Regulation of the Stability of Heat-Stable Antigen mRNA by Interplay between Two Novel *cis* Elements in the 3' Untranslated Region

QUNMIN ZHOU, YONG GUO, AND YANG LIU*

Michael Heidelberger Division of Immunology, Department of Pathology and Kaplan Comprehensive Cancer Center, New York University Medical Center, New York, New York 10016

Received 15 September 1997/Returned for modification 28 October 1997/Accepted 31 October 1997

The heat-stable antigen (HSA) is a costimulatory molecule for T-cell activation. Its expression is strictly regulated during lymphocyte development and differentiation. Recent studies using HSA-transgenic mice have demonstrated that this regulated expression is critical for normal development of T and B lymphocytes. However, the mechanisms that control the expression of HSA are largely unknown. HSA mRNA is comprised of a 0.23-kb open reading frame and a 1.5-kb 3' untranslated region (3'UTR). The function of the long 3'UTR has not been addressed. Here we investigate the role of the 3'UTR of HSA mRNA. We show that a 160-bp element, located in the region of nucleotides 1465 to 1625 in the 3'UTR of HSA mRNA, promotes RNA degradation and that this effect is neutralized by a 43-bp fragment approximately 1 kb upstream of the negative *cis* element. Both positive and negative *cis* elements in the HSA mRNA are distinct from other sequences that are known to modulate mRNA stability. These results provide direct evidence that the interplay between two novel *cis* elements in the 3'UTR of HSA mRNA determines cell surface HSA expression by modulating its RNA stability.

The heat-stable antigen (HSA) is a glycosyl-phosphatidylinositol-anchored cell surface protein (1, 5, 34, 39). Recent studies from several groups revealed that HSA plays a major role in regulating interaction between T and B lymphocytes (12, 19, 28, 29), T-cell clonal expansion (10, 18, 29), and induction of immunological memory (30, 41). The expression of HSA is largely restricted to cells of hematopoietic (1, 5, 34, 36) and neuronal (19, 36) origins. Among the hematopoietic cells, HSA is expressed on a variety of cell types, including erythrocytes (39), thymocytes (5), B cells (5, 19, 29, 36), macrophages (8), and Langerhans cells (10).

The expression of HSA is strictly regulated during the development of T and B lymphocytes (7, 27). In the T-cell lineage, HSA is expressed at high levels on a subset of CD4⁺CD8[−] thymocytes and all CD4⁺CD8⁺ immature thymocytes. The levels of HSA on CD4⁺ and CD8⁺ single-positive thymocytes are inversely correlated with their maturity (7). While HSA is not expressed on the majority of peripheral T cells, it is rapidly induced during T-cell activation (15). In the B-cell lineage, HSA is expressed at high levels on pro- and pre-B cells and at intermediate levels on peripheral B cells (27, 42), but it is absent on terminally differentiated plasma cells (27). The strict control of HSA expression suggests that it may also play an important role in lymphocyte development and differentiation. This is supported by findings that constitutive overexpression of HSA in lymphoid tissues leads to defective development of T and B cells (13, 14). However, the molecular basis for the regulated HSA expression has not been elucidated, although it is known that the amounts of cell surface HSA in different tissues and cell lines correlate with the steady-state levels of cellular HSA mRNA (17, 20–22, 44).

mRNA levels are determined by the rate of de novo RNA synthesis as well as the efficiency of posttranscriptional RNA processing, such as polyadenylation, splicing, transport, and degradation of mRNA. Accumulating data have suggested a major role of the 3' untranslated region (3'UTR) in determining the stability of mRNA (3, 9, 16, 18, 26, 40). Many cytokine and oncogene mRNAs have long 3'UTRs and are short-lived (2, 38), primarily due to active degradation by mechanisms involving AU-rich *cis* elements, such as multiple copies of AUUUA. Since HSA mRNA has a very short (0.23-kb) open reading frame (ORF) and a relatively long (1.5-kb) 3'UTR, we investigated whether HSA expression is controlled by its 3'UTR. Here we report that the interplay of *cis* elements in the 3'UTR of HSA mRNA determines cell surface HSA expression by modulating its mRNA degradation.

MATERIALS AND METHODS

Plasmid construction. All cDNA constructs described here were cloned into the mammalian expression vector pCDM8 (Invitrogen, San Diego, Calif.). HSA-Full contains a 1.8-kb fragment of full-length HSA cDNA originally cloned from lipopolysaccharide-stimulated murine spleen cells (28). HSA-DelA has a deletion of a 1,360-bp fragment within the 3'UTR (nucleotides [nt] 440 to 1800). It was generated by digesting HSA-Full with *EcoRI* and *NotI* and religating the remaining plasmid fragment with a 38-bp *EcoRI*-*NotI* linker derived from pBlue-script (Stratagene, San Diego, Calif.). HSA-DelB has a deletion of an 849-bp fragment within the 3'UTR between nt 440 and 1289. It was generated by digesting HSA-Full with *EcoRI*, removing an 849-bp *EcoRI* fragment, and religating the linear plasmid. HSA-pA-M is a mutant of HSA-Full, with the polyadenylation signal (AATAAA) at positions 993 to 998 changed to AgcAAA by PCR-based site-directed mutagenesis.

HSA-N1, -N2, -N3, -N4, -N5, -N6, and N7 consist of HSA nt 1 to 440 and a portion of nt 1289 to 1800. They were generated by inserting PCR fragments corresponding to different regions of the distal 3'UTR (nt 1289 to 1800) into HSA-DelA at *EcoRI* and *NotI* sites.

B7-2 cDNA in the pCDM8 vector has been reported before (43). It contains the entire 969-bp coding region of a costimulatory molecule, B7-2. B7-2-NE is composed of the B7-2 coding region and part of 3'UTR of HSA mRNA. It was generated by ligating a 0.51-kb distal 3'UTR fragment (nt 1289 to 1800) downstream of the B7-2 ORF.

Nine constructs, H3, AccS, AccL, and P1 to 6, were produced to identify the positive *cis* elements. These constructs all contain fragments spanning nt 1 to 440

* Corresponding author. Mailing address: Department of Pathology, New York University Medical Center, 550 First Ave., New York, NY 10016. Phone: (212) 263-7838. Fax: (212) 263-8179. E-mail: liuy01@mcrcr6.med.nyu.edu.

and 1289 to 1800 of HSA-Full. Portions of the fragment from nt 440 to 1289 were inserted between these two fragments. Inserts of various lengths were produced by either restriction enzyme digestion or PCR.

The sequences and orientations of all these cDNA constructs were confirmed by direct DNA sequencing. The primer sequences used for the study are available on request.

Cell culture and DNA transfection. COS cells were cultured in Dulbecco modified Eagle medium containing 5% fetal calf serum at 37°C and were used for transient transfection by the DEAE-dextran method. Briefly, 10⁶ COS cells were seeded in each 100-mm-diameter tissue plate. On the next day, COS cells were incubated with 5 ml of transfection medium (500 µg of DEAE-dextran per ml, 0.1 µM chloroquine, and 15 µg of plasmid DNA) for 2 to 3 h at 37°C. The cells were then shocked with 5 ml of 10% dimethyl sulfoxide in phosphate-buffered saline (PBS) for 2 min, washed twice with serum-free Dulbecco modified Eagle medium, and then cultured for 2 to 3 days. In some experiments, transfectants were split at 24 h after transfection and treated with either actinomycin D (ActD) (5 µg/ml) (Sigma, St. Louis, Mo.) or cycloheximide (CHX) (50 µg/ml) (Sigma) at 40 h after transfection.

Cell surface HSA expression determined by flow cytometry. Three days after transfection, COS cells were detached from plates by incubation with 5 mM EDTA-PBS solution. After being washed with PBS once, COS cells (5 × 10⁵/sample) were incubated with 100 µl of anti-HSA monoclonal antibody (MAb) M1/69 hybridoma supernatants (39) or the unrelated MAb GK1.5 as a control on ice for 30 min. After three washes with staining buffer (PBS containing 1% fetal calf serum), cells were incubated with 100 µl of a 1:100 dilution of fluorescein isothiocyanate-conjugated mouse anti-rat immunoglobulin G (Caltag, Mountain View, Calif.) for another 30 min. After unbound conjugates were removed, cells were fixed in 200 µl of 1% paraformaldehyde-PBS solution and cell surface fluorescence was measured with a FACScan (Becton Dickinson, Mountain View, Calif.).

Northern blot analysis. Cytoplasmic and nuclear RNAs were isolated as described previously (37). Total cellular RNA was isolated by the guanidium isothiocyanate extraction method (6). Poly(A)⁺ RNA was isolated with Oligotex mRNA kits (Qiagen, Santa Clarita, Calif.) according to the manufacturer's instructions. RNA (15 µg/sample) was separated on a 1.2% formaldehyde agarose gel by electrophoresis and transferred to nitrocellulose membranes. The membranes were prehybridized at 42°C for 4 to 10 h by incubation with prehybridization solution (50% formamide, 5× SSPE [1× SSPE is 0.18 M sodium chloride, 0.01 M sodium phosphate, 1 mM EDTA; pH 7.7], 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], and 100 µg of denatured salmon sperm DNA per ml). ³²P-labeled DNA probes were then added and incubated with the membranes at 42°C overnight. At the end of hybridization, membranes were washed once with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, twice with 2× SSC–0.1% SDS at 42°C, and once with 0.2× SSC–0.1% SDS at 60°C, with exchanges of the washing solution at intervals of 20 min. After the final wash, membranes were rinsed with 2× SSC, and then exposed to X-OMAT imaging films (Kodak, Rochester, N.Y.). In some experiments, the densities of the bands were quantified with a densitometer. The predicted sizes of mRNA were calculated based on the predicted transcription initiation site and the cleavage site for polyadenylation: predicted size = size of 5' vector sequence (0.1 kb) + size of cDNA insert + size of 3' vector sequence (0.67 kb).

Nuclear run-on assay. The nuclear run-on assay was performed as described previously (11). The run-on products (10⁷ cpm/sample) were hybridized to nitrocellulose membranes immobilized with DNA fragments corresponding to different portions of HSA cDNA or control DNA.

HSA-transgenic mice. The production of HSA-transgenic mice has been reported previously (44). Briefly, HSA cDNA was inserted into the p1017 cassette transgenic vector (kindly provided by Roger Perlmutter, University of Washington, Seattle). The expression of the HSA cDNA is controlled by a proximal *lck* promoter, and the 3' processing is aided by a portion of human growth hormone gene. Transgenic mice derived from founder A, which has approximately 40 copies of the HSA transgene, were used for the study. Total RNAs, isolated from the spleens and thymuses of HSA transgenic mice, their littermate controls, HSA-deficient mice (33), or C57BL/6j mice, were analyzed by Northern blotting.

RESULTS

Positive and negative regulation of steady-state levels of HSA mRNA by its 3'UTR. To investigate whether the 3'UTR of HSA mRNA contains *cis* elements that control HSA expression, we generated two deletion mutants of HSA cDNA: HSA-DelA and HSA-DelB (Fig. 1a). HSA-DelA has a deletion of a 1,360-bp fragment spanning nt 440 to 1800, while HSA-DelB has a deletion of an 849-bp fragment spanning nt 440 to 1289. These two deletion mutants and the full-length HSA cDNA were cloned in the expression vector pCDM8. Individual plasmids containing each of these HSA cDNAs were cotransfected

with the pCDM8 vector into COS cells by the DEAE-dextran method.

We first examined the HSA protein expression by flow cytometry. As shown in Fig. 1b, COS cells transfected with vector plus HSA-Full, but not those transfected with pCDM8 vector alone, expressed HSA. Interestingly, COS cells transfected with two deletion mutants had dramatically different levels of cell surface HSA: HSA-DelA yielded high levels of HSA, but HSA-DelB produced hardly detectable HSA protein. To determine if the cell surface HSA levels correlate with that of HSA mRNA, we performed Northern blot analyses with the HSA fragment from nt 38 to 440 as the probe (Fig. 1c, upper panel). Significant amounts of HSA mRNA were detected in COS cells transfected with HSA-Full but not in those transfected with vector alone. The highest levels of HSA mRNA were observed in HSA-DelA transfectants, while minimal amounts of HSA mRNA were detected in HSA-DelB transfectants. The differences are not due to transfection efficiency, because comparable levels of the cotransfected vector RNA were detected in all four transfectants (Fig. 1c, middle panel). In addition, the observed differences cannot be accounted for by variations in RNA loading (Fig. 1c, lower panel). These results demonstrate that the 3'UTR determines the levels of steady-state HSA mRNA, which in turn determine the amounts of HSA protein expressed. Since deletion of a middle portion in the 3'UTR (nt 440 to 1289) leads to a reduction in HSA mRNA and protein expression, positive *cis* elements may exist in this region. Moreover, a further deletion of the distal 3'UTR (nt 1289 to 1800) results in the highest levels of HSA expression; it is likely that negative *cis*-acting elements are present in the distal region.

The length of HSA mRNA detected does not correspond to that of HSA cDNA inserts, as three inserts of different lengths, ranging from 0.44 to 1.8 kb, give mRNAs of similar sizes, 1.1 to 1.2 kb. This apparent discrepancy can be explained by utilization of different polyadenylation sites in different constructs. The HSA-Full insert has a functional polyadenylation signal (AATAAA) at positions 993 to 998, utilization of which would lead to an mRNA of about 1.1 kb, as is the majority of HSA mRNA detected. In addition, a less abundant 2.6-kb HSA mRNA was detected, which is expected if the polyadenylation site on the vector is utilized. Since the HSA-DelA insert has no polyadenylation signal, it has to utilize the polyadenylation signal from the vector, which is 0.67 kb downstream. The mature HSA-DelA mRNA should be 1.2 kb. In order to verify this explanation, we mutated the polyadenylation signal in the 3'UTR of HSA-Full. As shown in Fig. 2a, mutation of the polyadenylation signal AATAAA to AgcAAA abrogated the accumulation of the 1.1-kb form of HSA mRNA and led to a preferential accumulation of the 2.6-kb HSA mRNA. These results demonstrate that the 1.1-kb HSA mRNA is due to utilization of the polyadenylation site in inserted HSA cDNA. In addition, since the 2.6-kb mRNA contains both positive and negative elements, it is likely that the function of the positive element dominates over that of the negative element. Moreover, the majority of HSA mRNA in the HSA-Full-transfected cells contained the putative positive but not the negative elements, yet its level was not higher than that in the COS cells transfected with HSA-DelA, which lacks both positive and negative *cis* elements. It is therefore likely that the sole function of the positive *cis* element is to neutralize the activity of the negative *cis* element. The HSA-pA-M and HSA-Full mRNAs were translated with comparable efficiencies, as similar levels of cell surface HSA were detected (Fig. 2b).

The 3'UTR of HSA mRNA does not affect the transcription rate. To determine the transcription rates of HSA-Full and

FIG. 1. Positive and negative control of HSA expression by the 3'UTR of its mRNA. (a) Diagrams of the expression vector pCDM8 and HSA cDNA constructs. The expression vector pCDM8 contains a 358-bp stuffer fragment. The compositions of the full-length HSA cDNA (HSA-Full) and its two deletion mutants, HSA-DelA and HSA-DelB, are also given. The filled bars represent the 231 bp of HSA coding region (bp 73 to 303), and the open bars depict either the 5'UTR (bp 1 to 72) or the 3'UTR (bp 304 to 1800). A polyadenylation signal (sequence AATAAA) is located at positions 993 to 998. HSA-DelA has a 1,360-bp deletion within the 3'UTR (bp 440 to 1800), whereas HSA-DelB has an 849-bp deletion within the middle of the 3'UTR (bp 440 to 1289). CMV, cytomegalovirus; SV40, simian virus 40. (b) The 3'UTR of HSA mRNA controls expression of cell surface HSA as determined by flow cytometry. Histograms of HSA expression are presented; the mean fluorescence values are indicated in each panel. (c) The 3'UTR of HSA mRNA controls the steady-state levels of HSA mRNA. COS cells were transfected with pCDM8 vector alone (Vec) or vector plus either HSA-Full, HSA-DelA, or HSA-DelB. Total cytoplasmic RNA was isolated, and the amount of HSA mRNA was measured by Northern blotting with a 32 P-labeled HSA cDNA fragment spanning bp 38 to 440 (top). As a control for the transfection efficiency, the same blot was hybridized with 32 P-labeled pCDM8 stuffer probe (middle). The amounts of RNA loaded are also shown (bottom).

HSA-DelB inserts in COS transfectants, we performed nuclear run-on assays with immobilized DNA fragments from different regions of HSA cDNA or control actin cDNA, to quantify nascent nuclear RNA. As shown in Fig. 3a, nascent RNA in HSA-Full-transfected COS cells hybridized to cDNA fragments corresponding to nt 38 to 440 and 440 to 1289, while those in HSA-DelB transfectants hybridized to HSA cDNA nt 38 to 440 but not HSA nt 440 to 1289, which is absent in HSA-DelB. Further experiments using other regions of HSA cDNA as probes (Fig. 3b) revealed that the transcripts from HSA-DelB transfectants contain all the HSA sequences in the construct. After being normalized with control actin, the

amounts of run-on products from both HSA-Full and HSA-DelB were almost identical. Thus, HSA-Full and HSA-DelB transfectants transcribed the HSA gene at comparable rates, and the lack of functional HSA mRNA in the HSA-DelB transfectants must be due to posttranscriptional mechanisms.

Stability of HSA mRNAs transcribed from HSA-full and HSA-DelB. As a preliminary approach to determining the mechanisms responsible for posttranscriptional regulation of HSA mRNA, we treated HSA cDNA transfectants with either ActD, which inhibits transcription, or CHX, which blocks both translating and RNA degradation, and then determined the levels of steady-state HSA mRNA by Northern blotting (Fig.

FIG. 2. Mutation of a polyadenylation site in the HSA cDNA alters the size of HSA mRNA but does not significantly affect the level of HSA expression. The polyadenylation signal (AATAAA) at nt 993 to 998 in HSA cDNA was changed to AgcAAA in HSA-pA-M. COS cells were transfected with either HSA-Full or HSA-pA-M. (a) Analyses of HSA mRNA by Northern blotting with an HSA DNA fragment from nt 38 to 440 as a probe. Note the essential elimination of the 1.1-kb band in the pA-M transfectants. (b) Analysis of cell surface HSA expression by flow cytometry. Histograms of HSA expression determined by staining with anti-HSA MAb M1/69 are shown. The mean fluorescence values are indicated in each panel.

4). As shown in Fig. 4, in the absence of either treatment, a high level of HSA mRNA was detected in HSA-Full transfectants but not in HSA-DelB transfectants. No significant decrease of HSA mRNA was observed in HSA-Full transfectants

after 4 h of incubation with ActD. This suggests that HSA mRNA in the HSA-Full transfectants is stable. Incubation with CHX increased HSA mRNA in HSA-Full transfectants by threefold, while the same treatment increased HSA-DelB RNA by ninefold. The major species of HSA mRNA detected in HSA-DelB transfectants were approximately 0.9 to 1.1 kb, much shorter than the 1.7 kb predicted from the construct.

The preferential increase in HSA-DelB RNA by CHX treatment suggests that the RNA is less stable. To test this possi-

FIG. 3. Transcription rates of transfected HSA-Full and HSA-DelB in COS cells as determined by nuclear run-on assay. Nuclei were isolated from COS cells transfected with either HSA-Full or HSA-DelB and were incubated with reaction buffer at room temperature for 20 min. Purified run-on products (10^7 cpm/sample) were hybridized to nitrocellulose membranes that had been immobilized with HSA cDNA fragments and a control actin DNA fragment. Results from two independent experiments using different immobilized fragments are presented in panels a and b.

FIG. 4. Effects of CHX and ActD on the accumulation of cytoplasmic HSA mRNA in COS transfectants. Aliquots of HSA-Full or HSA-DelB transfectants were either left untreated (Nil) or treated with ActD or CHX for 4 h. The amount of HSA mRNA was determined by Northern blotting with the HSA cDNA probe (nt 38 to 440). The relative amounts of the HSA mRNA were determined by densitometry. Numbers above the bottom panels are the relative amounts of HSA mRNA after the drug treatment. The amounts in untreated cells are defined as 1.0. ND, not determined.

FIG. 5. Kinetics of HSA mRNA decay in COS cells transfected with either the full-length HSA cDNA (HSA-Full) or its deletion mutant (HSA-DelB). Aliquots of COS transfectants were incubated with ActD (5 μ g/ml) for different periods starting at 40 h after transfection. Cytoplasmic RNA was then isolated, and the amount of HSA mRNA was determined by Northern blotting with the HSA fragment from nt 38 to 440. (a) Northern blot. The top panel is an autoradiograph of the hybridization. Note that in HSA-DelB transfectants, there are two bands of 1.1 and 0.9 kb, both of which are smaller than the predicted size of mature HSA RNA derived from the construct (1.7 kb). The bottom is a photograph of the nitrocellulose membrane after the transblot to illustrate the amounts of RNA loaded. (b) Kinetics of RNA decay. The amounts of HSA mRNA and total RNA loading in each lane were quantified by densitometry. After normalization by the amount of RNA loading, the relative amount of remaining HSA mRNA after ActD treatment was calculated as the percentage of that detected in untreated COS transfectants. The dominant 1.1-kb band in HSA-Full and the sum of 1.1- and 0.9-kb bands in HSA-DelB were used to calculate the decay kinetics.

bility directly, we incubated HSA-Full and HSA-DelA transfectants with ActD for different periods of time (0, 0.5, 1, 2, and 4 h) and then measured HSA mRNA. The amount of the predominant 1.1-kb HSA-Full RNA and the sum of two bands of 1.1- and 0.9-kb HSA-DelB mRNA were used to determine the decay kinetics of HSA mRNA. As shown in Fig. 5, HSA mRNA in HSA-Full transfectants was stable (half life, >4 h), while HSA-DelB mRNA was rapidly degraded (half-life, 0.5 h).

Evidence for an endonucleic cleavage of the HSA-DelB RNA. Since the HSA-DelB insert does not have a polyadenylation signal sequence with a correct downstream cleavage sequence, the mRNA transcript must use the signal and processing sequences from the vector for efficient polyadenylation. The mRNA transcript is predicted to be of 1.7 kb rather than the 0.9 and 1.1 kb protected by the CHX. To confirm that the 1.7-kb HSA mRNA is transcribed from the HSA-DelB construct, we prepared nuclear and cytoplasmic RNAs from the HSA-Full- and HSA-DelB-transfected COS cells and analyzed the size of the HSA RNA. As shown in Fig. 6a, nuclear RNA derived from HSA-Full was comprised of 1.2- and 2.6-kb species, corresponding to alternative polyadenylation. Both species were present in the cytoplasm, as predicted. HSA-DelB nuclear RNA was composed of 1.7- and >3-kb species. The 1.7-kb RNA is likely to be the full-length transcript of HSA-DelB that utilized the vector polyadenylation signal. The nature of the >3.0-kb species is not understood at present, but such larger-than-expected RNA has been reported to be associated with production of unstable mRNA (35). The 0.9- and 1.1-kb RNAs were not found in the nuclear RNA. Interestingly, the cytoplasmic HSA-DelB mRNA was selectively devoid of the 1.7-kb band while enriched for the 0.9- and 1.1-kb bands. These results strongly suggest that the 1.7-kb RNA is the precursor for the 0.9- and 1.1-kb RNAs in the cytoplasm.

To determine if the HSA-DelB RNA observed in the cytoplasm is polyadenylated, we divided the cytoplasmic RNA into two aliquots; one was left untreated, and the other was incubated with oligo(dT)-coated resin to obtain poly(A)⁺ RNA. As shown in Fig. 6b, all major species of HSA-Full RNA were recovered with high efficiency, while the overwhelming majority of the HSA-DelB RNA did not bind to the oligo(dT) resin. It is unclear at this stage whether the minute amount of the HSA-DelB RNA observed in the poly(A)⁺ fraction is due to incomplete removal of the poly(A)⁻ fraction in the experiment or due to existence of a small amount of polyadenylated HSA DelB RNA. These results demonstrate that most, if not all, of the HSA-DelB RNA lacks the poly(A) tail, which explains the short half-lives of these fragments. Moreover, while the ORF-containing HSA-DelB RNA was rapidly degraded, we have detected, in separate experiments, substantial amounts of HSA-DelB RNA in the cytoplasm by using the HSA (nt 1289 to 1625) and downstream vector sequence as probes (Fig. 6c). Taken together, the results presented in this section suggest that newly transcribed 1.7-kb RNA may be subject to rapid endonucleic cleavage, perhaps at multiple sites, to generate a spectrum of 0.9- to 1.1-kb products; the ORF-containing ones are devoid of poly(A) and are rapidly degraded, while the 3' fragments are polyadenylated and accumulate in a large quantity.

The distal 3'UTR (nt 1289 to 1800) of HSA mRNA promotes degradation of heterologous mRNA. To determine whether the distal 3'UTR fragment alters the stability of heterologous mRNA, we linked this fragment to the 3' end of the B7-2 ORF to produce B7-2-NE. Both B7-2 and B7-2-NE were transfected into COS cells. After drug treatment, RNAs were isolated and the amounts of B7-2 mRNA were measured by Northern blotting. As shown in Fig. 7, in the absence of any treatment, a strong 1.7-kb band was detected in B7-2 transfectants, while a

FIG. 6. Evidence for endonucleic cleavage of the HSA-DelB mRNA. (a) Distinct intracellular localization of the full-length and short HSA-DelB products. COS cells transfected with pCDM8 vector plus either HSA-Full or HSA-DelB were hypotonically lysed. After centrifugation, cytoplasmic RNAs were isolated from the supernatants, while the nuclear RNAs were prepared from the pellets from the same samples. After separation on an agarose gel, the HSA RNAs were detected by using the HSA(38-440) probe (top), while the mRNAs derived from the cotransfected vector were determined by using a stuffer probe (bottom). (b) Most, if not all, of the short, ORF-containing HSA-DelB RNA lacks a poly(A) tail. Equal aliquots of cytoplasmic RNA were either left untreated (cyto), or incubated with oligo(dT)-coated resin; after unbound RNAs were removed, the poly(A)⁺ RNAs were eluted from the column with soluble oligo(dT). The HSA RNAs were detected with the HSA(38-440) probe. (c) Composition of HSA mRNA in COS cell transfectants determined by Northern blotting with either HSA(38-440) (top) or HSA(1289-1625) (middle) and simian virus 40 (SV40) sequence (bottom). Note that in the HSA-DelB transfectant there is a preferential accumulation of the 3' portion of the mRNA detected by distal 3'UTR and downstream vector sequence.

weak 2.3-kb band, the predicted full-length B7-2-NE mRNA, was observed in the B7-2-NE transfectants. ActD decreased the levels of B7-2 and B7-2-NE mRNA, while CHX increased both levels. Both drugs were more effective for B7-2-NE mRNA than for B7-2 mRNA. Moreover, CHX-treated B7-2-NE transfectants had an additional 1.4-kb band, perhaps a truncated product of the 2.3-kb mRNA. These results indicate that the 3'UTR of HSA mRNA promotes degradation of B7-2 mRNA. It should be noted that the degradation of B7-2-NE mRNA is not as efficient as that of HSA-DelB mRNA. In particular, we have observed a significant protection of the intact B7-2-NE by CHX. Thus, the context of the ORF modulates the efficiency of NE-mediated RNA degradation.

Delineation of a negative *cis*-acting element in the distal 3'UTR. To further define the *cis* elements responsible for the accelerated RNA degradation, we generated HSA-N1 to -N4, each with a different deletion within the distal 3'UTR (nt 1289 to 1800) (Fig. 8a), and measured the RNA and protein expression of the mutants in COS transfectants (Fig. 8b and c). It was anticipated that deletions of negative *cis*-acting elements would restore HSA expression. However, in three deletion mutants, HSA-N1, -N2, and -N4, HSA expression was not restored as judged by the amounts of HSA mRNA and protein. In addition, much like the HSA-DelB mRNA, the majority of the HSA RNAs detected were either smaller or larger than predicted. However, one construct, HSA-N3, restored HSA expression to a level comparable to that in HSA-Full transfectants. The major mRNA species in N3 transfectants was 1.5 kb, the predicted size of mature N3 mRNA.

HSA-N3 has a 135-bp deletion from HSA-N4 in the region of nt 1465 to 1600, thus raising the possibility that this fragment may be largely responsible for the accelerated RNA degradation. Furthermore, since HSA-N2 and HSA-N4 share 30 bp, it

FIG. 7. The distal 3'UTR (nt 1289 to 1800) of HSA mRNA promotes degradation of heterologous mRNA. Northern blot analysis for B7-2 mRNA in COS cells transfected with either B7-2 or B7-2-NE is shown. The B7-2 insert is comprised of a 1.0-kb B7-2 cDNA ORF, while B7-2-NE is a chimera cDNA consisting of the B7-2 ORF and part of the 3'UTR (nt 1289 to 1800) of HSA mRNA. The sizes of the predicted mature mRNAs are 1.8 kb for B7-2 and 2.3 kb for B7-2-NE. Aliquots of COS cells transfected with B7-2 cDNA or B7-2-NE were left untreated (Nil) or treated with ActD or CHX for 4 h. The total RNA was then isolated, and the amount of B7-2 mRNA was determined by Northern blotting with a ³²P-labeled B7-2 probe. The loading of RNA is shown in the bottom panels. Numbers above the bottom panels are the relative amounts of B7-2 mRNA after the drug treatment. The amounts in untreated cells are defined as 1.0.

FIG. 8. Delineation of the negative *cis* elements within the 3'UTR of HSA mRNA. (a) Diagram of constructs, the predicted sizes of their mature RNAs, and relative expression of HSA protein. All constructs are composed of HSA nt 1 to 440 plus a portion of the nt 1289 to 1800 fragment. Cell surface HSA protein expression as determined by flow cytometry with anti-HSA MAb M1/69 is shown. The data presented are relative amounts of cell surface HSA expression based on the mean fluorescences from three independent experiments, with standard deviations (SD). The expression of HSA-Full is defined as 100. (b and c) Northern blot analysis of HSA mRNA in COS transfectants. COS cells were transfected with HSA-Full, HSA-DelB, or N1 to N6. The amounts of HSA mRNA in the cytoplasm were determined by using HSA(38-440) as a probe. The RNA loading is shown at the bottom. SV40, simian virus 40.

is tempting to suggest that this 30-bp fragment may be the negative *cis* element. We created HSA-N5, -N6, and -N7 to test these possibilities. As shown in Fig. 8c, deletion of nt 1465 to 1625, as in the N5 construct, restored HSA expression as judged by a selective increase of a 1.6-kb HSA mRNA and high levels of HSA protein. Thus, this region is necessary for efficient RNA degradation. Moreover, the lack of mature HSA mRNA in N6-transfected COS cells indicated that the fragment from nt 1465 to 1625 is sufficient to abolish accumulation of mature mRNA. Interestingly, significant amounts of two small HSA mRNAs were detected, which suggests that the fragment from nt 1465 to 1625 is only sufficient to convey

partial degradation of HSA mRNA. The 30-bp fragment shared by HSA-N2 and -N4 did not promote RNA degradation and has no effect on protein expression, as in the case of the N7 construct. Taken together, the results presented in this section demonstrate that a 160-bp fragment in the distal 3'UTR is both necessary and sufficient to promote HSA mRNA degradation.

A 43-bp element inhibits degradation of HSA mRNA. In order to delineate the positive *cis* element that neutralizes the function of the negative *cis* element, we generated a large series of deletion mutants that contain the HSA sequence from nt 1 to 440 and nt 1290 to 1800 and overlapping fragments that

FIG. 9. A 43-bp fragment is necessary and sufficient to neutralize the effect of the negative *cis* element residing within nt 1289 to 1800. (a) Diagram of the constructs and the relative amounts of cell surface HSA expression. All constructs contain the HSA nt 1 to 440 and 1289 to 1800 fragments. A portion of the putative positive regulatory region was inserted between the two fragments. Except for a reverse orientation of the H3 fragment, all fragments are inserted in their natural orientation. Cell surface HSA protein expression was determined by flow cytometry with anti-HSA MAb M1/69. The data presented are relative HSA expression based on the mean fluorescence of the samples, with standard deviations (SD), and are summaries of two or three independent measurements for each group. The expression of HSA-Full is defined as 100. The sequence of the minimal *cis* element is shown at the bottom. (b) Steady-state HSA mRNA levels in COS cells transfected with HSA-Full, DelB, AccS, P1, P2, P3, and AccL. Total RNA was used for the study. HSA(38-440) was used as the probe in the top panel. Bottom, the same blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to quantify the RNA loading. (c) As in panel b, except that HSA mRNAs in H3, P4, and P5 transfectants were determined. (d) A 43-bp oligonucleotide contains all necessary signal for neutralization of the negative *cis* element residing between nt 1289 and 1800. As in panels b and c, except a photograph of the gel is used to illustrate RNA loading (bottom).

cover all sequence between nt 440 and 1289 (Fig. 9a). These constructs were transfected into COS cells, and the cell surface HSA expression was determined by flow cytometry with anti-HSA MAb M1/69. The data presented in Fig. 9a show relative HSA expression, with that of HSA-Full defined as 100. Insertion into HSA-DelB of most fragments that contain a 43-bp fragment, such as AccL, P4, P5, and P6, restored the cell surface HSA expression to levels comparable to that of HSA-Full. These results indicate that extension of the 43-bp fragment in the 3' end does not increase the efficiency of the positive *cis* element. Further experiments revealed that removing the sequence adjacent to either end of the 43-bp fragment

had no effect on the fragment (data not shown). These results demonstrate that most, if not all, of the positive activity within the nt 440 to 1289 region resides within a 43-bp fragment between nt 440 and 482. The sequence of the 43-bp fragment is shown at the bottom of Fig. 9a. Moreover, the H3 fragment was inserted opposite to its natural orientation, which results in reversion of the 43-bp fragment. Since this fragment did not restore HSA expression, it is likely that the function of the 43-bp fragment is orientation dependent.

We have analyzed the RNA accumulation in COS cells transfected with all mutants listed in Fig. 9a. Total RNA was isolated, and the HSA mRNA was determined by Northern

FIG. 10. Preferential utilization of the distal 3' polyadenylation site in the endogenous HSA gene. (a) Sizes of the HSA mRNA in three B-cell lines, M12, A20, and CRCS, detected with the HSA(38-440) probe. RNA loading was determined with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (bottom). (b) Comparison of HSA mRNAs in HSA-transgenic mice (TG), their transgene-negative littermates (control) (WT), C57BL6/j mice (B6), and HSA-deficient mice (KO). Total RNAs isolated from spleens (left panel) and thymuses (right panel) were separated on an agarose gel and probed with either HSA(38-440) or GAPDH.

blotting. The results for all of the mutants are presented in Fig. 9b to d. Insertion of AccL and P4 to -6 fragments, but not P1 to -3 fragments, restored the HSA mRNA levels to that of HSA-Full. Two major species of HSA mRNA, of 1.1 and 2.3 kb, were observed in AccL transfectants, most likely because AccL contains a polyadenylation signal. The sizes of P4 to -6 were as predicted. As shown in Fig. 9d, a difference of a 43-bp fragment between HSA-DelB and P6 has a major effect on the amount of HSA mRNA accumulated. These results clearly demonstrate that the 43-bp *cis* element promotes accumulation of HSA mRNA by neutralizing the negative *cis* element residing within the nt 1289 to 1800 region.

Preferential utilization of the second polyadenylation site in the endogenous HSA gene. We have shown that HSA mRNA that is accumulated in the HSA-Full-transfected COS cells preferentially utilizes the first polyadenylation site, which results in an absence of the negative *cis* element identified in this study. Since the HSA cDNA used in this study lacks the necessary downstream element for polyadenylation (25), the preferential utilization of the first poly(A) signal is most likely due to inactivation of the second poly(A) site. In cell lines that express endogenous HSA, such as A20, M12, and CRCS, the majority of HSA mRNA was 1.9 kb, presumably due to utilization of the second polyadenylation site (Fig. 10a). A small fraction of HSA mRNA was 1.1 kb and thus utilized the first poly(A) signal. To directly compare the polyadenylation sites of the HSA cDNA and endogenous HSA gene, we compared the sizes of transgenic HSA mRNA with those of the endogenous HSA gene. The vector used for the HSA-transgenic mice consists of a proximal *lck* promoter, full-length HSA cDNA, and a portion of the human growth hormone gene for RNA processing. The mice used for the current study were derived from founder A, which had approximately 40 copies of the transgene (data not shown). Northern blot analysis revealed that the HSA transgene is expressed at a higher level in the thymus than in the spleen (Fig. 10b), which is consistent with the property of the vector. In the spleen, the transgene was expressed at a level comparable to that of the endogenous HSA gene. Most importantly,

while the major species of the HSA RNA in littermate control mice was 1.9 kb, the predominant species of the HSA derived from the transgene was 1.1 kb. Moreover, the majority of the endogenous HSA mRNA contain both positive and negative *cis* elements identified in the study. The fact that the mRNA derived from HSA cDNA in the transgenic mice cannot efficiently utilize the second polyadenylation signal at its distal 3' end can be due to a lack of downstream sequence. Alternatively, it is possible that there is a limiting factor that inhibits the utilization of the first polyadenylation signal in normal mice but that this putative inhibitor is titrated out due to increased HSA expression in the transgenic mice.

DISCUSSION

HSA cDNA consists of a short ORF (231 bp) and a long 3'UTR (1,497 bp). Partial deletion of the 3'UTR abolishes the mRNA accumulation in the cytoplasm and, consequently, the cell surface expression of HSA. Since further truncation of the 3'UTR restores HSA expression, we conclude that the region between nt 1289 and 1800 contains a negative *cis* element(s) that down-regulates the expression of HSA. The addition of a fragment from nt 440 to 1289 neutralizes the negative effect of the fragment from nt 1289 to 1800. These results indicate that the 3'UTR of HSA mRNA plays an important role in controlling HSA expression.

Several lines of evidence support the notion that these *cis* elements regulate the stability of HSA mRNA. First, the transcription rate of the HSA mRNA is not altered by changing the composition of the 3'UTR. Second, the accumulation of cytoplasmic HSA mRNA is significantly enhanced by treatment with CHX, a protein synthesis inhibitor that has been shown to increase RNA stability for a large number of genes studied. Most importantly, the half-life of RNA derived from HSA-DelB is at least eightfold shorter than that of RNA derived from the full-length HSA cDNA.

Similar to most unstable RNAs, the fragment from nt 1289 to 1800 of HSA mRNA contains multiple stretches of AU-rich elements, including one AUUUA motif. However, deletion analysis reveals that this AUUUA motif is neither necessary

a. Degradation pathway of HSA-delB RNA

b. Alternative polyadenylation and RNA stability

FIG. 11. (a) Mechanism of degradation of HSA-DelB mRNA. Newly synthesized full-length RNA transcripts were endonucleically cleaved by a CHX-independent mechanism. The 5' fragments that contain the HSA ORF but lack a poly(A) tail are rapidly degraded by a CHX-sensitive mechanism, while the 3' fragments that contain a poly(A) tail but not the ORF accumulate. CMV, cytomegalovirus. NE, negative *cis* element. (b) Implications of alternative polyadenylation on the mechanisms of posttranscriptional regulation of HSA gene expression. PE, positive *cis* element.

nor sufficient for promoting HSA mRNA degradation. Deletions including this motif (as in the cases of HSA-N1 and HSA-N2) do not restore expression of HSA. In contrast, HSA-N3, which retains this motif, still expresses HSA at a high level. Such a lack of effect of the AUUUA element on mRNA accumulation can be due to the fact that it is not multimerized, as in the case of proto-oncogenes and cytokines (2, 38). Alternatively, as suggested by recent studies, a longer motif, UUAUUUAUU, may be needed for RNA destabilization (24, 45).

Our deletion analyses have revealed that a 160-bp fragment is both necessary and sufficient for rapid degradation of HSA mRNA. Deletion of the element in HSA-N5 leads to accumulation of substantial amounts of HSA mRNA and cell surface HSA, while addition of this fragment to HSA-DelA abrogated accumulation of mature HSA mRNA and cell surface HSA

protein. Interestingly, this 160-bp fragment does not appear to contain all the activities of the fragment from nt 1289 to 1800 in the HSA-DelB construct. Thus, while HSA-N6 transfectants lack mature HSA mRNA, they do accumulate short HSA mRNA. It is therefore likely that other sequence within the nt 1289 to 1800 region may be responsible for rapid degradation of the apparently truncated HSA RNA. With a few notable exceptions (4, 23, 31, 32), RNA degradation in mammalian cells proceeds rapidly after initiation, and the intermediate degradation products are difficult to isolate. The substantial amount of the short RNA accumulated in the HSA-N6-transfected COS cells and CHX-treated HSA-DelB transfectants may facilitate the study of the mechanism of RNA degradation.

An important aspect of the current study is the identification

of a 43-bp fragment that can neutralize the negative *cis* element residing between nt 1289 and 1800. While multiple labile RNA contains negative *cis* elements (2, 24, 38, 45), the existence of positive *cis* elements has been rarely reported (3, 9). One such element has been reported for the transferrin receptor mRNA (4, 23, 31, 32). However, no obvious homology can be found between these two positive *cis*-acting elements. It is therefore likely that the two elements may act by different mechanisms. Moreover, in the published studies (4, 23, 31, 32) the positive *cis* element is adjacent to the negative *cis* element, whereas in the HSA mRNA the positive and negative *cis* elements are about 1 kb apart in the linear sequence. The positive *cis* element described in this study does not have homology with other sequences implicated in RNA regulation. It is therefore likely that it prevents RNA degradation by a novel mechanism.

The coexistence of stabilizing and destabilizing *cis*-acting elements within the 3'UTR, as described in this study, has been rarely reported (18). However, mRNA for the transferrin receptor possesses both of these two types of *cis* elements (4, 23, 31, 32). Recent studies indicate that cellular factors that interact with these distinct elements can regulate the expression of transferrin receptor mRNA in response to variation in iron concentration (23, 32). The presence of positive and negative *cis* elements within the 3'UTR of HSA mRNA suggests that the expression of HSA can be regulated by selectively activating or silencing the putative *trans* elements that act on the *cis* elements described in this paper. We show here that deletion within the 3'UTR of HSA selectively inactivates either positive or negative *cis* elements, which makes it possible to test the hypothesis by using a transgenic approach.

While the primary focus of the current study is to delineate the *cis* elements in the 3'UTR of the HSA mRNA that control its stability, several novel observations made in the study may have important implications for the mechanism of mammalian mRNA degradation, which has been difficult to study due to lack of degradation intermediates. Several species of HSA-DelB of approximate 1.0 kb are likely to be such intermediates. Thus, the full-length HSA-DelB transcript is detected in the nuclei but not in the cytoplasm. In contrast, the short pA⁻ ORF-containing fragments were observed in the cytoplasm but not in the nuclei. Moreover, a large amount of HSA-DelB RNA, which contains the 3'UTR plus downstream vector sequence but not the ORF, is detected in the cytoplasm. These findings lead us to propose a model for degradation of HSA-DelB RNA (Fig. 11a). First, the HSA transcript is cleaved at multiple sites to generate a spectrum of small fragments of approximately 1.0 kb. The 5' fragments, which contain the ORF but not poly(A), are subject to a rapid, CHX-sensitive degradation, while the 3' fragments, which contain the poly(A) tail, accumulate in a large amount. The relative speed and mechanism of the first endonucleic cleavage can be modulated by the ORF. For instance HSA-DelB cleavage is not prevented by CHX, while that for the B7-2-NE is CHX sensitive.

The HSA gene has two potential polyadenylation sites. Utilization of the first will produce a shorter mRNA devoid of the negative *cis* element. Thus, alternative polyadenylation will produce HSA mRNA subject to distinct posttranscriptional mechanisms (Fig. 11b). Nevertheless, most cell types in animals utilize the second polyadenylation site, and the mRNAs are thus likely to be subject to the posttranscriptional mechanism defined in the current study.

The expression of HSA is under stringent developmental control. In the T-cell lineage, HSA is expressed among 50% of the CD4⁻ CD8⁻ and all CD4⁺ CD8⁺ thymocytes (15). The level of HSA in T cells appears to correlate inversely with

T-cell maturity (7). Hough et al. (13) have recently demonstrated that transgenic mice that constitutively express HSA at high levels have a very small thymus and a selective defect in generating either CD4 or CD8 T cells. These results indicate that down-regulation of HSA is a necessary step for T-cell maturation in the thymus. Clearly, understanding of the strict regulation of HSA expression will provide insights into the mechanism of lymphocyte development and activation.

ACKNOWLEDGMENTS

Qunmin Zhou and Yong Guo contributed equally to the study.

We thank Peter Nielsen for HSA-deficient mice, Robert Schneider for helpful discussion, and Mary Lee and Fran Hitchcock for critical reading of the manuscript.

This study was supported by Public Health Service grant AI-32981 from the National Institutes of Health and by the Searle Scholar Program. Part of this study was performed when Y.G. was supported by NIH training grant CA09161-18.

REFERENCES

1. Alterman, L. A., N. Crispe, and C. Kinnon. 1990. Characterization of the murine heat-stable antigen: a hematomalymphoid differentiation antigen defined by the J11d, M1/69 and B2A2 antibodies. *Eur. J. Immunol.* **20**:1597-1602.
2. Asson-Batters, M. A., S. L. Spurgeon, J. Diaz, T. G. DeLoughery, and J. Bagby. 1994. Evolutionary conservation of the AU-rich 3' untranslated region of mRNA. *Proc. Natl. Acad. Sci. USA* **91**:1318-1322.
3. Beelman, C. A., and R. Parker. 1995. Degradation of mRNA in eukaryotes. *Cell* **81**:179-183.
4. Binder, R., J. A. Horowitz, J. P. Basillion, D. M. Hoeller, R. D. Klausner, and J. B. Harford. 1995. Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. *EMBO. J.* **13**:1969-1980.
5. Bruce, J., F. W. Symington, T. J. McKeane, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* **127**:2496-2501.
6. Chomczynski, P., and N. Sacchi. 1989. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
7. Crispe, I. N., and M. J. Bevan. 1987. Expression and functional significance of the J11d marker on mouse thymocytes. *J. Immunol.* **138**:2013-2018.
8. De Bruijn, M. L. H., P. A. Peterson, and M. R. Jackson. 1996. Induction of heat-stable antigen expression by phagocytosis is involved in vitro activation of unprimed cytotoxic T lymphocytes by macrophages. *J. Immunol.* **156**:2686-2692.
9. Decker, C. J., and R. Parker. 1995. Diversity of cytoplasmic function for the 3' untranslated region of eukaryotic transcripts. *Curr. Opin. Cell Biol.* **7**:383-392.
10. Enk, A., and S. Katz. 1994. Heat-stable antigen is an important costimulatory molecule on epidermal Langerhans' cells. *J. Immunol.* **152**:3264-70.
11. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (London)*. **311**:433-437.
12. Hahne, M., R. H. Wenger, D. Vestweber, and P. J. Nielsen. 1994. The heat-stable antigen can alter very late antigen 4-mediated adhesion. *J. Exp. Med.* **179**:1391-1395.
13. Hough, M., F. Takei, K. Humphries, and R. Kay. 1994. Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. *J. Exp. Med.* **179**:177-184.
14. Hough, M. R., M. S. Chapel, G. Sauvageau, F. Takei, R. Kay, and R. K. Humphries. 1995. Reduction of early B lymphocyte precursors in transgenic mice over-expressing the murine heat-stable antigen. *J. Immunol.* **156**:479-488.
15. Hubbe, M., and P. Altevogt. 1994. The heat-stable antigen/CD24 on mouse T lymphocytes: evidence for a costimulatory function. *Eur. J. Immunol.* **24**:731-737.
16. Izaurralde, E., and I. W. Mattaj. 1995. RNA export. *Cell* **81**:153-159.
17. Jackson, D., R. Waibel, E. Weber, J. Bell, and R. A. Stahel. 1992. CD24, a signal-transducing molecule expressed on human B cells, is a major surface antigen on small cell lung carcinomas. *Cancer Res.* **52**:5264-5270.
18. Jackson, R. J. 1993. Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region. *Cell* **74**:9-14.
19. Kadmon, G., M. Eckert, M. Sammar, M. Schachner, and P. Altevogt. 1992. Nectadrin, the heat-stable antigen, is a cell adhesion molecule. *J. Cell Biol.* **118**:1245-1258.
20. Karran, L., M. Jones, G. Morley, S. van Noorden, P. Smith, I. Lampert, and B. Griffin. 1995. Expression of a B-cell marker, CD24, on nasopharyngeal carcinoma cells. *Int. J. Cancer* **60**:562-566.
21. Kay, R., F. Takei, and R. Humphries. 1991. Expression cloning of a cDNA

- encoding M1/69-J11d heat-stable antigens. *J. Immunol.* **146**:1952–1959.
22. Kay, R., P. M. Rosten, and R. K. Humphries. 1991. CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosylphosphatidylinositol membrane anchor. *J. Immunol.* **147**:1412–1416.
 23. Klausner, R. D., T. A. Rousult, and J. B. Harford. 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell* **72**:19–28.
 24. Lagnado, C. A., C. L. Brown, and G. J. Goodall. 1994. AUUUA is not sufficient to promote poly(A) shortening and degradation of mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). *Mol. Cell. Biol.* **14**:7984–7995.
 25. Levitt, N., D. Briggs, A. Gil, and N. J. Proudfoot. 1989. Definition of an efficient synthetic poly(A) site. *Genes Dev.* **3**:1019–1025.
 26. Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated activation pathway. *Science* **249**:3399–3412.
 27. Linton, P.-J., D. J. Decker, and N. R. Klinman. 1989. Primary antibody-forming cells and secondary B cells are generated from separate precursor cell subpopulations. *Cell* **59**:1049–1059.
 28. Liu, Y., B. Jones, A. Aruffo, K. M. Sullivan, P. S. Linsley, and C. A. Janeway, Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* **175**:437–445.
 29. Liu, Y., B. Jones, W. Brady, C. A. Janeway, Jr., and P. S. Linsley. 1992. Costimulation for CD4 T cell growth: co-operation of B7 and the heat-stable antigen. *Eur. J. Immunol.* **22**:2855–2859.
 30. Liu, Y., R. H. Wenger, M. Zhao, and P. J. Nielsen. 1996. Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes. *J. Exp. Med.* **185**:251–262.
 31. Mullner, E. W., and L. C. Kuhn. 1988. A stem-loop in the 3'UTR mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. *Cell* **53**:815–825.
 32. Mullner, E. W., B. Neupert, and L. C. Kuhn. 1989. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell* **58**:373–382.
 33. Nielsen, P. J., B. Lorenz, A. M. Müller, R. H. Wenger, F. Brombacher, M. Simon, T. von der Weid, W. J. Langhorne, H. Mossmann, and G. Köhler. 1997. Altered erythrocytes and a leaky block in B-cell development in CD24/HSA-deficient mice. *Blood* **89**:1058–1067.
 34. Pierres, M., P. Naquet, J. Barbet, S. Marchetto, I. Marics, C. Devaux, M. Barad, R. Hyman, and G. Rougon. 1987. Evidence that murine hematopoietic cell subset marker J11d is attached to a glycosyl-phosphatidylinositol membrane anchor. *Eur. J. Immunol.* **17**:1781–1785.
 35. Pulak, R., and P. Anderson. 1993. mRNA surveillance by the *Caenorhabditis elegans* smg genes. *Genes Dev.* **7**:1885–1897.
 36. Rougon, G., L. A. Alterman, K. Dennis, X.-J. Guo, and C. Kinnon. 1991. The murine heat-stable antigen: a differentiation antigen expressed in both the hematolymphoid and neural cell lineages. *Eur. J. Immunol.* **21**:1397–1402.
 37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 38. Shaw, G., and G. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**:659–667.
 39. Springer, T., G. Galfre, S. Secher, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* **8**:539–551.
 40. Surdej, P., A. Riedl, and M. Jacobs-Lorena. 1994. Regulation of mRNA stability in development. *Annu. Rev. Genet.* **28**:263–282.
 41. Wang, Y.-C., L. Zhu, R. McHugh, K. W. Sell, and P. Selvaraj. 1995. Expression of heat-stable antigen on tumor cells provides co-stimulation for tumor-specific T cell proliferation and cytotoxicity in mice. *Eur. J. Immunol.* **25**:1163–1167.
 42. Wenger, R. H., M. Ayane, R. Bose, G. Kohler, and P. J. Nielsen. 1991. The genes for a mouse hematopoietic differentiation marker called the heat-stable antigen. *Eur. J. Immunol.* **21**:1039–1046.
 43. Wu, Y., Y. Guo, C. A. Janeway, Jr., and Y. Liu. 1995. Signaling by a new anti-Thy 1 monoclonal antibody inhibits T cell proliferation and interferes with T-cell-mediated induction of costimulatory molecule B7-2. *Cell. Immunol.* **165**:266–277.
 44. Zhou, Q., Y. Wu, P. J. Nielsen, and Y. Liu. 1997. Homotypic interaction is not responsible for costimulatory activity of the heat-stable antigen. *Eur. J. Immunol.* **27**:2524–2528.
 45. Zubiaga, A. M., J. G. Belasco, and M. E. Greenberg. 1995. The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol. Cell. Biol.* **15**:2219–2230.