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Gene 320 (2003) 49–58
GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES
www.elsevier.com/locate/gene

p63/73 homologues in surf clam: novel signaling motifs and implications for control of expression

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Received 9 May 2003; received in revised form 3 July 2003; accepted 31 July 2003

Received by A.J. van Wijnen

Abstract

To understand the role of *p53* gene family members during invertebrate embryonic development, we used polymerase chain reaction (PCR) to identify *p63/73* homologues in the marine mollusc *Spisula solidissima*. Here, we report the sequences of two distinct *p63/73*-like homologues, both cloned from *Spisula* embryos. The first, *Ssp63/73*α is 2699 nucleotide (nt); the second, *Spp63/73*β is 3920 nt. The nucleotide sequences of the two variants are nearly identical up to their stop codons but diverge in their 3′-untranslated regions (UTRs). The deduced amino acid sequence of both *Ssp63/73* variants is 597 amino acids, coding for a protein with predicted molecular weight of approximately 68 kDa. We conclude that the two unique transcripts, containing 3′ UTRs of variable lengths, represent tandem alternate polyadenylation sites for the *Ssp63/73* gene. While alternative splicing has been well documented in the *p63/73* gene family, this is the first report of alternate polyadenylation site choice as a control point for *p63/73* gene expression in any species.

In order to identify specific post-transcriptional as well as post-translational signals potentially involved in regulation of p63/73-like expression, we compared *Ssp63/p73* nucleotide and Ssp63/73 deduced amino acid sequences to corresponding regions of other mammalian and nonmammalian p63 and p73 homologues. Within the *Spisula* 3′ UTRs we identified multiple AU-rich elements (AREs) which may control translation activation. Within the deduced amino acid sequence, we identified potential sites for sumoylation, a post-translational process that has been identified in mammalian p63 and p73 proteins. Identification of these novel signaling sites provides information about potential mechanisms controlling expression of multiple p63/73 isoforms during development.

Keywords: Alternate polyadenylation; Embryonic development; *p53* gene family; Marine mollusc; Neuron; Post-transcriptional regulation; UTR

1. Introduction

Recently identified members of the *p53* gene family, *p63* (Yang et al., 1998) and *p73* (Kaghad et al., 1997) code for transcription factors that, in mammals, control p53-induced apoptosis (Flores et al., 2002) in response to DNA damage (Yuan et al., 1999), as well as neuronal (Pozniack et al., 2000); and epithelial (Yang et al., 1999) development. In mammals, the *p53*, *p63* and *p73* genes reside at distinct loci on different chromosomes (reviewed in Moll et al., 2001). The hallmark features of the p53 protein are shared by p63 and p73: an acidic amino-terminal transactivation domain (TA), a core DNA-binding domain (DBD), and a carboxy-terminal oligomerization (OLIGO) domain. However, in contrast to the *p53* gene, the *p63* and *p73* genes utilize multiple post-transcriptional processes. Alternate amino-terminal promoters generate distinct classes of p63 and p73 proteins, some of which contain, while others lack, the TA domain. Further complexity is generated at the carboxyl terminus by extensive differential splicing of exons, with some variants containing a steric alpha motif (SAM) and transactivation-inhibitory (TI) domains. These post-tran-

Abbreviations: ARE, AU-rich element; bp, base pair; DBD, DNA-binding domain; kb, kilo base (1000 bp); kDa, kilo dalton(s); PAS, polyadenylation signal; nt(s), nucleotide(s); SAM, steric alpha motif; Ssp63/73α, *Spisula* p63/p73 alpha variant; Ssp63/73β, *Spisula* 63/73 beta variant; SUMO, small ubiquitin-related modifier; TA, transactivation domain; TI, transactivation-inhibitory domain; UTR, untranslated region.

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doi:10.1016/j.gene.2003.07.001

scriptional modifications render multiple p63 and p73 isoforms with varied combinations of functional domains. This phenomenon has implications for specific cellular expression and function of the multiple p63 and p73 isotypes.

Despite the immediate relevance of the *p53* gene family to mammalian cell biology, little information is available regarding the forms and functions of *p53*, *p63* and *p73* in nonmammalian species. Genes described as *p53*-like, as well as *p63/p73*-like, are known to occur in various nonmammalian species, including fish, arthropods, and molluscs. However, whether these nonmammalian orthologues will map to separate loci with variation arising by multiple promoter use and alternative splicing is a question yet to be addressed.

In earlier reports, we identified a *p53*-like gene in the marine mollusc *Spisula solidissima* (surf clam) that is differentially expressed during embryonic development (Jessen-Eller et al., 2002). Here, we present full sequences for two unique *p63/73*-like transcripts, both cloned from *Spisula* embryo and adult foot tissue. They are defined as *Ssp63/73*. Ssp63/73 protein expression was confirmed by Western blot analyses using a *Spisula* specific p63/73 polyclonal antibody. Based on nucleotide and deduced amino acid sequence analysis, we believe that *Ssp63/73* is the same transcript as the previously reported *Spisula* p120 (Jessen-Eller et al., 2002).

To further understand mechanisms controlling *p63/73* gene expression during neurogenesis, we focused on identification of potential post-transcriptional, as well as post-translational signaling sites in the *Spisula* sequences. At the post-transcriptional level, we hypothesize that the two *Spisula p63/73* sequences, varying only in their 3′ noncoding regions, represent tandem alternate polyadenylation sites. Alternate polyadenylation is a process known to control differential gene expression during development (reviewed in Edwards-Gilbert et al., 1997). We also identified conserved *cis*-acting signaling sites within the 3′ untranslated regions (UTRs) of the two transcripts. These are AU-rich elements (ARE motifs) that may control stability and/or translatability of each p63/73 transcripts, having further impact on p63/73 expression. This is the first identification, in any species, of alternate polyadenylation site choice and 3′ UTR *cis*-acting signaling as control points in *p63/73* gene expression.

At the post-translational level, we identified a potential sumoylation site at the C-terminus of the *Spisula* p63/73 deduced amino acid sequence. Post-translational modification by sumoylation (Minty et al., 2000), or a similar mechanism, may control the complex expression pattern of multiple p63/73 isotypes during neurogenesis in *Spisula*.

Differential expression of multiple p53-like family members during embryonic development in *Spisula* (Jessen-Eller et al., 2002) suggests that the function of *Spisula* p63/73 parallels mammalian p73 as a regulator of neurogenesis (Pozniack et al., 2000). In the current study, we use a comparative approach to identify novel signaling motifs that may control the complex modulation of p53 family members during embryonic development.

2. Materials and methods

2.1. Organisms

Mature adult surf clams (*S. solidissima*) of both sexes were collected by divers off shore near Chatham, MA and from Menemsha Bay, Martha's Vineyard, MA. Animals were held until use in running seawater (15–18 °C) at the Aquatic Resources Division, Marine Biological Laboratory, Woods Hole, MA, USA.

2.2. Fertilization and culturing

Fertilization was carried out as described previously (Kreiling et al., 2000). Oocytes were collected from individual adult *S. solidissima*, washed gently and rinsed in Tris–HCl buffered artificial seawater (AFSW), pH 8.0. After filtering through cheesecloth, samples were allowed to settle and resuspended to a 1%-v/v density in AFSW. Exuded sperm were collected from gonadal tissue using a Pasteur pipette. Two drops of sperm were diluted in approximately 5 ml AFSW. The dilute sperm were added to the egg suspension at approximately one drop sperm mixture per 1 ml settled oocytes. Fertilization was confirmed microscopically by the occurrence of germinal vesicle breakdown. Batches showing >95% fertilization were used. Embryos were harvested 24 h post-fertilization by rinsing through cheesecloth, centrifuging, washing with AFSW and centrifuging again. Resulting pellets were resuspended in Trizol reagent, briefly sonicated and flash frozen in liquid nitrogen. Samples were stored at −80 °C.

2.3. Nucleic acid extraction

Extraction of RNA was performed on samples stored in Trizol at −80 °C, prepared as described above. These samples were further homogenized using an Ultra-Vax hand held homogenizer. Total RNA was extracted following Trizol manufacturer's guidelines; the only change in protocol being the addition of 4 M lithium chloride after precipitation in order to break down excess glycogen. Quantification of RNA was determined by UV absorption spectroscopy. Integrity of the RNA was monitored by gel electrophoresis on a 1% agarose/1.2% formaldehyde denaturing agarose gel.

2.4. Genome walking

Genomic DNA was extracted from gill tissue of two adult *S. solidissima* following the DNeasy protocol (QIAGEN). DNA was quantified by UV absorption spectroscopy. Integrity of the DNA was monitored by gel electrophoresis

(0.75% agarose). Following manufacturer's guidelines (Clontech), the Universal Genome Walker kit was used to construct a genome library by differential restriction enzyme digestion. DNA fragments were ligated to the adaptor (supplied by manufacturer, Clontech), and nested PCR was carried out using primers listed in Table 1. Nondegenerate primer design was based on the 3′ coding region of the *Spisula p53*-like gene sequence (Jessen-Eller et al., 2002).

2.5. Rapid amplification of cDNA ends (RACE)

RACE was performed on total RNA from *Spisula* embryos (prepared as described in Section 2.3). Following the SMART RACE cDNA amplification protocol (Clontech), 3′ RACE was performed using primers listed in Table 1. Design of RACE primers was based on results from genome walking (described above). For the primary amplification, the following conditions were used: 25 cycles of 94 °C for 5 s, 67 °C for 10 s, 72 °C for 90 s. The conditions for nested amplification were: 25 cycles of 94 °C for 5 s, 66 °C for 10 s, 72 °C for 90 s.

2.6. Reverse transcription polymerase chain reaction (PCR) amplification

First strand cDNA synthesis was carried out on total RNA extracted from *Spisula* embryos and *Spisula* adult foot tissue following manufacturer's guidelines for SMART RACE cDNA synthesis (Clontech). This was followed by PCR amplification, using specific primers designed from the 3′ RACE sequencing results (Table 1) and following the Advantage 2 PCR Enzyme system (Clontech). Amplifications were performed under the following conditions: initial melting at 95 °C for 1 min, 5 cycles of 94 °C for 30 s and 69.5 °C for 3 min; 5 cycles of 94 °C for 30 s, 68 °C for 30 s, 69.5 °C for 3 min; 5 cycles of 94 °C for 30 s, 66 °C for 30 s, 69.5 °C for 3 min; 25 cycles of 94 °C for 30 s, 64 °C for 30 s, 69.5 °C for 3 min; followed by a final extension at 72 °C for 7 min.

2.7. Cloning and sequencing

Amplification products were separated on 0.75–2% TAE agarose gels. Products of the predicted molecular weights were excised from gels, eluted from agarose, following the Geneclean protocol (Bio 101), and ligated into the pCR2.1 vector (Invitrogen). Ligation reactions were transformed using *Escherichia coli* TOP10F′ cells. Screening for transformants was performed on Luria Broth (LB) agar plates containing kanamycin (50 μg/ml), IPTG (100 mM) and Xgal (40 μg/ml). Positive colonies were grown in overnight cultures of LB with kanamycin (50 μg/ml). Plasmid DNA was prepared using the Plasmid Mini kit (QIAGEN), and sequenced on an ABI Prism 377 automated sequencer. M13 forward and reverse primers were used for sequencing all plasmid preparations. When PCR products exceeded the reading ability of M13, internal primers were designed. For reasons that remain unclear, the beta variant proved difficult to clone. Therefore, the beta PCR product was directly sequenced after gel purification using the QIAquick PCR Purification kit (QIAGEN).

2.8. Sequence analyses

The *Spisula* nucleotide sequences, in their entirety, were submitted to a nonrestricted BLAST search (Altschul et al., 1997). In addition, we performed a nucleotide alignment of the two *Spisula* variants, using CLUSTAL W 1.7 (Thompson et al., 1994). The alignment was edited in order to match potential poly-A-signaling sites. Deduced amino acid p63 and p73 sequences were compared to the *Spisula* p63/73 amino acid sequence, using the residue substitution matrix Blosum, in CLUSTAL W 1.7. Gap opening penalty was set at 10; gap extension penalty at 0.05; gap separation penalty at 8.

2.9. Relationship to previously described Spisula p120 sequence

The initial focus of the current study was to complete the sequencing of the previously described but incomplete *Spisula* p120 gene sequence (Jessen-Eller et al., 2002). Primers for genome walking were designed based on the p120 sequence. By genome walking, we obtained the sequence containing the stop codon. This result was confirmed by 3′ RACE, from which we obtained the two alternate 3′ UTRs described in this report. The entire gene sequences were confirmed by RT-PCR, using a 5′ forward primer containing the start codon, and 3′ reverse primers

Table 1
PCR primers

Primer	Forward (5′–3′)	Reverse (5′–3′)	Product size (nt)
RT-PCR	Ssp63/73F: ggcttccaggaaaaatgtccg	Revα: tactacgatttaaccagtaaataaac	2700
		Revb: ctgacagtgcaaacaaccaaataacac	4300
3′ RACE	1° RACE F: aagaactgtcccctccgacgaagaaa		ND[a]
	N-RACE F: actctacatactcttgcaaattgccc		2100
			850
Genome walking	1° GW 1: atttaggtctgggtgcatacattgaca		ND
	N-GW: aaggaacgagggtctgacagccataca		1500

[a] Molecular weight not determined.

located just upstream of the poly-A tail (described in Section 2.6).

2.10. Peptide antibody and SDS-PAGE and Western blot analyses

A peptide corresponding to amino acids *YEVTRYTFKHTISL*, encompassing the "HOMO-domain" (Fig. 2), was synthesized by New England Peptide and used to immunize two rabbits. An antibody was affinity-purified from the resulting combined antisera.

Tissue samples (~ 50 μg) were mixed with 1 ml (>20 volumes) of 2% sodium dodecyl sulfate (SDS) sample buffer containing 2 mM phenylmethylsulfonylfluoride, probe-sonicated for 30 s, heated to 100 °C for 3 min, and frozen at − 80 °C. The samples were thawed and spun at 45,000 × *g* for 15 min at room temperature. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on small format 7.5%T/2.5% C polyacrylamide gels (8 cm W × 6 cm H × 1.5 mm thick) using the discontinuous ionic system (Laemmli, 1970) and, when required, stained with Coomassie Blue by the equilibrium method (Fairbanks et al., 1971). For immunoblotting, the resolved proteins were transferred to nitrocellulose or PVDF membranes (Towbin et al., 1979; Dunn, 1986) and evaluated qualitatively by Ponceau S staining (Salinovich and Montelaro, 1986). Blots were blocked with 1% BSA, 1% goat serum, and 5% nonfat dry milk in Tris-buffered saline/0.05% Tween-20, pH 7.5, probed with the primary antibody for 1 h, washed in PBS, and subsequently probed with an alkaline-phosphatase labeled secondary antibody (Promega). Detection was colorimetric, using Western Blue (Promega). Reflectance video densitometry was used to quantify the relative absorbance signal of individual bands across lanes using integration methods described previously (Stephens, 1992).

3. Results and discussion

3.1. Two alternate 3′ untranslated regions

Two distinct products were generated by RT-PCR of *Spisula* embryo total RNA (Fig. 1) using specific p63/73 primers (Table 1). Following nomenclature initiated for *p53* gene family members in clams (Kelley et al., 2001), as well as for splice variants in mammals, we refer to these variants as *Ssp63/73*α (alpha) and β (beta). The α variant is 2699 nucleotides (nt); the β variant 3920 nt. Sequence analysis reveals that the alpha and beta nucleotide sequences are nearly identical up to their stop codons. Minor variation occurs in some third codon positions, and in two cases, this results in an amino acid change (Fig. 2). Their deduced amino acid sequence is 596 amino acids (Fig. 2), coding for a protein with a predicted molecular weight of 68 kDa. The sequences of the two variants diverge in their 3′ UTRs (Fig. 3). *Ssp63/73*α has a shorter 3′ UTR of 895 nt. The 3′ UTR of

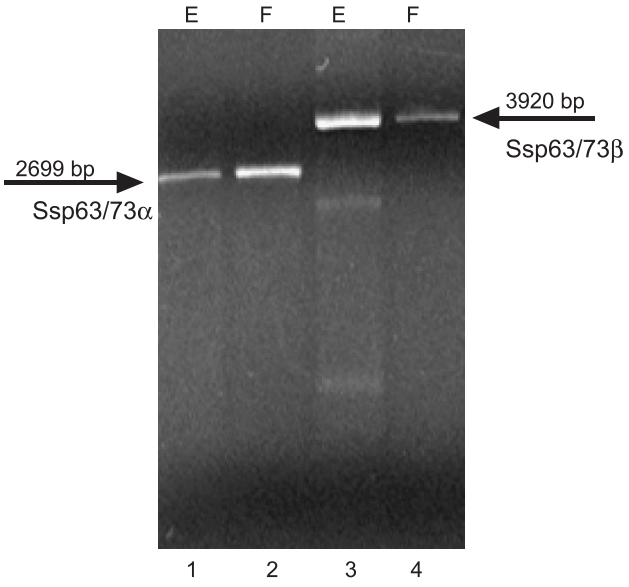

Fig. 1. Analysis of RT-PCR amplification products by gel electrophoresis through 1% agarose, followed by ethidium bromide staining. Lanes 1 and 2—*Spisula* p63/73α; lanes 3 and 4—*Spisula* p63/73β. Templates: lanes 1 and 3—*Spisula* 24 h embryo (E); lanes 2 and 4—*Spisula* adult foot (F). Total RNA (1–1.2 μg) was reverse transcribed using Clonetech SMART II A oligonucleotide. PCR was carried out using primers Spp63/73F and Revα or Revβ (see Table 1). For cycling parameters, see Section 2.6.

*Ssp63/73*β is 2122 nt. These lengths fall well within the range of 3′ UTR lengths (maximum 9142 nt, minimum 15 nt, average 444.5 nt) reported for invertebrate species (Pesole et al., 2001). The *Spisula p63/73* sequences have been deposited in Genbank (accession numbers, AY289767 and AY289768, respectively for the alpha and beta variants).

BLAST comparisons of the *Spisula* nucleotide sequences showed that the highest degrees of homology (second to our previously submitted *Spisula p53*-like sequence) were to *Mya arenaria*, the soft-shell clam *p53* and *p73*, and to *Danio rerio*, the zebra fish *p63* sequences. Regions of homology are restricted to the coding sequence. The 3′ UTRs of non-mammalian *p63* and *p73* genes appear to vary widely, most likely because these regions are under less evolutionary constraint than the coding regions (Grzybowska et al., 2001). The BLAST results show that orthologous relationships to *Spisula p63/73* may reflect phylogeny rather than function, possibly an artifact of the relatively small amount of sequence information available from nonmammalian species. However, functional domains in the p53-like sequences appear to be conserved across distantly related species. Assignment of specific invertebrate p53-like isoforms is difficult due to the current nomenclature, which relies too heavily on vertebrate homologues. Extensive sampling from a diversity of species will help to clarify this.

3.2. Polyadenylation site choice

This is the first report of multiple *p63/73* variants in any invertebrate species. We hypothesize that the use of alter-

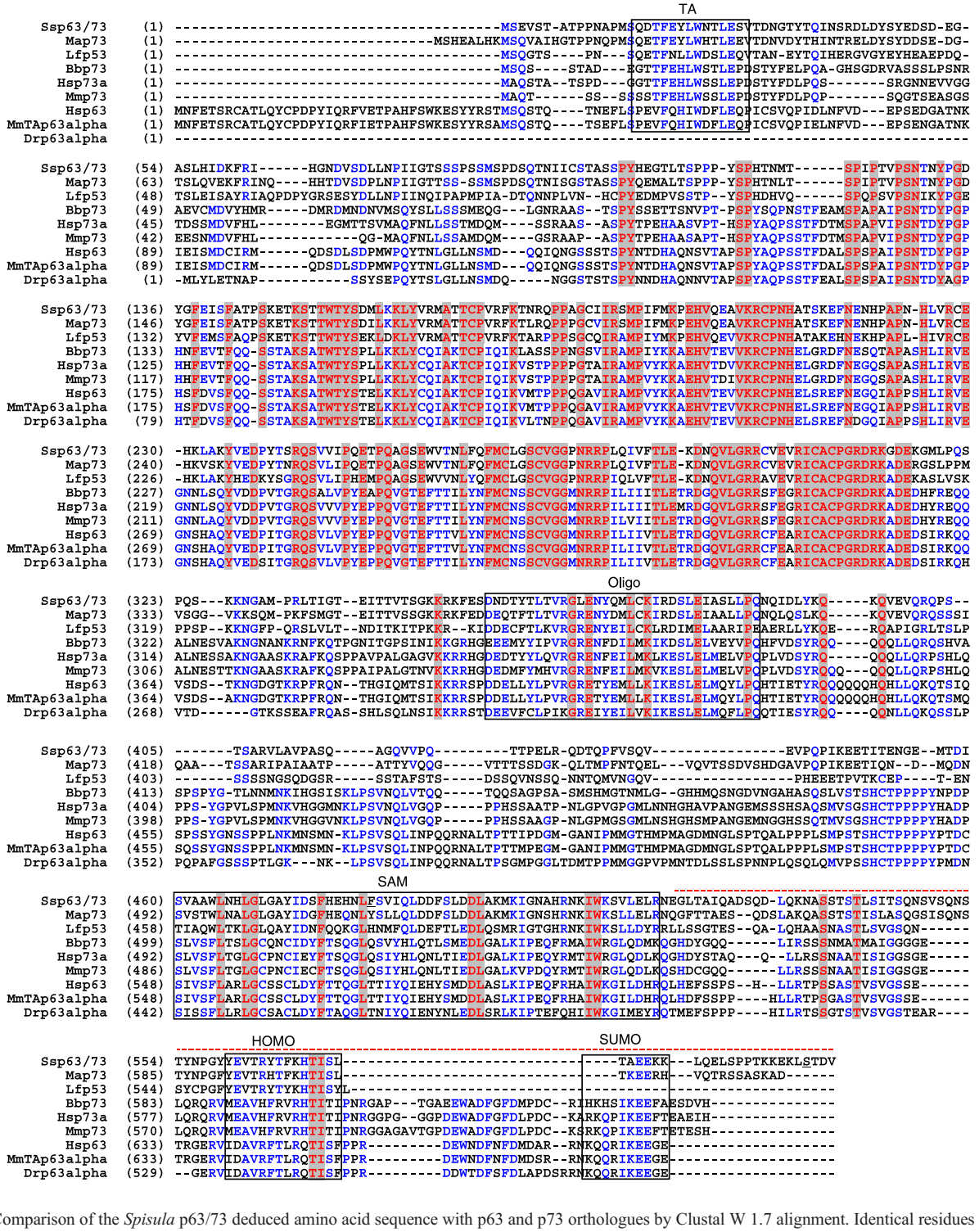

Fig. 2. Comparison of the *Spisula* p63/73 deduced amino acid sequence with p63 and p73 orthologues by Clustal W 1.7 alignment. Identical residues (red) are shown in blocks of gray. Conservative blocks of residues are shown in blue. TA, Oligo, SAM, HOMO domains and SUMO recognition site are indicated. TI domain is indicated by a red dashed line. Two amino acids differ between the alpha and beta variant. They are: amino acid 483 where a phenylalanine (F, underlined) in alpha is substituted for a tyrosine (Y) in beta; amino acid 593 where a serine (S, underlined) in alpha is substituted for a proline (P) in beta. Species and accession numbers are: *M. arenaria* (clam) Map73 (AAF67734); *Loligo forbesi* (squid) Lfp53 (AAA98563); *Barbus barbus* (fish) Bbp73 (AAD27752); *Homo sapien* (human) Hsp73a (CAA72220); *Mus musculus* (mouse) Mmp73 (CAB81953); *H. sapien* (human) Hsp63 (AAH39815B); *M. musculus* (mouse) MmTAp63alpha (AAC62641); *D. rerio* (zebra fish) Drp63alpha (AAM4810).

native polyadenylation sites accounts for the existence of the 2699 and 3920 base pair (bp) *p63/73* PCR products in *Spisula* embryo and foot (Fig. 1). These results indicate that the multiplicity of *p63/73* gene family members, well documented in vertebrates, extends to the invertebrates as well. In mammals, post-transcriptional processing at the 3′ end of the coding region gives rise to multiple p63 and p73 splice variants. From sequence analysis of the *Spisula* embryo variants, we found that the homologues diverge only in their 3′ non-coding regions, suggesting that they are tandem polyadenylation site variants. During RNA processing, when polyadenylation sites are formed, the choice of a particular site variant may confer stability to a particular transcript and, in the end, regulate specific isoform expres-

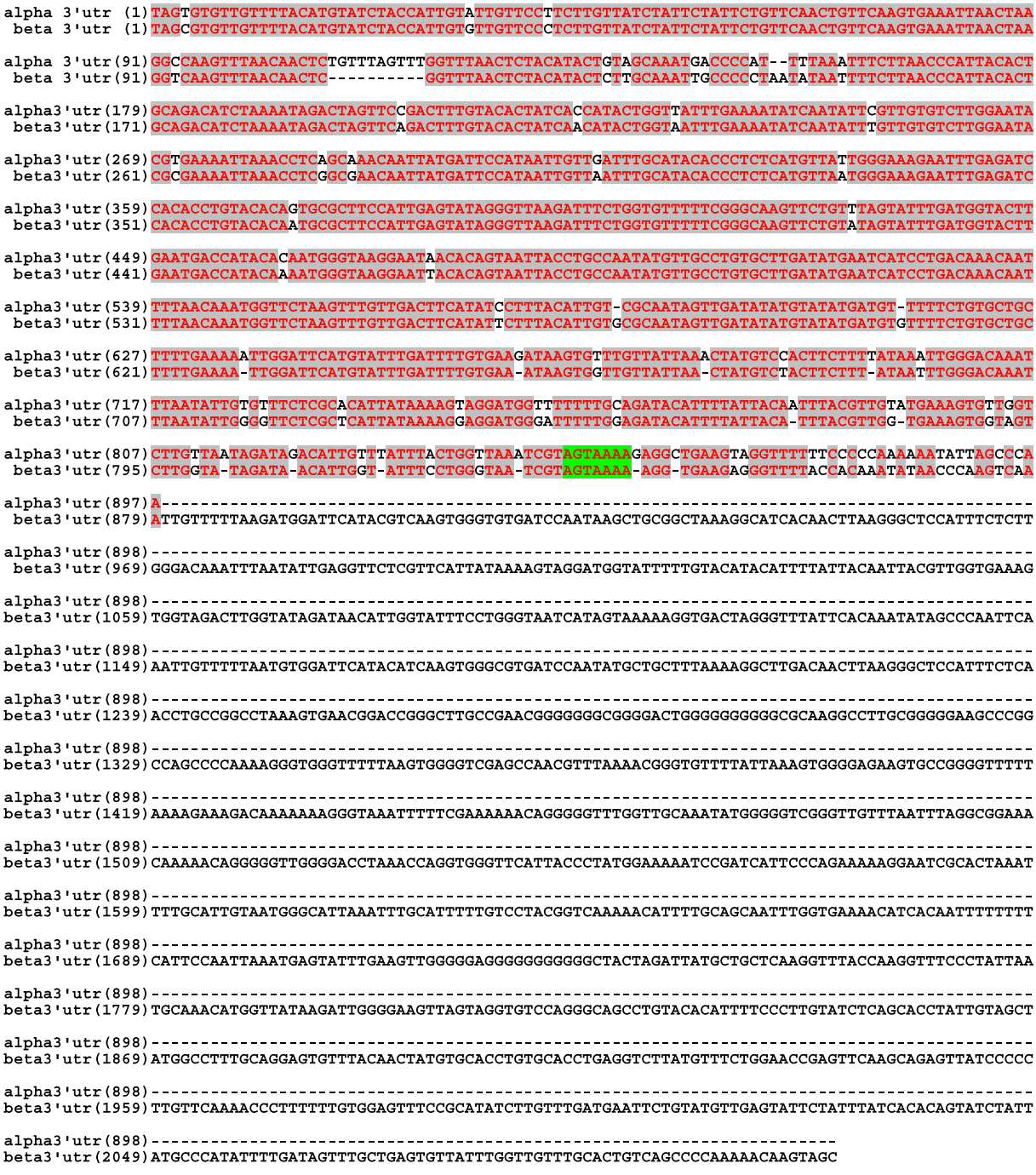

Fig. 3. Comparison of *Spisula p63/73* alpha and beta non-coding regions by Clustal W 1.7 alignment. Identical residues (red) are shown in blocks of gray. Potential "strong" poly-A site (*AGTAAA*) in alpha variant is highlighted in green. Sequence data from this article has been deposited with GenBank Database under accession numbers: AY289767 for *Ssp63/73*α and AY289768 for *Ssp63/73*β.

sion (Wickens et al., 1997). Since mRNAs often undergo alternative polyadenylation in a time specific manner, (Edwards-Gilbert et al., 1997), we hypothesize that during embryonic development in *Spisula*, the 3′ UTRs influence the differential p63/73 protein expression described earlier (Jessen-Eller et al., 2002).

Both 3′ UTRs contain the *ATTAAA* or *ATAAA* motifs, well conserved though less frequently used, hexanucleotide polyadenylation signals (PAS). The PAS determines the precise site of 3′ end cleavage and polyadenylation. In the *Spisula* 3′ UTRs, the distance between the putative PAS motifs and the poly-A tails is greater than the 11–30 nts, considered standard in mammalian transcripts (Edwards-Gilbert et al., 1997). This was also shown to be the case in the soft shell clam (*M. arenaria*) *p73* sequence, where the hexanucleotide sequence (PAS) occurs within 60 nts of the cleavage site (Kelley et al., 2001). In addition, within 30 nts of the *Spisula p63/73* alpha variant cleavage site, there is an *AGTAAA* signal (shown in Fig. 3), considered to be an alternate and, in some cases, a "stronger" poly-A signaling site (Legendre and Gautheret, 2003).

3.3. Cis-acting signaling motifs in the 3′ UTRs

The 3′ UTR has been identified as the location of multiple signaling sites controlling expression of genes involved in differentiation and development (Grzybowska et al., 2001). In addition, specific sequence motifs in the 3′ UTRs of mammalian *p53* genes appear to play a role in gene expression (D'Erchia et al., 1999; Fu et al., 1999). For these reasons, we chose to analyze the *Spisula p63/73* 3′ UTRs in order to locate specific motifs potentially controlling stability of mRNA species, and thus differential expression of the gene during development. A UTR search scan (found at bighost.area.ba.cnr.it/BIG/UTRHome) gave no hits, suggesting that the database is not representative of invertebrate motifs. However, we were able to identify relevant signaling motifs in both 3′ UTRs. The shorter alpha 3′ UTR contains 13 consecutive AT-rich elements (AREs), (ATTT, also referred to as "instability elements"). The longer beta variant of *Ssp63/73* contains 29 consecutive AREs in the 3′ UTR. These recognition signals specifically signal mRNA degradation and thus control mRNA processing for lymphokines, cytokines, and proto-oncogenes (Shaw and Kamen, 1986). While the minimum functional ARE sequence has not been identified, these highly conserved signaling elements are associated with differentially expressed transcripts (Asson-Batres et al., 1994) and may play a role in the differential expression of the *p63/73* gene.

We hypothesize that differential expression of p63/73 proteins during development of *Spisula* embryos (Jessen-Eller et al., 2002) may be partially controlled post-transcriptionally by *cis*-acting elements located in the 3′ UTR of the gene. The existence of two tandem polyadenylation site variants in *Spisula* supports the notion that mRNA stability and translation efficiency of p63/73 isotypes may be controlled by unique signaling elements in the 3′ non-coding regions of this gene.

3.4. Expression of multiple protein isoforms in Spisula

In order to characterize the expression of p63/73-like proteins in *Spisula*, we developed a polyclonal antibody against a highly conserved post-SAM domain region of p63 and p73 amino acid sequences. This region, shown in Fig. 2, is referred to as the "homology domain" (HOMO) (Jessen-Eller et al., 2002) and has no known function. However, the HOMO domain is contained within the transactivation-inhibitory (TI) domain (shown in Fig. 2), a region that acts as a negative inhibitor of p63 isoforms. The HOMO antiserum recognizes multiple bands on Western blots of *Spisula* neuron, embryos and foot (Fig. 4).

The most prominent band in *Spisula* neurons migrates at approximately 75 kDa (marked by arrow in Fig. 4A). However, another prominent band appears at 97 kDa and a less prominent pair of bands occurs at 120 and 140 kDa. In embryos, an immunoactive band at ~75 kDa is barely discernible, while three others, at 97, 120 and 140 kDa are prominent. The bands at 97 and 120 kDa correspond, respectively, to embryonic proteins detected with peptide antibodies generated against a common region of p53 (Kelley et al., 2001) and a unique region of *Spisula* p53-like sequences (Jessen-Eller et al., 2002), the latter being expressed temporally, in parallel with nervous system development. From our *Spisula* embryo p63/73 sequence data, we expected to see a more prominent band in the 65–75 kDa range on the *Spisula* embryo western blot. The lack of a prominent band in that molecular weight range suggests that mRNA processing, (as described in Sections 3.2 and 3.3), may control expression of the gene product on a temporal basis. This would help to explain our earlier results (Jessen-Eller et al., 2002) showing that the *Spisula* embryo *p53*-like gene is differentially expressed over time. As described in Section 2.9, we believe that the *p63/p73* transcript reported here and the *p120* transcript described previously (Jessen-Eller et al., 2002) are the same gene product. In *Spisula* foot (Fig. 4B), the antibody detects a 63 kDa protein in addition to the 75 and 97 kDa bands, with the 120/140 kDa bands being barely discernible. Thus, the HOMO-domain antibody recognizes a unique subset of higher molecular weight p53 family member proteins, p73/p97/p120, already identified separately in clams (Kelley et al., 2001; Stephens et al., 2001; Jessen-Eller et al., 2002), and may add a new ~140 kDa member as well.

The ~20 kDa incremental differences in molecular weight between the expected ~68 kDa protein(s) encoded by our sequences and these various higher molecular weight cross-reactive bands (p97, p120, p140) may be due to post-translational modification by sumoylation (Minty et al., 2000). As shown in Fig. 2, we have identified a putative

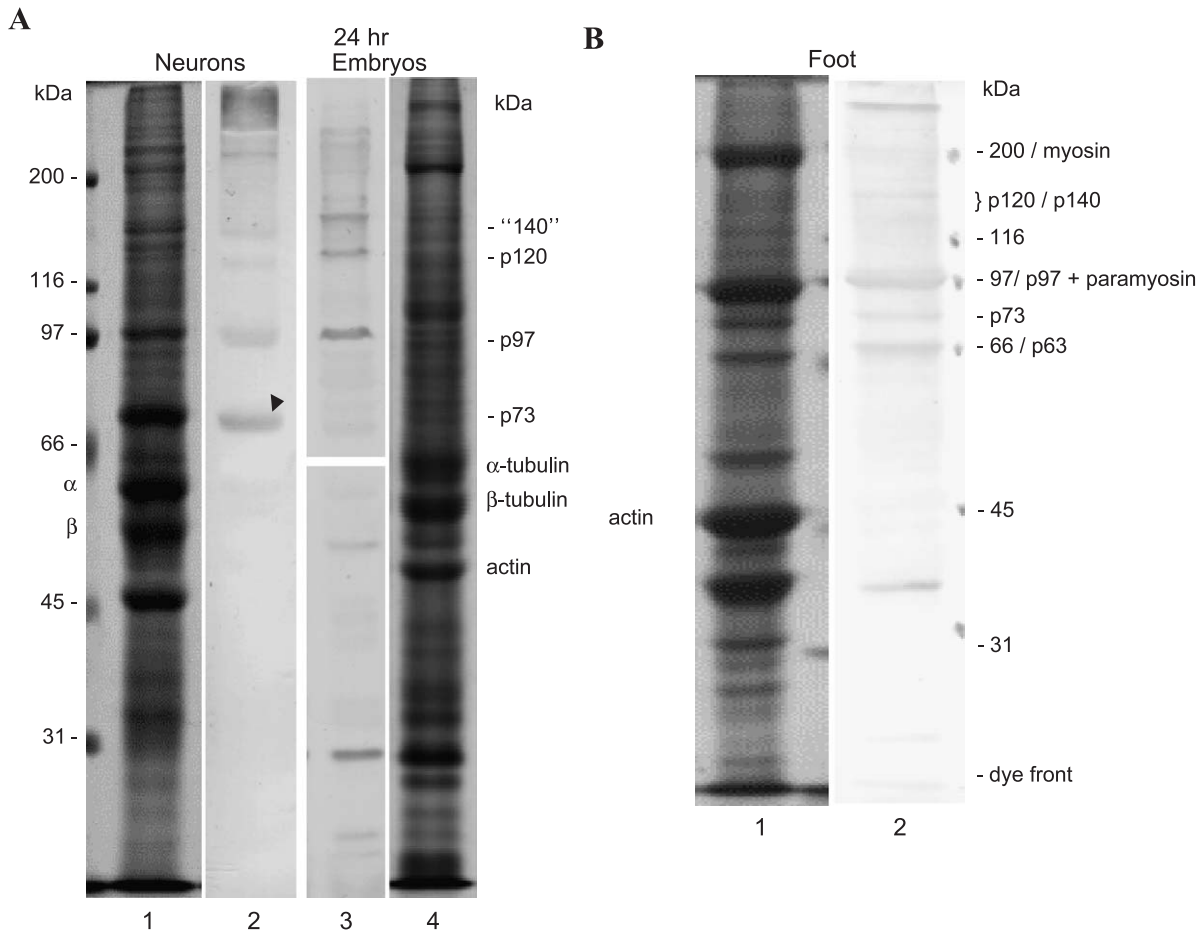

Fig. 4. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showing protein expression pattern for p63/73 family members in *Spisula*. Both blots used the rabbit p63/73 HOMO domain antibody. (A) *Spisula* adult neuron and embryo (24 h). Lanes 1 and 4—Coomasie blue stained gel; lanes 2 and 3—immunoblots with affinity-purified HOMO domain antibody. (B) *Spisula* foot tissue. Lane 1—Coomasie blue stained gel; lane 2—immunoblot with affinity-purified HOMO domain antibody. The low molecular weight bands visualized at 30 and 48 kDa (panel A) and 38 kDa (panel B) are variably detected and nonspecific, a result of incomplete blocking. Paramyosin runs as a 97 kDa band. Molecular mass markers are indicated.

sumoylation site at the C-terminal end of the *Spisula* p63/73 sequence. The position and relative band intensities would therefore reflect the relative degree of sumoylation of the primary p63/73 protein(s).

3.5. Comparative analysis of p63/p73

A question remains as to whether distinct *p63* and *p73* gene sequences will be characterized from nonmammalian species and whether these genes will be mapped to separate chromosomes. Initial information available from the invertebrates suggests that the nonmammalian *p53*-like orthologues thus far identified are splice variants of the same gene, rather than products of distinct genes, as in the case of mammals. The data reported here supports this hypothesis. Confirmation awaits the availability of more sequence from diverse species.

Phylogenetic analyses suggest that the ''primitive'' function of p63 and p73 was in development of the nervous system (Yang et al., 2002) and that over time, multiple, specialized functions evolved such as apoptosis and DNA-damage repair. *Spisula p63/73* is differentially expressed during embryonic development (Jessen-Eller et al., 2002) and we believe that mRNA processing as well as *cis*-acting signalling control *Spisula* p63/73 expression during development. Our identification of a dominant p63/73-like protein in *Spisula* neurons suggests that Ssp63/73 expression occurs during neurogenesis. Our future studies will focus on the location and control of *p63/73* gene expression in developing embryos.

A *p53*-like gene occurs in both *Caenorhabditis elegans* and *Drosophila*; however, these sequences lack the C-terminal SAM domain. The molluscan orthologues (*Mya* and *Spisula*) contain the SAM domain and thus share a greater degree of homology with vertebrate p63/73s than with their nonmammalian orthologues. These observations suggest that, as a model system for the study of *p63/p73* gene regulation, the molluscs are better suited than either *C.*

elegans or *D. melagonster*. In addition, due to the highly synchronous nature of their embryonic development, *Spisula* has been used extensively to examine mechanisms of centrosome formation, regulation of mRNA translation/polyadenylation, and development of the nervous system. These combined advantages clearly establish *Spisula* as a valuable model species for the comparative study of molecular mechanisms underlying regulation of p63/73 expression during embryonic and neural development.

4. Conclusions

(1) Two unique *p63/73* cDNA sequences, designated *Ssp63/73* alpha and beta, were identified in *S. solidissima*, the Atlantic surf clam.
(2) Primary sequence analysis suggests that the *Spisula p63/73* sequences represent alternate polyadenylation site variants. This is the first report, from any species, of polyadenylation site variants among the *p53* gene family. If these sequences represent functional site variants, then "site choice" represents a novel control point in *p63/73* gene expression during development.
(3) Novel *cis*-acting signaling motifs were identified in the 3′ UTRs of both *Spisula p63/73* variants. Molecular signaling during RNA processing may represent another level of control of *p63/73* gene expression.
(4) Western blot analysis with a *Spisula* specific p63/73 antibody demonstrates the expression of multiple immunoreactive bands in *Spisula* neuron, embryo and foot. We hypothesize that post-transcriptional and post-translational (small ubiquitin-related modifier (SUMO)-like) processing control the modulation in expression of p63/73 isotypes during development of the nervous system.

Acknowledgements

We thank Dr. Jill Kreiling for her expertise and careful review of the manuscript. This research was supported by Environmental Protection Agency Grant R82935901 to C.L.R. Molecular analyses were supported by a grant from the Alternatives Research and Development Foundation to C.L.R.

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