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Posttranscriptional Regulation in Lymphocytes: The case of CD154

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Abstract

The control of mRNA decay is emerging as an important control point and a major contributor to gene expression in both immune and non-immune cells. The identification of protein factors and *cis*-acting elements responsible for transcript degradation has illuminated a comprehensive picture of precisely orchestrated events required to both regulate and establish the decay process. One gene that is highly regulated at the posttranscriptional level is CD40 ligand (CD154 or CD40L). CD154 on $CD4^+$ T cells is tightly controlled by an interacting network of transcriptional and posttranscriptional processes that result in precise surface levels of protein throughout an extended time course of antigen stimulation. The activation-induced stabilization of the CD154 transcript by a polypyrimidine tract-binding protein (PTB)-complex is a key event that corresponds to the temporal expression of CD154. In this review, we discuss known and potential roles of major mRNA decay pathways in lymphocytes and focus on the unique posttranscriptional mechanisms leading to CD154 expression by activated $CD4^+$ T cells.

Progression of an immune response requires the coordinated expression of ligands, receptors and cytokines. The expression of these regulatory molecules is tightly controlled at multiple levels upon cell activation. At the epigenetic level these events are orchestrated by changes in chromatin conformation allowing binding of an array of transcription factors and rapid induction of gene expression.[1] Further control is modulated at the posttranscriptional level by regulating the decay rate and localization of transcripts such that the required level of translation is achieved. During the last two decades the study of posttranscriptional regulation of immune and non-immune related genes has advanced significantly and is now recognized as being central in regulating gene expression. [2–7]

Eukaryotic mRNAs transcribed by RNA polymerase II are made resistant from exonuclease degradation by incorporation of the 5′ 7-methylguanosine cap and the 3′ poly(A) tail.[8, 9] These two essential elements are added during transcription and their interaction with cytoplasmic eIF4E and poly(A)-binding protein (PABP) enhances both translation and protection from exonuclease activity.[10] Although these factors are common to every mRNA, a subset of transcripts are also susceptible to regulation mediated by RNA binding proteins interacting with *cis*-acting elements encoded within the 3′- or 5′-untranslated regions (UTRs). Binding of specific *trans*-acting factors to these regulatory elements has a wide range of consequences resulting in enhanced or diminished transcript stability and/or changes in the overall translational capacity of a specific mRNA.

Upon activation, lymphocytes must undergo a series of rapid phenotypic and functional changes that allow them to proliferate and express specific effector functions in order to

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neutralize pathogen. A balanced pattern of gene expression during such changes is an essential prerequisite to establish an appropriate immune response. Short-lived mRNA transcripts allow for a much more rapid response to changing environmental or developmental cues and can limit transcript availability at times when expression of a specific set of proteins would otherwise be detrimental.[11] Conversely, long-lived transcripts are a more energy efficient alternative when sustained expression of a protein is required.[12] Since lymphocyte activation is characterized by transitions between different checkpoints during which the fate of an immune response is decided, diversification at the level of mRNA stability of different transcripts provide a valuable tool to regulate the magnitude of a response. These changes in mRNA stability are controlled by the regulatory activity of many different RNA binding proteins whose function is dictated by signaling pathways during distinct stages of cell maturation and inflammation.[5, 13] This review will explore these mechanisms of posttranscriptional regulation of immune related genes with a focus on factors controlling CD154 mRNA stability.

AU-rich elements

The most studied transcript instability signature in lymphocytes is the AU-rich element (ARE).[14] This element is present in the 3′UTRs of many rapidly induced mRNAs and is often characterized by different length repeats of the *cis*-acting AUUUA pentamers organized in stem loop structures.[15] The number, length and surrounding sequences of ARE repeats are decisive factors for the recruitment of the family of ARE binding proteins (AUBP), which includes the AU-rich binding factor-1 (AUF1), tristetraprolin (TTP), KH splicing regulatory protein (KSRP), Human-antigen R (HuR) as well as others.[14, 16, 17] To date, the stability of several immune related-transcripts has been linked to AUBP binding including TNFα,18 IL-2,[19] IL-3,[20] IL-8,[21] IL-10,[21, 22] VEGF,23 COX224 and MMP13.[25] In addition to experimentally identified transcripts, microarray technology has established the cohort of ARE-containing genes that constitute the ARE-mRNA Database (ARED)[26] and provided a grouping of ARE containing mRNAs based on their role during immune cell activation.27

The mechanisms by which AUBPs regulate mRNA decay is under extensive investigation and is known to include mRNA targeting to the 3′–5′ cleavage activity of the cytoplasmic multiprotein component known as the exosome[28], or by formation of processing bodies (P-bodies); cytoplasmic messenger ribonucleoproteins foci characterized by the presence of decapping enzymes and 5′-3′ RNA processing proteins.[29] Differential AUBPs binding has also being shown to either increase or decrease endo-ribonuclease activity or translation of target mRNAs.3, 30, 31 Specifically, AUBPs binding to AREs can have opposite outcomes on the half-life ($t_{1/2}$) of transcripts either by recruitment of the deadenylation machinery and subsequent rapid degradation by the exosome[32, 33] or by masking the ARE to increase transcript stability and prevent endonuclease activity.[34] In some instances AUBPs with different properties compete for binding to the same *cis*-acting element. This is the case with TTP binding to an ARE within the TNFα 3′UTR that results in enhanced degradation by recruitment of the exosome.[35] However, TTP binding to the TNFα ARE can be out competed by HuR resulting in the increased stabilization of the transcript.[18] Also, the IL-2 mRNA, which has a 3′UTR rich in AREs, is stabilized by the NF90 AUBP following CD28 co-stimulation of $CD4^+$ T cells suggesting that NF90 binding prevents the interaction of other decay promoting AUBPs.[36] Finally, the same AUBP can function to stabilize or decay an mRNA which is the case with AUF1 which exits as four distinct isoforms and functions as a destabilizing factor in K562 cells[37] and a stabilizing factor in NIH 3T3 cells.[38]

GU-rich elements

Global analysis of mRNA degradation patterns in resting and activated T lymphocytes identified a sizeable number of transcripts exhibiting activation dependent changes in posttranscriptional regulation.39 Only a small minority of these transcripts was found to contain ARE sequences within their 3´UTRs. In contrast, the vast majority of mRNA regulated at the posttranscriptional level in T lymphocytes contained a GU-rich element (GRE). The presence of GU repeats in the 3´UTR corresponded to enhanced transcript instability[40] and the CUG-binding protein 1 (CUGBP1) was identified as a factor that bound GREs41 and stabilized transcripts.40 While specific studies have shown that CUGBP1 can recruit the deadenylation machinery42 others report that this factor functions to enhance translation of target genes.43 Therefore, it would appear that a subset of GREcontaining transcripts, like their ARE-containing counterparts, are targeted for stability or degradation through a GRE-specific pathway in response to varying cellular conditions.

CA-rich elements

The most common di-nucleotide motif in mammals is the CA repeat.44 This element is widely used in genetic linkage analyses and a correspondence between microsatelite CA polymorphisms within 3´UTRs and incidence of disease has been demonstrated in Type I diabetes, rheumatoid arthritis and systemic lupus erythematousus.45–48 The ubiquitously expressed heterogeneous ribonuclear protein (hnRNP)-L maintains a high affinity for ribo-CA-repeats in introns, exons and regulatory regions through the interaction of three distinct RNA binding domains (RBDs).[49] This factor is implicated in multiple steps of RNA processing including splice-site selectivity,50, 51 nuclear-cytoplasmic transport,52, [53] IRESmediated translation54 and RNA stability.51 In T cells, the expression of different spliced isoforms of CD45 is mediated through an activation-induced pathway of exon exclusion through selective binding of both hnRNP-L and a homologue hnRNP-LL.55–57 Also, hnRNP-L binding to 3'UTR CA-repeats has been shown to impact the stability of transcripts encoding endothelial isoform of nitric oxide synthase (eNOS),58 inducible nitric oxide synthase (iNOS) mRNA59, human vascular endothelial growth factor (VEGF) 60 and CD154.61

CU-rich elements

Two distinct CU-rich elements have been implicated in posttranscriptional regulation of multiple eukaryotic mRNA transcripts. These are the Differentiation Control Element (DICE) and the CU-Rich Element (CURE).62, 63 Although the distinction between these two elements is debatable,63 DICE is characterized by the consensus architecture (C/U)CCANx CCC(U/A)(C/U)y UC(C/U)CC.64 This sequence motif is bound by the heterogeneous ribonuclear protein (hnRNP) K (E2/E1, αCP, PCBP) resulting in the stabilization of the target molecule65 with a corresponding induction66 or repression67 of translation. Examples of DICE-directed post-transcriptional regulation include the modulation of 15-lipoxygenase mRNA stability by hnRNP-K and -E1.66 Also, DICE sequences within the 3´UTR of human p21-activated kinase 1 (Pak1) were shown to bind hnRNP-E1.[68]

A CURE binding protein that has been extensively characterized is the polypyrimidine tract-binding protein (PTB). PTB has been implicated in the post-transcriptional regulation of inducible nitric oxide synthetase (iNOS),[69] insulin,70 VEGF,71 and CD154.72, 73 In lymphocytes, this protein is present in two ubiquitously expressed isoforms of 50 and 55 kDa (PTB-1 and PTB-4), which contain four RNA Binding Domains (RBD) separated by unstructured linker sequences.74 Both PTB-1 and PTB-4 are shuttling proteins with a novel bipartite N-terminal nuclear localization sequence (NLS) that is bound by importin-α.75 This protein functions as a cytoplasmic receptor to promote nuclear localization of bipartite NLS-containing proteins.76 Translocation of PTB between the nuclear and cytoplasmic

compartments is controlled by phosphorylation at Ser-16 by 3′–5′ cAMP dependant protein kinase A (PKA)77 and this process *per se* appears to be uncoupled to RNA export.78 The activity of PTB in post-transcriptional regulation requires the formation of protein complexes that may include nucleolin,79 hnRNP-L,80 Cold Shock Domain (CSD)71 as well as additional heterologous RNA binding proteins (reviewed in ref. 81).

Posttranscriptional regulation of CD154 (CD40 ligand) mRNA

A critical immune regulatory protein that is controlled at the level of posttranscriptional control is CD40 ligand (CD40L or CD154). The interaction of transiently expressed CD154 on $CD4^+$ T cells with the constitutively expressed CD40 on antigen-presenting cells (APCs) generates critical thymus dependent (TD) responses and enhances a subset of innate responses to bacterial and viral pathogens.[82–84]

Several studies have shown that CD154 mRNA expression is regulated at both the transcriptional and posttranscriptional levels in response to T cell receptor (TCR) activation. Within 10 min of activation intracellular stores of CD154 protein are translocated to the extracellular surface and CD154 expression is quickly enhanced by increased gene transcription, which results in maximal mRNA levels occurring after 6 h of continuous stimulation. At approximately 12 h of stimulation the CD154 mRNA levels drop to a basal level that remains constant throughout the subsequent activation period.[85–87] Early expression analysis revealed that CD154 transcription is dependent on the activation of the Ca^{2+}/calmodulin pathway[88] whereas transcript stabilization is increased in response to treatment with cAMP analogues in ionomycin-stimulated peripheral blood mononuclear cells (PBMC).[89] Other reports indicated that co-culture of PHA-activated $CD4^+$ T cells with human endothelial cells (EC) causes direct changes in CD154 mRNA stability through an LFA-3-dependent process.[90] However, unlike its effect on ARE-mediated decay,[91, 92] co-stimulation through CD28 was shown to induce only a modest increase in CD154 transcript stability.93

The posttranscriptional mechanisms underlying CD154 expression have been extensively investigated.[73, 93, 94] In human $CD4^+$ T cells the CD154 transcript was found to decay with a $t_{1/2}$ of less than 40 min during the first 12 h of TCR activation and with a $t_{1/2}$ of approximately 2.2 h following 24 h of continuous stimulation.93 Biphasic posttranscriptional regulation of this mRNA was found to depend on the activation-induced binding of ribonucleoprotein (RNP) complexes containing PTB to a region of the CD154 3'UTR at extended times following activation.[94] A similar mechanism of posttranscriptional regulation was demonstrated in response to TLR9 stimulation in primary B cells where multiple transcripts were stabilized by the binding of a PTB-containing complex (B-cpx I) to CU-rich elements within their 3′UTRs.95 Thus, both T- and B-lymphocytes maintain a similar non-ARE pathway of mRNA stability that is directly linked to antigen activation.

cis-elements and *trans*-factors involved in CD154 mRNA regulation

A thorough analysis of *cis*- and *trans*-acting factors involved in CD154 mRNA decay revealed that two PTB containing complexes (Complex I and Complex II) bind to three distinct sequences spanning nucleotides 1300 to 1589 of CD154 mRNA (XbaI-E1, E1-E5 and E5-HaeIII), within the 3′UTR region defined by the *Xba* I and *Hae* III restriction sites (X-H) (Fig 1).[94] Further analysis revealed that PTB is the major RNA binding component of the RNP complexes[72, 73] and that nucleolin and hnRNP-L are additional components of Complex I and II, respectively.[61, 79, 80] Formation of these RNP complexes is only seen at extended times of activation corresponding to the increased stability of the transcript, suggesting their direct involvement in modulating activation-induced CD154 mRNA stability.[72, 94]

The minimal sequences required for each complex binding were identified in *in vitro* studies.[80] The contribution of each minimal sequence to stabilizing a heterologous transcript (*Renilla* luciferase) and modulating reporter activity was tested in Jurkat/D1.1 cells which constitutively express Complex I and II (Table I). The centrally located E1-E5 region (Fig 1, Site B), which is one of the two minimum Complex I binding sequences[94] was found to provide the highest level of transcript stability and activity.[80] A second unit of Complex I was found to bind to a region defined by *Xba* I and E1 (Fig. 1, Site A) and insertion of this sequence at the 3'UTR of *Renilla* luciferase was also shown to increase expression, although at a much lower level than that seen with Site B. Further analysis of Site A revealed that the region between Xba I and nucleotide 1351 was responsible for the reduced luciferase activity and that this reduction was caused by enhanced luciferase mRNA *instability* (Table I). Together these findings support a model where binding of Complex I to Sites A and B results in enhanced protection of the transcript from rapid degradation mediated by adjacent sequences between the *Xba* I site and nucleotide 1348 (Fig. 2). This model also explains the high level of mRNA decay at early times following T cell activation when there is an absence of Complex I binding to the CD154 transcript.[94]

Analysis of the 3′ sequence of the X-H region downstream of the E5 site identified the binding site for Complex II (Fig. 1, Site C) This region contains a contracted CU-rich stretch and a polymorphic CA repeat[72] and is the weakest of the three individual regions with respect to its ability to enhance luciferase expression (Table I). Interestingly, Complex II binding was shown to require the whole length of this region. In addition to PTB, hnRNP-L was identified as a protein that bound to this sequence through interactions with the CArepeats.[80] Hamilton and colleagues recently reported that the binding of hnRNP-L to the CD154 CA-repeat in HeLa cells correlated both with transcripts containing shortened poly(A) tails and increased translation of a heterologous transcript.[61] The allelic and genotypic distribution of the polymorphic CD154 repeat has been studied as a genetic marker in specific autoimmune diseases and the higher number of CA repeats correlate with disease incidence.[46, 47] Notably, deregulating CD154 expression is associated with an increase in autoantibodies in both mouse and humans and this may be achieved in part through posttranscriptional and translational processes that result in enhanced expression during an immune response.[96–100]

Analysis of CD154 transcript upon *in vivo* activation

Similar to the regulation of human, the mouse CD154 transcript maintains a biphasic pattern of stability that is activation-dependent, however, the $t_{1/2}$ values in both the early and late stages are considerably shortened (~23 min and ~45min) (Vavassori, et. al., submitted). The question of whether the activation induced program of mRNA stability functions *in vivo* following antigen challenge has been addressed by priming animals with antigen plus adjuvant or adjuvant alone and challenging the T cells *ex vivo* with the same antigen. Analysis of RNA $t_{1/2}$ profiles of the unprimed and primed lymphocyte populations demonstrated that several days following injection, CD154 mRNA decayed in unprimed T cells with a $t_{1/2}$ of approximately 30 min which increased two-fold to approximately 50 min in *in vivo* primed cells. These findings revealed that restimulating $CD4^+$ T cells with antigen resulted in an increase in CD154 transcript stability relative to cells that were exposed to antigen for the first time and strongly suggested a role for regulated CD154 mRNA stability *in vivo* (Vavassori et. al., submitted).

A comparison of CD154 mRNA decay with the overall pattern of CD154 regulation led us to propose that mRNA stability is largely responsible for appropriate protein levels at late times of activation. This is based on the unexpected finding that overall steady state levels of CD154 mRNA are inversely linked to transcript stability where at 6 h post-activation the message level is highest and the stability lowest and vice versa. Previous data suggested that purified $CD4^+$

T cells express CD154 for extended times of activation in the presence or absence of co-stimulation[101–105] and that expression is biphasic with an early peak detected at 6 h and a second peak at 24 h.[106–109] Since this pattern of expression closely corresponds to our biphasic pattern of mRNA stability it suggests that there is as yet an undefined role for enhanced CD154 expression at late times of T cell activation. Future work will focus on identifying the signaling pathways of CD154 mRNA stability and defining the functional consequences of this pattern of expression in both B cell and T cell activation.

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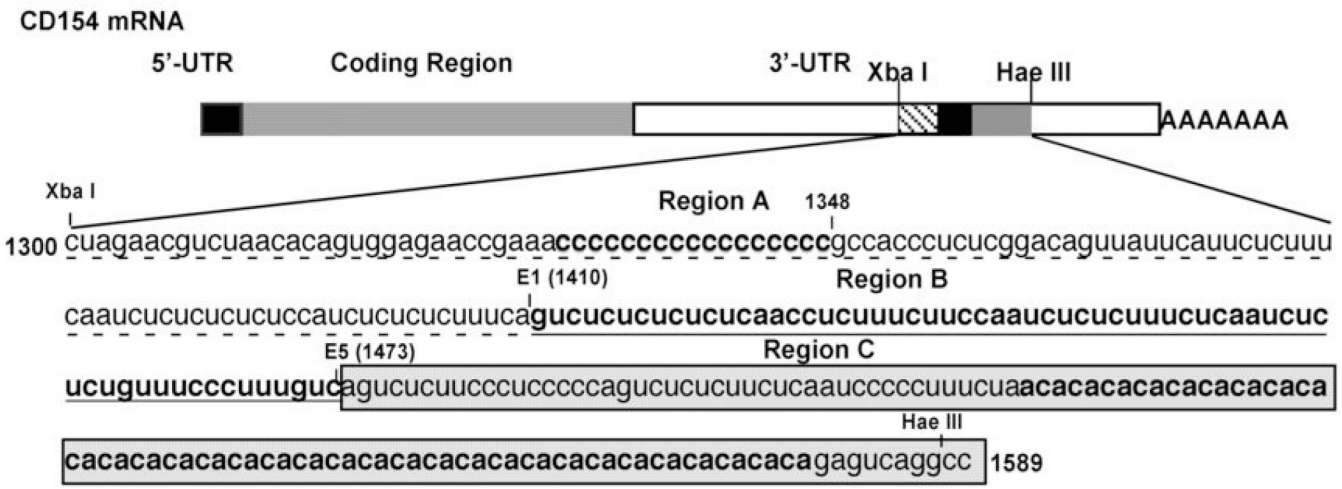


Figure 1.

Schematic diagram of the CD154 mRNA showing the three distinct Complex I/II binding sites in the 3'UTR. The CD154 stability element, defined by the restriction sites Xba I to Hae III (nt 1300 to 1589), is divided into three sub-regions: A (Xba I-E1, dashed underlined), B (E1–E5, underlined) and C (E5-Hae III, boxed). 5' A (1300–1348) is destabilizing and 1348-E1 is stabilizing and contains a Complex I binding site (see Table I) (*see Laughlin, et. al. 2008*).

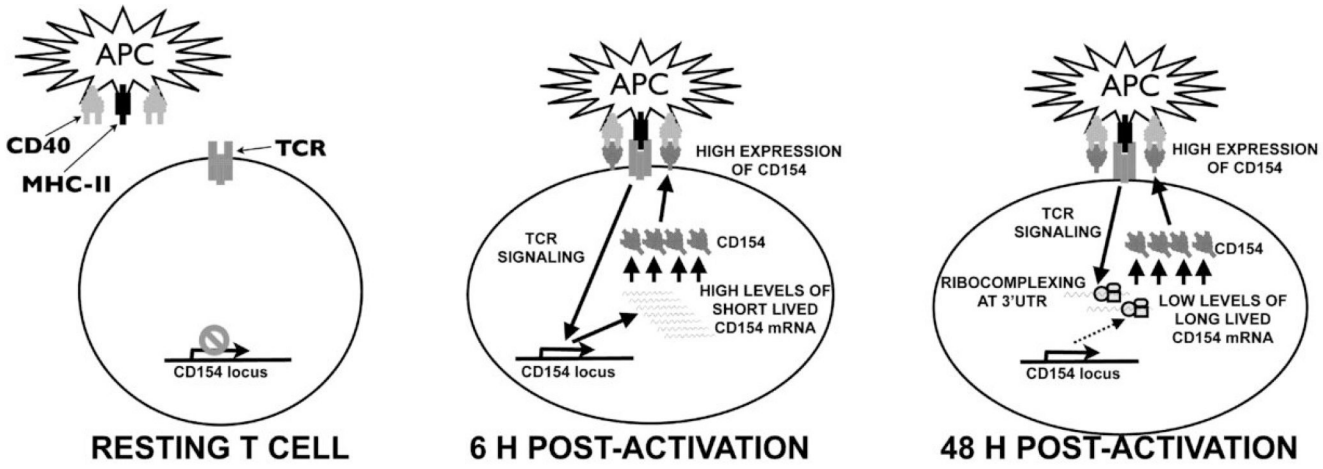
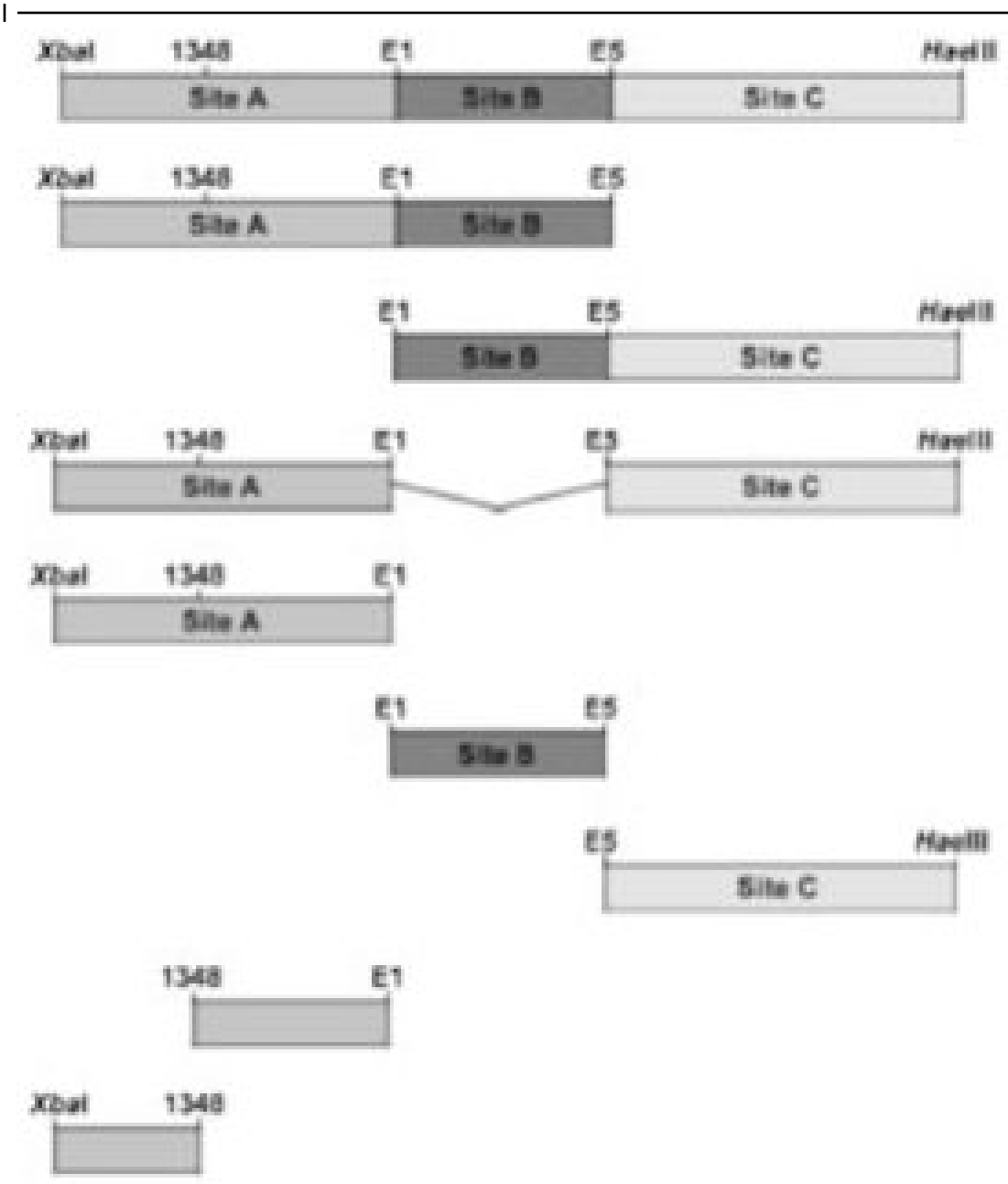


Figure 2.

Model of posttranscriptional regulation of CD154. Resting $CD4^+$ T cells retain low CD154-specific transcription, minimal amounts of short-lived CD154 mRNA and virtually little to no detectable surface expression of the protein. Within 6 h following antigen presentation by APCs a peak in transcription drives high levels of surface expression. At this time the CD154 message is being rapidly degraded. At extended times of activation, low levels of CD154 mRNA are stabilized by ribocomplexing on the 3'UTR which drives a second peak of surface expression.

TABLE I

Activity of the Different Regions of the CD154 Stability Element[1]

Region Tested	Luciferase Activity (Fold over pRLSV40)
	2.93+/−0.32
	3.1+/−0.29
	3.88+/−0.8
	0.26+/−0.05
	1.08+/−0.05
	4.52+/−0.55
	1.33+/−0.12
	2.25+/−0.11
	0.22+/−0.2

[1]The different regions of the CD154 stability regions were inserted into the 3′ UTR of the *Renilla* luciferase operon contained within the pRLSV40 vector. Jurkat D1.1 cells were transfected with the various constructs and luciferase activity measured 48 h later. Shown are the mean values +/− SEMs calculated for each construct over pRLSV40 vector alone (*see Laughlin, et. al. 2008*).