

# Small Nuclear RNAs Encoded by *Herpesvirus saimiri* Upregulate the Expression of Genes Linked to T Cell Activation in Virally Transformed T Cells

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## Summary

Seven small nuclear RNAs of the Sm class are encoded by *Herpesvirus saimiri* (HVS), a  $\gamma$  Herpesvirus that causes aggressive T cell leukemias and lymphomas in New World primates and efficiently transforms T cells in vitro [1–4]. The *Herpesvirus saimiri* U RNAs (HSURs) are the most abundant viral transcripts in HVS-transformed, latently infected T cells but are not required for viral replication or transformation in vitro [5]. We have compared marmoset T cells transformed with wild-type or a mutant HVS lacking the most highly conserved HSURs, HSURs 1 and 2. Microarray and Northern analyses reveal that HSUR 1 and 2 expression correlates with significant increases in a small number of host mRNAs, including the T cell-receptor  $\beta$  and  $\gamma$  chains, the T cell and natural killer (NK) cell-surface receptors CD52 and DAP10, and intracellular proteins—SKAP55, granulysin, and NKG7—linked to T cell and NK cell activation. Upregulation of three of these transcripts was rescued after transduction of deletion-mutant-HVS-transformed cells with a lentiviral vector carrying HSURs 1 and 2. These changes indicate an unexpected role for the HSURs in regulating a remarkably defined and physiologically relevant set of host targets involved in the activation of virally transformed T cells during latency.

## Results and Discussion

### T Cells Transformed by Wild-Type and Mutant HVS Lacking HSURs 1 and 2

*Herpesvirus saimiri* (HVS) reproducibly transforms primary T cells of the common marmoset (*Callithrix jacchus*) in vitro to continuous antigen- and IL-2-independent growth [1]. HVS-transformed T cells have a mature, activated phenotype and are predominantly CD8+ with natural killer (NK) cell markers and NK cell-like cytotoxic activity in both HVS tumors and in vitro-transformed marmoset peripheral blood lymphocytes (PBLs) [6, 7].

We investigated the function of *Herpesvirus saimiri* U

RNAs (HSURs) 1 and 2, the most highly conserved and the only small nuclear RNAs (snRNAs) encoded by the related *Herpesvirus ateles* (HVA) [8]. We used two sets of marmoset T cells (set A and set B [5], [Supplemental Data](#) and [Figure S1](#) available with this article online), each transformed with either wild-type HVS or a mutant HVS containing a 1379 kb deletion ([Figures 1A and S1](#)) that removes HSURs 1 and 2.

### HSURs 1 and 2 Upregulate Genes Implicated in T and NK Cell Activation

The HSURs are the only known Sm snRNAs encoded by a virus. They share no sequence similarity but have many other features characteristic of cellular Sm snRNAs ([Figure 1B](#)) [2]. Whereas most Sm snRNAs function in splicing, some snRNAs play more-diverse roles, such as in transcription initiation [9], transcription elongation [10], and telomere maintenance [11].

No phenotype has yet been described for HSUR deletions [5]. To supply clues to HSUR function, we used microarrays to compare host-gene expression in marmoset T cells latently infected with wild-type HVS or mutant HVS lacking HSURs 1 and 2. We were unable to meaningfully analyze uninfected host cells because HVS transforms a limited, incompletely defined subset of the PBLs isolated from the marmoset. Because *C. jacchus* genes exhibit ~90% identity to their human counterparts, arrays containing oligonucleotides complementary to 16,659 human genes were utilized.

Data from four independent microarrays for each cell set (a total of eight) were treated as replicates and statistical testing was applied to identify genes upregulated or downregulated  $\geq 2$ -fold. A p value of  $\leq 0.05$  was used as the limit for statistical significance; however, many changes had p values of  $\leq 0.01$ . Of the 2048 transcripts detected in both cell sets A and B, only eight genes were upregulated in the presence of HSURs 1 and 2 ([Table 1](#)), whereas one gene of unknown function was downregulated. No statistically significant differences in the expression of CD8 or NK markers were seen, demonstrating that HSURs 1 and 2 do not alter the subset of T cells transformed by HVS. Thus, the expression of approximately 0.5% of host genes was observed to be affected by the presence of HSURs 1 and 2, although additional altered genes may not have been detected in our analyses.

Genes encoding cell-surface receptors specific for T and NK cells are most highly upregulated with HSURs 1 and 2 ([Table 1](#)). TCR  $\beta$  chain mRNA is upregulated 9.8-fold on average for cell set A and 8.6-fold for B. The TCR  $\beta$  DNA oligonucleotide on the microarrays is complementary to the 3' UTR downstream of the marmoset TCR  $\beta$  constant region 1; this constant region and the corresponding 3' UTR are present in 79% of all full-length mRNAs from TCR  $\beta$  genes with functional rearrangements in T cells of *C. jacchus* [12]. The level of TCR  $\gamma$  chain mRNA is also upregulated; it is measured independently by two oligonucleotides complementary to the 3' UTR common to all TCR  $\gamma$  mRNAs (8.2-fold

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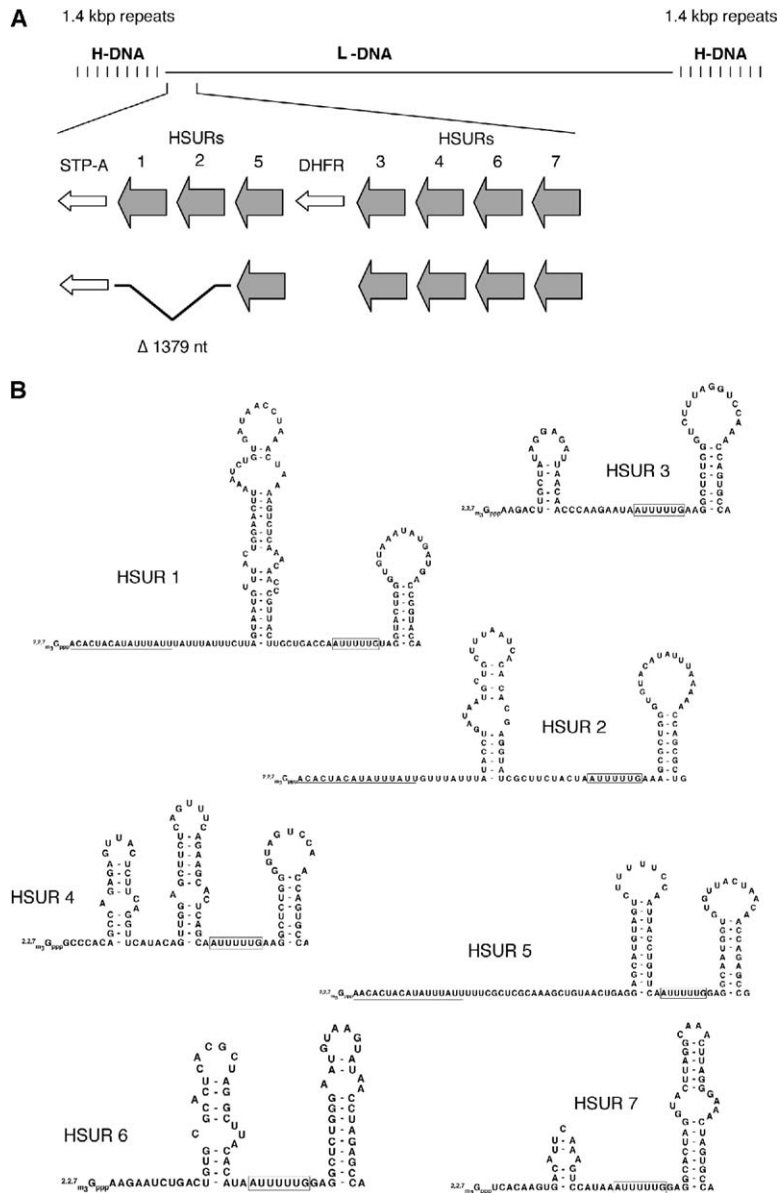


Figure 1. The *Herpesvirus saimiri* U RNAs

(A) The HVS A-11 genome consists of a 112 kb stretch of unique sequence containing 76 open reading frames [25]. The unique coding region has a low G + C content (L-DNA) and is flanked by repetitive terminal 1.4 kb repeats with a high G + C content (H-DNA) [25]. The schematic depicts the genomes of wild-type HVS A-11 and mutant HVS A-11 that were used to transform PBLs of the common marmoset in vitro, showing the location of the HSUR genes adjacent to the STP and DHFR (dihydrofolate reductase) genes at the leftmost end and the deleted 1379-nucleotide region containing HSURs 1 and 2. HSURs 3–7 remain intact in both viral genomes.

(B) The sequences and predicted secondary structures of HSURs 1–7 of HVS A-11; each of these RNAs exists at ~2,000 to 20,000 copies in latently infected T cells. Similarities between the HSURs and cellular Sm-class snRNAs include typical RNA polymerase II promoters, enhancers, and 3'-end formation signals; 5' trimethylguanosine caps; 3'-terminal stem loops; and canonical Sm protein binding site sequences (boxed) that assemble particles precipitable by autoantibodies against Sm proteins [2]. Conserved AU-rich sequences in HSURs 1, 2, and 5 are underlined.

and 3.4-fold) and the  $\gamma$  constant region 1 (5.8-fold and 3.9-fold). Signals for the  $\alpha$  and  $\delta$  loci were not sufficiently above background to be analyzed, likely reflecting differences between human and marmoset sequences. The TCR triggers complex intracellular-signaling events critical for T cell activation, proliferation, and effector functions.

HSUR 1 and 2 expression likewise correlates with increased mRNA levels for the cell-surface receptors CD52 (2.4-fold and 2.1-fold) and DAP10 (3.2-fold and 2.2-fold), which play costimulatory roles in T cell activation [13, 14]. Cross-linking of CD52 protein (CAMPATH-1 antigen), a small glycosylphosphatidylinositol-anchored molecule, with antibodies triggers activation of T cells in vitro [13] and induces many of the same tyrosine-phosphorylation events seen with signaling through the TCR [15]. DAP10 (DNAX-activation protein 10) is a

phosphatidylinositol 3-kinase adaptor membrane protein for the activating receptor NKG2 in CD8+  $\alpha/\beta$  T cells,  $\gamma/\delta$  T cells, and NK cells [16]. Costimulation of NKG2, through DAP10, and TCR on CD8+  $\alpha/\beta$  T cells induces IL-2 production leading to T cell proliferation [14].

Upregulation of intracellular genes linked to T cell activation is also associated with expression of HSURs 1 and 2 (Table 1). SKAP55 (upregulated 18.7-fold and 2.2-fold), a Src-kinase-associated protein of 55 kDa, positively influences TCR signaling by stimulating transcription of IL-2 [17], inducing mitogen-activated protein kinase phosphorylation [18], and binding and enhancing the activity of the Src-family kinase p59<sup>lyn</sup> [17]. Granulysin (upregulated 10.4-fold and 2.4-fold), a gene expressed late after T cell activation [19], accumulates in the cytotoxic granules of CD8+ T cells and NK cells and is exocytosed following TCR stimulation [19]. Sta-

Table 1. Genes Regulated by HSURs 1 and 2

Genes Upregulated in the Presence of HSURs 1 and 2						
	LocusLink ID	GenBank ID	Cell Set B		Cell Set A	
			Fold up <sup>a</sup>	p value	Fold up <sup>a</sup>	p value
<b>Transmembrane proteins</b>						
T cell-receptor beta	6957	X00437	8.6	0.0126	9.8	0.0001
T cell-receptor gamma constant 1	6966	M14998	3.9	0.0259	5.8	4.4E-05
T cell-receptor gamma locus	6965	M30894	3.4	0.0070	8.2	2.9E-05
DAP10 (PI3K adaptor protein)	10870	AL050163	2.2	0.0002	3.2	0.0019
CD52 (CAMPATH-1 antigen)	1043	X62466	2.1	0.0032	2.4	0.0072
Natural killer cell group 7	4818	S69115	2.0	0.0105	2.9	0.0208
<b>Signaling proteins</b>						
Src-family-associated phosphoprotein 1	8631	Y11215	2.2	0.0010	18.7	0.0491
<b>Secreted proteins</b>						
Granulysin	10578	NM_012483	2.5	0.0001	10.4	3.5E-07
<b>Proteins of unknown function</b>						
Fatty acid binding protein 5	2171	M94856	2.6	0.0001	12.8	1.4E-05
Genes Downregulated in the Presence of HSURs 1 and 2						
	LocusLink ID	GenBank ID	Cell Set B		Cell Set A	
			Fold down <sup>a</sup>	p value	Fold down <sup>a</sup>	p value
<b>Proteins of unknown function</b>						
CGI-38	51673	NM_016140	4.4	0.0472	5.7	0.0460

<sup>a</sup>“Fold up” and “Fold down” refer to *n*-fold increase and *n*-fold decrease, respectively. Fold-up and fold-down values represent the mean *n*-fold change across at least three microarrays for each cell set.

tistically significant mRNA increases are also seen for NKG7 (natural killer cell group 7; 2.9-fold and 2.0-fold), a cytotoxic-granule membrane protein expressed in activated NK and T cells and translocated with degranulation to the plasma membrane, where it may regulate T cell effector function [20].

**Microarray Results Are Validated by Northern Analyses**

We performed Northern blots of polyadenylated RNA isolated from both wild-type and mutant cell sets for six of the eight genes upregulated by HSURs 1 and 2 (Figure 2A). mRNAs for transportin-SR (TPN-SR) and vacuolar protein sorting-35 (VPS-35; data not shown), expressed at similar levels in the cell lines in microarray analyses, served as controls. After normalization to TPN-SR, the wild-type cells show upregulation of mRNA levels of approximately 2-fold in cell set A and

2-fold in B for CD52 (lanes 9–12), 4- and 5-fold for DAP10 (lanes 13–16), and 15- and 4-fold for SKAP55 (lanes 17–20). The dependence on HSURs 1 and 2 is even more striking for TCR β (lanes 1–4), TCR γ (lanes 5–8), and granulysin (lanes 21–24) mRNAs; the values for the mutant cell lines were so close to background levels that the relative magnitudes of the changes could not be calculated (~ infinity). We again did not detect TCR α and δ with probes to the constant region and 3' UTR, suggesting variability between the marmoset and human sequence in these regions.

To confirm that the levels of HSUR-dependent upregulated mRNA produce a corresponding increase in protein levels, we tested multiple commercially available antibodies against human SKAP55, TCR β, TCR γ, and CD52 proteins in both Western and FACS analyses. Of these, only anti-SKAP55 cross-reacted with the marmoset ortholog. SKAP55 protein is upregulated in wild-

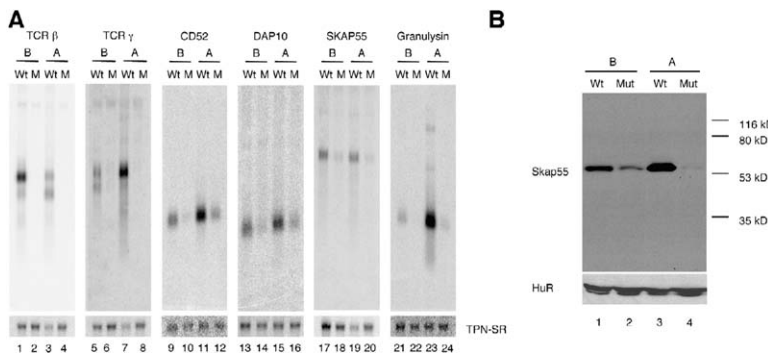


Figure 2. Northern and Western Analyses Confirm Microarray Data

(A) Northern blotting was performed on polyadenylated RNA isolated from wild-type (Wt)- and mutant (M)-HVS-transformed T cell lines of both cell sets A and B resolved in a 1% formaldehyde agarose gel. The following investigated genes were upregulated by HSURs 1 and 2 on the microarrays: TCR β, TCR γ, CD52, DAP10, SKAP55, and granulysin. The patterns provide no hints that RNA processing is altered in the absence of HSURs 1 and 2. The same blots were probed for TPN-SR as a loading control.

(B) Western blots were performed for SKAP55

on total-cell extract from wild-type (Wt)- and mutant (Mut)-HVS-transformed T cells of cell sets A and B, with anti-HuR serving as a loading control. Variation in the relative levels of expression in cell sets A and B in the Northern and Western analyses is likely due to the fact that the cells sets are derived from cells from different monkeys. The results are representative of those seen in multiple Northern blots and comparable to those obtained in the microarray analyses.

Table 2. HSURs 1 and 2 Rescue TCR  $\beta$ , SKAP55, and DAP10 Expression

Real-Time Calculations	Cell Sets for Comparison			
	Mutant cells ( $\Delta 2H$ )	Wild-type cells	Mut + GFP alone ( $\Delta 2H$ )	Mut +HSUR1, HSUR2, GFP
<b>TCR <math>\beta</math> Relative to TPN-SR</b>				
Average $C_T$				
TCR $\beta$	36.67 $\pm$ 0.71	33.49 $\pm$ 0.09	38.94 $\pm$ 1.25	34.61 $\pm$ 0.17
TPN-SR	31.69 $\pm$ 0.14	33.09 $\pm$ 0.06	32.08 $\pm$ 0.10	31.31 $\pm$ 0.09
$\Delta\Delta C_T = \Delta C_T - \Delta C_T(\Delta 2H)$	0 $\pm$ 0.72	-4.58 $\pm$ 0.10	0 $\pm$ 1.25	-3.55 $\pm$ 0.19
<b>TCR <math>\beta</math> Fold Up (<math>2^{-\Delta\Delta C_T}</math>)</b>	<b>1.0 (0.6–1.7)</b>	<b>24.0 (22.3–25.8)</b>	<b>1.0 (0.4–2.4)</b>	<b>11.7 (10.3–13.4)</b>
<b>SKAP55 Relative to TPN-SR</b>				
Average $C_T$				
SKAP55	33.34 $\pm$ 0.16	31.57 $\pm$ 0.57	32.84 $\pm$ 0.25	31.36 $\pm$ 0.05
TPN-SR	32.15 $\pm$ 0.08	31.55 $\pm$ 0.54	31.46 $\pm$ 0.18	30.53 $\pm$ 0.20
$\Delta\Delta C_T = \Delta C_T - \Delta C_T(\Delta 2H)$	0 $\pm$ 0.17	-1.18 $\pm$ 0.79	0 $\pm$ 0.31	-0.56 $\pm$ 0.20
<b>SKAP55 Fold Up (<math>2^{-\Delta\Delta C_T}</math>)</b>	<b>1.0 (0.9–1.1)</b>	<b>2.3 (1.3–3.9)</b>	<b>1.0 (0.8–1.2)</b>	<b>1.5 (1.3–1.7)</b>
<b>DAP10 Relative to TPN-SR</b>				
Average $C_T$				
DAP10	34.70 $\pm$ 0.21	32.32 $\pm$ 1.00	34.11 $\pm$ 0.00	31.99 $\pm$ 0.86
TPN-SR	32.15 $\pm$ 0.08	31.55 $\pm$ 0.54	31.46 $\pm$ 0.18	30.53 $\pm$ 0.20
$\Delta\Delta C_T = \Delta C_T - \Delta C_T(\Delta 2H)$	0 $\pm$ 0.22	-1.79 $\pm$ 1.13	0 $\pm$ 0.18	-1.20 $\pm$ 0.88
<b>DAP10 Fold Up (<math>2^{-\Delta\Delta C_T}</math>)</b>	<b>1.0 (0.9–1.2)</b>	<b>3.5 (1.6–7.6)</b>	<b>1.0 (0.9–1.1)</b>	<b>2.3 (1.2–4.2)</b>
<b>VPS-35 Relative to TPN-SR</b>				
Average $C_T$				
VPS-35	37.22 $\pm$ 0.11	37.08 $\pm$ 0.10	37.41*	35.66*
TPN-SR	37.90 $\pm$ 0.66	37.62 $\pm$ 1.05	38.66*	36.80*
$\Delta\Delta C_T = \Delta C_T - \Delta C_T(\Delta 2H)$	0 $\pm$ 0.67	0.14 $\pm$ 1.05	0*	0.11*
<b>VPS-35 Fold Up (<math>2^{-\Delta\Delta C_T}</math>)</b>	<b>1.0 (0.6–1.6)</b>	<b>0.9 (0.4–1.9)</b>	<b>1.0 (n.d.*)</b>	<b>0.9 (n.d.*)</b>

TCR  $\beta$ , SKAP55, DAP10, and VPS-35 expression was compared in cells lacking HSURs 1 and 2 to expression in cells in which HSUR 1 and 2 expression was present or restored by real-time RT-PCR via the comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method. Results shown are the average of two independent experiments performed on different days, except as noted below. The range of  $2^{-\Delta\Delta C_T}$  is based on standard deviation and shown in parentheses. Mut indicates mutant-HVS-transformed cell line (set B); Wild-type indicates wild-type-HVS-transformed cell line (set B); Mut + GFP alone indicates mutant-HVS-transformed cell line transduced with a lentiviral vector expressing only GFP; Mut + HSUR1, HSUR2, GFP indicates mutant-HVS-transformed cell line transduced with a lentiviral vector containing HSURs 1 and 2 and GFP;  $\Delta 2H$  indicates HVS-transformed cell lines lacking HSURs 1 and 2; and \* indicates that these VPS-35 negative control values are the result of one experiment, and thus a range cannot be calculated (n.d.). "Fold Up" refers to *n*-fold increase (calculated by the comparative  $C_T$  method).

type compared to deletion-mutant cells of both cell sets (Figure 2B, compare lanes 1 and 3 with lanes 2 and 4).

### Gene Expression in Mutant Cells Is Rescued by HSURs 1 and 2

We determined that the observed changes in host-gene expression in HSUR deletion mutants are not due to a difference in the expression of the adjacent viral gene, STP-A, or to lowered expression of HSURs 3–7 (see Supplemental Data, Figure S2). In fact, expression of the remaining five HSURs is 2- to 3-fold higher in mutant cells, which may contribute to the phenotype.

Finally, we confirmed that upregulation of T cell-activation genes is specifically attributable to the HSURs by using lentiviral vectors to restore HSUR 1 and 2 expression to the mutant transformed T cell line. HSURs 1 and 2 were cloned in tandem into a lentiviral vector containing enhanced green fluorescent protein (eGFP) [21], and the packaged vectors were transduced into the deletion-mutant cell line B. These and cells transduced in parallel with a vector expressing eGFP alone were sorted for GFP expression by FACS after 3 days in culture.

The levels of host mRNAs extracted from the transduced cells, as well as from wild-type and mutant cells

of set B, were assessed by real-time one-step RT-PCR. We analyzed TCR  $\beta$ , which exhibits the largest dependence on HSURs, as well as two genes with smaller effects, SKAP55 and DAP10. The average cycle threshold ( $C_T$ ) for each mRNA was determined, and the amount of each mRNA relative to TPN-SR was calculated by the comparative  $C_T$  method (Table 2). In the wild-type cells relative to the mutant cells, TCR  $\beta$  mRNA is 24.0-fold higher, whereas SKAP55 mRNA is 2.3-fold and DAP10 mRNA is 3.5-fold higher (each represents the average of two independent experiments; see Table 2). These results validate those obtained by microarray (Table 1) and Northern (Figure 2A) analyses.

When HSURs 1 and 2 were restored by lentiviral transduction of mutant cells, TCR  $\beta$  mRNA levels (relative to TPN-SR) were upregulated 11.7-fold, whereas SKAP55 and DAP10 mRNA levels were reproducibly upregulated 1.5-fold and 2.3-fold, respectively (each represents the average of two independent experiments; see Table 2). Restoration of HSURs 1 and 2 therefore upregulated TCR  $\beta$  expression to ~50% of wild-type levels and SKAP55 and DAP10 to ~65% of wild-type levels, whereas the control VPS-35 was not altered. The real-time RT-PCR data on the transduced cells (Table 2) therefore demonstrate that the upregulation of TCR  $\beta$ , SKAP55, and DAP10 mRNA levels in

HVS-transformed T cells is attributable to the expression of HSURs 1 and 2.

### The HSURs May Enhance the Activation Level of Latently Infected Host T Cells

These data suggest that during latency the HSURs act through the TCR and other host genes to further activate HVS T cells, and this activation may enhance their proliferation and persistence in the host or modulate the host immune response to the virus. We compared the expression of T cell-activation markers, proliferation rates, NF $\kappa$ B and NFAT activity, and tyrosine-phosphorylation profiles of cultured wild-type and mutant cells (data not shown), but we observed no interpretable changes. Although the assays used may not be sufficiently sensitive to detect differences in the relative levels of activation, a more likely possibility is that HSURs 1 and 2 allow a greater degree of activation and/or proliferation in vivo in response to extracellular signals or interactions with other cells in the host.

Our results differ from those published by other laboratories, which found that TCR  $\alpha/\beta$  levels were either unaffected [22] or decreased [23] in human T cells expressing HSURs or other genes of HVS strain C. These discrepancies likely reflect differences between marmoset and human cells, CD4 and CD8 T cells, and/or the transformation mechanisms and capacities of distinct HVS strains. Importantly, the activation state of HVS C-transformed human T cells (which are not transformed in vivo) differs from that of marmoset T cells because the human cells are dependent upon IL-2 for continuous growth [24]. Additionally, many of the changes we observe are specific for CD8+ and/or NK cells, suggesting that the function of the HSURs is specific to these T cell subsets.

### Conclusions

As the first phenotypic differences documented for HSUR deletions in latently HVS-infected marmoset T cells, the enhanced expression of a small set of host genes linked to T cell activation suggests that HSURs 1 and 2 specifically enhance the activation level of latently HVS-infected T cells in vivo. This genetically validated conclusion comprises a critical first step toward the elucidation of the molecular mechanism of action of the HSURs. Importantly, the in vitro-transformed T cells used for these studies are representative of those transformed by HVS in infected monkeys [6, 7], making the findings relevant to the biological activities of the HSURs in HVS infection. Our ability to rescue the expression of TCR  $\beta$ , SKAP55, and DAP10 in a mutant-transformed cell line by transducing HSURs 1 and 2 with a lentiviral vector (Table 2) indicates that the HSURs themselves are responsible for the observed changes in gene expression. Future transduction experiments can address the importance of perfectly conserved sequences at the 5' end of HSURs 1 and 2 (Figure 1B) by independently disrupting sequence motifs in this region. We are therefore poised to uncover the molecular mechanism of action of the HSURs. Such progress would contribute to our understanding of the range of cellular functions that can be accomplished by Sm snRNAs, of the persistence of viruses in the

host, and of the pathways regulating T cell activation and proliferation.

### Supplemental Data

Two figures and Supplemental Experimental Procedures are available with this article online at: <http://www.current-biology.com/cgi/content/full/15/10/974/DC1/>.

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