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Ectoderm nuclei from sea urchin embryos contain a Spec–DNA binding protein similar to the vertebrate transcription factor USF

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Summary

The Spec gene family of *Strongylocentrotus purpuratus* is expressed exclusively in aboral ectoderm cells during embryogenesis. To investigate the regulation of Spec gene activity, the region around the Spec1 transcriptional initiation site was analyzed for sites of protein–DNA interaction. One high-affinity site bound a factor termed SpF1 within the Spec1 5′ untranslated leader region at position +39 to +60. The core sequence recognized by SpF1, CACGTG, is the same as that of the upstream stimulatory factor (USF), a widely occurring vertebrate transcription factor containing a *myc*–HLH motif. A comparison of USF- and SpF1-binding activities suggested that SpF1 was a sea urchin version of USF. SpF1 activity was detectable only in ectoderm cells of the embryo, implying that it has a role as a cell type-specific transcription factor. SpF1-binding sites were also found upstream of the Spec2a and Spec2c genes in the same conserved sequence block as Spec1. Extracts from *Lytechinus pictus* embryos showed an SpF1-like activity, suggesting that SpF1 is conserved in sea urchins. Surprisingly, changes in the Spec1, Spec2a, or Spec2c genes that removed or modified the SpF1-binding site had no effect on expression when reporter gene fusions containing these mutations were injected into sea urchin eggs and analyzed for expression during embryogenesis. We propose that, while SpF1 may not be essential for expression of the exogenously introduced reporter genes, it may be required for proper regulation of the endogenous Spec genes.

Key words: sea urchin embryos, embryonic ectoderm cells, USF transcription factor, Spec genes.

Introduction

In sea urchins, cell lineages arising from the animal-most two-thirds of the unfertilized egg contribute solely to the embryonic ectoderm. By the larval pluteus stage (3 days after fertilization in *Strongylocentrotus purpuratus*), several ectodermal cell types are distinguishable. These include aboral ectoderm, a continuous sheet of approximately 470 cells covering the larval surface, and a variety of oral ectoderm cell types such as those constituting the neural ectoderm, ciliary band and stomadeum (Davidson, 1986). Because of its simplicity, the aboral ectoderm has been the focus of several investigations in recent years (Lynn *et al.* 1983; Hurley *et al.* 1989; Cameron *et al.* 1989; Nisson *et al.* 1989). It derives from 11 founder cells, one of which arises at the third, four at the fifth, and six at the sixth cleavage division (Cameron *et al.* 1987; 1989). Each of these founder cells contributes a distinct patch of cells on the larval surface; hence the aboral ectoderm is a composite of several invariant lineages (Cameron *et al.* 1987; 1989). However, in spite of this invariance, blastomeres destined to become aboral ectoderm in the normal embryo can be experimentally manipulated to form other cell types, including those associated with oral ectoderm and more vegetal cell types such as gut, muscle, pigment and skeletal tissue. (Horstadius, 1973; Davidson, 1989; Livingston and Wilt, 1989) The remarkable plasticity of aboral ectoderm precursors suggests that the mechanisms giving rise to this cell type in the early embryo cannot simply be cytoplasmic segregation of maternal factors. A recent review by Davidson (1989) addresses these issues in more detail.

Approaching the problem of aboral ectoderm specification requires the appropriate molecular markers, and many genes that are activated exclusively in this cell type have now been isolated and characterized (Cox *et al.* 1986; Hardin *et al.* 1988; Yang *et al.* 1989). We have been using the Spec genes as markers for aboral ectoderm differentiation. This small gene family, encoding intracellular calcium-binding proteins, is activated at the late cleavage–early blastula stage (Tomlinson and Klein, 1990). Spec mRNA accumulation is highly specific to aboral ectoderm cell lineages (Lynn *et al.* 1983; Carpenter *et al.* 1984; Hardin *et al.* 1988; Tomlinson and Klein, 1990). Our studies have been directed towards identifying the DNA sequence elements responsible for transcriptionally activating the Spec genes in aboral ectoderm cells and towards isolating the protein factors that recognize these elements.

Elucidating the ontogeny of such proteins and the mechanisms that activate them in the egg and early embryo would greatly increase our knowledge of both how the aboral ectoderm cell type originates and the basis of its plasticity.

Recently, we have focused our attention on the structure and expression of three Spec genes: Spec1, Spec2a, and Spec2c (Hardin *et al.* 1988; Gan *et al.* 1990*b*). By aligning these genes with a common upstream 600 bp repetitive DNA sequence element, termed RSR, we showed that a conserved DNA block of approximately 800 bp extends from the 3′ end of the first exon to the 5′ end of the RSR element. In Spec2a, the conserved sequence block is a continuous stretch of DNA, but in Spec1 and Spec2c, 2.4 to 2.9 kb of inserted DNA interrupts the conserved sequence block, thus changing the relative placement of the RSR element and other 5′ flanking DNA. Deletion of the 5′ half but not the 3′ half of the RSR element causes a significant decrease in the chloramphenicol acetyl transferase (CAT) activity induced by Spec–CAT reporter gene fusions injected into *Lytechinus pictus* eggs. Furthermore, this element has several properties suggesting that it is an enhancer-like element important for Spec gene expression (Gan *et al.* 1990*b*).

Here we continue our analysis of the Spec gene conserved sequence block. We demonstrate the presence of a DNA-binding protein in sea urchin embryos, termed SpF1, that by several criteria is similar to the human transcription protein called upstream stimulatory factor (USF). A single SpF1-binding site is located within the conserved sequence block of each Spec gene, but because of the unusual organization of the sequences flanking these genes, the SpF1-binding site is positioned very differently in each gene with respect to the site of transcriptional initiation. SpF1-binding activity was detected only in nuclear extracts from embryonic ectoderm cells; no activity was observed in endoderm or mesoderm cells despite the fact that these latter extracts contained other DNA–protein binding activities. Unexpectedly, mutations in any of the three Spec genes that abolished SpF1–DNA binding *in vitro* had no effect on expression of Spec–CAT reporter genes when these genes were microinjected into *L. pictus* eggs. Although SpF1 is clearly an embryonic ectoderm-specific DNA-binding protein, its ability to function as a transcription factor remains to be determined.

Materials and methods

Sea urchins

S. purpuratus and *L. pictus* were obtained from Marinus (Venice, CA). Gametes were collected from gravid adults by intracoelomic injection of 0.5 M KCl. Eggs were washed in artificial sea water and fertilized with dilute suspensions of sperm. Embryos were cultured at 15 °C with constant stirring at concentrations of 6–8000 embryos ml^{-1}.

Ectodermal cells were separated from endodermal/mesodermal cells according to the procedures of McClay (1986).

Oligonucleotides

The following oligonucleotides were synthesized in an Applied Biosystems 380B DNA synthesizer:

CTB: 5′AGCGTATGGTCACGTGCTTTAT3′
3′TCGCATACCAGTGCACGAAATA5′
CT1+2: 5′CTTTATTTCAGGAGTTATCACTC3′
3′GAAATAAAGTCCTCAATAGTGAG5′
CT3+4: 5′AGCAACGACATTCCTAGCGTA3′
3′TCGTTGCTGTAAGGATCGCAT5′
CTC-1: 5′ATTCCTAGCGTACAAGATCTCACTTTATTT-CAGG3′
CTC-2: 5′CCGCGCATGCTCAGATTG3′
CTD-1: 5′ATTCCTAGCATACAAGATCTCACTTTATTT-CAGG3′
CTD-2: 5′GCCCCCATTGTGCGCTCC3′

CTB corresponds to the sequence recognized by SpF1. CT1+2 and CT3+4 border and overlap six nucleotides on each side of CTB. CTC-1 and CTC-2 were used in the *in vitro* mutagenesis experiments for Spec1. CTD-1 and CTD-2 were used in the *in vitro* mutagenesis experiments for Spec2a. The underlined portions of CTC-1 and CTD-1 indicate the changed nucleotides, each of which contains a *Bgl*II sequence (AGATCT).

Plasmids

Clone 191H-S is a 191 bp *Hin*PI–*Sal*I fragment from −58 to +133 of the Spec1 gene. Clone 298N-S is a 298 bp *Nsi*I–*Sal*I fragment from −164 to +133 of Spec1 and clone 255R-N is a 255 bp *Eco*RI–*Nsi*I fragment from −420 to −165 of Spec1. Clone 102H-N is a 102 bp fragment from −73 to +18 of Spec2a plus 11 bp of polylinker from pUC18. Spec1 RNA was probed with a 390 bp cDNA insert extending from exons 2 through 5 of Spec1 (Carpenter *et al.* 1984). Endoderm/mesoderm RNA was probed with a 720 bp *Bam*HI–*Eco*RI DNA fragment representing a portion of the codogenic region of the cytoplasmic actin gene *Cylla* (Shott *et al.* 1984). Fragment B is a 151 bp *Hpa*II–*Bst*NI fragment that contains the SM50 factor B binding site (Davidson, 1989). The 'P6' clone is a 210 bp *Rsa*I fragment that contains the P6 binding site of the CyIIIa actin gene (Thèze *et al.* 1990).

Nuclear extract preparation

Nuclei were isolated from embryos at the indicated stages of development as described previously (Tomlinson and Klein, 1990). Nuclear extracts were prepared from the purified nuclei (Parker and Topol, 1984; Morris *et al.* 1986). The nuclei were suspended in 5 vol of buffer A (25 mM Hepes, pH 7.8, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF and homogenized with two strokes of a Dounce B pestle. The homogenate was transferred to a tube containing 0.1 vol of 4 M $(NH_4)_2SO_4$, gently rocked under ice for 30 min and then centrifuged at 136000 *g* for 60 min. The supernatant was transferred to a new tube containing 0.25 g $(NH_4)_2SO_4$ ml^{-1} extract and centrifuged at 136000 *g* for 15 min at 4 °C. The nuclear pellet was suspended in 1 ml of buffer C (15% glycerol, 25 mM Hepes, pH 7.8, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) with two strokes of a 2 ml Teflon pestle. The nuclei were dialyzed against 300 vol of buffer C for 1 h. The extract was cleared by a 2 min spin in a microfuge and stored at −70 °C in small volumes.

SpF1 proved to be a heat-stable protein, as is the case for USF. SpF1 was purified approximately 10-fold by utilizing a heat-treatment purification scheme performed as described by Sawadogo *et al.* (1988).

Band shift gel analysis

Radiolabeled DNA was prepared by the polynucleotide kinase procedure. Terminal phosphate groups were removed by incubating 2–4 μg DNA in 50 μl 10 mM Tris, pH 8.0, and 1 mM EDTA with 0.2 unit of bacterial alkaline phosphatase at 65°C for 1 h. Following extraction and precipitation, one hundred to three hundred nanograms of DNA was end-labeled in a total volume of 50 μl at 37°C for 30 min. The reaction mix contained 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 15 mM DTT, 0.33 μM ATP, 150 μCi [gamma-^{32}P]ATP (ICN; Irvine, CA) and 5 units of T4 polynucleotide kinase.

Protein-binding reactions were done on ice in a total volume of 20 μl. Nonspecific protein binding was eliminated by incubating 2–8 μg crude nuclear extract in 5 mM Hepes, pH 7.8, 87 mM NaCl, 2.5 mM KCl, and 1 mM $MgCl_2$ with 100 μg ml^{-1} dI-dC for 15 min. Competition reactions were performed by adding the indicated amounts of competitor to the reaction tubes for an additional 15 min. One to two nanograms of the appropriate radioactively labeled DNA (20–40 000 cts min^{-1}) was then added and incubated for 15 min. The reactions were loaded onto 5 % acrylamide gels in 0.25×TBE (1×TBE:89 mM Tris, 89 mM boric acid, and 2 mM EDTA) and run at 4°C in 0.25×TBE gel-running buffer. The gels were fixed in 10 % acetic acid and 10 % methanol and set up for autoradiography.

DNase I protection assays

The protein-binding reactions were carried out as described above for the band shift gels, except the reaction buffer also contained 1 mM DTT and 0.2 mM PMSF. At the end of the binding reaction, 3 μg DNase I was added to the tube while on ice. After 1 min, the DNase I was stopped with 30 μl of 0.6 M NaCl, 0.4 % SDS, 20 mM EDTA, 33 μg ml^{-1} tRNA, and 100 μg ml^{-1} proteinase K. The footprint reactions were extracted once with phenol/chloroform and precipitated with 0.5 vol 7.5 M ammonium acetate and 2 vol ethanol. The DNA pellet was washed once with 70 % ethanol, dried and loaded onto an 8 % acrylamide urea denaturing gel (Maniatis *et al.* 1982).

Phosphatase treatment of nuclear extracts

Between 0.05 and 1.25 units of bacterial alkaline phosphatase were added to 20 μl of blastula stage nuclear extract and incubated at ambient temperature for 10 min. The radiolabeled 191 bp *Hin*PI–*Sal*I fragment was then added and incubated at room temperature for an additional 15 min.

RNA blotting

Total RNA was denatured at 55°C for 15 min in a total volume of 100 μl containing 40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 50 % formamide, and 17.5 % formaldehyde solution (Maniatis *et al.* 1982). 5 μg of RNA per slot was loaded onto Hybond-N filter paper (Amersham; Arlington Heights, IL). Filter hybridization and washes were performed as described previously (Tomlinson and Klein, 1990).

Oligonucleotide-directed mutagenesis

4 μg of 0.8 Spec1–CAT and Spec2a–CAT plasmid DNA were digested with *Pvu*II, which cuts at a unique site in the CAT gene. The opened plasmids were digested at 37°C with 450 units of exonuclease III in 50 μl of 25 mM Tris, pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$, 100 μg μl^{-1} BSA, and 1 mM DTT for 30 s intervals from 30 s to 2 min. The reaction was stopped with 2 μl of 250 mM EDTA and extracted once with phenol/chloroform and once with chloroform. The DNA was precipitated with ammonium acetate and ethanol. The DNA hybridization reaction contained 40 ng of phosphorylated primer and mutated oligonucleotides and 1 μg of exonuclease III-digested Spec–CAT DNA in a total volume of 10 μl containing 25 mM Tris, pH 8.0, 10 mM $MgCl_2$, 100 μg ml^{-1} BSA, and 1 mM DTT. The hybridization reactions were incubated at 65°C for 10 min and then cooled slowly to 30°C. The DNA was repaired by adding 4 μl 2.5 mM dNTPs, 2 μl 10 mM ATP; 1 μl of a solution of 250 mM Tris, pH 8.0, 100 mM $MgCl_2$, 1 mg ml^{-1} BSA, and 10 mM DTT; 1 μl Klenow; and 1 μl T4 ligase in a total volume of 20 μl. The reaction tubes were incubated at 37°C for 2 h. 1 μl of T4 ligase was added, and the tubes were incubated at 37°C for an additional 30 min. Competent *Escherichia coli* HB101 cells were used for the transformation reactions. Altered sequences were confirmed by DNA sequence analysis.

CAT assays

Microinjections of sea urchin eggs were done as described previously (McMahon *et al.* 1985; Gan *et al.* 1990*b*). Plasmids derived from pSVo were linearized with *Bgl*I, which cuts within the pBR322 portion of the plasmid, and mixed with sperm DNA that had also been digested with *Bgl*I. Injected eggs were fertilized *in situ*, cultured at 18°C, and collected at blastula stage, 15 h after fertilization, or gastrula stage, 24 h after fertilization.

CAT assays were performed using extracts from embryos that had been injected with the 0.8 Spec1–CAT plasmid extending from −800 to +133 of Spec1 and the Spec2a–CAT plasmid extending from −1500 to +18 of Spec2a (Gan *et al.* 1990*b*). Injected embryos were collected along with approximately 1500 uninjected embryos of the same stage. One-half of the lysate was assayed for CAT activity as described by Gorman *et al.* (1982). The other half of the lysate was used for CAT DNA determination. The lysate was probed with a 500 bp fragment that contains the CAT gene coding region (Flytzanis *et al.* 1987).

Results

A protein binding site in the first exon of the Spec1 gene

The three Spec genes that have been analyzed in detail, Spec1, Spec2a and Spec2c, share about 800 bp of a strongly conserved sequence upstream of their translational initiation sites (Hardin *et al.* 1988; Gan *et al.* 1990*b*). The sequence match in this region is greater than 90 %. In Spec2a, the conserved sequences are continuous and include an RSR repetitive sequence element. Elsewhere, we have provided evidence that there is a transcriptional enhancer-like element contained within the RSR sequence (Gan *et al.* 1990*b*). In Spec1 and Spec2c, the conserved sequence block is interrupted by 2.4 or 2.9 kb of nonhomologous DNA. In addition, the transcriptional initiation site for Spec1 is positioned within the inserted element. These features alter the relative placement of the RSR element among the three genes as well as other 5′ flanking DNA. In the case of Spec1, homologous DNA that is 5′ flanking in Spec2a and Spec2c is part of the first exon of Spec1 (Hardin *et al.* 1988).

To identify proteins that bind to the conserved and nonconserved regions of the Spec genes, we undertook

an *in vitro* analysis of DNA–protein interactions by incubating various Spec1 gene fragments with nuclear extracts from *S. purpuratus* blastula stage embryos. One DNA fragment, a 191 bp *Hin*PI–*Sal*I fragment covering the region from −58 to +133 bp of the Spec1 gene (Fig. 1A), showed a strong-protein binding site by both band shift gel assays and DNaseI footprinting. The protein factor interacting with this site was called SpF1 for *Sp*ec gene *F*actor *1*.

Protein binding was evident by the two major bands (SpF1 complex A and B) shown on a band shift gel (Fig. 1B). Protein binding was specific because competition assays showed that 10- to 500-molar ratios of homologous competitor DNA (Fig. 1C, lanes 3–5) and a 300-fold molar ratio of the 298 bp *Nde*I–*Sal*I fragment (Fig. 1C, lane 6), a clone that encompasses the 191 bp *Hin*PI–*Sal*I fragment, inhibited the binding of proteins, but a 375-fold molar ratio of a 255 bp *Eco*RI–*Nde*I fragment, a DNA fragment of comparable size located immediately upstream of the 191 bp fragment, did not (Fig. 1C, lane 7). Protein-binding specificity was further verified by a DNase I footprint gel (Fig. 1D). Both the coding (CS) and noncoding (NCS) strands clearly showed that as the amount of blastula nuclear extract was increased (Fig. 1D, lanes 2–5 and 7–10), a single footprint region became observable at +39 to +60 bp, yet again, protein binding was inhibited by the homologous 191 bp *Hin*PI–*Sal*I fragment (Fig. 1D, lanes 5 and 10). As discussed below, the sequence –CACGTG– protected by SpF1 is the core sequence motif for the human transcription factor USF (Fig. 1A).

To test whether the sequence identified by the DNase I footprint gel was the correct SpF1 recognition site, a double-stranded complementary 22 bp oligonucleotide (CTB) corresponding to the footprint region was used in competition experiments (Fig. 1E). Two additional double-stranded oligonucleotides used as negative controls flank both ends of the region, a 23-mer homologous to the region immediately downstream (CT1+2), and a 21-mer immediately upstream (CT3+4). Both CT1+2 and CT3+4 overlap CTB by six nucleotides. Band shift gel analysis with blastula stage nuclear extracts incubated with the labeled 191 bp *Hin*PI–*Sal*I fragment showed that a molar ratio of 3.8×10^4 of CTB to labeled fragment inhibited SpF1 binding, but the same molar ratio of the bordering oligonucleotides CT1+2 and CT3+4 did not (data not shown). Further verification of the SpF1-binding site was shown by the DNase I footprint gel in Fig. 1E. Lanes 3 and 8 show that while 1.5 pmoles of CTB had little competitive effect, 15 pmoles (lanes 4 and 9) and 150 pmoles of CTB (lanes 5 and 10) completely inhibited the SpF1 footprint. These experiments demonstrated the presence of a strong DNA–protein interaction in the 5′ untranslated leader region of the Spec1 gene.

SpF1 has similar properties to human USF

USF is a transcription factor that binds the major late promoter of adenovirus (Sawadogo and Roeder, 1985) as well as a variety of other mammalian genes containing a core sequence of CACGTG (Sawadogo *et al.* 1988; R. Roeder, personal communication). Inspection of the SpF1-binding site in the Spec1 gene suggested that SpF1 may be similar to USF. USF has been purified to near homogeneity from HeLa cells (Sawadogo *et al.* 1988) and was found to be in two forms with apparent molecular weights of 43 000 and 44 000. Both forms show identical DNA-binding properties, though it is not clear what their relationship is to each other (Sawadogo, 1988).

To determine whether USF could recognize the SpF1-binding site on the Spec1 gene, purified USF was incubated with the 191 bp *Hin*PI–*Sal*I fragment and resolved on band shift gels (Fig. 2). Two complexes were apparent with very similar mobilities as observed for sea urchin embryo nuclear extracts. Thus, SpF1 complexes A and B may correspond to the same two molecular weight forms of USF. Increasing amounts of CTB oligonucleotide efficently inhibited USF binding (Fig. 2A, lanes 3–7); molar ratios of 200 totally eliminated USF binding (lane 4). USF-binding specificity was further demonstrated using the CT1+2 and CT3+4 oligonucleotides as negative controls (Fig. 2B), in that molar ratios of 200 had no discernable competitive effect (lanes 3 and 5) and molar ratios as high as 3.8×10^4 of CT1+2 and CT3+4 to labeled fragment (lanes 4 and 6) did not completely inhibit USF binding.

Sawadogo *et al.* (1988) have shown that USF is heat stable, as heating at 70°C did not significantly alter USF-binding activity. The sea urchin embryo nuclear extracts used in our experiments were subjected to a heat treatment purification protocol precisely the same as Sawadogo *et al.* (1988), implying that SpF1 was also heat stable. Thus, based on three criteria – sequence specificity, electrophoretic mobility of the DNA–protein complexes, and heat stability – SpF1 is similar to human USF.

SpF1 is an ectoderm-specific protein

The Spec1 gene is transcribed *in vitro* by nuclei prepared from ectoderm cells, but not by nuclei from endoderm/mesoderm cells (Tomlinson and Klein, 1990). These results suggested that ectoderm-specific transcription factors might exist that are involved in Spec1 gene transcription. We asked whether SpF1 was present only in the ectodermal cells of the embryo. Ectodermal and endodermal/mesodermal cells from *S. purpuratus* pluteus stage embryos were separated and purified (McClay, 1986). The degree of cross contamination was tested by isolating total RNA from each cellular fraction and spotting the RNA on two identical blots (Fig. 3A and B). The extent of ectodermal contamination in the endoderm/mesoderm fraction was monitored with a Spec1 codogenic probe and the extent of endodermal/mesodermal contamination in the ectoderm fraction with the endoderm/mesoderm-specific probe CyIIa (Cox *et al.* 1986). As shown in Fig. 3A and B, there was minimal contamination in both fractions.

Nuclear extracts prepared from the ectoderm and endoderm/mesoderm fractions were incubated with the 191 bp *Hin*PI–*Sal*I fragment of Spec1, with a 151 bp *Hpa*II–*Bst*NI fragment upstream of the SM50 gene,

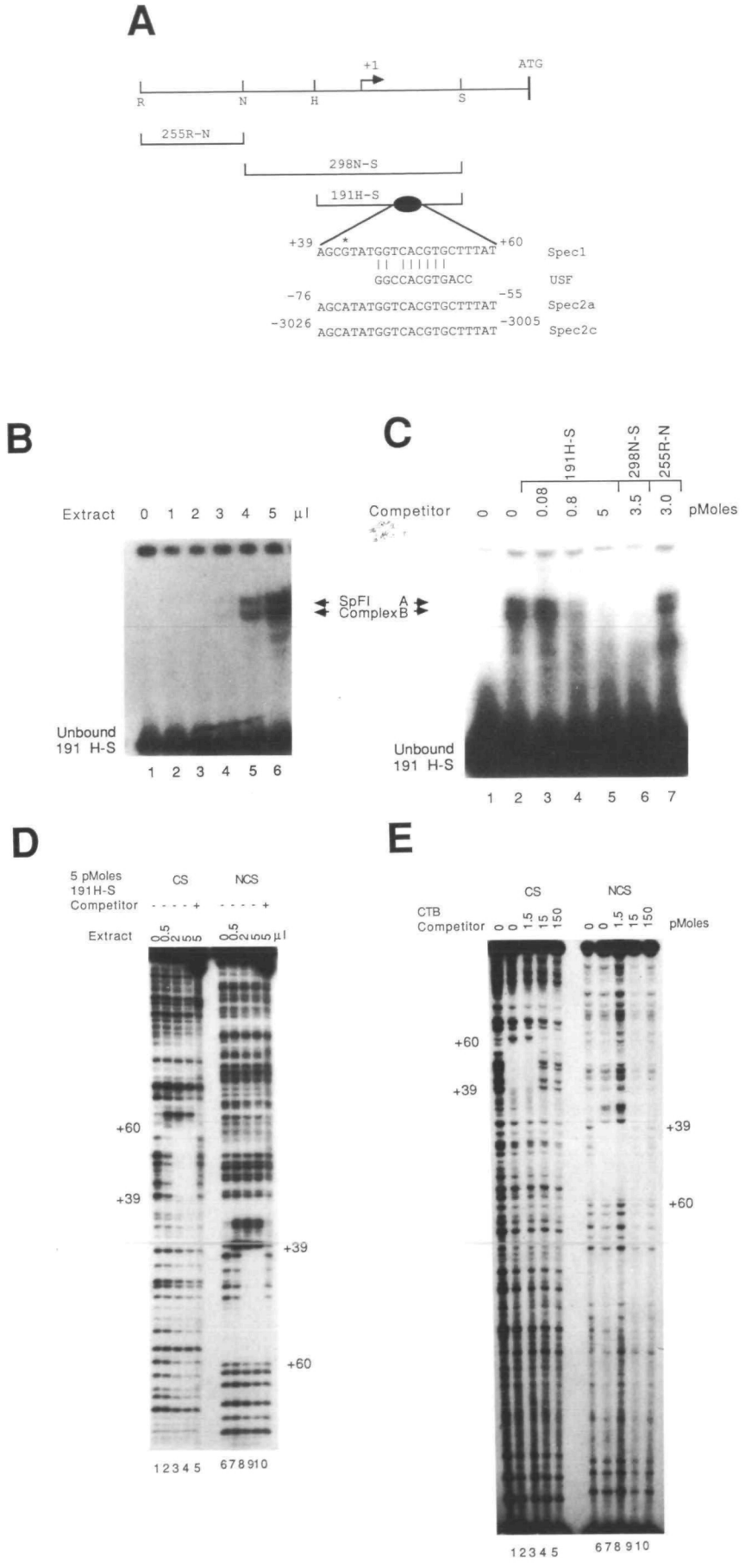

Fig. 1. *In vitro* protein–DNA binding analysis demonstrating the binding specificity of nuclear proteins to the 191 bp *Hin*PI–*Sal*I DNA fragment of Spec1. (A) Schematic illustration of the Spec1 promoter region. The start of transcription is represented by the arrow, and the start of translation is shown by ATG. The shorter solid lines show the relative positions of the 255 bp *Eco*RI–*Nsi*I, 298 bp *Nsi*I–*Sal*I, and 191 bp *Hin*PI–*Sal*I fragments used in this study. The solid oval on 191 H-S represents the SpF1-binding site. H, *Hin*PI; N, *Nsi*I; R, *Eco*RI; S, *Sal*I. The asterisk indicates the single base change from a guanine in Spec1 to an adenine in Spec2a and Spec2c. (B) Band shift gel with increasing amounts of blastula stage nuclear extracts and the 191 bp *Hin*PI–*Sal*I DNA fragment. No extract was present in lane 1. (C) Inhibition by homologous fragment. Equal amounts of nuclear extract from blastula stage embryos were used in lanes 2–7, no extract was used in lane 1. The indicated amounts of unlabeled competitor 191H-S DNA was used to test binding specificity. Approximately 1 pmole of labeled fragment was used. (D) DNase I protection assay with blastula stage nuclear extracts and the 191 bp *Hin*PI–*Sal*I fragment. The indicated amounts of nuclear extract were added. 4 pmoles of unlabeled 191 bp fragment as competitor were added to lanes 5 and 10. CS, coding strand; NCS, non-coding strand. (E) Additional DNase I protection assays with blastula stage nuclear extracts and the, 191 *Hin*PI–*Sal*I fragment. No extract was added to lanes 1 and 6; equal amounts of extract were added to the remaining lanes. The indicated amounts of oligonucleotide CTB was added as competitor to lanes 2–5 and 7–10.

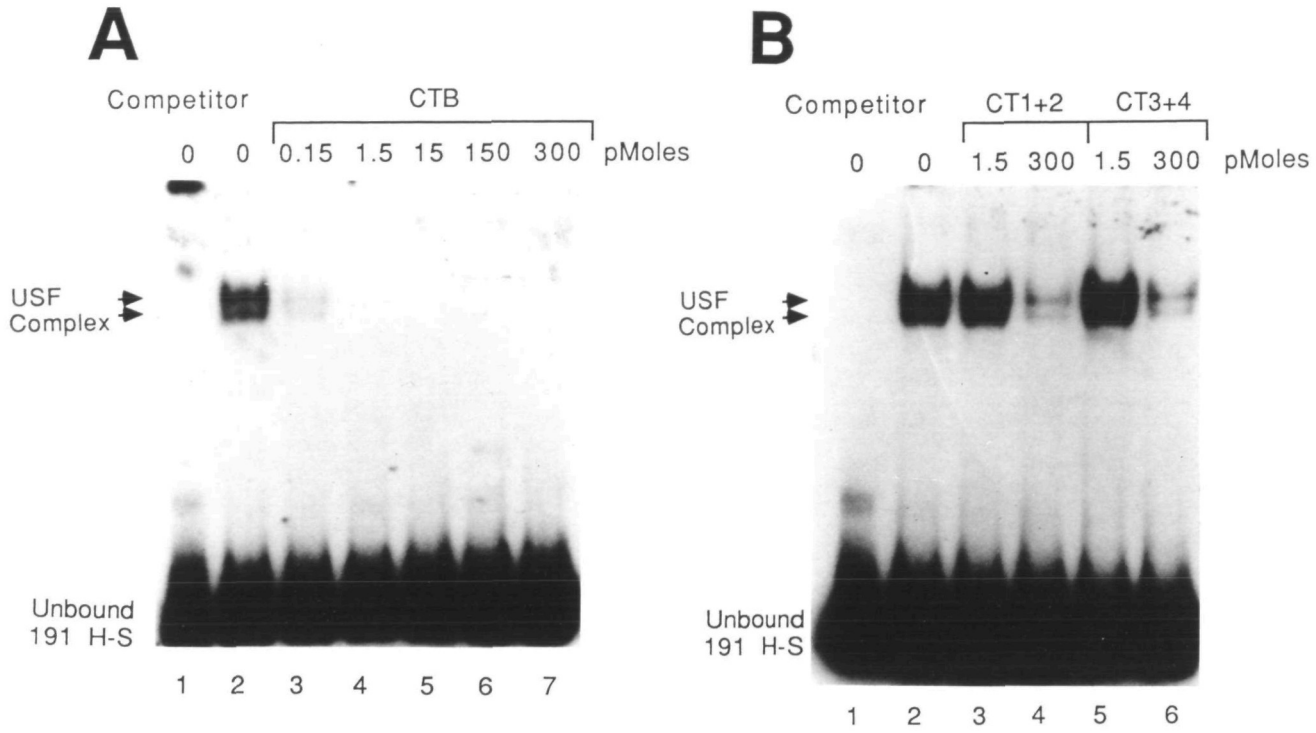

Fig. 2. USF binding to the 191 bp *Hin*PI–*Sal*I fragment of Spec1 was tested by band shift gel analysis (A, B). No USF was added to lanes 1 in A and B. Equal amounts of USF were added to the remaining lanes. The indicated amounts of competitor oligonucleotide CTB were added to A. The indicated amounts of the control oligonucleotide competitors CT1+2 and CT3+4 were added to verify the specificity of the USF binding sequence (B).

and with a 210 bp *Rsa*I fragment upstream of the CyIIIa actin gene. The SM50 gene is expressed only in the primary mesenchyme lineage and thus was used as a control for detection of protein binding of endodermal/mesodermal origin. At least one protein, termed factor B, is known to bind the 151 bp fragment (Davidson, 1989). The 210 bp *Rsa*I fragment from the CyIIIa actin gene was used as an additional control. It contains a 'P6' site that binds a protein known to be active at approximately equal levels in ectoderm and endoderm/mesoderm extracts (Thèzè *et al.* 1990; E.H. Davidson, personal communication). Equivalent amounts of heat-treated ectoderm and endoderm/mesoderm extracts were used in the band shift gels in Fig. 3C–F. SpF1 was greatly enriched and probably resides exclusively in ectodermal cells as shown in Fig. 3C. Both complexes, A and B, were observed. Increasing amounts of ectodermal extract resulted in increasing amounts of complexes (Fig. 3C, lanes 2–4). However, endoderm/mesoderm extracts revealed no complex formation, even at the highest extract level (Fig. 3C, lanes 5–7). Nevertheless, the nuclear proteins of the endoderm/mesoderm cells have binding capacity as shown in Fig. 3D. The 151 bp *Hpa*II–*Bst*NI fragment from the SM50 gene was capable of forming several protein complexes with the endoderm/mesoderm fraction (Fig. 3D, lanes 5–7).

SpF1 specificity was further demonstrated by adding increasing amounts of oligonucleotide CTB to the ectodermal nuclear extract (Fig. 3E, lanes 3–6). Competition was complete with the addition of molar ratios of 200 of CTB to labeled fragment (Fig. 3E, lane 4), yet molar ratios of 2.5×10^3 of the 151 bp SM50 fragment had no effect (Fig. 3E, lane 7). Conversely, increasing amounts of the 151 bp SM50 fragment inhibited any endoderm/mesoderm binding proteins from complex formation (Fig. 3F); e.g. 10 pmoles of the 151 bp fragment showed total competition (Fig. 3F, lane 5), yet 300 pmoles of CTB failed to effectively compete (Fig. 3F, lane 7). The lack of complex formation observed with the SM50 fragment and ectoderm nuclear extracts was different from that seen by Davidson and his co-workers, in that in their hands complexes were found in both fractions (E.H. Davidson, personal communication). The basis of this discrepancy may be that, in the experiment shown in Fig. 3C–F, extracts were heat treated before use. However, when non-heat-treated extracts were used, the same result was obtained with the Spec1 191 bp *Hin*PI-*Sal*I fragment; that is, ectoderm but not endoderm/mesoderm nuclear extracts were capable of forming complexes, although with non-heat-treated extracts, the bands were not as distinct as with the heat-treated extracts (Fig. 3G). In this experiment, the 210 bp *Rsa*I fragment from the CyIIIa actin gene formed the predicted 'P6' complex with nuclear extracts from either fraction (Fig. 3H).

Because phosphatases are prominent in gut tissue of sea urchin embryos, it was possible that the lack of complex formation with the Spec1 fragment was due to dephosphorylation of SpF1, rendering it inactive. We

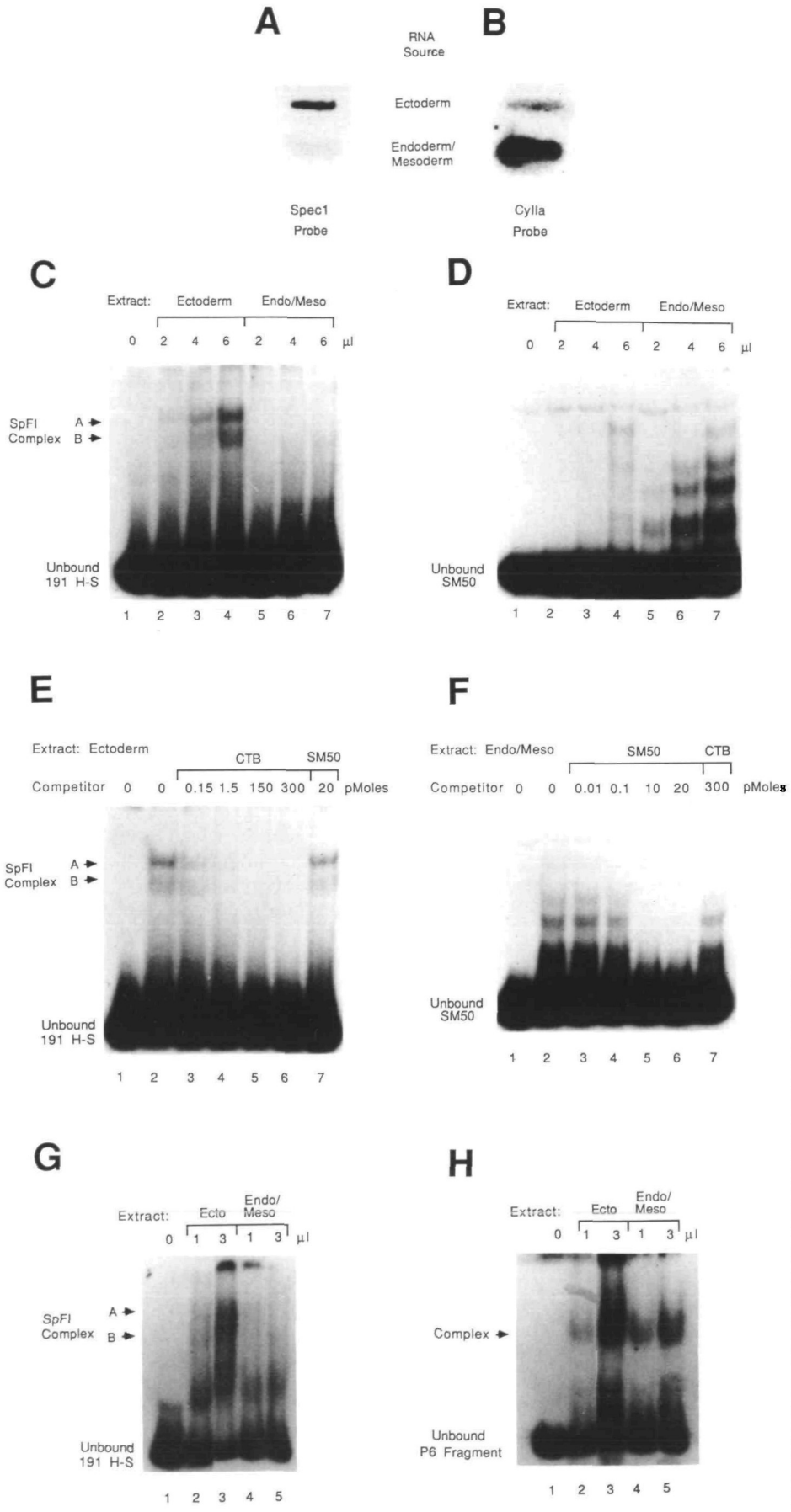

Fig. 3. Ectoderm specificity of SpF1 activity. (A, B) Slot blot of RNA isolated from the ectoderm and endoderm/mesoderm fractions hybridized with the Spec1 codogenic probe (A) or the CyIIa probe (B). (C, D) Pluteus stage nuclear heat-treated extracts of ectoderm and endoderm origin incubated with 191H-S (C) and the 151 bp SM50 DNA fragment (D) and subjected to band shift gel analysis. (E, F) Band shift gels demonstrating SpF1-binding specificity. No extract was added to the far left lanes of each gel. Oligonucleotide CTB and the 151 bp SM50 DNA fragment were added at the indicated amounts. (G, H) Band shift gels showing non-heat-treated ectoderm extracts shift both the 191H-S (G, lanes 2, 3) and 210 bp 'P6' fragments (H, lanes 2, 3) whereas the non-heat-treated endoderm extracts did not shift 191H-S fragment (G, lanes 4, 5) but did shift the 'P6' fragment (H, lanes 4, 5). No extract was added to lane 1 in either (G) or (H).

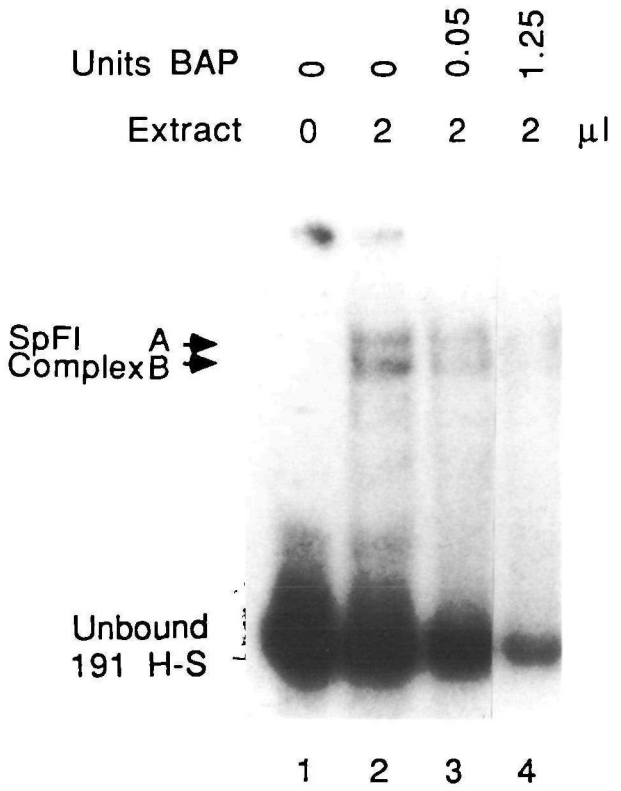

Fig. 4. SpF1 complex formation after bacterial alkaline phosphatase (BAP) treatment of blastula nuclear extracts.

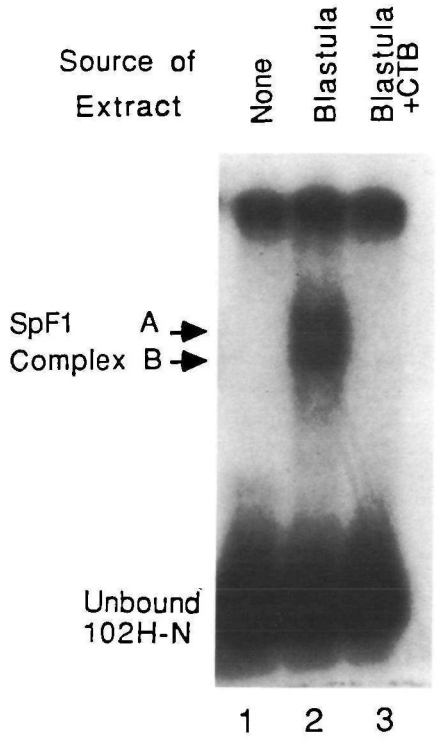

Fig. 5. SpF1 binding to the 102 bp *Hind*III–*Nsi*I fragment of Spec2a. Band shift gel analysis of *S. purpuratus* blastula stage extracts with the DNA fragment 102H-N of Spec2a. No extract was added to lane 1. 75 pmoles of the synthetic oligonucleotide CTB was added to lane 3 to demonstrate binding specificity.

incubated blastula extracts with varying amounts of bacterial alkaline phosphatase (BAP) and used these extracts in a band shift gel analysis. No decrease in the ratio of complexed to uncomplexed probe was observed, though the total amount of labeled probe decreased with increasing phosphatase due to dephosphorylation of the probe (Fig. 4, lanes 2–4). These experiments suggested that phosphorylated forms of SpF1 were not required for SpF1 binding. Similar conclusions have been drawn from experiments with the human USF (M. Sawadago, personal communication). Our results demonstrated that within the limits of our analysis, SpF1 resided exclusively in the ectodermal cells of the embryo.

SpF1 binds to the corresponding site of the Spec 2a gene

The SpF1-binding site found in the 5′ untranslated leader region of the Spec1 gene also occurs upstream of the Spec2a and Spec2c genes (Fig. 1A). The sequence is identical with the exception of a G→A change corresponding to position +40 of the Spec1 gene. As discussed above, these sequences are part of the conserved sequence block shared among the three Spec genes (Gan *et al.* 1990*b*). The CACGTG core sequence is located at positions −66 to −61 in Spec 2a and −3016 to −3011 in Spec 2c. We asked whether SpF1 had the ability to bind the SpF1-binding site of Spec 2a. A 102 bp *Hind*III–*Nsi*I fragment from Spec2a containing the SpF1-binding site was used in this experiment. SpF1 binding was demonstrated using heat-treated nuclear extracts prepared from blastula stage embryos. Fig.5, lane 2 shows that two complexes similar to those observed with the Spec1 fragment were seen with the Spec2a fragment. Futhermore, these complexes were eliminated with the competitor CTB oligonucleotide (Fig. 5, lane 3). Thus, at least *in vitro*, SpF1 bound the Spec2a site as efficiently as it did Spec1.

SpF1 is conserved among sea urchins

The sea urchin species *S. purpuratus* and *L. pictus* diverged 35 million years ago (Smith, 1988). The Spec genes of *S. purpuratus* and the Spec-like gene, LpS1, of *L. pictus* have diverged to a degree such that within the protein-coding region only the calcium-binding domains are recognizably similar (Xiang *et al.* 1988). However, in common with the Spec genes, LpS1 is expressed exclusively in the aboral ectoderm (Xiang *et al.* 1988; Tomlinson and Klein, 1990). To demonstrate the conservation of SpF1 in *L. pictus*, nuclear extracts were prepared from *L. pictus* blastulae and incubated with the 191 bp *Hin*PI–*Sal*I fragment of the *S. purpuratus* Spec1 gene. The band shift gel displayed in Fig. 6A demonstrated that an SpF1-like factor resided in *L. pictus*. Complex formation with *L. pictus* extracts (Fig. 6A, lane 2) appeared to be similar to that with *S. purpuratus* extracts because increasing amounts of the CTB oligonucleotide (Fig. 6A, lanes 3–7) efficiently prevented binding. Binding specificity was further demonstrated by the fact that neither control oligonuc-

leotides CT1+2 nor CT3+4 inhibited binding (lanes 8 and 9). DNase I footprinting with *L. pictus* extracts showed that the *L. pictus* protein bound the same site as observed with *S. purpuratus* extracts (Fig. 6B). Binding specificity was again demonstrated by lanes 5 and 10 in which the CTB oligonucleotide effectively inhibited binding. In both the band shift analysis and the DNase I footprinting, it was apparent that the *L. pictus* protein did not bind the SpF1 site on the Spec1 gene as tightly as the *S. purpuratus* protein. Nevertheless, these experiments demonstrated that *L. pictus* extracts contained SpF1 activity and strongly suggested that SpF1 is a conserved protein with identical function in both species.

Mutations in the SpF1-binding site do not affect reporter gene activity in microinjected embryos

Site-directed mutagenesis was employed to mutate the SpF1-binding site in 0.8 Spec1–CAT and Spec2a–CAT reporter gene plasmids. The mutations and plasmids are shown schematically in Fig. 7A and B. In these mutants, the core –CACGTG– sequence plus an additional 4 bp upstream have been replaced with a different 10 bp sequence. Fig. 7C and D show a band shift analysis where the Spec1 and Spec2a mutant fragments were incubated with heat-treated *S. purpuratus* blastula stage extracts. The wild-type control fragments yielded the expected complexes, but no significant complex formation was observed with the mutant fragments. These results support the conclusion that SpF1 binds to a specific sequence containing the –CACGTG– motif.

The wild-type plasmids depicted in Fig. 7A and B have been shown to be active when injected into the heterologous *L. pictus* egg (Gan *et al.* 1990*b*). In an earlier study, CAT activity resulting from microinjecting Spec1–CAT or Spec2a–CAT was seen in blastula, gastrula, prism and pluteus stage embryos but not in cleavage stage (Gan *et al.* 1990*b*). These data were consistent with nuclear run-on experiments showing the Spec genes are activated at the late cleavage – early blastula stage (Tomlinson and Klein, 1990). Moreover, many studies using *L. pictus* eggs have produced information on some of the elements needed for the correct expression of sea urchin histone genes for heterologous species. For example, Vitelli *et al.* (1988)

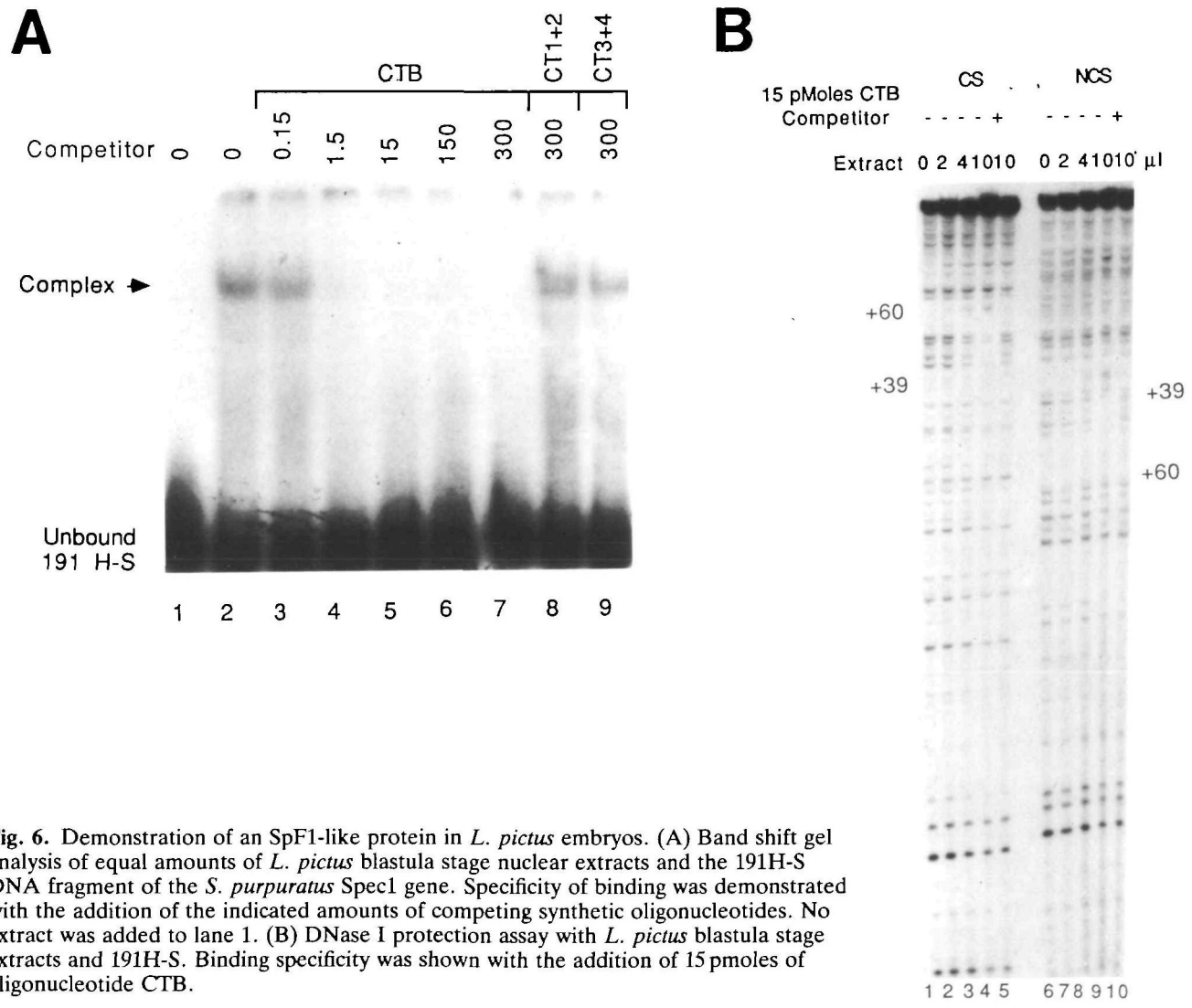

Fig. 6. Demonstration of an SpF1-like protein in *L. pictus* embryos. (A) Band shift gel analysis of equal amounts of *L. pictus* blastula stage nuclear extracts and the 191H-S DNA fragment of the *S. purpuratus* Spec1 gene. Specificity of binding was demonstrated with the addition of the indicated amounts of competing synthetic oligonucleotides. No extract was added to lane 1. (B) DNase I protection assay with *L. pictus* blastula stage extracts and 191H-S. Binding specificity was shown with the addition of 15 pmoles of oligonucleotide CTB.

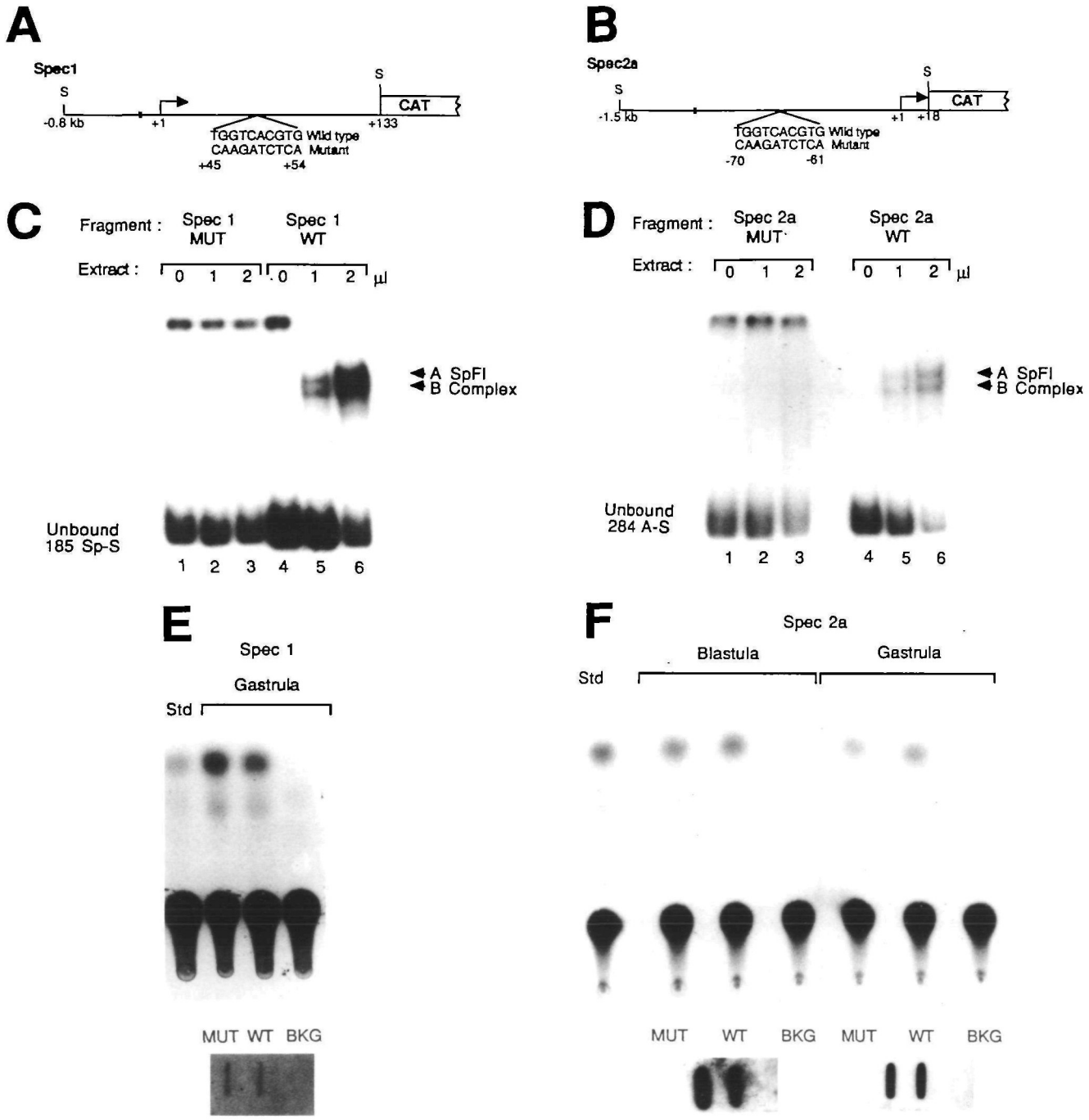

Fig. 7. CAT activity and band shift gel analysis of wild-type and mutant 0.8 Spec1–CAT (A, C, and E) and Spec2a–CAT (B, D, and F) plasmids. The 0.8 Spec1–CAT plasmid (A) extends from −800 to +133 of 5′ DNA from Spec1 and the Spec2a–CAT plasmid (B) contains approximately 1.5 kb of Spec2a 5′ upstream DNA ligated to pSV0 at +18 of Spec2a. The arrows at +1 show the start sites of transcription for the endogenous genes. SpF1 wild-type and mutant sequences used for microinjection are shown. (C and D) Band shift gel analysis using wild-type and mutant fragments from Spec1 and Spec2a–CAT plasmids with heat-treated blastula stage extracts. 185Sp-S is a 185 bp *Sph*I–*Sal*I fragment from −52 to +133 of Spec1. 284A-S is a 284 bp *Ava*II–*Sal*I fragment from −266 to +18 of Spec2a. (E and F) CAT activity of the wild-type and mutant plasmids. DNA slot blots centered below the CAT assays show that comparable amounts of injected plasmid were detected within each sample assayed for CAT activity and that hybridization background was negligible. The number of injected embryos in each sample was as follows: Spec1 mutant, 170; wild-type, 120; Spec2a (blastula) mutant, 100; wild-type 100; Spec2a (gastrula) mutant 40, wild-type 44. Standards (std.) represent 0.01 unit of bacterial CAT enzyme for the Spec1–CAT assay and 0.1 unit for the Spec2a–CAT assay.

have shown by SP6 RNA mapping that all five early histone genes of *Psammechinus miliaris* were properly expressed in *L. pictus* eggs. Colin *et al.* (1988), Lai *et al.* (1988, 1989), and Di Liberto *et al.* (1989) have identified sequences needed for the correct temporal and quantitative expression of *S. purpuratus* histone genes. The studies of Lai *et al.* (1988) have shown that a single base pair change within the 6 bp histone H1 Sp1-binding sequence can dramatically alter temporal and quantitative expression.

Because SpF1 activity was present in *L. pictus* extracts, we argued that *L. pictus* eggs were appropriate for testing the *in vivo* activity of the SpF1-binding site. Unexpectedly, however, as shown in Fig. 7E and F, no significant differences were ever observed between Spec1 or Spec2a mutant and wild-type plasmids when eggs were injected, fertilized, allowed to develop to the gastrula stage (Fig. 7E) or the blastula and gastrula stage (Fig. 7F), and subsequently analyzed for CAT activity and plasmid DNA content. We had previously made an internal deletion in the Spec2c 5′ flanking region that removed the SpF1-binding site plus substantial amounts of DNA both upstream and downstream from the binding site and found no differences in Spec2c–CAT activity (Gan *et al.* 1990*b*). These results do not imply a lack of general responsiveness of the expression system to mutations associated with *cis* transcriptional elements since several other regions including the 5′ half of the RSR could be deleted in these plasmids with drastic effects on Spec gene expression (Klein *et al.* 1990; Gan *et al.* 1990*b*). We have also repeated the microinjection experiments with the Spec1–CAT mutant using *S. purpuratus* eggs and have got the same result; that is, there was no effect on CAT activity when the SpF1 site was mutated (data not shown). From these results we conclude that the SpF1 protein–Spec DNA interaction is not essential for expression of the microinjected plasmids.

Discussion

SpF1 and aboral ectoderm specification

Our results clearly demonstrate the existence of a Spec–DNA binding protein whose activity at the pluteus stage is restricted to ectodermal cells. This protein binds to a known family of genes, the Spec genes and displays cell type specificity. A nuclear protein binding to Spec DNA might be expected to be an even earlier marker of aboral ectoderm than the Spec genes themselves. We have not proven that SpF1 activity is localized solely to aboral ectoderm lineages, but its tight association with the Spec genes suggests that this may be the case. If SpF1 synthesis precedes Spec gene activation, its temporal and spatial features should be highly interesting. In sea urchin development, the animal–vegetal axis is specified during oogenesis, and it is clear that more vegetal blastomeres can induce various fates in cells above them (Horstadius, 1973; Wilt, 1987; Davidson, 1989). Thus, in normal development, micromeres at the vegetal pole, which give rise to skeletal mesenchyme, probably induce gut, muscle and pigment tissue in the adjacent tiers. It is not clear what action vegetal blastomeres have on ectodermal differentiation. However, under certain experimental conditions, such as ectopic transplantation of micromeres (Horstadius, 1973) or treatment with vegetal inducing agents such as Li^+ (Livingston and Wilt, 1989), gut, muscle, pigment and skeletal tissue can be induced in animal blastomeres normally fated to become oral or aboral ectoderm. For SpF1 activity to occur only in the ectoderm, there must be differential expression or activation at some point during embryogenesis. However, if SpF1 is an early ectoderm marker, its activity must be reversible, since cells destined to become ectoderm can be converted to non-ectoderm cell types.

Recent studies with lineage tracers have shown that the second major embryological axis in sea urchins, the oral–aboral axis, is specified by at least the 2-cell stage (Cameron *et al.* 1989). Davidson (1989) has suggested that a short time after fertilization, a reorganization of the cytoplasm is responsible for setting up the oral–aboral axis in a fashion similar to what has been observed for the dorsal–ventral axis in amphibian embryos. Once this event takes place, it is likely, based on the regulative ability of the ectodermal cells, that a series of intercellular interactions results in the differential activation and inactivation of transcription factors along the axis. This in turn leads to the activation of various cell-type-specific genes and the differentiation of the oral and aboral ectoderm cell types. We argue that SpF1 activity appears only in the aboral ectoderm lineages and fits into the above scheme by being activated very early after oral–aboral axis specification in the aboral ectoderm region, or alternatively, by being inactivated in the oral and more vegetal regions. SpF1-binding activity appears to be absent in unfertilized eggs (C.R.T., unpublished results), suggesting that SpF1 must be activated in aboral ectoderm cells following fertilization.

SpF1 and USF

Whatever its role in Spec gene activation, SpF1 has properties that suggest it may be a sea urchin equivalent of the human USF. USF plays a key role in activating the major late promoter of adenovirus (Sawadogo and Roeder, 1985; Lee *et al.* 1988). It binds approximately 60 bp upstream of the adenovirus major late start site. *In vitro*, USF has been shown to interact with the TATA binding factor, TFIID, which in adenovirus binds 30 bp downstream from USF (Sawadogo *et al.* 1988; Workman *et al.* 1990). It has been proposed that USF functions in stimulating transcription by associating with TFIID. However, its precise role is not clear. Workman *et al.* (1990) have shown that under conditions of *in vitro* chromatin assembly, the fold stimulation of transcription by USF was several-fold greater than in the absence of chromatin assembly. These authors suggested that an important role for USF is to establish the transcriptional potential of the promoter during chromatin assembly by facilitating the binding of TFIID. An additional role is to enhance utilization of

the promoter in transcription by increasing the loading of RNA polymerase II and additional factors (Workman *et al.* 1990). USF has been shown to bind promoter regions from several mammalian genes besides adenovirus including those encoding mouse metallothionein, rat fibronectin, human growth hormone and several liver-specific genes (Sawadogo *et al.* 1988). A USF-binding site occurs in the intragenic region of the chicken and duck histone H5 genes and USF appears to activate expression of histone H5 in duck erythrocytes (During *et al.* 1990). Moreover, a USF-like protein has been purified from *Xenopus* oocytes, and there is a USF-binding site 250 bp upstream from the *Xenopus* TFIIIa gene (Scotto *et al.* 1989; Hall and Taylor, 1989).

Human and *Xenopus* USF cDNA clones have been isolated and sequenced, and they show the presence in their open reading frame of a basic region followed by amphipathic helices, termed the HLH (helix–loop–helix) domain by Murre *et al.* (1989). Sequence comparisons relate this 60 amino acid stretch to those found in several nuclear proteins including: C-*myc*, N-*myc*, L-*myc*, MyoD1, myogenin, myf5, daughterless, twist, achaete–scute and the immunoglobulin kappa chain enhancer binding proteins, E12 and E47. Some of these proteins are known to have essential roles as transcription factors involved in determining cell fate. For example, MyoD1, myogenin and myf5 control muscle differentiation in mammals (Davis *et al.* 1987; Wright *et al.* 1989; Edmondson and Olson, 1989; Braun *et al.* 1989), and achaete–scute is associated with the formation of the central and peripheral nervous system in *Drosophila* (Villares and Cabrera, 1987). It is possible that SpF1 belongs to this group of proteins and plays some role in the specification of aboral ectoderm lineages.

SpF1 as a transcription factor

We have presented circumstantial evidence that SpF1 is a transcription factor involved in activating Spec genes. There is a high affinity binding site for SpF1 in the Spec gene conserved sequence block, and the binding properties as well as the sequence of the binding sites are similar to USF (Sawadogo *et al.* 1988). However, we have not been able to demonstrate SpF1 activity *in vivo*. Mutations affecting the binding of SpF1 to Spec DNA have no effect in our expression system, either with *L. pictus* or *S. purpuratus* eggs. If SpF1 played a role in aboral ectoderm-specific expression, it would be expected to be a positive activator of transcription since its binding activity is found restricted to ectodermal cells where the Spec genes are active. However, SpF1 does not appear to be essential for expression of the microinjected Spec genes. Nevertheless it is still possible that SpF1 is actually required for correct endogenous Spec gene expression. For example, if the major role of SpF1 were to potentiate the Spec promoters by keeping them in an open configuration (similar to what may be occurring with USF), it might be that such a requirement is not necessary for the microinjected genes. The latter genes are present in the embryo in hundreds of copies per cell and the structural features of the chromatin may not be the same as exists in the endogenous genes. We have recently shown that the presence of an SpF1-binding site is not sufficient for conferring proper spatial expression from Spec promoters (Gan *et al.* 1990*a*). Spec-*lacZ* reporter genes containing 5′ flanking and 5′ untranslated leader sequences from Spec1, Spec2a and Spec2c were microinjected into *Strongylocentrotus or Lytechinus* eggs, and embryos were analyzed *in situ* for β-galactosidase activity. In the case of Spec2a, 1.5 kb of 5′ flanking DNA plus 18 bp of 5′ untranslated leader sequence was sufficient for correct aboral ectoderm expression (Gan et al. *1990a*). Unaccountably, Spec1-*lacZ* and Spec2c-*lacZ* reporter gene constructs containing highly similar regions were expressed in other cell types besides aboral ectoderm (Gan *et al.* 1990*a*). Yet both of these latter constructs contained unaltered SpF1-binding sites.

SpF1 and Spec gene structure

The Spec genes have evolved in an unusual manner. Insertions or deletions normally would not be tolerated in the 5′ flanking DNA of genes transcribed by RNA polymerase II. However, in the Spec genes of *S. purpuratus*, these events seemed to have occurred frequently. In addition, the Spec-related gene of *L. pictus*, LpS1, has undergone an internal duplication resulting in a protein of twice the normal molecular weight (Xiang *et al.* 1988). Whether the difference in placement of the SpF1-binding site (the 5′ untranslated leader of Spec1, the −60 bp region of Spec2a, and the −3.0 kb region of Spec2c) have any effect on Spec gene expression is unknown. In this regard, another part of the Spec gene conserved sequence block, the RSR repeat, contains an enhancer-like element that has a much stronger effect on the expression of Spec2a, where the repeat is only 100 bp upstream of the start of transcription, than on Spec1 or Spec2c, where it is 2.4 to 2.9 kb upstream (Gan *et al.* 1990*b*). With respect to the SpF1-binding sites, Spec2a most resembles the adenovirus major late promoter in that the SpF1/USF-binding sites and TATA boxes are positioned in a similar fashion. However, as discussed in the Results section, elimination of the SpF1-binding site in Spec2a does not affect expression.

It is likely that the USF-binding site exists on genes other than those in the Spec family. The expression properties of such genes would be interesting. It is also possible that there is a family of USF-like proteins or even unrelated proteins that recognize similar binding sites. Recently, Beckman *et al.* (1990) have reported the existence of another helix–loop–helix protein, distinct in sequence from USF, that binds to the same –CACGTG– sequence motif. Purification of SpF1 and the eventual cloning of the SpF1 gene should provide powerful tools for further investigation. The most crucial problem at present is ascribing a function to SpF1.

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