CHANGING U1A LEVELS REGULATE EXPRESSION OF IMMUNOGLOBULIN M AND THE TRANSCRIPTIONAL REPRESSOR ZHX1 DURING B CELL DIFFERENTIATION

by

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ABSTRACT OF THE DISSERTATION

Changing U1A levels regulate expression of IgM and the transcriptional repressor Zhx-1 during B cell differentiation

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During B cell differentiation U1A plays an important role in regulating the expression of the secretory poly(A) site by inhibiting both cleavage and polyadenylation. Previous work demonstrated that the inhibitory effect of U1A is alleviated in differentiated cells, which express the secretory poly(A) site, however, the mechanism underneath was unveiled. Using B cell lines representing different stages of B cell differentiation, here we show that U1A levels are reduced in differentiated cells. Undifferentiated B cells have more total U1A than differentiated cells and a greater proportion of U1A is not associated with the U1snRNP. We demonstrate that this non-snRNP associated U1A is available to inhibit poly(A) addition at the secretory poly(A) site. In addition, endogenous non-snRNP associated U1A—immunopurified from the different cell lines—inhibited poly(A) polymerase activity proportional to U1A recovered, suggesting that available U1A level alone is responsible for changes in its inhibitory effect at the secretory IgM poly(A) site.

It is known that U1A can regulate the expression of its own and IgM gene. Here we report that during mouse B cell differentiation U1A also regulates the expression of the transcriptional repressor, Zhx-1 (zinc fingers and homeoboxes 1), via alternative poly(A) site selection. Using affymetrix microarray analysis combined with RT-PCR techniques, we demonstrate that U1A binds to Zhx-1 mRNA in vivo. We show that the levels of Zhx-1 proteins and mRNA are negatively correlated with U1A levels in B cells and overexpression of U1A in HeLa cells significantly inhibits the expression of Zhx-1. Our in vitro and in vivo assays show that U1A regulates the expression of the upstream poly (A) site of Zhx-1 by binding to the five non-consensus motifs around the poly(A) site and inhibiting both poly(A) addition and cleavage. When the upstream poly(A) site of Zhx-1 is inhibited in mature B cells, the usage of the downstream poly(A) site of Zhx-1 results in the inclusion of ARE elements, which destabilize the mRNA transcript. As a result, less Zhx-1 RNA and protein are produced in mature B cells. We proposed one model about how U1A and ARE coordinately regulate the expression of Zhx-1 during B cell differentiation.

DEDICATION

This thesis is dedicated to my parents and my parents-in law

my sister and her family,

my wife, Lixia,

my daughter, Gloria,

my son, Victor,

for their unconditional love and support through all my life.

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Introduction

3′ end processing of nearly all eukaryotic pre-mRNAs is indispensable for nuclear export (Eckner et al., 1991) and stability of mRNA (Decker and Parker, 1994; Bernstein and Ross, 1989). Therefore, regulation of 3' end pre-mRNA processing plays an extremely important role in modulating expression of many genes in a tissue- or developmental stage-specific manner. Many eukaryotic and viral genes produce mRNAs with different 3' ends due to the choice between alternative poly(A) sites. One well-characterized model of alternative polyadenylation is IgM heavy chain mRNA. During B cell differentiation, IgM heavy chain is processed into either secretory form or membrane form depending on which poly(A) site is used. U1A protein has been known to regulate the 3' end formation of its own pre-mRNA as well as that of the IgM heavy chain during B cell differentiation (Gunderson et al., 1994 and 1997; Phillips et al., 2001 and 2004). This process of B cell differentiation usually consists of multiple steps, each with a distinct gene expression pattern (Igarashi et al., 2007). Aberrant expression patterns may cause B cell lymphoma, myeloma and malignancy (Kamio et al., 2003; Dalla-Favera et al., 1999; Sakane-Ishikawa et al., 2005). Therefore, studies on how U1A regulates the expression pattern of IgM heavy chain gene and whether it has some other target genes in B lymphoid cells will deepen our understanding of U1A's role in B cell differentiation. In this thesis, using established B cell lines representing the different stage of B cell development, we have found that (1) U1A levels decreases during B cell differentiation and the decreased U1A releases its inhibition of the IgM gene, (2) Zhx-1 (a transcriptional factor) is an additional target of U1A regulation. U1A regulates alternative polyadenylation of IgM heavy chain gene resulting in different proteins. In contrast, U1A

regulates Zhx-1 levels by inclusion or exclusion of ARE elements via alternative poly(A) site selection.

3'-end processing of mammalian pre-mRNAs

Most eukaryotic pre-mRNAs are subjected to a series of post-transcriptional processes which are essential for mRNA maturation. These processing events include 5' end capping, splicing and 3' end formation. The formation of the 3' end enhances transcription termination and transport of the mRNA from the nucleus, as well as the translation and stability of mRNA (Colgan and Manley, 1997; Eckner et al., 1991; Sachs and Wahle, 1993). Defects in mRNA 3' end formation can greatly change cell growth and development (Zhao and Manley, 1998; Takagaki and Manley, 1998). In humans, inappropriate or aberrant polyadenylation has been linked with some diseases such as lysosomal storage disorder, sporadic amyotrophic lateral sclerosis and thalassemias (Reviewed in Zhao et al., 1999a). To better understand the role of 3' end formation in cell growth and development, much effort has been made to explore the fundamental mechanism of mRNA 3' end formation and its regulation. Currently most of the factors involved in 3' end processing have been identified and much information has been obtained on how those factors and cis-elements in RNA interact with each other and how the basic polyadenylation machinery is regulated. Here we mainly focus on the 3' end formation of mammalian mRNA due to space limitation.

Cis-elements

In eukaryotes, the 3' end processing of most pre-mRNAs consists of two coupled steps: cleavage and polyadenylation. The processing efficiency is ultimately determined by the cis-elements on the RNA precursors. In mammalian cells, the core polyadenylation

signal is defined by three cis-elements— the poly(A) signal, the downstream elements (DSE) and the poly(A) site (Reviewed in Zhao et al., 1999a). For most genes the poly(A) signal is a highly conserved hexanucleotide sequence AAUAAA (canonical) or AUUAAA located 10-30 nucleotides upstream of the cleavage site (Proudfoot, 1991; Wahle and Kuhn, 1997) and the hexanucleotide is indispensable for both cleavage and polyadenylation. Poly (A) sites with single or two-base variants do occur in some genes, however, they are processed less efficiently (Beaudoing et al., 2000) and are often involved in alternative or tissue-specific polyadenylation (Hook and Kellems, 1988; Challoner et al., 1989). Downstream elements (DSEs) are located within ~30nts downstream of the poly(A) signal. It is poorly-conserved and can be a U-rich element or a GU-rich element or both. A poly(A) signal may have one DSE working alone or two DSEs working together (Chou et al., 1994; Gil and Proudfoot, 1987). The distance of the DSE to the poly(A) site is crucial for its function in affecting the cleavage site position and the cleavage efficiency (MacDonald et al., 1994; Gil and Proudfoot, 1987; McDevitt et al., 1986). However, the selection of the cleavage site (also called the poly(A) site) is mainly determined by the distance between the DSE(s) and upstream poly(A) signal (Chen et al., 1995). Although the local sequence surrounding the cleavage site varies, cleavage and polyadenylation occur after a CA dinucleotide for most genes (Sheets et al., 1990; Chen et al., 1995). Besides the above three cis-elements, some auxiliary sequences such as upstream elements (USE) have been found to modulate the activity of 3' end processing in some viral and cellular genes (Reviewed in Zhao et al., 1999a). In addition, the secondary structures in mRNA are also involved in affecting the use of certain poly(A) sites (Phillips et al., 1999; Hsieh et al., 1994; Klasens et al., 1999).

Trans-acting factors

Multiple trans-acting protein factors are also involved in the 3' end formation of mammalian mRNA. Cleavage/polyadenylation specificity factor (CPSF), Cleavage stimulatory factor (CstF), cleavage factors CFIm and CFIIm, RNA polymerase II (Pol II) and Poly(A) polymerase (PAP) participate in the cleavage step and CPSF, PAP and poly(A)-binding protein II (PABII) participate in the polyadenylation step (Zhao et al., 1999a). Mammalian CPSF recognizes and binds the poly(A) signal and it has multiple subunits: CPSF 160, 100, 73, 66 and 30 kDa (Murthy and Manley, 1995; Bienroth et al., 1991). CPSF-160 plays a key role in recognizing and binding AAUAAA, however, the other subunits of CPSF may facilitate the recognition (Murthy and Manley, 1995). In addition, CPSF-160 cooperatively interacts with PAP and the 77 kDa subunit of CstF, therefore facilitating the assembly of cleavage/polyadenylation complexes on the mammalian precursor RNA (Wahle and Kuhn, 1997). The exact function of CPSF-100 and CPSF-30 is unknown and it is thought that they may play a role in RNA binding and stabilize the polyadenylation complex (Edwalds-Gilbert and Milcarek, 1995; Chen et al., 1999). As for the CPSF-73, multiple evidence has shown that it is an endonuclease and may perform the actual cleavage reaction during 3' end formation (Mandel et al., 2006; Ryan et al., 2004; Callebaut et al., 2002). Recently, one new 66kDa factor called Fip1 has been identified in mammals as an integral subunit of CPSF (Kaufmann et al., 2004). Human Fip1 preferentially binds to the U-rich elements upstream of the poly(A) signal through its arginine-rich RNA binding motif. In addition, human Fip1 interacts with PAP and it can form a ternary complex with CPSF160 and PAP in vitro. Thus it may act

together with CPSF-160 in poly(A) site recognition and cooperatively recruit PAP to the RNA (Kaufmann et al., 2004).

Cleavage stimulatory factor CstF participates in both cleavage and polyadenylation steps and mammalian CstF has 3 subunits: 77, 64 and 50 kDa (Moreira et al., 1998; Zhao et al., 1999a). CstF64 has a classical RNA-binding domain near its amino terminus and it recognizes and binds GU- and U-rich sequences downstream of the poly(A) site (Takagaki and Manley, 1997; MacDonald et al., 1994). CstF77 bridges the CstF64 and CsfF50 and directly interacts with CPSF-160 to mutually stabilize the CPSF-CstF-RNA complex (Murthy and Manley, 1995; Takagaki and Manley, 2000). CstF50 can mediate protein-protein interaction through its seven transducin or WD-40 repeats and may be involved in the recruitment of pol II through its interaction with CTD of RNA polymerase II (McCracken et al., 1997).

Mammalian CFIm and CFIIm are two cleavage factors only required for the cleavage step. CFIm is a heterodimeric protein and preferentially binds a set of UGUAN (N=A>U>=C/G) sequences in pre-mRNAs (Brown and Gilmartin, 2003). It has been suggested that CFIm may stabilize the 3' end-processing complex and facilitate the recruitment of other processing factors in early steps by interaction with the subunit hFip1 of CPSF (Ruegsegger et al., 1996 and 1998). The binding of CFIm to pre-mRNA is not only important for cleavage and polyadenylation (Venkataraman et al., 2005; Kim and Lee, 2001) but also involved in coupling 3' processing and splicing (Awasthi and Alwine, 2003; Ruegsegger et al., 1998). In addition, human CFIm has been suggested to work as a regulator of poly(A) site selection (Brown and Gilmartin, 2003). CFIIm can be divided into two parts according to their activity for the cleavage reaction: one

essential (CFIIAm) part and one stimulatory (CFIIBm) part (De Vries et al., 2000). CFIIm can interact with CFIm and CPSF but the exact function of this interaction remains unclear.

Poly(A) polymerase (PAP) plays a key role in the 3' end formation of mammalian mRNA and it is required for both cleavage and polyadenylation. The vertebrate PAPs are highly homologous among species (Martin and Keller, 1996) and in each species it has multiple isoforms most likely generated by gene duplication, alternative RNA processing, or post-translation modification (Lee et al., 2000; Tupler et al., 2001; Raabe et al., 1991; Zhao and Manley, 1996; Colgan et al., 1996; Ballantyne et al., 1995). PAP II and PAPγ are the two main forms of PAP in human and PAP II is located in both the nucleus and the cytoplasm while PAPγ appears only in the nucleus (Thursson et al., 1994). They are highly conserved in the catalytic domains, ATP recognition domain and RNA binding domain. Both PAPs have poly(A) signal-dependent specific activity and poly(A) signal-independent nonspecific activity (Topalian et al., 2001; Kyriakopoulou et al., 2001; Thursson et al., 1994). However, the activity of PAP II not PAPγ is regulated by phosphorylation that regulates polyadenylation during the cell cycle (Colgan et al., 1996 and 1998; Zhao and Manley, 1998; Bond et al., 2000). Some other proteins such as U1A (discussed in more details later) (Gunderson et al., 1997) and 14-3-3ε (Kim et al., 2003) also regulate the activity of PAP II via the interaction with the C-terminal region of PAP II. The C-terminal region of PAP II is essential for its interaction with some cleavage factors such as CFIm (Kim and Lee, 2001) and splicing factors such as U2AF 65 (Vagner et al., 2000b). Such interactions may play a role in coupling the polyadenylation events with cleavage events and splicing events. Recently, a new cytoplasmic PAP called GLD-

2 has been identified in human, mouse and other organisms (Kwak et al., 2004; Rouhana et al., 2005; Barnard et al., 2004; Wang et al., 2002). This non-conventional PAP is responsible for cytoplasmic polyadenylation and may be involved in embryogenesis and germline development.

RNA Polymerase II (Pol 2) also directly participates in the formation of a stable cleavage complex through the interaction of the carboxyl terminal domain (CTD) of its largest subunit with CPSF and CstF (McCracken et al., 1997; Hirose and Manley 1998). The CTD of the Pol 2 large subunit consists of tandem heptad repeats which are conserved among species (Barron-Casella and Corden, 1992). In mammals it consists of 52 heptapeptides, of which 21 have the consensus sequence YSPTSPS and the remaining 31 have related sequences (Corden et al., 1985; Barron-Casella and Corden, 1992). CTD function depends on both its sequence and its length. In mammals 25 tandem heptad repeats plus a 10 amino acid motif at the C-terminus are the minimal requirements for its function in mRNA processing. Dynamic site-specific phosphorylation and dephosphorylation of these heptad repeats is also a critical mechanism for regulating CTD function (Komarnitsky et al., 2000). Deletion of CTD inhibits all three major pre-mRNA processing steps in vertebrate cells: capping, splicing, and cleavage of the poly(A) site (McCracken et al., 1997). Therefore, the Pol 2 CTD has been suggested to work as a landing pad for recruitment of RNA processing factors and so acts as a scaffold for two-way communication with the polymerase. (Bentley, 2005; Kotovic et al., 2003; Greenleaf, 1993).

Besides the above trans-acting factors, symplekin has recently been demonstrated to be a new member of the mammalian 3' end formation machinery. It can form a

complex with CstF and CPSF in the nucleus and form a complex with the cytoplasmic polyadenylation element binding protein (CPEB) and CPSF in the cytoplasm (Hofmann et al., 2002; Takagaki and Manley, 2000). Symplekin has been suggested to work as an assembly platform for the mammalian polyadenylation machinery but elucidation of its exact function in 3' end formation still requires further studies (Takagaki and Manley, 2000).

Polyadenylation and cleavage

In summary, 3' end processing factors such as CPSF and CstF are co-transcriptionally recruited to the pre-mRNA 3' end by the CTD of the largest subunit of Pol 2 (Ryan et al., 2002; McCracken et al., 1997; Hirose and Manley, 1998). Through the interaction of CPSF-160 and CstF-77 (Murthy and Manley, 1995; Wilusz et al., 1990), CPSF and CstF cooperatively bind at the AAUAAA poly(A) signal and the downstream U- or GU-rich sequence respectively (Bienroth et al., 1991; Keller et al., 1991; Murthy and Manley, 1992; Takagaki et al., 1990; MacDonald et al., 1994). The binding of CFIm to the pre-mRNA and its interaction with CPSF enhance the assembly of the cleavage complex in the early steps while the participation of CFIIm stimulates the cleavage reaction (Ruegsegger et al., 1996 and 1998; De Vries et al., 2000). PAP is recruited to the cleavage/ polyadenylation complex probably by its interaction with CPSF-160, CstF77 and/or CFIm (Kim and Lee, 2001; Murthy and Manley, 1995). Once the stable cleavage/ polyadenylation complex is formed, it is CPSF-73 that performs the cleavage at the cleavage site (Mandel et al., 2006; Ryan et al., 2004; Callebaut et al., 2002). After cleavage, CPSF and PAP remain bound to the cleaved RNA and PAP elongates the poly (A) tail to about 250 As in the presence of PAB II (Wahle et al., 1991).

Coupling 3' end formation with splicing and transcription

Many pre-RNA processing events in eukaryotes occur actually before transcription termination and some mRNA processing factors have protein-protein contacts with elongating Pol 2 in a complex called the "mRNA factory" which synthesizes, processes and packages the transcript (Reviewed in Zorio and Bentley, 2004). In other words, eukaryotes have developed a complicated and extensively coupled network instead of a simple linear assembly line to coordinate the transcription and mRNA processing events (Reviewed in Maniatis and Reed, 2002).

The idea that 3' end formation and transcription are kinetically coupled is based on the fact that a functional poly(A) site is required for efficient transcription termination (Logan et al., 1987; Whitelaw and Proudfoot, 1986; reviewed in Proudfoot, 1989). The recent discovery that the speed of transcription elongation influences the choice of poly(A) sites further supports such an idea (Cui and Denis, 2003). An early connection between general transcription initiation factors and 3' end formation originates from the discovery that RNA 3' end processing factors such as CPSF and CstF are recruited to the promoter by TFIID and then transferred to pol II during initiation (Hirose and Manley, 1997; Dantonel et al., 1997). Both the CTD of the Pol 2 large subunit and transcription elongation factors play critical roles in coupling transcription to pre-mRNA processing. The interactions of 3' end processing factors with some other components of the transcription machinery also coordinate co-transcriptional cleavage or polyadenylation. In addition, some factors involved in 3' end processing are also required for termination (Steinmetz and Brow, 2003; Birse et al., 1998; Dichtl et al., 2002; He et al., 2003; Proudfoot et al., 2002; Proudfoot, 2004). Generally speaking, coupling of transcription by

Pol 2 with 3’ end formation can influence both processes. Coupling affects 3’ end processing in two main ways. First, it positions mRNA 3’ end processing factors at the elongation complex, thus raising their local concentration around the nascent transcription. Second, the transcription rate can affect the choice of poly(A) sites (Cui and Denis, 2003). Two models have been proposed to explain how mRNA 3’ end formation affects transcription termination. One is called the “anti-terminator” model in which the extrusion of the polyadenylation sequences on the RNA cause a change in the factors associated with the polymerase. The other is called the “torpedo” model in which the cleavage of the transcript at the polyadenylation site generates a new uncapped 5’ end as an entry point for exonucleases to dissociate the polymerase from the transcript (Reviewed in Buratowski, 2005).

Splicing and 3'-end processing of vertebrate pre-mRNAs are also tightly coupled to coordinate gene expression. Splicing factors that associate with the terminal 3’ intron can interact with downstream polyadenylation factors and regulate the cleavage and polyadenylation reactions. There is evidence that SRm160, a coactivator of constitutive and exon enhancer-dependent splicing, participates in 3'-end formation. It binds specifically with CPSF and can efficiently stimulate the 3'-end cleavage of splicing-active pre-mRNAs in vitro (McCracken et al., 2002). The U2 snRNP Auxiliary Factor 65 kDa (U2AF 65) can interact with PAP and CFIm to stimulate cleavage and polyadenylation and therefore plays a direct role in coordinating 3’ end processing and splicing (Millevoi et al., 2006; Millevoi et al., 2002; Vagner et al., 2000b). The SR family protein SRp20, polypyrimidine tract-binding protein (PTB) and U1 snRNP components such snRNA, U1snRNP-A protein, U1 snRNP 70k protein are also implicated in 3’ end formation (Lou

et al., 1996 and 1998; Wassarman and Steitz, 1993; Lutz et al., 1996; Gunderson et al., 1998). In addition, U1 snRNP bound to a 5' splice site exhibits position-dependent inhibition of either cleavage or polyadenylation (Vagner et al., 2000). Reciprocally, cleavage/polyadenylation complexes can also affect the splicing of the upstream 3' terminal intron (Berget et al., 1995). According to Berget et al (1995), 3' terminal exon definition involves the coordinate recognition of a 3' splice site with the adjacent downstream cleavage and polyadenylation signals. A cleavage/polyadenylation site can stimulate splicing by facilitating the recognition of the adjacent upstream 3' splice site in a manner analogous to exon splicing enhancer (Vagner et al., 2000). However, in the case of the cleavage/polyadenylation site, it is the cleavage/polyadenylation machinery that recognizes the site and PAP fulfills the role of the SR proteins to establish the communication between the enhancer site and the 3' splice site.

Regulation of 3' end formation

Many eukaryotic and viral genes give rise to mRNAs that differ in their 3' ends due to the choice between alternative poly(A) sites. A large-scale bioinformatic study of ESTs has estimated that a great proportion of human (~54%) and mouse genes (~32%) have alternative polyadenylation sites (Tian et al., 2005). In addition, for many given poly(A) sites there are often multiple cleavage sites (Pauws et al., 2001; Tian et al., 2005), leading to heterogeneous 3' end formation for transcripts. As the selection of different poly (A) sites determines the final sequence and activity of those mRNA, the regulation of 3' end pre-mRNA processing has a significant potential to modulate expression of many genes in a tissue- or developmental stage-specific manner. In spite of this only a few examples of regulated polyadenylation are understood. Thus it is striking

that although substantial progress has been made in the characterization of the basic 3' end processing apparatus, its regulatory aspects are still only poorly understood (Barabino and Keller, 1999). The choice of the alternative poly(A) site has been shown to be regulated in five different ways as follows: (1) the intrinsic strength of the *cis*-acting sequence elements that define the cleavage site and the use of different poly (A) signals; and (2) changes in the concentration or the activity of constitutive polyadenylation factors (Takagaki et al., 1996; Colgan et al., 1998; Edwalds-Gilbert and Milcarek, 1995) and cleavage factors such as CFIm (Brown and Gilmartin, 2003); (3) expression of tissue or stage-specific regulatory factors (Phillips et al., 1996; Edwalds-Gilbert et al., 1997; Veraldi et al., 2001); (4) splicing factors, such as U1 snRNP and its associated proteins U1A and U1 70k (Gunderson et al., 1997; Lutz et al., 1996; Ko and Gunderson, 2002; Phillips et al., 2001 and 2004), polypyrimidine tract-binding protein (PTB) (Lou et al., 1999), SRp20 (Lou et al., 1996) and U2AF 65 (Millevoi et al., 2006; Millevoi et al., 2002; Vagner et al., 2000b); (5) the position of a poly(A) site relative to other poly(A) sites. If all the poly(A) sites are intrinsically equal in strength, the proximal site will be preferentially used. In this regard, it is interesting to note that the 5' most poly(A) sites use variant signals more often, while the 3'-most sites tend to use a canonical signal consistent with the idea that variant signals (including the common AUUAAA) are processed less efficiently than the canonical signal and could therefore be selected for regulatory purposes (Barabino and Keller, 1999).

U1A

U1A was originally discovered as one component of the U1 small nuclear ribonucleoprotein (U1 snRNP) involved in pre-mRNA splicing. It is a 32KD protein,

highly conserved in vertebrates and has two evolutionarily conserved RNA recognition motifs (RRM), which are connected by a linker region (aa 102-200) that includes a homodimerization region (aa 102-115) (Fig 1A). The RRM, also known as an RNA binding domain (RBD) has been found in proteins that participate in almost all steps of gene expression in all three kingdoms of life. Each RRM contains three highly conserved aromatic amino acids that contribute to the stacking interactions with RNA bases. Of the two RRMs of U1A, only the N-terminal RRM1 (aa 1-101) can interact specifically with the loop sequence AUUGCAC in hairpin 2 of U1 snRNA. The RRM2 (aa 201-282) has low affinity for RNA possibly due to lack of a stretch of eight highly conserved consensus amino acids (Scherly et al., 1989; Lutz-Freyermuth et al., 1990).

The U1A structure and its interaction with RNA

The N-terminal RRM of the U1A protein has been solved both by NMR and X-ray crystallography. It has a βαββαβ global fold and forms a four-stranded antiparallel β-sheet as the primary binding surface for RNA (Stump and Hall, 1995; Oubridge et al., 1994). The RNA loop lies across the β sheet and fits into a groove formed between loop 3 (connecting β3 and β2) and the C-terminal portion of the RRM domain. The β2 strand and the loop between β3 and β2 are crucial for RNA binding activity (Bentley and Keene, 1991; Scherly et al., 1990). However, additional flanking sequences in the form of a third α helix-helix C (aa 92-98) are also necessary (Jovine et al., 1996; Howe et al., 1998; Gubser and Varani, 1996). X-ray crystallography and NMR of the U1A (aa 2-98) / U1 snRNA complex have shown that the binding involves a short-range interaction of the side chain with the nucleotide base and an electrostatic long-range interaction through a

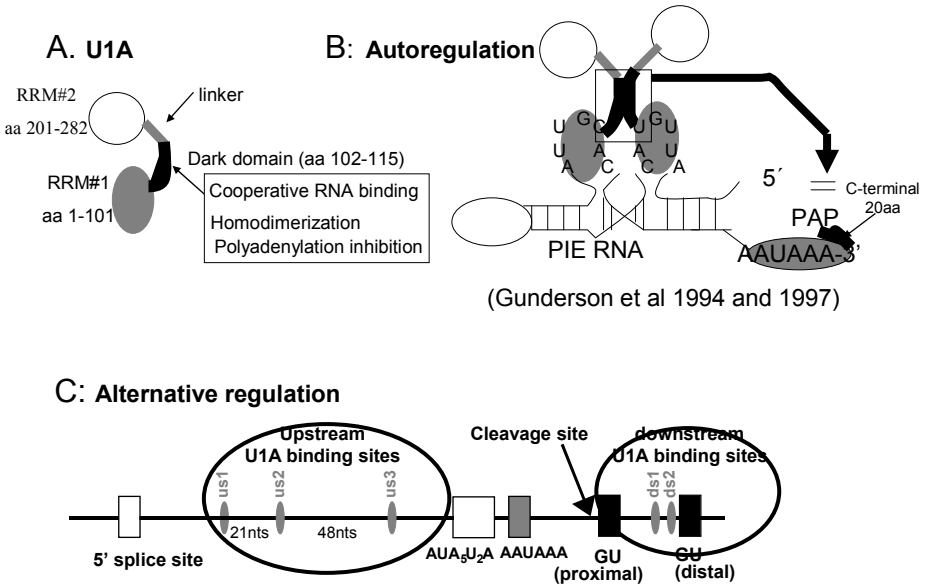


FIGURE 1. Diagram of U1A protein, autoregulation and alternative regulation

(A) Structure of U1A protein. (B) U1A autoregulation. The two loop sequences in PIE RNA are AUUGUAC and AUUGCAC. (C) Alternative regulation of U1A to IgM. The five non-consensus U1A binding sites are $AUUGC(N)_{1\text{-}3}C$.

sandwich-stacking motif (Guallar and Borrelli, 2005). The most conserved aromatic amino acids of the RRM located in the central two strands (β3 and β2) of the β sheet are likely to contribute primarily to the non-specific recognition of RNA, while the specific recognition is provided by the variable regions of the RRM and the cooperative binding of multiple RRMs in the same protein (Birney et al., 1993; Perez-Canadillas and Varani, 2001). Upon binding of U1A to U1 snRNA or its own RNA (PIE RNA), the helix C of U1A protein undergoes a 135° conformational change to stabilize the protein-RNA interaction and forms a surface for homodimerization (Hall, 1994; Gubser and Varani, 1996; Varani et al., 2000; Clerte and Hall, 2000).

The homodimerization domain (aa 102-105) is essential for autoregulation. This region has three biochemically defined activities: cooperative binding of two U1A proteins to PIE RNA, formation of a novel homodimerization surface and inhibition of polyadenylation (Klein-Gunnewiek et al., 2000). Mutation analysis has revealed that these three activities can be uncoupled and U1A autoregulation is selected for suboptimal inhibitory efficiency (Fei Guan et al., 2003).

U1A function

Although U1A is a component of U1 snRNP that participates in the formation of the spliceosome in an early step, it is dispensable for the splicing reaction (Will et al., 1996). However, the U1 snRNP-bound U1A has been suggested to play an important role in 5' and 3' communication (Tarn and Steitz, 1995; Gunderson et al., 1997). Besides the U1 snRNP-bound form, U1A also has been found to exist as two non-snRNP bound forms. In one non-snRNP bound form called SF-A or the RNA-free form, U1A forms a complex with some other proteins such as polypyrimidine-tract binding protein-associated factor

(PSF) etc (Lutz et al., 1998). In another non-snRNP bound form, U1A binds to its own RNA or heterogonous RNAs (Boelens et al., 1993; Philips et al., 2001 and 2004). It has been well known that non-snRNP bound U1A autoregulates its own expression level by a negative feedback mode where the polyadenylation of its own pre-mRNA is regulated by an "on-off" switch (Gunderson et al., 1994 and 1997; Boelens et al., 1993; Van Gelder et al., 1993) (Fig. 1B). Located in the 3' UTR of the human U1A pre-mRNA is a 50nt sequence conserved among vertebrates, called the polyadenylation inhibitory element (PIE RNA), which contains two AUUGYAC loop sequences. Although one loop has a 27-fold lower affinity for U1A than the other loop, two U1A molecules can cooperatively bind to PIE RNA with high affinity (Kd~0.1 nM) and the resulting $(U1A)_2$-PIE RNA complex inhibits the addition of poly(A) tail to the U1A pre-mRNA by specifically inhibiting poly(A) polymerase (PAP) (Klein-Gunnewiek et al., 2000; Van Gelder et al., 1993; Gunderson et al., 1994). This inhibition requires the essential interaction between the C-terminal 20 residues of PAP and residues 103-115 of U1A (Gunderson et al., 1997). To sum up, excess non-U1snRNP bound U1A will bind to its own pre-mRNA via the PIE RNA, thereby, inhibiting polyadenylation. Unpolyadenylated U1A pre-mRNA is unable to be exported to the cytoplasm and so less is available for translation and less U1A protein will be synthesized.

In addition, using a similar mechanism, non-U1snRNP bound U1A also regulates the expression of the secretory IgM heavy chain mRNA (Philips et al., 2001 and 2004). The IgM heavy chain gene, which is alternatively processed during B cell differentiation into mRNA, encodes either a membrane-bound receptor or a secreted antibody (Galli et al., 1988; Peterson and Perry, 1989). A promoter proximal poly(A) site (secretory) is not

expressed in undifferentiated cells resulting in the addition of two exons encoding a membrane tail. Upon differentiation the secretory poly(A) site is expressed and secreted antibody is produced. The production of secreted antibody is strictly controlled so as to ensure a rapid and specific response to infection while not overwhelming the body with potentially harmful antibodies. Regulation of the usage of the two poly (A) sites was shown to involve a change in the binding activity but not the amount of a general polyadenylation factor (CstF 64) in a B-cell stage-specific manner (Edwalds-Gilbert and Milcarek, 1995). When U1A protein regulates the expression of IgM heavy chain mRNA, it does not simply perform an "on-off switch" function of a single poly (A) site (Phillips et al., 2001). It selectively inhibits the use of secretory poly(A) site, therefore, modulating the competition between splicing and alternative polyadenylation. U1A can inhibit polyadenylation of the secretory IgM heavy chain pre-mRNA by directly binding to the three nonconsensus U1A binding motifs ($AUGC(N)_{1-3}C$) upstream of the poly(A) site (Phillips et al., 2001) and inhibit cleavage by directly binding to the two nonconsensus U1A motifs ($AUGC(N)_{1-3}C$) located in GU rich regions downstream of the poly(A) site (Phillips et al 2004) (Fig 1C). All five novel U1A binding motifs $AUGC\ (N)_{1-3}C$ are similar but not identical to the consensus, high affinity U1A binding sites on U1 snRNA and the 3'UTR of U1A, allowing a relatively weaker but more complicated regulation to the expression of secretory mRNA. Interestingly, Phillips et al (2001) also observed that U1A's capacity to inhibit the secretory poly (A) site changes in three B cell lines representing different B cell development stages. i.e the inhibitory capacity of U1A is greater in undifferentiated cells than differentiated cells, suggesting that the inhibitory effect of U1A is developmentally regulated. However, the mechanism of this is unclear.

According to Lutz et al (1996), U1A can also increase polyadenylation efficiency by directly binding to the 160 kDa subunit of CPSF and stabilizing the interaction of CPSF with the poly(A) signal-containing substrate RNA. In this case, since no specific RNA binding motifs seem to be involved, U1A has been suggested to play a more global role in RNA processing through its effect on polyadenylation.

In addition, it has been reported that U1A may play an important role in the initial step of the development of systemic lupus erythematosus (SLE), a systemic autoimmune disease with unknown aetiology (Yang et al., 2005). However, the exact role of U1A in SLE still remains unclear.

B cell differentiation

B cell differentiation is the last stage of B cell development which culminates in the formation of plasma cells (Fig. 2). It occurs when mature B cells receive the correct set of signals from antigens and T cells. At this stage mature B cells in peripheral lymphoid tissue undergo terminal differentiation into antibody-secreting plasma cells or become memory cells. This massive antibody production process accompanies a series of changes in cell functions such as induction of the secretory apparatus, loss of B cell identity, and greatly up-regulated transcription of antibody genes etc (reviewed in Igarashi et al., 2007).

Antigen-activated B cells can have multiple alternative fates. They can become IgM-secreting plasma cells rapidly in response to the antigen or plasma cells secreting isotype immunoglobulin or memory B cells. The latter two require undertaking a series of germinal center reactions including class switch and/or somatic hypermutation (Honjo et al., 2004; Muramatsu 2000; McHeyzer-Williams et al., 2001). The main or even sole

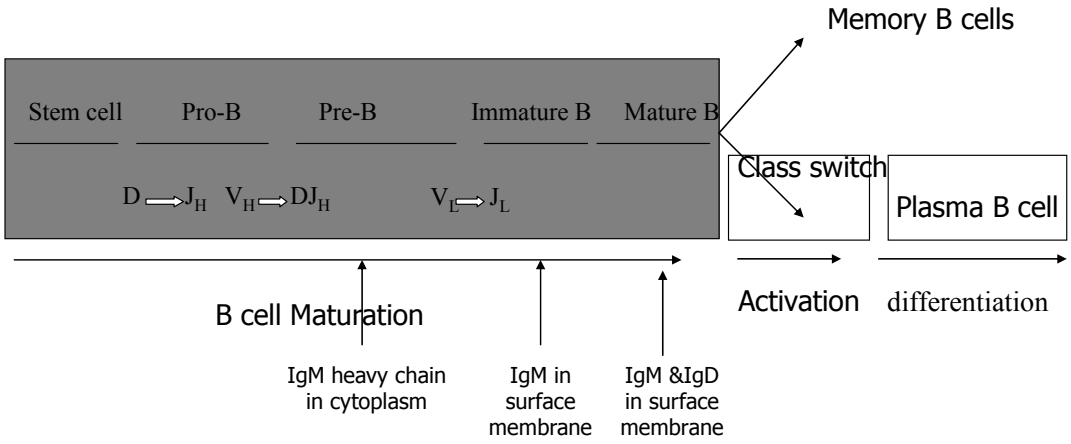


FIGURE 1. Diagram of B cell development

B cell development begins in bone marrow after birth. It can be divided into two stages: the maturation stage and the activation & differentiation stage. Stem cells receive signals from bone marrow stromal cells to undergo V, D-J rearrangement on the H chain chromosome to become pro-B. When the pro-B cells express membrane IgM heavy chains in the cytoplasm, they become pre-B cells. The pre-B cells undergo V-J rearrangement in one L chain chromosome. Once L chain is synthesized and expressed with heavy chain on the surface membrane, the cells are called immature B cells. Immature B cells are very sensitive to antigen binding. If they bind self antigen in the bone marrow, they die. Those not binding self antigen will express IgM and IgD in their surface membrane and leave the bone marrow and become mature B cells.

When the mature B cells encounter the antigen, it will be stimulated by T cells to turn on antibody production. The stimulated B cell undergoes repeated cell divisions, enlargement and differentiation to form a clone of antibody secreting plasma cells or become memory cells.

function of plasma cells is to secret immunoglobulin antibodies. During B cell differentation, Ig heavy and light chain mRNAs become over abundant due to increased transcription and mRNA stability (Chen-Bettecken et al., 1987; Jack and Wabl, 1988) and at the same time the ratio of secreted to membrane heavy chain mRNA increases as a result of changes in the competition between splicing and alternative poly (A) site choice (Galli et al., 1987). The post-transcriptional processing of immunoglobulin (especially IgM) heavy chain mRNA will be discussed later.

The processes of mature B-cell activation and plasma cell differentiation require the transition of transcription factor networks (Matthias and Rolink, 2005; Shapiro-Shelef and Calame, 2005). A series of transcription factors have been reported to regulate the process of B cell differentiation. Bach2 (BTB and CNC homology 2) and Pax5 (Paired box protein 5), two transcription repressors, are expressed from pro-B cells to mature B cells but are either silenced or absent upon plasma cell differentiation (Barberis et al., 1990; Oyake et al., 1996; Muto et al., 1998). Bach2 and Pax5 repress the expression of those genes required for plasma cells in the mature B cells (Horcher et al., 2001; Ochiai et al., 2006). In Pax5-deleted B cells and Bach2-deficient B cells, the expression of Blimp-1 (B lymphocyte-induced maturation protein 1) and XBP-1 (the transcription factor X-box binding protein 1), two key transcription factors for plasmacytic differentiation, is strongly up-regulated (Nera et al., 2006; Ochiai et al., 2006). Another transcription repressor, Bcl-6 (B-cell lymphoma 6), is essential for somatic hypermutation to produce high affinity immunoglobulin (Ye et al., 1997; Fukuda et al., 1997). During B cell differentiation, Bcl-6 antagonizes the role of AP-1 and STAT3 that function to activate the expression of the Blimp-1 gene (Vasanwala et al., 2002; Reljic et

al., 2000). In germinal center B cells, a primary function of Bcl-6 is to repress the Blimp-1 gene (Shaffer et al., 2000). However, as B cells differentiate into plasma cells, Bcl-6 is rapidly degraded in response to B cell receptor signaling (Niu et al., 1998). Upon terminal differentiation, the expression of Bcl-6 is repressed by Blimp-1 and this repression may terminate the germinal center (GC) cell function (Shaffer et al., 2002). Blimp-1, a transcriptional repressor, is a master regulator of terminal B cell differentiation. Overexpression of Blimp-1 is sufficient to drive the terminal differentiation of B cells to antibody-secreting plasma cells (Turner et al., 1994; Piskurich et al., 2000; Schliephake and Schimpl, 1996). The expression of Blimp-1 is strictly regulated and it is highly expressed in plasma cells whereas it is either low or absent in B cells (Kallies et al., 2004). Blimp-1 blocks a large set of genes and initiates a cascade of gene expression changes by directly repressing genes coding several transcription factors such as c-myc, Pax5, CIITA, Spi-B and id3 (Shaffer et al., 2002). For example, it represses c-myc to terminate cell cycle and proliferation (Lin et al., 1997; Eilers 1999); it represses CIITA to downregulate MHC Class II genes to extinguish a gene expression program specifying B cell identity (Silacci et al., 1994); and it represses Pax5 required for lineage commitment in the bone marrow and isotype switching in germinal center B cells (Lin et al., 2002; Nutt et al., 2001; Liao et al., 1994; Max et al., 1995). According to Shaffer et al (2002), Blimp-1 promotes plasmacytic differentiation by inhibiting the expression of those genes important for B cell receptor signaling, germinal center B cell function and cell proliferation while allowing the expression of some important plasma cell genes such as XBP-1. Although Blimp-1 is deemed as a master regulator of terminal differentiation, it alone is not sufficient to activate the complete program of plasmacytic

differentiation in transformed B cells (Wijdenes et al., 1996; Chilosi et al., 1999). XBP-1 is a bZip protein in the CREB/ATF family of transcriptional factors (Liou et al., 1990). The expression of XBP-1 initiates from pre-pro-B cells, continues in the mature B cells and culminates in plasma cells (Iwakoshi et al., 2003). It is required for plasma cell differentiation and the unfolded protein response. Blimp-1 only modestly induced the expression of Xbp-1 mRNA in transformed B cells (Shaffer et al., 2002) and it alone is not sufficient to achieve the high XBP-1 mRNA expression characteristic of plasma cells. Simply put, Blimp-1 is necessary but insufficient for complete up-regulation of XBP-1 mRNA, presumably by inhibiting PAX5, a known repressor of XBP-1 (Reimold et al., 1996). When B cells differentiate into plasma cells, the cytoplasm-to-nuclear ratio and the amounts of rough endoplasmic reticulum and secretory vacuoles increase to satisfy increased translation and secretion (Wiest et al., 1990; Geuze and Slot, 1980; Melchers 1971). The accumulation of an enormous number of unfolded proteins in the lumen of the ER triggers an ER stress or unfolded protein response (UPR) pathway (Ma and Hendershot, 2001; Morris et al., 1997). Once the ER transmembrane endoribonuclease and kinase (IRE1) senses the ER stress, it splices the XBP-1 mRNA and therefore produces transcriptionally active XBP-1 (XBP-1s) (Iwakoshi et al., 2003). XBP-1 in turn directly activates the transcription of the genes encoding chaperones and enzymes functioning in the ER secretory apparatus (Lee et al., 2003; Shaffer et al., 2004). XBP-1 has been deemed as one of the key regulators of the mammalian UPR pathway and is specifically required for the UPR-accompanying terminal plasma cell differentiation. In summary, transcription factors Pax5 and Bcl-6 block plasmacytic differentiation by inhibiting Blimp-1 and Xbp-1 to ensure that they are inactivated in the mature B cell.

When the repression of Blimp-1 is released by some unknown mechanism, such as stronger stimulation from BCR-mediated signals (Allman et al., 1996; Moriyama et al., 1997; Niu et al., 1998), Blimp-1 represses Bcl-6 and Pax5 and irreversibly promotes terminal differentiation. Thus, Blimp-1 forms a developmental regulatory loop with Bcl-6 and Pax5 to strictly control the process of B cell terminal differentiation.

Zhx-1

Zhx-1 belongs to the zinc finger (ZF) class of the homeodomain superfamily of transcription factors, which has been shown to regulate cellular commitment and differentiation in many species (Johnson and Mcknightm, 1989; Barthelemy et al., 1996; Yamada et al., 1999a; Hirano et al., 2002; Gehring et al., 1994). The ZF class contains both zinc-finger motifs of the Cys2-His2 type and homeodomains (HD) (Fortini et al., 1991). Through the interaction between HD and regulatory sequences on the target genes, homeotic proteins exert their effects on gene expression (Han et al., 1989).

The human, rat and mouse Zhx-1 are all composed of 873 amino acid residues. Each contains two highly conserved Cys2-His2-type zinc-finger (ZF) motifs and five highly conserved homeodomains (HDs) (Yamada et al., 1999b; Barthelemy et al., 1996; Hirano et al., 2002). The amino acid sequence of the human Zhx-1 shares a 91% and 93% similarity with that of the mouse and rat forms respectively. The human Zhx-1 gene is located on chromosome 8, the mouse Zhx-1 gene on chromosome 15 and the rat Zhx-1 on chromosome 7. The mouse Zhx1 gene spans approximately 29 kb and consists of five exons and four introns. Exons 1-3 and exon 5 contain the 5'- and 3'-noncoding sequence respectively, while exon 4 contains a part of the 5'-noncoding sequence, the entire coding sequence and a part of the 3'-noncoding sequence (Shou et al., 2003). The mouse Zhx-1

gene lacks a TATA box in the upstream region of the gene and this may account for the existence of multiple transcription initiation sites (Shou et al., 2003). The TATA box is indispensable for an accurate transcription initiation site and the transcription initiation site becomes variable for most TATA-less genes (Igarashi et al., 1997; de Launoit et al., 1997). An inhibitory region exists from -803 to -406 and the nucleotide sequence between -59 and +50 is required for the full promoter activity of the mouse Zhx-1 gene. The promoter of the mouse Zhx-1 gene is composed of at least two positive regulatory cis-acting elements; one is located between -47 and -42 (Box A) and the other between +22 and +27 (Box B). PEA3 and YY1 have been shown to bind to Box A and Box B in vitro respectively (Shou et al., 2003). PEA3 belongs to the Ets family which shares a highly conserved DNA-binding domain and recognizes similar nucleotide sequences having a centrally located 5'-GGAA-3' element. The Ets transcription factors are involved in tumorigenesis and developmental processes. The main target genes of PEA3 are involved in organogenesis and metastasis (de Launoit et al., 1997 and 2000). YY1 is also called the nuclear factor E1 or upstream conserved region binding protein. YY1 binds to the nucleotide sequence (5' AAGATGGCG-3') of Box B. It belongs to the GLI-Krüppel family of zinc-finger transcription factors, is ubiquitously expressed and regulates the transcription of several genes both positively and negatively (Thomas and Seto, 1999; Park and Atchison, 1991; Flanagan et al., 1992). It has been suggested that PEA3 and YY1, the two universal transcription factors, directly or indirectly interact with each other and synergistically control the transcriptional regulation of the mouse Zhx-1 gene ((Shou et al., 2003).

Zhx-1 mRNA is widely expressed in mouse, human and rat tissues (Barthelemy et al., 1996; Yamada et al., 1999b; Hirano et al., 2002). In humans, two major Zhx-1 transcripts composed of about 4.5 kb and 5 kb were observed ubiquitously (Yamada et al., 1999b). The 5-kb transcript is highly expressed in heart, brain, pancreas, kidney, placenta and skeletal muscle and in lower amounts in lung and liver, while the 4.5 kb transcript is highly expressed in skeletal muscle, heart and pancreas. In rat a major 4.7 kb Zhx-1 transcript was observed in all examined rat tissues such as heart, brain, spleen, lung, liver skeletal muscle, kidney and testis, although the intensity of the transcript varies among these tissues (Hirano et al., 2002). In mouse, a major transcript of about 4.5 kb was detected in some tissues. Mouse Zhx-1 is heavily expressed in brain, fairly expressed in lung, testis and spleen, expressed at very low level in liver and kidney, and was almost undetectable in heart and muscle. Besides the 4.5kb major band, in some mouse tissues there are two smaller bands below 4kb, which may represent different transcripts arising from alternative processing or transcription intitation sites (Barthelemy et al., 1996; Shou et al., 2003). Shou et al (2003) has shown that an alternative splicing of exon 3 in mouse Zhx-1 produces two species of Zhx-1 mRNA with or without Exon 3. Alternative splicing sometimes produces two or more closely related but distinct proteins from a single gene, thus playing a key role in cell- / tissue- specific or stage-specific expression of the gene (Yamada and Noguchi, 1999). However, this is not the case for the mouse Zhx-1 gene. The Zhx-1 protein sequence and structure are not affected by the alternative splicing since the entire coding region is located in exon 4 only. At present, the biological role of the alternative splicing of the mouse Zhx-1 gene is still unknown.

It was reported that Zhx-1 mRNA in mouse T cells can be regulated by IL-2 (Herblot et al., 1999). IL-2 is a primary growth factor of T cells and a potent modulator for T cell and NK cell function. It plays a major role in immune responses such as anti-tumor immunity and autoimmunity (Smith, 1988). IL-2 functions through its specific receptor (IL-2R) to activate intracellular transduction pathways and it induces the expression of certain genes (Leonard et al., 1990). Recent data have shown that IL-2 specifically induces the expression of mouse Zhx-1 in CTLL-2 cell line (IL-2 dependent cytotoxic T cell line) by increasing the stability of Zhx-1 mRNA (Shou et al., 2004). Both the Jak3/Stat5 pathway and the PI3K pathway are involved in this induction and both de novo RNA synthesis and proteins synthesis are required for this regulation. However, how IL-2 stabilizes the Zhx-1 mRNA is still a mystery.

Homeodomains (HDs)–containing proteins are known to bind AT-rich DNA sequences. Surprisingly, Zhx-1 seems not to bind DNA at all (Yamada et al., 1999a). It has been reported that Zhx-1 can directly interact with nuclear factor –Y (NF-Y) (Yamada et al., 1999a and b). Nuclear factor Y (also called CCAAT-binding protein (CBF)) is highly conserved among species and consists of three subunits NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C), each of which is necessary for DNA binding (Sinha et al., 1995; Maity and de Crombrugghe, 1998). The CCAAT box or Y box (an inverted CCAAT box) is one of many cis-acting DNA elements involved in transcriptional regulation in eukaryotic cells (Maity and de Crombrugghe, 1998). The binding of NF-Y to the Y box elements plays an important role in tissue-specific expression of MHC class II genes (Abdulkadir and Ono, 1995). Since NF-Y is required in cAMP-mediated transcription and no evidence shows that it is a phosphoprotein, there is

a high possibility that this requirement is a result of its capacity to interact with other transcription factors (Bellorini et al., 1997; Pise-Masison et al., 1997).

The amino acid sequence between 272 and 564 that contains the HD1 through HD2 region of human Zhx-1 is required for interaction with a glutamine-rich region of the NF-YA (Yamada et al., 1999a). These two HD regions can also interact with some other transcription factors or with proteins not considered as transcription factors. Therefore, the NF-YA-interacting domain of Zhx-1 may be a part of a transcription factor network. The N-terminal glutamine-rich domain of NF-YA interacts with Zhx-1, while the C-terminal domain of NF-YA interacts with NF-YB, NF-YC and DNA. Therefore, NF-YA has several domains for protein-protein interaction and probably participates in a complicated transcription factor network (Yamada et al., 1999a; Roder et al., 1997; Ueda et al., 1998). However, it remains unclear whether the interaction between Zhx-1 and NF-Y changes the promoter activity in target genes.

Zhx-1 has been reported as a ubiquitous transcription repressor localized in nuclei (Yamada et al., 2002). In human Zhx-1, the acidic region (aa 831-873) is a repressor domain while dimerization through HD1 (aa 272 -432) is a prerequisite for its full repressor activity. Recently, two novel zinc-finger and homeoboxes proteins, Zhx-2 and Zhx-3, have been identified (Kawata et al., 2003; Yamada et al., 2003). These two proteins form the Zhx family with Zhx-1 and both also contains two Cys2-His2 type zinc-finger motifs and five HDs. Human Zhx-2 and Zhx-3 not only form homodimers but heterodimers with Zhx-1 as well. Both Zhx-2 and Zhx-3 can interact with the activation domain of the NF-YA and work as ubiquitous transcriptional repressors like Zhx-1. The mouse Zhx-2 and Zhx-3 proteins consist of 836 and 951 amino acid residues respectively

and share a high similarity with their counterparts in human (Kawata et al., 2003). In mouse Zhx-2 can also form a heterodimer with Zhx-3.

Although Zhx-1 works as a transcription repressor, the exact biological role and transcriptional regulatory mechanism are unclear. It has been suggested that Zhx-1 may play an important role in the initiation of cell proliferation (Shou et al., 2004). Liu et al (2006) has demonstrated that Zhx proteins (Zhx-1, Zhx-2 and Zhx-3) are major transcriptional mediators of podocyte gene expression in primary glomerular disease. There is also a very early increase in the nuclear expression of Zhx-1 (and Zhx-2) that may be connected with the changes in gene expression for primary glomerular disease (Clement et al., 2007). Recently, Kim et al (2007) have shown that Zhx-1 enhances the transcriptional repression mediated by DNA methyltransferase (DNMT) 3B and Zhx-1 can interact with DNMT 3B in vitro and in vivo. DNA methylation is essential for transcriptional regulation, embryonic development and genomic stability and DNMT 3B is thought to primarily methylate DNA de novo, particularly during embryonic development (Li, 2002). In addition, Zhx-1 has also been shown to interact with BS69, a bifunctional transcription factor. Zhx-1 can suppress the transcriptional activation mediated by BS69 (Ogata-Kawata et al., 2007).

AREs and their regulation of mRNA stability

mRNA stability can be regulated by both cis-acting elements and trans-acting factors (Ross, 1995; Beelman and Parker, 1995). Of those characterized cis-elements, AU-rich RNA destabilizing elements called AREs have been said to be the most common determinant for RNA stability in mammalian cells (Xu et al., 1997; reviewed in Chen and Shyu, 1995). AREs consist of loosely defined AU-rich instability determinants typically

located in the 3' UTR of many highly labile mammalian mRNAs (Shaw and Kamen, 1986). They span from 50 to 150 nucleotides, exist in a wide variety of mRNAs such as those encoding nuclear transcriptional factors, cytokines, proto-oncoproteins and may play a significant role in the regulation of gene expression during cell growth and differentiation (reviewed in Chen and Shyu, 1995).

AREs can be divided into two groups (AUUUA-containing and non-AUUUA AREs) or three classes (class I, II, III) based on their sequence features and functional properties (Chen and Shyu, 1994 and 1995; Peng et al., 1996). Both class I and II AREs have various copies of AUUUA motifs while class III AREs do not have the pentanucleotide. Class I AREs are mainly located in those early-response gene mRNAs encoding transcriptional factors (c-fos, c-myc etc) and in some cytokine gene mRNAs such as IL-4 and IL-6 (Chen and Shyu, 1994; Shaw and Kamen, 1986). AREs in this class contain 1-3 copies of dispersed AUUUA motifs (domain I) and a high content of U and/or A residues (domain II). For example, the 69 nucleotide c-fos ARE has two structurally distinct and functional interdependent domains (domain I and II). The three copies of AUUUA motifs (domain I) confers a potent destabilizing ability, however the 20 nuceotide U stretch (domain II) can rescue the loss of the destabilizing effect of domain I by complementing the loss of U richness in domain I. Therefore, AUUUA motifs and U richness are two critical sequence features for class I AREs. The identified class II AREs are all located in cytokine gene mRNAs such as TNF-α, IL-3, GM-CSF and usually have multiple copies of AUUUA pentanucleotides clustering together. The cluster of multiple AUUUA pentanucleotides causes a high content of U residues and produces at least two overlapping nonamers UUAUUUA(U/A)(U/A) which define the

class II AUUUA-containing AREs. This nonamer is a key sequence motif necessary for directing rapid mRNA decay by class II AREs and it may specify the destabilizing function of class II AREs. Another defining feature of the class II AREs is an AU-rich region 20-30 nucleotides long immediately 5' to this cluster of AUUUA motifs, which can greatly enhance the destabilizing ability of the AUUUA cluster (Xu et al., 1997).

Although AUUUA motifs are present in both class I and class II AREs, an AUUUA motif does not always confer an ARE the destabilizing function (Chen and Shyu, 1995; Lagnado et al., 1994; Zubiaga et al., 1995). The AUUUA motifs have a destabilizing role only within a functional ARE (Chen et al., 1994; Stoecklin et al., 1994; Akashi et al., 1994). Therefore, neither the AUUUA motifs nor the nonamers are an indispensable part of all functional AREs. This idea was further confirmed by the existence of functional non-AUUUA AREs (class III) in the c-jun proto-oncogene mRNA (Peng et al., 1996). The class III non-AUUUA ARE consists of a couple of U stretches and a U rich domain. It can be divided into three structurally and functionally distinct regions (domain I, II and III). Domain I contains a 20 nucleotides alternative thymidylate and purine region, domain II contains a GU-rich sequence with four copies of GUUUG motifs and domain II has the least AU-rich sequence. Domain III and I are necessary and sufficient for the full destabilizing function of this non-AUUUA ARE while domain II can partially substitute for domain I. Although AUUUA-containing AREs and non-AUUUA AREs have no sequence homology, they seem to have domains that are structurally distinct but functionally overlapping and exchangeable. Therefore, the interplay of structurally distinct and functional interdependent domains most likely

determine whether an individual ARE element has destabilizing function or not (Peng et al., 1996).

All AREs (class I, II, and III) direct rapid deadenylation as the first step for mRNA degradation. The class I AUUUA-containing AREs and the class III non-AUUUA AREs direct synchronous (distributive kinetics) poly(A) shortening, while the class II AUUUA-containing ARE direct asynchronous (processive kinetics) poly(A) shortening with the formation of poly(A) minus intermediates (XU et al., 1997). According to Xu et al (1997), it is the clustering of multiple AUUUA motifs in class II AREs that dictates the processive deadenylation. The difference in deadenylation kinetics among those AREs suggests the presence of communication between the 3' poly(A) tail and the ARE. For class I and II AREs, the ARE-directed mRNA decay is tightly coupled to ongoing translation by the ribosome. However, for non-AUUUA AREs (class III) such as that in c-jun, the destabilizing function does not require the participation of the ribosome (Peng et al., 1996). Therefore, translation may be differentially required for AREs to work as a destabilizing element during cell growth and differentiation.

In the past decade, many trans-acting factors have been identified to regulate or participate in the ARE-directed rapid RNA decay. These include AUF1 (or hnRNP D) (Xu et al., 2001, Zhang et al., 1993), GAPDH (Nagy and Rigby, 1995), thiolase (Nanbu et al., 1993), HuR (Chen et al., 2002), HuC and HuD (Peng et al., 1998; Deschênes-Furry et al., 2006), T-cell intracellular antigen-1-related protein (TIAR) (Zhang et al., 2002), bata-catenin (a transcription factor) (Lee and Jeong, 2006), hnRNP A1 and C (Hamilton et al., 1993). Although those RNA-binding proteins can bind to AREs in vivo, for most of them, the functional consequences or the physiological significance for the

interaction remains unclear. HuR (in proliferating cells) and Hel-N1, HuC, HuD (in terminally differentiated neurons) can inhibit the C-fos ARE (class I)-mediated RNA decay but has no effect on the RNA decay mediated by c-jun ARE (class III) (Peng et al., 1998). Some proteins can bind to different regions of one given ARE. For example, HuR functions through its binding to the 5' AUUUA-containing domain and the 3' U-stretch-containing domain in the C-fos ARE, therefore exerting a stabilizing effect. Some ARE-binding proteins can have dual roles. For example, AUF1 (also termed as hnRNP D) recognizes a cluster of multiple AUUUA motifs or repeats in class II AREs (such as in most cytokines) and has both destabilizing and stabilizing effects (Xu et al., 2001). In addition, multiple trans-acting proteins can bind to the ARE in one given gene and synergistically regulate the mRNA stability. For example, multiple proteins such as HuR and beta-catenin (a transcription factor) can recognize and bind to the ARE in the 3' UTR of the cyclooxygenase-2 (COX-2) gene which is involved in regulating cellular proliferation, differentiation and tumorigenesis, thus stabilizing the expression of COX-2 mRNA. Beta-catenin induced the cytoplasmic localization of the RNA stabilizing factor HuR (Lee and Jeong, 2006). It has been also reported that some proteins can establish a cross talk between the ARE and the 3' end poly(A) tail. For example, mouse HuC displays specific RNA binding activity both for the ARE and the poly(A) signal. Although those abovementioned ARE-binding proteins can affect the deadenylation and decay kinetics displayed by different classes of AREs, the underlying mechanisms are still unknown.

Summary

Multiple transcription factors synergistically regulate the process of B cell differentiation. Therefore, it is important to know how each of those transcription factors itself is regulated. U1A protein as a trans-acting factor has been known to post-transcriptionally regulate the expression of itself and the IgM gene by inhibiting 3' end processing. The goals of this thesis are to discover other targets of U1A and how U1A regulates their expression.

Materials and methods

Plasmid constructs (Chapter I and II)

1. Plasmids used in chapter I. Plasmid pΔ3 containing the μ-heavy chain gene was a gift from Grosschedl and Baltimore. Plasmid p630 containing the human U1snRNA, plasmid pGEM3z + containing IgM1790–2030 mutss (spanning positions 1790–2030 of accession number V00818 and containing a mutated 5′ splice site, g/gtaaac to g/caaacc, shown to eliminate splicing to the membrane exons) (Peterson and Perry, 1989) and plasmid pGEM3z + containing IgM1730–2085 which includes the 5' splice site (1810) and the secretory poly(A) site (1998) were made in our lab.

2. Plasmids used in chapter II. To construct a series of pGEM 3Z+ plasmids used for in vitro transcription, a series of PCR products from the 3' UTR of mouse Zhx-1, containing wild type or mutated U1A motifs, wild type or mutated upstream poly(A) site (poly(A) site 1) and 5' EcoRI / 3' XbaI sites introduced as part of the synthetic primers, were cloned into the EcoRI and XbaI sites of pGEM 3Z+ containing a T7 promoter in the forward direction and a SP6 promoter in the reverse direction. To construct a series of pPKLT55 plasmids used for transfection, PCR products were cloned into the BglII and XbaI sites of pPKLT55 (Phillips et al., 1996 and 2004) containing the firefly luciferase cDNA, replacing the poly (A) site of the firefly luciferase. To construct a series of pRL/SV40 plasmids used for transfection, PCR products were cloned into the XbaI and BamHI sites of pRL/SV40 containing the renilla luciferase cDNA, replacing the poly (A) site of the renilla luciferase cDNA. The mutations in the U1A motifs and the AUUAAA (poly(A) signal) and the AUUUA motifs downstream of the poly(A) signal were incorporated using crossover PCR as previously described (Phillips et al., 1999).

Cell culture and whole cell extract preparation (Chapter I)

HeLa, J558L, and WEHI 231 cells were obtained from the European Collection of Animal Cell Cultures (ECACC). M12.4.1 cells were the gift from K.J. Kim (Kim et al., 1979). J558L, M12.4.1 and WEHI 231 were cultured in RPMI 1640 (Gibco) with 5% fetal calf serum (Gibco), 1×nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 55 mM mecaptoethanol (Gibco), and penicillin/streptomycin (100 units/mL). Cells were harvested during exponential growth, washed by 1× PBS twice, lysed and sonicated in SDS loading buffer, and then their proteins were subjected to 12% SDS-PAGE separation and western blotting.

Cytoplasmic extract and nuclear extract preparation (Chapter I)

Cells were harvested during exponential growth and counted using a Fischer scientific hemacytometer. Cell nuclear extracts were prepared using the extraction procedure originally developed by Dignam et al (1983) and modified for B lymphocytes by Virtanen and Chen (1990) with hypotonic buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 0.2% Triton X-100, and buffer C containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.35 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 0.4 u/μL RNase inhibitor (Promega). The protein concentrations in cytoplasmic extracts and nuclear extracts were determined by the Bio-Rad protein assay.

Western blot analysis (Chapter I and II)

Protein samples were resolved in a 12% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore) using a semi-dry blotting apparatus in the transfer buffer (192 mM glycine, 25 mM Tris, 20% v/v methanol) with 300 mA, 25 V and 10 W

overnight. The membrane was then blocked with 1x PBS, 0.1% triton, and 0.75%-1% w/v milk powder for 45 to 60 minutes. The membrane then was probed with the 1st antibody in a new blocking solution. Hybridizations were performed with rabbit anti-human U1A antibody 856 (1:5,000 home-made) or mouse anti-human GAPDH antibody (1:30,000 Chemicon) or rabbit anti-human ZHX1 antibody (1:4000 Bethyl) or rabbit anti-human NF-YA (1:2500 ProSci). After 1- 2 hours of shaking or rocking, the membrane was washed twice with 1x PBS, 0.1% triton (8 minutes each time). Then the membrane was incubated with corresponding anti-species specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000, Amersham) for 1 hr. After being washed twice with 1x PBS, 0.1% triton, 1% milk powder, twice with 1x PBS, 0.1% triton and once with 1x PBS (8 minutes each time), the membrane was visualized by ECL reagent (PerkinElmer) and exposed to X-ray film.

Total RNA preparation from B Cells (Chapter I and II)

Total RNA was extracted from cultured B cells with Qiagen RNeasy mini kit. Cells were harvested during exponential growth and counted using a Fischer scientific hemacytometer. To 10^7 cells, buffer RLT 600 µL was added to disrupt and lyses cells by vortexing. The lysate was homogenized by passing the lysate 5-10 times though a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. The homogenized lysate was mixed well with 1 volume of 70% ethanol, transferred to an RNeasy spin column placed in a 2 mL collection tube, centrifuged for 15 seconds at 10000 rpm and flow-through was discarded. The column was washed once by adding 350 µL buffer RW1, centrifuged for 15 seconds and the flow-through was discarded. 80 µL DNase I incubation mix (10 µL DNase I stock and 70 µL buffer RDD) was applied to the column

membrane, placed at room temperature for 15 minutes to remove DNA. Then the column was washed once with 350 μL buffer RW1 and twice with 500 μL buffer RPE as above. To eliminate any possible carryover of buffer RPE, the RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 additional minute. To elute RNA, 50 μL-100 μL RNase-free water was added to the column. After 5 minutes at room temperature, RNA was collected by spinning the column for 1 minute at 10000 rpm.

In vitro transcription with T7 or SP6 RNA polymerase (Chapter I and II)

The templates used for in vitro transcription were made by enzymatic digestion of plasmids containing the target genes fragments.

For making anti-U1 snRNA probe for northern blotting analysis, Rsa1-digested p630 plasmid containing the human U1 snRNA gene cloned into pGEM 3Z+ was used as a template and T7 as a polymerase (Gunderson et al., 1998).

For preparing ^{32}p-uniformly labeled μ-secretory IgM RNA substrate used for in vitro specific and non-specific poly(A) assays, XbaI-digested pGEM 3Z+ plasmid containing IgM1790–2030 mutss was used as a template and T7 as a polymerase.

For making ^{32}P-uniformly labeled μ anti-sense RNA probe used in RNase protection assays, EcoRI-digested pGEM 3Z+ plasmid containing IgM 2085-1730, which includes the 5′ splice site (1810) and the secretory poly(A) site (1998) was used as a template and SP6 as a polymerase.

For making ^{32}P-uniformly labeled Zhx-1 anti-sense RNA probes used in RNase protection assays, EcoRI-digested pGEM 3Z+ plasmids containing Zhx-1 28700-28044,

30132-29465 or 30215-29356 respectively were used as templates and SP6 as a polymerase.

For preparing ^{32}p-uniformly labeled Zhx-1 RNA substrates used for in vitro specific and non-specific poly(A) assays, UV Crosslinking assays and in vitro cleavage assay, XbaI-digested pGEM3 Z+ plasmids containing Zhx-1 29417-29764, 29417-29963 were used as templates and T7 as a polymerase.

RNA probes were prepared by in vitro transcription as previously described (Phillips and Virtanen 1997). For RNA substrates in the non-specific poly(A) assays, to one 1.5 mL eppendorff tube the following components were added at room temperature in the order as listed to a final volume of 20 µL: 4 µL transcription optimized 5x buffer (200 mM Tris-HCl pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2µL 100 mM DTT, 20-40 u recombinant RNasin® ribonuclease inhibitor, 2 µL low GU rNTP (4 mM each of rATP and rCTP, 1.5 mM each of rGTP and rUTP), 1 µL of the abovementioned linearized plasmid DNAs (0.2-1.0 mg/ml in water), 2 µL [α-32P] rUTP (15 µCi/µL), 15-20 u T7 RNA polymerase. For capped RNA substrates in the specific poly(A) assay and the cleavage assay, 1.5 uL 10 mM CAP analog was added. For RNA probes and substrates in other assays listed above such as RPA, northern blotting, UV crosslinking, 2 µL low GU rNTP was replaced with 3µL rAGC (10 mM each of rATP and rCTP) and 2 µL 250 µM rUTP, and 5µl [α-32P] rUTP (15µCi/µL) was used instead of 2 µL. Transcription was carried out for 1-2 hours at 37°C. Reaction products were separated on an 8% denaturing gel, recovered and eluted in 500 uL RNA elution buffer (100 mM Tris.HCl PH 8.0, 1 mM EDTA, 0.3 M NaOAC, 0.2-1% SDS). Eluted RNAs

were extracted with phenol/chloroform, precipitated by ethanol and resuspended in 20 µL DEPC-treated water and their radioactivity was measured with a scintillation counter.

Northern blot analysis (Chapter I)

RNA samples were proteinase K treated, phenol-chloroform extracted, and ethanol precipitated, separated by 8% urea-acrylamide denaturing PAGE, and electrotransfered to Amersham hybond-N+ membrane in 1xTBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) at 300 mA, 20 volts, 10 watts for 3 hours or overnight. The RNAs were cross-linked to the membrane in a UV Stratalinker 2400 apparatus. The dried membrane was prehybridized in prehybridization buffer (50% v/v formamide, 5 x SSC buffer [0.75 M NaCl, 75 mM sodium citrate, pH 7.0], 5 x Denhardts buffer [1 mg/mL BSA, 1 mg/mL PVP polypropyl, 1 mg/mL ficoll-400], 1% SDS, 0.1 mg/mL carrier DNA) at 42°C for at least 1 hour and hybridized against U1 snRNA probe (final concentration 4,000 dpm/µL) overnight. The membrane then was washed with 2× SSC and exposed to a PhosphorImager screen or Blue Lite autorad film (Bioexpress).

Trimethyl guanosine immunoprecipitation (Chapter I)

Nuclear extracts were mixed with 10 µL or 20 µL anti-2,2,7-trimethylguanosine (TMG) agarose beads (Oncogene) in 50 µL or 200 µL NET buffer (50 mM Tris at pH 7.6, 150 mM NaCl, 0.01% NP-40) and 0.4 U/µL RNasein (RNase inhibitor), incubated at 4° C for 1 hour, and supernatants were recovered by centrifugation at 14,000 rpm for 5 minutes. The supernatants were mixed again with beads, and the steps above were repeated once more to deplete the extracts of >95% of U1snRNA.

Recombinant proteins (U1A and PAP) (Chapter I and II)

The human U1A protein and bovine poly(A) polymerase (PAP) were tagged with C- terminal histidine to ensure that all the C-terminal residues were present after purification (Gunderson et al., 1997). U1A and PAP were expressed in and purified from BL21 cells as described in Gunderson et al (1998), first by nickel chromatography using NTA-agarose (Qiagen) followed by MonoS chromatography on an AKTA system (Pharmacia). Cells were cultured to OD_{595} 0.6-0.8 at 37^0c, treated by adding 0.1 M IPTG to a final concentration of 0.25 mM and cultured for another 3 hours at 30^0c to induce the expression of U1A or PAP. Cells were harvested, pelleted and resuspended on ice in (25 mL/liter of cell culture) Tris/KCl buffer (100 mM Tris-HCl pH 8.0, 300 mM KCl, 16% v/v glycerol, and 1 mM PMSF) containing 20 μg/mL benzamidine, 1 μg/mL leupeptin, 1 μg/mL DNase I, 10 μg/mL lysozyme, 1 μg/mL pepstatin A. Then cells were sonicated and centrifuged for 15 minutes at 15,000 rpm. The supernatant was mixed with Ni-NTA resin (0.8 mL/50 mL supernatant) in 300 mM KCl and 1.5 mM $MgCl_2$ for 2 hours at 4°C, and then washed twice in GTK buffer (20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) with 17 mM imidazole. Bound U1A or PAP was eluted with GTK buffer/400 mM imidazole. U1A-containing fractions were then pooled and purified on a 1 mL MonoS column in buffer containing 30 mM Tris (pH 9.5), 1.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT, in a 0-1M KCl gradient with U1A eluting at 350 mM KCl. Purified recombinant human U1A and PAP were separated on 12% SDS-PAGE gel and visualized by coomassie blue stain, silver stain or western blot.

In vitro specific poly (A) assay (Chapter I and II)

The polyadenylation assays in nuclear extracts were performed according to the protocol as previous described (Virtanen and Sharp, 1988; Gunderson et al., 1994 and

1997). For each reaction, 30,000 cpm of ^{32}p-labeled capped RNA substrates were used. Each reaction (total volume 20 μL) contained 14 mM Tris (pH 7.4), 0.66 mM $MgCl_2$, 6 mM DTT, 15 mM creatine phosphate, 4 units of RNasein (Promega), 0.1 μg/μL tRNA, 0.8 mM rATP, 10 μg HeLa cell nuclear extract. The reactions were prepared by first mixing IgM RNA substrates with 0–10 ng SL2 RNA and MS2 RNA (Klein Gunnewiek et al., 2000) or mixing Zhx-1 RNA substrates with recombinant U1A protein, then adding 10 μg nuclear extracts to initiate the polyadenylation reaction. The components were mixed gently and incubated at 30°C for 90 minutes. RNA products were extracted with phenol-chloroform, precipitated with ethanol, separated by denaturing PAGE gel and visualized by autoradiography.

Silver stain (Chapter I)

After purified non-snRNP-bound U1A was separated on a 12% SDS-PAGE gel, the gel was subjected to silver stain using BIO-RAD silver stain plus kit (Cat.161-0449). First, the gel was fixed in fixative enhancer solution (methanol: acetic acid: fixative enhancer concentrate: deionized distilled water=50%:10%:10%:30%) with gentle agitation for 20 minutes. Then the gel was rinsed twice in deionized distilled water with gentle agitation (10 minutes/each time). To stain and develop the 8 cmx10 cm gel, staining solution was first prepared by mixing 5 mL silver complex solution, 5 mL reduction moderator solution, 5 mL image development reagent with 35 mL deionized distilled water, then by adding 50 mL development accelerator solution immediately before staining. The gel was placed in stain solution with gentle agitation until the desired staining intensity was reached (normally 15-20 minutes) .The gel was placed in 5% acetic

acid solution for 5 minutes to stop the staining reaction and rinsed with deionized water for 5 minutes.

In vitro non-specific poly(A) assay (Chapter I and II)

Nonspecific poly(A) assays were performed as previously described (Phillips et al., 2001) with 50 ng of recombinant PAP and 30,000 cpm of ^{32}P-labeled μ-secretory RNA substrate IgM 1790–2030 mutss or Zhx-1 RNA substrates incubated for 30 minutes at 37°C.

Immunopurification of non-snRNP-bound U1A from nuclear extracts (Chapter I)

Rabbit polyclonal antibody 856 specific for U1A (Kambach and Mattaj, 1992) (kindly provided by Iain Mattaj, EMBL, Heidelberg) was coupled to CNBr-activated sepharose 4B bead according to the Amersham Bioscience's recommended protocol. Seven micrograms protein per milliliter of swollen beads was used.

U1 snRNP-associated U1A was removed from nuclear extracts by TMG immunoprecipitation as above. The TMG-depleted supernatant after centrifugation was adjusted to a final volume of 400 μL with NET buffer and pretreated with 20 μL uncoupled Sepharose 4B bead for 20 minutes at room temperature with gentle rotation. The pretreated supernatant was incubated with 20 μL U1A antibody-coupled Sepharose 4B beads at room temperature for 2 hrs with gentle rotation. Beads were then subjected to extensive washing as follows: twice with high salt NET buffer (500 mM NaCl) and once with NET buffer (150 mM NaCl).

Twenty microliters (v/v = 1:1) elution buffer (50 mM KCl, 150 mM glycine at pH 1.5, 0.1% Triton-X) was added to the washed bead and incubated for 5 minutes with gentle shaking. The eluted supernatant was neutralized by addition of 2 μL (1/10 volume)

of 1.0 M Tris (pH 8.0). Purified non-snRNP-bound U1A was separated on a 12% SDS-PAGE gel, visualized by western blotting or silver stain, scanned, and quantitated in ImageQuant.

Immunoprecipitation of RNA from B cell extracts by U1A protein (Chapter II)

M12.4.1 nuclear extracts were treated first with TMG beads, then with uncoupled Sepharose 4B beads and finally with U1A antibody-coupled Sepharose 4B beads following the protocol in the previous section. The pellet samples were digested by proteinase K, extracted by phenol/ chloroform and precipitated by ethanol. The RNA concentration was determined by spectrophotometry. The RNA was then analyzed by Affymetrix GeneChip microarray (Lockhart et al., 1996). The RNA sample treated with uncoupled Sepharose beads (in pretreated step) was used as a control.

RT-PCR (Chapter I)

RNA samples were analyzed by RT-PCR as follows.1 μg of RNA was mixed with 5 ng /mL random primers and 0.5 μM dNTP, placed at 70°c for 5 minutes, then quickly chilled on ice. Reverse transcription was carried out with home-made reverse transcription enzyme in 1x M-MLV buffer (Promega) first at room temperature for 10 minutes and then at 45° C for 1 hour. The reverse transcription products were amplified by PCR with exon-exon junction primer sets. U1A primer set: 5' - GGA GAC CAA CGA GCT CAT GCT CTC CAT GCT CTT CAA CC - 3' (7942, exon 6) and 5' - GGC TCT GAG AAG GTC CCT AAG GGG GAC TTA CCT TCA GG - 3' (9732, exon 7). GAPDH primer set: 5' - GCC AAG TAT GAT GAC ATC AAG AAG GTG GTG AAG CAG GC - 3' (3912) and 5' - CCA TGT AGG CCA TGA GGT CCA CCA CCC TGT TGC TGT AGC - 3' (4259). Zhx-1 primer set 1: 5' - GAA GAT CTG AGT TAG GTA

TAG AAT TAT TTG AGG AAA ATG - 3' (28469, exon 4) and 5' - GGC ACA ACA TCA AGT TCC ATT TCT TTT GGA CAT TGG - 3' (28444, exon 5). Zhx-1 primer set 2: 5' - GAA GAT CTG AGT TAG GTA TAG AAT TAT TTG AGG AAA ATG - 3' (28469, exon 4) and 5' - CCC ATC TTA CAC AGC AGT AAG CTG GAC TAG ATG TGA GG - 3' (28648, exon 5). PCR products were separated and visualized on a 1% agarose gel.

RNase protection assay (RPA) (Chapter I and II)

For IgM, wild type or mutant plasmids containing the IgM heavy chain gene were transfected into M12.4.1 cells in triplicate. Poly $(A)^+$ mRNA was extracted 17 hours later using a Quickprep micro mRNA preparation kit (Pharmacia). The mRNA levels were measured by RNase protection assay (RPA) according to Melton *et al*. (1984). Poly$(A)^+$ RNA was hybridized overnight at 45°C with 100 000 cpm uniformly ^{32}P-labeled wild-type or mutant antisense RNA spanning position 2085-1730, which includes the 5' splice site (1810) and the secretory poly(A) site (1998), in the presence of 5 µg of tRNA. Single-stranded RNA was digested using 50 U of RNase T1 and 1 µg of RNase A for 30 minutes at 37°C.

For endogenous Zhx-1, total RNA was prepared from cultured J558L and M12.4.1 cells using Qiagen micro RNA kits. RPA was performed with 10, 20, 30 µg total RNA from both cell lines. Hybridization was carried out overnight at 50°C with 100 000 cpm uniformly ^{32}P-labeled Zhx-1 antisense RNAs spanning position 28700-28044 and 30128-29465 respectively. Single-stranded RNA was digested using 100 U of RNase T1 for 30 min at 37°C. For GAPDH, the Zhx-1 antisense RNA probe was replaced with a ^{32}P-labeled 417nts GAPDH antisense RNA fragment spanning exon 8-5 produced by in

vitro transcription from XbaI/HindIII cleaved pTRI-GAPDH-Mouse antisense control template by SP6 and single-stranded RNA was digested using 100 U of RNase T1 and 1 μg of RNase A. The digestion reaction was stopped by 10% SDS and 10 mg/mL protease K. Products were quantitated by phosphorimagery.

U1A overexpression in HeLa cells (Chapter II)

Cell line stably expressing wild type Flag-tagged U1A protein was derived from HeLa Tet cells (Clontech) that stably express the tetracycline-controlled transactivator (tTA) in the presence of G418. The expression of transfected Flag U1A was under the control of the Tet response promoter as part of the Tet-off system. Cells were cultured in D-MEM, 10% fetal calf serum, antibiotics, 0.2 μg/mL G418, puromycin and/or doxycycline (DOX). In the absence of DOX, the Tet promoter is active because tTA binds the promoter and activates transcription. In the presence of DOX, tTA is released from the Tet promoter and so the promoter is inactive. Stable cells were selected in complete growth media containing 0.8 μg/mL puromycin whereas stable expression was maintained by growing in media containing 0.8 - 4 μg/mL puromycin depending on the level of expression desired. Note that puromycin was omitted when DOX was present in the media. The level of expression of the tagged U1A protein, endogenous U1A protein and Zhx-1 proteins were measured by western blotting.

UV crosslinking assay (Chapter II)

UV crosslinking assays were performed as previously described (Phillips et al., 1997 and 2004). For crosslinking assays to test the binding of recombinant U1A protein, U1A (0~150 ng) was incubated with 100,000 cpm of uniformly ^{32}P-labelled Zhx-1 substrates with wild type or mutated U1A motifs in 8 mM HEPES (pH 7.9), 100 mM

NaCl, 2 mM EDTA, 2 mM DTT, 0.16 µg/µL tRNA, 8% glycerol, 0.06% Triton-x 100 in a total volume of 12.5 µL. PIE RNA and mutated PIE were used as positive and negative controls respectively. For crosslinking assays to test the inhibition of U1A to CstF64 binding, recombinant GSTCstF64KRBD (2 µM) and increasing amounts of U1A (0-500 ng) were used. The protein and RNA were crosslinked first on ice under a handheld UV lamp at 245 nm for 10 minutes and then were crosslinked twice in a UV stratlinker 2400 (Stratagene) (1200 microjoules each time). The crosslinking products were incubated with 1µL (10 µg/µL) RNase A at 30^0c for 30 minutes and immediately subjected for separation by a 12% SDS-PAGE gel. The crosslinking products were visualized by phosphorimagery.

In vitro cleavage assay (Chapter II)

In vitro cleavage was performed in HeLa nuclear extract according to Moore and Sharp (1984 and 1985). U1A (0-600 nM) was mixed with ^{32}P-labeled RNA substrate 100,000 cpm in a buffer containing 1.25 mM 3' dATP, 0.7 mM $MgCl_2$, and 3% polyvinyl alcohol. To start the cleavage reaction, 7 µL HeLa cell nuclear extract (2.5 µg of total protein/µL) was added to the above mixture to make a final volume of 20 µL and incubated for 2 hours at 30°C. The reaction product was treated with proteinase K, extracted with phenol-chloroform, precipitated with ethanol, resuspended in 95% formamide loading buffer and run on an 8% denaturing PAGE gel.

Dual-luciferase reporter assay (Chapter II)

The constructed plasmid pPKLT55-Zhx-1 was co-transfected with plasmid pRLSV-40 expressing renilla luciferase into J558L and M12.4.1 cells in log phase using Qiagen Superfect at 10 µL/10^6 cells. After 20-24 hours, the firefly and renilla luciferase

activities were measured using the dual-luciferase reporter assay system (Promega). Cells were harvested, washed with 1x PBS, then resupended and lysed with 1x passive lysis buffer in 1.5 mL eppendorff tubes and frozen in -20°C for 30 minutes or overnight. The lysates were thawed to room temperature and centrifuged to precipitate cell debris before being subjected to the measurement of luciferase activity with the Promega "DLR-O-INJ" protocol in a $20/20^{n}$ luminometer (Turner BioSystem). For each sample, to one 1.5 mL tube the supernatant of cell lysate 10 μL and 50 μL of LAR II (luciferase assay reagent II) were mixed to measure the firefly luciferase activity first, then 50 μL Stop & Glo reagent was added to measure the renilla luciferase activity. The relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

CHAPTER I: Non-snRNP U1A levels decrease during mammalian B-cell differentiation and release the IgM secretory poly(A) site from repression.

Ma J, Gunderson SI, Phillips C. RNA Journal. 2006 Jan; 12(1):122-32

Summary

Upon B cell differentiation a regulated conversion from the production of membrane to secretory forms of Immunoglobulin M (IgM) mRNA occurs due to the activation of an upstream secretory poly(A) site. U1A plays a key role in inhibiting the expression of the secretory poly(A) site by inhibiting both cleavage at the poly(A) site and subsequent poly(A) tail addition. The inhibitory effect of U1A is alleviated in differentiated cells, which express the secretory poly(A) site, however, the mechanism underneath was unclear. Using B cell lines representing different stages of B cell differentiation, we demonstrated that the amount of U1A available to inhibit the secretory poly(A) site is reduced in differentiated cells. Undifferentiated B cells have more total U1A than differentiated cells and a greater proportion of U1A is not associated with the U1snRNP. We showed that this non-snRNP associated U1A is available to inhibit poly(A) addition at the secretory poly(A) site using cold competitor RNA oligos to de-repress poly(A) addition in nuclear extracts from the respective cell lines. In addition, endogenous non-snRNP associated U1A—immunopurified from the different cell lines—inhibited poly(A) polymerase activity proportional to U1A recovered, suggesting that available U1A level alone is responsible for changes in its inhibitory effect at the secretory IgM poly (A) site.

Introduction

B cell development begins in bone marrow after birth. Stem cells undergo a series of rearrangements of heavy chain and light chain to become immature B cells that express intact IgM receptor in surface membrane. The immature B cells that survive negative selection migrate to the periphery lymphoid tissue to become mature B cells. If they receive the correct set of signals from antigen and T cells, mature B cells will proliferate, undergo class switching and antibody affinity maturation, and then differentiate into antibody-secreting plasma cells (reviewed in Goldsby and Osborne, 2000).

The IgM precursor mRNA contains two alternatively used poly(A) sites: an upstream (secretory) poly(A) site and downstream (membrane) poly(A) site (Alt et al., 1980; Early et al., 1980). In immature B cells and mature B cells, the membrane mRNA form is produced, and the secretory poly(A) site is inactive (Lamson and Koshland, 1984). Upon differentiation, the secretory poly(A) site is activated and the secretory form of mRNA is expressed (Galli et al., 1987 and 1988; Peterson et al., 1991; Takagaki et al., 1996). In addition, the secretory mRNA's stability is increased (Mason et al., 1988; Cox and Emtage, 1989). U1A protein has been shown to inhibit activation of the secretory poly(A) site both on the level of cleavage and of poly(A) addition, the latter resulting in destabilization (Phillips et al., 2001 and 2004). To inhibit cleavage it binds to two novel U1A binding motifs downstream of the secretory poly(A) site, thereby occluding the binding of Cleavage stimulatory Factor (CstF) to downstream GU-rich motifs and inhibiting formation of the cleavage/polyadenylation complex (Phillips et al., 2001 and 2004). To inhibit poly(A) addition, it binds three novel U1A binding motifs upstream of the secretory poly(A) site and inhibits poly(A) polymerase activity (Phillips et al., 2001;

Phillips and Gunderson, 2003). Thus U1A is a key regulator of the expression of the secretory poly(A) site.

Evidence from in vivo studies with reporter constructs suggests that the inhibitory effect of U1A decreases upon B cell differentiation (Phillips et al., 2001). This would fit with a model in which the secretory poly(A) site is inhibited by U1A in mature cells that produce mRNA encoding the heavy chain of a transmembrane receptor and then activated or de-repressed in differentiated cells that produce secretory mRNA encoding the heavy chain of secreted antibody.

Not all U1A in the cell is available to inhibit poly(A) polymerase. The majority of U1A in the cell is associated with the U1snRNP and unable to bind mRNA (Gunderson et al., 1997). A decrease in the ratio of U1A to the snRNP associated protein, B' has been observed during differentiation, suggesting that less U1A is available in differentiated cells (Milcarek et al., 2003). Furthermore, U1A has been reported to exist in a complex with other proteins, which may modulate its effect (O'Connor et al., 1997; Lutz et al., 1998). Therefore, a thorough investigation of the availability of U1A to inhibit poly(A) addition during B cell differentiation is necessary to understand the mechanism for the decrease in the inhibitory effect of U1A during differentiation.

We used B cell lines that represent different stages of B cell differentiation to examine the changing availability and capacity of U1A to inhibit poly(A) polymerase during B cell differentiation. We demonstrated here that not only the absolute level of U1A decreases relative to GAPDH, but also the proportion of U1A that is not snRNP-bound decreases as B cells differentiate. Using competition with cold RNA oligos that de-repress inhibition by U1A in nuclear extracts from the different cell types, we showed

that de-repression is proportional to the amount of non-snRNP-bound U1A levels. Furthermore, endogenous non-snRNP-bound U1A immunoprecipitated from the different cells lines with their individual U1A associated factors inhibited recombinant poly(A) polymerase activity in proportion to the amount of U1A recovered. Taken together, these result show that changing levels of the U1A protein itself regulate the inhibition of secretory poly(A) site expression during B cell differentiation.

Result

B cell lines that represent different stages of B cell differentiation produce a graded ratio of secretory to membrane μ-mRNA

To investigate the changing availability and capacity of U1A to inhibit poly(A) addition during B cell differentiation, we used B cell lines that represent different stages of B cell differentiation. These are J558L, M12.4.1, and WEHI 231, which we used previously (Phillips et al., 2001). J558Ls are a plasmacytoma cell line that has lost endogenous IgM heavy chain (μ) and produce only the secretory form of μ chain mRNA from a transfected gene construct (Oi et al., 1983; Mason et al., 1988), M12.4.1 cells produce IgG2a and aproximately twofold secretory versus membrane μ-mRNA from a transfected μ chain construct (Kim et al., 1979) and WEHI 231 is an immature B cell line that produces more endogenous membrane than secretory μ mRNA (Mason et al., 1988).

We first confirmed that the cell lines used in this study produced a graded ratio of secreted to membrane μ-mRNA and thus adequately represent differing levels of inhibition of expression of the secretory poly(A) site. For this we used RNase protection assays (RPA) of poly(A)$^+$ RNA extracted from the respective cell lines. J558L and M12.4.1 cells do not produce endogenous μ-heavy chain. We therefore transfected these

with the pΔ3 plasmid containing the μ-heavy chain gene (Grosschedl and Baltimore, 1985). Eight micrograms of plasmid were transfected into 10^6 cells, respectively, and poly(A)$^+$ mRNA was extracted 24 h later. WEHI 231 cells produce endogenous μ-heavy chain and poly(A)$^+$ RNA was extracted from these cells without transfection. RPA was performed using an in vitro transcribed uniformly ^{32}P-labeled anti-sense probe spanning positions 2085–1730 of the μ-heavy chain and the secretory poly(A) site at 1997 and the 5' splice site for splicing of the membrane exons at 1812, and includes 34nt of polylinker sequence. Figure 1A shows the regions spanned by the probe and the positions of the protected fragments representing the membrane and secretory mRNA. After overnight hybridization of the poly(A)$^+$ RNA with the probe and subsequent RNase digestion to remove single-stranded RNA, the resulting protected fragments were resolved on 8% denaturing PAGE and quantitated by PhosphorImager analysis (Fig. 3B). Ratios of secretory to membrane mRNA were calculated after adjustment for the length of the protected fragments and these results ± *SD* are presented below the lanes of each respective sample in Figure 3B. J558L cells produce 38 ± 8 times more secretory than membrane mRNA from the transfected plasmid pμΔ3 containing the immunoglobulin M heavy chain gene, M12.4.1 produce 2.3 ± 0.3 times more secretory than membrane mRNA from pμΔ3 while WEHI 321 endogenously produce less than half the amount of secretory mRNA to membrane mRNA, with a ratio of 0.4 ± 0.1. This confirms that the cell lines used in this study produce a graded ratio of secretory to membrane μ-mRNA that correlates with the stage of differentiation, with the least differentiated producing the lowest ratio and the most differentiated producing a very high ratio.

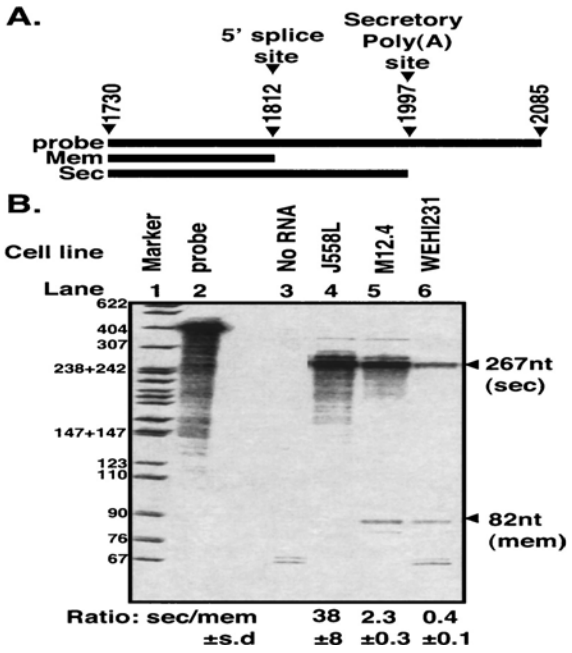


FIGURE 3. B cell lines that represent different stages of B cell differentiation produce a graded ratio of secretory to membrane m-mRNA.

RNase protection assays using an in vitro–transcribed, uniformly 32P-labeled anti-sense probe spanning positions 2085–1730 of the μ-heavy chain, and the secretory poly(A) site at 1997 and the 5' splice site for splicing of the membrane exons at 1812. (*A*) Schematic diagram of the probe and the protected fragments representing the membrane (mem) and secretory (sec) mRNA. (*B*) RPA with poly(A)+ RNA extracted from the respective cell lines. Eight micrograms of p 3 plasmid containing the μ-heavy chain gene were transfected into 106 J558L and M12.4.1 cells 24 h previous to harvest. For WEHI 231 the results are for the endogenous μ-heavy chain. Protected fragments were resolved on 8% denaturing PAGE and quantitated by phosphorimager analysis. Ratios of secretory to membrane mRNA (sec:mem) were calculated after adjustment for the length of the protected fragments and results of triplicates ± *SD* are presented *below* each lane, respectively.

The total amount of U1A decreases with stage of differentiation

As a first approach, we measured the total U1A levels in J558L, M12.4.1, and WEHI 231 cell lines. Whole cells were lysed and sonicated in SDS-loading buffer at the concentration of 10^4/μl and run on 12% SDS-PAGE. The samples were eletrotransfered and immunostained by rabbit poly-clonal antibody specific for U1A and mouse polyclonal antibody specific for GAPDH. The bands were visualized using horseradish peroxidase conjugated anti-rabbit (for U1A) or anti-mouse (for GAPDH) secondary antibody and ECL reagents.

In order to accurately compare the U1A levels in the different cells lines we first titrated cell extracts to determine the linear range of the assay. We found that 1 μl, 2 μl, and 3 μl for each cell extract was linear (see Fig. 4A, lanes 1–3 [J558L], lanes 4–6 [M12.4.1], and lanes 7–9 [WEHI 231] both U1A and corresponding comparable GAPDH level). These bands were scanned and quantitated in Image-Quant. We calculated the ratio of U1A:GAPDH in triplicate ± *SD* for each cell line and normalized these to the value for J558L (i.e., J558L was set to 1). We found that the mature (M12.4.1, 1.39 ± 0.11 *SD*) and immature B cell (WEHI 231, 2.2 ± 0.31 *SD*) lines both had significantly more total U1A relative to GAPDH than differentiated cells (J558L = 1.0) and that WEHI 231 had significantly more U1A than M12.4.1 (Fig. 4A, cf. M12.4.1 [lanes 4–6]/WEHI 231 [lanes 7–9] and J558L [lanes 1–3] and quantitation thereof in the bar graph). Thus, the amount of U1A is ranked according to differentiation stage WEHI 231 > M12.4.1 > J558L. This is in line with the inhibitory effect of U1A on poly(A) site expression found in previous in vivo studies that showed the same ranking (Phillips et al., 2001). This

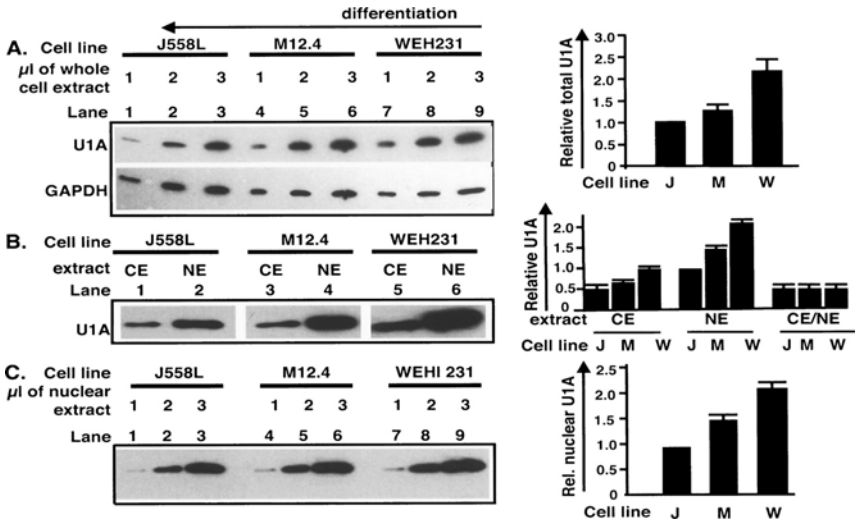


FIGURE 4. U1A levels decrease upon differentiation

(*A*) In whole cell extracts: Western blotting using U1A antibody 856 and anti-GAPDH antibody as control. (Lanes *1–3*) J558L, (lanes *4–6*) M12.4.1, (lanes *7–9*) WEHI 231. Bands were quantitated by PhosphorImager analysis, and U1A values were calculated relative to GAPDH in each case as shown in the bar graph. The relative total U1A level in J558L was set as 1.0. Triplicates ± *SD*. Cell lines are indicated. (J) J558L, (M) M12.4, (W) WEHI231. (*B*) U1A protein compartmentalization during B cell differentiation. U1A protein in nuclear extracts (NE) and cytoplasmic extracts (CE) was detected by Western blotting using U1A antibody 856. (Lanes *1*, *2*) CE and NE of J558L, (lanes *3*, *4*) CE and NE of M12.4.1, (lanes *5*, *6*) CE and NE of WEHI 231. For comparison, the loaded cytoplasmic extracts (CE) and nuclear extracts (NE) have the same amount of total protein. Bands were quantitated by PhosphorImager analysis and normalized to J558L NE (set as 1.0) in each case, and the ratio of CE:NE was calculated for each cell line as shown in the bar graph. Cell lines are indicated. (J) J558L, (M) M12.4, (W) WEHI231. Triplicates ± *SD* (*C*) Nuclear U1A protein decreases upon differentiation. Western

Blotting using anti-U1A antibody 856. (Lanes *1–3*) J558L, (lanes *4–6*) M12.4.1, (lanes *7–9*) WEHI 231. Loaded were 1, 2, and 3 μL of each nuclear extract containing the same amount of total protein. The U1A bands were quantitated by phosphorimagery, and values were normalized to J558L (set as 1.0) as shown in the bar graph. Cell lines are indicated. (J) J558L, (M) M12.4, (W) WEHI231. Triplicates ± *SD*.

suggests that changing U1A levels between the cell lines may be responsible for the changing inhibitory effect.

U1A retains the same distribution between the nucleus and cytoplasm at different stages of differentiation

As RNA processing occurs in the nucleus and U1A is known to shuttle between the cytoplasm and nucleus (Kambach and Mattaj, 1992), we next determined the availability of U1A to the RNA processing apparatus by comparing U1A levels in nucleus and cytoplasm of the different cell lines. Cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared from ~2 x 10^7 cells using a modified Dignam et al. (1983) method specific for a small number of lymphoid cells (Virtanen and Chen, 1990). The CE and NE of each cell line with equal amounts of total protein were loaded in 12% SDS-PAGE for comparison. Western blotting shows that the nucleus has more U1A than the cytoplasm for all three B cell lines (Fig. 4B, cf. lanes 1 and 2, 3 and 4, and 5 and 6). Bands were quantitated by phosphorimagery and normalized to J558L NE (set as 1.0) in each case and the ratio of CE:NE was calculated for each cell line (see bar graph in Fig. 4B). Upon differentiation, the U1A protein level in both the nucleus and cytoplasm decreases (Fig. 4B, cf. CE: WEHI 231, M12.4.1, and J558L [lanes 5, 3, 1] and NE: WEHI 231, M12.4.1, and J558L [lanes 6, 4, 2 and bar graph]). However, the proportion of U1A in CE to that in NE remains almost equivalent (Fig. 4B, CE:NE). Thus there is no selective accumulation of U1A in either compartment during B differentiation and we can rule out selective accumulation in the cytoplasm as a means of restricting the availability of U1A to the RNA processing apparatus.

U1A in nuclear extracts from the different cells lines follows the same differentiation-specific decreasing pattern as seen for whole cells

In order to accurately calibrate the relative U1A protein level in nuclear extracts of the above lymphoid cell lines and HeLa cell lines, we once again loaded 1 μL, 2 μL, and 3 μL of each nuclear extract containing the same amounts of total protein (determined to be in the linear range of the assay) onto 12% SDS-PAGE and carried out Western blotting as before (see Fig. 4C). The bands in Figure 4C were quantitated and, once again, normalized to J558L (set as 1.0). Our results showed that U1A levels decreased in nuclear extracts from cell lines from immature (WEHI 231, 2.12 ± 0.17), through mature (M12.4.1, 1.48 ± 0.10) to differentiated B cell (J558L, 1.0) following the B cell differentiation pathway (Fig. 4C, cf. WEHI 231 [lanes 7–9], M12.4.1 [lanes 4–6], and J558L [lanes 1–3], and corresponding bar graph). In addition, we found that B cells at all stages of differentiation have more nuclear U1A than HeLa cells (0.55 ± 0.02) (data not shown).

The ratio of U1A to U170K and to U1 snRNA decreases as B cells differentiate

We wanted to investigate how much U1A is available to regulate polyadenylation of IgM secretory mRNA. U1snRNP associated U1A is not available to regulate poly-adenylation (Gunderson et al., 1997). In the fully assembled U1snRNP, U1A binds to loop 2 and U170K binds to loop 1 (Will et al., 1993), and the stoichiometry of U1A/70K is 1:1 (Will and Luhrmann, 2001). U1A that is available to regulate mRNA poly(A) addition binds mRNA directly and will not be present in the U1snRNP. Thus an investigation of

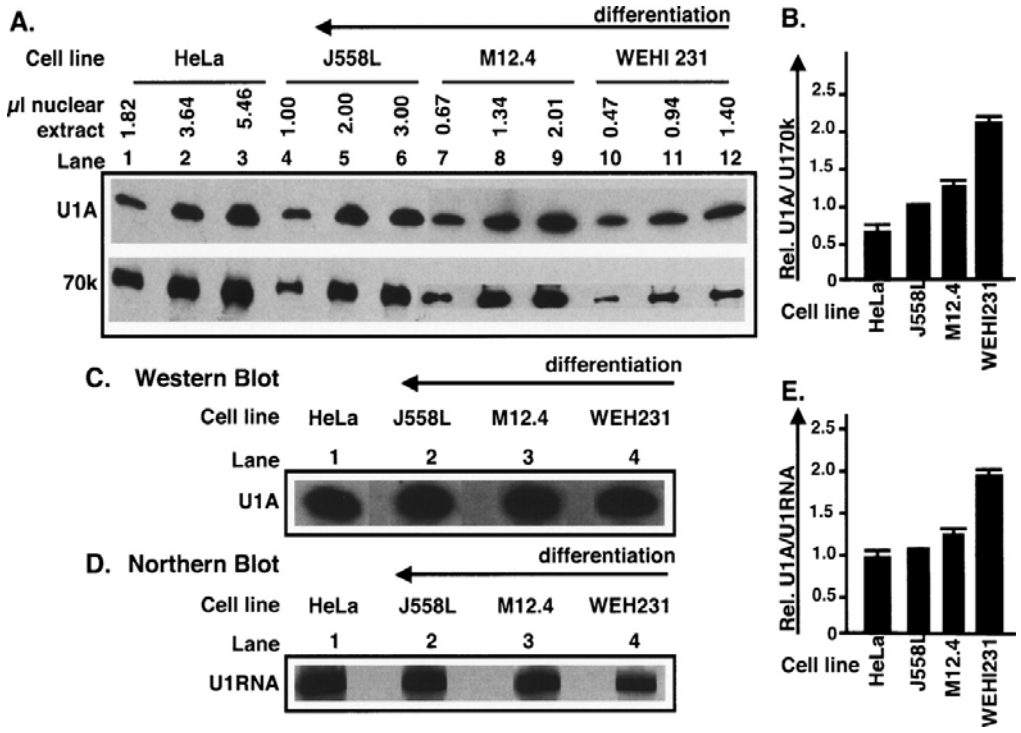


FIGURE 5. Undifferentiated cells have a greater ratio of nuclear U1A to U170K and to U1snRNA.

(*A*) Western blotting using anti-U1A and anti-U170K. (Lanes *1–3*) HeLa, (lanes *4–6*) J558L, (lanes *7–9*) M12.4.1, (lanes *10–12*) WEHI 231. Triplicates ± *SD* (*B*) Quantitation of *A*. The U1A and 70K bands in *A* were quantitated in ImageQuant and values were normalized to J558L (set as 1.0). Cell lines are indicated. Triplicates ± *SD* (*C*) Western blotting against U1A and (*D*) Northern blotting against U1snRNA in nuclear extracts from the cell lines as indicated. (*E*) Quantitation of *C* and *D*. The bands in *C* and *D* were quantitated in ImageQuant and the ratios of nuclear U1A to U1snRNA were calculated and normalized to J558L (set as 1.0). Triplicates ± *SD*.

the ratio of U1A/U170K and U1A/U1snRNP in the various cells lines should provide clues as to whether the proportion of U1A that is not snRNP-associated changes during B cell differentiation.

To more accurately compare the differences in the ratio of U170K and U1snRNA to U1A between nuclear extracts from the cell lines representing different stages of B cell differentiation, we artificially adjusted the loading of the samples so that amounts of nuclear extracts contained equal amounts of U1A. In other words, we added 1.82, 3.64, and 5.46 μL of HeLa extracts; 1, 2, and 3 μL of J558L extracts; 0.67, 1.34, and 2.01 μL of M12.4.1; and 0.47, 0.94, and 1.4 μL of WEHI 231 extract. Bands were scanned and quantitated in Image-Quant as before. The ratios of nuclear U1A to U170k for the different cell extracts were normalized to J558L (set as 1.0). First we noticed that all B cells have a greater amount of U1A relative to 70K than HeLa cells (U1A:70K ratio = 0.71 ± 0.03 *SD*) (Fig. 5A, cf. HeLa [lanes 1–3] and J558L [lanes 4–6], M12.4.1 [lanes 7–9], and WEHI 231 [lanes 10–12]). Furthermore, the ratio of U1A to 70K decreased with differentiation stage (Fig. 5A, cf. lanes 10–12 and 7–9 and 4–6). WEHI 231 have a relative U1A to 70K ratio of 2.06 ± 0.11 *SD*; M12.4.1, 1.25 ± 0.06 *SD*; while the differentiated cells have a normalized ratio of 1.0 (Fig. 5B). Thus the stoichiometry of U1A relative to 70K is greater in mature B cells. As the stoichiometry in U1snRNP is 1:1, these results suggest that there is more U1A that is not snRNP-bound in mature B cells and therefore more likely to be available to bind and regulate polyadenylation of mRNA.

We next examined the stoichiometry of U1A to U1snRNA. When we compared extracts with equal protein amounts, we found U1snRNP levels to be equivalent, showing that it is the U1A levels that change, not the U1snRNA levels (data not shown). However,

to more accurately determine the U1A to U1snRNA ratio, we once again artificially adjusted the loading of the amount of extract so that the samples contained equal amounts of U1A (Fig. 5C, U1A content by Western blotting, lanes 1–4). We measured the U1snRNA level in the different B cell lines by Northern blotting. Titration was performed to check that the bands fell within the linear range before quantitation by phosphorimage and normalization to J558L (set to 1.0). As can be seen in Figure 5D, the U1snRNA amount in the artificially adjusted volumes increases upon differentiation (Fig. 5D, cf. lanes 4, 3, 2) showing that the relative ratio of U1A to U1snRNA decreases. The quantitation is shown in Figure 5E. The relative U1A to U1snRNA ratio is 1.95 ± 0.12 for WEHI 231 and 1.18 ± 0.05 for M12.4.1, and J558L is 1.0. A value of 0.9 ± 0.01 was obtained for HeLa cells, once again showing that B cells in general have a higher ratio of U1A to U1snRNP than HeLa cells (Fig. 5E, HeLa cells). This is consistent with results obtained above for the U1A to 70K ratio and further suggests that undifferentiated B cells have a greater proportion of U1A that is not snRNP bound and therefore available to regulate polyadenylation of mRNA.

Undifferentiated cells have a greater proportion of non-snRNP bound U1A than differentiated cells

To directly measure the proportion of non-snRNP bound U1A in the different extracts, we used the fact that all snRNAs (except U6) have a unique nucleoside 2,2,7 tri-methylguanosine cap (TMG) (Will and Luhrmann, 2001), which can be specifically recognized by anti-TMG antibodies. We employed an immunoprecipitation strategy to separate U1A that is snRNP bound from that which is not. We used anti-TMG-coupled agarose beads to immunoprecipitate U1snRNP-associated U1A from the nuclear extracts

and leave the non-snRNP-bound U1A in supernatant. This way we could directly measure the proportion of non-snRNP U1A in the different cell extracts. We found that two rounds of immunoprecipitation with 20μL of TMG beads were sufficient to deplete >95% of U1snRNA as tested by Northern blotting of the supernatant (S) and comparing it with U1snRNA present in nondepleted extracts (T) (Fig. 6A % S/T). With this optimized protocol, we compared the proportion of U1A that was not bound in the U1snRNP (i.e., non-snRNP associated U1A (N) remaining in the supernatant) with total input U1A (T) for HeLa, J558L, M12.4.1, and WEHI 231 nuclear extracts of equal amounts of total protein. As can be seen in Figure 4, the nuclear U1A (T) decreases upon differentiation, confirming the results obtained above (cf. Fig. 6B, lanes 7, 5, 3, 1). The non-snRNP-associated U1A (N) also decreases upon differentiation but to a greater extent (Fig. 4B, cf. lanes 8, 6, 4, 2). The relative non-snRNP-bound U1A levels were quantitated and normalized to J558L (Fig. 6C). We found 6.95 ± 0.14-fold more and 2.0 ± 0.2-fold more non-snRNP associated U1A in WEHI 231 (immature) and M12.4.1 (mature undifferentiated), respectively, than in J558L (differentiated) cells.

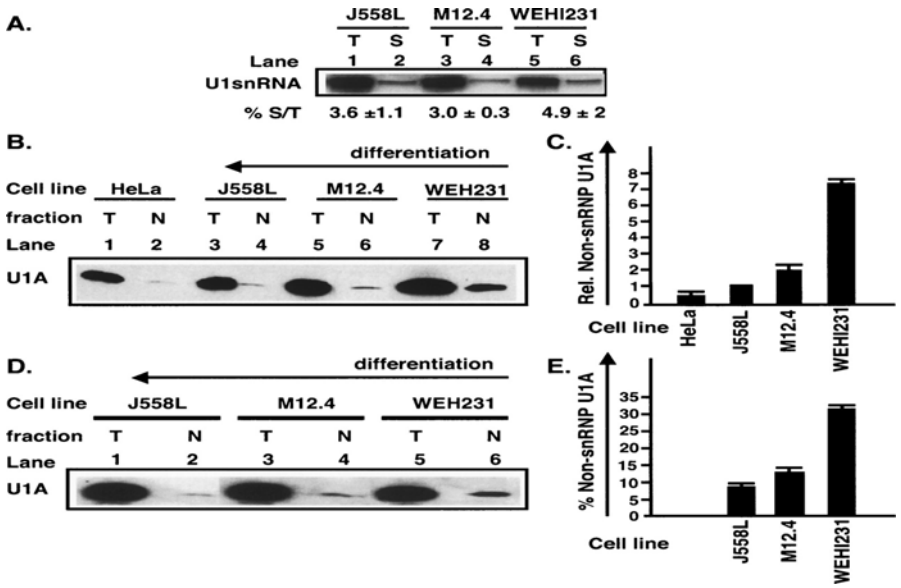


FIGURE 6. Undifferentiated B cells have more non-snRNP-bound U1A and a greater proportion of all U1A is non-snRNP-associated.

(*A*) Northern blotting against U1snRNA. (Lanes *1*, *3*, *5*) T, inputted U1snRNA from J558L, M12.4.1, and WEHI 231 nuclear extracts. (Lanes *2*, *4*, *6*) S, U1snRNA left in supernatant after immunoprecipitation. (*B*) Undifferentiated B cells have more non-snRNP-bound U1A. TMG immunoprecipitation starting with nuclear extracts of same amount of total proteins. (Lanes *1*, *3*, *5*, *7*) T, inputted nuclear U1A from HeLa, J558L, M12.4.1, and WEHI 231. (Lanes *2*, *4*, *6*, *8*) Non-snRNP-bound U1A left in supernatant after immunoprecipitation (N). U1A bands are detected by Western blotting. (*C*) Quantitation of *B*. The non-snRNP-associated U1A (N) bands were quantitated in ImageQuant and normalized to the non-snRNP-associated U1A from J558Ls. Triplicates ± *SD* (*D*) A greater proportion of all U1A is non-snRNP associated in undifferentiated cells. TMG immunoprecipitation starting with artificially adjusted volume of nuclear extract to contain equal amounts of nuclear U1A. (Lanes *1*, *3*, *5*) T, inputted nuclear U1A from J558L, M12.4.1, and WEHI 231. (Lanes *2*, *4*, *6*).

N, non-snRNP-bound U1A left in supernatant after immunoprecipitation (*E*) Quantitation of *D*. The percentage of non-snRNP-bound U1A relative to total U1A was calculated and normalized to the value for J558L. Triplicates ± *SD*.

To obtain an accurate comparison of the ratios of non-snRNP-bound U1A to total U1A for each extract, we again artificially adjusted the input amount of the three B cell nuclear extracts to equalize the starting amount of U1A. We subjected these to two rounds of TMG-beads immunoprecipitation followed by Western blotting as before (Fig. 6D) and quantitated the proportion of non-snRNP-bound U1A (N/T) in each case (see Fig. 6E). We found that the proportion of non-snRNP-bound U1A in the various cell lines decreased upon differentiation (31.6 ± 0.6% for WEHI 231, 13 ± 0.4% for M12.4.1, and 8.45 ± 0.2% for J558L) (Fig. 6D, cf. lanes 5, 3, 1 and 6, 4, 2). Taken together, these results show that both the proportion and the total amount of non-snRNP U1A decreases upon differentiation. Thus the pool of U1A that may be available to regulate mRNA polyadenylation correlates with the level of expression of the μ-secretory poly(A) site. This fits with the model that the inhibitory effect of U1A is greatest in undifferentiated cells and is relieved upon differentiation allowing expression of the μ-secretory poly(A) site and production of the secreted antibody.

Endogenous U1A in undifferentiated B cell nuclear extracts has a greater inhibitory effect on polyadenylation than that in differentiated cells

We next investigated if endogenous U1A is available to inhibit poly(A) addition. For this we tested whether we could de-repress the inhibitory effect of U1A on polyadenylation in nuclear extracts from the various cell lines. We also wanted to know if the level of de-repression achieved in each cell extract correlated with differentiation stage and therefore the amount of non-snRNP-associated U1A. For this we performed in vitro specific poly(A) assays in nuclear extracts from the different cell types and used a cold competitor to de-repress poly(A) activity in each extract respectively. For

polyadenylation we used an α-^{32}P-labeled μ-secretory RNA substrate (1790–2030) representing pre-cleaved mRNA that was synthesized by in vitro transcription as previously described (Phillips et al., 2001). For the cold competitor we used a synthesized 30-mer RNA oligo (SL2) that spans stem-loop 2 of U1snRNP and includes the consensus U1A binding site (Klein Gunnewiek et al., 2000). To control for nonspecific effects of addition of stem-loop RNA oligos to the reaction we used the MS2 stem-loop RNA that does not bind U1A.

We first compared the poly (A) efficiency using HeLa cell and B cell nuclear extracts and 30,000 cpm (50 fmol) radio-labeled mRNA substrate. Nuclear extracts from HeLa cells polyadenylated the RNA substrate to a greater extent than nuclear extracts from B cells in general, and nuclear extracts from undifferentiated B cells (WEHI 231 and M12.4.1) polyadenylated a greater percentage of the RNA substrate than those from differentiated B cells (J558L) (data not shown). The poor performance of nuclear extracts from differentiated cells has been previously reported (Virtanen and Sharp., 1988). We nevertheless obtained sufficient poly-adenylation to be able to measure de-repression in each case. In vitro specific polyadenylation assays were performed using equal amounts of total protein in the nuclear extracts (8 μg). We found that we could achieve specific de-repression with 5 and 10 ng of SL2 RNA, and that beyond 20 ng nonspecific effects came into operation as judged by an effect of MS2 RNA at this concentration (data not shown). Five (5) and 10 ng of SL2 RNA represents a 10-and 20-fold molar excess of cold competitor, respectively, over the radiolabeled RNA polyadenylation substrate. We found that we could specifically de-repress all three cell nuclear extracts significantly with 5 and 10 ng of SL2 RNA. However, the extent of de-repression was significantly lower for

J558L (differentiated) than for either M12.4.1 (mature undifferentiated) or WEHI 231 (immature undifferentiated) nuclear extracts, and M12.4.1 significantly lower than WEHI231 (Fig. 7A, cf. J558L [lanes 1–3], M12.4.1 [lanes 4–6], and WEHI 231 [lanes 7–9]). It can be seen that for this range, MS2 RNA has no effect (Fig. 7A; WEHI 231 MS2 control [lanes 10–12]). Unreacted probe is in lane 13 (no extract). We quantitated the poly(A) tails by PhosphorImager analysis and normalized the set of values obtained to that from "zero cold competitor" for each nuclear extract. For 5 and 10 ng of SL2 cold competitor we found the results to be 1.25 ± 0.06 *SD* and 1.15 ± 0.04 for J558L, 1.40 ± 0.10 and 1.60 ± 0.04 for M12.4.1, and 2.03 ± 0.04 and 2.29 ± 0.10 *SD* for WEHI 231 (see Fig. 7). In other words, we could de-repress WEHI 231 approximately fourfold more than J558L and twofold more than M12.4.1. These results correlate with differentiation stage, with undifferentiated cells being the most repressed, and differentiated cells with almost no discernable repression. From this we conclude that endogenous U1A is available to inhibit poly(A) addition in context with other factors present in nuclear extracts. Furthermore, taken together with the findings in Figure 4, we conclude that the level of repression correlates with the differentiation stage and the amount of non-snRNP-associated U1A in each case.

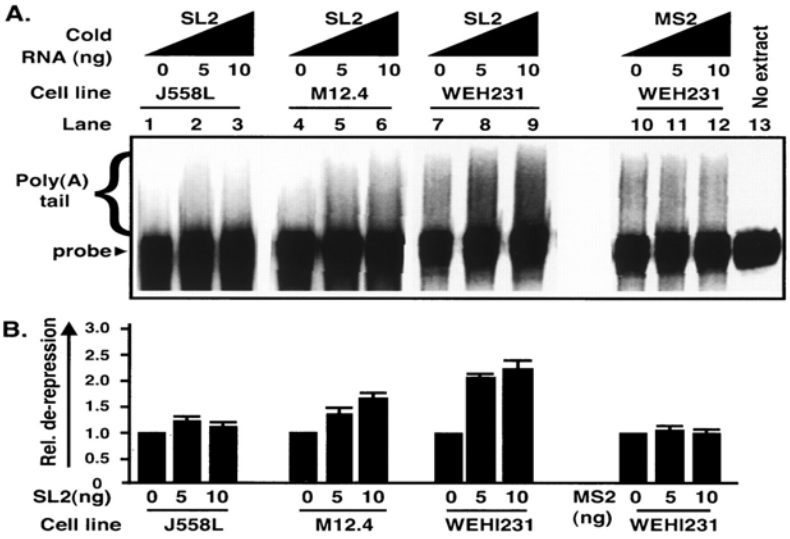


FIGURE 7. The extent of de-repression with SL2 RNA is larger in undifferentiated cells.

In vitro specific poly(A) assays in nuclear extracts from the different cell types as indicated. SL2 is a synthesized RNA 30-mer oligo that spans stem-loop 2 of U1snRNP and includes the consensus U1A binding site. This was used to de-repress poly(A) activity in each extract, respectively. To control for nonspecific effects of addition of stem-loop RNA oligos to the reaction the MS2 stem-loop RNA that does not bind U1A was used. (*A*) In vitro specific polyadenylation with nuclear extracts from the indicated cell lines. Zero (0), 5, and 10 ng SL2 representing a 0-, 10-, and 20-fold molar excess of cold competitor, respectively, over the radiolabeled RNA polyadenylation substrate, were added to consecutive samples. (Lanes *1–3*) J558L, (lanes *4–6*) M12.4.1, and (lanes *7–9*) WEHI 231. Controls: WEHI 231 NE, MS2 RNA added (lanes *10–12*) and probe alone, no extract (lane *13*). (*B*) Quantitation of *A*. Comparison of the increased percentage of polyadenylation with added SL2 RNA. Poly(A) tails were quantitated by phosphorimagery. Values were normalized to the value from the poly(A) tail with no SL2 RNA added for each set of samples from the same extract. Triplicates ± *SD*.

Non-snRNP-associated U1A's ability to inhibit poly(A) polymerase activity correlates with differentiation stage

To directly test whether endogenous non-snRNP-associated U1A complexes could inhibit poly(A) polymerase activity, we added the non-snRNP-associated U1A obtained from the nuclear extracts from the cells representing different stages of differentiation, as in Figure 4D, into nonspecific poly(A) assays with recombinant poly(A) polymerase. For this assay we once again started with volumes of HeLa and the three B cells nuclear extracts that contained the same amount of nuclear U1A to test if a greater proportion of U1A is available to inhibit poly(A) addition in undifferentiated cells. The amount of inputted U1A was estimated to be ~300 ng in each case by comparison with a standard curve (data not shown). After the snRNP-associated U1A was removed by two rounds of TMG immunoprecipitation as before, the non-snRNP-bound U1A that remained in the supernatant was immunopurified with the anti-U1A 856 antibody bound to Sepharose beads (see Materials and Methods). This non-snRNP-bound U1A was then eluted from the beads and neutralized to give a final volume of 13.5 μL. The amount of eluted non-snRNP-bound U1A was measured by Western blotting and compared to a standard curve. Eight, 18, 27, and 66 ng were obtained for HeLa, J558L, M12.4.1, and WEHI 231, respectively, confirming the differentiation stage progression in the proportion of non-snRNP-bound U1A between the cell lines (see Fig. 8A). We also did a silver stain to see what other proteins were coprecipitated in complex with U1A (see Fig. 6B). We found a prominent band at 32 kDa (U1A), which was not present in the no-extract lane (lane 10) and increased from HeLa (lane 11), J558L (lane 12) to M12.4.1

(lane 13) to WEHI 231 (lane 14) (Fig. 8B, cf. lanes 11–14). There were a number of prominent bands that were also present in the no-extract control. These did not inhibit poly(A) polymerase activity (see Fig. 8C, lane 7) and therefore were deemed nonspecific antibody-associated bands. There were two bands in the 45 kDa region that were not present in the no-extract lane (lane 10) but were present to the same degree in the extract lanes (lanes 11–14). However, a 45 kDa was visible in the extract only, no antibody lanes (lanes 1–4), suggesting that this too is a nonspecific protein that binds beads alone. This also did not inhibit poly(A) polymerase activity (Fig. 8C, lane 8). We therefore conclude that U1A is the major specific protein eluting under this protocol.

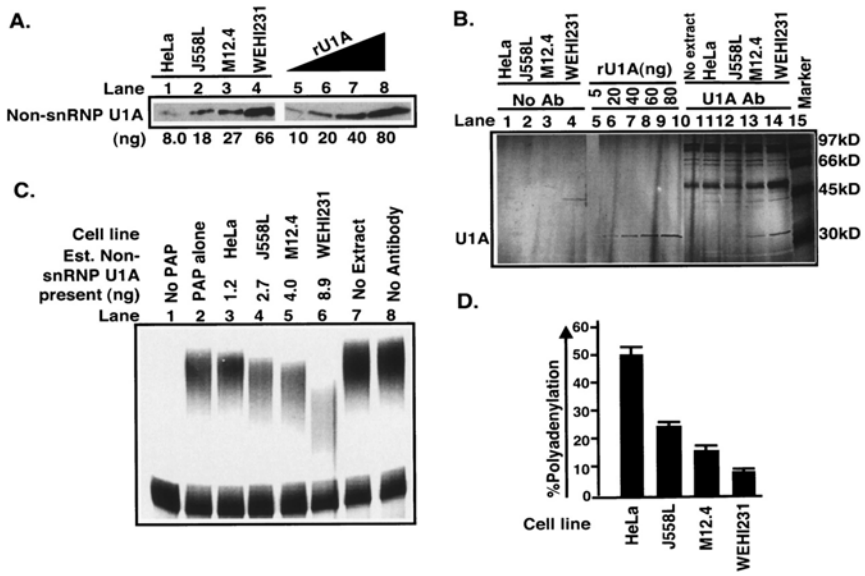


FIGURE 8. The percentage of polyadenylated IgM RNA tail in nonspecific poly(A) assay correlates with the proportion of non-snRNP-bound U1A immunopurified from the nuclear extracts.

Starting amounts of extract were artificially adjusted to contain equal amounts of input nuclear U1A. (*A*) Western blot and quantitation of non-snRNP-bound U1A from the different nuclear extracts (lanes *1–4*) by comparison with a standard curve of recombinant U1A (rU1A) (lanes *5–8*) after immunodepletion with anti-TMG antibodies, immunoprecipitation with anti-U1A 856 antibody, and elution. (*B*) Silver stain of proteins obtained after immunoprecipitation and elution. (*C*) Nonspecific poly(A) assay with immunopurified samples from nuclear extracts and recombinant poly(A) polymerase. (Lane *1*) No PAP, (Lanes *2–8*) 50 ng PAP. Lane *2*, PAP alone; lanes *3–6*, PAP plus 2µL of eluate from HeLa (lane *3*), J558L (lane *4*), M12.4.1 (lane *5*), and WEHI 231 (lane *6*); lane *7*, PAP plus eluate from a mock immunoprecipitation with no extract; lane *8*, PAP plus eluate from a mock immunoprecipitation with no antibody. (*D*) Quantitation of *C*. Poly(A) tails were quantitated by phosphorimagery and expressed as percentage of the poly(A) tail obtained with the mock immunoprecipitated and eluted sample (no antibody control, lane *8*). Triplicates ± *SD*.

For the nonspecific polyadenylation assay, we found that 50 ng recombinant bovine poly(A) polymerase (PAP) produced a robust poly(A) tail on the 30,000 rpm radiolabeled IgM secretory RNA substrate (Fig. 8C, lane 2). We then introduced 2 μL of the eluted complexes from the different cell lines into the assay to assess the extent of inhibition induced in each case. We found that HeLa eluted complexes were able to induce a very small difference in the length and abundance of the poly(A) tail compared with the "no extract" control and no antibody control (Fig. 8B, cf. lanes 3 and 7,8). The effect of the eluted complexes from the B cell lines was in general greater than for HeLa cells, with the equal amounts of eluate inducing a progressively greater inhibition of poly(A) tails production from J558L to M12.4.1 to WEHI 231, respectively (Fig. 8C, cf. lanes 3–6). The processed and unprocessed RNA substrates were quantitated by phosphorimagery and the amount of poly(A) tail was calculated and expressed as a percentage of the unreacted probe (see lane 1). An average of 83.6% ± 0.5% of IgM secretory RNA substrate is processed when mock eluted samples were added (lanes 7, 8). Two microliters of HeLa eluted samples can reduce the percentage to 47.1% ± 2.5%, J558L to 25.9% ± 1.0%, M12.4.1 to 16.9% ± 0.8%, and WEHI 231 to 6.5% ± 0.4% (Fig. 8). From these results we conclude that eluted complexes from undifferentiated B cells inhibit poly(A) polymerase to a greater extent than differentiated cells and that the inhibitory effect correlates with the proportion of non-snRNP-bound U1A from the B cells in their respective stages of differentiation.

Discussion

U1A plays a key role in the regulation of the expression of the secretory poly(A) site. Therefore, it is essential to discover whether U1A available to regulate

polyadenylation at the secretory poly(A) site changes during B cell differentiation. We have shown that (1) total U1A levels decrease upon differentiation both in whole cells and in the nucleus, (2) the proportion of U1A that is not bound in U1snRNP and therefore available to inhibit the expression of the secretory poly(A) site is reduced upon differentiation, and (3) the U1A not bound to U1snRNP is able to inhibit poly(A) addition to the secretory poly(A) site in vitro.

Total U1A as well as the proportion that is non-snRNP associated decreases with B cell differentiation

The alternative processing of the IgM heavy chain pre-mRNA is finely balanced with a weak secretory poly(A) site in competition with a weak splicing reaction to produce the secretory or membrane form of mRNA, respectively. A twofold change in strength of either reaction can result in a complete change in mRNA product even in cells that normally produce the opposite mRNA (Peterson and Perry 1989; Peterson 1992). We show here that the total U1A levels decrease upon differentiation both in whole cells and in the nucleus. Mature undifferentiated (M12.4.1, 1.39 ± 0.11 *SD*) and immature B cell (WEHI 231, 2.2 ± 0.31 *SD*) lines both had significantly more total U1A relative to GAPDH than differentiated cells (J558L = 1.0). Thus we found a 55% decrease from immature to differentiated B cells and a 30% decrease from mature undifferentiated to differentiated B cells. The latter are in agreement with results from mature human B cells induced to differentiate with IL-6 where a 29% decrease in U1A relative to an snRNP protein was observed (Milcarek et al. 2003). However as a large proportion of the cell's U1A is bound up in the U1snRNP and unavailable to bind the secretory poly(A) site, it is deceiving to examine changes in total U1A alone. We found that not only did the level of

total U1A decrease but also the proportion of U1A that is not snRNP associated decreases upon differentiation. When the amount of non-snRNP-bound U1A was compared, the changes were much more dramatic with immature B cells (WEHI 231) having sevenfold and maturing undifferentiated (M12.4.1) having twofold more non-snRNP-associated U1A than differentiated cells (J558L). As the alternative processing is finely balanced (Peterson and Perry 1989; Peterson, 1992), this amount of change in the abundance of a polyadenylation inhibitory factor alone could conceivably change the mRNA product from secreted to membrane mRNA. However, other changes in factors affecting the process have been documented that would presumably also contribute to the switch in mRNA product during B cell differentiation.

We examined a number of other parameters to document the change in non-snRNP-associated U1A. We found a change in stoichiometry of U1 snRNA and the snRNP-associated 70K protein relative to U1A without a change in U1snRNA levels in these cells. This is similar to the approach taken by Milcarek et al. (2003) in mature human B cells, and our results are in agreement with theirs. However, they did not measure non-snRNP-associated U1A as we have done in the Trimethylcap immunodepletion experiments.

The U1A not bound to U1snRNP is able to inhibit poly(A) addition to the secretory poly(A) site in vitro

Although U1A protein predominantly exists as a component of U1snRNP, it enters the nucleus by a separate Ran independent route (Kambach and Mattaj, 1992). Once in the nucleus, it can presumably bind either U1snRNP or mRNA, either its own or heterologous mRNA, such as the IgM secretory mRNA. As the proportion of U1A

theoretically available to bind to mRNA is not necessarily a measure of U1A activity on an mRNA, we also wanted to investigate if endogenous U1A from the various cell lines is able to inhibit poly(A) addition at the secretory poly(A) site and if this inhibition correlates with the proportion of non-snRNP-associated U1A. We demonstrated with the de-repression experiments in Figure 7 that there is a proportion of U1A that actively inhibits polyadenylation at the secretory poly (A) site in its native context in nuclear extracts. Furthermore, non-snRNP-associated U1A immunoprecipitated from each of the extracts is able to inhibit recombinant poly(A) polymerase activity on the secretory poly(A) site substrate. Once again, the inhibition correlates with the proportion of U1A recovered. Thus these results show that endogenous U1A actively suppresses polyadenylation at the secretory poly(A) site and that this suppression is alleviated upon differentiation.

The next question is whether U1A acts alone or in conjunction with other proteins. In the de-repression experiments, U1A is present in the context of other endogenous proteins in the various cell extracts, and the inhibitory effect could be mediated or modulated by accessory proteins. Others have reported that RNA-free U1A is present in a complex with other proteins (O'Connor et al. 1997; Lutz et al., 1998). We therefore considered the possibility that U1A that binds mRNA might also be in complex with other proteins that might modulate its activity on that mRNA. We anticipated that these accessory proteins would co-precipitate with U1A obtained from the various cell extracts in Figure 8 and would continue to affect U1A activity. However, our silver staining of the products of the immunoprecipitations show U1A as the major band that is not also present in the "no extract" and "no antibody" control, suggesting that U1A is the

major player in this inhibitory effect. Furthermore, we find that the inhibitory effect correlates with the proportion of U1A obtained, diminishing the possibility that a developmentally induced factor excessively modulates U1A activity at a particular stage of differentiation. Thus, the results we obtained suggest a more direct mechanism: The level of U1A available to bind the secretory mRNA is reduced upon differentiation, thus alleviating its inhibitory effects on both cleavage and poly(A) addition. We cannot completely rule out the possibility that an accessory protein that facilitates the effect of U1A may not be coprecipitated with our protocol or visible by silver staining. Nevertheless, the inhibitory effect correlates with the abundance of the U1A protein, and its levels change during differentiation, thus offering a straightforward explanation of the data.

We have shown that both total U1A and the proportion of non-snRNP U1A decreases upon differentiation, offering a simple mechanism for how U1A inhibition of the secretory poly(A) site is alleviated upon B cell differentiation, leading to the activation of this poly(A) site and the production of secreted antibody.

CHAPTER II: U1A regulates levels of the transcriptional repressor, Zhx-1, during B cell differentiation via alternative poly(A) site selection.

Summary

In chapter I, we showed that changing U1A levels are responsible for regulation of the poly(A) site choice in the immunoglobulin M heavy chain gene during B cell differentiation. In this chapter, we present strong evidence that U1A also regulates the expression of the transcriptional repressor, Zhx-1 (zinc fingers and homeoboxes 1), which has two alternative poly(A) sites in its 3'UTR and 5 copies of the AUGCN(1-3)C motif surrounding the upstream poly(A) site, a similar structure as the IgM secretory poly(A) site. We demonstrate that U1A protein can bind to Zhx-1 mRNA in vivo and Zhx-1 protein level, total mRNA level and the upstream poly(A) site of Zhx-1 are up-regulated in Ig-secreting cells that have a decreased amount of U1A. Overexpression of U1A in HeLa cells greatly reduced the expression of endogenous Zhx-1 protein. In addition, recombinant U1A inhibited both poly(A) addition and cleavage of the upstream Zhx-1 poly(A) site in vitro and mutation of the five U1A motifs released the inhibition of U1A. We also show that when the downstream poly(A) site of Zhx-1 is used, the expression of the resulting RNA transcript is affected by the inclusion of ARE elements. Therefore, we proposed one model about how U1A and ARE coordinately regulate the expression of Zhx-1 during B cell differentiation. As Zhx-1 binds the activation domain of the transcription factor, NFYA, which plays a key role in the expression of ER stress responsive element (ERSE)-containing genes, this provides a potential mechanism by which U1A controls the response to ER stress, an essential component of B cell differentiation.

Introduction

U1A protein was originally found as a component of U1 snRNP which is involved in RNA splicing (reviewed in Lührmann et al., 1990; Kramer, 1996). Although U1A exists predominantly as a U1 snRNP-bound form, a small percentage of U1A can function in polyadenylation independent of other U1 snRNP components (Boelens et al., 1993; Lutz et al., 1998). The percentage of non-U1 snRNP bound U1A varies when stimulated by cytokines (Milcarek et al., 2003) and in different cell types (Ma et al., 2006). In HeLa cells, only 4% of U1A exists independent of U1snRNP, while the ratio increases to 8.45 ± 0.2%, 13 ± 0.4 % and 31.6 ± 0.6 % in differentiated B cells, mature B cells and immature B cells respectively (Ma et al., 2006). U1A protein has 238 amino acids and contains two conserved RNA recognition motifs (RRM) characteristic of the largest family of RNA binding proteins (reviewed in perez-Canadillas et al, 2001; Varani and Nagai, 1998; Burd and Dreyfuss, 1994). In human U1 snRNP, U1A binds with higher specificity to stem-loop 2 of the 164-nucleotide U1snRNA at the consensus sequence AUUGCAC through its N-terminal 101 residues (RRM #1) (Lutz-Freyermuth et al., 1990; Scherly et al., 1989). U1A not bound to U1snRNP can exist as an mRNA-bound form such as binding to U1A pre-mRNA (Gunderson et al., 1994 and 1997) and IgM pre-mRNA (Phillips et al., 2001) or an RNA-free form such as SF-A (Lutz et al., 1998; O'connor et al., 1997).

Although U1snRNP is involved in splicing, the function of U1snRNP-bound U1A in splicing remains unclear (Will et al., 1996). Non-snRNP bound U1A can autoregulate its own production by binding its own pre-mRNA and inhibiting polyadenylation (Boelens et al., 1993). Two U1A molecules can bind with high affinity and specificity to

the two-7nt loops (one AUUGCAC, one AUUGUAC) in the 3' untranslated region (UTR) of human U1A pre-mRNA, dimerize and inhibit poly(A) addition by interacting with PAP (Boelens et al., 1993; Van Gelder et al., 1993). Non-snRNP bound U1A can also regulate expression of the IgM heavy chain gene by directly binding to the five non-consensus U1A motifs (AUGC(N)1-3C) surrounding the secretory poly(A) sites and inhibiting poly(A) addition and cleavage (Phillips et al., 2001 and 2004).

In chapter I, we have shown that total U1A levels as well as the proportion of non-snRNP bound U1A decreases upon B –cell differentiation, offering a simple mechanism for how U1A inhibition of the secretory poly(A) site is alleviated in differentiated B cells, resulting in the activation of IgM secretory poly(A) site and production of secreted antibody (Ma et al., 2006). Given that U1A plays a crucial role in regulation of expression of the IgM secretory poly (A) site and changing U1A levels developmentally regulate its inhibitory effect during B cell differentiation, it is possible that U1A might regulate some other genes during B cell differentiation. Several observations strongly support our view. 1. U1A levels change among different B cell lines representing different stage of differentiation (Ma et al., 2006). 2. U1A levels change upon IL-6 stimulation of B cell lines (Milcarek et al., 2003). 3. U1A is an RNA-binding protein which can bind to the consensus motif AUUGCAC with high affinity and specificity (K_d, ~0.1M) (Boelens et al., 1993; Van Gelder et al., 1993) or the non-consensus motif AUGC(N)1-3C with a relatively weaker affinity (Phillips et al., 2001 and 2004). The existence of multiple relatively weaker motifs may offer a complicated and finer-scale regulation. 4. Although two consensus motifs rarely appear around the poly(A) sites in the 3' UTR of heterogenous mRNAs, the non-consensus motif has a

higher frequency of 1/375 if randomly distributed. Of the 11366 mouse genes containing at least one poly(A) site with non-consensus U1A motifs, about 4.8% (551) of the genes have at least two upstream non-consensus motifs within 100 nucleotides upstream of the poly(A) site and 17.7% (2019) of the genes have at least one motif within 60 nucleotides downstream of the poly(A) site (Dr. Liu unpublished data). In addition, analysis of the 18145 poly(A) sites of mouse genes with non-consensus motifs revealed that about 3.1% (564) of the poly(A) sites have at least two upstream non-consensus motifs within 100 nucleotides upstream and 12.1 % (2202) of the genes have at least one motif within 60 nucleotides downstream (Dr. Liu, unpublished data).

Mouse Zhx-1 consists of 873 amino acids residues and contains two Cys_2–His_2-type zinc-finger (Znf) motifs and five homeodomains (HDs) and it is highly similar (91%) in amino acid sequence with human Zhx-1 (Yamada et al, 1999a; Barthelemy et al., 1996). Zhx-1, Zhx-2 and Zhx-3 are members of the *z*inc-fingers and homeobo*x*es (Zhx) family and these proteins not only form homodimers but heterodimers and act as ubiquitous transcriptional repressors (Yamada et al., 2003; Kawata et al., 2003; Hirano et al., 2002; Yamada et al., 2002). Zhx-1 can interact with the activation domain of the A subunit of nuclear factor-Y (NF-Y) which binds to the CCAAT box or Y box (an inverted CCAAT box) as a ubiquitous transcriptional activator (Yamada et al., 1999b; Mantovani, 1999). In addition, Zhx-1 has been reported to interact with the BS69 co-repressor and ataxia-related proteins (Yamada et al., 2003). The Mouse Zhx-1 gene exhibits alternative splicing. The longer mRNA transcript (NM_001042438 5212bp) contains five exons while the shorter transcript (NM_009572 5086bp) lacks exon 3. Both transcripts encode the same protein product, because the coding region is only located in exon 4.

Since Zhx-1 is a ubiquitious transcription repressor, research on how its levels are regulated in B cells might provide some clue about its precise function in the process of B cell differentiation. In this study we reported that during mouse B cell differentiation Zhx-1 levels are regulated by U1A through alternative poly(A) site selection. Using affymetrix microarray analysis combined with RT-PCR techniques, we identified that U1A can bind to Zhx-1 mRNA in vivo. Here we reported that the levels of Zhx-1 proteins and mRNA are negatively correlated with U1A levels in B cells and overexpression of U1A in HeLa cells significantly inhibits the expression of Zhx-1. Our in vitro and in vivo assays showed that U1A regulates the expression of the upstream poly(A) site of Zhx-1 by binding to the five non-consensus motifs around the poly(A) site and inhibiting both poly(A) addition and cleavage. When the upstream poly(A) site of Zhx-1 is inhibited in mature B cells, the usage of the downstream poly(A) site of Zhx-1 results in the inclusion of ARE elements, which destabilize the mRNA transcript. As a result, less Zhx-1 RNA and protein are produced in mature B cells.

Result

Zhx-1 mRNA binds to U1A in vivo

To determine which genes bind to U1A in vivo, we performed immunoprecipitation with anti-U1A antibody combined with microarray analysis (diagramed in Fig.9). U1 snRNP-bound U1A was removed from mouse M12.4.1 nuclear extracts by TMG antibody as previously described (Ma et al., 2006), and the RNA species specifically bound to non-U1snRNP bound U1A were pulled down by U1A antibody and subjected to an Affymetrix GeneChip microarray analysis (Lockhart et al 1996). The non-specific binding RNA sample eluted from Sepharose 4B bead (in the

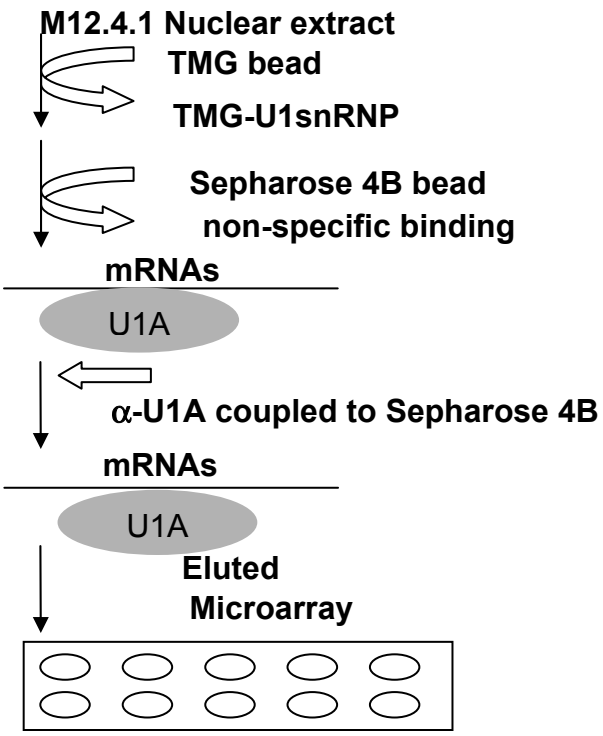


FIGURE 9. Diagram of immunoprecipitation by U1A antibody

TMG treatment was performed as previously described. The non-specific binding mRNAs sample in the pretreated step was used as a control for microarray and RT-PCR. U1A antibody was coupled to Sepharose 4B according to the Amersham's protocol.

Table 1: Classified genes pulled down by U1A antibody.

# of genes	Gene property
11	Transcription –related
6	Response to shock
27	Involved in cell cycle
12	Ribosome protein
18	Protein modification and transport
8	Electron transport / energy
3	Translation
3	snRNP associated / mRNA binding (including U1A mRNA)

Table 2. Pulled-down genes ranked by the fold enrichment.

Gene	Index	Gene	Index
Zhx-1	2.4	YY1	1.2
Zf207	1.8	CAC	1.0
Slbp	1.4	Not7	1.0
TAR	1.3		

Memo: Two fold (index 1.0) is used as a cut-off value.

pretreated step) was used as a control (see Fig.9). Using 2 fold (index 1.0) as a cut-off value, we identified 147 genes which can be classified into following categories (Table 1). U1A mRNA was also identified and so served as a positive control for this assay. We ranked the genes according to the fold enrichment in the microarray and found that the top candidate is the transcriptional repressor, Zhx-1 which has the highest value of 5.28 fold (index 2.4) (Table 2).

The mouse Zhx-1 has two reported poly(A) sites and sequence analysis revealed that it has 5 non-consensus U1A binding motifs around the reported upstream poly(A) site (29742) (Fig.10). Of them, three are located 68nt, 179nt and 242nt upstream of the poly(A) site respectively and two 62nt, 146nt downstream of the poly(A) site, respectively (Dr. Tian, unpublished data), a structure similar to IgM. No U1A binding motifs are found around the reported downstream poly(A) site (30044). Mouse Zhx-1 mRNA shares high homology with its counterpart in human (Yamada et al., 1999a). The human Zhx-1 mRNA also has two reported poly(A) sites and 5 U1A non-consensus motifs around the reported upstream poly(A) site (26140). By aligning the 3' UTR of mouse Zhx-1 and human ZHX1, we found one far upstream poly(A) signal (Denoted as PAx) (28496, AUUAAA) near the stop codon which may be used in mouse, based on the fact that 48 EST clonies were observed for that potential poly(A) site in human (27498) (Eric Ho, unpublished data).

To confirm that Zhx-1 mRNA is present in the U1A antibody-pulled down samples, we performed RT-PCR with specific exon-junction primers (Fig. 11A). We used two sets of primers to detect Zhx-1. The forward primer (24689) is the same for both sets and located in exon 4. But for set 1, the reverse primer (28444) is just before the putative

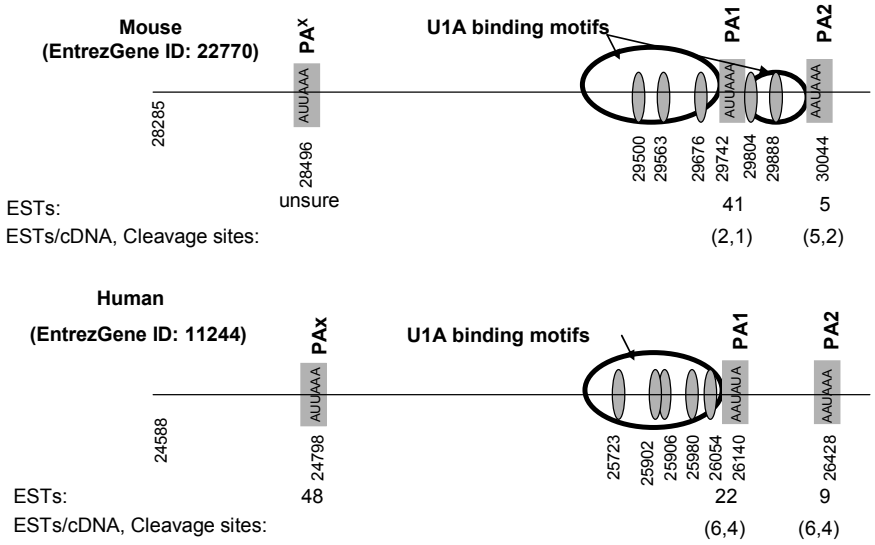


FIGURE 10. Diagram of 3' UTR of mouse and human Zhx-1

The gray rectangles represent the putative (PAx) and reported (PA1 and PA2) poly(A) sites. The gray ovals show non-consensus U1A binding motifs. PA1 and PA2 are documented in the Ensemble website. Underneath each reported poly(A) site are the number of ESTs provided by Eric Ho in our lab and the number of EST/cDNA and Cleavage sites from website http://polya.umdnj.edu/polyA_DB1.

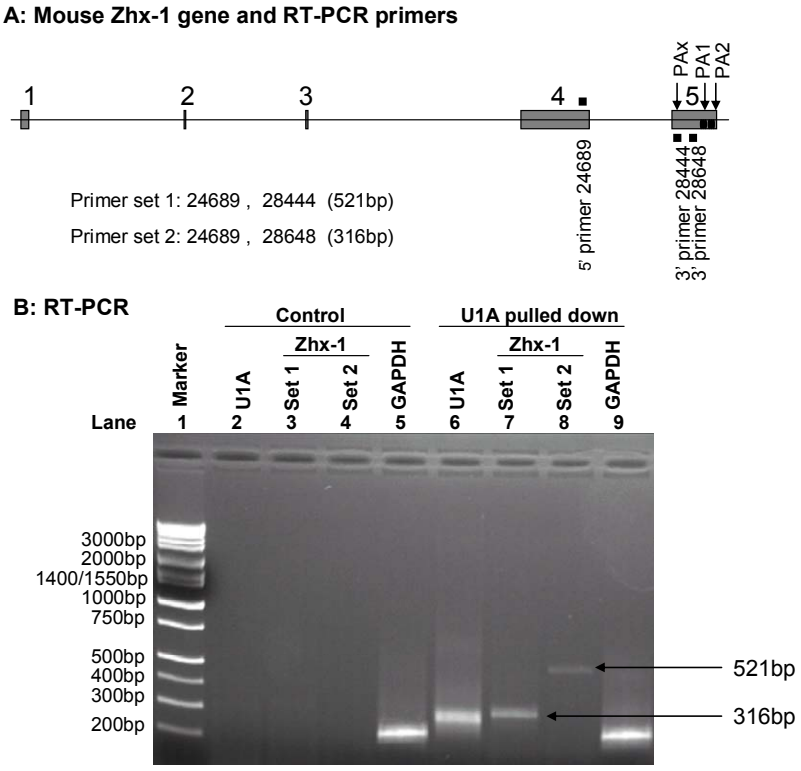


FIGURE 11. Mouse Zhx-1 mRNA binds to U1A in vivo.

RT-PCR of mRNA in the U1A antibody-pulled down samples. (A) The design of RT-PCR primers. Rectangles represent exons and arrows show the positions of poly(A) sites. The 5' primer is located in exon 4 and both 3' primers are in exon 5. (B) RT-PCRs with microarray samples of equal amount of total RNA. (Lane 1) DNA marker, (Lanes 2-5) control, (Lanes 6-9) U1A-pulled down samples. RT-PCRs with different primer sets were

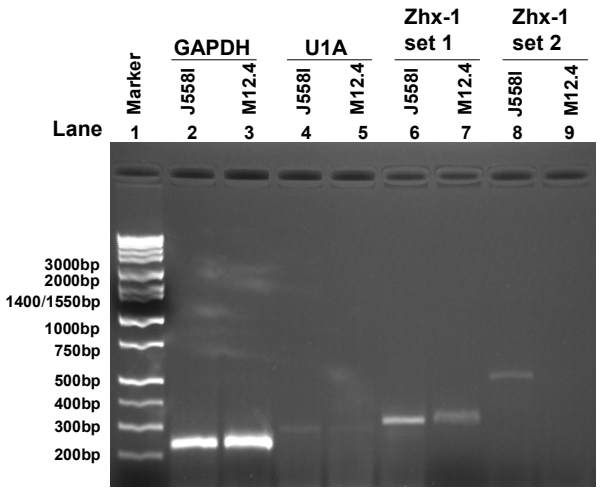


FIGURE 12. Differentiated B cells have a relatively higher amount of Zhx-1 mRNA.

RT-PCRs were performed with equal amounts of total RNA from J558L and M12.4.1 cells. (Lane 1) Marker, (Lanes 2, 4, 6, 8) J558L, (Lanes 3, 5, 7, 9) M12.4.1. RT-PCRs with different primer sets are indicated.

poly(A) site (PAx) and for set 2, the reverse primer (28648) is between the putative poly(A) site (PAx, 28496) and the reported upstream poly(A) site (PA1, 29742). The RT-PCR was performed with equal amount of total RNA (Fig 11B). Our RT-PCR showed that U1A mRNA as a positive control was specifically pulled down only by the U1A antibody (lane 6), while GAPDH mRNA as a negative control appeared in both samples (lanes 5 and 9). Zhx-1 mRNA only appears in U1A-pulled down samples. This experiment demonstrated that U1A protein does bind Zhx-1 mRNA in vivo.

Differentiated B cells have a relatively higher amount of Zhx-1 mRNA

Given that U1A levels decrease upon B cell differentiation, if U1A does regulate the expression of mouse Zhx-1, we expect to observe the difference in the Zhx-1 mRNA level between mature B cells and plasma cells. Therefore, we compared the Zhx-1 mRNA level both in differentiated (J558L) and mature (M12.4.1) B cells by semi-quantitative RT-PCR (Fig.12). Compared with J558L, M12.4.1 did not show higher levels of U1A mRNA (Fig.12, cf. lanes 4 and 5) although it has higher U1A protein (Ma et al., 2006). In fact, the decline in U1A protein level during B cell differentiation is mainly from translational repression not due to the change of transcription rate or post-transcriptional processing (Dr. Phillips, unpublished data). As predicted, J558L has more Zhx-1 mRNA than M12.4.1 (cf. lanes 6, 7, 8, 9), whereas GAPDH mRNA levels are similar in both cells. Therefore, we conclude that Zhx-1 mRNA specifically increases upon B cell differentiation.

The expression of Zhx-1 first poly(A) site is up-regulated in differentiated B cells which have a decreased amount of U1A

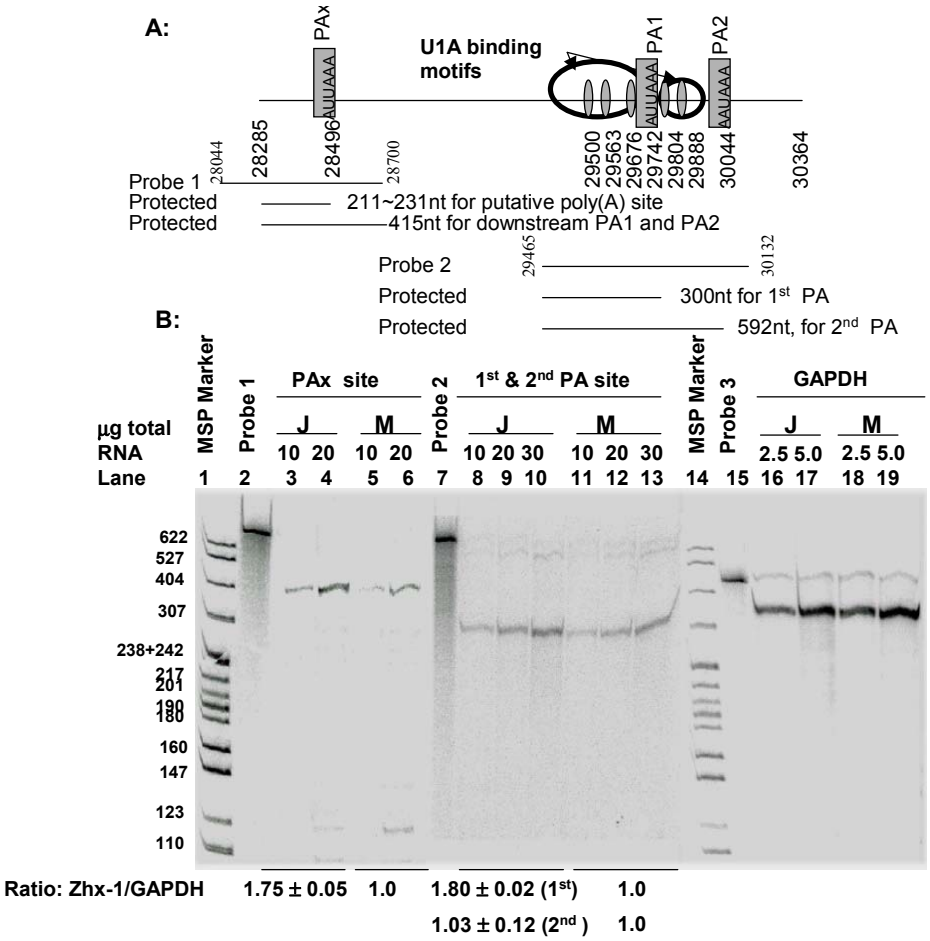


FIGURE 13. The expression of Zhx-1 1st poly(A) site of is up-regulated in differentiated B cells.

(A) Diagram of probe design for RNase protection assay (RPA). (B) RPA with total RNA from (J) J558L and (M) M12.4.1. Protected fragments were resolved by an 8% denaturing PAGE gel. (Lanes 1, 14) MSP marker, (Lanes 2, 7, 15) unreacted probes, (Lanes 3-4, 8-10,16-17) J558L, (Lanes 5-6,11-13,18-19) M12.4.1, (Lanes 3-6) RPA with probe 1, (Lanes 8-13) RPA with probe 2, (Lanes 16-19) RPA with probe 3 (anti-GAPDH). Ratios of Zhx-1 to GAPDH were calculated and the ratio value in M12.4.1 was artificially set to 1.0. These results ± *SD* are presented below the lanes of each respective sample.

Since RT-PCR is semi-quantitative, it can't accurately measure Zhx-1 mRNA levels, not mention the relative level of each transcript representing the usage of each poly(A) site in different cell lines. Thus, RNase protection assay (RPA) using total RNA from J558L or M12.4.1 was performed with two probes designed to specifically detect transcripts from the usage of each poly (A) site. Probe 1 was designed to detect the usage of the far upstream putative poly(A) site and a 211–231nt protected band is expected if that poly(A) site is used. In the case that the putative poly(A) site is not expressed, the 415nt band protected due to the usage of the two downstream reported poly(A) sites (PA1 and PA2) will measure Zhx-1 total mRNA level. Probe 2 was designed to detect the expression of PA1 and PA2. We expect one protected 300nt band for the usage of PA1 and one 592nt band for the usage of PA2 (Fig.13A). Failure to observe the specific 211-231nt band confirmed that the putative poly(A) site is not expressed in both B cells (Fig. 13B, lanes 3-6). As a result, the observed 415nt band represents the overall Zhx-1 mRNA level. Ratios of Zhx-1 (415nt bands in lanes 3-6) to GAPDH (bands in lanes 16-19) were calculated and the ratio value in M12.4.1 was artificially set to 1.0. These results ± *SD* are presented below the lanes of each respective sample in Figure 13B. The Zhx-1 total mRNA level in J558L cells is about 1.75 ± 0.05 times that in M12.4.1 (Fig. 13B, cf. lanes 3, 5 and lanes 4, 6). This further confirmed our RT-PCR result that differentiated B cells have a relatively higher amount of Zhx-1 mRNA. In addition, we did observe a strong protected band (300nt) from the usage of the 1st poly (A) site and a much weaker protected band (592nt) from the usage of the 2nd poly(A) site (Fig. 13B, lanes 8-13). We calculated the ratios of the 592nt protected band to the 300nt protected band after

adjustment for the length of the protected fragments and found that although both 1st and 2nd poly(A) sites are expressed in B cells, the 1st one is predominately used (90% to 95%). ImageQuant quantitation of the 300nt band revealed that the 1st poly(A) site is up-regulated in J558L (the protected band intensity ratio J558L:M12.4.1= 1.80±0.02) (Fig. 13B, cf. lanes 8-10, 11-13, Cf. the ratios below the lanes of each respective sample) and its ratio approximately reflects the Zhx-1 total mRNA ratio (1.75 ± 0.05) in J558L and M12.4.1. In contrast, the expression of the 2nd poly(A) site in J558l does not show any significant difference with that in M12.4.1 (the 592nt protected band intensity ratio J558L:M12.4.1= 1.03 ± 0.12) (Fig. 13B, cf. lanes 8-10, 11-13, Cf. the ratios below the lanes of each respective sample).

Zhx-1 protein is also up-regulated in differentiated B cells

So far, we have shown that endogenous U1A can pull down Zhx-1 mRNA and differentiated B cells have more Zhx-1 mRNA than mature B cells. The protein levels of Zhx-1, its interacting partner NF-YA and GAPDH were measured by western blot of whole cell extracts from the same number of J558L and M12.4.1 cells. Whole cells were lysed and sonicated in SDS-loading buffer at a concentration of 10^5 cells/μL and run on 12% SDS-PAGE. The samples were electrotransfered and probed with rabbit polyclonal antibody for human Zhx-1, rabbit polyclonal antibody for human NF-YA and mouse polyclonal antibody for GAPDH respectively. The bands were visualized using horseradish peroxidase conjugated anti-rabbit (for Zhx-1 and NF-YA) or anti-mouse (for GAPDH) secondary antibody and ECL reagents (Fig. 14). The visualized bands were

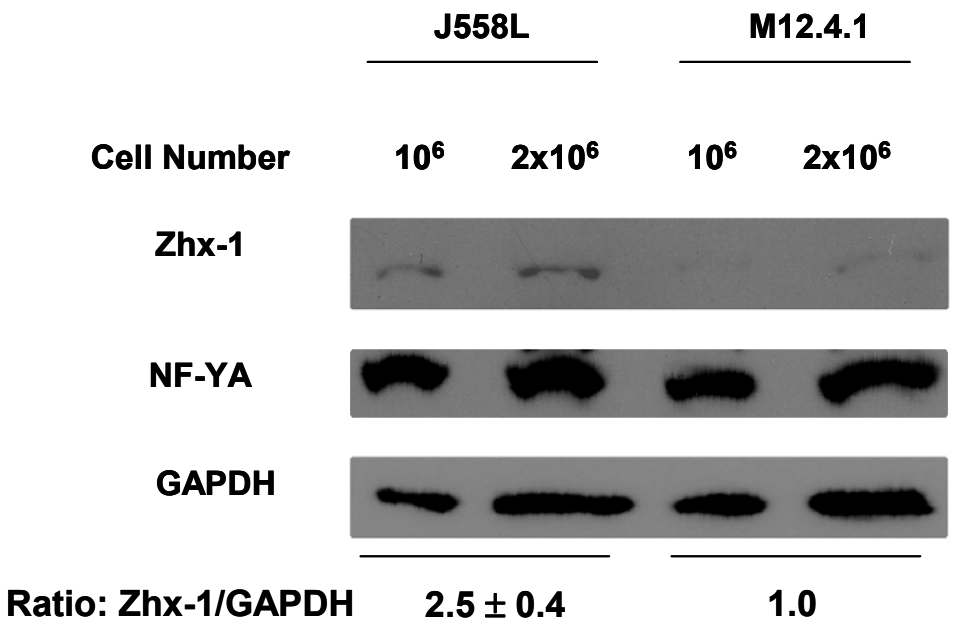


FIGURE 14. Zhx-1 protein is up-regulated in differentiated B cells. Western blot was performed with total cell extracts from J558L and M12.4.1. Cells were suspended and sonicated in 1xSDS loading buffer. The supernatants were analyzed by a 12% SDS-PAGE gel. Protein bands were electrotransferred to a membrane and probed with mouse anti-human GAPDH, rabbit anti-human Zhx-1 and rabbit anti-human NF-YA. (Lanes 1, 2) J558L, (Lanes 3, 4) M12.4.1. Cell number is indicated for each lane. The ratios of Zhx-1 to GAPDH were calculated and normalized to the value for M12.4.1 (i.e M12.4 was set to 1.0). Results of triplicates ±SD are presented below the lanes of each respective sample.

scanned and quantitated in ImageQuant. We calculated the ratio of Zhx-1 to GAPDH and the ratio of NF-YA to GADPH in triplicate ±SD for each cell line and normalized these to the value for M12.4.1 (i.e M12.4 was set to 1.0). We found that differentiated B cells (J558L, 2.5±0.4 SD) have significantly more Zhx-1 protein relative to GAPDH than mature cells (M12.4.1 =1.0). The ratio of NF-YA to GAPDH was similar in both cell lines (quantitation data not shown).

Overexpression of U1A inhibits the production of Zhx-1 protein in HeLa cells

If U1A does regulate the expression of Zhx-1, we expected that overexpression of U1A in vivo would inhibit the production of Zhx-1 protein. To overexpress U1A, we used a HeLa Tet- regulatable cell line (Tet-Off) (Clontech) designed for use with Clontech's tetracycline-regulatable gene expression system. These cell lines stably express tTA (tetracycline transactivator). To create a Tet-regulatable expression system for U1A, the U1A binding sites in the 3' UTR of human U1A were inactivated by mutation and then the U1A cDNA was placed under the control of a tetracycline-responsive promoter (e.g. TRE) (diagrammed in Fig 15A). The U1A expression construct used to make stable cell lines was derived from the pIRESPuro3 plasmid (Clontech), in which the constitutively active cytomegalovirus (CMV) promoter was replaced by the Tet responsive promoter (Guan et al., 2003). pIRESPuro3 is a bicistronic expression vector that can produce two polypeptides from one messager RNA. One is U1A protein, and the other is the puromycin resistance protein. Tet-Off cell lines express tTA (tetracycline transactivator) that binds and activates the expression of U1A in the absence of Doxycycine (Dox). In the presence of doxycycline, tTA is maintained in an inactive status, which prevents tTA protein from binding to the Tet responsive promoter.

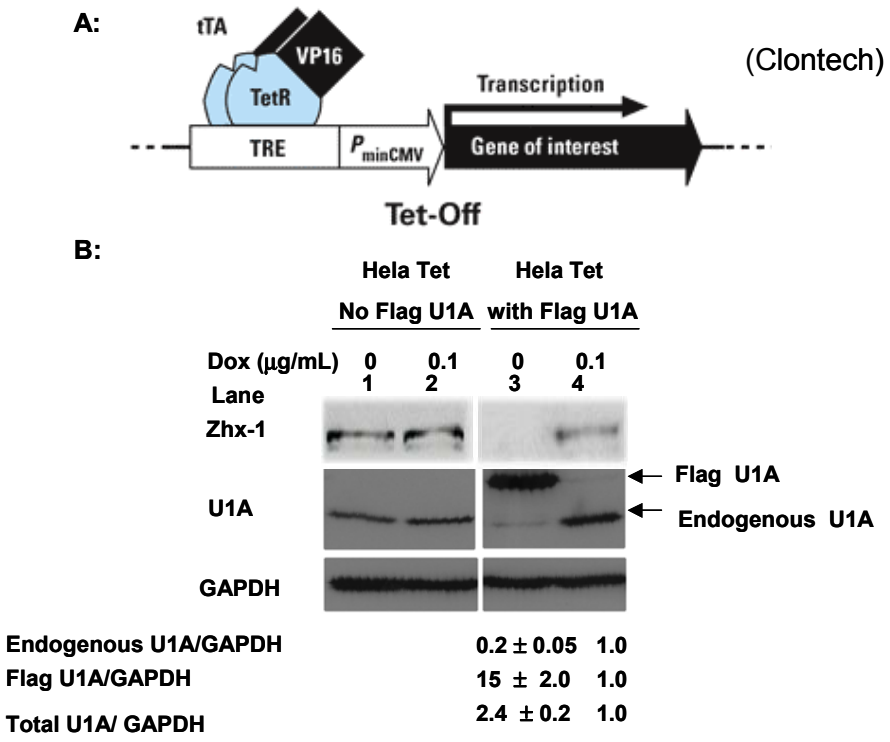


FIGURE 15. Overexpression of U1A inhibits the production of Zhx-1 protein.

(A) Diagram of the Tet-off gene expression system for overexpression of U1A. The U1A binding sites in the 3' UTR of human U1A were removed from the U1A cDNA to abolish autoregulation and then the U1A cDNA was placed under the control of a tetracycline-responsive promoter (e.g. TRE). The construct was transfected into the Tet-Off HeLa cell line expressing tTA (tetracycline transactivator) and Stable cell lines were isolated that stably express U1A-Flag protein. (B) Western blot was performed with total cell extracts from equal amounts of HeLa Tet cells with or without transfection. The blot was probed with mouse anti-human GAPDH, rabbit anti-human Zhx-1 and rabbit anti-human U1A. (Lanes 1, 2) HeLa Tet (no U1A-Flag transgene), (Lanes 3, 4) HeLa Tet stably expressing U1A-Flag protein. The concentration of Dox is indicated above each lane. The ratios of U1A to GAPDH were calculated and normalized to the value when DOX is present (Lane 4).

Cultured HeLa Tet-Off cells were harvested and lysed by heat in SDS-loading buffer at a concentration of 10^5 cells/μL. Protein samples were separated by a 12% SDS-PAGE gel and electrotransferred. Western blot was performed as abovementioned and the protein bands were scanned and quantitated in Image-Quant. We calculated the ratio of U1A:GAPDH in triplicate ± *SD* for each cell line and normalized these to the value when DOX is present (lane 4, set to 1.0). For HeLa Tet without stably expressed Flag U1A, we did not observe any visible change in endogenous U1A levels or in Zhx-1 levels when Dox was added (Fig. 15B, cf. lanes 1 and 2, quantitation data not shown). However, in the absence of DOX, the overexpression of Flag U1A caused the endogenous U1A to decrease 4-6 fold (0.2 ± 0.05) (Fig. 15B, cf. lanes 3 and 4, endogenous U1A) and this is consistent with its autoregulation. This demonstrated that our Flag U1A is active. The stably expressed Flag U1A level was about 2.4 fold over the level of endogenous U1A in the control HeLa Tet cells (Fig. 15B, cf. lanes 3 and 4). As a result, in the absence of DOX, the total U1A level increased (2.4 ± 0.2) while Zhx-1 almost disappeared (quantitation data not shown). Therefore, we conclude that overexpression of U1A inhibits the expression of Zhx1.

U1A binding to the motifs upstream of the first poly(A) site inhibits poly(A) addition

To explore the mechanism of the U1A inhibition of Zhx-1 expression, we did a bioinformatic analysis to identify the location of the poly(A) sites, the U1A motifs, the ARE elements and the GU rich regions in the 3' UTR of mouse Zhx-1. Interestingly, in addition to the five U1A motifs surrounding the upstream poly(A) site, we found three ARE motifs in the context of the two poly(A) sites and two of them are located between the two poly(A) sites (diagrammed in Fig. 16). For the upstream poly(A) site, the RPA

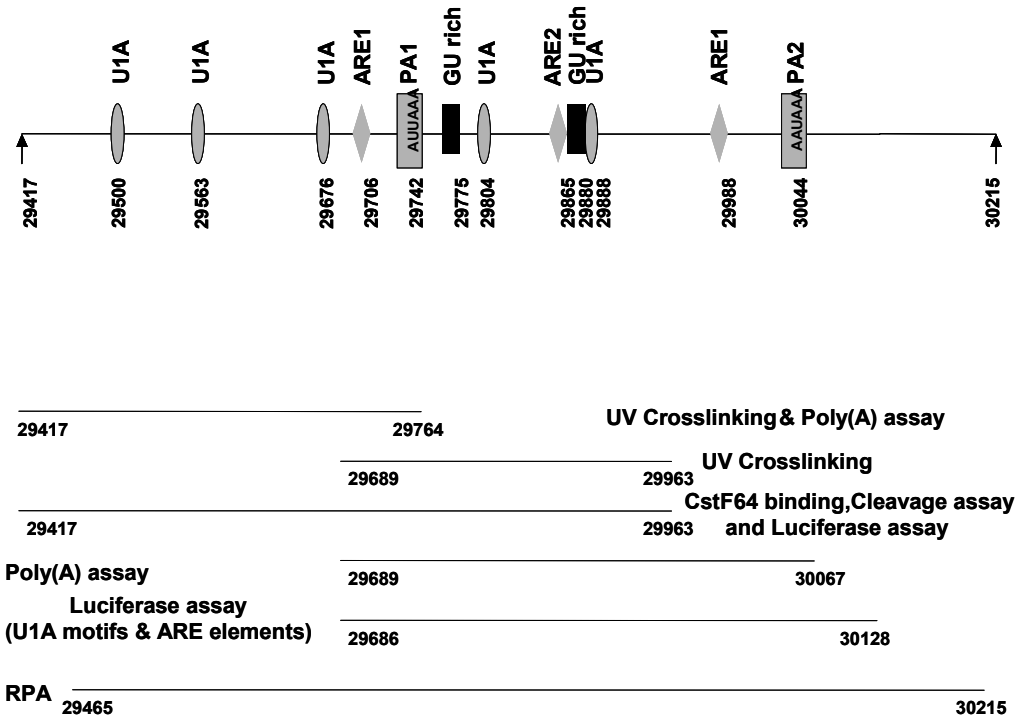


FIGURE 16. Diagram of the location of the poly(A) sites, the U1A motifs, the ARE elements and the GU-rich regions in the Zhx-1 3' UTR and plasmids made to test these elements

Gray rectangles represent poly(A) sites and gray ovals show the non-consensus U1A motifs. Classic ARE elements (ATTTA) and GU-rich elements are indicated as gray diamonds and black rectangles respectively. The numbers underneath show the position of each element. The types of assays that each cloned fragment is used for are indicated.

data (Fig. 13B) shows that its expression is up-regulated in differentiated cells (J558L:M12.4.1= 1.80±0.02). It is possible that U1A binds to those 5 motifs and inhibits the expression of that poly (A) site in mature B cells. In differentiated B cells, U1A levels decrease, thereby releasing the inhibition. For the downstream poly(A) site, the RPA data (Fig.13B) shows that its expression is extremely low in both cell lines. It is possible that this is due to the ARE elements. To investigate all these possibilities, we made a series of constructs for in vitro and in vivo analysis (Fig.16).

To test if U1A can bind to the three upstream non-consensus motifs, we performed UV cross-linking assay as previously described (Phillips et al., 2004 and 1997). We used ^{32}P-labeled wild type PIE RNA (PIE WT) and PIE with two mutated U1A motifs (PIE Δ1/2) as positive and negative controls respectively. As U1A increased from 0 to 150 ng, a specific 32kd cross-linked band was observed both in PIE WT (Fig.17A, lanes 2-5) and in Zhx-1 29417-29764 WT (Fig.17A, lanes 10-13). Mutation of U1A motifs greatly reduced the binding (Fig.17A, lanes 6-9 and 14-17. quantitation in Fig. 17B). Therefore, recombinant U1A can specifically bind to the three non-consensus U1A motifs in Zhx-1 but to a weaker degree than the two consensus motifs in PIE WT (Fig. 17A, cf. lanes 11-13 and lanes 3-5. See quantitation data in Fig. 17B), which is similar to the previous result in IgM (Phillips et al., 2001 and 2004).

Next, we wanted to determine whether the binding is functional. In vitro nonspecific poly(A) assays were performed as previously described (Phillips et al., 2001) with 50 ng of recombinant PAP and 30,000 cpm of ^{32}P-labeled RNA substrate. The polyadenylation reaction was carried out for 30 minutes at 37°C. PIE wt and PIE Δ1/2 were used as controls as before. We observed that increasing concentrations of U1A (0-

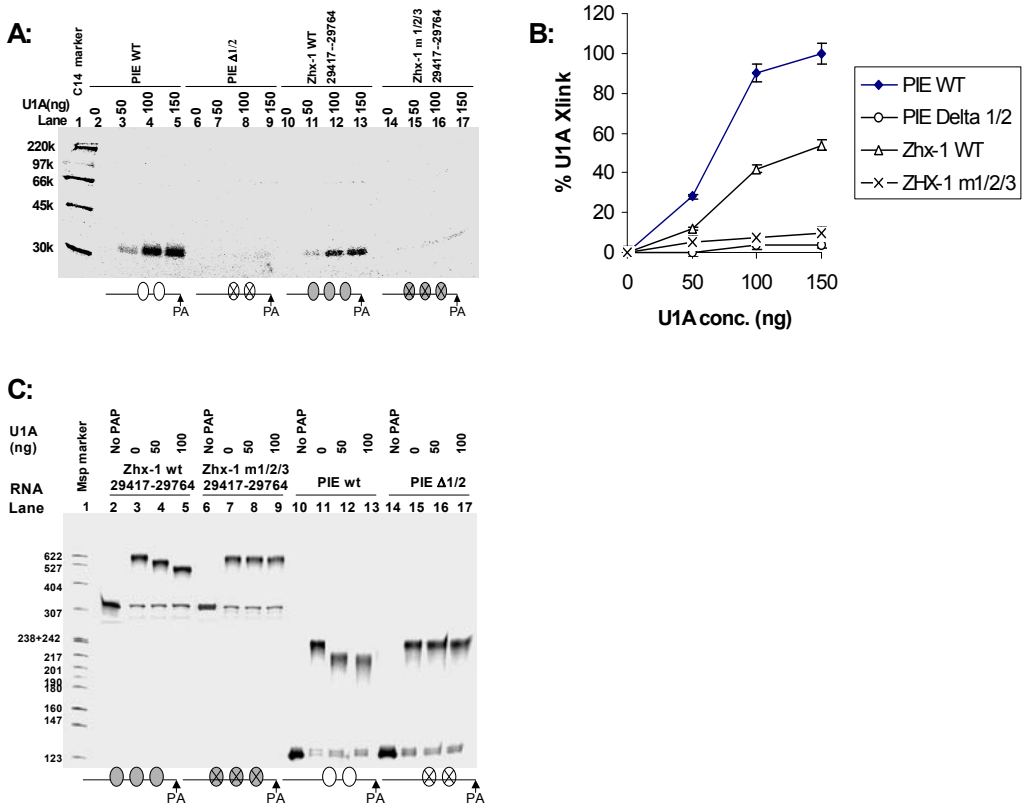


FIGURE 17. U1A binding to the three motifs upstream of the 1st poly(A) site inhibits poly(A) addition.

The RNA substrates and U1A amount are indicated above each lane. The consensus and non-consensus U1A motifs are shown as blank oval and gray ovals respectively. Wt means wild type and m1/2/3 represents a triple mutation. (A) UV crosslinking assay with 32p-labelled RNA substrates. Crosslinked products were separated by 12% SDS-PAGE. (B) Phosphorimager quantitation of panel A. Results are expressed a percentage of 150ng U1A binding the PIE wild-type substrate (set as 100%) (Lane 5). Data are means of triplicates from three separate cross-linkings ± SE. (C) In vitro non-specific poly(A) assay with 32p-labelled RNA substrates, recombinant U1A and recombinant PAP. Reaction products were separated by 8% denaturing PAGE.

100 ng) inhibited the poly(A) addition of Zhx-1 29417-29764 wild type (Fig. 17C, lanes 3-5) and PIE wild type (Fig. 17C, lanes 11-13) but not Zhx-1 29417-29764 m1/2/3 (Fig. 17C, lanes 7-9) or PIE Δ1/2 (Fig.17C, lanes 15-17). Therefore, U1A binding to the upstream 3 motifs inhibited the poly(A) addition to the 1st poly(A) sites.

The proximal upstream U1A motif plays a key role in inhibiting poly(A) addition of the 1st Poly(A) sites

To determine whether the three U1A motifs IN Zhx-1 play equal roles in inhibiting the polyadenylation of Zhx-1, we mutated each motif one by one and then performed specific polyadenylation assays in nuclear extracts according to the protocol as previously described (Virtanen and sharp, 1988). For wild type Zhx-1 29417-29764 substrate (Fig.18A, lanes 2-5), 100ng U1A reduced the poly(A) addition to approximately half (56% ± 4%) (Fig.18B). When we mutated all three motifs, no inhibition was observed (Fig.18A, lanes 18-21. Fig.18B). When the first motif was mutated, 100ng U1A reduced the poly(A) addition to approximately three-quarters (73% ± 3%) (Fig.18B). Thus, U1A still inhibited the poly(A) addition but to a less degree (Fig.18A, cf. lanes 7-9 and lanes 3-5.). When the second motif or third motif was mutated, there was almost no visible inhibition (Fig.18A, lanes 11-13, 15-17. Fig.18B). Thus, the proximal site (66nt upstream) plays a more important role in inhibiting polyadenylation of the upstream poly(A) site.

U1A binding to the two downstream motifs inhibits the binding of CstF64 and the cleavage at the 1st poly(A) site

To investigate whether recombinant U1A can bind to the two downstream sub-optimal motifs (29804, 29888), we mutated each motif and performed UV crosslinking

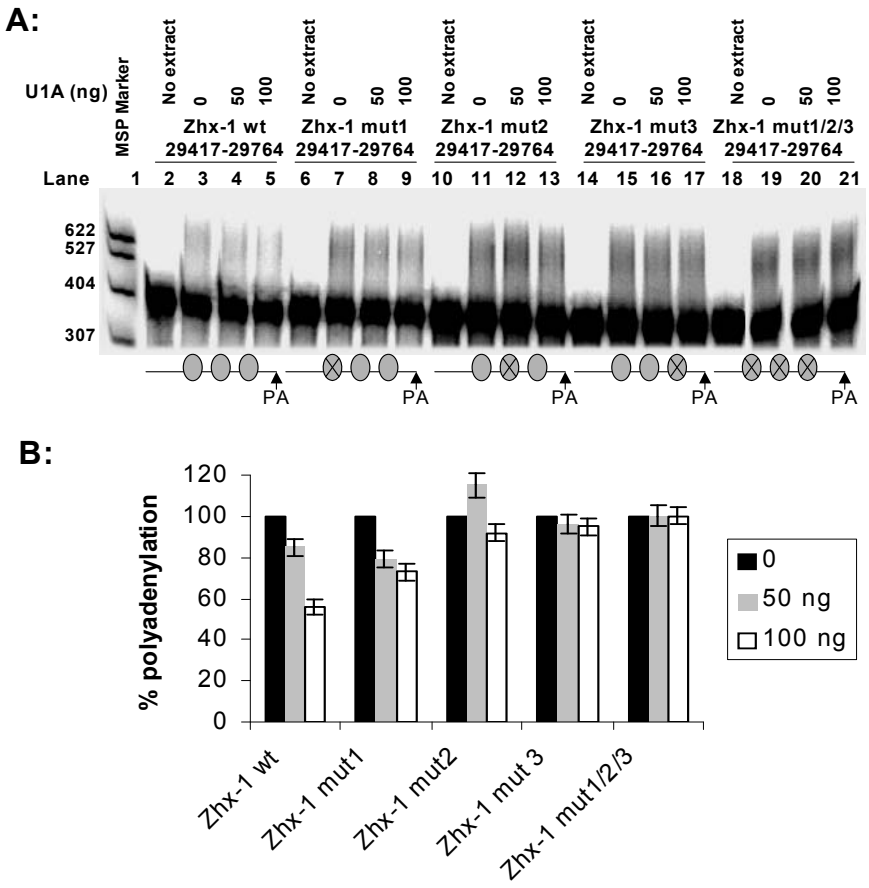
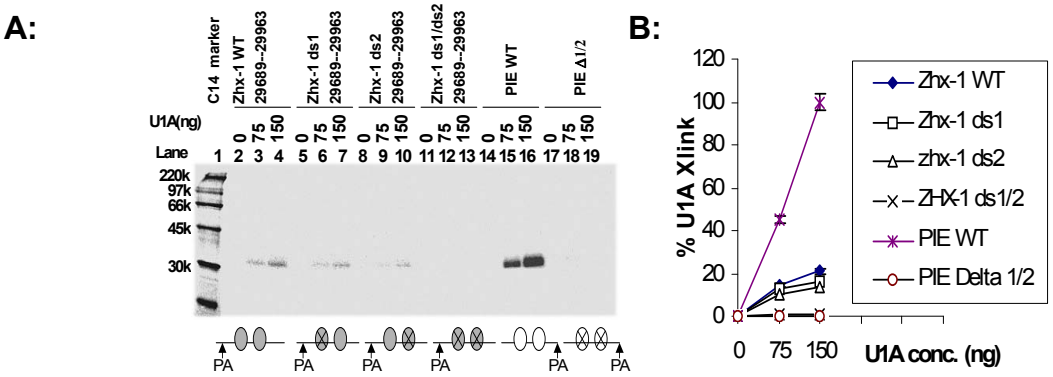


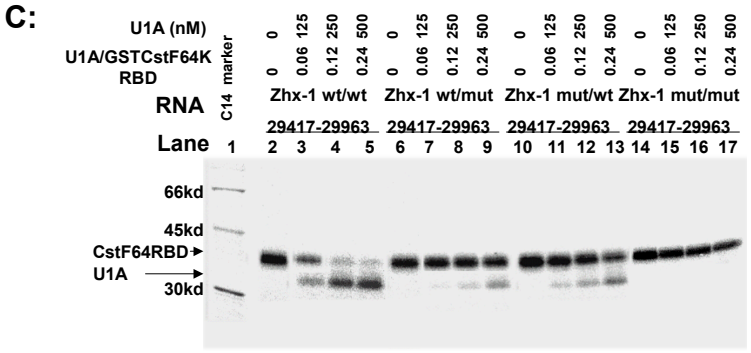
FIGURE 18. The proximal upstream U1A motif plays a key role in inhibiting the poly(A) addition of 1st poly(A) site.

(A) In vitro specific poly(A) assays were performed in HeLa nuclear extracts with 32p-labeled Zhx-1 29417-29764 substrates. Reaction products were separated by 8% denaturing PAGE. The RNA substrates and U1A amount are indicated above each lane. The non-consensus U1A motifs are shown as gray ovals. (B) Quantitation of *A*. Poly(A) tails were quantitated by phosphorimagery and expressed as percentage of the poly(A) tail obtained with no U1A added (Lanes 3,7,11,15,19). Triplicates ± *SD*.

assays with ^{32}P-labeled Zhx-1 RNA and PIE RNA substrates (Fig.19A). PIE wild type (PIE WT) (Fig.19A, lanes 14-16) and double mutated form (PIE Δ1/2) (Fig.19A, lanes 17-19) are the positive and negative controls as in Fig.17A. Crosslinked products were separated by a 12% SDS-PAGE gel. We observed a specific 32kd crosslinking band with increasing intensity as U1A increased from 75 ng to 150 ng for Zhx-1 29689-29963, wild type (lanes 2-4), single mutation ds1 (lanes 5-7) and ds2 (lanes 8-10) but not for the double mutation ds1/2 (lanes 11-13). Each single mutation reduced the binding of U1A (150 ng) from 21% ± 1% (WT) to 15.9% ± 0.7% (ds1) or 13.5% ± 0.6% (Fig. 19A, cf. lanes 2-4, 5-7 and 8-10, Fig. 19B). The double mutation completely abolished the binding (Fig. 19A, lane 11-13. Fig.19B). Therefore, U1A does bind to the two downstream motifs.

Sequence analysis revealed that the downstream two U1A motifs and two GU rich regions overlap. To test whether U1A binding to those two motifs inhibits CstF64 binding, UV crosslinking assays were performed as in Fig. 17A and Fig.19A. Full length CstF64 contains domains partially blocking its binding to RNA, it by itself can't bind well and requires simultaneous binding of CPSF to an AAUAAA hexanucleotide motif (Takagaki and Manley, 1997). Therefore, we used recombinant GSTCstF64kRBD that has been previously shown to bind RNA on its own (Phillips et al., 2004; Takagaki and Manley, 1997). In this assay, 32p-labelled RNA substrates were incubated with GSTCstF64RBD first, then an increasing amount of recombinant U1A was added to compete off the binding of CstF64. To see if the upstream U1A motifs have some effect, we used the Zhx-1 RNA substrate 29417-29963 spanning the 3 upstream U1A motifs and


A:
C14 marker
Zhx-1 WT 29689--29963
Zhx-1 ds1 29689--29963
Zhx-1 ds2 29689--29963
Zhx-1 ds1/ds2 29689--29963
PIE WT
PIE Δ1/2
U1A(ng)
0 75 150
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
220k
97k
66k
45k
30k
PA
B:
% U1A Xlink
U1A conc. (ng)
Zhx-1 WT
Zhx-1 ds1
zhx-1 ds2
ZHX-1 ds1/2
PIE WT
PIE Delta 1/2


C:
U1A (nM)
U1A/GSTCstF64K RBD
C14 marker
RNA
Zhx-1 wt/wt
Zhx-1 wt/mut
Zhx-1 mut/wt
Zhx-1 mut/mut
29417-29963
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
66kd
45kd
CstF64RBD
U1A
30kd

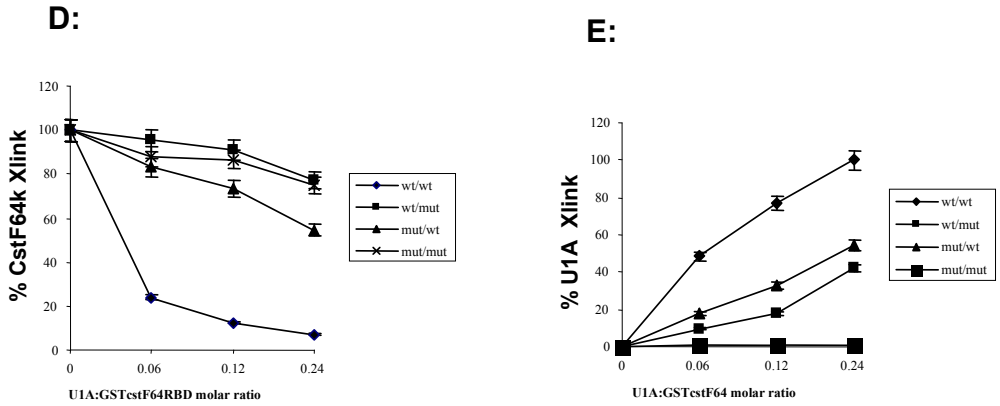

D:
% CstF64k Xlink
U1A:GSTcstF64RBD molar ratio
wt/wt
wt/mut
mut/wt
mut/mut
E:
% U1A Xlink
U1A:GSTcstF64 molar ratio

FIGURE 19. U1A binding to the two downstream motifs inhibits the binding of CstF64.

The 32p-labelled RNA substrates and U1A amount are indicated above each lane. The consensus and non-consensus U1A motifs are shown as blank oval and gray ovals respectively. (A) UV crosslinking assay with recombinant U1A (0~150 ng). Zhx-1 RNA substrates 29689-29963 spanning the two downstream U1A motifs were used. Crosslinked products were separated by 12% SDS-PAGE and visualized by Phosphorimagery. (B) Phosphorimager quantitation of panel A. Results are expressed a percentage of 150ng U1A binding the PIE wild-type substrate (set as 100%) (Lane 16). Data are means of triplicates from three separate cross-linkings ± SE. (C) UV crosslinking assay with 2 mM GSTCstF64RBD and increasing concentrations of U1A (0-500 nM). Zhx-1 RNA substrates 29417-29963 spanning all five U1A motifs were used. RNA substrates were incubated with GSTCstF64RBD first then an increasing amount of U1A was added. Products were separated by 12% SDS-PAGE and cross-links were visualized by phosphorimagery. Wt/wt denotes that both the upstream and the downstream motifs are wild type; wt/mut means that the downstream 2 motifs are mutated; mut/wt represents that the upstream 3 motifs are mutated; mut/mut means all 5 motifs are mutated. (D) Quantitation of the binding of GSTCstF64kRBD in B. The binding of CstF64kRBD in the absence of U1A (lanes 2, 6, 10 and 14) were set to 100%. (E) Quantitation of the binding of U1A in B. The binding percentage of U1A to Zhx-1 29417-29963 wt/wt when 500 nM U1A was added (lane 5) was set as 100%.

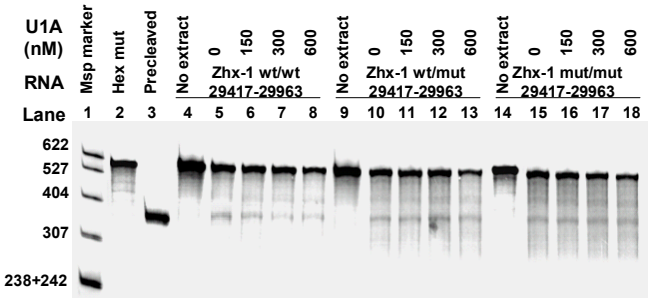
the 2 downstream motifs. We mutated either the three upstream U1A motifs (denoted as mut/wt) or the two downstream U1A motifs (denoted as wt/mut) or all five motifs (mut/mut).

We found that a relatively low amount of U1A (0.06-0.24 U1A/GSTCst64kRBD molar ratio) inhibited the CstF64kRDB binding for Zhx-1 wt/wt 29417-29963 substrate (Fig.19C, lanes 2-5) and Zhx-1 mut/wt 29417-29963 substrate (Fig.19C, lanes 10-13). Mutation of the two downstream U1A motifs greatly reduced or abolished the inhibitory effect of U1A (Fig.19C, cf. lanes 6-9, 14-17 and 2-5, quantitation in Fig.19D), These results are similar to that seen with IgM (Phillips et al., 2004). In addition, we observed that when the upstream U1A motifs were mutated, recombinant U1A via its binding to the two downstream motifs inhibited the binding of CstF64RBD but to a lesser degree (Fig.19C, cf. lanes 10-13 and 2-5, quantitation in Fig.19D), which is different from what was previously reported for IgM (Phillips et al., 2004). We think the presence of the upstream U1A motifs might enhance the binding of U1A molecules to the two downstream motifs, thereby enhancing the inhibitory effect, but the upstream motifs themselves have no direct inhibitory effect on CstF64 binding (Fig.19C, lanes 6-9). The percentages of U1A binding to each RNA substrate were quantitated in Fig.19E. The binding of U1A to Zhx-1 29417-29963 wt/wt when 500nM U1A was added (Fig.19C, lane 5) was set as 100%. We observed that mutation of either the upstream 3 U1A motifs or the downstream 2 U1A motifs greatly reduced the binding of U1A (Fig.19C, lanes 6-9 and 10-13, quantitation in Fig.19E) and mutation of all 5 motifs completely abolished the binding of U1A (Fig.19C, lanes 14-17, quantitation in Fig.19E). We also note that there

may be cooperative binding of U1A to the five motifs (Fig.19C, cf. lanes 2-5, 6-9 and 10-13. Fig.19E, cf. the additive value of wt/mut and mut/wt with that of wt/wt).

Next, we investigated whether U1A binding to the GU-rich regions affects the cleavage at the first poly(A) site. We performed an in vitro cleavage assay in HeLa nuclear extracts (Moore and sharp, 1984 and 1985). We added 3' dATP in the reaction mixture to block the polyadenylation reaction, therefore the cleaved products could be observed as discrete bands. In this assay the unreacted substrate is 564 nts (29417-29963) and the two cleavage products are 347nts and 217nts. Reaction products were separated by 8% denaturing PAGE and visualized by Phosphorimagery. The smaller cleaved product runs off the gel. In this assay an in vitro transcribed pre-cleaved substrate was used as a reference (Fig.20A, lane 3) and unreacted wild type and mutated substrates were included as controls (Fig.20A, lanes 4, 9 and 14). No cleavage band (Fig.20A, lane 2) was observed when the hexanucleotide AUUAAA was mutated, demonstrating that the cleavage is hexanucleotide specific (Fig.20A, lanes 5-8, 10-13 and 15-18). For Zhx-1 wild type and mutated substrates, about 23%~27% of the substrate was cleaved in HeLa nuclear extracts when no U1A was added (Fig.20A, lanes 5, 10 and 15, quantitation in Fig. 20B). Increasing amount of U1A significantly inhibited the cleavage of the wild type substrate (Fig. 20A, lanes 4-8, Fig. 20B) but not that of substrates with the downstream U1A motifs mutated (Fig. 20A, lanes 10-13 and 15-18, Fig. 20B) or all 5 U1A motifs mutated (Fig. 20A, lanes 10-13, Fig. 20B). In conclusion, U1A binding to the GU-rich regions inhibits cleavage of the first poly(A) site of Zhx1.

A:



B:

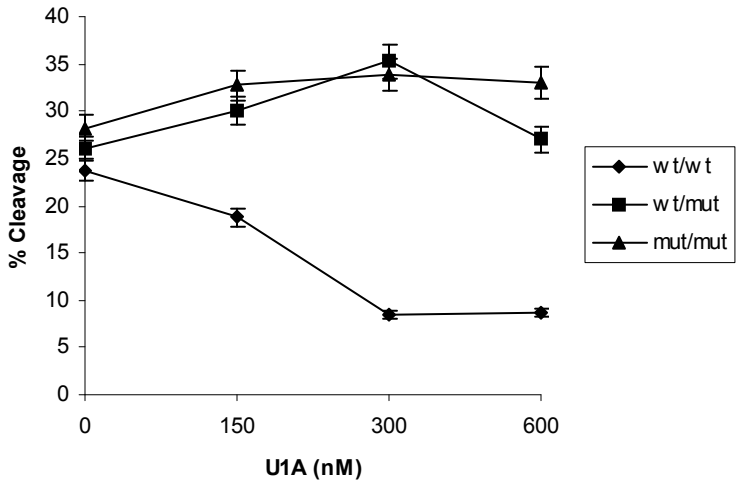
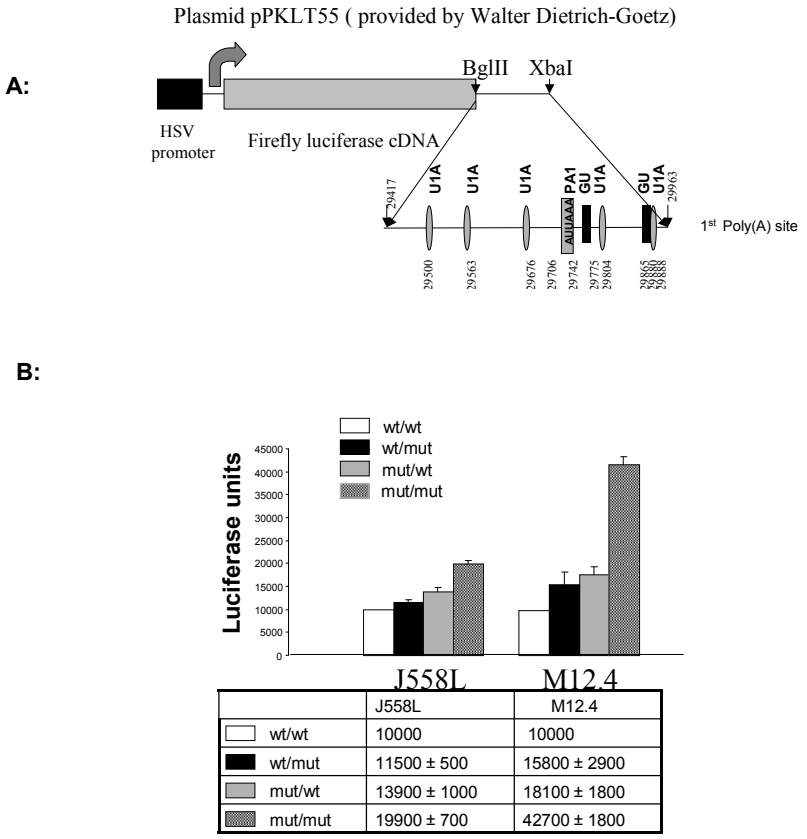


FIGURE 20. U1A inhibits cleavage at the upstream poly(A) site of Zhx-1.

(A) In vitro cleavage assay was performed in HeLa nuclear extracts with 32p-labeled Zhx-1 29417-29963 substrates and increasing concentrations of recombinant U1A. Reaction products were separated by 8% denaturing PAGE. The RNA substrates and U1A amount are indicated above each lane. (Lane 1) MSP marker, (Lane 2) Zhx-1 29417-29963 substrate with hexanucleotide mutated as control, (Lane 3) pre-cleaved Zhx-1 29417-29764 substrate, (Lanes 4, 9 and 14) no extracts were added, (Lanes 5-8, 10-13 and 15-18) 7 µL HeLa nuclear extract (2.5 µg total proteins/ µL were added. (B) Phosphorimager quantitation of A. Results are expressed as percentage of cleavage. Data represent the averages of triplicates from three separate cleavage assays ± SE.

Mutation of the U1A motifs releases U1A inhibition of the usage of Zhx-1 1st poly(A) site

If U1A functions in inhibiting polyadenylation and cleavage through the non-consensus U1A motifs, we expected that mutation of those motifs should release the U1A inhibition of the in vivo expression of Zhx-1. To test this, we used a plasmid pPKLT55 with a HSV promoter as a transfection vector and a dual luciferase reporter assay system. Zhx-1 constructs 29417-29963 with wild type and/or mutated U1A motifs (as used in cleavage assay) were inserted downstream of the firefly luciferase reporter gene in plasmid pPKLT55 to replace the poly(A) site of firefly luciferase (diagrammed in Fig.21A). Constructs were transfected in triplicate into the plasmacytoma J558L and mature M12.4.1 cells along with a reference plasmid expressing renilla luciferase. Cells were harvested 20-24 hours after transfection and luciferase activity was measured using the Promega dual luciferase kit. The firefly luciferase activity for each mutant was corrected for transfection efficiency and expressed as a relative value of the corresponding wild-type construct. Therefore, any release of the inhibition by mutation of U1A binding motifs would be measured as an increase in luciferase activity (Fig. 21B).



	J558L	M12.4
wt/wt	10000	10000
wt/mut	11500 ± 500	15800 ± 2900
mut/wt	13900 ± 1000	18100 ± 1800
mut/mut	19900 ± 700	42700 ± 1800

FIGURE 21. U1A inhibition of the in vivo expression of Zhx-1 1st PA site is developmentally regulated.

(A) Diagram of plasmid construct for luciferase reporter assay. Zhx-1 3' UTR (29417-29963) containing the first poly(A) site and all five U1A motifs was inserted into downstream of firefly luciferase gene under the control of HSV promoter in plasmid pPKLT55. The poly(A) site of firefly luciferase was replaced by Zhx-1 poly(A) site. (B) Constructs were transfected in triplicate into the plasmacytoma J558L and undifferentiated M12.4.1 cells along with a reference plasmid expressing renilla luciferase. Luciferase activity was measured using the Promega dual luciferase kit. w/w: all five U1A motifs are wild type. w/m: the downstream 2 motifs mutated. m/w: the upstream 3 motifs mutated. m/m: all five motifs mutated. w/w values in J558L and M12.4.1 are artificially set as 10000.

We denoted letter wt as wild type and letter mut as mutant. For example, wt/wt means that both the upstream 3 U1A motifs and the downstream 2 U1A motifs are wild type. For convenient comparison, we artificially set firefly luciferase value in wt/wt as 10000 for both J558L and M12.4. In fact, the absolute luciferase values in J558L were always higher (~2 fold) than those in M12.4.1, which is consistent with our RPA result for the expression of that upstream poly(A) site (Fig. 13B, lanes 8-10 and 11-13). Mutation of the downstream motifs resulted in a slight increase in luciferase activity (15 ± 5%) in J558L and relatively greater increase (58 ± 29%) in M12.4.1 and mutation of the upstream motifs caused 39 ± 10% and 81 ± 18% increase in luciferase activity in J558L and M12.4.1 respectively. When all 5 motifs were mutated, a two fold and 4-fold increase in luciferase activity were observed in J558L and M12.4.1 respectively.

Therefore, we can conclude both the upstream and the downstream U1A motifs contribute to the inhibitory effect in vivo and mutations released the inhibition. In addition, we observed that there is always a larger increase in luciferase activity value in M12.4.1 for all the mutations (Fig.21B, cf. J558L column and M12.4.1 column). This is consistent with our data that mature B cells have a relatively higher level of total U1A and non-snRNP U1A (Ma et al., 2006).

The expression of the 2nd poly(A) site of Zhx-1 is affected by the inclusion of ARE elements and U1A has a minor effect on its expression

Given that the two poly(A) sites of Zhx-1 are only 300nt away from each other and the two U1A motifs between them are just 150-250nt upstream of the 2nd poly(A) site (one 156nt and another 240nt away), it is possible that these two U1A motifs also

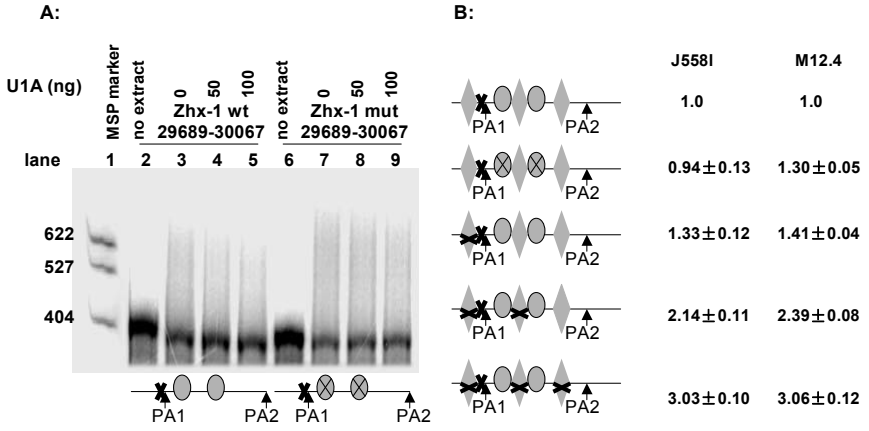


FIGURE 22. The expression of the 2nd poly(A) site is affected by the inclusion of ARE elements and U1A has a minor effect on its expression.

(A) In vitro specific poly(A) assay in Hela nuclear extracts with radiolabelled Zhx-1 29689-30067 substrates (ΔPA1) in which the upstream poly(A) site was mutated. wt: two intact U1A motifs, mut: two mutated U1A motifs. (B) Luciferase assay. Zhx-1 3' UTR 29689-30128 (ΔPA1) with wild type or double mutated U1A motifs or with single, double or triple mutated ARE motifs were inserted into downstream of firefly luciferase gene under the control of HSV promoter in plasmid pPKLT55. The poly(A) site of firefly luciferase was replaced by Zhx-1 poly(A) site. The constructed plasmids were transfected in triplicate into J558L and M12.4.1 along with a reference plasmid expressing renilla luciferase. Cells were harvested 20-24 hours after transfection and luciferase activity was measured using the Promega dual luciferase kit as previously. The luciferase values for wild types were set as 1.0 both in J558L and M12.4.1.

regulate the polyadenylation of the downstream (2^{nd}) poly(A) site. To investigate this possibility, we employed in vitro specific poly(A) assay to analyze the inhibitory effect of recombinant U1A on the Zhx-1 substrates 29689-30067 (ΔPA1) which has the upstream poly(A) site mutated. We found that U1A is capable of inhibiting the poly(A) addition of the downstream poly(A) site (Fig.22A, lanes 2-5). Mutation of the two U1A motifs almost abolished the inhibition (Fig.22A, lanes 6-9). To confirm the poly(A) assay result and to analyze if the three ARE elements (diagrammed in Fig.16) upstream of the second poly(A) site affect the expression of that poly(A) site, we performed dual luciferase assay as before. Zhx-1 3' UTR 29689-30128 (ΔPA1) with wild type or double mutated U1A motifs or with single, double or triple mutated ARE motifs (AUUUA → AUGGA) were inserted into the downstream of firefly luciferase gene in pPKLT55 to replace the poly(A) site of luciferase gene. The plasmids were transfected in triplicate into J558L and M12.4.1 and luciferase activities were measured using promega dual luciferase kit (Fig.22B). The luciferase values for wild type were set to 1.0 both in J558L and M12.4.1. Mutation of the two downstream U1A motifs caused a 30% increase in luciferase activity in M12.4.1 (1.30±0.05) but almost no effect in J558L (0.94±0.13). Previously we had shown by luciferase assay that for the first poly(A) site, mutation of the upstream 3 U1A motifs alone resulted in an 81% and 39% increase in M12.4.1 and J558L respectively and mutation of all 5 U1A motifs released the inhibition up to 4 fold and 2 fold in M12.4.1 and J558L respectively. Therefore, U1A inhibits the expression of the first poly(A) site much more than it does to the downstream one.

ARE mutation analysis revealed that single, double, triple mutation increases luciferase activity by 33%, 114%, 203% in J558L and 41%, 139%, 206% in M12.4.1 respectively (Fig.22B). Therefore, we think that the expression of the 2nd poly(A) site is affected mainly by ARE elements. There is no significant difference in ARE regulation between differentiated B cells and mature B cells.

To further confirm that the ARE elements are functional, we inserted the Zhx-1 3' UTR (29465-30215) containing both poly(A) sites into the renilla luciferase plasmid pRLSV-40, thereby replacing the 3' UTR and poly(A) site of the renilla gene (diagrammed in Fig. 23A). Constructs were transfected in triplicate to M12.4 cell lines and harvested. Total RNA was extracted and RNase protection assay was performed with 200,000 cpm/reaction anti-sense Zhx-1 probe 30215-29356 which can detect both poly(A) sites and distinguish the usage of the endogenous and the exogenous poly(A) sites. We kept the 1st poly (A) site intact and compared the expression level of the 2nd poly(A) site when the two AUUUA motifs between the two poly(A) sites are intact (denoted as letter W) or mutated (denoted as letter M). Equal amount (15 μg) of total RNA were used. Unfortunately we could not detect any expression of the exogenous 2nd one for both cases and only the expression of the exogenous 1st one was observed (Fig. 23B), as demonstrated the SV40 promoter is even still not strong enough here. Therefore, we mutated the 1st poly(A) site AUUAAA to AGGAAA, constructed the plasmid containing Zhx-1 3' UTR 29465-30215 ΔPA1 and transfected it into M12.4.1 cell lines, then compared the expression level of the 2nd poly(A) site when the two AUUUA motifs between the two poly(A) sites are intact (denoted as letter W) or mutated (denoted as letter M). Here we observed that mutation of the AUUAAA to AGGAAA completely

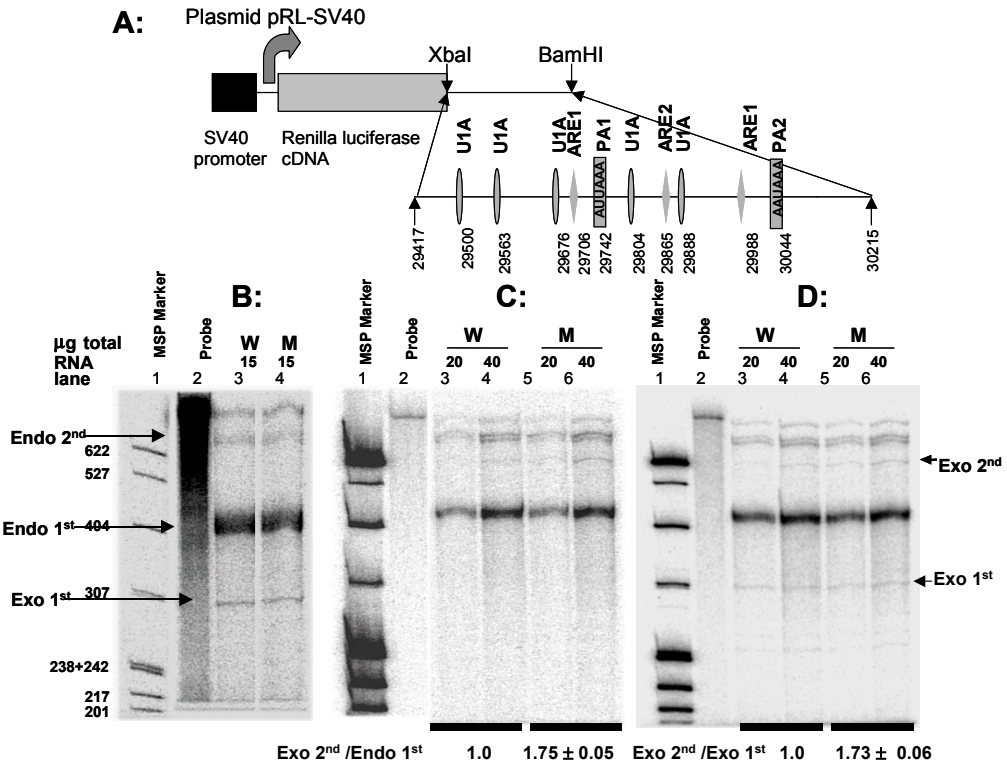


FIGURE 23. ARE elements affect the expression of Zhx-1 when the 2nd poly(A) site is chosen.

(A) Diagram of plasmid construct used in RNase protection assay for analyzing the effect of ARE elements to the expression of Zhx-1 2nd poly(A) site. (B), (C) and (D) RPA assays using total RNAs from M12.4.1 cell lines. 200,000 cpm/reaction of anti-sense Zhx-1 probe (30215-29356) was used to detect both poly(A) sites and distinguish the usage of the endogenous and the exogenous poly(A) sites. (B) Cells transfected with plasmid pRLSV-40 carrying Zhx-1 3' UTR (29465-30215) with two intact poly(A) sites. (C) Cells transfected with plasmid pRLSV-40 carrying Zhx-1 3' UTR (29465-30215) with the intact 2nd poly(A) site. (D) Cells transfected with two plasmids pRLSV-40. One carries Zhx-1 3' UTR with two intact poly(A) sites (internal control), and one carries Zhx-1 3' UTR with the intact 2nd poly(A) site. The ratio of exo 2nd/endo 1st and exo 2nd/exo 1st were calculated and shown below the lanes of respective samples. Triplicates ± SD.

abolished the usage of that poly(A) site while mutation of that two AUUUA motifs increased the expression of 2nd poly(A) site nearly 75% (The value of exo 2nd /endo 1st =1.75 ±0.05, if W was set as 1.0) (Fig. 23C, cf. lanes 3 and 5, lanes 4 and 6). When we mutated all three AUUUA motifs, we observed two fold increase measured by the abovementioned luciferase assays. For correcting any difference in transfection efficiency, we cotransfected the constructed plasmid pRLSV-40 having Zhx-1 3' UTR 29465-30215 with the intact 1st poly(A) site as an internal control and observed the similar result ((The value of exo 2nd /exo 1st =1.73 ±0.06, if W was set as 1.0) (Fig. 23D, cf. lanes 3 and 5, lanes 4 and 6).

Our RPA result further confirmed that mutation of the U1A motifs releases U1A inhibition of the usage of Zhx-1 1st poly(A) site

The plasmid pRLSV-40 with a stronger promoter enabled us to observe the exogenous expression of Zhx-1 1st poly(A) site (Fig. 23B and D) and mutation of the 1st poly(A) enables us to analyze the exogenous expression of the downstream (2nd) poly(A) site. Therefore, this allowed us to analyze the effect of U1A motifs on the in vivo expression of Zhx-1 1st poly(A) by RNA protection assay. Plasmid pRLSV-40 containing Zhx-1 3' UTR (29465-30215, 2 intact poly(A) sites) with 5 intact or mutated U1A motifs was constructed and transfected into M12.4.1 cells as before. RPA assay was performed with 200,000 cpm/reaction anti-sense Zhx-1 probe 30215-29356 using total RNAs from transfected M12.4.1 as previously described. We investigated the effect of mutating all 5 U1A motifs on activity of the 1st poly(A) site and found that the mutation caused a 3 fold (3.2 ± 0.2) increase in the exogenous expression of the 1st poly(A) site (Fig. 24, cf. lanes 3 and 4), consistent with the luciferase assay in M12.4.1.

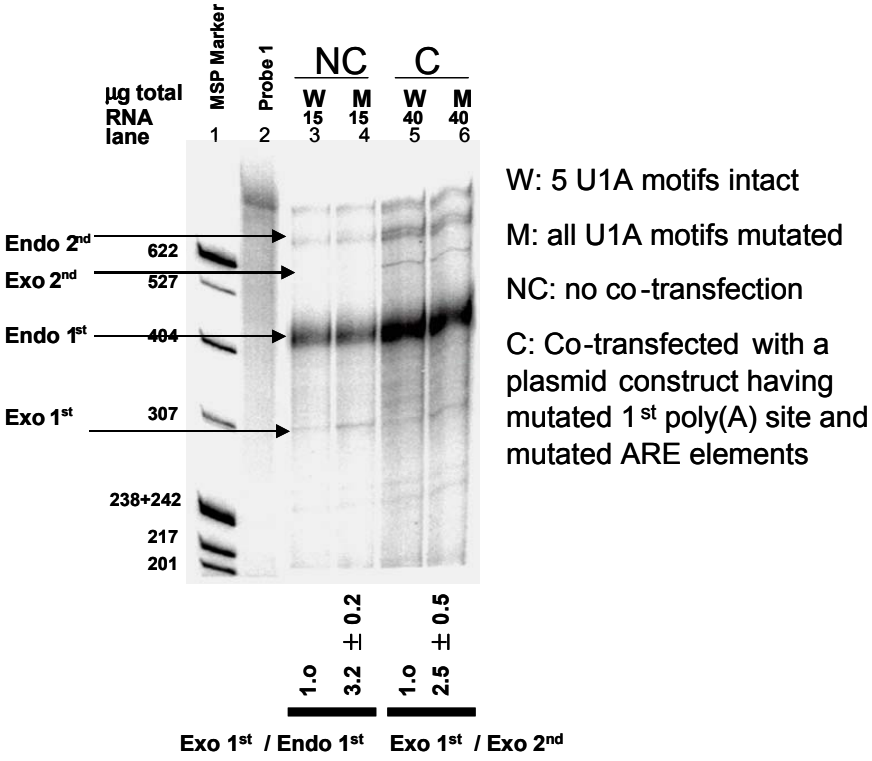


FIGURE 24. Mutation of the U1A motifs releases U1A inhibition of the usage of Zhx-1 1st poly(A) site.

Plasmid construct used in RNase protection assay for analyzing the effect of U1A motifs to the expression of Zhx-1 1st poly (A) site is similar to that in Fig. 16(A). The 5 U1A motifs instead of the two ARE element are either mutated (Lanes 4, 6) or kept intact (Lanes 3, 5). 200,000 cpm/reaction of anti-sense Zhx-1 probe (30215-29356) was used to detect both poly(A) sites and distinguish the usage of the endogenous and the exogenous poly(A) sites. (Lane 3 and 4) total RNAs from cells transfected with plasmid pRLSV-40 carrying Zhx-1 3' UTR (29465-30215) with two intact poly(A) sites. (Lane 5 and 6) total RNAs from cells transfected with two plasmids pRLSV-40. One carries Zhx-1 3' UTR with two intact poly(A) sites and one carries Zhx-1 3' UTR with the mutated 1st poly(A) site and the two mutated ARE elements (internal control). The ratio of exo 1st /endo 1st and exo 1st /exo 2nd were calculated and shown below the lanes of respective samples. Triplicates ± SD.

To correct for any differences in transfection efficiency, we cotransfected into M12.4.1 cells the plasmid with Zhx-1 3' UTR 29465-30215 having the mutated 1st poly(A) site and the two mutated ARE elements and observed a similar result (2.5 ± 0.5) (Fig. 24, cf. lane 5 to lane 6). Thus, the RPA data is consistent with the previous luciferase assay data (Fig. 21).

Discussion

In this chapter we have identified that mouse Zhx-1, a transcriptional repressor, is regulated by U1A protein during B cell differentiation, as supported by several lines of evidence. First, a microarray analysis of mRNAs bound to U1A revealed that Zhx-1 mRNA was pulled down by U1A in vivo (Table 1 and 2), as was confirmed by RT-PCR with specific exon-exon junction primers (Fig. 11B). Second, we found that the upstream poly(A) site of Zhx-1 was more highly expressed in Ig-secreting cells than mature cells (Fig. 13B) as well as overall Zhx-1 mRNA (Fig. 12 and 13B) and protein level (Fig. 14), consistent with its inhibition by U1A in mature B cells. Third, overexpression of U1A in HeLa cells greatly reduced the expression of Zhx-1 protein (Fig. 15). Fourth, recombinant U1A inhibited both the poly(A) addition (Fig. 17C and Fig. 18) and the cleavage (Fig. 20) of the upstream Zhx-1 poly(A) site in vitro and the inhibition was lost when the U1A motifs were mutated. Fifth, transfection assays demonstrated that mutation of the U1A motifs (both upstream and downstream of the upstream poly(A) site) released the inhibition of U1A resulting in a significant increased luciferase activity (Fig.21) and increased protected band intensities in RPA assays (Fig. 24).

U1A as a trans-factor regulates the production of Zhx-1 mainly by inhibiting the usage of Zhx-1 1st poly(A) site

Both mouse and human Zhx-1 genes have two reported poly(A) sites and produce two corresponding transcripts, however, U1A motifs are only located around the first reported poly(A) site (Fig. 10). Although both human and mouse Zhx-1 have one putative poly(A) site located upstream of the two reported ones, we have demonstrated that the putative poly(A) site is not expressed at all in mouse B cell lines (Fig. 13B). The frequency of the usage of these two poly(A) sites is determined by the position, the relative strength of each poly(A) signal and a series of transacting factors. Our RPA data has shown that PA1 is predominatly expressed both in mouse differentiated and mature B cells (Fig. 13B), which is the only poly(A) site documented in Ensemble. Interestingly, we found that in Ensemble, the RNA transcript (ENSMUST00000070143) arising from the usage of this poly(A) site lacks exon 3. There is no evidence, however, for a correlation between the poly(A) site usage and the inclusion/ exclusion of exon 3 (splicing). Northern blot analysis of the expression pattern of Zhx-1 in different mouse tissues revealed that a major band of 4.5 kb was detected in brain, lung, spleen and testis (Barthelemy et al., 1996). This 4.5 kb RNA transcript may arise from the usage of the upstream poly(A) site, but this has yet to be firmly elucidated.

We demonstrated that U1A levels and Zhx-1 levels are inversely correlated, i.e when U1A protein levels are higher (in mature B cells such as M12.4.1), Zhx-1 mRNA and protein levels are lower; when U1A is lower (in differentiated B cells such as J558L), Zhx-1 mRNA and protein levels are higher (Fig. 12, 13B and 14). However, the direct evidence that U1A regulates the production of Zhx-1 is from our overexpression experiment. The stable overexpression of Flag tagged U1A in HeLa cells greatly reduced the production of the endogenous U1A (Fig. 15B), which demonstrated that the Flag

U1A is highly active and can bind to the PIE element in the 3' UTR of the endogenous U1A. Recently, the Martha Peterson lab (Peterson et al 2006) claimed that U1A has no effect on processing of the IgM secretory poly(A) signal in an intact IgM gene, which is in conflict with our previous findings (Phillips et al., 2001 and 2004; Ma et al., 2006). Peterson obtained from Dr. Carol Lutz HeLa cells over-expressing TAP-tagged U1A and HeLa cells stably expressing the empty TAP-tagged vector. Their TAP tag is located in N-terminal and therefore may interfere the RNA–binding function of N-terminal RRM of U1A. Previous work from Dr. Gunderson (Gunderson et al 1997) has shown that N-terminal epitope tagging (Flag tag) of U1A protein inhibits the ability of the N-terminal RRM to bind to RNA thereby resulting in an inactive U1A protein.

Given that Dr. Lutz's TAP-tag was placed also at the N-terminus it was possible that this resulted in an inactive protein and so would explain why the Peterson lab saw no effect with their TAP-tagged U1A. Indeed, inspection of Figure 1B in Dr. Lutz's publication (Liang and Lutz, 2006) indicates that the TAP-tagged U1A had no effect on the levels of endogenous U1A suggesting the TAP-tagged U1A was inactive for autoregulation of endogenous U1A. To investigate this further, we obtained Dr. Lutz's stable cell lines that express the TAP-tagged U1A and the empty TAP-tagged vector as a control and did a series of experiments to compare them with our HeLa cell lines overexpressing C-terminal Flag-tagged U1A.

We repeated the western blots and confirmed that TAP-U1A does not downregulate endogenous U1A whereas our Dox-regulatable Flag-U1A does downregulate endogenous U1A (data not shown). We then did a series of transfections to measure the inhibitory activity of U1A in these cell lines by comparing expression of a

Renilla reporter plasmid having a wild type PIE (RL-wtPIE) reporter versus a matching RL-mtPIE reporter that has a mutated PIE (see Figure 25). For a given cell line grown either with Doxycline or not, we define the "Inhibitory Index" to be the ratio of expression of the RL-mtPIE to that of RL-wtPIE reporter. For regular HeLa Tet cells (having no stably expressed U1A protein) the inhibitory index is 3.3 and that value is not significantly affected by addition of doxycline. For HeLa Tet cells stably expressing high levels of wild type Flag-U1A, the inhibitory index is elevated to 5.7. This level is reduced to 3.0 when doxycycline is added that reduces the levels of wild type Flag-U1A to below detection. In contrast to these data, the Dr. Lutz cell lines that stably express TAP-U1A have an inhibitory index of 4.0 that is the same as the matching cell line that expresses the empty TAP vector. These data clearly demonstrate that the TAP-tagged U1A is inactive for autoregulation and polyadenylation inhibition and therefore they explain why Dr. Peterson was unable to observe inhibition of IgM expression. Simply put, Dr. Peterson was trying to inhibit IgM expression with a "dead" U1A protein that had been inactivated by having a TAP tag. Therefore the conclusions in her 2006 publication are incorrect.

U1A regulates the upstream poly(A) site of Zhx-1 in a similar manner as it does to the secretory poly(A) site of IgM. They both have five non-consensus U1A motifs (3 upstream and 2 downstream), which allows a fine scale regulation instead of simply switching on or off expression. However, the cleavage and the poly(A) addition reaction of IgM secretory poly(A) site is in direct competition with a splicing reaction (Peterson, 1992; Peterson and Perry, 1989), i.e the membrane and secretory poly(A) sites in IgM are mutually exclusive. In contrast, for Zhx-1, no reported alternative splicing reaction exists

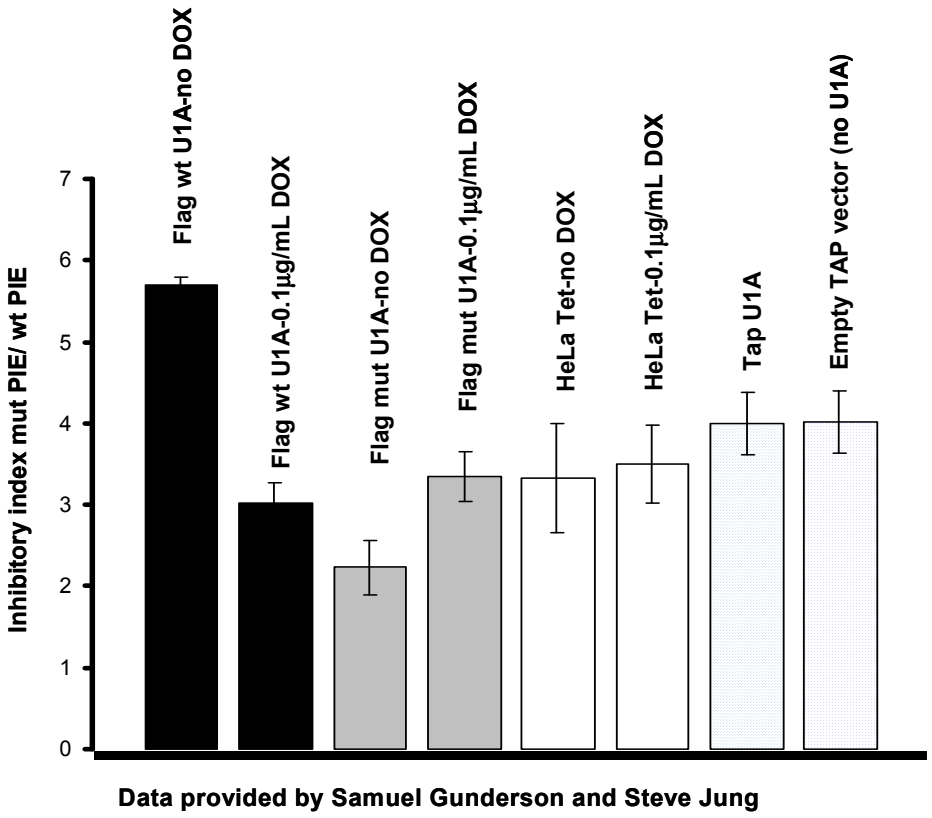


FIGURE 25. C-terminal tagged Flag does not affect the function of U1A whereas N-terminal tagged TAP does.

HeLa Tet-off gene expression system for overexpression of wild type (wt) or mutant (mut) C-terminal Flag tagged U1A protein was used as in Fig.15. HeLa cell lines constitutely overexpressing N-terminal TAP tagged U1A and HeLa cell lines carrying empty TAP vector are from Dr. Lutz. Each of those HeLa cell lines was transiently transfected with a renilla luciferase plasmid pRLSV-40 carrying wild type PIE or mutant PIE and a firefly luciferase plasmid (as an internal control). The relative luciferase value for each transfected cell line was measured as the ratio of renilla to firefly. U1A inhibitory index is calculated by normalizing the cell line with mut PIE to the matching cell line with wt PIE.

for the last exon and even if the upstream poly(A) site is not used, it is still in the terminal exon.

ARE elements enhance the inhibition effect of U1A on Zhx-1

The first poly(A) site is a predominant one and the second poly(A) site is a minor one in both the differentiated and mature B cell lines (Fig. 13B). Therefore, when the first poly(A) site is inhibited in mature B cells (M12.4.1), it is most likely that the RNA transcript will be cleaved and polyadenylated at the second poly(A) site. As a result, we are supposed to observe the increased usage of the 2nd poly(A) site in M12.4.1, compared to that in J558L. In fact, that is not the case. Two reasons may account for that. First, this poly(A) site is also inhibited by U1A through its binding to the two identified U1A motifs (Fig.22A) although the inhibition effect of U1A to this poly(A) site is relatively much weaker in M12.4.1 (Fig. 22B). Second, the usage of this second poly(A) site produces an mRNA with three AUUUA motifs that are the critical sequence feature of classic AU-rich RNA-destabilizing elements (ARE) which play a key role in regulation of gene expression during cell growth and differentiation (reviewed in Chen and Shyu, 1995). AREs range in size from 50 -150 nucleotides and generally contain multiple copies of the pentanucleotide AUUUA and are typically located in the 3‘ UTR of many highly labile mammalian mRNAs (Peng et al., 1996). However, it is also reported that not all AUUUA motifs are functional and the presence of AUUUA motif(s), even in an AU-rich region, does not guarantee a destabilizing function (Zubiaga et al., 1995; Lagnago et al., 1994; Chen and Shyu, 1994). By using luciferase assays and RNA protection assays, we have identified that those three AUUUA motifs in Zhx-1 3‘ UTR are functional most likely through regulating mRNA

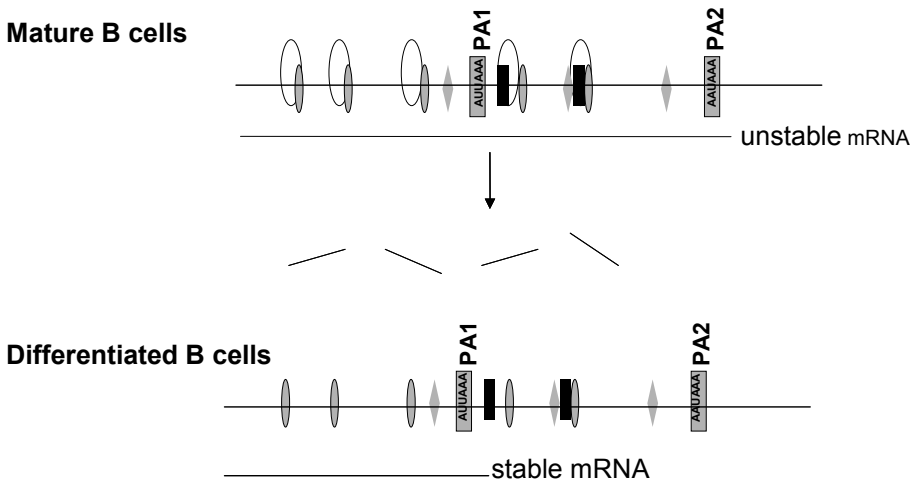


FIGURE 26. Diagram of one model about how U1A and ARE elements coordinately regulate the expression of mouse Zhx-1

In mature B cells (M12.4.1), U1A binds to the motifs around the 1st poly(A) site of Zhx-1 and inhibits the polyadenylation and cleavage of this poly(A) site. As a result, the downstream poly(A) site is used and a longer transcript with three ARE elements produced. This transcript is unstable and fast degraded. While in differentiated B cells (J558L), U1A level decreases and releases the inhibition to the 1st poly(A) site of Zhx-1. Therefore, a shorter and more stable transcript is produced. As a result, The Zhx-1 mRNA level are up-regulated.

stablity as they do in other cytokine genes.

Base on the findings we have in this chapter, we proposed one model (Fig. 26) that when U1A inhibits the upstream poly(A) site in mature B cells, the use of the downstream poly(A) site results in inclusion of the AREs in the final mRNA and its degradation. As U1A levels decrease upon B cell differentiation, activation of the upstream poly(A) site excludes the AREs and the mRNA is stablized thus raising Zhx-1 mRNA levels in Ig-secreting cells.

To sum up this chapter, we demonstrated U1A regulates Zhx-1 in a manner similar to what it does to IgM seceretory mRNA. This is the first time to identify that U1A can regulate one gene other than itself and the IgM gene. Since Zhx-1 is a ubiquitous transcriptional repressor, it might provide a scaffold or pathway for U1A to function during B cell differentiation. In addition, the finding of the new U1A target greatly raises the possibility that other unknown gene candidates might be discovered in the near future. The job to further screen the other possible genes such as Zf207, SLBP, YY1 (Table 2) is will be the focus of future work.

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