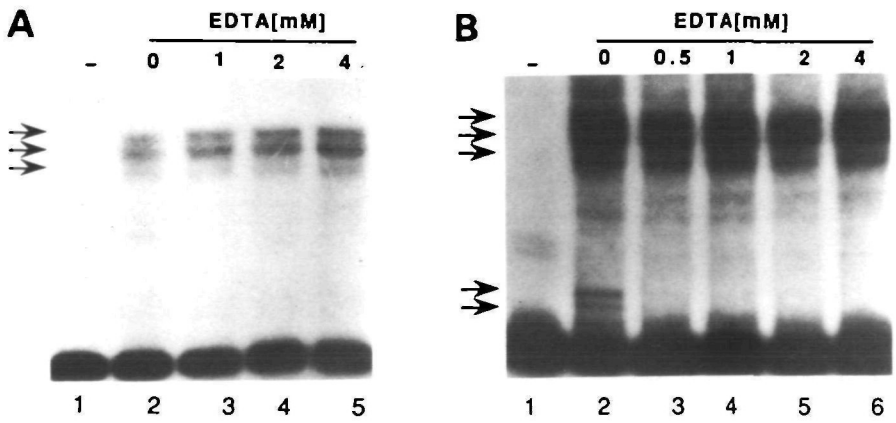

Fig. 6. Effect of EDTA concentration on DNA-binding of the G-string factors. Radiolabelled fragment *a* was used for electrophoretic mobility shift analysis with *L. pictus* ectoderm nuclear extracts (A), and blastula stage nuclear extracts (B) in the presence of 0–4 mM of EDTA as indicated.

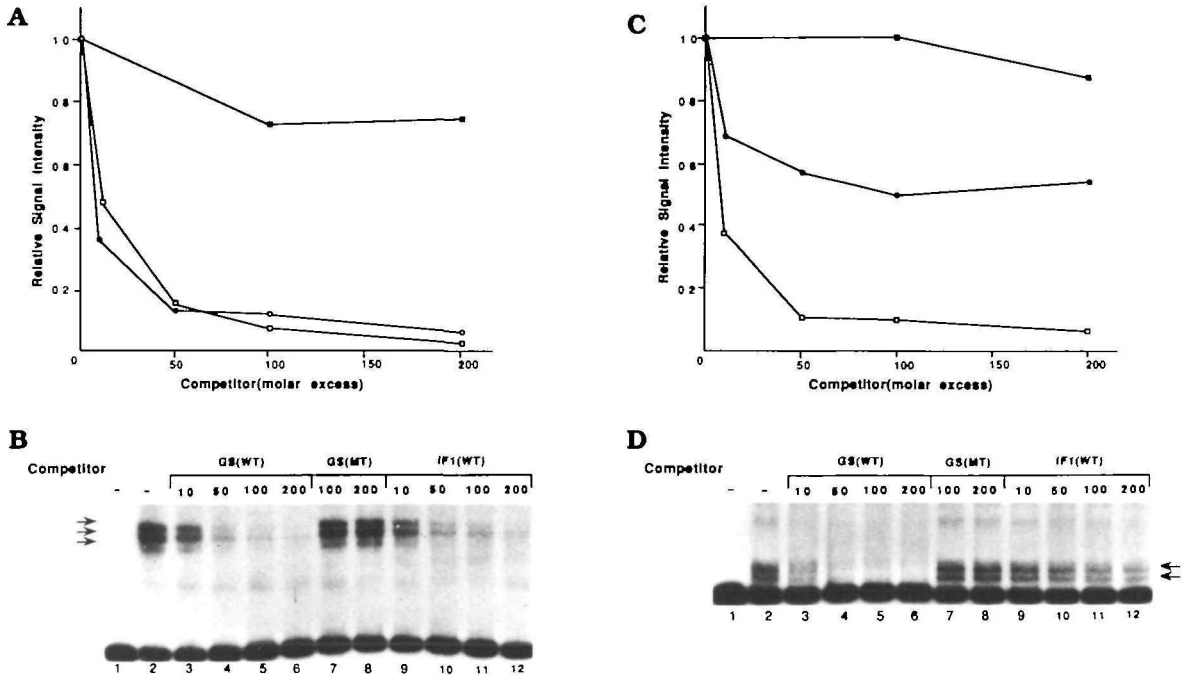

Fig. 7. Differential DNA-binding specificity of the two tissue-specific G-string factors. Radiolabelled fragment *a* was used for electrophoretic mobility shift analysis with ectoderm nuclear extracts (B) and endoderm/mesoderm nuclear extracts (D). The indicated molar excess of oligonucleotides GS(WT), GS(MT), and IF1(WT) were utilized for competition experiments. The intensity of the DNA–protein complexes was measured and quantified by densitometry, and the relative intensities are shown in A and C. The open and filled squares represent competition with GS(WT) and GS(MT), respectively. The open circles represent IF1(WT) competition.

DNA (Emerson *et al.* 1985; Calzone *et al.* 1988). Using the electrophoretic mobility shift data from Fig. 7A (lanes 2–6) and Fig. 7B (lanes 2–6), we generated Scatchard plots for the ectoderm and endoderm/mesoderm G-string factors, respectively. The slopes of these plots yielded Kr values of 2×10^5 for both factors. These values indicated that the ectoderm and endoderm/mesoderm factors bound to the G-string region of fragment *a* with high affinity relative to nonspecific DNA-binding and that the affinity of both factors for the G-string target site was very similar. From the x-intercept of the Scatchard plots, we also estimated that there were 2×10^5 copies per embryo of the ectoderm G-string factor and 10^5 copies per embryo of the endoderm/mesoderm factor (Calzone *et al.* 1988). These values implied that both factors were present at a few hundred copies per nucleus.

To summarize, our data suggested that the ectoderm factor had different target site specificity than the endoderm/mesoderm factor and that while the ectoderm factor was capable of binding the G-strings in fragments *a*, *c*, and the IF1 oligonucleotide, the endoderm/mesoderm factor would bind only the G-string of fragment *a*. Both factors appeared to bind fragment *a* with comparable affinities. As indicated in Table 1, the sequences surrounding the G-strings of fragment *a* and *c* and of oligonucleotide IF1 were all different. We have not yet determined the bases that are critical for factor binding. In this regard, mutating the G-string oligonucleotide from $(G)_6C$ to $(G)_3C(G)_3$ (Table 1), inhibited complex formation of fragment *a* with both the ectoderm and endoderm/mesoderm factors indicating that the G in position 4 of the $(G)_6$ string was not required for either factor to bind fragment *a* (data not shown). In addition, poly(dG-dC) weakly competed complex formation with the ectoderm factor and fragment *a* (data not shown; Table 1) suggesting that this factor has weak affinity for simple runs of G residues.

The ectoderm factor shares DNA-binding properties with IF1

IF1 is a mammalian transcription factor that binds to the mouse $\alpha1$(I) and $\alpha2$(I) collagen promoters and acts as an inhibitor of transcription (Karsenty and de Crombrugghe, 1990, 1991). Based on the sequence homology within their respective target sites (a match of 10 out of 12 bp, Table 1), their similar salt requirements for DNA-binding and their insensitivity to EDTA, it was possible that the sea urchin ectoderm factor had similar DNA-binding properties to IF1. To test this possibility, we performed a series of competition experiments, using a partially purified preparation of IF1 and radiolabelled oligonucleotides containing either the IF1 or ectoderm factor binding site (G-string oligonucleotide). When we used the IF1 oligonucleotide as a probe, we observed a complex similar to that previously reported (Fig. 8A, lane 3; Karsenty and de Crombrugghe, 1990, 1991). As expected, the formation of this complex was inhibited

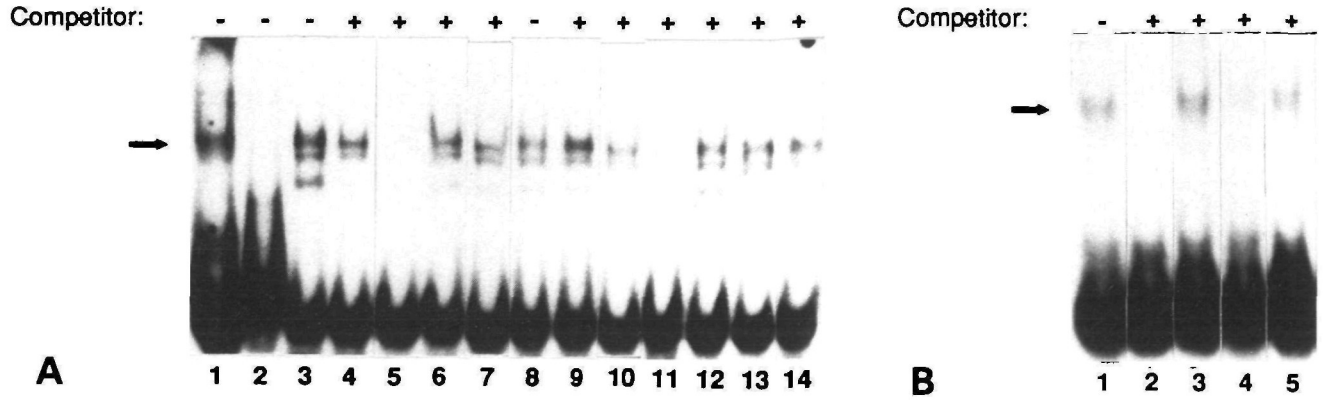

Fig. 8. Binding of IF1 and G-string oligonucleotides to DNA affinity purified IF1. Partially purified IF1 was used in all lanes. (A) Lane 1, G-string oligonucleotide as probe, lane 2 mutant G-string oligonucleotide as probe. Lanes 3–14, IF1 oligonucleotide as probe with no competior (lanes 3, 8); 10- and 25-fold molar excess of IF1 oligonucleotide (lanes 4, 5); or mutant IF1 oligonucleotide (lanes 6, 7); 10-, 25- and 100-fold molar excess of G-string oligonucleotide (lanes 9–11); or mutant G-string oligonucleotide (lanes 12–14). (B) G-string oligonucleotide as probe (lane 1); 50-fold molar excess of G-string oligonucleotide (lane 2); or mutant G-string oligonucleotide (lane 3); or IF1 oligonucleotide (lane 4); or mutant IF1 oligonucleotide (lane 5). Arrows indicate the specific complexes.

by a 50-fold molar excess of unlabelled IF1 oligonucleotide but not by the same molar excess of a mutant IF1 oligonucleotide which cannot bind IF1 (Karsenty and de Crombrugghe, 1990) (Fig. 8A, lanes 4–7). This complex was also inhibited by a 100-fold molar excess of the G-string oligonucleotide containing the binding site for the factor present in the ectoderm nuclear extracts (Fig. 8A, lanes 9–11). In contrast, the G-string mutant oligonucleotide was unable to compete for binding (Fig. 8A, lanes 12–14).

When we used the G-string oligonucleotide as a probe with the partially purified IF1 preparation, we observed a complex migrating at the same location as the complex formed between IF1 and the IF1 oligonucleotide (Fig. 8A, lane 1; Fig. 8B, lane 1). The formation of this complex was inhibited by a 50-fold molar excess of the G-string oligonucleotide, (Fig. 8B, lane 2), but not by a 50-fold molar excess of the mutant G-string oligonucleotide (Fig. 8B; lane 3). The mutant oligonucleotide did not bind any protein present in the DNA affinity purified IF1 preparation (Fig. 8A, lane 2). The G-string–IF1 complex was also competed significanty by a 50-fold molar excess of IF1 oligonucleotide but not by the same molar excess of the mutant IF1 oligonucleotide (Fig. 8B, lanes 4, 5). Taken together these experiments suggest that the factors that were purified using an IF1 binding site as an affinity resin substrate shared DNA-binding properties with the ectoderm G-string factor. While these experiments are by no means definitive, they suggest that IF1 and the ectoderm factor may be related proteins.

Discussion

The above experiments were aimed at identifying *cis*-regulatory elements and *trans*-acting factors associated with the LpS1β gene promoter that could play a role in aboral ectoderm specific expression. We characterized at least one *cis*-regulatory element at −70 to −75 bp with six guanines as its core sequence. Mutations that removed these guanines abolished both the promoter activity and its ability to interact with two distinct nuclear factors, one present in ectoderm cells and one in endoderm/mesoderm cells. A second, more distal G-string motif at −726 to −719 bp appeared to interact only with the ectoderm G-string factor. The role of this second G-string motif is uncertain, though a deletion from −762 to −511 bp, which removed the G-string, had one third the promoter activity and altered spatial expression. Based on the results presented here, the proximal G-string element is a strong positive regulatory site for LpS1β. A simple interpretation of our data is that the ectoderm G-string factor serves as a transcriptional activator by binding to the proximal G-string element.

LpS1β is active only in aboral ectoderm cells (Xiang *et al.* 1988, 1991; Tomlinson and Klein, 1990), but embryo fractionation yields a mixture of aboral ectoderm and oral ectoderm cell types, along with minor amounts of pigment cells (Table 2). If the ectoderm G-string factor was an activator of LpS1β only in aboral ectoderm cells, then this factor must be absent or, if present, unable to activate LpS1β in the other cell types included in the ectoderm fraction.

The role of the endoderm/mesoderm G-string factor is less easy to interpret. Since virtually no DNA-binding activity, as determined by the presence of the fast-migrating complexes, was observed in the ectoderm fraction, the endoderm/mesoderm factor was either absent from ectoderm cell types or converted to the slower migrating complexes. Such interconversion does not readily take place *in vitro* since whole embryo nuclear extracts from blastula, gastrula or prism stage routinely show the presence of both sets of complexes, as does mixing equal amounts of ectoderm and endoderm/mesoderm nuclear extracts. Moreover, the biochemical characteristics of the two factors, particu-

Table 2. *Properties of ectoderm and endoderm/mesoderm G-string factors*

Factor[1]	Possible cell types[2]	Sequence affinity[3]	DNA-binding optimum (M KCl)	EDTA sensitivity (mM)
ecto GSF	AOE, OE, Pigment cells	*a*, *c*, IF1 G_3CG_3, poly(dG-dC)	0.2	>4
endo/meso GSF	PMC, SMC EN	*a*, G_3CG_3	0.1	≤0.5

[1] ecto GSF is the ectoderm G-string factor; endo/meso GSF is the endoderm/mesoderm G-string factor.
[2] AOE, aboral ectoderm; OE, oral ectoderm including ciliary band cells, diverse cells of the oral surface and neuronal sensory cells; PMC, primary mesenchyme cells; SMC, secondary mesenchyme cells including pigment and muscle cells; EN, endoderm.
[3] See Table 1 for a description of these sequences.

larly the differences in EDTA sensitivity, suggest the factors are separate proteins.

Although the endoderm/mesoderm G-string factor could bind to the proximal G-string element on LpS1β, it cannot serve as an activator because, as we have shown previously, this gene is not transcriptionally active in endoderm/mesoderm nuclei (Tomlinson and Klein, 1990). We also showed that the DNA-binding specificity of the endoderm/mesoderm factor is distinct from the ectoderm factor (Table 2). Thus, the endoderm/mesoderm factor may either be a repressor at this target site or be non-functional with respect to LpS1β. The rapid migration of the endoderm/mesoderm G-string factor complexes in electrophoretic mobility shift experiments suggests that this factor is a low molecular weight protein. Similar rapidly migrating complexes have been observed with GC-rich regions from the murine MCK gene promoter and nuclear extracts of C2 myoblasts (E. N. Olson, personal communication) and from the *neu* oncogene promoter and a breast cell carcinoma nuclear extract (Zhao and Hung, 1992). In addition, rapidly migrating complexes have been observed for nonGC-rich *cis*-regulatory elements, for example, the complex formed between the positive regulatory domain II of the human interferon β gene promoter and the C-terminal fragment of the zinc-finger DNA-binding protein PRDII-BF1 (Fan and Maniatis, 1988).

L. pictus has two LpS1 genes. The LpS1α gene is closely related to LpS1β and its 5′ flanking DNA sequence is virtually identical to LpS1β for 326 bp (Xiang *et al.* 1991). Thus, LpS1α has the proximal G-string element but not the distal one. In *S. purpuratus*, the 5′ flanking DNA of the aboral ectoderm specific Spec1 and Spec2 genes have little in common with the LpS1 genes (Hardin *et al.* 1985, 1988; Gan *et al.* 1990*b*; Xiang *et al.* 1991). The Spec2a gene promoter contains a strong enhancer-like region between −800 bp and −400 bp (Gan *et al.* 1990*a*,*b*). This enhancer-like region appears to consist of multiple elements and is itself within an 800 bp conserved repetitive sequence block shared among all the *S. purpuratus* Spec1 and Spec2 genes analyzed thus far (Gan *et al.* 1990*a*,*b*). Within the enhancer region of Spec2a is a $(C)_2G(C)_5$ stretch at −645 bp to −638 bp. A 5′ deletion to −613 bp in the Spec2a promoter shows 50 % less promoter activity than a deletion to −658 bp, suggesting that this GC-rich region may also function in the Spec2a promoter as a positive *cis*-regulatory element (L. Gan and W. Klein, unpublished observations). Recently, Shimada and his co-workers have identified nuclear proteins that bind to G-strings in the 5′ flanking DNA of the aboral ectoderm-specific arylsulfatase gene of *Hemicentrotus pulcherrimus* (Sasaki *et al.* 1988; Akasaka *et al.* 1990; H. Shimada, personal communication). Thus, G-string elements may regulate the expression of unrelated genes in the aboral ectoderm.

Several nuclear proteins that bind to G-rich sequences have been characterized previously. Sp1, a well-described, ubiquitous, zinc finger transcription factor binds to the consensus sequence motif G/T G/T GGCG G/T G/A G/A C/T (Dynan and Tjian, 1985). The Krox-20 gene product contains three Zn^{2+} fingers similar to those of Sp1 and is a serum-inducible transcriptional activator possibly involved in hindbrain development (Wilkinson *et al.* 1989). An erythrocyte-specific nuclear factor of unknown function, BGP1, is immunologically related to but distinct from Sp1 and binds to the poly(dG) region of the chicken β-globin gene promoter (Lewis *et al.* 1988). Another Sp1-like factor is ETF, which binds G-rich regions on the epidermal growth factor receptor gene and apparently binds GC elements not recognized by Sp1 (Kageyama *et al.* 1988*a*,*b*). It is likely that the genes encoding many of these proteins will constitute a multigene family of zinc finger DNA-binding proteins.

In contrast, a human DNA-binding factor, called GCF, binds to G-rich promoter elements present in the epidermal growth factor receptor, β-actin and calcium-dependent protease gene promoters and acts as transcriptional repressor (Kageyama and Pastan, 1989). The sequence of the cDNA clone for GCF reveals that it is unrelated to Sp1 and that it is not a zinc finger protein (Kageyama and Pastan, 1989). Our results indicate that the ectoderm G-string factor does not require metal ions to bind to DNA. The sequence of its binding site, its salt requirement and insensitivity to EDTA are features shared with the mammalian DNA-binding protein IF1 (Karsenty and de Crombrugghe, 1990, 1991), which binds to a G-rich sequence present in the promoter region of the mouse α1(I) and α2(I) collagen genes. DNA affinity purified IF1 preparations bind to both the IF1 and LpS1 G-string oligonucleo-

tides. Because EDTA was used in purifying IF1, the preparation should be devoid of metalloproteins requiring metal ions to bind DNA (G.K. unpublished observations). The competition experiments shown in Fig. 8 indicate that at least one factor present in the IF1 DNA affinity purified preparation binds to both the IF1 and G-string oligonucleotide and that the DNA–protein complexes migrate to the same location with both oligonucleotides. While not proven, IF1 and the ectoderm G-string factor may be related.

A major difference between the two factors derives from functional assays. We have shown here that a mutation in the proximal G-string of the LpS1β promoter decreased the affinity of the ectoderm G-string factor for the promoter and correspondingly abolished promoter activity suggesting that the ectoderm factor activates transcription. However, results of transfection experiments using either wild-type α1(I) or α2(I) CAT chimeric genes or mutant α1(I) or α2(I) CAT chimeric genes suggest that IF1 is an inhibitor of transcription (Karsenty and de Crombrugghe, 1990, 1991). Thus, IF1 and the ectoderm G-string factor could have different biological roles and be different proteins recognizing similar binding sites. Alternatively it is possible that the two factors are identical and that their mechanism of action is different on different promoters. This has been demonstrated in the case of the ultrabithorax protein in *Drosophila* which activates its own promoter but inhibits the antennapedia promoter (Krasnow *et al.* 1989). Similarly, the glucocorticoid receptor, usually an activator of transcription, can act as a transcriptional repressor of the bovine prolactin gene (Sakai *et al.* 1988). At their present stage of characterization, it is not possible to determine more precisely the relationship between IF1 and the ectoderm G-string factor. Questions regarding their relationship can be approached when these factors are purified to homogeneity or when cDNA clones are available.

Our original interest in attempting to identify cell-type specific *trans*-acting factors associated with LpS1 and Spec gene promoters was to determine the mechanisms by which such factors become spatially restricted during embryogenesis. Preliminary experiments show that the ectoderm G-string factor is not present in extracts of unfertilized eggs but can be observed in blastula stage nuclear extracts (data not shown; Fig. 6B), whereas the endoderm/mesoderm factor appears to be present in unfertilized eggs. A major question to be addressed in future experiments is how these factors become non-uniformly distributed during embryogenesis.

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