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Viral small nuclear ribonucleoproteins bind a protein implicated in messenger RNA destabilization

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ABSTRACT ***Herpesvirus saimiri*** **(HVS) is one of several primate viruses that carry genes for small RNAs. The five *H. saimiri*-encoded U RNAs (HSURs) are the most abundant viral transcripts expressed in transformed marmoset T lymphocytes. They assemble with host proteins common to spliceosomal small nuclear ribonucleoproteins (snRNPs). HSURs 1, 2, and 5 exhibit sequences at their 5′ ends identical to the AUUUA motif, which targets a number of protooncogene, cytokine, and lymphokine mRNAs for rapid degradation. We show that a 32-kDa protein previously demonstrated to bind to the 3′ untranslated region of several unstable messages can be UV crosslinked specifically to HSUR 1, 2, and 5 transcripts *in vitro*, as well as to endogenous HSUR snRNPs. Our results suggest an unusual role for these viral snRNPs: HSURs may function to attenuate the rapid degradation of certain cellular mRNAs, thereby facilitating viral transformation of host T lymphocytes.**

Herpesvirus saimiri (HVS) is a γ herpesvirus that causes lymphomas or leukemias in certain species of New World primates (reviewed in ref. 1). The virus targets T cells and is able to transform them both *in vivo* and *in vitro* (ref. 2; reviewed in ref. 3). Five small RNAs called HVS U RNAs (HSURs), which range in size from 76 to 143 nucleotides, are encoded in a 7.4-kilobase-pair region of the HVS genome implicated in viral transformation (4, 5). Based on the following characteristics, HSURs are classified as U RNAs (6–8): (*i*) their assembly into small nuclear ribonucleoproteins (snRNPs) directed by an AU_5G sequence, which binds proteins displaying the Sm epitope; (*ii*) a 2,2,7-trimethylguanosine cap structure (m_3G); (*iii*) the presence of conserved U RNA expression signals in the genes encoding them; (*iv*) high abundance (2000–20,000 copies per cell) relative to mRNA levels. Although HSURs were originally identified and characterized in 1670 cells (a marmoset T-cell line transformed by HVS subgroup A), HSUR genes are also carried by the highly oncogenic HVS subgroup C and display 60–70% homology between the two subgroups (9). Since HSURs are expressed in virally immortalized lymphocytes or tumors, but not during lytic viral growth (5), they are implicated in HVS transformation.

Within a conserved region at their 5′ ends (6, 7), HSUR 1, HSUR 2, and HSUR 5 display an A+U-rich sequence that contains, respectively, three, two, or one copy of the pentamer AUUUA (Fig. 1). In the 3′ untranslated region (UTR) of short-lived mRNAs coding for cytokines, lymphokines, and protooncogene products such as granulocyte–monocyte colony-stimulating factor (GM-CSF) and c-fos are similar highly conserved 20- to 60-nucleotide-long A+U-rich stretches containing multiple copies of the AUUUA motif. Insertion of the A+U-rich sequence of GM-CSF into the 3′ UTR of β-globin mRNA targets this normally stable message for rapid degradation (10). A+U-rich sequences containing AUUUA motifs are thus the best characterized of several signals that dictate mRNA instability (12–18).

Recently, using either RNA bandshift assays or UV crosslinking analyses, a number of investigators have identified human proteins that specifically recognize the A+U-rich mRNA destabilization signal *in vitro* (19–24). These polypeptides range in size from 15 to 45 kDa. Their relationships to one another remain unclear, as do their exact roles in mRNA degradation. In one case, however, the binding of a 32-kDa protein in HeLa cell nuclear extract to transcripts containing mutated A+U-rich sequences was correlated with the mutant sequences' ability to function as mRNA destabilization signals *in vivo* (24). This particular protein is therefore strongly implicated as a component of the mRNA degradation machinery.

In this report, we present evidence that HSURs 1, 2, and 5 bind the same 32-kDa protein as described by Vakalopoulou *et al.* (24). Binding occurs with both the naked RNAs and HSURs assembled into snRNPs and requires the single-stranded 5′ region containing one or more iterations of the pentamer AUUUA. The association of viral small RNAs with this protein suggests a model for HSUR 1, 2, and 5 function in transformed T lymphocytes.

MATERIALS AND METHODS

Cell Culture and Nuclear Extracts. 1670 cells were cultured and nuclear extracts were prepared as described (6).

Cloning and Transcription. HSUR clones were created by using overlapping oligonucleotides and inserted into psp64 (Promega) by standard cloning procedures (25). Transcription driven by a bacteriophage T7 RNA polymerase promoter yielded RNAs that differ from the published sequences (6, 7) as follows: all five transcripts have two extra G residues at their 5′ ends to facilitate efficient transcription, the HSUR 1 transcript (149 nucleotides) has AGCU added at the 3′ end, the HSUR 2 (121 nucleotides) and HSUR 5 (120 nucleotides) transcripts have UCGA added at their 3′ ends, and the HSUR 3 (78 nucleotides) and HSUR 4 (108 nucleotides) transcripts have the final residue changed from G to C. The c-fos 3′ UTR is a 270-nucleotide-long transcript including sequences between the *Rsa* I/*Tth* III sites of the human c-fos mRNA subcloned into pGEM3, which was the generous gift of Thomas Shenk (Princeton University) (24). The human β-globin cDNA clone, yielding a 759-nucleotide transcript, was originally obtained from Richard Spritz (University of Wisconsin, Madison) and is described in ref. 26. All transcriptions were performed as described (27) and were primed with G(5′)ppp(5′)G.

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Abbreviations: HSUR, *Herpesvirus saimiri*-encoded U RNA; snRNP, small nuclear ribonucleoprotein; HVS, *Herpesvirus saimiri*; UTR, untranslated region; GM-CSF, granulocyte–monocyte colony-stimulating factor.

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Human GM-CSF 3' UTR	UAAUAUUUAUAUAUUUAUAUUUUUAAAAU AUUUAUUUAUUUAUUUAUUUAA
Human c-fos 3' UTR	UUUUUAAUUUAUUUAUUAAGAUGGAUUCUC AGAUAUUUAUAUUUUUAUUUUAUUUUU
HSUR 1 5' end	$^{2,2,7}m_3$GpppACACUACAUAUUUAUUUAUUUAUUUCUUA
HSUR 2 5' end	$^{2,2,7}m_3$GpppACACUACAUAUUUAUUGUUUAUUUAUA
HSUR 5 5' end	$^{2,2,7}m_3$GpppACACUACAUAUUUAUUUUU

FIG. 1. 5' end sequences of HSURs are similar to A+U-rich mRNA degradation signals. A+U-rich sequences in the 3' UTR of two short-lived mRNAs, GM-CSF and c-fos, are compared with HSUR 1, 2, and 5 sequences 5' to the first putative stem–loop (6, 7). The AUUUA motif is indicated by boldface type. These and other A+U-rich sequences found in the 3' UTR of labile mRNAs are tabulated in refs. 10 and 11.

UV-Light Induced Crosslinking. Three hundred thousand cpm of transcript labeled to a specific activity of 106 kcpm/ng was incubated for 30 min in 60% nuclear extract (from 5×10^6 cells) and supplemented with 3.2 mM $MgCl_2$, 2 mM ATP, 20 mM creatine phosphate, and 5 μg of *Escherichia coli* tRNA (final reaction vol, 18 μl). The mixture was then irradiated on ice for 15 min at a distance of 3–5 cm with 254-nm light. RNase A was added to a final concentration of 1 mg/ml and the mixture was incubated at 37°C for 15 min and analyzed on a SDS/12.5% polyacrylamide gel. In competition experiments, unlabeled competitor RNA was added with the labeled transcript at the beginning of the procedure.

Partial Proteolytic Digestion Analyses. Proteins were excised from polyacrylamide gels and exposed to increasing amounts of V8 protease exactly as described (28).

Antisense 2'-*O*-Methyl Oligonucleotide Competitions. 2'-*O*-methyl RNA oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (29) using phosphoramidite monomers purchased from EMBL: 5' end, 5'-AAUAAAUAUGUAGUGUBBBBC; internal, 5'-BBBBUCACAGAUUUAAG; NS, 5'-BBBBCUGUAUGAACCUG. B indicates modified 2'-deoxycytidine residues, which serve as possible sites for biotinylation, but they were not biotinylated in this experiment. The 2'-*O*-methyl oligoribonucleotides were added to 300,000 cpm of HSUR 1 transcript in 5 μl of H_2O, heated to 60°C for 5 min, and incubated at 30°C for 30 min before addition to 1670 nuclear extract, crosslinking, and analysis as described above.

Crosslinking of Proteins to Endogenous Particles. Nuclear extracts were incubated as described above except that 1 unit of DNase I (Boehringer Mannheim) was added and radiolabeled transcript was omitted. Immunoprecipitations were performed as described (6) using monoclonal Y12 antibody [anti-Sm (30)] or patient antiserum [anti-(U1)RNP and anti-m_3G (Ru; characterized to have properties similar to those described in ref. 31)] except that, after binding the snRNPs to the antibody-bound protein A-Sepharose, the pellets were washed with IPP buffer (500 mM NaCl/10 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40). The pellets were then incubated for 30 min at 37°C in TE (10 mM Tris, pH 7.5/1 mM EDTA) containing 5 mg each of RNases A and T1 per ml followed by another 30-min incubation at 37°C with 300 nM [γ-^{32}P]ATP (3000 Ci/mmol), polynucleotide kinase, DNase I (1 unit; Boehringer Mannheim), 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, and 0.1 mM EDTA.

RESULTS

To ask whether proteins implicated in mRNA destabilization bind to the HSURs, we incubated *in vitro* transcribed ^{32}P-labeled RNAs in 1670 cell nuclear extract. After exposure to 254-nm UV light to mediate covalent crosslinks between the RNA and associated proteins, excess RNA was digested with RNase A. Analysis of the resulting labeled proteins on an SDS/polyacrylamide gel (Fig. 2*A*) reveals that the protein most prominently crosslinked to HSURs 1, 2, and 5 is ≈32 kDa in size (lanes 1–4, 9, and 10). This is also the major

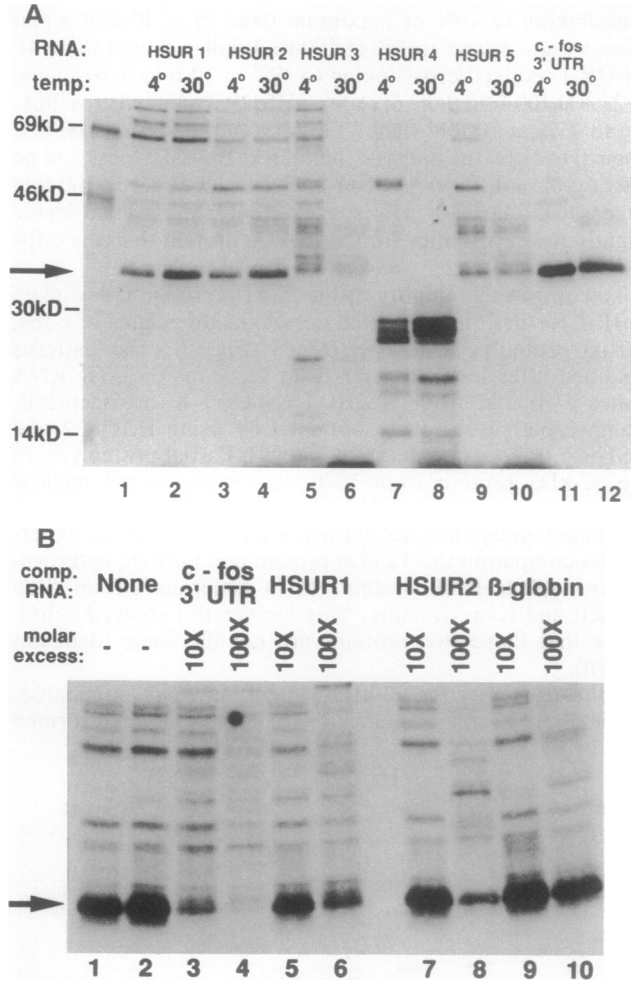

FIG. 2. UV-induced crosslinking of a 32-kDa protein to HSUR and c-fos transcripts *in vitro*. RNAs transcribed using [α-^{32}P]UTP *in vitro* were incubated in 1670 cell nuclear extract and irradiated with UV light to induce RNA–protein crosslinks. The reaction mixture was digested with RNase A and fractionated on a SDS/12.5% polyacrylamide gel. (*A*) Transcripts containing a HSUR sequence or the 3' UTR of c-fos were incubated at the indicated temperature (°C) before crosslinking. (*B*) Radiolabeled c-fos 3' UTR RNA was incubated with various unlabeled competitor RNAs at the molar excess indicated above before crosslinking. Lane 1 was incubated at 4°C; lanes 2–10 were incubated at 30°C. Arrow denotes the 32-kDa protein.

species that crosslinks to a transcript containing 250 nucleotides from the 3′ UTR of human c-fos mRNA (lanes 11 and 12), which includes the A+U-rich destabilization signal (Fig. 1). The efficiency of crosslinking is enhanced by incubation at 30°C relative to 4°C. HSURs 3 and 4, which do not contain an A+U-rich sequence (6), do not transfer significant label to the 32-kDa protein (Fig. 2*A*, lanes 5–8). Likewise, experiments using transcripts of small RNAs U1, U3, and EBER 2 (encoded by Epstein–Barr virus), or human β-globin mRNA indicated that none of these can be crosslinked to the 32-kDa protein (data not shown).

Evidence that the 32-kDa protein crosslinked to HSUR 1, 2, and 5 is the same as that previously characterized as recognizing the 3′ UTR of c-fos (24) was obtained in competition experiments. Fig. 2*B* shows that excess unlabeled HSUR 1 (lanes 5 and 6) or HSUR 2 (lanes 7 and 8) significantly reduces the amount of protein that crosslinks to labeled c-fos 3′ UTR RNA (compare with lane 2), while human β-globin mRNA (lanes 9 and 10) has little effect. Quantitation of the data was performed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA): c-fos reduced crosslinking to 19% of maximum (lane 2) at 10-fold molar excess (lane 3) and to 6% at 100-fold molar excess (lane 4); HSUR 1 reduced crosslinking to 49% at 10-fold (lane 5) and to 27% at 100-fold (lane 6) excess; HSUR 2 reduced crosslinking to 75% at 10-fold (lane 7) excess and to 20% at 100-fold (lane 8) excess. In contrast, β-globin at 10-fold excess had no effect (102%; lane 9) and had only a small effect at 100-fold excess (63%; lane 10). This suggests that HSURs 1 and 2 have slightly lower affinities for the 32-kDa protein than the c-fos message.

To confirm the identity of the 32-kDa protein crosslinked to HSURs and the A+U-rich destabilization signal of c-fos, partial proteolysis was performed (Fig. 3). The patterns obtained after label transfer from the c-fos 3′ UTR RNA (lanes 1–4) and from HSUR 1 (lanes 5–8) are identical. Comparable results were obtained by using HSUR 2 and HSUR 5 transcripts (data not shown). Partial proteolysis of the 32-kDa protein from 1670 versus HeLa cell nuclear extract further indicated that the simian and human proteins are homologous (data not shown; see ref. 24). Similar experiments comparing the 32-kDa protein and purified heterogeneous nuclear RNP protein A1 (a generous gift of Stan Barnett and Ken Williams, Yale University) showed definitively that these two proteins are not the same (data not shown).

To test whether the 32-kDa protein binds, as anticipated, to the 5′ A+U-rich sequence of the HSURs, we performed

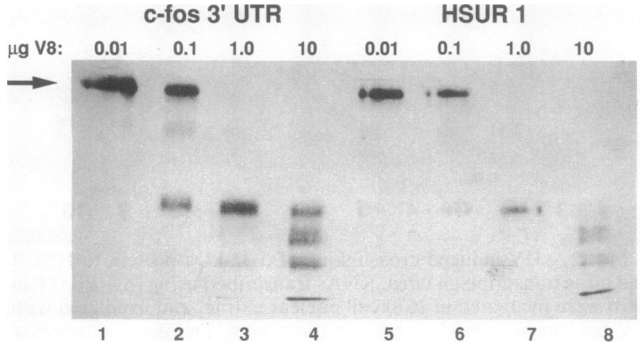

FIG. 3. Partial proteolytic digestion of the 32-kDa protein. The 32-kDa protein crosslinked either to radiolabeled c-fos 3′ UTR RNA (see Fig. 2*A*, lane 12) or to HSUR 1 RNA (see Fig. 2*A*, lane 2) as indicated was gel purified and subjected to digestion with increasing amounts of V8 protease. Three major digestion products of 17, 13, and 8 kDa are detected. Arrow indicates the full-length 32-kDa protein.

crosslinking in the presence of antisense 2′-*O*-methyl oligoribonucleotides. Three oligonucleotides (Fig. 4*B*) were prehybridized with labeled HSUR 1 transcript before crosslinking. The oligonucleotide that is complementary to the destabilization-like signal near the 5′ end of HSUR 1 substantially reduced transfer of label to the 32-kDa protein (Fig. 4*A*, lanes 2 and 3), while neither of the other two oligonucleotides had an effect (lanes 4–7). We also found that a minimal substrate consisting of only the first 30 nucleotides of HSUR 1 is sufficient to transfer label to the 32-kDa protein (data not shown), confirming that the protein interacts with the 5′ A+U-rich sequence.

Finally, we asked whether the 32-kDa protein can be found associated with HSURs assembled into Sm snRNPs *in vivo*. 1670 cell nuclear extract containing endogenous HSUR snRNPs was incubated at 30°C without added transcript, irradiated with 254-nm UV light, and then immunoprecipitated with various antibodies directed against snRNPs. The RNAs in the immunoprecipitates were next digested to completion with RNases A and T1, and the resulting crosslinked RNA fragments were labeled with [γ-^{32}P]ATP and polynucleotide kinase before fractionation of the proteins on a SDS/polyacrylamide gel (Fig. 5). Antibodies directed against the Sm proteins (lane 1) or the m_3G cap (lane 3) specifically immunoprecipitate a band that approximately comigrates with the 32-kDa protein crosslinked *in vitro* (lane M); a slightly altered mobility is not unexpected, since this postlabeling protocol adds one more phosphate to the protein than the direct label transfer procedure. In contrast, this band is not seen in precipitates using an antibody specific for the U1 snRNP (lane 2) or in HeLa nuclear extracts, which lack HSURs (lanes 4–6). When the 1670 extract was incubated with increasing amounts of the 2′-*O*-methyl oligoribonucleotide that hybridizes to the 5′ end of HSURs 1, 2, and 5 (Fig. 4*B*), the amount of labeled 32-kDa protein detected in anti-Sm immunoprecipitates decreased, while the nonspecific oligonucleotide had no effect (data not shown). We conclude that the 32-kDa protein becomes labeled because it has been crosslinked to endogenous HSUR 1, 2, and 5 snRNPs in the 1670 cell nuclear extract.

DISCUSSION

We have shown that a 32-kDa protein present in virally transformed marmoset T cells binds to the 5′ end of HSURs 1, 2, and 5. This protein appears to be the homolog of a HeLa cell protein characterized by Vakalopoulou *et al.* (24). They demonstrated that this protein binds to RNAs displaying ≥1 copy of the AUUUA pentamer in a U-rich context and that transcripts containing three pentamers bind better than those with only one pentamer. Their mutational analyses showed that multiple U to G changes within the AUUUA motif eliminated binding entirely and that the relative affinities of mutant sequences for the 32-kDa protein correlated with their ability to serve as destabilizing signals in the 3′ UTR of β-globin mRNA *in vivo*. Our results further suggest that the 32-kDa protein requires a single-stranded region for RNA binding since sequestering the 5′ A+U-rich sequence of HSUR 1 in a duplex inhibits label transfer (Fig. 4). Oligonucleotide-directed RNase H cleavage of HSUR snRNPs previously demonstrated that the 5′ A+U-rich sequence of HSURs 1, 2, and 5 is single stranded and accessible for interactions within the particle (6, 7).

Other human proteins that specifically recognize the A+U-rich mRNA destabilization signal *in vitro* have also been reported. AUBF, detected by UV crosslinking and band-shift assay in cytoplasmic extracts of Jurkat cells, migrates with an apparent molecular mass of 44 kDa on nonreducing SDS gels and as 15-, 17-, and 19-kDa polypeptides under reducing conditions; it is up-regulated in cells treated with mitogens

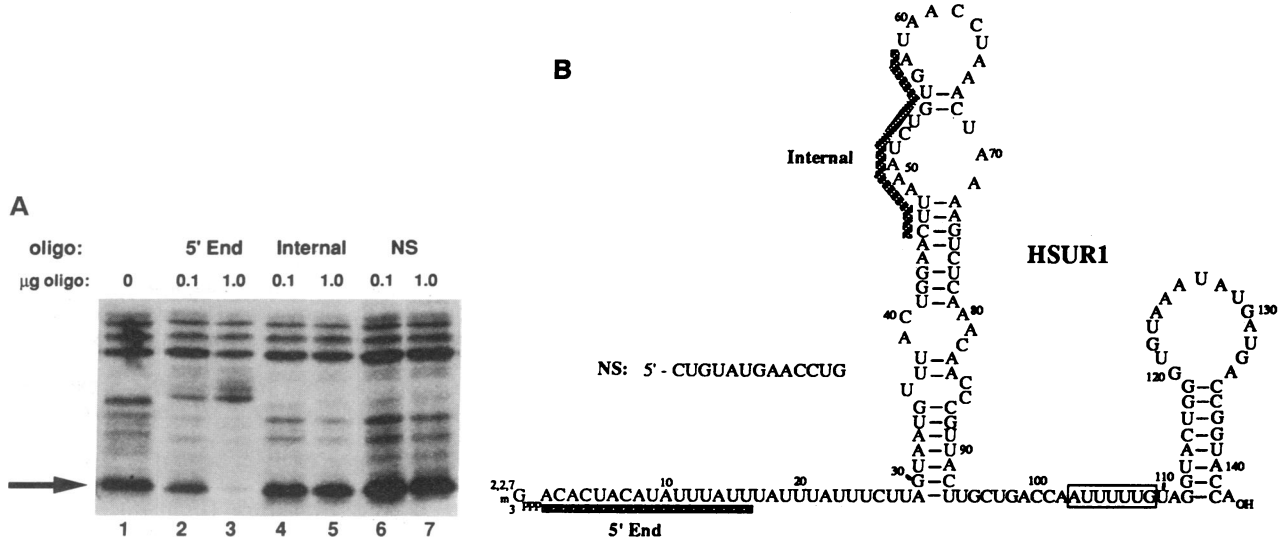

FIG. 4. Inhibition of crosslinking of the 32-kDa protein to HSUR 1 by an oligonucleotide complementary to the 5′ A+U-rich sequence. (*A*) Radiolabeled HSUR 1 transcript was hybridized with either 0.1 or 1 μg of 2′-*O*-methyl oligoribonucleotide before incubation in 1670 cell nuclear extract. Arrow indicates the 32-kDa protein. (*B*) HSUR 1 is displayed in a putative secondary structure with the nucleotides complementary to the 2′-*O*-methyl oligoribonucleotides indicated by stippled bars and the Sm binding site boxed. NS, nonspecific 2′-*O*-methyl oligoribonucleotide.

known to stabilize labile messages (19–21). AU-A (estimated size, 34 kDa) and a slightly smaller protein, AU-B, have been identified by band shift analyses in T-cell cytoplasmic extracts; AU-A is expressed constitutively and binds the 3′ UTR of lymphokine as well as protooncogene mRNAs, while AU-B is present only in extracts of proliferating T cells and binds only lymphokine mRNAs (23). Fractionation of a S130 cytoplasmic extract of erythroleukemia cells has yielded a fraction called Auf, which stimulates *in vitro* degradation of endogenous labile mRNAs, exhibits specific binding to A+U-rich sequences, and contains two polypeptides of 37 and 40 kDa (22). The relationship of these proteins to the nuclear 32-kDa protein studied by Vakalopoulou *et al.* (24) and ourselves is unknown.

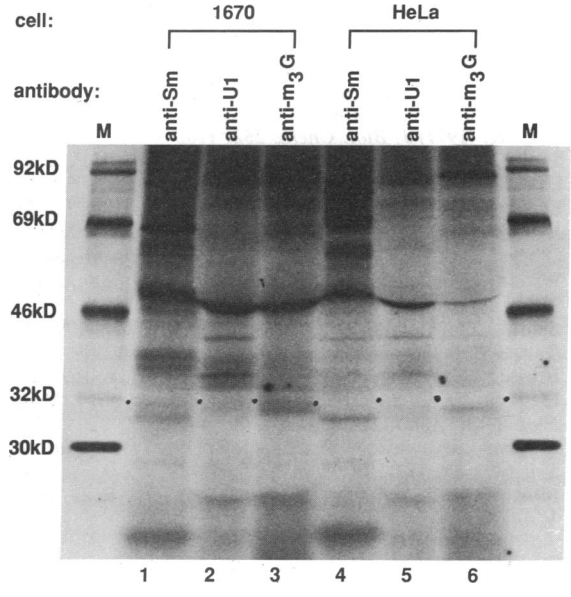

FIG. 5. Crosslinking of the 32-kDa protein to endogenous HSUR snRNPs. Nuclear extracts of 1670 cells (lanes 1–3) or HeLa cells (lanes 4–6) containing equal amounts of the abundant U RNAs were incubated without radiolabeled transcript at 30°C and then crosslinked. snRNPs were immunoprecipitated by using anti-Sm (lanes 1 and 4), anti-(U1)RNP (lanes 2 and 5), or anti-m_3G cap (lanes 3 and 6) antibodies followed by digestion with RNases A and T1. The resulting RNA fragments crosslinked to proteins were labeled with [γ-^{32}P]ATP and polynucleotide kinase and fractionated on a SDS/12.5% polyacrylamide gel. As a marker, the 32-kDa protein crosslinked *in vitro* to HSUR 1 (see Fig. 2*A*, lane 2) was fractionated with prelabeled markers (Amersham) in parallel (lane M). Dots indicate mobility of the postlabeled 32-kDa protein.

Our experiments suggest that HSURs 1, 2, and 5 function to stabilize certain cellular or viral messages normally targeted for rapid decay by competitively inhibiting the cellular pathway that degrades mRNAs containing the A+U-rich destabilization signal. Like cellular U RNAs, HSURs are stable transcripts displaying half-lives of >8 hr (data not shown). In transformed cells, HSURs are 100- to 1000-fold more abundant than any given short-lived mRNA, and our UV crosslinking experiments demonstrate that at least 70% of the label transfer from the c-fos substrate can be blocked by competition with a 100-fold molar excess of either HSUR 1 or 2 (Fig. 2*B*). Thus, HSURs, being complex nuclear particles, could stably sequester the 32-kDa protein, preventing its normal action in mRNA degradation but themselves escaping destruction.

Attempts to design experimental systems to test this idea *in vivo* have not yet yielded definitive answers. Steady-state levels of β-globin mRNA containing the GM-CSF A+U-rich sequence (Fig. 1), expressed from an integrated gene, have been measured in HeLa cell lines stably expressing each individual HSUR without detecting reproducible differences. However, because all stably expressing HeLa cell lines obtained contain 20- to 50-fold fewer HSURs than 1670 cells (data not shown), such levels may not be high enough to effectively inhibit the degradation pathway. In contrast, HSURs can be transiently expressed in HeLa cells at levels comparable to those found in 1670 cells (8), but the only reliable transient system for studying the effect of the A+U-rich sequence on mRNA half-life utilized NIH 3T3 cells (14) and cannot be applied to HeLa cells (data not shown). Thus, testing our model for HSUR 1, 2, and 5 function *in vivo* must await development of an inducible HSUR expression system that will minimize toxicity to the host cell, yet produce high levels of viral snRNPs.

Since U RNAs are nuclear, the function we propose for HSURs 1, 2, and 5 is reasonable only if the recognition of the A+U-rich destabilization signal can occur in the nucleus.

Although studies linking the destabilization signal in the 3′ UTR to deadenylylation of mRNA (32, 33) and to nuclease action in polysome washes (34) suggest that the actual degradation process is cytoplasmic, they do not eliminate the added possibility of degradation or recognition of the destabilization signal before a newly synthesized mRNA exits the nucleus. Indeed, the insertion of A+U-rich sequences into the 3′ UTR of β-globin transcripts reduces levels of both unspliced and spliced mRNAs, arguing that the signal targets both nuclear and cytoplasmic RNAs for rapid decay (24). Moreover, the crosslinking activity of the 32-kDa protein is enriched in nuclear relative to cytoplasmic fractions (24), an observation with which we concur.

Stabilizing labile mRNAs with resulting overexpression of cellular growth factors or oncogenic proteins (10, 35, 36) could contribute significantly to the transforming potential of HVS. The conservation of HSUR genes between oncogenic HVS subgroups (9) argues that these snRNPs are functionally important in virally transformed cells. Detailed genetic analysis of HVS subgroup A has revealed an open reading frame (specifying a protein called STP-A11) required for immortalization of marmoset peripheral blood lymphocytes in culture (37). However, neither a mRNA encoding STP-A11 nor the protein itself could be detected in HVS transformed cells, leading Murthy *et al.* (37) to suggest that STP-A11 may be necessary for the induction but not the maintenance of transformation. Viruses lacking HSUR 1, 2, and 5 genes do immortalize T cells in culture, but these transformed cells grow significantly slower, doubling in 1 week as compared to 1.5 days for cells transformed by wild-type HVS (37). It remains to be seen whether the other abundant viral transcripts, HSURs 3 and 4, or perhaps the newly detected HSURs 6 and 7 (J. C. Albrecht, B. Biesinger, and B. Fleckenstein, personal communication), all of which lack the 5′ A+U-rich sequence, collaborate with HSURs 1, 2, and 5 or fulfill a different role in transformed cells. Our results provide a testable hypothesis for how HSURs 1, 2, and 5 could function in the maintenance of viral transformation.

Because of the nature of their contributions, both V.E.M. and S.I.L. should be considered as primary authors. We thank the members of the Steitz lab, especially Dr. Susan Baserga, Karen Montzka-Wassarman, David Toczyski, Dr. Kazimierz Tyc, and David Wassarman, for thoughtful insights, critical evaluation, and essential materials. Also, John Flory synthesized all oligonucleotides, Mei-Di Shu prepared the Y12 antibody, and Dr. Anita Gilliam characterized the anti-m_3G patient antiserum. This work was supported in part by Grant CA 16038 from the National Institutes of Health.

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