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SCIENCE DIRECT

Genomics 85 (2005) 92–105

GENOMICS

www.elsevier.com/locate/ygeno

Identification and characterization of RNA sequences to which human PUMILIO-2 (PUM2) and deleted in Azoospermia-like (DAZL) bind

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Received 13 August 2004; accepted 5 October 2004
Available online 6 November 2004

Abstract

Members of the Pumilio and DAZL family of RNA binding proteins are required for germ cell development in *Drosophila, Xenopus,* and *Caenorhabditis elegans*. Here, we report identification and characterization of RNA sequences to which PUM2 and DAZL bind. We established that human PUM2 specifically recognized the *Drosophila* Pumilio RNA target (the NRE or Nanos regulator element sequence); single nucleotide changes in the NRE abolished PUM2 binding. Then, we used coimmunoprecipitation to isolate human transcripts specifically bound by PUM2 and DAZL and subsequently identified those that contain NRE-like sequence elements. We confirmed that the interacting proteins, PUM2 and DAZL, are capable of binding the same RNA target and further characterized mRNA sequences bound by both proteins in the 3′ UTR of human *SDAD1* mRNA. Taken together, the results define sequences to which these germ cell-specific RNA binding proteins may bind to promote germ cell development.

Keywords: PUM2; DAZL; Germ cells; SDAD1; RNA; Ribonucleoprotein; Spermatogonia; Stem cells

In recent years, a number of genes that encode RNA binding proteins, such as VASA, NANOS-1, NANOS-2, NANOS-3, PUMILIO-1 (PUM1), PUMILIO-2 (PUM2), and members of the Deleted in Azoospermia (DAZ) family, have been identified in evolutionarily divergent organisms [1–7]. The central role of these proteins in germ cell development is illustrated in genetic studies. In all cases in which disruption of these genes has been reported, in flies, worms, frogs, mice, and human, severe defects in germ cell formation and differentiation are associated with null mutations [8–25]. More recently, reports have demonstrated that several of these conserved RNA binding proteins may interact in mammalian germ cells [2,5]. In a screen for proteins that interact with the human DAZ and DAZL (DAZ-like) proteins, five additional proteins that contain RNA-binding motifs were identified as interacting partners [5,26,27]. One of these, human PUM2 protein, shares 80% identity with *Drosophila* Pumilio over more than 280 amino acids that define the RNA-binding domain [2,5]. The homology of human PUM2 to *Drosophila* Pumilio was of particular interest given the well-defined role of *Drosophila* Pumilio as a translational repressor that is required for both anterior–posterior patterning and germ cell development in the fly embryo and adult [28–33]. In addition, other data from the evolutionarily distant roundworm, *Caenorhabditis elegans,* indicated that two Pumilio homologs, FBF-1 and FBF-2, act together to regulate germ-line stem cell maintenance by interacting with the 3′ UTR of *gld-1* mRNAs [34]. A third Pumilio homolog in *C. elegans*, PUF-8, also acts during germ cell development to regulate completion of meiosis [35]. Taken together, data from model organisms and the identification and characterization of human DAZ, DAZL, and PUM2 proteins as interacting factors suggest that the actions of these proteins may be conserved in germ-

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doi:10.1016/j.ygeno.2004.10.003

line stem cell maintenance and meiosis in diverse organisms including humans [2,5].

Although data are accumulating regarding the interactions and functions of germ-cell-specific RNA-binding proteins, less is known of the RNAs that they regulate. In model organisms, Pumilio protein may bind at least two different mRNAs, the NRE (Nanos regulating element) sequences of the 3′ UTR of *hunchback* mRNA during embryonic abdominal patterning in *Drosophila* [29] and the NRE-like sequences of the 3′ UTR of *cyclin B* mRNA during oocyte maturation in *Xenopus* [36]. Similarly, mouse Dazl protein may bind several mRNAs, including Cdc25A and Tpx1 [37]. Yet, it is unlikely that genes such as these are the only targets of Dazl protein given that *Cdc25A* is not essential for germ cell development and *Tpx1* is expressed only in the testis [38], whereas *Dazl* is essential for both male and female germ cell development [22]. Thus, we sought to explore further the identity of mRNAs that may be regulated by these RNA-binding proteins. We took advantage of the observations that the PUM2 and DAZ/DAZL proteins can form a stable complex on the same mRNA and that they colocalize in germ cells [5,26].

Results

Copurification of novel mRNA substrates bound by PUM2 and DAZL

Previous results demonstrated that PUM2 and DAZL proteins interact and form a complex on RNA [5]. Thus, we sought to identify mRNAs to which both the PUM2 and the DAZL proteins bind. Immobilized PUM2 or DAZL fusion proteins were mixed with purified human testis mRNAs. The mRNA bound to each fusion protein was then extracted and amplified via RT-PCR. RT-PCR products were cloned and 200 colonies corresponding to mRNA products derived from each DAZL and PUM2 experiment were evaluated. To enhance specificity, serial copurifications were performed for each protein and resulting mRNAs were reverse-transcribed, radiolabeled, and used to screen colonies identified in the first screen. mRNAs that were potentially bound by both the DAZL and the PUM2 fusions were identified as positives in at least two rounds of screening. These screens resulted in the identification of 61 mRNAs that are potential targets (Table 1). Many of the mRNAs are translated into proteins involved in growth regulation; a few may have roles in spermatogenesis. We used these mRNAs to characterize PUM2 and DAZL consensus binding sites, in more detail.

Analysis of PUM2 binding sites by yeast three-hybrid assay

Coimmunoprecipitation allowed identification of potential mRNA targets but did not provide information regarding binding sites. We previously demonstrated that human PUM2 binds to the *Drosophila* NRE sequence in the presence or absence of DAZL protein [5]. Given the degree of conservation between the *Drosophila* and the human Pumilio proteins, we sought additional information regarding requirements for PUM2 to bind the *Drosophila* RNA target, the NRE. The minimal NRE binding site as recently described [39] was cloned into the plasmid pIIIA/MS2-2. This plasmid allows expression of hybrid RNA molecules in yeast. We mutagenized 11 nucleotides, 5 nucleotides within Box A (GUUGU) and 6 nucleotides within Box B (AUUGUA), and analyzed the ability of these constructs to recruit binding of the PUM2 RNA-binding protein fused to the *GAL4* activation domain. RNA fusion constructs and RNA-binding fusion constructs were cotransfected into yeast and selected for on medium lacking uracil and leucine. The interaction between the RNA targets and PUM2 was assayed for by confirming activation on the *lacZ* reporter by β-galactosidase activity assays. Based on the results of this mutational analysis, we identified a minimal sequence (GNNNNNNNNNNUGUA) that can support PUM2 binding (Fig. 1A). No interaction with the NRE sequence by DAZ, DAZL, or the GAL4 activation domain of the vector was detected (data not shown), thus confirming that the RNA–protein interaction was specific to PUM2. We next analyzed the sequences of the transcripts that we had identified for the presence of this sequence. Twenty elements were identified in 12 transcripts, which were then screened for PUM2 binding in the yeast three-hybrid system (Fig. 1B). Of the 12 elements screened, only 2 were recruited PUM2. We focused on the mRNA of an uncharacterized protein (FLJ10498), SDAD1, which is homologous to the *sda1* (severe depolymerization of actin) gene in yeast.

Expression of SDAD1

For PUM2 and DAZL to regulate an mRNA posttranscriptionally in vivo they must be expressed within the same cell type, the germ cell. We examined expression of *SDAD1* by Northern and RT-PCR analysis. As shown by Northern analysis, *SDAD1* is highly expressed in the testis, kidney, spleen, and brain (Fig. 2A). Lower expression was also detected in heart, lung, liver, small intestine, ovary, uterus, breast, and placenta (Fig. 2A). To extend these results, RT-PCR was also used to determine if *SDAD1* was expressed in human embryonic stem cells and various fetal and adult tissues (Fig. 2B). Results confirmed that *SDAD1* is widely expressed in all of the embryonic, fetal, and adult tissues studied to date.

Identification of PUM2 and DAZL binding sites on the SDAD1 3′ UTR

Based on the observation that PUM2 and DAZL can form a stable complex and that PUM2 may specifically bind a sequence located within the 3′ UTR of *SDAD1,* we