Title: METHODS AND COMPOSITIONS FOR REGULATION OF GENE EXPRESSION THROUGH MODULATION OF mRNA TURNOVER

Abstract: The present invention provides means to modulate gene expression by destabilizing or stabilizing mRNA species. The invention provides compositions and methods for altering the level of RNA, and for screening test agents that alter RNA levels. The invention’s compositions and methods are useful in, for example, increasing the level of an mRNA and conversely in decreasing the level of mRNA by impacting the level of degradation of the mRNA.
METHODS AND COMPOSITIONS FOR REGULATION OF GENE EXPRESSION THROUGH MODULATION OF mRNA TURNOVER

This application claims priority to U.S. Provisional Application Serial No. 60/334,712 to Michael Karin et al., filed November 15, 2001, the contents of which are incorporated herein in their entirety.

FIELD OF THE INVENTION

The present invention provides means to modulate gene expression by destabilizing or stabilizing mRNA species. In particular, the invention relates to altering the level of an mRNA by impacting the level of degradation of the mRNA.

BACKGROUND OF THE INVENTION

A multitude of diseases such as immune disease (e.g., allergic disease, and autoimmune disease), inflammatory disease, cancer, hormone deficiency diseases (e.g., Turner syndrome, dystrophy, and osteoporosis) involve the presence of inappropriately elevated or depleted levels of cytokines, proto-oncogenes and growth factors. Gene expression is controlled at the transcriptional and post-transcriptional levels. An important post-transcriptional control is exerted on mRNA stability, which varies considerably from one mRNA species to another and can be modulated by extracellular stimuli [Ross (1995) Microbiol. Rev. 59:423-450; Caponigro et al. (1996) Microbiol. Rev. 60:233-249; and Wilusz et al. (2001) Nat. Rev. Mol. Cell Biol. 2:237-246]. The rate of mRNA decay is determined by cis-acting elements within the mRNA molecule, which are recognized by trans-acting factors that act through ill-defined mechanisms. The most common cis element responsible for rapid mRNA decay in mammalian cells is the AU-rich element. Indeed, inherently unstable mammalian mRNAs contain AU-rich elements (AREs) within their 3′ untranslated regions (UTRs). In particular, these elements are found within the UTRs of short-lived cytokine, proto-oncogene and growth factor mRNAs [Shaw et al. (1986) Cell
46:659-667; Chen et al. (2000) Genes Dev. 14:1236-1248; and Bakheet et al. (2001) Nucleic Acids Res. 29:246-254]. Although these elements were discovered approximately 15 years ago, the means by which AREs dictate rapid mRNA has not been clear. In yeast, 3' to 5' mRNA degradation is mediated by the exosome, a multisubunit particle.


Thus, there is a need to identify compositions that are involved in the stabilization and/or destabilization of the degradation of RNA. In particular, there is a need for compositions and methods for altering the level of RNA, and for screening test agents that alter RNA levels.

SUMMARY OF THE INVENTION

The invention provides compositions and methods for altering the level of RNA, and for screening test agents that alter RNA levels. The invention's compositions and methods are useful in, for example, increasing the level of an mRNA
and conversely in decreasing the level of mRNA by impacting the level of degradation of the mRNA. Thus, the present invention provides means to modulate gene expression by destabilizing or stabilizing-mRNA species.

In particular, the present invention provides the human exosome. As indicated herein, the human exosome was purified and characterized using mass spectrometry. Also as indicated herein, the composition of the human exosome is similar to its yeast counterpart. During the development of the present invention and described in greater detail herein, a cell-free RNA decay system was used to determine that the mammalian exosome is required for rapid degradation of ARE-containing RNAs, but not for poly(A) shortening. The mammalian exosome does not recognize ARE-containing RNAs on its own. ARE recognition requires certain ARE-binding proteins that can interact with the exosome and recruit it to unstable RNAs, thereby promoting their rapid degradation.

Thus, in one embodiment, the invention provides a purified mammalian exosome. In a preferred embodiment, the mammalian exosome comprises at least one of GIVILMVDEK (SEQ ID NO:24) and LTEQLAGPLR (SEQ ID NO:25). In an alternative embodiment, the mammalian exosome further comprises YIGEVGDIVVGR (SEQ ID NO:2), LDSVLLLSSMNLPGGELR (SEQ ID NO:3), NCIISLVTQR (SEQ ID NO:4), LSVENVPCIVTLCK (SEQ ID NO:5), GGDDLGETEIANTLYR (SEQ ID NO:6), GSDLDPESIFEMMETGKR (SEQ ID NO:7), TTTVNIGSISTADGSLVK (SEQ ID NO:8), AEFAAPSTDAPDK (SEQ ID NO:9), MAEPASVAAESLAGSR (SEQ ID NO:10), GDHVIGIVTAK (SEQ ID NO:11), VVCGBPGLR (SEQ ID NO:12), GGGPAGAGGEAPAALR (SEQ ID NO:13), ELALALQEALEPAVR (SEQ ID NO:14), LYPVLQQSLVR (SEQ ID NO:15), VLCAVSGPR (SEQ ID NO:16), QTFDEAIATQHLPR (SEQ ID NO:17), MAGLELLSDQGYR (SEQ ID NO:18), ATLEVILRPK (SEQ ID NO:19), AVLTFALDSVER (SEQ ID NO:20), VHLITYGSMPLK (SEQ ID NO:21), SSGNALPVPVSVVR (SEQ ID NO:22), and HGYIFSSLAGCLMK (SEQ ID NO:23). More preferably, the mammalian exosome further comprising ISEEHWYDLPELK (SEQ ID NO:26) and YETLVGTIGK (SEQ ID
ID NO:27). In an alternative embodiment, the purified mammalian exosome is a human exosome.

Also provided herein is a mammalian exosome purified by the process of: a) providing: i) a host mammalian cell; ii) an expression vector comprising a reporter gene operably linked to a nucleic acid sequence encoding a hRrp4p polypeptide; b) transforming the host mammalian cell with the vector to produce a transformed cell expressing a protein the comprises hRrp4p polypeptide operably linked to a reporter polypeptide; and c) purifying the protein from the transformed cell, wherein the purified protein comprises a purified mammalian exosome. In one preferred embodiment, the host mammalian cell is a Jurkat cell. In another embodiment, the purifying comprises isolating the protein from the cytoplasm of the transformed cell or from the nucleus of the transformed cell. Alternatively, the purifying comprises contacting the protein from the transformed cell with an agent that specifically binds to the reporter polypeptide, and purifying the agent that is bound to the reporter polypeptide.

The invention further provides an exosome purified from a transgenic cell line designated JurkatRrp4-TAP.

Also provided by the invention is a transgenic cell line designated JurkatRrp4-TAP.

Also provided herein is a method for purifying a mammalian exosome in a sample, comprising: a) providing: i) an AUBP capable of specifically binding to a mammalian exosome; and ii) a sample suspected of comprising a mammalian exosome; b) contacting said AUBP and said sample such that said AUBP binds to said mammalian exosome; and c) purifying the AUBP that is bound to said mammalian exosome, thereby purifying said mammalian exosome.

In yet another embodiment, the invention provides a method for purifying an RNA sequence comprising an adenylate uridylate-rich element (ARE), comprising: a) providing: i) a purified mammalian exosome; ii) an AUBP capable of specifically binding to said mammalian exosome; and iii) a sample suspected of comprising said RNA sequence; b) contacting said exosome, said AUBP, and said sample such that
said AUBP binds to said mammalian exosome, and said mammalian exosome binds to said RNA sequence; and c) purifying the AUBP that is bound to said mammalian exosome bound to said RNA sequence, thereby purifying said RNA sequence.

The invention additionally provides a method for altering the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising an adenylate uridylate-rich element (ARE); ii) an AUBP capable of specifically binding to the ARE; and ii) an agent that alters the level of binding of the ARE to the AUBP; and b) contacting the RNA, the AUBP, and the agent such that the level of binding of the ARE to the AUBP is altered, and the level of the RNA is altered.

In another embodiment, the invention provides method for reducing the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising an adenylate uridylate-rich element (ARE); ii) an AUBP capable of specifically binding to the ARE; and ii) an agent that increases the level of binding of the ARE to the AUBP; and b) contacting the RNA, the AUBP, and the agent such that the level of binding of the ARE to the AUBP is increased, and the level of the RNA is reduced.

In a further embodiment, the invention provides a method for increasing the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising an adenylate uridylate-rich element (ARE); ii) an AUBP capable of specifically binding to the ARE, wherein the AUBP is not AUFI; and ii) an agent that reduces the level of binding of the ARE to the AUBP; and b) contacting the RNA, the AUBP, and the agent such that the level of binding of the ARE to the AUBP is reduced, and the level of the RNA is increased.

In a preferred embodiment, the RNA is not β-adrenergic receptor mRNA.

With respect to any of the methods disclosed herein, the sample may be in vitro or in vivo in a subject. In a preferred embodiment the subject is human or a mouse. In another embodiment, the subject is capable of developing a pathological condition associated with altered expression of the RNA in a tissue. Alternatively, subject is suffering from a pathological condition. In one embodiment, the contacting is before,
concomitant with, or after manifestation of one or more symptoms of the pathological condition. In a preferred embodiment, the pathological condition is an immune disease. In one embodiment, the immune disease is an allergic disease selected from pollinosis and atopic dermatitis. In an alternative embodiment, the immune disease is an autoimmune disease selected from Sjogren’s disease, type I diabetes, insulin dependent diabetes mellitus, scleroderma, polymyositis, chronic active hepatitis, mixed connective tissue disease, primary biliary cirrhosis, pernicious anemia, autoimmune hemolytic anemia, autoimmune thyroiditis, idiopathic Addison’s disease, vitiligo, gluten-sensitive enteropathy, Grave’s disease, myasthenia gravis, neutropenia, idiopathic thrombocytopenia purpura, pemphigus vulgaris, autoimmune infertility, Goodpasture’s disease, bullous pemphigoid, discoid lupus, systemic lupus erythematosus, dense deposit disease, Hashimoto’s disease, fibromyalgia, arthritis selected from rheumatoid arthritis, gouty arthritis, and juvenile rheumatoid arthritis, an autoimmune disease of blood vessels selected from necrotizing angitis, and granulomatous angitis, or an autoimmune disease of kidney selected from nephritis, glomerulonephritis, and systemic lupus erythematosus. In an alternative embodiment, the pathological condition is inflammatory disease, and preferably, the inflammatory disease is sepsis, septic shock, endotoxic shock, inflammatory bowel disease such as Crohn’s disease and ulcerative colitis, multiple sclerosis, inflammatory diseases involving acute or chronic inflammation of bone and/or cartilage in a joint, anaphylactic reaction, nephritis, asthma, conjunctivitis, inflammatory gum disease, systemic lupus erythematosus, insulin dependent diabetes mellitus, pulmonary sarcoidosis, ocular inflammation, allergy, emphysema, ischemia-reperfusion injury, fibromyalgia, an inflammatory cutaneous disease selected from psoriasis and dermatitis, or an arthritis selected from rheumatoid arthritis, gouty arthritis, juvenile rheumatoid arthritis and osteoarthritis. In another alternative, the pathological condition is cancer, and more preferably, the cancer is in a tissue selected from the trachea, esophagus, colon, epidermis, anus, rectum, lymph node, spleen, lung, breast, prostate, bladder, uterus, mouth, throat, pancreas, kidney, stomach, ovary, brain, liver, larynx, thyroid, testis, and cervix. In a further alternative, the pathological condition is
hormone deficiency, more preferably the pathological condition is Turner Syndrome, infertility, dystrophy, osteoporosis, lactation failure, growth hormone deficiency in adults or in children, short stature in children, and thyroid hormone deficiency.

In one embodiment, the AUBP is K homology-type splicing regulatory protein (KSRP), tristetraprolin (TTP), AUF1, butyrate response factor-1 (BRF1), HuR, or HuB. In another embodiment, the RNA of interest is encoded by a gene that encodes a cytokine, proto-oncogene or growth factor. In a preferred embodiment, the RNA of interest is encoded by a gene that encodes a cytokine, proto-oncogene or growth factor. More preferably, the cytokine is a tumor necrosis factor (TNF), an interferon (IF), a colony stimulating factor (CSF), or an interleukin (IL). Yet more preferably, the tumor necrosis factor is TNFα; the interferon is IF-α, IF-β, or IF-γ; the a colony stimulating factor is granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), or macrophage colony stimulating factor (M-CSF); the interleukin is IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, or IL-15; and the proto-oncogene is gsp, gip2, fos or pituitary tumor-transforming gene, AKT-2, c-erbB-2, mdm-2, c-myc, c-myb, c-ras, c-src, c-yes, or HER-2/neu. In another embodiment, the RNA sequence of interest is encoded by a gene that encodes neutrophil activating protein (NAP), macrophage chemoattractant and activating factor (MCAF), RANTES, macrophage inflammatory peptide-1a (MIP-1a), macrophage inflammatory peptide-1b (MIP-1b), or an accessory molecule, such as B7.1, B7.2, ICAM-1, 2 or 3 and cytokine receptors. OX40 and OX40-ligand (gp34), lymphotactin, CD40, CD40L; or complement components and their receptors.

In another embodiment, the agent is an anti-AUBP antibody, anti-mammalian exosome antibody, an AUBP antisense sequence, mammalian exosome antisense sequence, AUBP ribozyme, or mammalian exosome ribozyme.

The invention further provides a method for altering the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising an ARE; ii) an AUBP capable of specifically binding to the ARE; iii) a purified mammalian exosome capable of specifically binding to the
AUBP; and iv) an agent that alters the level of binding of the exosome to the AUBP; and b) contacting the RNA, the AUBP, the exosome and the agent such that the level of binding of the exosome to the AUBP is altered, and the level of the RNA is altered. In one embodiment, the exosome is cytoplasmic or nuclear. In another embodiment, the exosome is human.

Also provided by the invention is a method for altering the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising a first nucleotide sequence; ii) a protein comprising: 1) a first peptide sequence capable of specifically binding with a mammalian exosome; and 2) a second peptide sequence capable of specifically binding with the first nucleotide sequence; and iii) a mammalian exosome; and b) contacting the RNA with the protein and with the exosome such that the first peptide sequence binds to the first nucleotide sequence, and the exosome binds to the second peptide sequence, thereby altering the level of the RNA.

The invention additionally provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) an AUBP capable of specifically binding to the ARE; and iii) a test agent; b) contacting the RNA, the AUBP, and the test agent to produce a test sample; c) contacting the RNA and the AUBP to produce a control sample; and d) detecting an altered level of binding of the RNA to the AUBP in the test sample compared to the level of binding of the RNA to the AUBP in the control sample, thereby identifying the test agent as altering the level of RNA degradation. In a preferred embodiment, the AUBP is immobilized on a solid support. In an alternative embodiment, the RNA is labeled with a detectable label.

In an alternative embodiment, the invention provides a method for screening a test agent as reducing the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) an AUBP capable of specifically binding to the ARE; and iii) a test agent; b) contacting the RNA, the AUBP, and the test agent to produce a test sample; c) contacting the RNA and the AUBP to produce a control sample; and d) detecting an increased level of binding of the RNA to the AUBP in the test sample.
compared to the level of binding of the RNA to the AUBP in the control sample, thereby identifying the test agent as reducing the level of RNA degradation.

Also provided is a method for screening a test agent as increasing the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) an AUBP capable of specifically binding to the ARE, wherein the AUBP is not AUF1; and iii) a test agent; b) contacting the RNA, the AUBP, and the test agent to produce a test sample; c) contacting the RNA and the AUBP to produce a control sample; and d) detecting a reduced level of binding of the RNA to the AUBP in the test sample compared to the level of binding of the RNA to the AUBP in the control sample, thereby identifying the test agent as increasing the level of RNA degradation. In a preferred embodiment, the RNA is not β-adrenergic receptor mRNA.

Also provided herein is a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an AUBP; ii) a purified mammalian exosome capable of specifically binding to the AUBP; and iii) a test agent; b) contacting the AUBP, the exosome and the test agent to produce a test sample; c) contacting the AUBP and the exosome to produce a control sample; and d) detecting an altered level of binding of the AUBP to the exosome in the test sample compared to the level of binding of the AUBP to the exosome in the control sample, thereby identifying the test agent as altering the level of RNA degradation. In one preferred embodiment, the AUBP is immobilized on a solid support. In another embodiment, exosome is fluorescently labeled with a label selected from DAPI, ANS, Bis-ANS, ruthenium red, cresol violet, and DCVJ.

The invention further provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) a mammalian cell extract; and iii) a test agent; b) incubating the RNA, the cell extract, and the test agent to produce a test sample; c) incubating the RNA and the cell extract to produce a control sample; and d) detecting an altered level of the RNA in the test sample compared to the level of the RNA in the control sample, thereby identifying the test agent as altering the level of RNA degradation.
Furthermore, the invention provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an expression vector that expresses an RNA comprising an ARE; ii) a mammalian cell; and iii) a test agent; b) transforming the mammalian cell with the vector to produce a transfected cell expressing the RNA comprising the ARE; c) incubating the transfected cell with the test agent to produce a test transfected cell; and d) detecting an altered level of the RNA in the test transfected cell compared to the level of the RNA in the transfected cell, thereby identifying the test agent as altering the level of RNA degradation.

Additionally provided herein is a kit for screening for a test agent as altering the level of RNA degradation, wherein the kit comprises one or more containers comprising a purified mammalian exosome. In one embodiment, the kit further comprises an AUBP capable of specifically binding to the exosome. More preferably, the AUBP is K homology-type splicing regulatory protein (KSRP), tristetraprolin (TTP), AUF1, or butyrate response factor-1 (BRF1). In an alternative preferred embodiment, the AUBP is attached to a solid surface.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows that PM-Scl associated proteins are required for ARE-mediated RNA decay. (A) Degradation of an ARE-RNA. ARE\textsuperscript{50s} RNA, 5' or 3' labeled using T4 polynucleotide kinase and γ\textsuperscript{32P}-ATP or T4 RNA ligase and 5',32P-pCp, respectively, was incubated with Jurkat cell S100. At indicated times, RNA was isolated and analyzed on denaturing polyacrylamide gels. Arrow-intact RNA; arrowhead-decay intermediates. Decay products at the bottom of left panel (indicated by a vertical line) represent inefficiently degraded short RNA fragments. Asterisks-decay products which may be free ribonucleotides. (B) Immunoblot analysis of S100s depleted with normal human serum (NHS) or anti-PM-Scl serum. Proteins were probed with antibodies to human exosome components as indicated. (C) RNA decay in exosome-depleted S100. Internally 32P-labeled RNAs (0.5 ng) including ARE-containing 3' UTR of IL-2 and ARE\textsuperscript{50s} or non-ARE-containing GAPDH(580-810) and a short random sequence (E4) were synthesized with (lanes 1-8) or without (lanes 9-
16) a 60 nt poly(A) tail. The RNAs were incubated with mock- (NHS) or exosome-
(anti-PM-Scl) depleted S100s and their decay analyzed as above. PolyA⁺ and polyA⁻
RNAs are indicated. The lower bands marked "con" is external RNA added to the
completed reactions to control for recovery and loading. (D) Decay intermediates
observed in exosome-depleted S100 represent deadenylated RNAs. Polyadenylated IL-2
3'UTR RNA was incubated with mock- and exosome-depleted S100s for 45 min,
isolated, and further analyzed by RNase H digestion in the absence or presence of
oligo(dT).

Figure 2 shows that the exosome is required for rapid degradation of ARE⁶⁶⁶⁶
RNA. (A and B) S100s were depleted by incubation (4X) with protein A-agarose
beads loaded with either normal rabbit serum (NRS), anti-hRrp40p, anti-hRrp46p, or
anti-hRrp46p plus His-tagged hRrp40p. The depleted S100s were fractionated and
immunoblotted (IB) with anti-hRrp40p (A) or anti-hRrp46p (B) antibodies. (C and D)
The stability of ARE⁶⁶⁶⁶ (C) or E4 (D) RNAs was examined in the immunodepleted
S100s.

Figure 3 shows purification and composition of the human exosome. (A)
Cytoplasmic (CE) or nuclear (NE) control and exosome fractions isolated from cells
expressing the TAP fragment only (TAP) that is shown in Figure 10, or TAP-tagged
hRrp4p (hRrp4p) were fractionated by SDS-PAGE and silver stained. Proteins
identified by MALDI and/or nano-electrospray tandem mass spectrometry are indicated
on the right. (B) Purified nuclear or cytoplasmic exosome preparations were
immunoblotted with polyclonal antibodies to PM-Scl100 [Alderuccio et al. (1991) J.
Exp. Med. 173:941-952] (left panel) or PM-Scl75 (right panel) [Alderuccio et al.
(1991) supra]. (C) Nanoelectrospray tandem mass spectrum of a double charged
peptide extracted from band p11. Sequence tag was assembled from a series of
fragment ions and used to search for a matching pattern. (D) Nucleotide and predicted
amino acid sequences of hMTR3. Peptide sequences identified by mass spectrometry
are underlined. (E) Amino acid sequence alignment of mammalian and yeast Mtr3p.

Figure 4 shows that the purified exosome promotes degradation of ARE-RNA.
(A) Reconstitution of ARE-RNA decay in exosome-depleted S100s. Mock-depleted
(lane 1, NHS) or anti-PM-Scl-depleted (lanes 2-6, anti-PM-Scl) S100s were supplemented or not (lane 2) with either cytoplasmic (lane 4) or nuclear (lane 6) exosomes or control preparations isolated from cytoplasmic (lane 3) or nuclear (lane 5) extracts of cells that express TAP tag only. 32P-labeled AREf1s or E4 RNAs were added and incubated for 45 min. The levels of remaining RNA were determined. (B) Isolated exosomes reconstitute AREf1s degradation. Cytoplasmic exosomes (top panel) used in (A) or precipitates of normal rabbit serum (NRS), anti-hRrp40p, or anti-hRrp46p (bottom panel) were added to exosome-depleted S100 and RNA decay assays were performed. The amounts of added exosome components were equivalent to those present in mock-depleted S100. (C) The purified exosome supports rapid AREf1s decay. AREfox or E4 RNAs (1 ng) were incubated with 2.5, 5 or 10 µl of purified cytoplasmic (CE) or nuclear (NE) exosomes or control fractions isolated from cytoplasmic and nuclear extracts of cells expressing TAP fragment only. RNA decay was assayed in the presence of 10 mM Na2HPO4. Ten µl of purified exosome fraction are equivalent to 50% of the amount present in 15 µg of S100. (D) Immunopurified exosome supports AREf1s degradation. 32P-labeled AREf1s or E4 RNAs were incubated with NRS, anti-hRrp40p, or anti-hRrp46p immunoprecipitates in the presence of 10mM Na2HPO4 and their decay was examined.

Figure 5 shows physical interactions between AUBPs and the exosome. (A) Co-immunoprecipitation of AUBPs with the exosome Jurkat cytoplasmic extracts were incubated with 32P-labeled AREf1s RNA and UV crosslinked. Proteins were immunoprecipitated with NRS, NHS, anti-hRrp46p, anti-hRrp40p, or anti-PM-Scl, separated by SDS-PAGE and autoradiographed. 5% of the total UV crosslinked extract was loaded onto lane 1. (B) Identification of AUBPs that bind AREf1s RNA. Proteins crosslinked to AREf1s RNA were immunoprecipitated with antibodies to KSRP, HuR, or AUF1 and analyzed as described in (A). (C) KSRP and AUF1, but not HuR, copurify with the exosome. Proteins purified by TAP procedure from either control (TAP) or hRrp4p-TAP-expressing cells were immunoblotted with antibodies to KSRP, AUF1 or HuR. Total Jurkat cytoplasmic extract was loaded onto lane 3. (D) Cytoplasmic extracts of hRrp4p-TAP-expressing cells were either untreated (lane 1) or
treated (lane 2) with RNase A (0.5 mg/ml) for 30 min at 37 °C. hRrp4p-TAP and associated proteins were precipitated with calmodulin beads (CB) or not and analyzed as indicated. 5% of non-precipitated extracts were loaded onto lane 3. (E) TTP coprecipitates with the exosome. Cytoplasmic or nuclear extracts of 293 cells transiently expressing myc-TTP were immunoprecipitated with NHS or anti-PM-Sc1 and analyzed by immunoblotting with anti-myc antibody. 10% of the unprecipitated input were loaded onto lanes 2 and 5. (F) KSRP binds AREfos RNA. Recombinant KSRP (50 or 100 ng) was incubated with either 32P-labeled AREfos or E4 RNA probes and UV crosslinking assays were performed. (G) Specific binding of KSRP to AREfos RNA. KSRP (100 ng) was incubated with 32P-labeled AREfos RNA in the presence of different amounts (10- or 50-fold excess) of cold AREfos or E4 RNAs and analyzed by UV crosslinking.

Figure 6 shows that AUBPs are required for exosome-mediated ARE-RNA decay. (A) Depletion of AUBPs by poly(U)-agarose. Jurkat S100s were incubated with either poly(G)- or poly(U)-agarose beads. Supernatants were examined by UV crosslinking assays using 32P-labeled AREfos RNA. (B) Exosome components are not removed by either poly(G)- or poly(U)-agarose. Poly(G)- or poly(U)-depleted supernatants were analyzed by immunoblotting with antibodies to exosome components. (C) Degradation of AREfos RNA is AUBP dependent. 32P-labeled AREfos or E4 RNAs were incubated with poly(G)- (lanes 1 and 2) or poly(U)- (lanes 3-14) depleted S100s supplemented or not with 25 or 100 ng of recombinant AUBPs, as indicated. Levels of remaining RNA were examined. (D) KSRP and TTP restore degradation of AREfos RNA. 32P-labeled AREfos RNA was incubated with poly(G)- or poly(U)-depleted S100s in the presence of GST (100 ng), KSRP (100 ng), or GST-TTP (25 ng) and its decay was examined. (E) KSRP and TTP stimulate degradation of ARE- but not of non-ARE-RNAs. 32P-labeled wtAREmfr, mAREmfr, IL-2 3’UTR or GAPDH RNAs were incubated with poly(U)-depleted S100 supplemented or not with 25 or 100 ng of recombinant AUBPs, as indicated. Levels of remaining RNA were examined. (F) KSRP or TTP stimulate degradation of AREfos by the exosome. 32P-labeled AREfos or E4 RNAs (6 ng) were incubated with purified cytoplasmic exosome
in the absence or presence of AUFI, TTP or KSRP (12.5 or 25 ng) and levels of remaining RNA were examined.

Figure 7 provides a brief schematic working model of the pathways involved in the present invention.

Figure 8 provides a general model for methods involving specific mRNA degradation.

Figure 9 provides data showing that the destabilizing AUBPs, TTP and KSRP, specifically stimulate degradation of ARE$_{tor}$ RNA by the exosome, but have no effect on E4 (i.e., without ARE).

Figure 10 shows the amino acid sequence (SEQ ID NO:28) of the TAP fragment in the plasmid pcDNA3-hRRP4-TAP that was used to the generate transgenic cell line JurkatRrp4-TAP.

Figure 11 shows the amino acid sequence (SEQ ID NO:29) of hRrp4p in the plasmid pcDNA3-hRRP4-TAP that was used to the generate transgenic cell line JurkatRrp4-TAP.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "sample" and "specimen" are used in their broadest sense and encompass samples or specimens obtained from any source, including buffer solutions, saline solutions, cell culture media, etc. As used herein, the term "biological samples" refers to samples or specimens obtained from animals (including humans, domestic animals, as well as feral or wild animals, such as ungulates, bear, fish, lagamorphs, rodents, etc.), and encompasses cells, fluids, solids, tissues, and gases. In preferred embodiments of this invention, biological samples include tissues (e.g., biopsy material), cell lines, cells isolated from tissue (whether or not the isolated cells are cultured after isolation from tissue), fixed cells (e.g., fixed for histological and/or immunohistochemical analysis), cerebrospinal fluid (CSF), serous fluid, blood, and
blood products such as plasma, serum and the like. However, these examples are not
to be construed as limiting the types of samples which find use with the present
invention.

As used herein, the term "subject" refers to any animal, including humans.

As used herein, the term "animal" refers to any animal, preferably a mammal.
A "mammal" includes, without limitation, human and non-human animals such
simians, rodents, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines,
canines, felines, aves, etc. Preferred non-human animals are members of the Order
Rodentia (e.g., mouse and rat), sheep, pig, rabbit or cattle.

A "non-human animal" refers to any animal which is not a human and includes
vertebrates such as rodents, non-human primates, ovines, bovines, ruminants,
lagomorphs, porcines, caprines, equines, canines, felines, aves, etc. Preferred non-
human animals are selected from the order Rodentia (particularly a mouse), sheep, pig,
rabbit and cattle.

The "non-human animals having a genetically engineered genotype" of the
invention are preferably produced by experimental manipulation of the genome of the
germline of the non-human animal. These genetically engineered non-human animals
may be produced by several methods including the introduction of a "transgene"
comprising nucleic acid (usually DNA) into an embryonal target cell or integration
into a chromosome of the somatic and/or germ line cells of a non-human animal by
way of human intervention, such as by the methods described herein. Non-human
animals which contain a transgene are referred to as "transgenic non-human animals".
A transgenic animal is an animal whose genome has been altered by the introduction
of a transgene.

The term "transgene" as used herein refers to a foreign gene that is placed into
an organism by introducing the foreign gene into newly fertilized eggs or early
embryos. The term "foreign gene" refers to any nucleic acid (e.g., gene sequence)
which is introduced into the genome of an animal by experimental manipulations and
may include gene sequences found in that animal so long as the introduced gene does
not reside in the same location as does the naturally-occurring gene.
The term "compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Compounds comprise both known and potential therapeutic compounds. A compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (e.g., through animal trials or prior experience with administration to humans) to be effective in such treatment. In other words, a known therapeutic compound is not limited to a compound efficacious in the treatment of disease (e.g., cancer).

As used herein, the term "cell culture" refers to any in vitro culture of cells. Included within this term are continuous cell lines (e.g., with an immortal phenotype), primary cell cultures, finite cell lines (e.g., non-transformed cells), and any other cell population maintained in vitro.

A "transformed cell" is a cell or cell line that has acquired the ability to grow in cell culture for many multiple generations, the ability to grow in soft agar and the ability to not have cell growth inhibited by cell-to-cell contact. In this regard, the term "transformation" as used herein refers to the introduction of a transgene into a cell. Transformation of a cell may be stable or transient. The term "transient transformation" or "transiently transformed" refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell’s genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the transgenes. Alternatively, transient transformation may be detected by detecting the activity of the protein (e.g., β-glucuronidase) encoded by the transgene (e.g., the uid A gene) as demonstrated herein [e.g., histochemical assay of GUS enzyme activity by staining with X-gluc which gives a blue precipitate in the presence of the GUS enzyme; and a chemiluminescent assay of GUS enzyme activity using the GUS-Light kit (Tropix)]. The term "transient transformant" refers to a cell which has transiently incorporated one or more transgenes. In contrast, the term
"stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the transgenes. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify transgene sequences. The term "stable transformant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene.

As used herein, the term "kit" is used in reference to a combination of reagents and other materials. It is contemplated that the kit may include reagents such as antibodies, control proteins, as well as testing containers (e.g., microtiter plates, etc.). It is not intended that the term "kit" be limited to a particular combination of reagents and/or other materials.

The terms "label" and "reporter molecule" refer to a molecule that is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems.

The terms "Western blot," "Western immunoblot," "immunoblot," and "Western" refer to the immunological analysis of protein(s), polypeptides or peptides that have been immobilized onto a membrane support. The proteins are first resolved by acrylamide gel electrophoresis to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to an antibody having reactivity towards an antigen of interest. The binding of the antibody (i.e., the primary antibody) is detected by use of a secondary antibody which specifically binds the primary antibody. The secondary antibody is typically conjugated to an enzyme which permits visualization by the production of a colored reaction product or catalyzes a luminescent enzymatic reaction (e.g., ECL reagent, Amersham).
The term "solid support" refers to any solid surface, such as a glass bead, planar glass, controlled pore glass, plastic, porous plastic metal, or resin to which the molecule may be adhered. One of skill will appreciate that the solid supports may be derivatized with functional groups (e.g., hydroxyls, amines, carboxyls, esters, and sulfhydryls) to provide reactive sites for the attachment of linkers or the direct attachment of the component(s).

The term "Southern blot," refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size followed by transfer of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, NY, pp 9.31-9.58 [1989]).

As used herein, the term "ELISA" refers to enzyme-linked immunosorbent assay. Numerous methods and applications for carrying out an ELISA are well known in the art, and provided in many sources (See, e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, NJ [1998]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; and Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]).

In one embodiment of the present invention, a "direct ELISA" protocol is provided, where an antigen is first bound and immobilized to a microtiter plate well. In an alternative embodiment, a "sandwich ELISA" is provided, where the antigen is attached to the stationary phase by capturing it with an antibody that has been previously bound to the microtiter plate well. The ELISA method detects an immobilized antigen by use of an antibody-enzyme conjugate, where the antibody is
specific for the antigen of interest, and the enzyme portion allows visualization and quantitation by the generation of a colored or fluorescent reaction product. The conjugated enzymes commonly used in the ELISA include horseradish peroxidase, urease, alkaline phosphatase, glucoamylase or β-galactosidase. The intensity of color development is proportional to the amount of antigen present in the reaction well.

As used herein, the term "in vitro" refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments consist of, but are not limited to, controlled laboratory conditions. The term "in vivo" refers to the natural environment (e.g., within an organism or a cell) and to processes or reactions that occur within that natural environment.

As used herein, the term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated as the "normal" or "wild-type" form of the gene. In contrast, the term "modified" (or "mutant") refers to a gene or gene product which displays modifications in sequence and or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

"Nucleic acid sequence," "nucleotide sequence," and "polynucleotide sequence" as used herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

As used herein, the terms "oligonucleotides" and "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe or amplimer.

"Amino acid sequence," "polypeptide sequence," "peptide sequence," and "peptide" are used interchangeably herein to refer to a sequence of amino acids.
As used herein, the terms "peptide," "polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent "peptide linkages." In general, a peptide consists of a few amino acids, typically from 2-50 amino acids, and is shorter than a protein. The term "polypeptide" may encompass either peptides or proteins. A peptide, polypeptide or protein may be synthetic, recombinant or naturally occurring. A synthetic peptide is a peptide which is produced by artificial means in vitro (e.g., was not produced in vivo).

The term "portion" when used in reference to an amino acid sequence and nucleotide sequence refers to fragments of that amino acid sequence and nucleotide sequence, respectively. Fragments of nucleotide sequences may range in size from 5, preferably from 10, nucleotide residues to the entire nucleotide sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide sequence comprises from five (5) contiguous nucleotide residues of the nucleotide sequence to the entire nucleotide sequence. Fragments of an amino acid sequence may range in size from 3, preferably 5, amino acids to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of" an amino acid sequence comprises from three (3) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence.

An oligonucleotide sequence which is a "homolog" of a first nucleotide sequence is defined herein as an oligonucleotide sequence which exhibits greater than or equal to 50% identity, and more preferably greater than or equal to 70% identity, to the first nucleotide sequence when sequences having a length of 10 bp or larger are compared.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of another
mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects that transcription proceeds in a 5' to 3' direction along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "cloning" as used herein, refers to the process of isolating a nucleotide sequence from a nucleotide library, cell or organism for replication by recombinant techniques.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

The term "transfection" as used herein refers to the introduction of foreign DNA into cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybren mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (i.e., particle bombardment) and the like.

As used herein, the terms "complementary" or "complementarity" are used in reference to "polynucleotides" and "oligonucleotides" (which are interchangeable terms that refer to a sequence of nucleotides) related by the base-pairing rules. For example, the sequence "5'-CAGT-3'," is complementary to the sequence "5'-ACTG-3'." Complementarity can be "partial" or "total." "Partial" complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules, for
example from 51% to 99%, preferably from 70% to 99%, more preferably from 80%
to 99%, and most preferably from 90% to 99%, matched. "Total" or "complete"
complementarity between nucleic acids is where each and every nucleic acid base is
matched with another base under the base pairing rules (i.e., 100% matched). The
degree of complementarity between nucleic acid strands may have significant effects
on the efficiency and strength of hybridization between nucleic acid strands. This may
be of particular importance in amplification reactions, as well as detection methods
which depend upon binding between nucleic acids.

The terms "homology" and "homologous" as used herein in reference to
nucleotide sequences refer to a degree of complementarity with other nucleotide
sequences. There may be partial homology or complete homology (i.e., identity). A
nucleotide sequence which is partially complementary (i.e., "substantially
homologous") to a nucleic acid sequence is one that at least partially inhibits a
completely complementary sequence from hybridizing to a target nucleic acid
sequence. The inhibition of hybridization of the completely complementary sequence
to the target sequence may be examined using a hybridization assay (Southern or
Northern blot, solution hybridization and the like) under conditions of low stringency.
A substantially homologous sequence or probe will compete for and inhibit the binding
(i.e., the hybridization) of a completely homologous sequence to a target sequence
under conditions of low stringency. This is not to say that conditions of low
stringency are such that non-specific binding is permitted; low stringency conditions
require that the binding of two sequences to one another be a specific (i.e., selective)
interaction. The absence of non-specific binding may be tested by the use of a second
target sequence which lacks even a partial degree of complementarity (e.g., less than
about 30% identity); in the absence of non-specific binding the probe will not
hybridize to the second non-complementary target.
As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. "Stringency" typically occurs in a range from about $T_m$°C to about 20°C to 25°C below $T_m$. As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" the nucleotide sequence portions thereof, will hybridize to its exact complement and closely related sequences.

Low stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH$_2$PO$_4$•H$_2$O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1 % SDS, 5X Denhardt's reagent (50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)) and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 2.0X SSPE, 0.1% SDS at room temperature when a probe of about 100 to about 1000 nucleotides in length is employed.

High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 68°C in a solution consisting of 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, and 0.1% SDS at 68°C when a probe of about 100 to about 1000 nucleotides in length is employed.

It is well known in the art that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol), as well as components of the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, conditions which promote
hybridization under conditions of high stringency (e.g., increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.) are well known in the art.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 50% to 70% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 50% to 70% homology to the first nucleic acid sequence.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize either partially or completely to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize (i.e., it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids using any process by which a strand of nucleic acid joins with a complementary strand through base pairing to form a hybridization complex. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the $T_m$ of the formed hybrid, and the G:C ratio within the nucleic acids.
As used herein the term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bounds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g., C\text{gel} or R\text{gel} analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized to a solid support (e.g., a nylon membrane or a nitrocellulose filter as employed in Southern and Northern blotting, dot blotting or a glass slide as employed in \textit{in situ} hybridization, including FISH (fluorescent \textit{in situ} hybridization)).

As used herein, the term "$T_m$" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the $T_m$ of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the $T_m$ value may be calculated by the equation: $T_m = 81.5 + 0.41(\% \text{ G + C})$, when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization, in \textit{Nucleic Acid Hybridization} [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of $T_m$.

The term "heterologous nucleic acid sequence" or "heterologous DNA" are used interchangeably to refer to a nucleotide sequence which is ligated to a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (e.g., proteins which confer drug resistance), \textit{etc}. 

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"Amplification" is defined herein as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (see, e.g., Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY [1995]). As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. The length of the amplified segment of the desired target sequence is determined by the relative positions of two oligonucleotide primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (e.g., hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of $^{32}$P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

The terms "reverse transcription polymerase chain reaction" and "RT-PCR" refer to a method for reverse transcription of an RNA sequence to generate a mixture of cDNA sequences, followed by increasing the concentration of a desired segment of the transcribed cDNA sequences in the mixture without cloning or purification. Typically, RNA is reverse transcribed using a single primer (e.g., an oligo-dT primer) prior to PCR amplification of the desired segment of the transcribed DNA using two primers.
As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (i.e., in the presence of nucleotides and of an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (i.e., a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that it is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double- or single-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "an oligonucleotide having a nucleotide sequence encoding a gene" means a nucleic acid sequence comprising the coding region of a gene (i.e. the nucleic acid sequence which encodes a gene product). The coding
region may be present in either cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (i.e., the sense strand) or double-stranded. Suitable control elements such as enhancers, promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers, splice junctions, intervening sequences, polyadenylation signals, etc. or a combination of both endogenous and exogenous control elements.

Transcriptional control signals in eukaryotes comprise "enhancer" elements. Enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis et al., Science 236:1237 [1987]). Enhancer elements have been isolated from a variety of eukaryotic sources including genes in plant, yeast, insect and mammalian cells and viruses. The selection of a particular enhancer depends on what cell type is to be used to express the protein of interest.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, pp. 16.7-16.8 [1989]). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts
lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene.

The term "promoter," "promoter element," or "promoter sequence" as used herein, refers to a DNA sequence which when placed at the 5' end of (i.e., precedes) an oligonucleotide sequence is capable of controlling the transcription of the oligonucleotide sequence into mRNA. A promoter is typically located 5' (i.e., upstream) of an oligonucleotide sequence whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and for initiation of transcription.

The term "promoter activity" when made in reference to a nucleic acid sequence refers to the ability of the nucleic acid sequence to initiate transcription of an oligonucleotide sequence into mRNA.

As used herein, the terms "nucleic acid molecule encoding," "nucleotide encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA).

As used herein, the term "structural gene" or "structural nucleotide sequence" refers to a DNA sequence coding for RNA or a protein which does not control the expression of other genes. In contrast, a "regulatory gene" or "regulatory sequence" is
a structural gene which encodes products (e.g., transcription factors) which control the expression of other genes.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements include splicing signals, polyadenylation signals, termination signals, etc.

As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene. A "gene" may also include non-translated sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into heterogenous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the
gene. The 3' flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

As used herein, the terms "auto-antibody" or "auto-antibodies" refer to any immunoglobulin that binds specifically to an antigen that is native to the host organism that produced the antibody (i.e., the antigen is not synthetic and/or has not been artificially supplied to the host organism). However, the term encompasses antibodies originally produced in response to the administration or presence of a foreign and/or synthetic substance in the host, but also cross-react with "self" antigens. The presence of auto-antibodies is termed "autoimmunity."

As used herein, the term "antigen" is used in reference to any substance that is capable of reacting with an antibody. It is intended that this term encompass any antigen and "immunogen" (i.e., a substance which induces the formation of antibodies). Thus, in an immunogenic reaction, antibodies are produced in response to the presence of an antigen or portion of an antigen.

The term "antigenic determinant" or "epitope" as used herein refers to that portion of an antigen that makes contact with a particular antibody variable region. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants.

A "cultured cell" is a cell which has been maintained and/or propagated in vitro. Cultured cells include primary cultured cells and cell lines.

"Primary cultured cells" are primary cells which are in in vitro culture and which preferably, though not necessarily, are capable of undergoing ten or fewer passages in in vitro culture before senescence and/or cessation of proliferation.

The terms "cell line" and "immortalized cell" refer to a cell which is capable of a greater number of cell divisions in vitro before cessation of proliferation and/or senescence as compared to a primary cell from the same source. A cell line includes, but does not require, that the cells be capable of an infinite number of cell divisions in
culture. Cell lines include, but are not limited to, finite cell lines and continuous cell lines. As used herein, the term "finite cell line" refers to a cell line which is capable of a limited number (from about 1 to about 50, more preferably from about 1 to about 40, and most preferably from about 1 to about 20) of cell divisions prior to senescence.

The terms "neoplasm" and "tumor" refer to a tissue growth. Often, though not necessarily, neoplasms are characterized, in part, by angiogenesis. Neoplasms may be benign and are exemplified, but not limited to, a hemangioma, glioma, teratoma, and the like. Neoplasms may alternatively be malignant, for example, a carcinoma, sarcoma, glioblastoma, astrocytoma, neuroblastoma, retinoblastoma, and the like.

The terms "malignant neoplasm" and "malignant tumor" refer to a neoplasm which contains at least one cancer cell. A "cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described [H.C. Pitot (1978) in "Fundamentals of Oncology," Marcel Dekker (Ed.), New York pp 15-28]. The features of early, intermediate and advanced stages of neoplastic progression have been described using microscopy. Cancer cells at each of the three stages of neoplastic progression generally have abnormal karyotypes, including translocations, inversion, deletions, isochromosomes, monosomies, and extra chromosomes. A cell in the early stages of malignant progression is referred to as "hyperplastic cell" and is characterized by dividing without control and/or at a greater rate than a normal cell of the same cell type in the same tissue. Proliferation may be slow or rapid but continues unabated. A cell in the intermediate stages of neoplastic progression is referred to as a "dysplastic cell." A dysplastic cell resembles an immature epithelial cell, is generally spatially disorganized within the tissue and loses its specialized structures and functions. During the intermediate stages of neoplastic progression, an increasing percentage of the epithelium becomes composed of dysplastic cells. "Hyperplastic" and "dysplastic" cells are referred to as "pre-neoplastic" cells. In the advanced stages of neoplastic progression a dysplastic cell become a "neoplastic" cell. Neoplastic cells are typically invasive i.e., they either invade adjacent tissues, or are shed from the primary site and circulate through the
blood and lymph to other locations in the body where they initiate one or more secondary cancers, i.e., "metastases." Thus, the term "cancer" is used herein to refer to a malignant neoplasm, which may or may not be metastatic.

The term "cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described [H.C. Pitot (1978) in "Fundamentals of Oncology," marcel Dekker (Ed.), New York pp 15-28]. The features of early, intermediate and advanced stages of neoplastic progression have been described using microscopy. Cancer cells at each of the three stages of neoplastic progression generally have abnormal karyotypes, including translocations, inversion, deletions, isochromosomes, monosomies, and extra chromosomes. A cell in the early stages of malignant progression is referred to as "hyperplastic cell" and is characterized by dividing without control and/or at a greater rate than a normal cell of the same cell type in the same tissue. Proliferation may be slow or rapid but continues unabated. A cell in the intermediate stages of neoplastic progression is referred to as a "dysplastic cell." A dysplastic cell resembles an immature epithelial cell, is generally spatially disorganized within the tissue and loses its specialized structures and functions. During the intermediate stages of neoplastic progression, an increasing percentage of the epithelium becomes composed of dysplastic cells. "Hyperplastic" and "dysplastic" cells are referred to as "pre-neoplastic" cells. In the advanced stages of neoplastic progression a dysplastic cell become a "neoplastic" cell. Neoplastic cells are typically invasive i.e., they either invade adjacent tissues, or are shed from the primary site and circulate through the blood and lymph to other locations in the body where they initiate secondary cancers. The term "cancer" or "neoplasia" refers to a plurality of cancer cells.

The term "epithelial cell" refers to a cuboidal-shaped, nucleated cell which generally located on the surface of a tissue. A layer of epithelial cells generally functions to provide a protective lining and/or surface that may also be involved in transport processes. An epithelial cell is readily distinguished from a non-epithelial cell (e.g., muscle cell, nerve cell, etc.) using histological methods well known in the art.
The term "control" as used herein when in reference to a sample, cell, tissue, animal, etc., refers to any type of sample, cell, tissue, animal, etc. that one of ordinary skill in the art may use for checking the results of another sample, cell, tissue, animal, etc., by maintaining the same conditions except in some one particular factor, and thus inferring the causal significance of this varied factor.

**DESCRIPTION OF THE INVENTION**

The invention provides compositions and methods for altering the level of RNA, and for screening test agents that alter RNA levels. The invention’s compositions and methods are useful in, for example, increasing the level of an mRNA and conversely in decreasing the level of mRNA by impacting the level of degradation of the mRNA.

The invention was based, in part, on the inventors’ discovery that the mammalian exosome is required for rapid ARE-directed RNA decay and that the exosome is required for decay of non-ARE-RNAs (Figures 7 and 8). For example, the inventors made the surprising discovery that the exosome discriminates between ARE-RNAs and non-ARE-RNAs. In particular, data provided herein demonstrates that the purified mammalian exosome restores rapid degradation of ARE-RNAs when added to exosome-depleted extracts and that similar activity was exhibited by purified cytoplasmic and nuclear exosomes in the absence of other added components. The inventors also made the surprising discovery that although only a small amount of AUBPs is associated with mammalian exosome, the AUBPs are nonetheless functional, accounting for the exosome’s ability to preferentially target ARE-RNAs. Data provided herein shows that depletion of AUBPs with poly(U) beads prevented preferential degradation of ARE-RNAs by the exosome. Furthermore, the use of higher amounts of RNA substrates in the decay experiments, which is likely to titrate residual AUBPs, resulted in stabilization of ARE-RNAs. Under these conditions, addition of at least two recombinant AUBPs (the exemplary KSRP and TTP) to purified exosome preparations, restored the preferential and rapid degradation of certain ARE-RNAs.
For further clarity, the invention is further described under (A) mRNA Turnover, (B) The Mammalian Exosome Is Involved In mRNA Turnover, (C) AUBPS Are Involved In mRNA Turnover, (D) Methods For Altering RNA Levels By Recruiting The Exosome To The ARE, (E) Methods For Altering RNA Levels By Recruiting The Exosome To Sequences Other Than The ARE, (F) Agents The Alter Binding of ARE to AUBP and/or Alter Binding of AUBP to A Mammalian Exosome, (G) Altering The Level of mRNA In A Subject, (H) Screening Agents That Alter the Level of Degradation of RNA, and (I) Kits For Screening For Compounds That Alter the Level of Degradation of mRNA.

A. mRNA Turnover

The rate of mRNA degradation is controlled via cis-acting elements within the mRNA molecule (Ross, [1995]). The major cis-element responsible for rapid turnover of inherently unstable mammalian mRNAs is the ARE [Shaw et al. (1986) supra; and Chen et al. (1995) Trends Biochem. Sci. 20:465-470]. Understanding the means by which mRNA turnover is regulated requires the identification of the enzymatic machinery for mRNA degradation. In *Saccharomyces cerevisiae*, two mRNA decay pathways exist [Tucker et al. (2000) Annu. Rev. Biochem. 69:571-595]. Both pathways initially involve shortening of the poly(A) tail. Subsequently, the mRNA is degraded in either 5’ to 3’ or 3’ to 5’ directions. In 5’ to 3’ decay, which is the major mRNA degradation pathway in yeast, removal of the cap structure by the Dep1p decapping enzyme occurs after poly(A) shortening [LaGrandeur et al. (1998) EMBO J. 17:1487-1496]. Following decapping, the transcript is rapidly degraded by the Xrn1p 5’ to 3’ exonuclease [Hsu et al. (1993) Mol. Cell. Biol. 13:4826-4835]. The minor 3’ to 5’ decay pathway depends upon a complex of exonucleases, termed the "exosome" [Mitchell et al. (1997) Cell 91:457-466]. The yeast exosome, present in both the nucleus and cytoplasm, is composed of at least 10 subunits, all of which are 3’ to 5’ exoribonucleases and/or RNA-binding proteins [Mitchell et al. (1997) supra; Allmang et al. (1999a) EMBO J. 18:5399-5410; Allmang et al. (1999b) supra; van Hoof et al.
The nuclear exosome, which includes an additional component, Rrp6p, is involved in
processing of small nuclear and nucleolar RNAs, ribosomal RNAs, and degradation of
pre-rRNA spacers and unspliced pre-mRNAs [Mitchell et al. (1997) supra; Allmang et
al. (1999a) supra; Allmang et al. (2000) Nucleic Acids Res. 28:1684-1691; Bousquet-
20:441-452]. The cytoplasmic exosome participates in mRNA turnover [Jacobs et al.
8243]. The exosome appears to be conserved in higher eukaryotes, where it also
appears to be a multiprotein complex [Allmang et al. (1999b) supra; and Brouwer et

Two putative human exosome components, PM-Scl100 and PM-Scl75, are part
of the PM-Scl particle, a protein complex recognized by autoimmune sera from
collagen-induced overlap syndrome patients [PM-Scl; Alderuccio et al. (1991)
J. Exp. Med. 173:941-952; Ge et al. (1992) J. Clin. Invest. 90:559-570]. To date,
eight putative human exosome components have been cloned, six of which are found
within a multiprotein complex [Allmang et al. (1999b) supra; Brouwer et al. (2001)
supra]. Two additional human genes KIAA0116 (Genbank Accession No. D29958)
and OIP-2 (Genbank Accession No. AF025438) are homologues to the yeast RRP45
gene [Allmang et al. (1999b) supra], but three yeast exosome subunits, Rrp42p,
Rrp43p, and Mtr3p, have not been assigned any mammalian homolog. Both yeast and
human exosome components display sequence similarity to E. coli RNase, PH, RNase
D, or RNase R [Allmang et al. (1999b) supra; and Mitchell et al. (2000) supra].

The first step in mammalian mRNA decay also appears to be poly(A)
shortening [Shyu (1991) supra]. However, the subsequent steps in mammalian mRNA
decay are not as well defined as in yeast. For example, it is not even clear whether
RNA degradation proceeds via a 5' to 3' or 3’ to 5' pathway. In vitro data suggest
that mammalian mRNA decay occurs via a 3’ to 5’ pathway [Brewer (1998) J. Biol.
Chem. 273:34770-34774]. However, the enzyme responsible for this pathway was not heretofore identified.

B. The Mammalian Exosome Is Involved In mRNA Turnover

The mechanism by which the ARE acts remains elusive. Using a cell-free RNA decay system and purified components, AREs are shown to recruit the mammalian exosome, a 3'-to-5' exonuclease, to affect rapid mRNA degradation. This function requires recognition of AREs by AUBPs, several of which have been shown to interact with the exosome. To establish the mechanism of ARE-promoted mRNA decay, the pathway that mediates decay of adenylated and deadenylated ARE-RNA were examined in the cell-free system (Figure 1A). Although ARE-RNAs were not shown to be degraded via a 3'-to-5' pathway, immunodepletion of the exosome resulted in almost complete stabilization of ARE-RNAs (Figure 1C, 2C). Given the 3'-to-5' exonuclease activity of the exosome, these results strongly indicate that the 3'-to-5' decay pathway is responsible for rapid degradation of ARE-RNAs in this system. Interestingly, removal of the exosome did not substantially interfere with deadenylation. These results are consistent with the role of the cytoplasmic exosome in mRNA turnover in yeast [Jacobs et al. (1998) supra; and van Hoof et al. (2000b) supra]. Although AREs promote deadenylation in vivo [Shyu (1991) supra; and Xu et al. (1997) supra], deadenylation was not observed in this system (Figure 1C). Using HeLa cell extracts and substrates with long poly(A) tails (e.g., 200 nucleotides), AREs were reported to slightly enhance deadenylation in vitro [Ford et al. (1999) supra]. However, using RNAs with shorter polyA tails (e.g., 60 nucleotides), the same investigators could not detect a reproducible effect on the ARE on deadenylation. The lack of strong ARE-facilitated deadenylation in vitro may be due to titration of poly(A)-binding protein (PABP) by the large amount of poly(A) added to the reactions, which renders the poly(A) tail freely accessible to deadenylating enzymes. This suggests that ARE-enhanced deadenylation may involve binding of PABP to the poly(A) tail. Regardless, the present results demonstrate that the exosome is required
for rapid ARE-directed RNA decay and that the exosome is required for decay of non-ARE-RNAs, although the latter is inefficient and requires prolonged incubation.

Based on sequence similarity between individual yeast and human exosome components, it was postulated by the inventors that the mammalian particle is functionally homologous to the yeast exosome and therefore, responsible for 3' to 5' mRNA decay, including rapid turnover of ARE-RNAs. To confirm this hypothesis, experiments were conducted during the development of the present invention. In these experiments, the human exosome was purified, the composition was determined, and its role in decay of ARE-RNAs using an in vitro mRNA turnover system [Ford et al. (1999) supra] was determined. This turnover system faithfully replicates the regulation of ARE-directed mRNA degradation [Chen et al. (2000) supra].

The following is a brief description of the purification of the exemplary human exosome. By incorporating a tagged hRrp4p into the human exosome, the exosome could be purified so that its composition could be determined by mass spectrometry. The inventors discovered that the human exosome is similar in structure to its yeast counterpart, containing at least 10 subunits. With the exception of hDis3p, human homologs of all of the yeast exosome subunits were identified. Two proteins, previously known as KIAA0116 and OIP-2, were identified as the human homologs of Rrp42p and Rrp43p, respectively. Another newly identified human exosome component is likely to be the homolog of Mtr3p. All three of these human proteins exhibit similarity to E. coli RNase PH and are therefore likely to be bona fide exosome subunits. The relative paucity of high molecular weight subunits in the purified exosome (Figure 3A) could be due to their proteolysis during isolation, as several bands recognized by antibodies to each PM-Sc1 antigen were found (Figure 3B). Also identified were at least two exosome-associated proteins. One of which, encoded by the KIAA0052 open reading frame, is homologous to yeast Ski2p and Mtr4p, which are believed to interact with the exosome and provide it with RNA helicase activity. The other protein, MPP-6, was previously identified as a protein phosphorylated during mitosis.
As disclosed herein, the purified exosome restores rapid degradation of ARE-RNAs when added to exosome-depleted extracts. Similar activity was exhibited by purified cytoplasmic and nuclear exosomes in the absence of other added components.

As indicated herein, the human exosome contains at least 10 subunits and is responsible for exonucleolytic degradation of the RNA body, but is not required for poly(A) shortening. The mammalian exosome is tightly associated with a putative RNA helicase, whose yeast homologs, Ski2p, and Mtr4p, have been genetically implicated in mRNA turnover and RNA processing [de la Cruz et al. (1998) EMBO J. 17:1128-1140; and Jacobs et al. (1998) supra].

C. AUBPS Are Involved In mRNA Turnover

The ability of the exosome to discriminate between ARE-RNAs and non-ARE-RNAs was unexpected, and indeed additional experiments have shown that discrimination depends on the presence of AUBPs, several of which copurify with the exosome. The presence of AUBPs in purified exosome preparations was demonstrated by UV crosslinking and immunoblotting experiments. However, it should be noted that no AUBP could be identified as an abundant exosome-associated protein by mass spectrometry. In fact, immunoprecipitation experiments have revealed the association of the exosome with only a fraction of the total pool of KSRP and even a smaller portion of total AUF1. However, the small amounts of associated AUBPs are functional and account for the exosome’s ability to preferentially target ARE-RNAs. Indeed, depletion of AUBPs with poly(U) beads prevented preferential degradation of ARE-RNAs by the exosome. Furthermore, the use of higher amounts of RNA substrates in the decay experiments, which is likely to titrate residual AUBPs, results in stabilization of ARE-RNAs. Under these conditions, addition of at least two recombinant AUBPs, KSRP and TTP, to purified exosome preparations, restored the preferential and rapid degradation of certain ARE-RNAs. Interestingly, the effect of either KSRP or TTP on exosome-mediated degradation of certain ARE-RNAs was biphasic. Most likely, small amounts of either protein suffice for effective recruitment through titration of AUBP-binding sites on both the exosome and the RNA substrate.
KSRP was originally identified as a component of protein complex assembled on an intronic c-src enhancer required for neuronal-specific splicing [Min et al. (1997) supra]. It was then found that KSRP was also an exosome-interacting AUBP capable of promoting ARE-mediated decay at least in vitro. The other protein shown to have such an activity, TTP, is a well established stimulator of ARE-mediated mRNA turnover in vivo [Lai et al. (1999) supra; Stoecklin et al. (2000) Mol. Cell. Biol. 20:3753-3763; and Ming et al. (2001) Mol. Cell. Biol. in press]. Both KSRP and TTP do not stimulate degradation of substrates lacking functional AREs. Surprisingly, recombinant AUFI did not enhance ARE-mediated mRNA decay in the reconstituted system, although it had been reported to do so in vivo [Loflin et al. (1999) supra]. However, the possibility that the recombinant form of AUFI was not properly folded or post-translationally modified could not be excluded, even though it had ARE binding activity. The structural basis for the interactions of AUBPs with the exosome has not yet been determined. These interactions, although RNase independent, could be direct or indirect and may be mediated by a specific exosome targeting sequence motif. At least one AUBP, HuR, was shown to promote RNA stabilization rather than decay [Fan et al. (1998) supra; Peng et al. (1998) supra; and Ford et al. (1999) supra]. No HuR could be detected in the purified exosome preparation (Figure 5C and D). Thus, the stabilizing activity of HuR may be due to its inability to recruit the exosome to ARE-RNAs.

Whether the exosome is involved in 3'-to-5' decay of ARE-RNA in vivo awaits further investigation, but preliminary results suggest that partial inhibition of exosome subunit synthesis using anti-sense RNA results in stabilization of ARE-RNA in living cells. Most importantly, the cell-free decay system used during development of the present invention has proven to be invaluable for investigating the mechanistic aspects of mammalian mRNA turnover. This system is able to reproduce several aspects of mRNA turnover observed in vivo, such as ARE-facilitated mRNA decay and decapping, as well as signal-induced mRNA stabilization [Ford et al. (1999) supra;

Based on the data obtained during the development of the present invention, a simple working model that is consistent with currently available results is provided at Figure 7. Without limiting the invention to any particular mechanism, and recognizing that an understanding of the mechanism of action of the invention's compositions is not necessary for patentability, it is nonetheless the inventors' view that binding of some AUBPs to AREs may alter the interactions between PABP and the poly(A) tail or between the cap binding proteins and the 5' cap, thereby providing access to the poly(A) ribonuclease (PARN) and the decapping enzyme [Gao et al. (2000) supra; Gao et al. (2001) supra; and Wilusz et al. (2001) supra]. Alternatively, AUBPs may recruit PARN and the decapping enzyme to the RNA substrate. At the same time, AUBPs such as TTP and KSRP, recruit the exosome to the vicinity of ARE-RNAs to initiate degradation of the RNA body after deadenylation is completed. Alternatively, AUBPs recruit a large, but loosely assembled, degradative machinery containing PARN, the decapping enzyme, and the exosome. This recruitment-based mechanism explains why ARE-RNAs are preferentially degraded and why they are stabilized upon ARE removal. The results presented herein also suggest that AUBPs may be important targets for some of the signaling pathways by which extracellular stimuli stabilize ARE-RNAs.

Similar to yeast, decay of mammalian mRNAs is initiated via deadenylation [Shyu (1991) supra]. Unlike yeast, in which both 5' to 3' and 3' to 5' decay pathways exist [Tucker et al. (2000) supra], the subsequent steps of mammalian mRNA turnover have not been elucidated in vivo. However, as indicated herein, the exosome is responsible for 3' to 5' decay of ARE-RNAs in vitro. It is contemplated that these observations are applicable to live cells as well (i.e., in vivo). Thus, it is likely that the 3' to 5' pathway may be the predominant decay pathway of ARE-RNAs.
As recent results suggest that ARE-mediated mRNA decay may exist in yeast [Vasudevan et al. (2001) Mol. Cell 7:1191-1200], the present invention suggests that AUBPs may be involved in this phenomenon in several organisms. It is contemplated that the availability of a biochemical system based on purified components and genetic studies provided by the present invention will facilitate the analysis of AUBP function and determination of the molecular basis for both stabilizing and destabilizing activities.

Although the purified exosome per se, does not exhibit ARE-binding activity, the inventors determined that it is recruited to ARE-RNAs through interactions with several AUBPs. As described herein, AREs function through binding of AUBPs, such as KSRP and TTP, which in turn recruit the exosome to affect 3' to 5' RNA degradation. Indeed, although most of the experiments described herein were conducted in a cell-free RNA decay system, there are clear indications that the exosome is also responsible for degradation of short-lived mRNAs in vivo. By disrupting the interaction between specific mRNAs and by targeting the exosome to specific mRNAs via specific targeting proteins, the expression of certain genes can be shut off.

Modulation of gene expression by destabilizing or stabilizing mRNA species provides new means to regulate the levels of gene expression and facilitates the development of new treatments for immune diseases, inflammatory diseases, cancer, hormonal deficiencies and other abnormalities. The modulation of mRNA stability is achieved either by directly or indirectly controlling the activity of ribonucleases responsible for mRNA decay. Indeed, the present invention provides means for up and down-modulation of the levels of various cytokines, proto-oncogenes, and growth factors. In addition, it is contemplated that the present invention will find use in the development of new ways to specifically control the abundance of any mRNA molecule by either targeting the exosome to stable mRNAs or inhibiting recruitment of the exosome to unstable mRNAs. These advancements are significant, as with the exception of antisense RNA or sequence-specific ribozymes, there are no generally applicable methods to modulate the expression of specific mRNA molecules.
AREs function to stimulate the degradation of specific mRNAs based on the binding of specific AUBPs to AREs, which in turn recruit the exosome to the RNA through direct interactions with this particle. Exosome recruitment results in rapid degradation of ARE-containing mRNAs (See, Figure 7). Thus, there are a number of ways to prevent binding of specific AUBPs to specific mRNAs (See, "A" in Figure 7) or prevent the interactions between AUBPs and the exosome (See, "B" in Figure 7), and thereby stabilize and elevate expression of specific mRNAs when needed. This also provides means to recruit the exosome to any given mRNA through chimeric proteins containing an exosome-binding domain derived from a destabilizing AUBP and a sequence-specific mRNA binding domain (See, Figure 8).

As indicated in Figure 9, although no significant degradation of ARE-RNA can be achieved by the isolated exosome itself (compare lane 2 to lane 1) after 30 minutes of incubation in the RNA decay system used during the development of the present invention, addition of recombinant AUBPs, such as TTP (lanes 5 and 6) or KSRP (lanes 7 and 8), but not AUF (lanes 3 and 4) to the isolated exosome promotes degradation of ARE-containing RNA (e.g., ARE\textsuperscript{\textasciitilde}; See, Figure 9, upper panel), but has no effect on ARE-lacking RNA (e.g., E4; See, Figure 9, bottom panel). The data obtained during the development of the present invention indicate that interactions between the ARE, destabilizing AUBPs and the exosome are required for rapid degradation of ARE-containing mRNAs and can be used to target any RNA to rapid degradation, as needed and/or desired.

D. Methods For Altering RNA Levels By Recruiting The Exosome To The ARE

The invention provides methods for altering the level of an RNA of interest in a sample by recruiting the exosome to the ARE in the RNA as exemplified by Figure 7. In particular, the invention provides methods for altering the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising an adenylate uridylate-rich element (ARE); ii) an AUBP capable of specifically binding to the ARE; and ii) an agent that alters the level of
specific binding of the ARE to the AUBP; and b) contacting the RNA, the AUBP, and the agent such that the level of binding of the ARE to the AUBP is altered, and the level of the RNA is altered. As represented schematically in Figure 7A, the invention's methods are used to increase or decrease the level of ARE-RNA by increasing or decreasing the level of interaction of AUBP to ARE. These methods are useful for reducing mRNA expression in diseases where increased expression of the mRNA is associated with disease, thereby reducing disease. Conversely, the invention's methods are also useful for increasing the level of a desirable mRNA, (e.g., those encoding growth factors, cytokines) thereby reducing disease.

The term "altering the level of an RNA" as used herein refers to an increase or decrease in the quantity of the RNA. The terms "increase the level of an RNA" and "reduce the level of degradation of an RNA" when in reference to the level of RNA in a first sample relative to a second sample, mean that the quantity of RNA in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. Preferably the quantity of RNA in the first sample is at least 10% greater than, more preferably at least 50% greater than, yet more preferably at least 75% greater than, even more preferably at least 90% greater than, the quantity of the same RNA in a second sample. The terms "reduce the level of an RNA" and "increase the level of degradation of an RNA" when in reference to the level of RNA in a first sample relative to a second sample, mean that the quantity of RNA in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. Preferably the quantity of RNA in the first sample is at least 10% lower than, more preferably at least 50% lower than, yet more preferably at least 75% lower than, even more preferably at least 90% lower than, the quantity of the same RNA in a second sample.

RNA levels in a sample may be determined using methods known in the art, and disclosed herein. For example, as shown in Figure 1A, RNA may be 5' or 3' labeled using T4 polynucleotide kinase and γ-32P-ATP, or using T4 RNA ligase and 5'-32P-pCp, respectively. Labeled RNA is isolated and analyzed on denaturing
polyacrylamide gels. The level of intact RNA (compared to a control), of decay RNA fragments (relatively lower molecular weight than intact RNA) and free ribonucleotides is determined. An increase in the proportion of decay fragment and/or free ribonucleotides relative to the intact RNA indicates a reduced level of RNA, i.e., an increased level of RNA degradation.

Alternatively, RNA level in a sample may be determined as shown in Figure 1D, by isolating $^{32}$P-labeled RNA from the sample, incubating the isolated RNA with RNase H in the presence of oligo(dT) in order to remove adenylated RNA, and analyzing the isolated RNA on denaturing polyacrylamide gels. Observing a reduction (compared to a control sample) in the level of deadenylated RNA fragments indicates that there is a reduction in RNA degradation, which in turns means that there is an increase in RNA level.

1. **Mammalian Exosome**

In one embodiment, the invention provides a purified mammalian exosome. The term "exosome" as used herein, refers to a multiprotein complex that binds specifically to an AUBP, which in turn binds specifically to an ARE-RNA. Binding of the exosome to the AUBP alters (i.e., increase or decreases) the level of the RNA sequence that contains the ARE by increasing or decreasing the rate of degradation of the RNA. The proteins that form the multiprotein complex of the exosome include exonucleases (such as 3' to 5' exoribonucleases), RNA-binding proteins, proteins that participate in mRNA turnover, and proteins (such as Rrp6p) that are involved in processing of small nuclear and nucleolar RNAs, ribosomal RNAs, and degradation of pre-rRNA spacers and unspliced pre-mRNAs.

The terms "purified," "to purify," "purification," "isolated," "to isolate," "isolation," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one contaminant from a sample. For example, an exosome is purified by at least a 10%, preferably by at least 30%, more preferably by at least 50%, yet more preferably by at least 75%, and most preferably by at least 90%, reduction in the amount of undesirable proteins, such as proteins present in a nuclear
and/or cytoplasmic cell extract. Thus purification of an exosome results in an "enrichment," *i.e.*, an increase in the amount, of exosome protein(s) in the sample. The mammalian exosome may be purified using methods disclosed herein, such as the TAP procedure or immunoprecipitation with antibodies.

In one preferred embodiment, the mammalian exosome contains at least one of GIVILMVDEK (SEQ ID NO:24) and LTEQLAGPLR (SEQ ID NO:25), which are portions of the proteins that were purified in the exemplary human p1 (Table 2) (Figure 3A), and which further distinguish the exemplary human exosome from the yeast exosome that does not contain p1 proteins.

In an alternative embodiment, the mammalian exosome further comprises YIGEVDIVVGR (SEQ ID NO:2), LDSVLLSSSMNLPGEGLR (SEQ ID NO:3), NCIISLVTQR (SEQ ID NO:4), LSVENVPCIVTLCK (SEQ ID NO:5), GGDDLGETIANTLYR (SEQ ID NO:6), GSDLPSIFEMMETGKR (SEQ ID NO:7), TTVNIGSISTADGSALVK (SEQ ID NO:8), AEFAAPSTDAPDK (SEQ ID NO:9), MAEPASVAEAESLAGSR (SEQ ID NO:10), GDHVGIVTAK (SEQ ID NO:11), VVCWPGLR (SEQ ID NO:12), GGGPAGAGGAPAAPL (SEQ ID NO:13), ELALAQEALEPAV (SEQ ID NO:14), LYPVLQQSLVR (SEQ ID NO:15), VLCAVSGPR (SEQ ID NO:16), QTFEAAILTQLHPR (SEQ ID NO:17), MAGLELLSDQGYR (SEQ ID NO:18), ATLEVILRPK (SEQ ID NO:19), AVLTFALDSVER (SEQ ID NO:20), VHILYVGSMLK (SEQ ID NO:21), SSGEGALPVVSVVR (SEQ ID NO:22), and HGYFSSLAGCLMK (SEQ ID NO:23). These sequences represent portions of the proteins that were present in the exemplary purified human core proteins p7-p13 (Table 2) (Figure 3A). In a more preferred embodiment, the mammalian exosome further comprises ISEEHWYLDLPELK (SEQ ID NO:26) and YETLVGTIGK (SEQ ID NO:27), which are portions of the proteins that were present in the exemplary purified human p14 protein (Table 2) (Figure 3A).

While it is not intended that the invention be limited to any particular type of mammalian exosome, in one preferred embodiment, the purified mammalian exosome is a human exosome.
In another preferred embodiment, the purified mammalian exosome is purified from the transgenic cell line designated JurkatRrp4-TAP.

In yet another embodiment, the invention provides a mammalian exosome purified by the process of: a) providing: i) a host mammalian cell; ii) an expression vector comprising a reporter gene operably linked to a nucleic acid sequence encoding a hRrp4p polypeptide; b) transforming the host mammalian cell with the vector to produce a transformed cell expressing a protein the comprises hRrp4p polypeptide operably linked to a reporter polypeptide; and c) purifying the protein from the transformed cell, wherein the purified protein comprises a purified mammalian exosome. This method is exemplified herein by the transformation of Jurkat cells with pcDNA3-hRRP4-TAP which express the human exosome.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "operably linked," "in operable combination," and "in operable order" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term The term "reporter gene" refers to a gene which encodes a reporter molecule (e.g., RNA, polypeptide, etc.) which is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. Exemplary reporter genes include, for example, green fluorescent protein gene, E. coli β-galactosidase gene, human placental alkaline phosphatase gene, and chloramphenicol
acetyltransferase gene. It is not intended that the present invention be limited to any particular detection system or label. However, in a preferred embodiment, the reporter gene encodes TAP (SEQ ID NO:28) (Figure 10) and is in the expression vector pcDNA3-TAP. The individual steps for purifying TAP labeled hRrp4p-TAP and associated proteins are known in the art [Rigaut et al. (1999) supra].

While it is not intended that the invention be limited to any particular type or source of mammalian cell, in one preferred embodiment, the host mammalian cell is a Jurkat cell.

It is also not intended that the protein be purified from any particular compartment of the cell. In one embodiment, the purified protein is isolated from the cytoplasm of the transformed cell or from the nucleus of the transformed cell, since data herein demonstrates that the expressed exosome is present in both the cytoplasmic and nuclear compartments.

In one preferred embodiment, the purification is by affinity purification, which comprises contacting the protein from the transformed cell with an agent that specifically binds to the reporter polypeptide, and purifying the agent that is bound to the reporter polypeptide. The terms "an agent that specifically binds to the reporter polypeptide" and "probe" when used in reference to a polypeptide refers to a polypeptide which is capable of interacting with the reporter polypeptide. Polypeptide probes may be produced synthetically or by recombinant means. Chemicals methods for polypeptide synthesis are known in the art, such as those described by Cartuthers et al., (1980) Nuc. Acids Res. Symp. Ser 215-23, and by Horn et al. (1980) Nuc. Acids Res. Symp. Ser 225-32. Alternatively, a polypeptide probe may be produced by expression in an expression vector of polynucleotides (i.e., a sequence of nucleotides) encoding the probe. Probes are useful in the detection, identification and isolation of particular polypeptide sequences. It is contemplated that the polypeptide probes of the invention may be, but are not necessarily, used in combination with a label. The polypeptide probes of the present invention may be labelled with any "reporter molecule," so that the polypeptide reporter is detectable in any detection system,
including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label. The polypeptide probe may be fused to another molecule capable of binding to a ligand, and the polypeptide isolated by binding the fused composition to the ligand. The ligand may be immobilized to a solid support to facilitate isolation of the fused polypeptide. Ligand-binding systems useful for the isolation of polypeptides are commercially available and include, for example, the staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to Ni²⁺), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), etc. It is not intended that the polypeptide probes of the present invention be limited to any particular isolation system.

The invention further provides an exosome purified from a transgenic cell line designated JurkatRrp4-TAP. The term "transgenic cell" refers to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene. Transgenic cells may be produced using methods known in the art, such as those used herein.

2. **AU-Rich Element (ARE)**

In one embodiment, the compositions and methods of the invention serve to alter the level of and RNA comprising an adenylate uridylate-rich element. The terms "adenylate uridylate-rich element," "AU-rich element," and "ARE" are used interchangeably herein to refer to a nucleotide sequence from 4 to 500 nucleotides, more preferably from 4 to 150 nucleotides, yet more preferably from 4 to 100 nucleotides, even more preferably from 4 to 50 nucleotides, an yet most preferably from 4 to 10 nucleotides, that contains at least 20%, more preferably at least 40%, even more preferably at least 60%, more preferably at least 70%, and yet more preferably at least 80%, uridine residues, adenine residues, or a combination of uridine and adenine residues. In one preferred embodiment, an ARE contains one or more of
the following motifs: AUUUA (SEQ ID NO:1), UUAUUUAUU (SEQ ID NO:2), UUAUUUAAA (SEQ ID NO:3), UUAUUUUAAU (SEQ ID NO:4), UUAUUUAUA (SEQ ID NO:5), UUAUUUAUU (SEQ ID NO:6), UUAUUUAAA (SEQ ID NO:7), UUAUUUAU (SEQ ID NO:8), UUAUUUAUA (SEQ ID NO:9), AUAUUUAUU (SEQ ID NO:10), AUAUUUAAA (SEQ ID NO:11), AUAUUUAU (SEQ ID NO:12), AUAUUUAUA (SEQ ID NO:13), AAAUUUAUU (SEQ ID NO:14), AAAUUUAAA (SEQ ID NO:15), AAAUUUAU (SEQ ID NO:16), AAAUUUAUA (SEQ ID NO:17), Group I Cluster (AUAUUUAUUUAUUUAUUUA) (SEQ ID NO:18) (see Table 1); Group II Cluster (AUAUAULTUAUUUAUUUA) (SEQ ID NO:19) (see Table 1), Group III Cluster (WAUUUAUUAUUUAUW) (SEQ ID NO:20) (see Table 1), Cluster IV Group (WWWUUUAUUUAUWW) (SEQ ID NO:21) (see Table 1), Cluster V Group (WWWUUUAUUAWWWW) stretch (SEQ ID NO:22), wherein W is A or U. These motifs have been found to play a role in mRNA stability [Chen et al. (1995) Trends Biochem. Sci 20:465-470; Bakheet et al. (2001) Nucleic Acids Res. 29:246-254]. In one embodiment, an ARE may contain only uridine residues or only adenine residues. Alternatively, an ARE may contain at least 20% uridine residues with the remainder of the residues being adenine residues, or may contain at least 20% adenine residues with the remainder of the residues being uridine residues. Also, an ARE may contain one or more copies of any one or more of the motifs described herein and/or any one or more of these motifs may be present in tandem or be overlapping in an ARE.

The terms "ARE-mRNA," "ARE-RNA," "ARE-containing mRNA, and "ARE-containing RNA" are used interchangeably herein to refer to an RNA sequence containing an AU-rich element.

ARE sequences may readily be introduced into an RNA sequence using chemical synthesis or standard molecular biological techniques, such as those disclosed herein. Moreover, the prepense of an ARE in an RNA sequence may readily be determined by detecting hybridization of the RNA to a probe that is complementary to the ARE.
3. **AU-Rich Element-Binding Protein (AUBP)**

In one embodiment, the compositions and methods of the invention serve to alter the level of an ARE-RNA using an ARE-binding protein. The terms "adenylate uridy late-rich element-binding protein," "AU-rich element-binding protein," "ARE-binding protein" and "AUBP" are used interchangeably herein to refer any protein that is capable of specifically binding to an ARE.

The terms "specifically binds" and "specific binding" and grammatical equivalents when used in reference to the binding of a first protein (such as an ARE-binding protein) to an ARE or to a second protein (such as a mammalian exosome) refer to an interaction of the first protein with one or more nucleotide sequences in the ARE or with one or more peptide sequences in the second protein, respectively, where the interaction is dependent upon the presence of a particular structure and/or sequence in the ARE or in the second protein, respectively. For example, if a first protein (such as an ARE-binding protein) is specific for nucleotide sequence "A" in an ARE, then the presence of an unlabeled RNA sequence containing the nucleotide sequence A (or the presence of unlabeled nucleotide sequence A) in a reaction containing labeled nucleotide sequence "A" and the first protein will reduce the amount of labelled nucleotide sequence A that is bound to the first protein. Similarly, if a first protein (such as an ARE-binding protein) is specific for peptide sequence "A" in a second protein (such as a mammalian exosome), then the presence of an unlabeled protein containing the peptide sequence A (or the presence of unlabeled peptide sequence A) in a reaction containing labeled peptide sequence "A" and the first protein will reduce the amount of labelled peptide sequence A that is bound to the first protein.

The specificity of binding of an AUBP to an ARE may be determined using methods known in the art such as by examining UV cross-linking of the ARE to the AUBP as disclosed in Zhang et al. (1993) *supra* and U.S. Patent No. 6,436,636, incorporated in its entirety.

The specificity of binding of an AUBP to an exosome may be determined by routine methods, such as those taught in Mitchell *et al.* (1997) *supra*; Allmang *et al.*

In a preferred embodiment, the AUBP is K homology-type splicing regulatory protein (KSRP) [Min et al. (1997) supra]. In another embodiment, the AUBP is tristetraprolin (TTP), which is also known as Nup475, TIS11, or G0S24 [Lai et al. (1999) supra], and which binds to AREs of TNFα and other cytokine mRNAs to promote their deadenylation and decay. In a further embodiment, the AUBP is AUFI [Zhang et al. (1993) supra]. In yet another embodiment, the AUBP is butyrate response factor-1 (BRF1) [Stoecklin et al. (2002) The EMBO J. 21:44709-4719]. In a further embodiment, the AUBP is HuR [Ma et al. (1996) J. Biol. Chem. 271:8144-8151], which is also called HuA [Keene (1999) Proc. Natl. Acad. Sci USA 96:5-7] and is a mammalian Hu protein required for neuronal differentiation in the mammalian nervous system [Akamatsu et al. (1999) Proc. Natl. Acad. Sci. USA 96:9885-9890]. In yet another embodiment, the AUBP is HuB, which is also known as Hcl-N2 [Levine et al. (1993) Mol. Cell. Biol. 13:3494-3504; Akamatsu et al. (1999) supra].

In some embodiment, it may be desirable that the AUBP is not AUFI. The term "not" when preceding, and made in reference to, any particularly named molecule (such as AUFI, β-adrenergic receptor mRNA, etc.) means that only the particularly named molecule is excluded.

While the invention is illustrated using the exemplary AUBPs of KSRP, TTP, AUFI, BRF1, HuR, and HuB, it is not intended that the scope of the invention be limited to these exemplary AUBPs, but rather to include any protein that is capable of specifically binding to an ARE. Several methods for identifying ARE-binding proteins are known in the art. For example, ARE-binding proteins with an established function in mRNA turnover have been identified by purification of an in vitro decay activity [AUFI; Zhang et al. (1993) supra]; by homology cloning [HuR; Ma et al. (1996) supra]; by examining the pathology of knockout mice [TTP; Carballo et al., (1998) Science 281:101-1005]; by purification of an RNA-binding activity [KSRP; Chen et al. (2001) Cell 107:451-454]; and by using a diploid reporter cell line where the element
directs decay of a hybrid reporter transcript, isolating a mutant with a recessive defect correctable by cell fusion, in combination with observing phenotypic reversion by gene transfer [BRF1; Stoecklin et al. (2002) supra]

4. RNA Sequences Of Interest

The invention's compositions and methods may be applied to alter the level of any RNA sequence of interest. The term "RNA sequence of interest" refers to any RNA sequence, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. In a preferred embodiment, the RNA sequence of interest contains an ARE. Such ARE-RNA sequences include, but are not limited to, mRNA sequences that are encoded by genes that encode a cytokine, proto-oncogene or growth factor as described in Shaw et al. (1986) Cell 46:659-667, Chen et al. (1995) Trends Biochem. Sci 20:465-470, and Bakheet et al. (2001) Nucleic Acids Res. 29:246-254. A list of exemplary ARE-mRNA Sequences is provided in Table 1.

Table 1
Exemplary ARE-mRNA Sequences

<table>
<thead>
<tr>
<th>ARE-mRNA Sequence</th>
<th>GenBank #</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I Cluster (AUUUAAUUUAUUAUUAUUAUUA) (SEQ ID NO:23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMSET type I (MMSET)</td>
<td>AF071594</td>
<td>Cell growth/differentiation</td>
</tr>
<tr>
<td>GDP-L-fucose:β-D-galactoside 2-α-L-fucosyltransferase</td>
<td>M35531</td>
<td>Metabolism, carbohydrate</td>
</tr>
<tr>
<td>Cellular growth-regulating protein</td>
<td>L10844</td>
<td>Cell growth</td>
</tr>
<tr>
<td>Gro-β (melanoma stimulating growth factor)</td>
<td>M57731</td>
<td>Cell growth</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Accession</td>
<td>Function</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Pim-1</td>
<td>M16750</td>
<td>Signal transduction, oncogenes</td>
</tr>
<tr>
<td>Neuron-specific γ-2 enolase</td>
<td>M22349</td>
<td>Development/differentiation</td>
</tr>
<tr>
<td>Nuclear matrix protein NRP/B (NRPB)</td>
<td>AF059611</td>
<td>DNA transcription/differentiation</td>
</tr>
<tr>
<td>Natural killer cell stimulatory factor (IL-12)</td>
<td>M65200</td>
<td>Hematopoiesis, immune response</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
<td>M11220</td>
<td>Hematopoiesis</td>
</tr>
<tr>
<td>Adipogenesis inhibitory factor (IL-11)</td>
<td>X58377</td>
<td>Hematopoiesis, metabolism</td>
</tr>
<tr>
<td>Natural resistance-associated macrophage protein</td>
<td>D50402</td>
<td>Immune response</td>
</tr>
<tr>
<td>Interleukin 1-β</td>
<td>M15330</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>X01394</td>
<td>Inflammation</td>
</tr>
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<td><strong>Group II Cluster (AUUUAULTUAUUUAUUA) stretch (SEQ ID NO:24)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis-inducing (TRAIL) receptor 2</td>
<td>AP016849</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Death receptor 5 (DR5)</td>
<td>AF012535</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>K-ras oncogene protein</td>
<td>M54968</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td>S79851</td>
<td>Metabolism, redox</td>
</tr>
<tr>
<td>Interleukin-10 receptor</td>
<td>U00672</td>
<td>Immune regulation, receptors</td>
</tr>
<tr>
<td>Tyrosine kinase (ELKI) oncogene</td>
<td>M25269</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Accession Number</td>
<td>Function</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------</td>
<td>----------</td>
</tr>
<tr>
<td>6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFKFB)</td>
<td>AF056320</td>
<td>Metabolism, carbohydrate</td>
</tr>
<tr>
<td>Inducible 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (IPFK-2)</td>
<td>AF109735</td>
<td>Metabolism, carbohydrate</td>
</tr>
<tr>
<td>MOPI, basic helix-loop-helix PAS</td>
<td>U29165</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>Cytokine-inducible SH2-containing protein (G18)</td>
<td>AF132297</td>
<td>Signal transduction, inhibitors</td>
</tr>
<tr>
<td>K-Cl cotransporter KCC4</td>
<td>AF10536</td>
<td>Nutrient transport</td>
</tr>
<tr>
<td>Dishevelled 1 (DVL1)</td>
<td>AF006011</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>Guanine nucleotide regulatory factor (LFP40)</td>
<td>U72206</td>
<td>Cellular motility/biogenesis</td>
</tr>
<tr>
<td>Zinc finger containing protein ZNF157 (ZNF157)</td>
<td>U28687</td>
<td>DNA transcription</td>
</tr>
<tr>
<td>Tubulin-folding cofactor C</td>
<td>U61234</td>
<td>Cellular motility/biogenesis</td>
</tr>
<tr>
<td>Interferon (LyIFN-α-1)</td>
<td>E00102</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>α-interferon Gx-1 124</td>
<td>E00124</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Interferon-α</td>
<td>V00542</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Leukocyte interferon-α, clone pIFN105</td>
<td>M28585</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Interferon α J</td>
<td>E0052</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Messenger RNA for human leukocyte interferon</td>
<td>V00542</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Angiotensin/vasopressin receptor AI/AVP</td>
<td>AF054176</td>
<td>Signal transduction, receptors</td>
</tr>
<tr>
<td>c-fos proto-oncogene</td>
<td>V01512</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
<td>---------------------------</td>
</tr>
</tbody>
</table>

**Group III Cluster (WAUUAUUUUAUUUAU) stretch (SEQ ID NO:25)**

<table>
<thead>
<tr>
<th>Interferon (IFN-α-MI)</th>
<th>M27318</th>
<th>Immune response, innate</th>
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<tbody>
<tr>
<td>B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene</td>
<td>M13994</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Chondroitin 6-sulfotransferase</td>
<td>AB017915</td>
<td>Metabolism, sulphate</td>
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<tr>
<td>Sodium bicarbonate cotransporter 3 (SLC4A7)</td>
<td>AF047033</td>
<td>Metabolism, transport</td>
</tr>
<tr>
<td>Cyclooxygenase-2 (Cox-2)</td>
<td>M90100</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Neuralized mRNA</td>
<td>AF029729</td>
<td>Development, neural</td>
</tr>
<tr>
<td>Gro (growth regulated) gene</td>
<td>J03561</td>
<td>Cellular growth</td>
</tr>
<tr>
<td>Epiregulin</td>
<td>D30783</td>
<td>Cellular growth</td>
</tr>
<tr>
<td>A-kinase anchor protein</td>
<td>U17195</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>CREB-binding protein</td>
<td>U47741</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>L-type amino acid transporter subunit LAT1</td>
<td>AF104032</td>
<td>Nutrient accumulation</td>
</tr>
<tr>
<td>Zinc finger DNA-binding motifs (IA-1)</td>
<td>M93119</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>Interleukin 3</td>
<td>M20137</td>
<td>Hematopoiesis</td>
</tr>
<tr>
<td><strong>E16</strong></td>
<td><strong>M80244</strong></td>
<td><strong>Signal transduction, receptors</strong></td>
</tr>
<tr>
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<td>------------</td>
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</tr>
<tr>
<td>2-oxoglutarate dehydrogenase</td>
<td>D10523</td>
<td>Metabolism, energy</td>
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<td>SIRP-β1</td>
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<td>Putative sulphate transporter (PDS)</td>
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<td>Interleukin 2 (IL2)</td>
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<tr>
<td>Post-synaptic density protein 95 (PSD95)</td>
<td>U83192</td>
<td>Neural regulation</td>
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<tr>
<td>Dihydrolipoamide dehydrogenase-binding protein</td>
<td>AF001437</td>
<td>Metabolism, energy</td>
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<td>Type 2 iodothyronine deiodinase</td>
<td>AF993774</td>
<td>Metabolism, hormones</td>
</tr>
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<td>RGP G-protein 3</td>
<td>U27655</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>α-Endosulfine</td>
<td>AF067170</td>
<td>Nutrient transport, regulation</td>
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<tr>
<td>Tumor necrosis factor α inducible A20 zinc finger protein</td>
<td>M59465</td>
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<td>Vascular endothelial growth factor</td>
<td>AF022375</td>
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<tr>
<td>Inhibitor of apoptosis protein-1 (MIHC)</td>
<td>AF070674</td>
<td>Apoptosis, regulation</td>
</tr>
<tr>
<td>A28-RGS14p</td>
<td>U70426</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>TNFR2-TRAF signaling complex protein</td>
<td>L49432</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>Fibrillin-2</td>
<td>U03272</td>
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</tr>
<tr>
<td>Gene/Protein</td>
<td>Accession</td>
<td>Function</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Farnesylated-proteins converting enzyme 1</td>
<td>Y13834</td>
<td>Protein modification</td>
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<td>D-1 dopamine receptor</td>
<td>X58987</td>
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<td>HFB helix-loop-helix protein (HEB)</td>
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<td>IFN-α 1</td>
<td>A12140</td>
<td>Immune response, innate</td>
</tr>
<tr>
<td>Interferon β</td>
<td>X04430</td>
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</tr>
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<td>Lymphotoxin</td>
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<td>Immune response</td>
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<td>Musashi/Nrp-1</td>
<td>AB012851</td>
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<td>Thiamine carrier 1 (TC 1)</td>
<td>AF153330</td>
<td>Metabolism, nutrient transport</td>
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<td>Transcription factor (HTF4A)</td>
<td>M83233</td>
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<td>Phospholipase C-β-2</td>
<td>M95678</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>Onconeural ventral antigen-1 (Nova-1)</td>
<td>U04840</td>
<td>Development/differentiation, neural</td>
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<td>Protein tyrosine kinase mRNA</td>
<td>M59371</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>Tyrosine kinase receptor p145TRK-B (TRK-B)</td>
<td>U12140</td>
<td>Signal transduction, receptors</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td>U27193</td>
<td>Signal transduction, inhibitors</td>
</tr>
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<td>Transcriptional regulatory protein p54</td>
<td>AF045451</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>cAMP phosphodiesterase PDE7 (PDE7A1)</td>
<td>L12052</td>
<td>Signal transduction</td>
</tr>
</tbody>
</table>
In one preferred embodiment, the cytokine is a tumor necrosis factor (TNF), an interferon (IF), a colony stimulating factor (CSF), or an interleukin (IL). In a preferred embodiment, the tumor necrosis factor is TNFα, which is a cytokine shown to play a very important role in rheumatoid arthritis. TNFα can also cause septic shock syndrome and cancer cachexia and play a crucial part in inflammatory diseases such as rheumatoid arthritis.

In another embodiment, the cytokine is an interferon exemplified by interferon-α (IF-α), interferon-β (IF-β), and interferon-γ (IF-γ). The term "interferon" or "IFN" as used herein means the family of highly homologous species-specific proteins that inhibit viral replication and cellular proliferation and modulate immune response. Interferons are grouped into three classes based on their cellular origin and antigenicity, namely, alpha-interferon (leukocytes), beta-interferon (fibroblasts) and gamma-interferon (immunocompetent cells). Recombinant forms and analogs of each
group have been developed and are commercially available. Subtypes in each group
are based on antigenic/structural characteristics.

In a particularly preferred embodiment the interferon is IFα. The terms "IFα,"
"IFNα," "alpha-interferon," "alpha interferon," "interferon alpha," and "human
leukocyte interferon" are used interchangeably herein to describe members of a group
of at least 24 interferon alphas (grouped into subtypes A through H) having distinct
amino acid sequences and that have been identified by isolating and sequencing DNA
encoding these peptides [Viscomi, 1996 Biotherapy 10:59-86]. Both naturally
occurring and recombinant alpha interferons, including consensus interferon such as
that described in U.S. Pat. No. 4,897,471, the contents of which are incorporated by
reference in its entirety, may be used in the practice of the invention. Human
leukocyte interferon prepared in this manner contains a mixture of human leukocyte
interferons having different amino acid sequences. Purified natural human alpha
interferons and mixtures thereof which may be used in the practice of the invention
include but are not limited to Sumiferon RTM interferon alpha-n1 available from
Sumitomo, Japan; Welflerong interferon alpha-n1 (Ins) available from
Glaxo-Wellcome Ltd., London, Great Britain; and Alferon RTM interferon alpha-n3
available from the Purdue Frederick Co., Norwalk, Conn.

In another embodiment, the cytokine is a colony stimulating factor exemplified
by, but not limited to, granulocyte-macrophage colony stimulating factor (GM-CSF),
granulocyte colony stimulating factor (G-CSF), and macrophage colony stimulating
factor (M-CSF).

In an alternative embodiment, the cytokine is an interleukin selected from any
one or more of IL-1 through IL-15 (i.e., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8,
IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, or, IL-15). In a particularly preferred
embodiment, the interleukin is IL-5 which is implicated in inflammation of the
pulmonary airways, especially in asthma. IL-5 is one of the commonest cytokines
which are produced by T-helper cells. It is particularly implicated in control of
eosinophil functions, including selective chemotaxis, growth differentiation,
proliferation, release of cytotoxic proteins and the survival of these cells. IL-5 also
enhances T-cell adhesion to vascular endothelial cells. IL-5 has been detected at the site of the allergic inflammation and in the peripheral blood of patients, its level correlating to the extent of eosinophilia, the severity, and the response of the treatment of asthma. Animal models from murine to monkey have demonstrated the involvement of IL-5 in asthma and the beneficial effects due to the inhibition of the cytokine. In another particularly preferred embodiment, the interleukin is IL-15, which is a T-cell growth factor that can cause excessive cellular proliferation.

In another alternative embodiment, the RNA of interest is encoded by a proto-oncogene. The term "proto-oncogene" refers to a cellular gene which when modified (i.e., by one or more deletion, insertion, or substitution) and/or expressed at increased levels becomes an "oncogene," i.e., a gene that is implicated in transformation of a normal cell to a tumor cell.

For example, the transforming ability of a number of retroviruses has been localized in individual viral oncogenes (generally v-onc). Cellular oncogenes (generally c-onc) present in many species are related to viral oncogenes. It is generally believed that retroviral oncogenes may represent escaped and/or partially metamorphosed cellular genes that are incorporated into the genomes of transmissible, infectious agents, the retroviruses. Some c-onc genes intrinsically lack oncogenic properties, but may be converted by mutation into oncogenes whose transforming activity reflects the acquisition of new properties, or loss of old properties. Amino acid substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the c-ras proto-oncogene family (H-ras, N-ras and K-ras) can give rise to a transforming oncogene by a single base mutation. Other c-onc genes may be functionally indistinguishable from the corresponding v-onc, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is c-myc. Changes in MYC protein sequence do not appear to be essential for oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of c-myc appears to stem from insertion of a
retroviral genome within or near the c-myc gene, or translocation to a new
environment. A common feature in the translocated loci is an increase in the level of
c-myc expression. Gene amplification provides another mechanism by which
oncogene expression may be increased. Many tumor cell lines have visible regions of
chromosomal amplification. For example, a 20-fold c-myc amplification has been
observed in certain human leukemia and lung carcinoma lines. The related oncogene
N-myc is five to one thousand fold amplified in human neuroblastoma and
retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the
proto-oncogene c-myb is amplified five to ten fold. While established cell lines are
prone to amplify genes, the presence of known oncogenes in the amplified regions, and
the consistent amplification of particular oncogenes in many independent tumors of the
same type, strengthens the correlation between increased expression and tumor growth.

The following is a partial list of representative proto-oncogenes, the
overexpression of which has been associated with one or more malignancies. AKT-2
(ovarian and pancreatic cancers), c-erbB-2, also known as HER2/neu (bladder cancer,
breast cancer, lung cancer, and ovarian cancer), mdm-2 (leukemia), c-myb (colon
cancer), c-myc (breast, gastric, colorectal, lung, nasopharyngeal, ovarian, and prostate
cancers), c-ras (lung and ovarian cancers), s-src (breast, colon, and colorectal cancers),
c-yes (colon cancer). Other proto-oncogenes within the scope of this invention, in
addition to those in Table 1, supra, include, without limitation, gsp, gip2, and fos.

While the invention is illustrated using the exemplary above-discussed mRNAs
that are encoded by genes that encode cytokines, proto-oncogenes and growth factors,
the invention nonetheless contemplates any other mRNA which contains an ARE,
including, for example, mRNAs encoding neutrophil activating protein (NAP),
macrophage chemoattractant and activating factor (MCAF), RANTES, macrophage
inflammatory peptide-1a (MIP-1a), macrophage inflammatory peptide-1b (MIP-1b), or
an accessory molecule, such as B7.1, B7.2, ICAM-1, 2 or 3 and cytokine receptors.
OX40 and OX40-ligand (gp34), lymphotactin, CD40, CD40L; or complement
components and their receptors.
E. **Methods For Altering RNA Levels By Recruiting The Exosome To Sequences Other Than The ARE**

The invention provides methods for altering the level of an RNA of interest in a sample by recruiting the exosome to sequences other than the ARE as exemplified by Figure 8. In particular, the invention provides methods for altering the level of an RNA of interest in a sample, comprising: a) providing: i) a sample comprising an RNA of interest, the RNA comprising a first nucleotide sequence; ii) a protein comprising: 1) an exosome recruitment domain capable of specifically binding with a mammalian exosome; and 2) an RNA binding domain capable of specifically binding with the first nucleotide sequence; and iii) a mammalian exosome; and b) contacting the RNA with the protein and with the exosome such that the RNA binding domain binds to the first nucleotide sequence, and the exosome binds to the exosome recruitment domain, thereby altering the level of the RNA.

The terms "exosome recruitment domain" and "peptide sequence capable of specifically binding with a mammalian exosome" refers a peptide sequence (such as at least a portion of an AUBP) that specifically binds with a mammalian exosome. Methods for isolating portions of an AUBP that specifically bind to an exosome are known, and are similar to the methods that were used to characterize the specificity of binding of full length AUBPs [Mitchell *et al.* (1997) *supra*; Allmang *et al.* (1999a) *EMBO J.* 18:5399-5410; Allmang *et al.* (1999b) *supra*; van Hoof *et al.* (1999) *Cell* 99:347-350; and Mitchell *et al.* (2000) *Nat. Struct. Biol.* 7:843-846] with the exception that portions of the AUBPs will be used.

In addition, protein-protein interaction of the exosome recruitment domain of an AUBP may be determined by using co-immunoprecipitation analysis. Briefly, referring to determination of the exosome recruitment domain of the exemplary ARE-binding protein, KSRP, expression vectors that express epitope (Xpress)-tagged polypeptides containing a series of N-terminal or C-terminal deletion mutations of KSRP may be constructed and transiently transfected into HeLa or 293 cells. Cytoplasmic extracts of transfected cells may be prepared and subjected to immunoprecipitation using antibodies against the exosome subunits, such as human
anti-PM-Scl serum, and rabbit anti-hRrp40p and anti-Rrp46p sera, which were previously shown to efficiently precipitate the exosome complex (Chen et al., 2001). Exogenous Xpress-tagged KSRP polypeptides that coprecipitate with the exosome complex may be identified in the precipitates by immunoblot analysis using an anti-Xpress monoclonal antibody. Specific interactions may be verified further using control antisera, such as normal human serum and normal rabbit serum, which should not precipitate any Xpress-tagged KSRP polypeptides. Once a candidate region able to interact with the exosome complex is identified, the minimal region of KSRP required for the interaction may be further defined by shortening the polypeptide from the N-terminus or the C-terminus.

Yet another approach may be used for co-immunoprecipitation analysis. Briefly, referring to determination of the exosome recruitment domain of the exemplary ARE-binding protein, KSRP, expression vectors encoding various portions of KSRP may be used to transfect a previously established stable cell line expressing a tagged exosome component (hRrp4p) (Figure 11) (SEQ ID NO:29) containing calmodulin binding peptide and IgG binding domain of protein A at its C-terminus. The inventors have shown that this tagged hRrp4p was incorporated into the entire exosome complex and allowed purification and determination of the human exosome components. Extracts from the transfected stable cells may be prepared and incubated with calmodulin- or IgG-agarose beads to precipitate the exosome complex. Similarly, the coprecipitated and tagged KSRP polypeptides may be identified by immunoblot analysis as described above. Clear results may be obtained in the precipitation of the exosome complex using calmodulin-agarose beads which do not contain IgG molecules.

It may also be desirable, though not necessary, to verify the nature of the exosome recruitment domain by identifying the exosome component(s) which interacts with the exosome recruitment domain of the exemplary ARE-binding protein, KSRP. The exosome complex contains core subunits, accessory factors, and possibly unidentified factors. The observed interactions between KSRP and the exosome in cells may be indirect via a non-exosomal subunit. Once the interaction between KSRP
and the exosome complex is established and a responsible domain is determined in intact cells, it may be important to determine whether the interaction is direct and which core exosome component(s) directly interacts with KSRP. The cDNAs encoding all ten human exosome core subunits are available in the PI's laboratory. Individual core components may be translated and labeled with $^{35}$S in a reticulocyte lysate system, and incubated with GST or GST-KSRP. The labeled exosome components that specifically associate with GST-KSRP, but not with GST, may be pulled down using GSH-agarose beads, separated by SDS-PAGE, and visualized by autoradiography. If direct interaction is observed in the pull down assays, the minimal region of KSRP identified in the transfection experiments described above and required for interaction with the exosome complex may be further verified in this GST pull down assay. A direct protein-protein interaction between KSRP and an exosome subunit may be further verified in this assay using purified recombinant exosomal protein expressed and purified from E. coli, which would rule out the possibility that contaminated proteins in reticulocyte lysate are responsible for the observed interaction.

The terms "RNA binding domain," "RNA binding polypeptide," and "peptide sequence capable of specifically binding with a nucleotide sequence" refer to a peptide sequence (such as at least a portion of an AUBP or any protein from a cell extract) that specifically binds with an RNA sequence of interest.

Methods for isolating proteins (such as portions of an AUBP) that specifically bind to an RNA sequence of interest are known, such as by examining UV cross-linking of the RNA sequence of interest to cell extracts [Zhang et al. (1993) supra]. Briefly, to isolate an RNA binding polypeptide that binds to an RNA sequence of interest that is expressed in a cell of interest, a cell extract is prepared using the cell of interest, and is incubated with $^{32}$P-labeled RNA sequence of interest isolated from the cell of interest. Binding of the $^{32}$P-labeled RNA sequence of interest to a protein in the cell extract is determined by known methods, such as RNA gel shift analysis or UV cross linking analysis [Zhang et al. (1993) supra]. The protein(s) that bind to the RNA of interest may then be purified (e.g., column fractionation and RNA affinity
chromatography). Optionally, the purified protein may be further characterized by mass spectrometry as disclosed herein.

F. Agents The Alter Binding of ARE to AUBP and/or Alter Binding of AUBP to A Mammalian Exosome

The methods of the invention employ agents that alter the level of binding of an AUBP to an ARE and/or to a mammalian exosome. The term "alters the level of binding" when in reference to the binding of a first molecule (such as an AUBP) to a second molecule (such as an ARE or an exosome) refers to an increase or decrease in the level of binding of the first molecule to the second molecule. The term "increase the level of binding" when in reference to the level of binding of a first molecule to a second molecule in a first sample relative to a second sample, means that the quantity of binding in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. Preferably, the quantity of binding of the first molecule to the second molecule in the first sample is at least 10% greater than, more preferably at least 50% greater than, yet more preferably at least 75% greater than, even more preferably at least 90% greater than, the quantity of binding of the first molecule to the second molecule in the second sample. The term "reduce the level of binding" when in reference to the level of binding of a first molecule to a second molecule in a first sample relative to a second sample, means that the quantity of binding in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. Preferably, the quantity of binding of the first molecule to the second molecule in the first sample is at least 10% lower than, more preferably at least 50% lower than, yet more preferably at least 75% lower than, even more preferably at least 90% lower than, the quantity of binding of the first molecule to the second molecule in the second sample.

The level of binding of an AUBP to an ARE may be determined using routine methods in the art. For example, the quantity of UV cross-linking of the ARE to the AUBP may be determined as disclosed in Zhang et al. (1993) supra. Alternatively,
fluorescently labeled ARE may be incubated with the AUBP (where the AUBP is either in solution or, more preferably, is immobilized on a solid support), and the quantity of fluorescent RNA that is bound to the AUBP determined.

The level of binding of a AUBP to an exosome may be determined using routine methods, such as those taught in Mitchell et al. (1997) supra; Allmang et al. (1999a) EMBO J. 18:5399-5410; Allmang et al. (1999b) supra; van Hoof et al. (1999) Cell 99:347-350; and Mitchell et al. (2000) Nat. Struct. Biol. 7:843-846.

Alternatively, fluorescently labeled exosome may be incubated with the AUBP (where the AUBP is either in solution or, more preferably, is immobilized on a solid support), and the quantity of fluorescent exosome that is bound to the AUBP determined.

The terms "agent," "test agent," "molecule," "test molecule," "compound," and "test compound" as used herein refer to any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic, and inorganic molecule, etc.) obtained from any source (for example, plant, animal, and environmental source, etc.), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, etc.). Agents are exemplified by, but not limited to, antibodies, nucleic acid sequences, and other agents as further described below.

1. Antibodies

In one preferred embodiment, the agent that alters the level of binding of the AUBP to the ARE and/or to the exosome is an antibody that specifically binds to an AUBP or to an exosome. The terms "antibody" and "immunoglobulin" are interchangeably used to refer to a glycoprotein or a portion thereof (including single chain antibodies), which is evoked in an animal by an immunogen and which demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies,
chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, including, for example, Fab, F(ab')2, Fab fragments, Fd fragments, and Fv fragments of an antibody, as well as a Fab expression library. It is intended that the term "antibody" encompass any immunoglobulin (e.g., IgG, IgM, IgA, IgE, IgD, etc.) obtained from any source (e.g., humans, rodents, non-human primates, caprines, bovines, equines, ovines, etc.). The term "polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Monoclonal and polyclonal antibodies may or may not be purified. For example, polyclonal antibodies contained in crude antiserum may be used in this unpurified state.

Antibodies useful in the instant invention include, without limitation, "anti-AUBP antibodies," i.e., antibodies that specifically bind to an AUBP polypeptide, and "anti-mammalian exosome antibodies," i.e., antibodies that specifically bind to a mammalian exosome.

Naturally occurring antibodies may be generated in any species including murine, rat, rabbit, hamster, human, and simian species using methods known in the art. Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as previously described [Huse et al., Science 246:1275-1281 (1989)]. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

The term "specifically binds" and "specific binding" are in reference to the binding of an antibody to an AUBP or to mammalian exosome refer to an interaction of the antibody with one or more epitopes on the AUBP or the mammalian exosome,
where the interaction is dependent upon the presence of a particular structure on the AUBP or on the mammalian exosome.

In one embodiment, the invention's antibodies are characterized by having specific binding activity for the AUBP or exosome of at least about $1 \times 10^5 \text{M}^{-1}$, more preferably at least about $1 \times 10^6 \text{M}^{-1}$, and yet more preferably at least about $1 \times 10^7 \text{M}^{-1}$.

Those skilled in the art know how to make polyclonal and monoclonal antibodies which are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one preferred embodiment, the peptide is conjugated to an immunogenic carrier (e.g., diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin [KLH]). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of monoclonal antibodies directed toward molecules of interest in the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (See, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature 256:495-497 [1975]), as well as the trioma technique, the human B-cell hybridoma technique (See e.g., Kozbor et al. Immunol. Today 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 [1985]). In some particularly preferred
embodiments of the present invention, the present invention provides monoclonal antibodies of the IgG class.

In additional embodiments of the invention, monoclonal antibodies can be produced in germ-free animals utilizing technology such as that described in PCT/US90/02545. In addition, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 [1983]) or by transforming human B cells with EBV virus in vitro (Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96 [1985]).

Furthermore, techniques described for the production of single chain antibodies (See e.g., U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce single chain antibodies that specifically recognize a molecule of interest (e.g., at least a portion of an AUBP or mammalian exosome, as described herein). An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a particular protein or epitope of interest (e.g., at least a portion of an AUBP or mammalian exosome).

The invention also contemplates humanized antibodies. Humanized antibodies may be generated using methods known in the art, including those described in U.S. Patent Numbers 5,545,806; 5,569,825 and 5,625,126, the entire contents of which are incorporated by reference. Such methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes.

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab
expression libraries (Huse et al., Science, 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (e.g., radioimmunoassay, ELISA [enzyme-linked immunosorbent assay], "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays [e.g., using colloidal gold, enzyme or radiisotope labels], Western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays, etc.), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. As is well known in the art, the immunogenic peptide should be provided free of the carrier molecule used in any immunization protocol. For example, if the peptide was conjugated to KLH, it may be conjugated to BSA, or used directly, in a screening assay.

2. Nucleic Acid Sequences

In an alternative embodiment, the agent that alters the level of binding of the AUBP to the ARE and/or to the exosome is a nucleic acid sequence. The terms
"nucleic acid sequence" and "nucleotide sequence" as used herein refer to two or more nucleotides which are covalently linked to each other. Included within this definition are oligonucleotides, polynucleotide, and fragments or portions thereof, DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Nucleic acid sequences which are particularly useful in the instant invention include, without limitation, antisense sequences and ribozymes.

1. Antisense

Antisense sequences have been successfully used to inhibit the expression of several genes [Markus-Sekura (1988) Anal. Biochem. 172:289-295; Hambor et al. (1988) J. Exp. Med. 168:1237-1245; and patent EP 140 308], including the gene encoding VCAM1, one of the integrin αβ1 ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. The terms "antisense DNA sequence" and "antisense sequence" as used herein interchangeably refer to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus, an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA" (i.e., a ribonucleotide sequence whose sequence is complementary to a "sense mRNA" sequence). The designation (-) (i.e., "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (i.e., "positive") strand.

Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter which permits the synthesis of antisense RNA. The transcribed antisense RNA strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation.
Any antisense sequence is contemplated to be within the scope of this invention if it is capable of reducing the level of expression of an AUBP or exosome to a quantity which is less than the quantity of the AUBP or exosome expression in a corresponding control tissue which is (a) not treated with the AUBP antisense sequence or anti-mammalian exosome antisense sequence, respectively, (b) treated with a corresponding sense AUBP sequence or mammalian exosome sense sequence, or (c) treated with a nonsense sequence.

The terms "reducing the level of expression," "diminishing expression" and grammatical equivalents thereof when in reference to expression of an AUBP or exosome, refer to reducing the level of AUBP or exosome expression to a quantity which is preferably at least 20% less than the quantity in a corresponding control tissue, more preferably is at least 50% less than the quantity in a corresponding control tissue, yet more preferably is at least 90% less than the quantity in a corresponding control tissue, and most preferably is at the background level of, or is undetectable by, a Western blot analysis of AUBP or exosome, by immunofluorescence for detection of AUBP or exosome, by reverse transcription polymerase chain (RT-PCR) reaction for detection of AUBP or exosome mRNA, or by in situ hybridization for detection of AUBP or exosome mRNA, respectively. When a background level or undetectable level of AUBP or exosome peptide or mRNA is measured, this may indicate that the AUBP or exosome is not expressed. A reduced level of AUBP or exosome need not, although it may, mean an absolute absence of expression of AUBP or exosome. The invention does not require, and is not limited to, AUBP antisense or exosome antisense sequences which eliminate expression of AUBP or exosome, respectively.

In one preferred embodiment, the antisense sequence is an AUBP antisense sequence. The term "AUBP antisense sequence" refers to an antisense sequence that is capable of hybridizing with at least a portion of an AUBP cDNA under high stringency or low stringency conditions, more preferably under high stringency conditions. The term "AUBP antisense sequence" also can refer to an antisense sequence that has at least 51%, preferably at least 70%, more preferably at least 80%,
yet more preferably at least 90%, and most preferably 100%, homology to the sense strand of dsDNA encoding the AUBP.

In another embodiment, the antisense sequence is a mammalian exosome antisense sequence. The term "mammalian exosome antisense sequence" refers to an antisense sequence that is capable of hybridizing with at least a portion of a mammalian exosome cDNA under high stringency or low stringency conditions, more preferably under high stringency conditions. The term "mammalian exosome antisense sequence" also can refer to an antisense sequence that has at least 51%, preferably at least 70%, more preferably at least 80%, yet more preferably at least 90%, and most preferably 100%, homology to the sense strand of dsDNA encoding any one of the proteins in a mammalian exosome.

Antisense sequences within the scope of this invention may be designed using approaches known in the art. In a preferred embodiment, the antisense sequences are designed to be hybridizable to at least a portion of an mRNA that is encoded by the coding region of the AUBP gene or mammalian exosome genes. Alternatively, antisense sequences may be designed to reduce transcription by hybridizing to upstream nontranslated sequences of the AUBP gene or mammalian exosome genes, thereby preventing promoter binding to transcription factors.

In a preferred embodiment, the antisense oligonucleotide sequences of the invention range in size from about 8 to about 100 nucleotide residues. In yet a more preferred embodiment, the oligonucleotide sequences range in size from about 8 to about 30 nucleotide residues. In a most preferred embodiment, the antisense sequences have 20 nucleotide residues.

However, the invention is not intended to be limited to the number of nucleotide residues in the oligonucleotide sequence disclosed herein. Any oligonucleotide sequence which is capable of reducing expression of an AUBP and/or mammalian exosome is contemplated to be within the scope of this invention. For example, oligonucleotide sequences may range in size from about 3 nucleotide residues to the entire AUBP and/or mammalian exosome cDNA sequence. The art skilled know that the degree of sequence uniqueness decreases with decreasing length, thereby
reducing the specificity of the oligonucleotide for AUBP mRNA and/or exosome mRNA.

The antisense oligonucleotide sequences which are useful in the methods of the instant invention may comprise naturally occurring nucleotide residues as well as nucleotide analogs. Nucleotide analogs may include, for example, nucleotide residues which contain altered sugar moieties, altered inter-sugar linkages (e.g., substitution of the phosphodiester bonds of the oligonucleotide with sulfur-containing bonds, phosphorothioate bonds, alkyl phosphorothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates and short chain alkyl or cycloalkyl structures), or altered base units. Oligonucleotide analogs are desirable, for example, to increase the stability of the antisense oligonucleotide compositions under biologic conditions since natural phosphodiester bonds are not resistant to nuclease hydrolysis. Oligonucleotide analogs may also be desirable to improve incorporation efficiency of the oligonucleotides into liposomes, to enhance the ability of the compositions to penetrate into the cells where the nucleic acid sequence whose activity is to be modulated is located, in order to reduce the amount of antisense oligonucleotide needed for a therapeutic effect thereby also reducing the cost and possible side effects of treatment.

Antisense oligonucleotide sequences may be synthesized using any of a number of methods known in the art, as well as using commercially available services (e.g., Genta, Inc.). Synthesis of antisense oligonucleotides may be performed, for example, using a solid support and commercially available DNA synthesizers. Alternatively, antisense oligonucleotides may also be synthesized using standard phosphoramidate chemistry techniques. For example, it is known in the art that for the generation of phosphodiester linkages, the oxidation is mediated via iodine, while for the synthesis of phosphorothioates, the oxidation is mediated with 3H-1,2-benzodithiole-3-one,1,-dioxide in acetonitrile for the step-wise thiation of the phosphite linkages. The thiation step is followed by a capping step, cleavage from the solid support, and purification on HPLC, e.g., on a PRP-1 column and gradient of acetonitrile in triethylammonium acetate, pH 7.0.
ii. **Ribozyme**

In some alternative embodiments, the agent that alters the level of binding of the AUBP to the ARE and/or to the exosome is a ribozyme. Ribozyme sequences have been successfully used to inhibit the expression of several genes including the gene encoding VCAM1, which is one of the integrin α4β1 ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference].

The term "ribozyme" refers to an RNA sequence that hybridizes to a complementary sequence in a substrate RNA and cleaves the substrate RNA in a sequence specific manner at a substrate cleavage site. Typically, a ribozyme contains a "catalytic region" flanked by two "binding regions." The ribozyme binding regions hybridize to the substrate RNA, while the catalytic region cleaves the substrate RNA at a "substrate cleavage site" to yield a "cleaved RNA product." The nucleotide sequence of the ribozyme binding regions may be completely complementary or partially complementary to the substrate RNA sequence with which the ribozyme binding regions hybridize. Complete complementarity is preferred, in order to increase the specificity, as well as the turnover rate (i.e., the rate of release of the ribozyme from the cleaved RNA product), of the ribozyme. Partial complementarity, while less preferred, may be used to design a ribozyme binding region containing more than about 10 nucleotides. While contemplated to be within the scope of the claimed invention, partial complementarity is generally less preferred than complete complementarity since a binding region having partial complementarity to a substrate RNA exhibits reduced specificity and turnover rate of the ribozyme when compared to the specificity and turnover rate of a ribozyme which contains a binding region having complete complementarity to the substrate RNA. A ribozyme may hybridize to a partially or completely complementary DNA sequence but cannot cleave the hybridized DNA sequence since ribozyme cleavage requires a 2'-OH on the target molecule, which is not available on DNA sequences.

The ability of a ribozyme to cleave at a substrate cleavage site may readily be determined using methods known in the art. These methods include, but are not limited to, the detection (e.g., by Northern blot analysis as described herein, reverse-
transcription polymerase chain reaction (RT-PCR), in situ hybridization and the like) of reduced in vitro or in vivo levels of RNA which contains a ribozyme substrate cleavage site for which the ribozyme is specific, compared to the level of RNA in controls (e.g., in the absence of ribozyme, or in the presence of a ribozyme sequence which contains a mutation in one or both unpaired nucleotide sequences which renders the ribozyme incapable of cleaving a substrate RNA).

Ribozymes contemplated to be within the scope of this invention include, but are not restricted to, hammerhead ribozymes [See e.g., Reddy et al., U.S. Patent No. 5,246,921; Taira et al., U.S. Patent No. 5,500,357, Goldberg et al., U.S. Patent No. 5,225,347, the contents of each of which are herein incorporated by reference], Group I intron ribozyme [Kruger et al. (1982) Cell 31: 147-157], ribonuclease P [Guerrier-Takada et al. (1983) Cell 35: 849-857], hairpin ribozyme [Hampel et al., U.S. Patent No. 5,527,895 incorporated by reference], and hepatitis delta virus ribozyme [Wu et al. (1989) Science 243:652-655].

A ribozyme may be designed to cleave at a substrate cleavage site in any substrate RNA so long as the substrate RNA contains one or more substrate cleavage sequences, and the sequences flanking the substrate cleavage site are known. In effect, expression in vivo of such ribozymes and the resulting cleavage of RNA transcripts of a gene of interest reduces or ablates expression of the corresponding gene.

For example, where the ribozyme is a hammerhead ribozyme, the basic principle of a hammerhead ribozyme design involves selection of a region in the substrate RNA which contains a substrate cleavage sequence, creation of two stretches of antisense oligonucleotides (i.e., the binding regions) which hybridize to sequences flanking the substrate cleavage sequence, and placing a sequence which forms a hammerhead catalytic region between the two binding regions.

In order to select a region in the substrate RNA which contains candidate substrate cleavage sites, the sequence of the substrate RNA needs to be determined. The sequence of RNA encoded by a genomic sequence of interest is readily determined using methods known in the art. For example, the sequence of an RNA
transcript may be arrived at either manually, or using available computer programs (e.g., GENWORKS, from IntelliGenetic Inc., or RNADRAW available from the internet at ole@mango.mef.ki.se), by changing the T in the DNA sequence encoding the RNA transcript to a U.

Substrate cleavage sequences in the target RNA may by located by searching the RNA sequence using available computer programs. For example, where the ribozyme is a hammerhead ribozyme, it is known in the art that the catalytic region of the hammerhead ribozyme cleaves only at a substrate cleavage site which contains a NUH, where N is any nucleotide, U is a uridine, and H is a cytosine (C), uridine (U), or adenine (A) but not a guanine (G). The U-H doublet in the NUH cleavage site does not include a U-G doublet since a G would pair with the adjacent C in the ribozyme and prevent ribozyme cleavage. Typically, N is a G and H is a C. Consequently, GUC has been found to be the most efficient substrate cleavage site for hammerhead ribozymes, although ribozyme cleavage at CUC is also efficient.

In a preferred embodiment, the substrate cleavage sequence is located in a loop structure or in an unpaired region of the substrate RNA. Computer programs for the prediction of RNA secondary structure formation are known in the art and include, for example, "RNADRAW" [ole@mango.mef.ki.se], "RNAFOLD" [Hofacker et al. (1994) Monatshefte F. Chemie 125:167-188; McCaskill (1990) Biopolymers 29:1105-1119]. "DNASIS" (Hitachi), and The Vienna Package.

In addition to the desirability of selecting substrate cleavage sequences which are located in a loop structure or an unpaired region of the substrate RNA, it is also desirable, though not required, that the substrate cleavage sequence be located downstream (i.e., at the 3'-end) of the translation start codon (AUG or GUG) such that the translated truncated polypeptide is not biologically functional.

It is known in the art that the specificity of ribozyme cleavage for a substrate RNA molecule is determined by the sequence of nucleotides which flank the substrate cleavage site and which hybridize with the ribozyme binding regions. Thus, ribozymes can be designed to cleave at different locations within a substrate RNA molecule by altering the sequence of the binding regions that surround the ribozyme catalytic region.
of the ribozyme such that the binding regions hybridize with any known sequence on the substrate RNA.

In addition to varying the sequence of the binding regions to effect binding to different locations on the RNA substrate, the number of nucleotides in each of the ribozyme binding regions may also be altered in order to change the specificity of the ribozyme for a given location on the RNA substrate. The number of nucleotides in a binding region is preferably between about 5 and about 25 nucleotides, more preferably between about 11 and about 15 nucleotides, yet more preferably between about 7 nucleotides and about 10 nucleotides.

One of skill in the art appreciates that it is not necessary that the two binding regions which flank the ribozyme catalytic region be of equal length. Binding regions which contain any number of nucleotides are contemplated to be within the scope of this invention so long as the desirable specificity of the ribozyme for the RNA substrate and the desirable cleavage rate of the RNA substrate are achieved. One of skill in the art knows that binding regions of longer nucleotide sequence, while increasing the specificity for a particular substrate RNA sequence, may reduce the ability of the ribozyme to dissociate from the substrate RNA following cleavage to bind with another substrate RNA molecule, thus reducing the rate of cleavage. On the other hand, though binding regions with shorter nucleotide sequences may have a higher rate of dissociation and cleavage, specificity for a substrate cleavage site may be compromised.

It is well within the skill of the art to determine an optimal length for the binding regions of a ribozyme such that a desirable specificity and rate of cleavage are achieved. Both the specificity of a ribozyme for a substrate RNA and the rate of cleavage of a substrate RNA by a ribozyme may be determined by, for example, kinetic studies in combination with Northern blot analysis or nuclease protection assays.

In a preferred embodiment, the complementarity between the ribozyme binding regions and the substrate RNA is complete. However, the invention is not limited to ribozyme sequences in which the binding regions show complete complementarity with
the substrate RNA. Complementarity may be partial, so long as the desired specificity of the ribozyme for a substrate cleavage site, and the rate of cleavage of the substrate RNA are achieved. Thus, base changes may be made in one or both of the ribozyme binding regions as long as substantial base pairing with the substrate RNA in the regions flanking the substrate cleavage sequence is maintained and base pairing with the substrate cleavage sequence is minimized. The term "substantial base pairing" means that greater than about 65%, more preferably greater than about 75%, and yet more preferably greater than about 90% of the bases of the hybridized sequences are base-paired.

It may be desirable to increase the intracellular stability of ribozymes expressed by an expression vector. This is achieved by designing the expressed ribozyme such that it contains a secondary structure (e.g., stem-loop structures) within the ribozyme molecule. Secondary structures which are suitable for stabilizing ribozymes include, but are not limited to, stem-loop structures formed by intra-strand base pairs. An alternative to the use of a stem-loop structure to protect ribozymes against ribonuclease degradation is by the insertion of a stem loop at each end of the ribozyme sequence [Sioud and Drlica (1991) Proc. Natl. Acad. Sci. USA 88:7303-7307]. Other secondary structures which are useful in reducing the susceptibility of a ribozyme to ribonuclease degradation include hairpin, bulge loop, interior loop, multibranch loop, and pseudoknot structure as described in "Molecular and Cellular Biology," Stephen L. Wolfe (Ed.), Wadsworth Publishing Company (1993) p. 575. Additionally, circularization of the ribozyme molecule protects against ribonuclease degradation since exonuclease degradation is initiated at either the 5'-end or 3'-end of the RNA. Methods of expressing a circularized RNA are known in the art [see, e.g., Puttaraju et al. (1993) Nucl. Acids Res. 21:4253-4258].

Once a ribozyme with desirable binding regions, a catalytic region and nuclease stability has been designed, the ribozyme may be produced by any known means including chemical synthesis. Chemically synthesized ribozymes may be introduced into a cell by, for example, microinjection electroporation, lipofection, etc. In a
preferred embodiment, ribozymes are produced by expression from an expression vector which contains a gene encoding the designed ribozyme sequence.

In a preferred embodiment, the ribozyme is an "AUBP ribozyme" which refers to a ribozyme whose substrate cleavage sequence is designed to partially or completely hybridize with at least a portion of the AUBP mRNA. An "AUBP ribozyme" also refers to a ribozyme that has at least 51%, preferably at least 70%, more preferably at least 80%, and most preferably 100% homology to at least a portion of the AUBP mRNA.

In another embodiment, the ribozyme is a "mammalian exosome ribozyme" which refers to a ribozyme whose substrate cleavage sequence is designed to partially or completely hybridize with at least a portion of the mammalian exosome mRNAs. An "mammalian exosome ribozyme" also refers to a ribozyme that has at least 51%, preferably at least 70%, more preferably at least 80%, and most preferably 100% homology to at least a portion of the mammalian exosome mRNAs.

3. Other Agents


G. Altering The Level of mRNA In A Subject

With respect to administration of the agents of the invention to a subject, it is contemplated that the subject is capable of developing a pathological condition or is suffering from a pathological condition.

As used herein, the terms "pathological condition" and "disease" are used interchangeably to a state, signs, and/or symptoms that are associated with any impairment of the normal state of a living animal or of any of its organs or tissues that interrupts or modifies the performance of normal functions, and may be a response to environmental factors (such as malnutrition, industrial hazards, or climate), to specific infective agents (such as worms, bacteria, or viruses), to inherent defect of the organism (such as various genetic anomalies, or to combinations of these and other factors.

As used herein, the terms "capable of developing a pathological condition" and "at risk for a pathological condition" refers to a subject (e.g., a human) that is predisposed to experiencing a particular disease. This predisposition may be genetic (e.g., a particular genetic tendency to experience the disease, such as heritable disorders), or due to other factors (e.g., environmental conditions, exposures to detrimental compounds present in the environment, etc.). Thus, it is not intended that the present invention be limited to any particular risk, nor is it intended that the present invention be limited to any particular disease.

As used herein, the term "suffering from disease" refers to a subject (e.g., a human) that is experiencing a particular disease. It is not intended that the present invention be limited to any particular signs, symptoms, or disease. Thus, it is intended that the present invention encompass subjects that are experiencing any range of disease, from sub-clinical infection to full-blown disease, wherein the subject exhibits
at least some of the indicia (e.g., signs and symptoms) associated with the particular disease.

Exemplary pathological conditions whose symptoms may reduced (i.e., delayed, reduced in severity, reduced in duration, or prevented), that may be ameliorated by the invention methods and compositions include, without limitation, immune diseases, autoimmune diseases, inflammatory diseases, cancer, and hormone deficiency.

Immune diseases are exemplified by the allergic diseases of pollinosis and atopic dermatitis. Exemplary autoimmune diseases include Sjogren’s disease, type I diabetes, insulin dependent diabetes mellitus, scleroderma, polymyositis, chronic active hepatitis, mixed connective tissue disease, primary biliary cirrhosis, pernicious anemia, autoimmune hemolytic anemia, autoimmune thyroiditis, idiopathic Addison’s disease, vitiligo, gluten-sensitive enteropathy, Grave’s disease, myasthenia gravis, neutropenia, idiopathic thrombocytopenia purpura, pemphigus vulgaris, autoimmune infertility, Goodpasture's disease, bullous pemphigoid, discoid lupus, systemic lupus erythematosus, dense deposit disease, Hashimoto’s disease, fibromyalgia, arthritis selected from rheumatoid arthritis, gouty arthritis, and juvenile rheumatoid arthritis, an autoimmune disease of blood vessels selected from necrotizing angiitis, and granulomatous angiitis, or an autoimmune disease of kidney selected from nephritis, glomerulonephritis, and systemic lupus erythematosus.

Inflammatory diseases are exemplified by sepsis, septic shock, endotoxic shock, inflammatory bowel disease such as Crohn’s disease and ulcerative colitis, multiple sclerosis, inflammatory diseases involving acute or chronic inflammation of bone and/or cartilage in a joint, anaphylactic reaction, nephritis, asthma, conjunctivitis, inflammatory gum disease, systemic lupus erythematosus, insulin dependent diabetes mellitus, pulmonary sarcoidosis, ocular inflammation, allergy, emphysema, ischemia-reperfusion injury, fibromyalgia, an inflammatory cutaneous disease selected from psoriasis and dermatitis, or an arthritis selected from rheumatoid arthritis, gouty arthritis, juvenile rheumatoid arthritis and osteoarthritis.

Cancer is exemplified by, but not limited to, cancer in the trachea, esophagus, colon, epidermis, anus, rectum, lymph node, spleen, lung, breast, prostate, bladder,
uterus, mouth, throat, pancreas, kidney, stomach, ovary, brain, liver, larynx, thyroid, testis, and cervix.

Hormone deficiency diseases are also contemplated to be within the scope of diseases that are amenable to treatment with the invention compositions and methods. The terms "hormones" and "growth factors" include hormone releasing hormones such as growth hormone, thyroid hormone, thyroid releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), leuteinizing hormone, leuteinizing hormone-releasing hormone (LHRH, including the superagonists and antagonists, such as leuprolide, deltirelix, gosorelin, nafarelin, danazol, etc.) that are obtained from natural, human, porcine, bovine, ovine, synthetic, semi-synthetic, or recombinant sources. Hormones and growth factors also include somatostatin analogs such as octreotide (Sandostatin). Other agents in this category of biotherapeutics include medicaments that are used for uterine contraction (e.g., oxytocin), diuresis (e.g., vasopressin), neutropenia (e.g., GCSF), medicaments for respiratory disorders (e.g., superoxide dismutase), RDS (e.g., surfactants, optionally including apoproteins), and the like. Hormone deficiency diseases include, without limitation, Turner Syndrome, infertility, dystrophy, osteoporosis, lactation failure, growth hormone deficiency in adults or in children, short stature in children, and thyroid hormone deficiency.

Is it further contemplated that the invention's agents be administered in a "pharmacologically effective amount." The terms "pharmacologically effective amount," "therapeutically effective amount," and "therapeutic amount" are used interchangeably herein to refer to an amount which is sufficient to achieve a desired result. In particular, a therapeutic amount is that amount that results in altering the level of an mRNA of interest in the treated subject. More preferably, the therapeutic amount also results in reducing one or more symptoms of a pathological conditions associated with overexpression or underexpression of an mRNA of interest in the subject. For example, with respect to tumors, the agents preferably result in reducing a tumor. The term "reducing a tumor" and grammatical equivalents as used herein refer to delaying the onset of tumor development and/or reducing the number, weight, volume, and/or growth rate of the tumor. Preferably, though not necessarily, "reducing a tumor"
refers to complete elimination of the onset of tumor development, and/or complete elimination of an increase in weight, volume, and/or growth rate of the tumor.

One of ordinary skill recognizes that a therapeutically effective amount varies depending on the therapeutic agent used, the subject’s age, condition, and the extent of the disease in the subject. Generally, the dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. The dosage can also be adjusted by the individual physician or veterinarian to achieve the desired therapeutic goal.

As used herein, the actual amount encompassed by the term "pharmacologically effective amount" will depend on the route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the art will recognize.

The dosage amount and frequency are selected to create an effective level of the agent without substantially harmful effects. When administered orally or intravenously, the dosage of a agent will generally range from 0.001 to 1000 mg/Kg/day, more preferably from 0.01 to 100 mg/Kg/day, and most preferably from 0.1 to 10 mg/Kg/day. To achieve these concentrations in the subject, when intended for oral administration, the weight of the agent may be from 0.01% to 90%, more preferably from 0.1% to 50%, and most preferably from 0.1% to 70% of the total weight of the composition. Preferred parenteral dosage units contain from 0.001% to 10%, more preferably from 0.01% to 10%, and most preferably from 0.01% to 1% by weight of any one of the agents.

A pharmaceutically effective amount may be determined using in vitro assays or using in vivo assays (e.g., animal models) known in the art.

Methods of administering a pharmaceutically effective amount of the invention's agents are well known in the art and include, without limitation,
administration in parenteral, oral, intraperitoneal, and sublingual routes. Parenteral routes of administration include, for example, subcutaneous, intravenous, intramuscular, intrastomal injection, and infusion routes.

The agents of the invention may be administered before, concomitantly with, and/or after manifestation of one or more symptoms of a pathological condition. The term "concomitant" when in reference to the relationship between administration of a compound and a disease symptoms means that administration occurs at the same time as, or during, manifestation of the disease symptom. Also, the invention's agents may be administered before, concomitantly with, and/or after administration of another type of drug or therapeutic procedure (e.g., surgery, chemotherapy, radiotherapy, etc.).

Pharmaceutical compositions preferably comprise one or more agents of the present invention associated with one or more pharmaceutically acceptable carrier, diluent or excipient. In preparing such compositions, the active ingredients are usually mixed with or diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule or sachet in which the coating may be gelatin, sugar, shellac, and other enteric coating agents. When the excipient serves as a diluent, it may be a solid, semi-solid, or liquid material which acts as a vehicle, carrier, or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, elixirs, suspensions, emulsions, solutions, syrups, soft and hard gelatin capsules, suppositories, sterile injectable solutions and sterile packaged powders. Examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose.

Pharmaceutically acceptable carriers are known in the art such as those described in, for example, Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). Exemplary pharmaceutically acceptable carriers are sterile saline, phosphate-buffered saline at physiological pH, polyethylene glycols, polypropylene copolymers, and water soluble gels. Other agents that may be included with the invention's compositions include, for example, diluents, fillers, salts, buffers, preservatives (e.g., sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid),
stabilizers, dyes, antioxidants, flavoring agents, lubricating agents (such as talc, magnesium stearate and mineral oil), wetting agents, emulsifying and suspending agents, preserving agents such as methyl- and propylhydroxybenzoates, sweetening agents and/or flavoring agents.

The pharmaceutically acceptable carriers may be liquid, with the compositions being, for example, an oral syrup or injectable liquid. Compositions in solid or liquid form may include an agent which binds to the active component(s) and thereby assists in the delivery of the active components. Suitable agents which may act in this capacity include a monoclonal or polyclonal antibody, a protein or a liposome.

When intended for oral administration, the composition is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following adjuvants may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin, a flavoring agent such as peppermint, methyl salicylate or orange flavoring, and a coloring agent.

In one preferred embodiment, the subject to whom the agents are administered is "capable of developing a pathological condition" in a tissue. This term encompasses any mammal that may be deemed by one of skill in the art to be capable, for any reason (such as family history, genetic profile, expression of a proto-oncogene, exposure to environmental and chemical agents, etc.) of developing one or more symptoms of the pathological condition or disease.

For example, according to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are
selected for administration of the invention's agents that reduce RNA levels. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabeled, e.g., by a radioisotope such as $^{125}$I so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-immunoglobulin or protein A) may be radiolabeled or covalently coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner, but taken from normal as opposed to tumor tissue.

H. Screening Agents That Alter the Level of Degradation of RNA
The invention further provides methods for screening agents that alter the level of RNA in a sample by altering the level of RNA degradation. In particular, the present invention (i.e., briefly shown schematically in Figure 7), provides means to develop drugs and other compositions that are capable of modulating gene expression through control of mRNA turnover. For example, drugs that increase binding affinity between the ARE and destabilizing AUBPs (See, step A in Figure 7) or that strengthen the interaction between the AUBPs and the exosome (See, step B in Figure 7) would promote degradation of ARE-containing mRNAs encoding cytokines, proto-oncogenes, and growth factors. On the other hand, drugs that inhibit interactions between the ARE and destabilizing AUBPs or between the exosome and AUBPs would elevate expression of essential cytokine encoding mRNAs. In addition, the present invention provides a general means to target any mRNA for rapid degradation. All of these methods and compositions find use in the treatment of a wide variety of diseases and pathologies caused by aberrant (i.e., either too much or too little) mRNA expression.

As discussed above, the agents include, for example, antibodies, antisense sequences, ribozymes, molecules from oligonucleotide libraries, peptide libraries, peptidomimetic libraries, oligosaccharide libraries, lipoprotein libraries, glycoprotein or glycolipid libraries, or from chemical libraries. In particular, the invention’s methods screen for agents that interfere with the binding of AUBP to an ARE, and/or interfere with the binding of an AUBP to a mammalian exosome. For example, drugs that increase binding affinity between the ARE and destabilizing AUBPs (See, step A in Figure 7) or that strengthen the interaction between the AUBPs and the exosome (See, step B in Figure 7) would promote degradation of ARE-containing mRNAs encoding cytokines, proto-oncogenes, and growth factors. On the other hand, drugs that inhibit interactions between the ARE and destabilizing AUBPs or between the exosome and AUBPs would elevate expression of essential cytokine encoding mRNAs.

Thus, in one embodiment represented in Figure 7A, the invention provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) an AUBP capable of
specifically binding to the ARE; and iii) a test agent; b) contacting the RNA, the AUBP, and the test agent to produce a test sample; c) contacting the RNA and the AUBP to produce a control sample; and d) detecting an altered level of binding of the RNA to the AUBP in the test sample compared to the level of binding of the RNA to the AUBP in the control sample, thereby identifying the test agent as altering the level of RNA degradation. In one embodiment, the AUBP is immobilized on a solid support.

In another embodiment represented in Figure 7B, the invention provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an AUBP; ii) a purified mammalian exosome capable of specifically binding to the AUBP; and iii) a test agent; b) contacting the AUBP, the exosome and the test agent to produce a test sample; c) contacting the AUBP and the exosome to produce a control sample; and d) detecting an altered level of binding of the AUBP to the exosome in the test sample compared to the level of binding of the AUBP to the exosome in the control sample, thereby identifying the test agent as altering the level of RNA degradation. In one embodiment, the AUBP is immobilized on a solid support. In another embodiment, the exosome is fluorescently labeled with a label selected from DAPI, ANS, Bis-ANS, ruthenium red, cresol violet, and DCVJ.

Having identified lead agent(s) that interfere with binding of the AUBP to an ARE and/or to a mammalian exosome, it may be desirable, though not necessary, to further determine whether the lead agent(s) impact the level of RNA degradation in vitro or in vivo. Alternatively, agents that interfere with the binding of the AUBP to an ARE and/or to a mammalian exosome, may be initially screened using the following in vitro and in vivo tests.

Thus in one embodiment, where in vitro screening is desired, the invention provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an RNA comprising an ARE; ii) a mammalian cell extract; and iii) a test agent; b) incubating the RNA, the cell extract, and the test agent to produce a test sample; c) incubating the RNA and the cell extract to produce a control sample; and d) detecting an altered level of the RNA in the test sample compared to
the level of the RNA in the control sample, thereby identifying the test agent as altering the level of RNA degradation. In one preferred embodiment, the RNA can be inserted into a vector, and the vector used as a substrate in an in vitro transcription system. In another preferred embodiment, the RNA may be labeled in vitro with $^{32}$P to facilitate quantitation of its levels.

In another embodiment, where in vivo screening is desired, the invention provides a method for screening a test agent as altering the level of RNA degradation, comprising: a) providing: i) an expression vector that expresses an RNA comprising an ARE; ii) a mammalian cell; and iii) a test agent; b) transforming the mammalian cell with the vector to produce a transfected cell expressing the RNA comprising the ARE; c) incubating the transfected cell with the test agent to produce a test transfected cell; and d) detecting an altered level of the RNA in the test transfected cell compared to the level of the RNA in the transfected cell, thereby identifying the test agent as altering the level of RNA degradation. In one preferred embodiment, in vivo transcribe RNA may be labeled with $^{32}$P to facilitate quantitation of its levels.

In one embodiment, the assays of this invention are facilitated by the use of databases to record assay results. Maintenance and management of data produced by large-scale screening systems, (e.g., screening of combinatorial libraries) is aided by methods automated information retrieval (e.g., a computer database).

Such a database is useful for a variety of functions, including, but not limited to library registration, library or result display, library and/or result specification, documentation, and data retrieval and exploratory data analysis. The registration function of a database provides recordation/registration of combinatorial mixtures and assay results to protect proprietary information in a manner analogous to the registration/protection of tangible proprietary substances. Library and assay result display functions provide an effective means to review and/or categorize relevant assay data. Where the assays utilize complex combinatorial mixtures for test agents, the database is useful for library specification/description. The database also provides
documentation of assay results and the ability to rapidly retrieve, correlate (or conduct other statistical analysis), and evaluate assay data.

Thus, in some preferred embodiments, the assays of this invention additionally involve entering test agent(s) identified as positive (i.e., having an effect on microtubule polymerization, and/or depolymerization, and/or severing) in a database of "positive" compounds and more preferably in a database of therapeutic lead compounds.

The database can be any medium convenient for recording and retrieving information generated by the assays of this invention. Such databases include, but are not limited to manual recordation and indexing systems (e.g., file-card indexing systems). However, the databases are most useful when the data therein can be easily and rapidly retrieved and manipulated (e.g., sorted, classified, analyzed, and/or otherwise organized). Thus, in a preferred embodiment, the databases of this invention are most preferably "automated" (e.g., electronic [e.g., computer-based]) databases. The database can be present on an individual "stand-alone" computer system, or a component of or distributed across multiple "nodes" (processors) on a distributed computer systems. Computer systems for use in storage and manipulation of databases are well known to those of skill in the art and include, but are not limited to "personal computer systems," mainframe systems, distributed nodes on an inter- or intra-net, data or databases stored in specialized hardware (e.g., in microchips), and the like.

I. Kits For Screening For Compounds That Alter the Level of Degradation of mRNA

The invention further provides kits for screening for a test agent as altering the level of RNA degradation, wherein the kit comprises one or more containers comprising a purified mammalian exosome. In one preferred embodiment, the kit further comprises an AUBP capable of specifically binding to the exosome, more preferably, the AUBP is K homology-type splicing regulatory protein (KSRP), tristetraprolin (TTP), AUF1, or Butyrate Responding Factor (BRF1). In one embodiment, AUBP is attached to a solid surface.
In still another embodiment, this invention provides kits for the practice of any of the methods described herein. The kits comprise one or more containers containing one or more of the assay components described herein. Such components include, but are not limited one or more of the following components: mammalian exosome, AUBPs, ARE-RNA, test agent, reaction media, solid supports (e.g., microtitre plates) with attached components, buffers, labels, and other reagents as described herein.

The kits may optionally include instructional materials containing directions (i.e., protocols) for carrying out any of the assays described herein. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BSA (bovine serum albumin); CFA (complete Freund’s adjuvant); IFA (incomplete Freund’s adjuvant); IgG (immunoglobulin G); IM (intramuscular); IP (intraperitoneal); IV (intravenous or intravascular); SC (subcutaneous); H$_2$O (water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl$_2$ (magnesium chloride); NaCl (sodium chloride); OD$_{280}$ (optical density at 280 nm); OD$_{600}$ (optical density at 600 nm); PAGE
(polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PCR (polymerase chain reaction); PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); RT-PCR (reverse transcription PCR); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); YAC (yeast artificial chromosome); Amersham (Amersham Life Science, Inc. Arlington Heights, IL); ICN (ICN Pharmaceuticals, Inc., Costa Mesa, CA); Amicon (Amicon, Inc., Beverly, MA); ATCC (American Type Culture Collection, Rockville, MD); BioRad (BioRad, Richmond, CA); Clontech (CLONTECH Laboratories, Palo Alto, CA); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Invitrogen (Invitrogen Corp., San Diego, CA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Sigma (Sigma Aldrich Chemical Co., St. Louis, MO); and Stratagene (Stratagene Cloning Systems, La Jolla, CA).

Experimental Procedures

The following procedures were used to carry out the experiments described in the following Examples.

1. Plasmids

DNAs used to produce RNA substrates were subcloned into pCY2, a derivative of pSP64poly(A) (Promega), in which a linker [5'-GGTACCCTCGAG(AAAAAA)_{10}GAATTC-3'] was inserted between the SacI and EcoRI sites. DNA fragments containing the 3' UTR of IL-2 (nt 590-780) or GAPDH (nt 580-810) were subcloned between the HincII and KpnI or HindIII and XbaI sites of pCY2, respectively. ARE^{50} and E4 were constructed by inserting linkers containing the ARE of c-fox mRNA and a non-ARE sequence (5'-TTATGGTGATATATAGTATCTATGTAGATTGTTACTAAACT-3') (SEQ ID NO:1) between the HindIII and SacI sites and PstI and XbaI sites of pCY2,
respectively. Linkers containing the ARE of TNFα mRNA, 5’-ATTATTATTATTATTATTATTATTATTATTATTATTTTAATTTT-3 (SEQ ID NO:28), or its mutant version, in which the underlined TT dinucleotides were changed to GG, were subcloned between the HindIII and XhoI sites of pCY2. DNA fragment encoding calmodulin-binding peptide, TEV protease recognition sequences, and two IgG binding units of protein A was PCR amplified from pBS1479 (from B. Seraphin at EMBL-Heidelberg) and subcloned between the XhoI and XbaI sites of pcDNA3 to obtain pcDNA3-TAP, into which a PCR fragment containing the coding region of hRRP4 was subcloned in frame between the EcoRV and XhoI sites. The myc-tagged TTP vector was described [Stoecklin et al. (2000) supra]. Construction of plasmids expressing GST-AUF1 and GST-TTP are described in Ming et al. (2001) supra.

pBac-KSRP was constructed by subcloning EcoRI-NotI fragment, lacking the first 46 amino acids of human KSRP [Min et al. (1997) supra], into the same sites of pAcHLTA (Pharmingen).

2. Preparation of substrates and in vitro RNA decay and UV crosslinking analysis

Plasmids were linearized with EcoRI or XhoI and used to produce internally \(^{32}\)P-labeled RNA substrates [Chen et al. (2000) supra]. When EcoRI-linearized plasmids were used, RNAs containing a poly(A) tail of 60 residues followed by a G were obtained. XhoI-linearized plasmids yield identical RNAs lacking a poly(A) tail. For 5’ or 3’ end-labeling, unlabeled RNA was prepared, and following alkaline phosphatase treatment was labeled as described [Caruccio et al. (1994) J. Biol. Chem. 269:31814-31821] and gel purified. In vitro analysis of RNA decay and S100 preparation from Jurkat cells were described [Chen et al. (2000) supra]. Typically, \(^{32}\)P-labeled RNA (0.5 ng) was incubated with S100 (15 μg) in 25-μl buffer containing 100 mM KCH\(_3\)COOH, 2 mM Mg(CH\(_3\)COOH)\(_2\), 10 mM Tris (pH 7.6), 2 mM DTT, 10 mM creatine phosphate, 1 μg creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine. UV crosslinking was performed by incubating extract (10-20 μg) and
$^{32}$P-labeled RNA in a 20-μl RNA binding buffer [Chen et al. (2000) supra] at room
temperature for 20 min. 100 U of RNase T1 were added and the samples incubated at
37°C for 15 min, followed by UV irradiation, treatment with RNase A (200 µg per
reaction) and SDS-PAGE separation or immunoprecipitation.

3. Immunodepletion

The exosome was removed from Jurkat S100 by four 2-hr rounds of
immunoprecipitation at 4°C with antisera pre-bound to Protein A-Sepharose. Equal
amounts of supernatants from normal serum- and antiserum-depleted reactions were
analyzed by immunoblotting and subsequently used in RNA decay assays. Precipitates
were washed and used for reconstitution and RNA decay analysis. AUBPs were
depleted by incubating (3 rounds of 2h at 4°C) extracts with poly(U)- and poly(G)-
agarose beads (Sigma).

4. Protein purification and sequence determination

Jurkat cells were transfected with pcDNA3-hRRP4-TAP and stable transfectants
(referred to JurkatRrp4-TAP cells) were selected and pooled. hRrp4p-TAP and
associated proteins were purified using the TAP procedure [Rigaut et al. (1999)
supra]. Cytoplasmic extracts were prepared by lysing frozen cells at 4°C in lysis
buffer [10 mM Hepes (pH7.6), 3 mM MgCl$_2$, 10 mM KCl, 5% glycerol, 0.5% NP-40,
10 µg/ml of aprotinin and leupeptin, 1 mM PMSF]. Nuclei were removed by
centrifugation, washed twice with lysis buffer and extracted with lysis buffer
containing 400 mM KCl. After centrifugation, KCl concentration of supernatants was
adjusted to 100 mM. Cytoplasmic and nuclear extracts were subjected to two-step
affinity purification. After 2-hr incubation with IgG Sepharose (200 µl) at 4°C, the
beads were washed three times with IPP150 [10 mM Tris (pH8.0), 150 mM NaCl,
0.1% NP-40], once with TEV buffer (IPP150 containing 0.5 mM EDTA, 1mM DTT),
and incubated in 1 ml TEV buffer containing 100 U of TEV protease (GIBCO) at RT
°C for 2 hrs. Supernatants were diluted to 4 ml with calmodulin binding buffer [10
mM β-mercaptoethanol, 10 mM Tris-Cl (pH8.0), 150 mM NaCl, 1 mM Mg-acetate, 1
mM imidazole, 2 mM CaCl₂, 0.1% NP40], 100 μl of calmodulin beads (Stratagene) were added, and incubated at 4 °C for 2 hr. After washing, bound proteins were eluted (5 times, 200 μl each) with calmodulin elution buffer [10 mM β-mercaptoethanol, 10 mM Tris-Cl (pH8.0), 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM EGTA, 0.1% NP40].

The 78-kD RNA-binding protein (p78) was purified using established procedures [Chen et al. (2000) supra]. Jurkat cytoplasmic extract (250 mg protein) was loaded on a 5-ml HiTrap SP column. After washing, proteins were eluted with 100 ml buffer with a linear 100 mM to 1 M KCl gradient and p78 was identified by UV crosslinking assay using IL-2 3’UTR RNA as a probe. Fractions containing p78 were pooled and subjected to gel filtration chromatography. Peak p78 fractions were pooled and subjected to RNA affinity chromatography using IL-2 3’UTR as a ligand [Chen et al. (2000) supra].

Purified proteins were concentrated by Centricon YM-10 (Amicon) and analyzed by SDS-PAGE, followed by silver staining. Protein bands were excised and subjected to in-gel reduction, alkylation and tryptic digestion. Digests were desalted and concentrated on a microcolumn packed into GELoader tips [Wilm et al. (1996a) supra]. Peptides were eluted with 1 μl 50% methanol in 5% formic acid directly into a nanospray needle and the eluate subjected to MS and MS/MS analysis on a QSTAR Pulsar quadrupole time-of-flight tandem mass spectrometer (AB/MDS-Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics). Proteins were identified by searching peptide sequence tags, derived from fragment ion spectra of selected peptides, against the non-redundant protein database maintained and updated regularly at the European Bioinformatics Institute (EBI, Hinxton, UK) using the program PepSea (MDS Proteomics).

5. Recombinant proteins and antibodies

GST- and histidine-tagged proteins were produced in BL21(DE3) cells and purified on GSH-Sepharose 4B (Pharmacia Biotech) and Ni-NTA (Qiagen) resins, respectively. Histidine-tagged KSRP was expressed in SF9 cells and purified by Ni-NTA
chromatography. Anti-hRrp4p, anti-hRrp40p, anti-hRrp41p, anti-hRrp46p, and anti-KSRP were described [Min et al. (1997) supra; Mitchell et al. (1997) supra; Brouwer et al. (2001) supra]. Autoimmune sera from PM-Scl patients and polyclonal anti-PM-Scl75 antibodies were also described [Alderuccio et al. (1991) supra]. Monoclonal antibody to HuR and anti-AUF1 were provided by H. Furneaux and G. Brewer, respectively.

EXAMPLE 1

ARE-RNAs Are Degraded Via the Mammalian Exosome

A cell-free RNA decay system was used to assess the degradation pathway mediated by AREs within cytokine and proto-oncogene mRNAs. First, experiments were conducted to examine whether an RNA containing the c-fos ARE (ARE\(^{\text{c-fos}}\)) was degraded via a 3' to 5' pathway, as previously suggested [See, Brewer (1998) supra]. The RNA was 5' or 3' end-labeled, added to a cytosolic extract (S100) of Jurkat cells [Chen et al. (2000) supra], and decay products were analyzed on a denaturing polyacrylamide gel (See, Figure 1, Panel A). The 3'-labeled substrate was rapidly degraded and no decay intermediates were observed (See, Figure 1, Panel A, right panel). In contrast, at least one degradation intermediate and a smear of smaller products were observed with the 5'-labeled substrate (See, Figure 1, Panel A, left panel). The smear most likely corresponds to short RNAs, which are not efficiently degraded. These results suggest that the decay is processive, with some stalling, as no prominent degradation intermediates could be detected. Although the involvement of other pathways in the decay of ARE\(^{\text{c-fos}}\) in this system could not be ruled out, the detection of decay intermediate only with a 5' end-labeled substrates indicates the involvement of a 3' to 5' pathway.

To determine whether the mammalian exosome was responsible for degradation of ARE-RNAs in this system, the exosome was removed by sequential immunoprecipitation with autoimmune PM-Scl serum. Immunoblot analysis with
antibodies against human exosome components including PM-Scl75, hRrp4p, hRrp40p, hRrp41p, and hRrp46p, indicated that the PM-Scl serum removed these exosome subunits (See, Figure 1, Panel B). Capped, either adenylated (See, Figure 1, Panel C, lanes 1-8) or non-adenylated (See, Figure 1, Panel C, lanes 9-16), and uniformly $^{32}$P-labeled RNAs, including two ARE-RNAs (IL-2 3’ UTR and c-fos ARE), and two non-ARE RNAs (a fragment of GAPDH and a non-ARE random sequence [E4]) were produced in vitro and their stability examined in depleted and mock-depleted extracts. As reported [Brewer (1998) supra; and Ford et al. (1999) supra], deadenylation preceded decay of the RNA body in the mock-depleted extract (See, Figure 1, Panel C, lanes 1-40). The presence of an ARE promoted degradation of the RNA body, but little degradation occurred with non-ARE RNAs. However, partial degradation of E4 RNA was observed after a 3-hour incubation (data not shown). Importantly, the ribonucleases responsible for degradation of ARE-RNAs appear not to require a poly(A) tract and the ARE-facilitated turnover of the RNA body is independent of the ARE location, which can be very close to the 3’ end, as in ARE$^{fes}$, or 200 nucleotides away from it, as in IL-2 3’ UTR.

Most importantly, whereas IL-2 3’ UTR and ARE$^{fes}$ RNAs were rapidly degraded in the mock-depleted extract, they were relatively stable in the PM-Scl-depleted extract (See, Figure 1, Panel C, lanes 5-8 and 13-16). The immunodepletion also prevented partial degradation of E4 RNA observed after prolonged incubation (data not shown). Interestingly, accumulation of prominent decay intermediates of adenylated ARE-RNAs identical in size to the non-adenylated ARE-RNAs was observed in the immunodepleted extract, but not in the mock-depleted extract (See, Figure 1, Panel C, lanes 5-8 vs. lanes 1-4). Furthermore, deadenylation of non-ARE-RNAs was not impaired by exosome removal, while deadenylation of ARE-RNAs was only slightly retarded. To verify that the decay intermediates represent deadenylated products, polyadenylated IL-2 3’ UTR RNA was incubated with RNase H in the presence of oligo(dT). This treatment resulted in the disappearance of adenylated RNA and appearance of deadenylated products (See, Figure 1, Panel D, lane 7).
smaller of the two RNA species observed after incubation with the depleted extract (See, Figure 1, Panel D, lane 3), was not affected by RNAS H treatment and was identical in size to deadenylated RNA (See, Figure 1, Panel D, lane 9). That form is deadenylated RNA and it can be concluded that the exosome is not required for deadenylation in this system, but is necessary for degradation of deadenylated ARE-RNA bodies.

To exclude the stabilizing effect of anti-PM Sc1 is due to removal of proteins other than the exosome complex, S100s were immunodepleted with antibodies raised to the exosome components hRrp40p and hRrp46p [Brouwer et al. (2001) supra]. Uniformly labeled non-adenylated RNA substrates were used in these experiments. Either antiserum completely removed both hRrp40p and hRrp46p (See, Figure 2, Panels A and B), and caused ARE-RNA stabilization (See, Figure 2, Panel C). As a control, anti-hRrp46p was incubated with His-tagged hRrp46p prior to immunodepletion. Immunoblot analysis showed that His-hRrp46p blocked depletion of both hRpr40p and hRrp46p (See, Figure 2, Panels A and B). However, a considerable amount of His-hRrp46p was present in the resulting supernatant (See, Figure 2, Panel B). Importantly, preincubation of anti-hRrp46p with His-hRrp46p prevented the stabilization of ARE\textsuperscript{eva} RNA (See, Figure 2, Panel C). No effect on stability of E4 RNA was observed (See, Figure 2, Panel D).

**EXAMPLE 2**

**Purification of the Human Exosome and Molecular Identification of Its Components**

Several components of the PM-Sc1 particle, including PM-Sc1100 and PM-Sc175, are similar to yeast exosome subunits (See, Allmang et al. (1999b) supra). To biochemically analyze the mammalian exosome, it was purified from Jurkat cell extracts using the tandem affinity (TAP) procedure [See, Rigaut et al. (1999) Nat.
Biotechnol. 17:1030-1032]. The TAP tag (Figure 10) (SEQ ID NO:28) was introduced at the C-terminus of hRrp4p (Figure 11) (SEQ ID NO:29); the fusion protein was stably expressed in Jurkat cells. The complex containing hRrp4p-TAP was similar in size to the endogenous complex, as analyzed by gel filtration chromatography (data not shown). hRrp4p-TAP-associated proteins were isolated from either cytoplasmic or nuclear extracts by two-step affinity purification, concentrated, fractionated by SDS-PAGE, and detected by silver staining (See, Figure 3, Panel A). This analysis revealed 14 polypeptides ranging in size from 20 to 120 kDa, labeled as p1 to p14, that were not present in control preparations isolated form cells expressing the TAP tag alone. The relative levels of p1 to p14 varied significantly with the more abundant species migrating between 25 and 40 kDa.

The relevant bands were excised from the gel and analyzed by mass spectrometry (See, Table 2).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide sequence</th>
<th>Match</th>
<th>Yeast homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Core Components</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p7</td>
<td>YIGEVGDIVVGR (SEQ ID NO:2) LDSVLSSMNLPPGELR (SEQ ID NO:3) NCIIISLVTQR (SEQ ID NO:4)</td>
<td>hRrp4p</td>
<td>Rrp4p 43% (52%)</td>
</tr>
<tr>
<td>p8</td>
<td>LSVENVPCTVLCK (SEQ ID NO:5) GGDDLGEIANTLYR (SEQ ID NO:6) GSLDPEIFEMMETGKR (SEQ ID NO:7)</td>
<td>hRrp42p (KIAA0116)</td>
<td>Rrp42p 25% (51%)</td>
</tr>
<tr>
<td>p9</td>
<td>TTTVNISSIDGSAVLK (SEQ ID NO:8) AEFAPSTDAPDK (SEQ ID NO:9)</td>
<td>hRrp43p (OIP-2)</td>
<td>Rrp43p 23% (37%)</td>
</tr>
<tr>
<td>p10</td>
<td>MAEPASVAAESLAGSR (SEQ ID NO:10) GDHVIGIVTAK (SEQ ID NO:11) VVCQPGLR (SEQ ID NO:12)</td>
<td>hRrp40p</td>
<td>Rrp40p 30% (46%)</td>
</tr>
</tbody>
</table>
Several proteins previously believed to be part of the human exosome were identified: p7 is hRrp4p-TAP, whereas p10 and p13 and hRrp40p and hCs14p [Baker et al. (1998) Genetics 149:73-85; and Brouwer et al. (2001) supra], respectively. Two distinct proteins, hRrp41p and hRrp46p [Brouwer et al. (2001) supra], were present within band p12. Bands p8 and p9 contained previously identified gene products KIAA0116 (Genbank Accession No. D29958) and OIP-2 (Genbank Accession No. AF025438), respectively. Several peptide sequences were obtained from band p11 using electrospray tandem mass spectrometry [Wilm et al. (1996a) Anal. Chem. 68:1-8; Wilm, M. et al. (1996) Nature 379:466-469]. One of the peptide and its tandem mass spectrum are shown (See, Figure 3, Panel C). The sequences of the peptides did not match any previously known protein, but were encoded by a segment of human genomic clone AC009060 (See, Figure 3, Panel D). Subsequently, a mouse gene
(Genbank Access. No. AK01201020 that encodes a highly similar protein (92% identity) was found. The two mammalian proteins exhibit sequence similarity to yeast Mtr3p (See, Figure 3, Panel E). Two polypeptides, p1 and p14, were identified that did not exhibit homology to any yeast exosome components. p1 is a previously identified gene product (KIAA0052; Genbank Access. No. P42285) that exhibits striking similarity to yeast Ski2p and Mtr4p [Dangel et al. (1995) Nucleic Acids Res. 23:2120-2126]. Ski2p and Mtr4p were implicated, based on genetic evidence in mRNA turnover and RNA processing, but were not directly shown to associated with the exosome. p14 was found to be M-phase phosphoprotein 6 (MPP-6; Genbank Access. No. BC005242). The identity of p2, p3, p4, p5, and p6 could not be established by mass spectrometry, probably due to insufficient amounts of material in these bands. However, bands p2, p3 and p4, contained PM-Scl100-, whereas bands p5 and p6 contained PM-Scl175 immunoreactive material, as determined by immunoblotting (See, Figure 3, Panel B). PM-Scl100 was previously shown to be predominantly localized in the nucleus [Allman et al. (1999)] supra; and [Brouwer et al. (2001)] supra], and its presence in the cytoplasmic fraction is probably due to leakage of nuclear components. In summary, human homologs were identified for 10 of the yeast exosome subunits, except for a human homolog of yeast Dis3p.

EXAMPLE 3

The Exosome Promotes Rapid Degradation of ARE-RNAs

Experiments were conducted to determine whether the human exosome isolated by either the TAP procedure or immunoprecipitation with antibodies could restore RNA decay in exosome-depleted extracts. Cytoplasmic or nuclear exosomes isolated from hRrp4p-TAP expressing cells, but not control preparations isolated from cells expressing the TAP tag alone (See, Figure 3, Panel A), reconstituted degradation of AREs RNA, but did not affect E4 RNA (See, Figure 4, Panels A and B). Similar restoration of ARE-RNA degradation was observed with precipitates of anti-hRrp40p or anti-hRrp46p, but not control rabbit serum (See, Figure 4, Panel B). The amounts of exosome used for reconstitution experiments were equivalent to those present in mock-depleted S100 (See, Figure 4, Panel A, lane 1). Next,
experiments were conducted to examine whether isolated exosomes discriminate between ARE- and non-ARE-RNAs in the absence of an S100 extract. Extensive degradation of ARE\textsuperscript{fus} RNA was observed with three different amounts of TAP-isolated exosome (See, Figure 4, Panel C). In contrast, at most 50% degradation of E4 RNA was achieved at the highest exosome input. A similar level of E4 RNA degradation was observed only after a 3 hour incubation with non-depleted S100 (data not shown). The purified exosome also exhibited preferential degradation of other ARE-RNAs, including the ARE of TNF\alpha mRNA (wtARE\textsuperscript{inf}) and IL-2 3’ UTR, but did not significantly degrade non-ARE-RNAs, including a mutant version of ARE\textsuperscript{inf} (mARE\textsuperscript{inf}) and GAPDH (data not shown). Similarly ARE\textsuperscript{fus}, but not E4 RNA, was completely degraded upon incubation with anti-hRrp40p or anti-hRrp46p immunoprecipitates (See, Figure 4, Panel D).

EXAMPLE 4

Interaction of AUBPs with the Exosome and Their Role
in Exosome-Mediated Decay of ARE-RNA

The ability of isolated exosomes to preferentially degrade ARE-RNAs was rather surprising. Experiments were then conducted to examine whether the ARE-directed ribonuclease activity reflects an inherent ability of some exosomal components or whether it is due to small amounts of AUBPs that copurify with the exosome. First, experiments were conducted to check for AUBPs by UV crosslinking experiments using ARE\textsuperscript{fus} RNA as a probe. After crosslinking, the products were treated with ribonuclease and immunoprecipitated with anti-exosome antibodies. Several AUBPs were detected in the crude extract (See, Figure 5, Panel A, lane 1, indicated as "Input" shows all of the proteins that are labeled by crosslinking to the ARE\textsuperscript{fus} probe) and most of them, with the notable exception of an abundant 34 kD species, were precipitated by anti-Rrp40p, anti-hRrp46p, or anti-PM-Scl, but not by control sera (See, Figure 5, Panel A). As none of these proteins seemed to be exosome subunit, it was determined whether they correspond to known AUBPs. In the course of identifying proteins that interact with the 3’ UTR of IL-2 mRNA, a 78 kD RNA-binding protein was purified and identified as KSRP, a K-type RNA-binding
protein [Min et al. (1997) Genes Dev. 11:1023-1036]. The 78 kD AUBP was specifically precipitated by anti-KSRP antibodies (See, Figure 5, Panel B), whereas the 40 kD and 34 kD AUBPs were precipitated by antibodies to AUFI and HuR, respectively (See, Figure 5, Panel B). These data suggest that KSRP and AUFI, but not HuR, may interact with the exosome. Immunoblotting analysis confirmed this conclusion, in which AUFI and KSRP, but not HuR, were found in exosome preparations purified from cells expressing hRrp-TAP (See, Figure 5, Panel C). The interaction between the exosome and KSRP or AUFI was further confirmed by coprecipitation experiments using RNase treated extracts. KSRP and AUFI coprecipitated with the exosome components, regardless of RNase treatment (See, Figure 5, Panel D). However, it should be noted that a considerably higher fraction of KSRP was exosome-associated than AUFI. Two other RNA binding proteins, La and nucleolin, were not precipitated (See, Figure 5, Panel D). These results indicate that the observed interactions are specific and RNA independent. Similar results were obtained by gel permeation chromatography (data not shown). To investigate the interaction between the exosome and TTP, also an AUBP, 293 cells were transiently transfected with a vector expressing epitope-tagged TTP. Immunoblotting analysis indicated that TTP was precipitated by anti-PM-Scl, but not by control sera (See, Figure 5, Panel E). Among the AUBPs that interact with the exosome, DSRP was not previously known as such. Experiments were then conducted to determine whether recombinant KSRP specifically recognizes an ARE-RNA in UV crosslinking assays. KSRP bound ARE<sup>fos</sup>, but not E4 RNA, in a concentration-dependent manner (See, Figure 5, Panel F). The binding of KSRP to ARE<sup>fos</sup> RNA was efficiently competed for by unlabeled ARE<sup>fos</sup>, but not E4 RNA (See, Figure 5, Panel G).

Next, experiments were conducted to determine whether any of these proteins is responsible for targeting the exosome to ARE-RNAs. AUBPs were removed from the S100 by incubation with poly(U)-agarose, as most AUBPs were removed by this treatment (See, Figure 6, Panel A). Immunoblotting analysis confirmed that KSRP, AUFI, and HuR were completely removed (data not shown), whereas some AUBPs were not depleted (See, Figure 6, Panel A). In contrast to AUBPs, no significant depletion of exosome components was
observed (See, Figure 6, Panel B). Thus, although the AUBPs interact with the exosome, they are not tightly associated with it. Alternatively, only a small fraction of the exosome associated with AUBPs. Most importantly, ARE\textsubscript{fos} RNA was much more stable in the poly(U)-depleted S100 than in the poly(G)-depleted S100 (See, Figure 6, Panels C and D). Therefore, AUBPs are required for degradation of ARE-RNAs.

Recombinant AUBPs were added to the poly(U)-treated S100 and their effect on ARE-directed RNA decay was examined. While no significant degradation of ARE\textsubscript{fos} RNA was observed upon addition of HuR or AUFI, addition of KSRP or TTP reconstituted degradation of ARE\textsubscript{fos}, but had no effect on E4 RNA (See, Figure 6, Panels C and D). Similarly, addition of KSRP or TTP, but not AUFI to the poly(U)-depleted S100 restored degradation of wtARE\textsuperscript{wt} and IL-2 3’ UTR RNAs, but had no effect on mARE\textsuperscript{wt} and GAPDH RNAs (See, Figure 6, Panel E). To further examine the role of KSRP and TTP in ARE-directed RNA decay, similar experiments were performed using isolated exosomes. By increasing the RNA input (6 times more than the amount used in the results shown in Figure 4, Panel C), no significant decay of ARE\textsubscript{fos} was detected (See, Figure 6, Panel F), indicating that the amount of AUBPs copurifying with the exosome specifically stimulated the degradation of ARE\textsubscript{fos}, although higher inputs of either protein were less effective (See, Figure 6, Panel F). These data strongly indicate that KSRP and TTP are involved in decay of ARE-RNA, probably through their ability to recruit the exosome to the RNA.

From the above, it is clear that the invention provides compositions and methods for altering the level of RNA, and for screening test agents that alter RNA levels. The inventions’s compositions and methods are useful in, for example, increasing the level of an mRNA and conversely in decreasing the level of mRNA by impacting the level of degradation of the mRNA.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in
connection with specific preferred embodiment, it should be understood that the invention as claimed should not be unduly limited to such specific embodiment. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the present invention.
CLAIMS

1. A purified mammalian exosome.

2. The exosome of Claim 1, wherein said exosome comprises at least one of GIVILMVDEK (SEQ ID NO:24) and LTEQLAGPLR (SEQ ID NO:25).

3. A mammalian exosome purified by the process of:
   a) providing:
      i) a host mammalian cell;
      ii) an expression vector comprising a reporter gene operably linked to a nucleic acid sequence encoding a hRrp4p polypeptide;
   b) transforming said host mammalian cell with said vector to produce a transformed cell expressing a protein the comprises hRrp4p polypeptide operably linked to a reporter polypeptide; and
   c) purifying said protein from said transformed cell, wherein the purified protein comprises a purified mammalian exosome.

4. An exosome purified from the transgenic cell line JurkatRrp4-TAP.

5. A transgenic cell line designated JurkatRrp4-TAP.

6. A method for reducing the level of an RNA sequence of interest in a sample, comprising:
   a) providing:
      i) a sample comprising an RNA sequence of interest, said RNA sequence comprising an adenylate uridylate-rich element;
      ii) an AU-rich element-binding protein capable of specifically binding to said adenylate uridylate-rich element; and
ii) an agent that increases the level of binding of said adenylate uridylylate-rich element to said AU-rich element-binding protein; and

b) contacting said RNA sequence, said AU-rich element-binding protein, and said agent such that the level of binding of said adenylate uridylylate-rich element to said AU-rich element-binding protein is increased, and the level of said RNA sequence of interest is reduced.

7. The method of Claim 6, wherein said sample is in vivo in a subject.

8. The method of Claim 7, wherein said subject is human.

9. The method of Claim 7, wherein said subject is capable of developing a pathological condition associated with increased expression of said RNA sequence in a tissue.

10. The method of Claim 9, wherein said pathological condition is an immune disease, inflammatory disease, or cancer.

11. The method of Claim 6, wherein said AU-rich element-binding protein is K homology-type splicing regulatory protein (KSRP) or tristetraprolin (TTP).

12. The method of Claim 6, wherein said RNA sequence of interest is encoded by a gene that encodes a cytokine, proto-oncogene or growth factor.

13. A method for increasing the level of an RNA sequence of interest in a sample, comprising:

a) providing:
i) a sample comprising an RNA sequence of interest, said RNA sequence comprising an adenylate uridylate-rich element;

ii) an AU-rich element-binding protein capable of specifically binding to said adenylate uridylate-rich element, wherein said AU-rich element-binding protein is not AUFI; and

ii) an agent that reduces the level of binding of said adenylate uridylate-rich element to said AU-rich element-binding protein; and

b) contacting said RNA sequence, said AU-rich element-binding protein, and said agent such that the level of binding of said adenylate uridylate-rich element to said AU-rich element-binding protein is reduced, and the level of said RNA sequence of interest is increased.

14. The method of Claim 13, wherein said sample is in a subject capable of developing a hormone deficiency.

15. A method for altering the level of an RNA sequence of interest in a sample, comprising:

a) providing:

i) a sample comprising an RNA sequence of interest, said RNA sequence comprising an adenylate uridylate-rich element;

ii) an AU-rich element-binding protein capable of specifically binding to said adenylate uridylate-rich element;

iii) a purified mammalian exosome capable of specifically binding to said AU-rich element-binding protein; and

iv) an agent that alters the level of binding of said exosome to said AU-rich element-binding protein; and

b) contacting said RNA sequence, said AU-rich element-binding protein, said exosome and said agent such that the level of binding of said
exosome to said AU-rich element-binding protein is altered, and the level of said RNA sequence of interest is altered.

16. The method of Claim 15, wherein said exosome is human.

17. A method for altering the level of an RNA sequence of interest in a sample, comprising:

   a) providing:
      i) a sample comprising an RNA sequence of interest, said RNA sequence comprising a first nucleotide sequence;
      ii) a protein comprising:
            1) a first peptide sequence capable of specifically binding to a mammalian exosome; and
            2) a second peptide sequence capable of specifically binding to said first nucleotide sequence; and
      iii) a mammalian exosome; and
   b) contacting said RNA sequence with said protein and with said exosome such that said first peptide sequence binds to said first nucleotide sequence, and said exosome binds to said second peptide sequence, thereby altering the level of said RNA sequence of interest.

18. A method for screening a test agent as reducing the level of RNA sequence degradation, comprising:

   a) providing:
      i) an RNA sequence comprising an adenylate uridylate-rich element;
      ii) an AU-rich element-binding protein capable of specifically binding to said adenylate uridylate-rich element; and
      iii) a test agent;
b) contacting said RNA sequence, said AU-rich element-binding protein, and said test agent to produce a test sample;

c) contacting said RNA sequence and said AU-rich element-binding protein to produce a control sample; and

d) detecting an increased level of binding of said RNA sequence to said AU-rich element-binding protein in said test sample compared to the level of binding of said RNA sequence to said AU-rich element-binding protein in said control sample, thereby identifying said test agent as reducing the level of RNA sequence degradation.

19. A method for screening a test agent as increasing the level of RNA sequence degradation, comprising:

a) providing:
   i) an RNA sequence comprising an adenylate uridylate-rich element;
   ii) an AU-rich element-binding protein capable of specifically binding to said adenylate uridylate-rich element, wherein said AU-rich element-binding protein is not AUFI; and
   iii) a test agent;

b) contacting said RNA sequence, said AU-rich element-binding protein, and said test agent to produce a test sample;

c) contacting said RNA sequence and said AU-rich element-binding protein to produce a control sample; and

d) detecting a reduced level of binding of said RNA sequence to said AU-rich element-binding protein in said test sample compared to the level of binding of said RNA sequence to said AU-rich element-binding protein in said control sample, thereby identifying said test agent as increasing the level of RNA sequence degradation.
20. A method for screening a test agent as altering the level of RNA sequence degradation, comprising:

a) providing:
   i) an AU-rich element-binding protein;
   ii) a purified mammalian exosome capable of specifically binding to said AU-rich element-binding protein; and
   iii) a test agent;

b) contacting said AU-rich element-binding protein, said exosome and said test agent to produce a test sample;

c) contacting said AU-rich element-binding protein and said exosome to produce a control sample; and

d) detecting an altered level of binding of said AU-rich element-binding protein to said exosome in said test sample compared to the level of binding of said AU-rich element-binding protein to said exosome in said control sample, thereby identifying said test agent as altering the level of RNA sequence degradation.

21. A method for screening a test agent as altering the level of RNA sequence degradation, comprising:

a) providing:
   i) an RNA sequence comprising an adenylate uridylate-rich element;
   ii) a mammalian cell extract; and
   iii) a test agent;

b) incubating said RNA sequence, said cell extract, and said test agent to produce a test sample;

c) incubating said RNA sequence and said cell extract to produce a control sample; and
d) detecting an altered level of said RNA sequence in said test sample compared to the level of said RNA sequence in said control sample, thereby identifying said test agent as altering the level of RNA sequence degradation.

22. A method for screening a test agent as altering the level of RNA sequence degradation, comprising:
   a) providing:
      i) an expression vector that expresses an RNA sequence comprising an adenylate uridylate-rich element;
      ii) a mammalian cell; and
      iii) a test agent;
   b) transforming said mammalian cell with said vector to produce a transfected cell expressing said RNA sequence comprising said adenylate uridylate-rich element;
   c) incubating said transfected cell with said test agent to produce a test transfected cell; and
   d) detecting an altered level of said RNA sequence in said test transfected cell compared to the level of said RNA sequence in said transfected cell, thereby identifying said test agent as altering the level of RNA sequence degradation.

23. A kit for screening for a test agent as altering the level of RNA sequence degradation, wherein said kit comprises one or more containers comprising a purified mammalian exosome.

24. The kit of Claim 23, further comprising an AU-rich element-binding protein capable of specifically binding to said exosome.
25. The kit of Claim 24, wherein said AU-rich element-binding protein is K homology-type splicing regulatory protein (KSRP), tristetraprolin (TTP), AUF1, butyrate response factor-1 (BRF1), Hur, or Hub.

26. The kit of Claim 24, wherein said AU-rich element-binding protein is attached to a solid surface.
FIGURE 2
**FIGURE 7**

**A**
Inhibit AUBP binding to RNA

**B**
Inhibit the interaction between exosome and AUBP

**mRNA degradation**

**ARE**

**AUBPs**

**The exosome**