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 LpSla and J 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 a1(I) and a2(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/LpSl 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; Kołowski 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 LpSβ gene promoter. We show that a guanosine-rich, or ‘G-string’, motif located 70 to 75 base pairs upstream of the LpSβ 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 LpSβ 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 a1(I) and a2(I) collagen promoters (Karsenty and de Crombrugghe, 1990, 1991).

**Materials and methods**

**Plasmid constructs**

The −511 LpSβ-CAT and −108 LpSβ-CAT plasmid constructs were generated from the Bluescript vector (Stratagene) and pSVβ-CAT (Gorman et al. 1982) as follows: A 1635-bp HindIII–BamHI fragment containing the CAT reporter gene and SV40 polyadenylation site was isolated from pSVβ-CAT and cloned into the HindIII and BamHI sites of Bluescript II SK(+) to create pCATo. A 5' deletion containing LpSβ 5' flanking regions from +17bp to −511 bp (Xiang et al. 1991) was then inserted in a forward orientation into the SalI site of pCATo to generate −511 LpSβ-CAT. The −108 LpSβ-CAT construct was subsequently produced by digestion of −511 LpSβ-CAT with MluI (a single site at −108bp) and KpnI (a single site in pCATo).

**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 LpSβ-CAT using an M3K07 helper phage (Promega, Biotec.). A G-string mutant oligonucleotide, GS(MT), containing LpSβ sequence from −57 to −88 bp with the GGGGGG motif mutated to an AT-rich site, was phosphorylated and annealed to the T7 DNA polymerase using 5-methyl-dCTP, and ligated with T4 DNA ligase. The unmethylated parental DNA strands were subsequently cut by MspI and digested with exonuclease III. The circular, methylated mutant DNA strands were used to transform Escherichia coli SDM cells. Isolated mutant −511LpSβ-CAT plasmids were confirmed by BglII 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. 1990a; 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. pCATo and −511 LpSβ-CAT were linearized with XhoI and KpnI. The −108 LpSβ-CAT construct was released from −511 LpSβ-CAT by digestion with MluI and KpnI and was gel purified before injection.

Microinjected embryos were harvested at mesenchyme blastula, gastrula or prism/pluteus stage and with 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. 1990a).

**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-32P] ATP and T4 polynucleotide kinase. Approximately 5×10^6 cts min^{-1} of radiolabelled DNA (1–5 fmols 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 μg 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% non-denaturing 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 10^3 cts min^{-1} (5 fmols) 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 mM KCl, 0.1 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin and were electrophoresed on a 5% non-denaturing polyacrylamide gel.

**Methylation interference assay**

A 5' end labelled EcoRI–HindIII fragment containing the
LpSIβ upstream sequence from +17 to −762 bp was digested with Ddel (at −683 bp) to generate the EcoRI–Ddel fragment c with only the coding strand labelled at its 5′ end (Fig. 1A). The 5′ end-labelled fragment c was then cut with TaqI (at −745 bp) to create a fragment with only the non-coding strand labelled at its 5′ end. Approximately 1×10^4 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 1×10^4 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 purified 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 μg ml^{-1} and leupeptin 5 μg 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 μg 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 LpSIβ gene promoter

In a previous study, we microinjected LpSIβ 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 LpSIβ 5′ deletion constructs. (A) Schematic illustration of the LpSIβ promoter region. The arrow indicates the transcription initiation site, +1. Fragment a is a 120-bp HincII–Rsal fragment containing the LpSIβ sequence from +17 to −103 bp and fragment c represents the 80-bp Ddel–EcoRI 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 LpSIβ deletions −511, −368 and −108 used in a previous report (Xiang et al. 1991) and in this report are marked. D, Ddel; E, EcoRI; H, HincII; R, RsaI. (B) Diagram of the relative CAT activities of LpSIβ–CAT constructs. (C) Comparison of CAT activity between −108 LpSIβ–CAT and −511 LpSIβ–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.

LpSIβ gene expression apparently were between −108 bp and +17 bp. To investigate the LpSIβ promoter further, we monitored DNA–nuclear protein interactions in vitro by mixing DNA fragments generated from +17 bp to −762 bp of the LpSIβ gene with nuclear extracts from
Table 1. DNA sequences that inhibit fragment a binding to the ectoderm G-string factor

<table>
<thead>
<tr>
<th>Oligonucleotide or fragment</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>a, G-string</td>
<td>...TCTTCGAGCATG<em>GGC</em>GCTG...</td>
</tr>
<tr>
<td>c1</td>
<td>...CATAACAGCTTACGGCGGCGGCGG...</td>
</tr>
<tr>
<td>IF1</td>
<td>...TTTGGGGAGGGGGCGCTGGGTG...</td>
</tr>
<tr>
<td>G3CG3</td>
<td>...TCTTCGAGCATG<em>GGC</em>GCTG...</td>
</tr>
<tr>
<td>Poly(dG-dC)</td>
<td>...GGGGGGGGGGGGGGGGGGGGGG...</td>
</tr>
</tbody>
</table>

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. Asterisks 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-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 BglII 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 pSVα-CAT derivative. Activity from this construct also remained very low compared to the wild-type construct (data not shown).
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).

![Diagram](image-url)
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 cofactor 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 cofactor 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°C 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 IFl (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 IFl 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 IFl 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 BglII 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 Cyt IIIa actin gene promoter. In this treatment, a parameter, K_r, 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 \(K_r\) values of \(2 \times 10^5\) 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 \(2 \times 10^5\) 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)\(_6\)C to (G)\(_3\)C(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 \(a1(I)\) and \(a2(I)\) 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...
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 significantly 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 LpSlβ 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 LpSlβ. 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.

LpSlβ 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 LpSlβ only in aboral ectoderm cells, then this factor must be absent or, if present, unable to activate LpSlβ 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

<table>
<thead>
<tr>
<th>Factor</th>
<th>Possible cell types</th>
<th>Sequence affinity</th>
<th>DNA-binding optimum (M KCl)</th>
<th>EDTA sensitivity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ecto GSF</td>
<td>AOE, OE, Pigment cells</td>
<td>a, c, IF1</td>
<td>0.2</td>
<td>&gt;4</td>
</tr>
<tr>
<td>endo/meso GSF</td>
<td>PMC, SMC EN</td>
<td>a, G3CG3</td>
<td>0.1</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

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.

G-string nuclear DNA-binding factors

Laurel 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 LpSlβ, 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 LpSlβ. 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 /3 gene promoter and the C-terminal fragment of the zinc-finger DNA-binding protein PRDII-BF1 (Fan and Maniatis, 1988).

L. pictus has two LpSl genes. The LpSlα gene is closely related to LpSlβ and its 5' flanking DNA sequence is virtually identical to LpSlβ for 326 bp (Xiang et al. 1991). Thus, LpSlα 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 LpSl genes (Hardin et al. 1985, 1988; Gan et al. 1990b; Xiang et al. 1991). The Spec2a gene promoter contains a strong enhancer-like region between -800 bp and -400 bp (Gan et al. 1990a,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. 1990a,b). Within the enhancer region of Spec2a is a (C)3G(C)3 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 -688 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/A C/T (Dyman and Tjian, 1985). The Krox-20 gene product contains three Zn2+ 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. 1988a,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 IFI (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 IFI preparations bind to both the IFI and LpSl 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 LpSlβ 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 (Krasnock et al. 1989). Similarly, the glucocorticoid receptor, usually an activator of transcription, can act as a transcriptional repressor of the bovine prolactin gene (Krasnow et al. 1990b). Regulatory elements from the related Spec genes of Strongylocentrotus purpuratus yield different spatial patterns with a lac Z reporter gene. Devi Biol. 142, 346–359.

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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References


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