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2012

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Characterizing the Molecular Basis for mRNA Targeting and Destruction by the Kaposi's
Sarcoma-Associated Herpesvirus (KSHV) Protein SOX

By

Sergio Covarrubias

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Britt Glaunsinger, Chair

Professor Kathleen Collins

Professor Matthew Welch

Spring 2012

Abstract

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Professor Britt Glaunsinger, Chair

Lytic infection with gamma herpesviruses such as Epstein Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) results in a global depletion of cellular mRNA, which manifests in a general suppression of host gene expression, termed host shutoff. The importance of targeting cellular gene expression at the posttranscriptional level is highlighted by the observation that many changes in important cellular gene programs result in drastic changes in RNA stability. Hence, virus targeting of host gene expression at this level likely represents an efficient way to change the cellular environment into one that supports maximal viral production. In KSHV, the host shutoff phenotype is caused by virally encoded multifunctional nuclease, SOX, that is conserved throughout all gamma-herpesviruses. Here I provide detailed mechanistic insight into how SOX targets host messages to enact host shutoff activity in cells. I show that within the cytoplasm, SOX specifically targets conserved mRNA features, which normally promote their efficient expression. Furthermore, I demonstrate that this mechanism of mRNA targeting is conserved across various other viruses, likely demonstrating an evolutionarily convergent strategy. Characterizing how viruses co-opt specific cellular factors involved in gene expression will yield a better understanding of the signals that initiate activation of RNA turnover pathways needed for viral replication success.

Dedicated to my family.
Thank you for believing in me.

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Acknowledgements

I would like to thank Britt Glaunsinger for being a wonderful and supportive mentor. Throughout the years, Britt has fostered an environment that has promoted active discussion of science in a way that allowed me to thrive, never being afraid of proposing the craziest ideas. Furthermore, her emotional support consistently motivated me to get through the storm of failed experiments (a natural product of science) and personal problems in my life. I will always be grateful to her for believing in me even when I had seriously doubted myself.

Thanks to all current and past Glaunsinger Lab members for creating a scientifically enriching environment, while at the same time creating a fun atmosphere. I want to specifically thank Dr. Karen Clyde for always being available to share her expertise and being open to discussion ideas and crude experimental designs. Additionally, I am grateful to Dr. Marta M. Gaglia for always being available for a good coffee break to discuss ideas for developing my project. I am grateful to Ian Van Trump and Marta M. Gaglia for providing constructive feedback on this thesis.

During the beginning of my graduate school career, Dr. Alex Sabogal took me under his wing and connected me with the biochemistry expertise of the Rio Lab. All members of the Rio Lab were always welcoming to my many naïve biochemistry questions and also provided countless reagents.

I am extremely grateful to Dr. Robert Beatty, who gave me the opportunity and provided the expertise to help me successfully make anti-SOX monoclonal antibodies as part of his immunology lab class. This reagent will be invaluable for years to come.

A sincere thanks is due to my thesis committee for providing insightful input during our various meetings. I am grateful to Emily Egan from the Collins lab for sharing her knowledge through various emails and for generously providing reagents.

All of the polysome profile analysis would not be possible without Dr. Andy Jackson, who was kind enough to take the time to train me in these techniques and was a great source of knowledge. I appreciate his extraordinarily great sense of humor that made the training that much more enjoyable.

Thanks to my aunt Olga Plateado and uncle Freyr Thor for all their support and for helping me resolve personal issues, which allowed me to start graduate school on time.

Throughout the years, my wife Debbie Covarrubias has offered her unconditional support. She was always there behind the scenes telling me the right things when I was feeling the most beat down. She always believed that I would be successful and spent a lot of time convincing me that things were going to work out. She was right.

Chapter 1:

Introduction

The success of a viral infection is determined by the ability of the virus to modulate the host environment such that viral replication and dissemination is maximized while eradication by the immune system is minimized. At the cellular level, this is achieved by directly interfacing with cellular gene expression machinery.

A viral infection is often accompanied by a reprogramming of cellular gene expression in order to alter the cellular environment to accommodate efficient mass production of virions. Gene regulation at the mRNA stability level is a significant contributor to global gene expression (78). It is therefore not surprising that various divergent viruses have evolved convergent strategies of specifically targeting RNA stability in order to modulate host gene expression. Among these viruses is the most recently identified human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), which destabilizes mRNA through the activity of the viral SOX protein, resulting in a suppression of host gene expression. The SOX protein is a multifunctional nuclease whose host shutoff function is conserved in all gammaherpesviruses. Here I provide extensive molecular characterization of the mechanism of SOX host shutoff (HS), providing insight into how SOX selectively targets cellular messages in cells. Herpesviruses represent one of the most ancient human-associated viruses. Suppression of host gene expression likely represents an evolutionary conserved strategy for directly modulating the cellular environment in order to optimize viral production and ensure its survival in the host.

Herpesviruses

Human herpesviruses (HV) have a long co-evolutionary history with humans, highlighted by their stringent species specificity as well as the observation that over 30% of herpesviral genes are not required for viral replication in tissue culture and are instead proposed to function in immune evasion (27, 95). The evolutionary connection between virus and host is further highlighted by the fact that many viral genes have highly conserved homology to human genes. Many of these homologous genes function in a variety of important cellular processes presumed to be important for viral replication (2, 74). Furthermore, HVs infect individuals for life, which suggests that the virus must have evolved sophisticated mechanisms of evading detection/elimination by the host immune system, while ensuring the continual propagation of virus within the host.

HVs are characterized by their ability to engage in two alternative cycles, latent and lytic replication. During the passive state of latency, very few genes are expressed and no virions are produced. Latency is therefore thought to be one way in which HVs are able to persist in the host while remaining immunologically invisible. Lytic infection is essential for pathogenesis and is characterized by expression of the full complement of viral genes, many of which function to manipulate the host environment for the benefit of the virus (2). Lytic infection also serves as a way to reseed new cells with virus to counter clearance by the immune system. Because HVs infect individuals for life, even in their latent state there is always potential for reactivation, which is kept in check by the immune system. In fact, the suppression of the immune system has been shown to be a trigger for herpesviral lytic replication and subsequent disease (70).

The herpesviridae family is subdivided into three subfamilies: alpha, beta and gamma, based on replication length, cell tropism, and genome structure (31). The best-characterized subfamily is the alpha subfamily, and particular the Herpes Simplex Virus (HSV), the causative agent of genital and oral herpes lesions. In addition, the alpha subfamily has a second well-known member, the Varicella-Zoster Virus, the causative agent of chicken pox. The beta subfamily member, Cytomegalovirus (CMV) has been implicated in a variety of birth defects when acquired during pregnancy (42, 64). Representatives of the gamma subfamily include the well known Epstein-Barr Virus (EBV), as well as the most recently discovered human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV). EBV is known to cause infectious mononucleosis and is causative agent of certain types of B cell lymphoproliferative diseases. Similarly, KSHV is also implicated in certain B cell lymphomas as well as causing an atypical skin cancer commonly seen in immunocompromised AIDS patients (75).

Gene Expression During Lytic KSHV Infection

It has been established that KSHV infection is necessary for the development of such diseases as Kaposi's sarcoma (KS) lesions, as well as certain types of B cell lymphomas. Early analysis of the diseased tissue revealed that the majority of cells were latently infected, with lytic infection making up approximately 1-3% of the total infected cells (28). This observation led to the conclusion that latent viral infection was the major player in propagating disease. However, an epidemiological study assessing KS treatment with ganciclovir (which blocks viral DNA replication during the lytic cycle) revealed that treatment with the drug resulted in a decrease of new KS lesion formation (85). The ganciclovir observations led to the conclusion that, in addition to latency, the lytic replication cycle plays a critical role in the development of disease. It has since been proposed that lytic infection may be important for providing autocrine and paracrine factors necessary for tumor survival (12, 13, 68). Additionally, lytic infection provides a new source of viral progeny to re-infect the next round of susceptible cells.

At the cellular level, infection begins with the binding of the virion to the cell via various receptors including heparin sulfate (HS) (8), integrins (34), and the cystine/glutamate transporter (xCT) (102). Following binding, a membrane fusion reaction delivers the capsid and tegument to the cell cytosol. The capsid is then transported to the nuclear membrane where it docks at the nuclear pore (19). The dsDNA genome is then transported into the nucleus (89) where it is circularized and acquires histone modifications that will facilitate cellular RNA Polymerase II (Pol II) recruitment for initiating transcription (reviewed in (61)).

Once the viral genome reaches the nucleus, two programs of gene expression are possible: latent and lytic cycles. For KSHV, the latent program appears to predominate in most examples of infections of various cell types (6). Only a hand-full of genes are expressed during latency. Among these is the latency-associated nuclear antigen protein (LANA), involved in the maintenance of the viral genome, which physically tethers the viral DNA to the host chromosome (20). In addition, LANA has been demonstrated to play a role in inhibiting the lytic program by directly binding and inactivating the lytic replication and transcriptional activator (RTA) (described below) (63).

Reactivation from latency initiates with temporally regulated waves that can be divided into immediate early (IE), delayed early (DE), and late (L) gene expression. This "wave" pattern of expression allows for the synthesis of groups of proteins with similar functions. The RTA IE viral gene has been demonstrated to be sufficient to induce entry into the lytic cycle (40). Furthermore, the RTA gene product is characterized as a classical viral

transcriptional activator possessing an N-terminal DNA binding domain (DBD) and a C-terminal activation domain (AD) (104). RTA directly binds a consensus sequence upstream of several viral promoters of DE genes, which includes genes involved in DNA replication as well as genes involved in modulating gene expression.

The DE group of genes involves two main classes of factors: 1) DNA replication-associated proteins including DNA polymerases, ssDNA binding proteins and DNA exonucleases, which promote efficient production of new viral genomes (92). 2) The other type of genes expressed includes factors that alter gene expression. The viral ORF57 or mRNA transcript accumulation (MTA) protein is thought to promote enhanced expression of L genes by directly influencing splicing, RNA export, and translation (reviewed in (18)). Interestingly, the SOX protein (the focus of this thesis) belongs to the DE group and can be categorized as a factor involved in both DNA replication and modulation of gene expression (this will be discussed below). In this manner each temporal cluster of expression drives the next set of genes propagating the entire lytic program.

Traditional and New Roles For The Viral Alkaline Exonuclease (AE) Proteins

Early studies demonstrated that the alkaline exonuclease (AE) in herpes simplex virus I (HSV I) is essential for viral replication, as an AE deletion resulted in the accumulation of unresolved complex DNA structures in the nucleus that normally have to be processed to yield individual linear dsDNA genomes (38). Studies in other related herpesviruses have underscored the high conservation of this gene, demonstrating a similar essential role in viral replication (22, 105). *In vitro*, several studies have established AE as 5'→3' DNA exonuclease with the ability to target a variety of linear ds and ssDNA substrates (5, 10, 69, 90, 96). Additionally, AE was shown to target supercoiled and nicked dsDNA implicating an additional endonucleolytic activity (10, 90, 96). Assessment of intrinsic RNase activity led to the conclusion that AE degrades RNA poorly, if at all (56, 77).

Although there is consensus on the enzymatic activity of AE, its exact *in vivo* role is less clear. The high structural similarity to lambda phage exonuclease suggests that AE could function similarly, degrading ssDNA from the 5' end, thereby creating 3' overhangs that can be bound by ssDNA binding proteins and promote recombination (98). In support of this, AE has been shown to interact with the viral ssDNA binding protein, ICP8 (99). *In vitro*, strand exchange reactions (first step in recombination) can be recapitulated with a DNA substrate in the presence of both AE and ICP8 supporting their role in recombination during viral infection (82). In addition to these activities, the gamma-HV AE has acquired a novel function of promoting RNA turnover, resulting in suppression of host gene expression (37). The additional HS function could be genetically separated from its DNase activity, suggesting that the gamma-HV AE truly possesses two distinct functions (36). Furthermore, in KSHV the gene was renamed SOX for its dual Shutoff and DNA exonuclease activities (the AE gene will be referred to as SOX from here on). All SOX homologs localize to the nucleus consistent with their roles as DNA AEs, however in gamma-HVs, SOX possesses an additional cytoplasmic localization (36). This difference in localization potentially explains why only gamma-HV SOX possessed HS activity.

The search for the molecular basis of host shutoff has resulted in three recent crystal structures of the gamma-HV HS protein SOX from KSHV (4, 23) and BGLF5 from EBV (10). Several interesting observations were made by these studies in connection to their HS activity. First, SOX homologs possess RNase activity, although this activity is significantly weaker than their DNase activity (requiring 30X more protein) and is preferentially enhanced with Mn²⁺

instead of Mg^{2+} (10). Second, the core catalytic residues (Mn/ Mg^{2+} binding residues) are required for both DNase and RNase activities *in vitro* (10). Third, the affinity of SOX for RNA is noted as weak (K_d of 87 μM), which is inconsistent with SOX binding RNA directly (23). Fourth, it was noted that the previously characterized single function HS- mutants (36) had WT *in vitro* RNase activity, which does not reflect their HS defect seen in cells (23). Additionally, the majority of HS- mutations map to outside the catalytic interior towards the N-terminal portion of SOX (23). The conclusions made by these studies suggest that the RNase activity directly contributes to the HS activity seen in cells. However, HS defects observed in cells were not reflected *in vitro*, suggest that additional cellular cofactors could account for the SOX's HS activity ((10), unpublished observations). Furthermore, these findings are in line with our hypothesis that cellular factors contribute to the specificity and enhancement of HS in cells.

Role of Host Shutoff During Herpesviral Infection

At the cellular level, HS has been proposed to aid in freeing up cellular machinery from competing cellular mRNAs in order to enhance translation of viral messages (100). *In vivo* studies have suggested that HS plays a vital role in preventing clearance by the immune system (93, 94, 97). Similar to KSHV, the alpha-HV member, herpes simplex virus (HSV), promotes HS during viral infection. However, since HSV SOX homologs lack HS activity, these viruses instead use a separate unrelated viral product called the virion host shutoff (vhs), which similarly destabilizes cellular mRNAs. Previous studies had suggested that vhs could function in promoting transitions from E to L viral gene expression, since it also appeared degrade viral mRNAs (62). Surprisingly, a vhs deletion mutant replicated to equivalent levels as wild-type virus in tissue culture, suggesting little role in viral replication (62). In contrast, during mouse infections the vhs mutants demonstrated a severe defect in pathogenesis and in its ability to establish latent infection (97). This defect was attributed in part to an inability to evade immune responses (97), in part due to the inability to downregulate the interferon response, which is a major branch of the antiviral immune response (26).

Both KSHV and EBV induce HS during a viral infection, which is mediated by their SOX homologs. Assessment of the *in vivo* role of HS for both KSHV and EBV has been challenging because of their untractable genetics due in part to their default latent program. Additionally, infection for both viruses is restricted to humans. However, using a closely related mouse gamma-HV, murine herpesvirus 68 (MHV68), we recently demonstrated in mouse infections that a HS defective virus was severely defective in latency establishment (83). It was observed that the HS defective mutant reactivated at a higher efficiency compared to WT MHV68 (83). This observation highlights the possibility that HS could have a role in maintaining the latent/lytic balance needed for efficient latency establishment (83). Additionally, we have recently observed that the HS defective virus may have a cell specific replication defect (E. Abernathy, unpublished observations), which adds another layer to the many potential roles of HS during viral infection.

Gamma-Herpesvirus Host Shutoff

Regulation at the RNA stability level is an important contributor to global cellular gene expression, as it is estimated that more than 40% of mRNA abundance is due to changes in stability (29, 78). Protein levels are often proportional to mRNA levels and therefore controlling the abundance of mRNA directly affects protein synthesis (7). Changes in RNA stability have been demonstrated to play crucial roles in various cellular processes such as cellular stress (29),

activation (14), and signaling (41) where tight control of gene expression is anticipated to be important. Basal RNA turnover involves the activity of highly conserved RNases that can degrade RNA in either the 5' → 3' direction by the ribonuclease 1 (Xrn1) or in the 3' → 5' direction by the complex called the exosome (reviewed in (7, 33)). RNA is degraded in a regulated and ordered fashion beginning with the rate-limiting deadenylation of the poly(A) tail, which is followed by a decapping reaction. Degradation by Xrn1 is thought to be the major pathway to bulk degradation of mRNA in the cytoplasm.

KSHV infection results in the destruction of nearly the entire cellular transcriptome, which culminates in a global halt of host gene expression, mediated by the multifunction viral nuclease, SOX. As discussed in previous sections, SOX homologs are conserved across all HVs where they function in DNA processing, however only in the gamma-HV has SOX acquired a novel genetically separable HS function of promoting degradation of mRNA. HS likely serves to assist in immune evasion and pathogenesis in order to promote successful establishment of latency (83). In addition to RNA degradation, HS involves two other downstream events. First, upon SOX expression, nascent cellular mRNAs undergo poly(A) tail extension (hyperadenylation), and are likely retained in the nucleus (66). Although hyperadenylation has not been characterized in human cells, in yeast it is thought to be a quality control mechanism arising as a consequence of aberrant mRNA 3'-end processing or nuclear export defects (45, 49, 67). The second SOX-induced phenotype is a striking relocalization of cytoplasmic PABPC into the nucleus. PABPC has prominent roles in cytoplasmic mRNA stability and translation, and its nuclear relocalization by SOX during KSHV infection is coincident with turnover mRNAs in the cytoplasm (66).

Direct activation of one or more degradation pathways would likely be an efficient way for the virus to promote large changes in the cellular expression program. We have recently uncovered further insight into how messages are destabilized by SOX in the cytoplasm. Interestingly, the mechanism involves the coordinated activities of SOX and the cellular Xrn1, where SOX-induces a site-specific endonucleolytic cleavage on mRNA providing an accessible end for Xrn1-mediated degradation of the mRNA body (21). We anticipate that further characterizing how the virus co-opts specific cellular degradative pathways could lead to the identification of novel cellular proteins that are essential for the regulation of this process.

Outline

Herpesviruses have a long co-evolutionary history with humans, which has resulted in finely-tuned strategies to efficiently alter host gene expression in order to promote the production of factors necessary for the viral life cycle. Regulation at the RNA stability level is an important contributor to cellular gene expression and distinct viruses have now been shown to target mRNA stability as a direct way to alter host gene expression. Among the viruses causing extensive mRNA turnover is gamma herpesviruses, which promote a general suppression of host gene expression caused by conserved virally encoded multifunctional nuclease, SOX. Although we have recently shown that SOX utilizes RNA turnover pathways to mediate host shutoff, it was unclear how SOX specifically targets mRNAs in cells.

Here I provide detailed mechanistic insight into how SOX specifically targets host mRNAs to mediate shutoff activity in cells. I show that SOX's cytoplasmic localization is critical for its HS activity. In the cytoplasm, SOX specifically targets conserved features on mRNAs that normally ensure their efficient expression. I demonstrate that this mechanism of targeting is conserved across various other divergent viruses, which likely demonstrates an

evolutionarily convergent strategy of targeting common stages of gene expression. Characterization of how different viruses co-opt specific factors involved in gene expression will allow a better understanding of the signals that initiate the activation of cellular RNA turnover pathways needed for viral replication success.

Chapter 2:

Host Shutoff Is a Conserved Phenotype of Gammaherpesvirus Infection and Is Orchestrated Exclusively from the Cytoplasm

Background

The alkaline exonuclease is an essential protein widely conserved across all herpesviruses, where it participates in the resolution of complex branched structures that arise during rolling circle replication of the viral DNA genome (39, 73, 81, 82). In KSHV and EBV, this protein has also evolved a genetically separable host shutoff function (36, 106). While the exact cellular mRNA turnover pathways targeted by SOX or BGLF5 remain incompletely characterized, we have observed that SOX induces two prominent phenotypes directly related to host shutoff. First, upon SOX expression, nascent cellular mRNAs undergo poly(A) tail extension (hyperadenylation), and as a consequence they are likely retained in the nucleus (59, 66). The second SOX-induced phenotype is a striking relocation of cytoplasmic poly(A) binding protein (PABPC) into the nucleus. PABPC has prominent roles in cytoplasmic mRNA stability and translation, and its nuclear relocation by SOX during KSHV infection is coincident with turnover of mRNAs in the cytoplasm (66). Thus, SOX may prevent export of nascent nuclear messages and deplete preexisting cytoplasmic mRNAs via distinct mechanisms. SOX and BGLF5 localize predominantly to the nucleus but are also detectable to a lesser extent in the cytoplasm of transfected cells. The nuclear population is anticipated to participate in the viral genome processing function, but it has been proposed that the cytoplasmic fraction of SOX and BGLF5 contributes to mRNA turnover in that locale (36, 86). At the time, it was unclear whether PABPC import and hyperadenylation activities were carried out by cytoplasmic SOX. This point was particularly relevant for understanding the mechanism by which SOX induces nuclear hyperadenylation, as direct modulation of the RNA 3'-end processing machinery by SOX should require its presence in the nucleus.

Using spatially restricted variants of KSHV SOX and the MHV68 murine SOX (muSOX), I show that all host shutoff-related activities, including nuclear mRNA hyperadenylation, are orchestrated exclusively by the cytoplasmic pool of SOX and muSOX. These results have important implications for the mechanisms underlying gamma-HV-induced host shutoff.

Results

SOX homologs of gamma-herpesviruses localize to both the nucleus and cytoplasm

All herpesvirus AE homologs examined to date localize either predominantly or exclusively to the nucleus, in agreement with roles in processing and packaging newly replicated viral DNA (39, 73, 81, 82). However, several lines of evidence suggest that cytoplasmic localization of the gamma-HV SOX homologs plays an important role in host shutoff. First, KSHV SOX and EBV BGLF5 localize both to the nucleus and cytoplasm of cells, whereas HSV-

1 AE, which lacks host shutoff activity, is constrained to the nucleus (37, 86). Second, a SOX nuclear localization signal (NLS) mutant retains wildtype (WT) mRNA turnover activity in transfected cells (36). Finally, mRNA half-life studies demonstrated that cytoplasmic messages are destabilized in SOX-expressing cells (66).

I predicted that a related gamma-HV homolog, MHV68 muSOX, would also exhibit partial cytoplasmic localization, which might be integral to its host shutoff function. Indeed, similar to its KSHV and EBV homologs, haemagglutinin (HA)-tagged muSOX was detected in both the nucleus and cytoplasm of transfected 293T cells, whereas HSV-1 AE remained exclusively nuclear (Figure 2.1A). The absence of antibodies against any gamma-HV SOX homologs that function in immunofluorescence (IF) had previously prevented confirmation of this partial cytoplasmic SOX localization during viral infection. Given that plasmid-based overexpression does not always faithfully mimic endogenous protein localization, we generated both monoclonal antibodies (MAbs) against KSHV SOX and affinity-purified polyclonal antibodies (pAbs) against MHV68 muSOX to monitor their localization throughout the course of infection. The localization and kinetics of expression of SOX and muSOX were examined by IF in HFF and 3T3 cells lytically infected with KSHV and MHV68, respectively (Figures 2.1B and 2.1C). Expression of both SOX and muSOX was detected beginning at 8 h postinfection, in agreement with previous results (Figures 2.1 B and 2.1C) (37), and expression was maintained throughout the lytic cycle. Importantly, SOX and muSOX localized to both the nucleus and the cytoplasm during viral infection, although the majority of SOX and muSOX exists in the nucleus (Figures 2.1B and 2.1C; a magnified view of representative muSOX-expressing cells is shown in the bottom panels of Figure 2.1C).

Restriction of muSOX to the nucleus abolishes its activity

Having shown that both SOX and muSOX exhibit partial cytoplasmic localization during lytic infection, I next sought to determine whether this cytoplasmic fraction was necessary for host shutoff. A SOX nuclear localization signal mutant (SOX-NLSmut) had previously been shown to be predominantly cytoplasmic and to retain WT mRNA turnover activity (36). I initially attempted to generate a similar NLS mutant in muSOX to explore the level of phenotypic conservation between these homologs. However, I found that unlike the monopartite SOX NLS, muSOX possesses a bipartite NLS (aa 314 to 318 and aa 409 to 412), the latter component of which resides in a highly conserved region of the protein that cannot be mutated in either SOX or muSOX without destroying both DNase and host shutoff activity (unpublished observations).

I pursued the converse approach of restricting muSOX to the nucleus by fusing it to a Nuclear Retention Signal (NRS) derived from the heterogeneous nuclear ribonucleoprotein C1 (HA-NRSmuSOX) (91). As a control, I fused muSOX to a mutant NRS peptide of identical length that is incapable of nuclear restriction (HA-ΔNRS-muSOX). IF staining confirmed that the HA-NRS-muSOX fusion was constrained to the nucleus, whereas the WT HA-muSOX and the HA-ΔNRS-muSOX fusion were present both in the nucleus and the cytoplasm (Figure 2.2A). I then measured host shutoff by testing the ability of these fusions to block expression of green fluorescent protein (GFP). I observed no shutoff of GFP expression in cells coexpressing HA-NRSmuSOX, whereas GFP protein expression was strongly inhibited in cells coexpressing HA-muSOX or HA-ΔNRS-muSOX (Figure 2.2B). Northern blot analysis of the GFP message confirmed that the inability of HA-NRS-muSOX to block GFP protein expression was due to its failure to promote mRNA turnover, even at high concentrations (Figure 2.2C). Western blotting

of the muSOX variants shows that transfecting increased amounts of HA-muSOX or HA-ΔNRS-muSOX does not lead to a significant increase in their protein levels, presumably because they are inducing degradation of their own transcripts (Figure 2.2C). This is not the case with HA-NRS-muSOX, whose protein levels continually increase as more plasmid DNA is transfected, further supporting the observation that this protein is defective for RNA turnover. Notably, the inability of HA-NRS-muSOX to promote RNA turnover is not due to gross misfolding of the protein, since this fusion retains enzymatic activity in DNase assays (Figure 2.2D). As this was not the case for NRS-tagged SOX from KSHV, which lost all activity upon fusion to any large tag, I was unable to perform similar experiments with SOX (unpublished observations). Thus, I conclude that the nuclear fraction of muSOX does not contribute appreciably to mRNA turnover, whereas the cytoplasmic fraction is critical for this function.

An alphaherpesivirus SOX homolog localized to the cytoplasm does not induce host shutoff

Given that cytoplasmic localization is critical for host shutoff activity, I considered the possibility that HSV-1 AE fails to promote mRNA turnover simply because it is not present in the cytoplasm. To test this hypothesis, I mutated the NLS of HSV-1 AE to cause localization in the cytoplasm resembling that of its gamma-HV homologs. An HSV-1 AE NLS has previously been mapped to the N terminus of the protein (aa 1 to 126) (81). Two point mutations (in bold) were introduced into the predicted AE NLS (33PP**K**RPR38 to 33PPA**A**PR38) to generate HA-AE-NLSmut, and the protein localization was monitored by IF in 293T cells (Figure 2.3A). The HA-AE-NLSmut protein exhibited partial cytoplasmic localization, very similar to WT SOX. Despite its presence in the cytoplasm, this mutant was unable to promote host shutoff, as measured by GFP protein and mRNA accumulation (Figure 2.3B and C). HA-AE-NLSmut retained WT enzymatic activity in DNase assays (Figure 2.3D), indicating that failure of this mutant to induce host shutoff was not likely due to gross misfolding of the protein. I therefore concluded that cytoplasmic localization of the AE homologs, while critical for activity, is not the sole determinant of host shutoff and that the gamma-HV SOX proteins have indeed evolved additional functions not present in HSV-1 AE.

The cytoplasmic fraction of SOX and muSOX drives hyperadenylation of nuclear messages and the relocalization of the poly(A) binding protein

We had previously shown that SOX induces aberrant hyperadenylation of nascent cellular messages within the nucleus (66). The mechanism by which SOX causes such mRNA 3'-end processing defects has not been completely elucidated, yet this activity is dependent on its host shutoff function (66). I observed that muSOX similarly promotes mRNA hyperadenylation, as measured both by oligo(dT) staining to detect accumulation of endogenous nuclear poly(A) RNA and by Northern blotting to show an increased mRNA length (Figure 2.4A and B). Similar to our previous observations with KSHV SOX (66), the increased poly(A) RNA signal in muSOX-expressing cells occurred only in the nucleus, whereas the cytoplasm was depleted of poly(A) RNA (Figure 2.4A, right). To confirm that the GFP mRNA size difference observed in the presence of muSOX was due to hyperadenylation, I removed the mRNA poly(A) tails by hybridizing the RNA to oligo(dT) and digesting with RNase H. Indeed, after deadenylation, the size of the GFP mRNA was identical in the presence or absence of muSOX (Figure 2.4C).

Given that hyperadenylation occurs within the nucleus, I reasoned that direct modulation of the cellular mRNA processing machinery would involve the nuclear fraction of muSOX or SOX. Conversely, if the nuclear population of SOX were dispensable for hyperadenylation, this

would indicate of SOX indirectly alters mRNA 3'-end processing in the nucleus via one or more of its cytoplasmic activities. I therefore assessed the ability of the muSOX NRS fusions, as well as that of the SOX NLS mutant, to promote mRNA hyperadenylation. Surprisingly, I observed that nucleus-restricted HA-NRS-muSOX was deficient for nuclear hyperadenylation, as measured by both oligo(dT) in situ hybridization and Northern blotting (Figure 2.4A and B). In contrast, the cytoplasmic NLS mutant of SOX and the control HA- Δ NRS-muSOX retained WT nuclear hyperadenylation activity (Figure 2.4A and B). RNase H-mediated poly(A) tail removal confirmed that the increased GFP mRNA size in the presence of HA- Δ NRS-muSOX, SOX, and SOX-NLSmut was due to hyperadenylation (Figure 2.4C). These data indicate that SOX and muSOX do not directly target nuclear mRNA processing machinery, but instead must indirectly alter its regulation to promote hyperadenylation in the nucleus.

An additional prominent activity of SOX is to promote the relocalization of PABPC from the cytoplasm into the nucleus of cells in a host shutoff-dependent manner (66). I first confirmed by IF that muSOX expression also induces nuclear relocalization of endogenous PABPC (Figure 2.4D). I then explored whether PABPC relocalization was dependent on the cytoplasmic or nuclear activities of muSOX. Again, I observed that HA-NRS-muSOX was unable to alter PABPC localization, whereas HA- Δ NRS-muSOX and SOX-NLSmut induced PABPC nuclear import as efficiently as WT SOX and muSOX (Figure 2.4D). In summary, our data support the conclusion that all known host shutoff-related activities of the gamma-HV proteins are orchestrated exclusively from the cytoplasm of cells.

Discussion

Homologs of SOX are found throughout the HVs family, yet only in gamma-HVs have these proteins evolved the ability to induce cellular mRNA turnover. A notable difference between these proteins in alpha-HV versus gamma-HVs is their subcellular localization; gamma-HV SOX proteins are present in both the nucleus and the cytoplasm, whereas alpha-HV AE is exclusively nuclear. However, while cytoplasmic localization is clearly necessary for the host shutoff function of these proteins, partial redirection of HSV-1 AE to the cytoplasm is not sufficient to induce mRNA turnover. Thus, gamma-HV SOX homologs have evolved additional activities required for host shutoff. Both SOX and HSV-1 AE have *in vitro* RNase activity indicating that this function is not sufficient to mediate host shutoff in cells. I hypothesize that SOX must interface with one or more cytoplasmic cellular factors not targeted by HSV-1 AE to trigger mRNA turnover. Thus far, no functionally significant cellular proteins interactions have been identified for either SOX or muSOX during infection (unpublished observations). Although PABPC is specifically targeted for relocalization by the gamma-HV SOX proteins and not by HSV-1 AE, we have not detected an interaction between SOX and PABPC (R. Kumar, B. Glaunsinger, unpublished observations). Recent findings have shown that destruction of mRNA in the cytoplasm, by other host shutoff RNases from other viruses, also induces relocalization of PABP by exposing a non-canonical nuclear localization signal that is normally occluded during RNA binding (60). Furthermore, we have also recently shown that nucleus-restricted PABPC can directly promote hyperadenylation of messages in the absence of SOX (59). These recent findings explain the connection between cytoplasmic SOX and nuclear hyperadenylation that was not known at the time. Nevertheless, the exact mechanism by which PABPC induces hyperadenylation remains unclear at this point.

Normal mRNA polyadenylation is carried out by cellular poly(A) polymerase II (PAPII) in concert with nuclear poly(A) binding protein (PABPN) (53, 54, 103). PABPN is proposed to

participate in tail formation and length control by facilitating interactions between PAP^{II} and the cleavage and polyadenylation specificity factor complex over a defined tail length (58). Similar to cellular mRNAs, SOX-induced hyperadenylation requires both PAP^{II} and PABPN, and the resulting aberrantly processed mRNAs fail to be exported for translation (66). In yeast, hyperadenylated mRNAs accumulate at their site of transcription in mutants defective in either 3'-end processing or export (45, 49, 67). Hyperadenylation may therefore be viewed as a quality control signal to retain improperly processed messages in the nucleus and, additionally, may occur on mRNAs whose nuclear biogenesis and export do not take place in a timely manner.

Our results demonstrate that hyperadenylation and PABPC relocalization are both orchestrated by SOX and muSOX from the cytoplasm. We have gone on to show that the relocalization of PABPC is directly caused by the depletion of cytoplasmic messages, which explains the link between nuclear PABPC and cytoplasmic SOX (59). Additionally, nuclear PABPC induces hyperadenylation of nascent transcripts resulting in a second wave of host shutoff by preventing export and expression of these messages (59). It is unclear at this point whether PABPC relocalization would be advantages to the either the virus or the cell. For example relocalized PABPC could have roles in viral replication, which occurs in the nucleus. In support of this, we see that PABPC localizes to distinct nuclear bodies during late stages of viral infection (G. R. Kumar, unpublished observations). Additionally, its relocalization could represent a cellular stress response to host shutoff, allowing the cell to itself shutoff expression to limit viral replication. In fact, PABPC relocalization has been observed during other stresses such as heat shock (72) and transcriptional blocks (1), which support its role as a general sensor of stress.

Identification of the cellular factors involved in regulating mRNA turnover will undoubtedly shed light on mechanisms of global repression of gene expression, either during viral infection or in response to other physiological stimuli. Additionally, we anticipate that SOX and its homologs will be powerful tools to help dissect RNA metabolism in metazoans.

Materials & Methods

Plasmids. Primers and restriction sites used for plasmid construction are shown in Table 1. A hemagglutinin (HA) tag was introduced onto the N terminus of muSOX by using PCR methods and cloned into the EcoRI/NotI sites of pCDEF3, creating pCDEF3-HAmuSOX. The pCDEF3-SOX-NLS mutant (NLSmut) construct has been previously described (36). An HA tag was introduced onto the N terminus of the wild-type (WT) nuclear retention signal (NRS) of hnRNP C1 (amino acids [aa] 88 to 165) or mutant NRS (aa 98 to 146) (kindly provided by Jens Lykke-Andersen) by standard PCR methods. To ensure that the mutant NRS was the same size as the WT, an additional 29 aa from green fluorescent protein (GFP) were incorporated to compensate for the deleted NRS sequence. These PCR products were cloned into the N terminus of muSOX using KpnI/EcoRI sites to generate pCDEF3-HA-NRS-muSOX and pCDEF3- HA-ΔNRS-muSOX. An N-terminally tagged HSV-1 alkaline exonuclease (AE) nuclear localization signal (NLS) mutant with alanines at amino acid residues 35 and 36 (within the predicted NLS) (81) was generated by overlap extension PCR and cloned into the EcoRI/NotI sites of pCDEF3 to generate pCDEF3-HA-AENLSmut.

Primers

HA-NRS	KpnI-HA-NRS-F	GGGGTACCATGGCTTACCCATACGATGTACCTGACTATGCGCCAAAA GTGAACCGAGGAAAA
HA-NRS	EcoRI-NRS-R	CGGAATTCTCCACTCTTAGAATTGAAGCC
HA-ΔNRS	KpnI- HA-ΔNRS -F	GGGGTACCATGGCTTACCCATACGATGTACCTGACTATGCGAAACGAT CTGCAGCGGAG
HA-ΔNRS	EcoRI- HA-ΔNRS -R	CGGAATTCTTTTCGAGGGGCACTACAGCCCCG
GFP-29aa	EcoRI-GFP-29aa-F	GGGAATTCCAGGATGATGGCACGCTGCCC
GFP-29aa	EcoRI-GFP-29aa-R	CGGAATTCATTGATCCTAGCAGAAGCACA
HA-UL12	EcoRI-UL12-F	GGAATTCATGGCTTACCCATACGATGTACCTGACTATGCGATGGAGTC CACGGTAGGC
HA-UL12	NotI-UL12-R	ATAGTTTAGCGGCCGCTCAGCGAGACGACCTCCC
UL12-NLSmut	UL12-NLSmut-F	CGTGGCCCCGACAGCCCCCGCGGCCCCCGCCCTAACAGTCTTCCG
UL12-NLSmut	UL12-NLSmut-R	CGGAAGACTGTTAGGGCGGGGGCGCGGGGGGGCTGTCGGGGCCAC G

Cells, transfections, and viruses. 293T, NIH 3T3, NIH 3T12, and human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). BHK-21 cells (clone 15) were propagated in RPMI 1640 medium (Invitrogen) supplemented with 5% FBS. 293T cells were transfected with Effectene (Qiagen), following the manufacturer's protocol. BHK and 3T3 cells were transfected with SuperFect (Qiagen). KSHV stocks were prepared from induced BCBL-1 cells as described previously (6). KSHV infections of HFFs were carried out in the presence of 8 µg/ml Polybrene, and lytic reactivation was induced by superinfection with an adenoviral vector expressing RTA (Ad-RTA) as described previously (6). Bacterial artificial chromosome (BAC)-derived MHV68 was generated by transfecting 2 µg of BAC DNA per well of a six-well plate of NIH 3T3 using SuperFect (Qiagen) and then propagated in NIH 3T12 cells, and the titer was determined by using a plaque assay on NIH 3T3 cells. To harvest the virus, cells were passed through a Dounce homogenizer and cellular debris was removed by pelleting and passing the supernatant through a 0.45-µm filter.

DNase assays. Proteins analyzed for DNase activity were in vitro transcribed and translated (IVT) using the rabbit reticulocyte lysate system (Promega). From each reaction mixture, 8 µl of protein was incubated with 200 ng of NotI-linearized pCDEF3 plasmid DNA in 42 µl of degradation assay buffer (0.1 M MgCl₂, 0.5 M Tris, pH 9.0, 100 µg/ml bovine serum albumin, 5 mM β-mercaptoethanol) at 37°C for the indicated time period and then phenol-chloroform extracted. Pellets were resuspended in 20 µl of water, resolved on a 1% agarose gel, and visualized by ethidium bromide staining. One-third of each IVT reaction was also resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and gels were fixed, dried, and visualized by autoradiography to verify equivalent protein expression.

Cell extracts, Western blots, and Northern blots. Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) containing protease inhibitor cocktail (Roche) and quantified by the Bradford assay (Bio-Rad). Equivalent masses of each sample were resolved by SDS-PAGE and either dried and exposed to film or transferred to a polyvinylidene difluoride membrane and Western blotted with either HA 12CA5 monoclonal antibodies (MAbs) (1:4,000; Abcam), SOX MAbs (1:1,000), or muSOX polyclonal antibodies (pAbs) (1:250) and either horseradish peroxidase-conjugated goat anti-

rabbit or goat antimouse secondary antibodies (1:5,000; Southern Biotechnology Associates).

For Northern blotting, total RNA was harvested using RNA-Bee (Tel-Test) and analyzed by agarose-formaldehyde gel electrophoresis. RNAs were transferred to a 0.45- μ m nylon membrane and probed with ³²P-labeled DNA probes generated using the Rediprime II random prime labeling system (GE Healthcare). RNase H digests were performed by combining 5 μ g of RNA with 500 pmol of oligo(dT)15 primer in a 25.8 μ l reaction volume and then incubating at 65°C for 8 min. Samples were then incubated at 37°C for 30 min in the presence of 1 U RNase H (New England Biolabs), 1XRNase H buffer, and 40 U RNasin (Promega); reactions were terminated by the addition of 1 μ l of 0.5 M EDTA pH 8.0, and the RNA was ethanol precipitated.

IF and oligo(dT) in situ hybridization. 293T cells grown on coverslips following transfection and were fixed in 4% formaldehyde and processed for IF as described previously (71). In situ samples were processed as described previously (<http://www.singerlab.org/protocols>) using 2 ng/ μ l of Alexa Fluor 546-labeled oligo(dT)15 (Molecular Probes). After oligonucleotide hybridization, samples were incubated with either anti-SOX or anti-HA (Abcam) MAbs at a 1:100 or 1:500 dilution, respectively, in 2XSSC (1XSSC is 0.15MNaCl plus 0.015Msodium citrate) and 0.1% Triton X-100 for 3 h at 37°C and subsequently with Alexa Fluor 488-labeled goat antimouse secondary antibodies (Molecular Probes) and mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing VectaShield mounting medium (Vector Labs, Inc.). IF not involving in situ hybridization was done as described previously (71) using anti-SOX MAbs (1:100 dilution), rabbit polyclonal anti-PABPC pAbs (1:25 dilution) (Cell Signaling Technology), or rabbit anti-muSOX pAbs (1:25 dilution) and Alexa Fluor 488- or 546-labeled goat anti-rabbit or anti-mouse secondary antibodies (1:1,500 dilution) (Invitrogen).

Figures

Figure 2.1

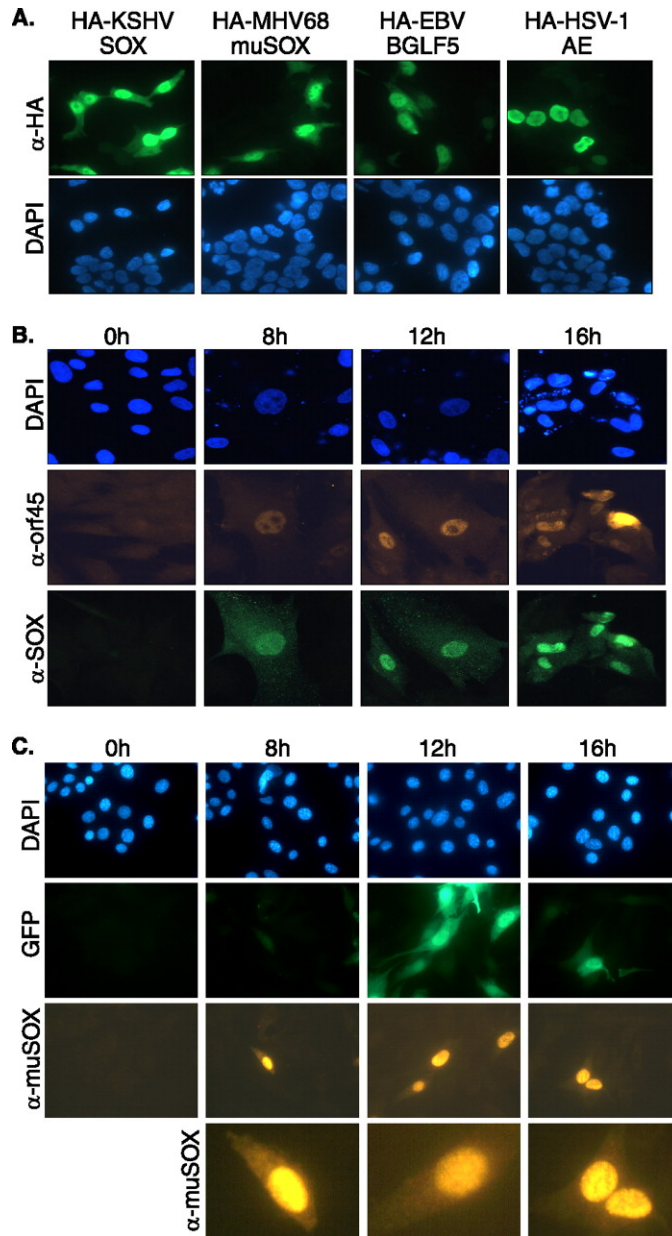


Figure 2.1 SOX homologs of gamma-HVs localize to both the nucleus and cytoplasm. (A) 293T cells were transfected with empty vector or HA-tagged SOX (KSHV), muSOX (MHV68), BGLF5 (EBV), and AE (HSV-1). Twenty-four hours posttransfection, cells were subjected to IF with anti-HA antibodies. (B) HFF cells were either mock infected or infected with KSHV and lytically reactivated with an adenoviral vector expressing RTA. At the indicated times, samples were subjected to IF with anti-ORF45 (KSHV lytic marker) (center panels) and anti-SOX (bottom panels) antibodies. (C) 3T3 cells were either mock infected or infected with BAC-derived MHV68. At the indicated times, samples were subjected to IF with anti-muSOX antibodies. GFP is encoded by the BAC and serves

as a marker of infection. Bottom panels show a magnified view of representative muSOXpressing cells at each time point. All samples were costained with DAPI to visualize nuclei. α , anti.

Figure 2.2

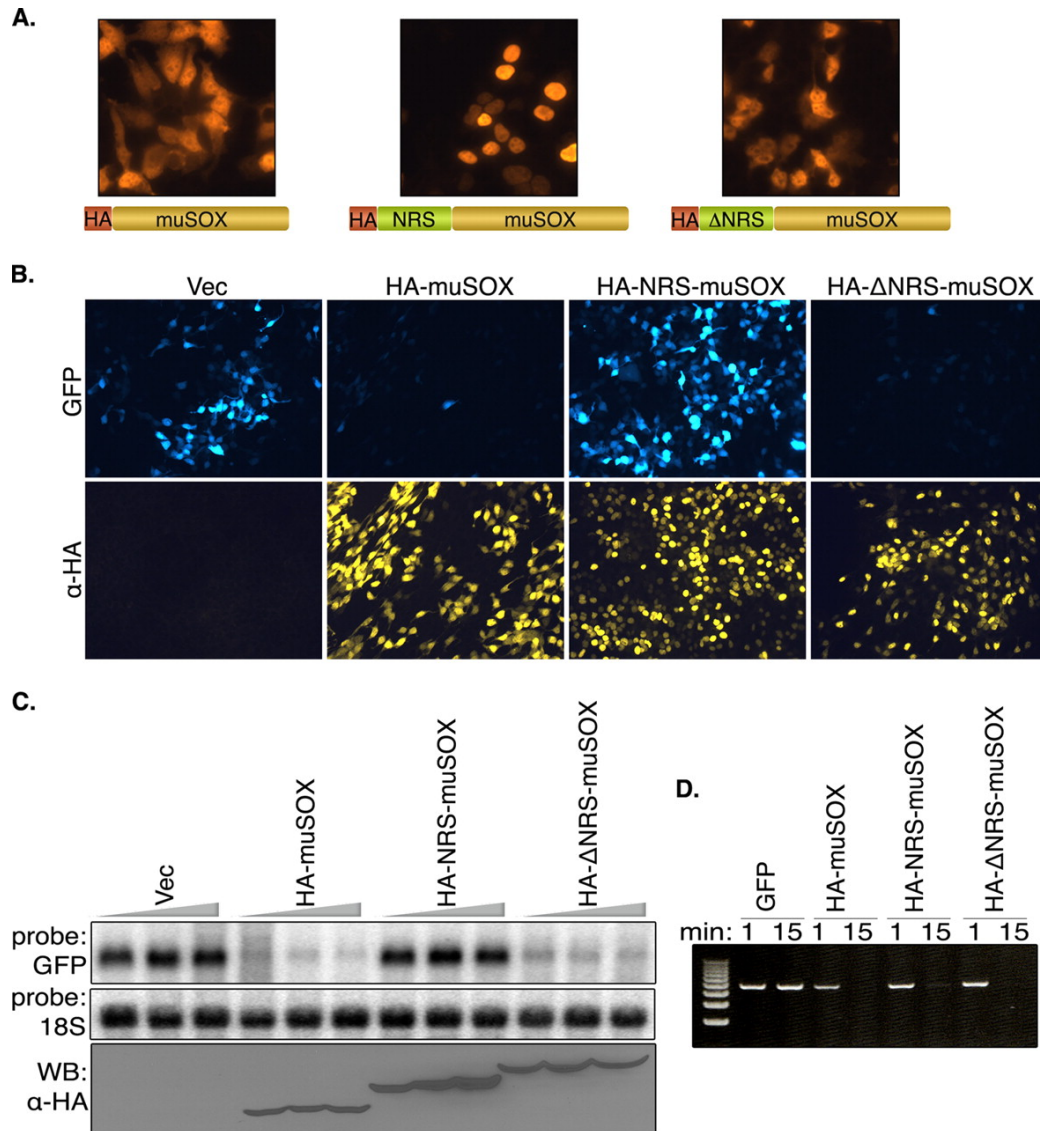


Figure 2.2 Host shutoff is orchestrated from the cytoplasm. (A) 293T cells were transfected with the indicated plasmid expressing HA-tagged muSOX or muSOX fused to a WT or mutant NRS, as diagramed. Localization of each muSOX protein was visualized by IF with anti-HA antibodies. (B) The host shutoff activity of each muSOX variant was assessed by cotransfection of a GFP reporter with either empty vector or the indicated muSOX-expressing plasmid. At 24 h posttransfection, cells were subjected to IF with anti-HA antibodies. (C) 293T cells were transfected with increasing amounts of the indicated muSOX constructs (100 to 300 ng). Total RNA was harvested 24 h posttransfection and Northern blotted with GFP and 18S probes. Protein levels were assessed by Western blotting with anti-HA antibodies. (D) Linearized pCDEF3 plasmid was incubated with aliquots of the indicated IVT protein for 1 or 15 min in degradation assay buffer at 37°C. The DNA was then extracted, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. α , anti; Vec, vector.

Figure 2.3

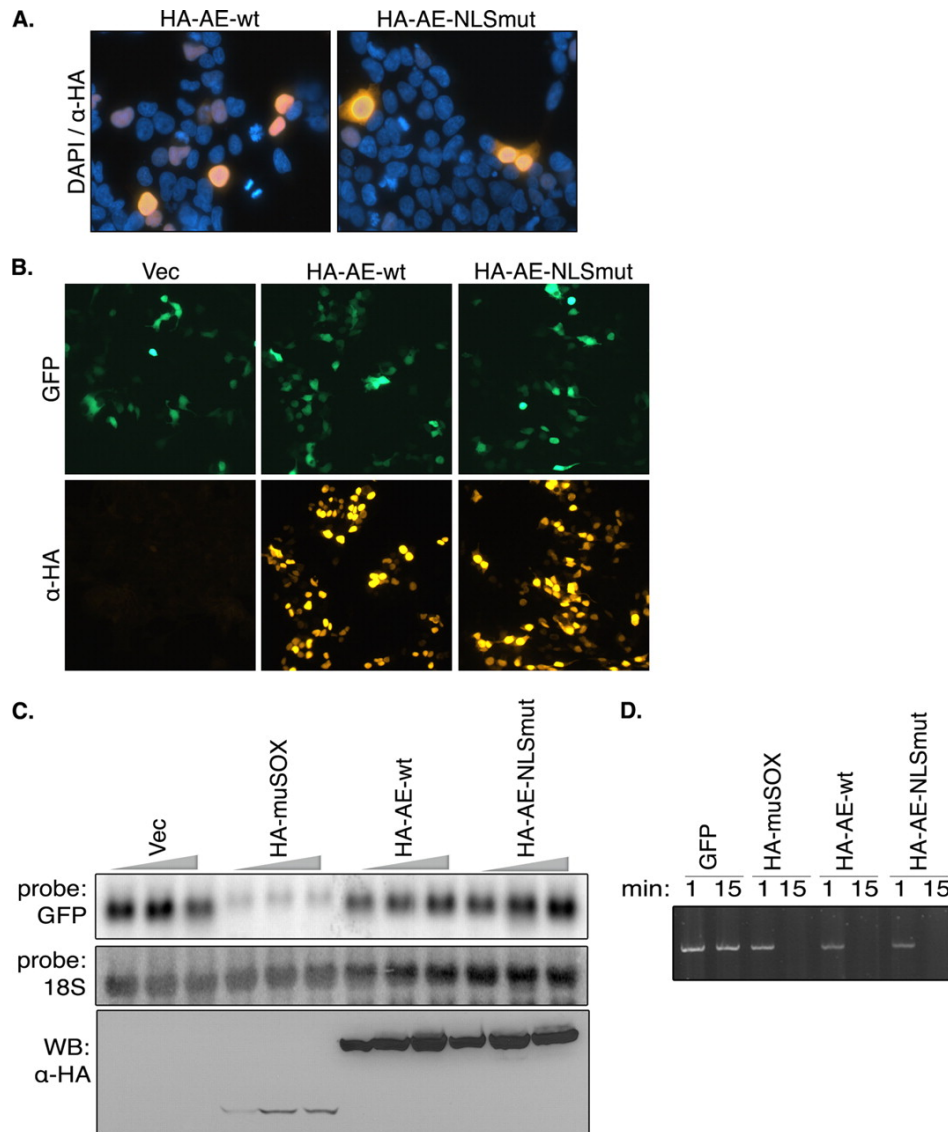


Figure 2.3 Partial relocation of HSV-1 AE into the cytoplasm is not sufficient to induce mRNA turnover.

(A) 293T cells were transfected with plasmids expressing either WT HSV-1 AE or AE-NLSmut for 24 h and then subjected to IF with anti-HA antibodies. DAPI staining was used to visualize nuclei. (B) 293T cells were transfected with a GFP reporter plasmid alone or together with the indicated AE construct for 24 h and then subjected to IF with anti-HA (α -HA) antibodies. (C) 293T cells were transfected with increasing quantities of the indicated plasmids (100 to 300 ng). Total RNA was harvested 24 h posttransfection and Northern blotted with GFP and 18S probes. AE and muSOX levels were assessed by Western blotting with anti-HA antibodies. (D) The linearized pCDEF3 plasmid was incubated with aliquots of the indicated IVT protein for 1 or 15 min in degradation assay buffer at 37°C. The DNA was then extracted, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. Vec, vector.

Figure 2.4

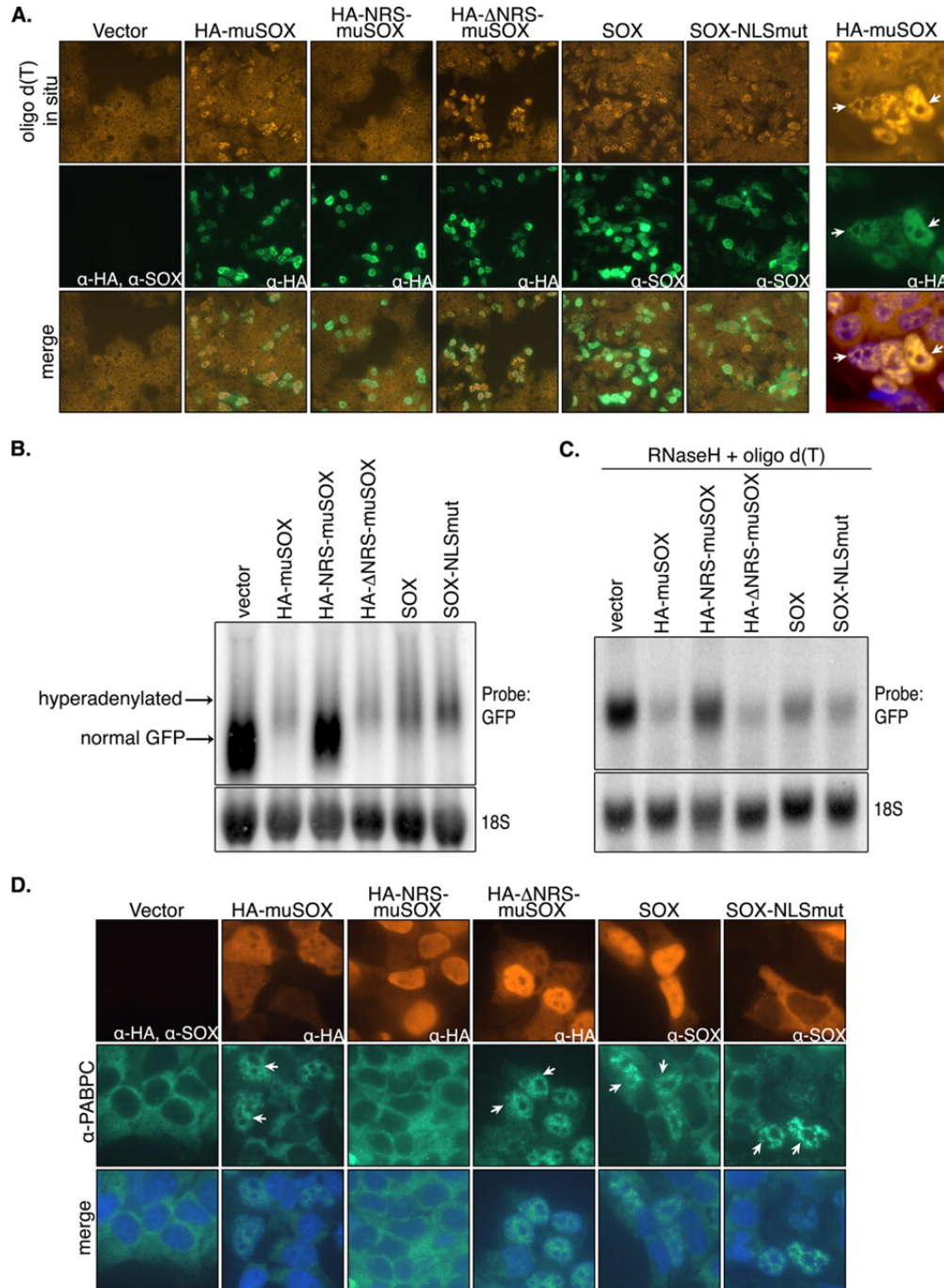


Figure 2.4 The cytoplasmic fractions of muSOX and SOX drive mRNA hyperadenylation and PABPC nuclear import. (A) 293T cells were transfected with the indicated plasmids for 24 h and then subjected to oligo(dT) in situ hybridization (top panels), followed by staining with anti-SOX Mabs (SOX samples), anti-HA antibodies (muSOX samples), or both anti-HA and anti-SOX antibodies (vector sample) (center panels). The

bottom panels show a merge of the in situ and IF staining. The far right panels show a magnified view of a field of muSOX-transfected cells, the bottom right panel showing a merge of the in situ signal and DAPI-stained nuclei. Arrows identify select muSOX-expressing cells. (B) 293T cells were transfected with a GFP reporter plasmid alone or together with the indicated muSOX- or SOX-expressing plasmid. At 24 h posttransfection, samples were treated with 5 ng/ml leptomycin B for 6 h to stabilize hyperadenylated RNAs. Total RNA was then harvested and Northern blotted with GFP and 18S probes. (C) A fraction of each RNA sample from that shown in panel B was incubated with oligo(dT) to bind poly(A) tails and then subjected to RNase H digestion to deadenylate the mRNAs prior to agarose-formaldehyde gel electrophoresis and Northern blotting. (D) 293T cells were transfected with indicated plasmids for 24 h and then subjected to double-label IF with polyclonal anti-PABPC antibodies (center panels) and anti-HA or anti-SOX antibodies (top panels). The bottom panel shows a merge of the PABPC-stained and DAPI-stained nuclei. Arrows point to representative cells exhibiting PABPC nuclear import.

Chapter 3:

The Gammaherpesvirus SOX Protein Targets Translationally Competent Cellular mRNAs for Destruction During Early Stages of Translation Initiation

Background

In vitro SOX homologs have been demonstrated to possess robust DNase activity along with a weaker RNase activity (52, 56). Given that the SOX protein triggers RNA destabilization, it seems logical to infer that the RNase activity could have a potential role in the host shutoff (HS) activity. However, the exact connection is unclear since even homologs that lack HS activity in cells are capable of degrading RNA *in vitro* to WT levels (23). In addition, SOX single function point mutants that are host shutoff defective demonstrate WT RNase activity *in vitro*. I hypothesized that the HS activity in cells likely required interfacing with one or more cellular factors that could enhance the turnover efficiency of RNA.

At the outset of this study, two existing lines of evidence supported a link between translation and SOX mediated turnover. First, we have observed that nonpolyadenylated transcripts lacking a 3' UTR are neither translated nor targeted by SOX for turnover (66). Addition of templated poly(A) sequences or a random 3'UTR partially restores translation and SOX-mediated turnover (66). Second, we have previously demonstrated that cells expressing SOX exhibit nuclear relocalization of cytoplasmic poly(A) binding protein (PABPC), a protein with key roles in mRNA stability and translation. Specifically, SOX targets the population of PABPC that is engaged in translation complexes (60). I therefore hypothesized that translating cellular mRNAs are targeted by SOX during KSHV infection, coincident with removal of PABPC from the cytoplasm. Here I characterize an important component of the SOX-induced mechanism of host shutoff by demonstrating a preference for targeting of translationally competent Pol II mRNAs. I go on to show that targeting of mRNA occurs during early stages of translation, not requiring significant 40S scanning or AUG recognition. Incorporating previous data, we present a model for host shutoff in which messages are targeted by a two-step process involving an initial SOX cleavage during early stages of translation with subsequent destruction by the cellular exoribonuclease Xrn1.

Results

SOX depletes polysomes and co-sediments with 40S subunits

I sought to determine how SOX targets cytoplasmic mRNAs. *In vitro*, SOX exhibits relatively weak affinity for RNA (K_d of 87 μ M), suggesting that in cells it is recruited to mRNAs

via associations with host factors (4). In cellular quality control pathways such as NMD, translation is required for error recognition and recruitment of factors that promote a primary endonucleolytic cleavage (35, 47). In addition, it has recently been reported that the vast majority of mRNAs in the cytoplasm are polysome-associated (44), suggesting that targeting mRNAs engaged in translation would be an efficient mechanism to clear host messages during host shutoff.

To examine the effects of host shutoff on translating mRNAs, I performed polysome profiling of a B cell line (BCBL-1) derived from a patient with primary effusion lymphoma, which harbors KSHV in a latent state. I used a line of BCBL-1 cells bearing an inducible version of the KSHV major lytic transactivator RTA (TREx BCBL-1-RTA) (79) to allow efficient lytic reactivation following RTA induction. Upon chemical stimulation of lytic KSHV replication in these cells (when SOX is expressed from the viral genome), I observed a significant decrease in the polysome population and a corresponding increase in 80S monosomes, consistent with degradation of actively translating mRNAs (Figure 3.1A). It should be noted that the level of polysome depletion during viral infection is likely underestimated, as ~20% of induced cells generally fail to enter the lytic cycle. The depletion of polyribosomes was not due to chemical treatment alone, because treatment of the KSHV negative BJAB B cell line did not result in a similar decrease in polysomes (unpublished observations). I next looked for the presence of SOX in gradient fractions from the corresponding polysome profiles of lytically reactivated BCBL-1 cells. As controls, I also blotted for PAIP2A, a protein not found in polysomes (84), as well as the ribosomal protein RPS3 (Figure 3.1B). SOX appeared to sediment primarily with the ribonucleoprotein (RNP), 40S, and monosome fractions, and exhibited partial overlap with RPS3 (Figure 3.1B). Puromycin treatment disrupted polysomes but failed to alter the SOX sedimentation profile, arguing against a specific association with the 80S and polysome fractions (unpublished observations). To more accurately determine the sedimentation profile, I increased the resolution of the lighter molecular weight complexes using lower density sucrose gradients. These experiments revealed that SOX indeed sediments in both the RNP and 40S fractions, similar to Xrn1 (Figure 3.1C). Its sedimentation profile also overlapped with the eIF3j and eIF2 α components of the 40S pre-initiation complex, which are recruited to the 5' cap prior to ribosomal scanning (Figure 3.1C). This is in contrast to the sedimentation of PABPC, which remains bound to the mRNA throughout the polysome fractions (Figure 3.1C; unpublished observations).

Given that SOX is the dominant effector of host shutoff during KSHV infection, I hypothesized that polysome depletion in BCBL-1 cells was a consequence of SOX activity. I therefore tested the effects of transient SOX expression on the endogenous translating mRNA pool through polysome profiling of 293T cells. The sedimentation profile of both wild-type SOX (Figure 3.2A) and the SOX D221S catalytic mutant (Figure 3.2B), were similar to those of endogenous SOX in virally infected cells. This indicates that the catalytic site of the protein is unlikely to be involved in its recruitment to target RNAs. In addition, expression of wild-type SOX alone caused a significant depletion of polysomes relative to vector-transfected cells (Figure 3.1D). In contrast, expression of the SOX catalytic mutant D221S, or of the single function SOX P176S mutant (which lacks host shutoff but not DNase activity) had little effect on the polysome levels of 293T cells (Figure 3.1D). These results additionally confirm that the catalytic activity of SOX is required to initiate widespread turnover of endogenous host messages.

SOX specifically targets translationally-competent Pol II transcripts

We had previously observed that a translationally incompetent version of the GFP mRNA, which terminates by ribozyme cleavage and lacks a poly(A) tail (GFP-HR) (Figure 3.3E), escaped SOX-mediated turnover (66). Although this mRNA is inefficiently exported, subcellular fractionation experiments confirmed that even the exported cytoplasmic population of GFP-HR was not subject to SOX-induced turnover (Figure 3.3F). Thus, the failure of SOX to degrade this mRNA in the cytoplasm could be due to its translational incompetence.

To further explore a role for translation in SOX-induced mRNA turnover, I examined SOX turnover of RNAs transcribed by Pol I and Pol III. Pol I and Pol III transcription does not result in the addition of the cap and poly(A) tail, mRNA modifications critical to translation initiation specifically associated with Pol II transcription. Using a pure population of cells expressing GFP-SOX or GFP alone, I found that neither the endogenous Pol III-generated Y3 and 7SL cytoplasmic non-coding RNAs, nor the 18S rRNA transcribed by Pol I undergo turnover in the presence of SOX (Figure 3.3A). In contrast, I observed significant depletion of endogenous mRNAs transcribed by Pol II, including GAPDH, β -actin, and stearoyl-CoA desaturase (SCD) in SOX-expressing cells (+) relative to vector-expressing cells (-) (Figure 3.3A). In principle, the inability of SOX to degrade the non-Pol II transcripts could be due to the absence of an open reading frame (ORF), or the presence of RNP complexes that could prevent proper recruitment of SOX. To exclude these possibilities, I expressed the GFP reporter under the control of Pol I or Pol III promoters and found that in both cases, the GFP RNA was not targeted by SOX (Figure 3.3B). I confirmed using subcellular fractionation experiments that the inability of SOX to promote degradation of these RNAs was not due to a failure of the RNAs to be exported (Figure 3.3C-D). Collectively, these data indicate that RNAs must be translationally competent to be targeted by SOX.

mRNAs are targeted during early stages of translation

We previously demonstrated that SOX induces destabilization of mRNA by inducing a sequence-specific internal cleavage. This process creates 3' degradation intermediates that are normally cleared by cellular Xrn1 and can be visualized upon Xrn1 knock-down (21). To determine whether mRNA degradation by SOX required ribosomal passage over or near the putative initial cleavage site, I designed a reporter dsRed2 mRNA harboring a termination codon upstream of the putative SOX cleavage site (dsRed2-100stop). This prevented production of full-length dsRed2 protein (Figure 3.4A). Interestingly, in cells depleted of Xrn1, dsRed2-100stop was cleaved similarly to wild-type dsRed2 upon SOX expression (Figure 3.4B), indicating that ribosomal passage over the cleavage site is not necessary for its recognition.

To determine whether 60S joining is required for cleavage, I made use of a modified version of the encephalomyocarditis virus internal ribosomal entry site (IRES), termed Δ EMCV, which cannot promote cap-independent translation but is highly structured and decreases translation initiation *in vitro* (15). Indeed, insertion of Δ EMCV into the GFP 5' UTR 30 nt from the cap significantly reduced GFP protein accumulation (Figure 3.4C). However, in Xrn1-depleted cells, SOX still induced cleavage of this mRNA with similar efficiency as wild-type GFP, and at the same site (Figure 3.4D). In agreement with the Δ EMCV-GFP data, I observed that SOX could also promote turnover of a GFP mRNA with a 60 nt adenylate tract inserted in the 5' UTR (5'A60-GFP) (Figure 3.4F), which similarly reduced GFP protein production (Figure

3.4E). A 50–70 nt adenylate-rich tract in the 5' UTR of PABPC has likewise been shown to repress translation of its message in an autoregulatory manner as a consequence of PABPC protein binding to this region (24). Consistent with the sedimentation profile of SOX, these data suggest that in SOX-expressing cells, mRNAs are targeted for cleavage at an early step during translation, perhaps prior to AUG recognition.

To monitor directly where cleavage occurs, I performed sucrose density gradient centrifugation on Xrn1-depleted cells expressing SOX and the GFP reporter. In agreement with our above findings, the cleaved intermediate preferentially accumulates in the 40S fraction (Figure 3.5A). The degradation intermediate is absent in all fractions from cells expressing the SOX catalytic mutant D221S (Figure 3.5B). These results lead us to favor a model in which mRNAs are recognized by SOX during formation of the 40S preinitiation complex, at which point they undergo SOX-induced endonucleolytic cleavage and subsequent destruction by Xrn1.

Discussion

Our results support the involvement of an early stage of translation in the targeting of mRNAs by SOX. This would allow the virus to selectively eliminate competing host mRNAs, while sparing regulatory RNAs that may be necessary for its own gene expression. Eukaryotic translation initiates with the recruitment of the 40S ribosomal subunit to the cap via interactions with translation initiation factors, followed by scanning to the AUG codon and recruitment of the 60S subunit. Two observations indicate that recognition and turnover in SOX-expressing cells are initiated prior to 60S joining. Specifically, insertion in the 5' UTR of elements that presumably inhibit or prematurely terminate 40S scanning, and thus significantly reduce protein production, does not affect SOX activity. Moreover, the degradation intermediate accumulates preferentially in the 40S fraction. Our finding that SOX cosediments with the 40S initiation complex suggests that an association with translation initiation machinery directs it to mRNAs. Supporting this prediction is the failure of SOX to target translationally incompetent reporter and endogenous RNAs transcribed by RNA polymerase I or III.

I hypothesize that SOX targeting requires recognition of the mRNA 5' end, likely via associated translation initiation factors, and that this recruitment somehow allows SOX access to the cleavage site(s) within the mRNA. Interestingly, we previously showed that cleavage can occur hundreds of nucleotides away from the site of translation initiation (21). One interesting parallel to these seemingly discrepant observations is that of the *E. coli* endonuclease RNase E, which can cleave anywhere along the length of the RNA (preferably within AU-rich sequences), but requires a monophosphate at the mRNA 5' end. Thus, similar to our proposed model for SOX activity, RNase E can simultaneously recognize two non-adjacent regions of the primary RNA sequence (11). These observations can be reconciled by the fact that RNAs adopt secondary and tertiary structures within a cell that could presumably enable such sequences to be juxtaposed. Alternatively, this distance could be bridged if there was an additional interaction of SOX with a host protein co-factor bound to the cleavage element.

It is interesting to note the parallels between the translation-linked mechanism of mRNA targeting by SOX and the cellular mRNA quality control pathways like NMD, which also trigger endonuclease-mediated destruction of target messages. In addition to the enhanced rate of decay afforded by an endonucleolytic mechanism, the advantage for the virus may be that this strategy of host shutoff generates intermediates that look indistinguishable from products of quality control pathways. Such intermediates would then be readily degraded by the core degradation

machinery and might not be recognized as aberrant, perhaps avoiding activation of stress or innate immune responses. Thus, understanding how viral endonucleases interface with host pathways may provide insight into how manipulation of these pathways contributes to disease, as well as helping elucidate the events that regulate cellular RNA decay

Materials and Methods

Cell Extracts, Western blots, and Northern Blots

Protein lysates were either prepared in RIPA buffer [50 mM Tris-HCl (pH8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS)], or fractionated using the NE-PER kit (ThermoScientific). Western blots were performed with either mouse monoclonal anti-GFP (1:2000, BD Biosciences), mouse monoclonal anti-dsRed (1:500, Clontech), mouse monoclonal anti-HA (1:2000, Invitrogen), mouse monoclonal anti-RPS3 (1:1000, Abcam), mouse monoclonal anti-Flag (1:1000, Sigma), rabbit polyclonal anti-PAIP2A (1:2000, kindly provided by N. Sonenberg (55)), rabbit polyclonal anti-hXrn1 (1:5000, kindly provided by J. Lykke-Andersen), rabbit polyclonal anti-hDcp1a (1:5000, kindly provided by J. Lykke-Andersen), rabbit polyclonal anti-hDcp2 (1:400, kindly provided by M. Kiledjan), rabbit polyclonal anti-hRrp41 (1:1000, see below), rabbit polyclonal anti-SOX J5803 (1:5000, (36)), mouse monoclonal hnRNPC1/C2 (1:2000, Abcam), mouse monoclonal anti-Hsp90 (1:3000, Stressgen Bioreagents), mouse monoclonal anti-tubulin (1:3000, Sigma Aldrich), goat polyclonal anti-actin (1:5000, Santa Cruz Biotechnology), mouse monoclonal 10e10 anti-PABPC (1:2000, Santa Cruz Biotechnology), rabbit polyclonal anti-eIF2 α (1:1000, Cell Signaling) or rabbit polyclonal anti-eIF3j (1:1000, Cell Signaling). Rabbit polyclonal antibodies were raised against recombinant maltose binding protein (MPB)-fused hRrp41 purified from *E. coli*.

Total cellular RNA was isolated for Northern blotting using RNA-Bee (Tel-Test). Where indicated, the NE-PER kit (ThermoScientific) was used for cellular fractionation prior to RNA extraction. Northern blots were probed with ³²P-labeled DNA probes made using RediPrime II (GE Healthcare). Results in each figure are a representative of at least 3 independent replicates of each experiment. Image J (<http://rsbweb.nih.gov/ij/>) was used for quantification of Northern and Western blots.

Polysome profiling

Profiles were obtained from uninduced or lytically reactivated TREx BCBL-1-RTA cells, or from 293T cells transfected with the indicated plasmids. Polysome profiles were carried out as described in Jackson and Larkins (48), except that cells were treated with 100 μ g/ml CHX for 30 minutes prior to harvesting. BCBL-1 and 293T extracts were pelleted through 60% sucrose before layering or were directly layered on a 15–60% sucrose gradient containing 100 μ g/ml CHX. 24 h post transfection or induction, cells were treated with 100 mg/ml cycloheximide for 30 minutes prior to harvesting in polysome extraction buffer [100 mM Tris-CL (pH8.0), 20 mM MgCl₂, 200 mM KCl, 5 mM EGTA, 1 mM DTT, 1% (v/v) Triton X-100, 100 mg/ml cycloheximide, protease inhibitors cocktail (Roche)]. The soluble portion of the cell lysate was overlaid onto 15-60% sucrose gradients in gradient buffer [40mM Tris-CL (pH 7.4), 10 mM MgCl₂, 20 mM KCl, 100 mg/ml cycloheximide], and centrifuged at 37,000 rpm in a SW41 rotor at 4°C for 100 min. Where indicated, extracts were pelleted through 60% sucrose using a Ti 60

rotor before layering on the gradient. Resolution of the RNP/40S fractions was carried out as described above, but using a 5%-20% gradient of sucrose and centrifuging for 120 min. One ml fractions were collected and analyzed using an ISCO model D density gradient fractionator attached to an ISCO model UA5 absorbance monitor at 254 nm absorbance. For Western blots, 50-100 μ l of each fraction was used directly for SDS-PAGE. For Northern blots, total RNA was isolated from 500 μ l of each fraction.

Plasmid Constructs

Plasmids pCDEF3-SOX (37), pd2eGFP-HR (66) and pCDNA3.1-GFP-SOX (17) were described previously. Plasmids pd2eGFP-N1 and pDsRed2-N1 were purchased from Clontech. Plasmid pd2-5'A60-GFP-N1 was constructed by cloning a stretch of 60 adenosines into the 5' UTR of GFP-N1 via the EcoRI/KpnI sites. The pd2- Δ EMCV-GFP-N1 construct was made by XhoI/EcoRI digestion of the SV40 dual luciferase plasmid pR/ Δ E/F(C-53), kindly provided by P. Sarnow (50). The Δ EMCV fragment was then subcloned into the 5' UTR of GFP-N1 using XhoI/EcoRI. To generate the RNA Pol I-driven GFP construct, we subcloned the GFP open reading frame into the BsmBI sites of the pHH21 vector (kindly provided by J. Doudna) which contains the human RNA Pol I promoter and the mouse RNA Pol I terminator (80). In order to express GFP via a Pol III promoter, the Y3 gene was first excised from the pBSU6-Y3IP construct (kindly provided by K. Collins, (46)) using PstI/BamHI. GFP was then amplified with primers containing NsiI/BglII restriction sites and subcloned into the empty vector. A premature termination codon at DsRed2 amino acid 100 was introduced using QuikChange to generate pDsRed2-100stop. The SOX point mutants were generated from pCDEF3-T7-SOX using QuikChange. Wild-type SOX was cloned into the EcoRI/SalI sites of pBMN-IP to generate pBMN-SOX. pBMN-D221S was generated using Quikchange. QuikChange primer sequences are listed below in Table S1. Cloning primer sequences are listed in Table S2.

Primers

Construct	Primer sequence
dsRed2-100stop	CGCGTGATGAACTTCTAGGACGGCGGCGTG
Pol I GFP	F: GCGTCTCATATTATGGTGAGCAAGGGCGAG R: GCGTCTCAGGGGCTACACATTGATCCTAGCAGAAGCA
Pol III GFP	F: AGTCGATGCATATGGTGAGCAAGGGCGAG R: AGTCAGATCTCTACACATTGATCCTAGCAGAAGCA
pBMN-SOX	F: CGGAATTCATGGAGGCCACCCCA R: ACTGTCGACCTACGGGCTGTGAGGGACG

Cells, Transfections, and Lytic Reactivation

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). For knockdown experiments, 293T cells were transfected twice with a final concentration of 70 nM siRNA target specific or DS scrambled negative control oligos (IDT), or 1 μ g/ml of shRNA constructs (kindly provided by O. Mühlemann) using Lipofectamine 2000 (Invitrogen) at 96 and 48 hours prior to DNA transfection. Sequences of the siRNA oligos and shRNAs are listed in Table S4. For DNA transfections, reporter constructs (0.1 or 0.2 μ g/ml) were transfected either alone or in combination with 0.2 μ g/ml of the indicated SOX construct, using Effectene reagent (Qiagen) or Lipofectamine 2000 following the manufacturers' protocol. Where specified, the Dcp2 E148Q dominant negative construct was also transfected at 0.2 μ g/ml. Protein and RNA samples were collected 16-24 hours following DNA transfection as described below.

The KSHV-positive B cell line TREx BCBL1-RTA (79) (kindly provided by J. Jung and I. Mohr) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 200 μ M L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), and 50 μ g/ml hygromycin B (Omega Scientific). To induce lytic reactivation of KSHV, TREx BCBL1-RTA cells were split to 2×10^5 cells/ml and, 24 h later, induced with 20 ng/ml 2-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma), 1 μ g/ml doxycycline (BD Biosciences), 500 ng/ml ionomycin (Fisher Scientific) (3). To obtain pure fractions of GFP-SOX or GFP-expressing cells for the analysis of endogenous transcripts, HEK 293T cells transfected with pCDNA3.1-GFP-SOX (17) or pd2eGFP-N1 and were sorted on a Dako-Cytomation MoFlo High Speed Sorter at the Cancer Research Laboratory Flow Cytometry Facility at the University of California, Berkeley.

Figures

Figure 3.1

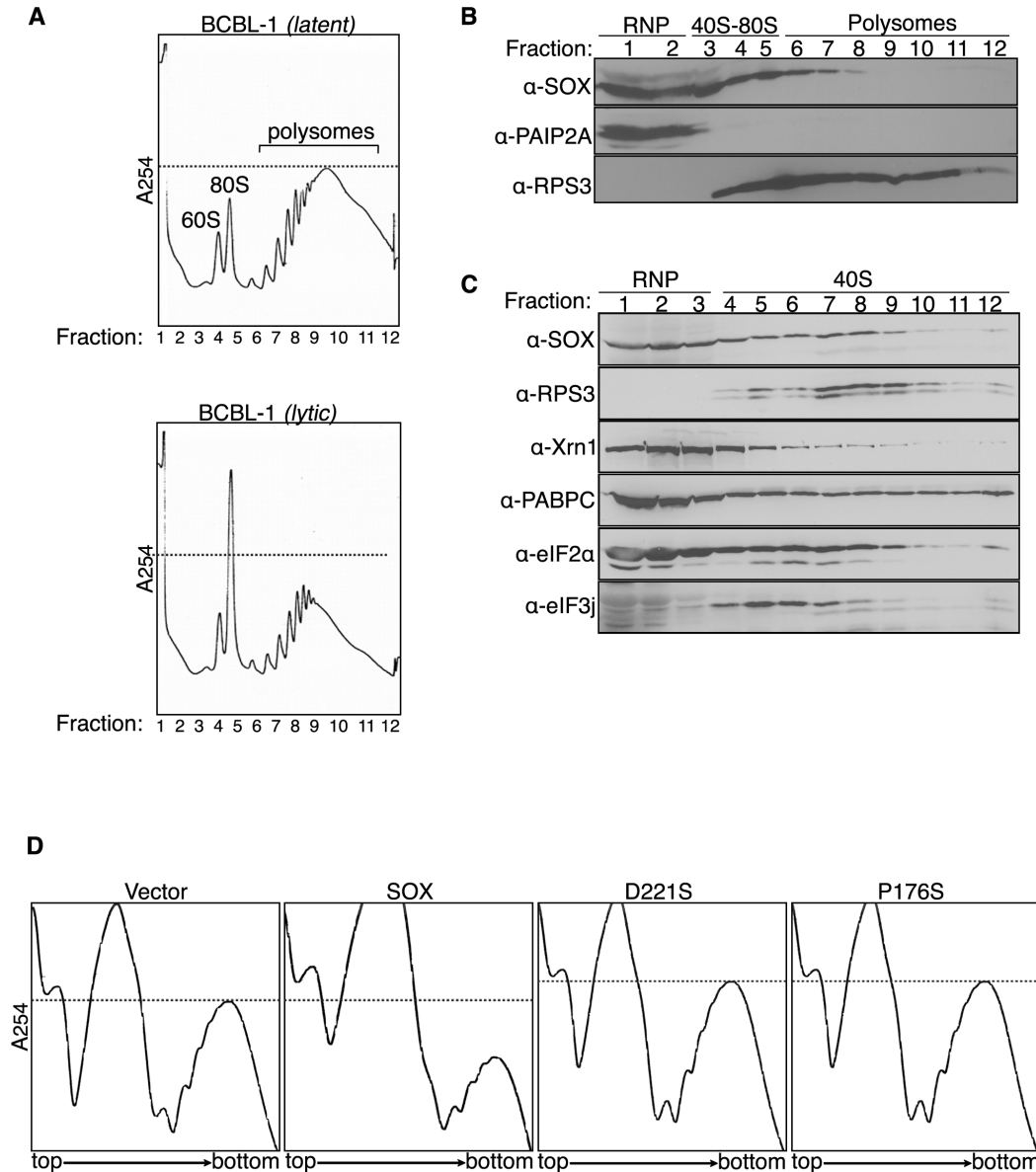


Figure 3.1 SOX depletes polyribosomes and cosediments with 40S subunits.

(A) TREx BCBL-1-RTA (BCBL-1) cells were mock treated (*latent*) or induced (*lytic*) with 1 μ g/ml doxycycline, 500 ng/ml ionomycin, and 20 ng/ml 2-*O*-tetradecanoylphorbol-13-acetate for 24 h to stimulate KSHV replication, then subjected to sucrose gradient fractionation using a 15–60% sucrose gradient in order to monitor the abundance of translating polysomes. (B) Fractions collected from the induced BCBL-1 gradients shown in panel A were Western blotted with the indicated antibodies. (C) Lysate from induced BCBL-1 cells was fractionated using a 5–20% sucrose gradient and analyzed by Western blot with indicated antibodies. (D) 293T cells were transfected with the indicated construct and subjected to sucrose gradient fractionation to obtain polysome profiles. Dashed lines indicate polysome levels of either latent cells (A) or vector expressing cells (D).

Figure 3.2

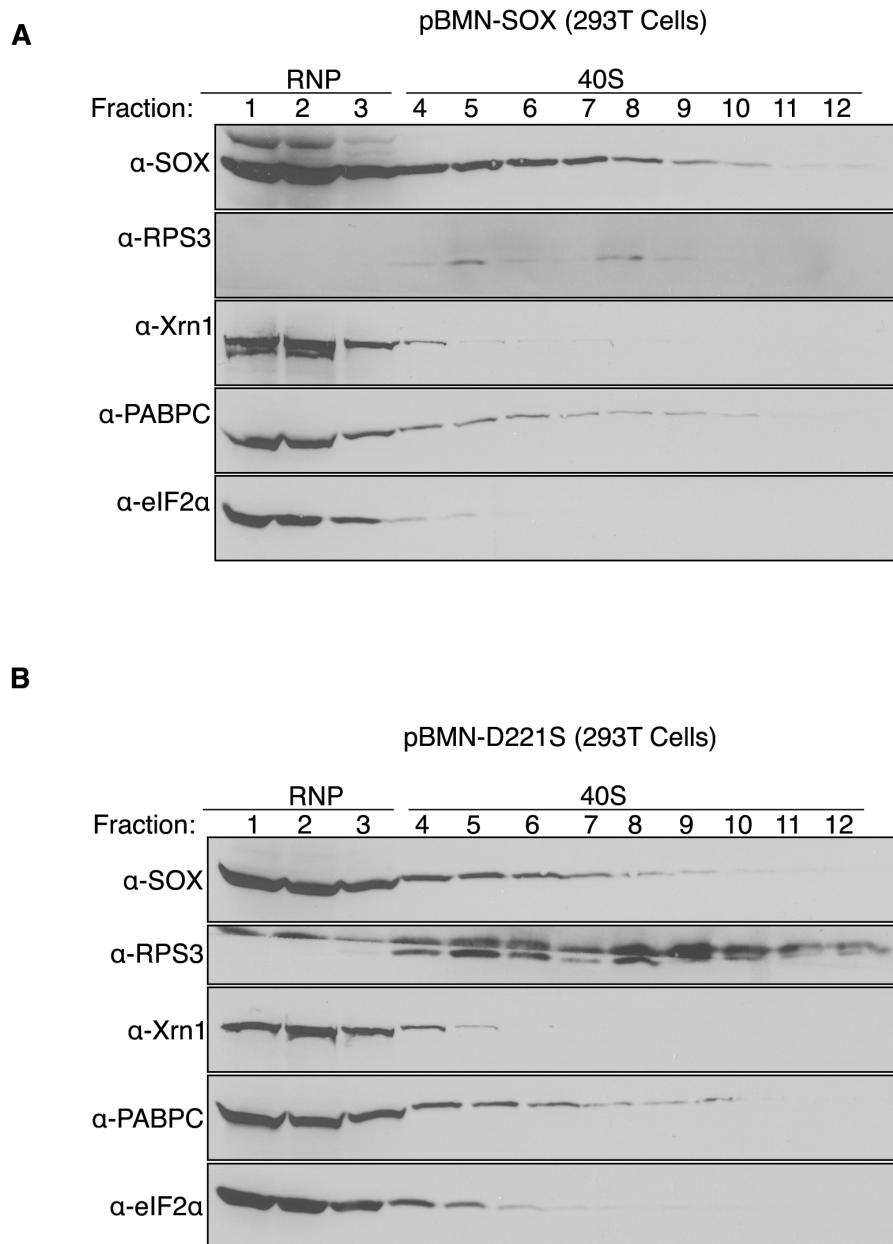


Figure 3.2 Transiently expressed SOX in 293T cells cosediments with translation initiation factors. Lysates from 293T cells transfected with wild-type SOX (A) or the catalytic mutant SOX D221S (B) were analyzed by sucrose gradient fractionation through a 5–20% sucrose gradient. SOX proteins were expressed to near-physiological levels using the 3' Moloney murine leukemia virus long terminal repeat (LTR) promoter in the pBMN vector. Fractions were analyzed by Western blot with indicated antibodies.

Figure 3.3

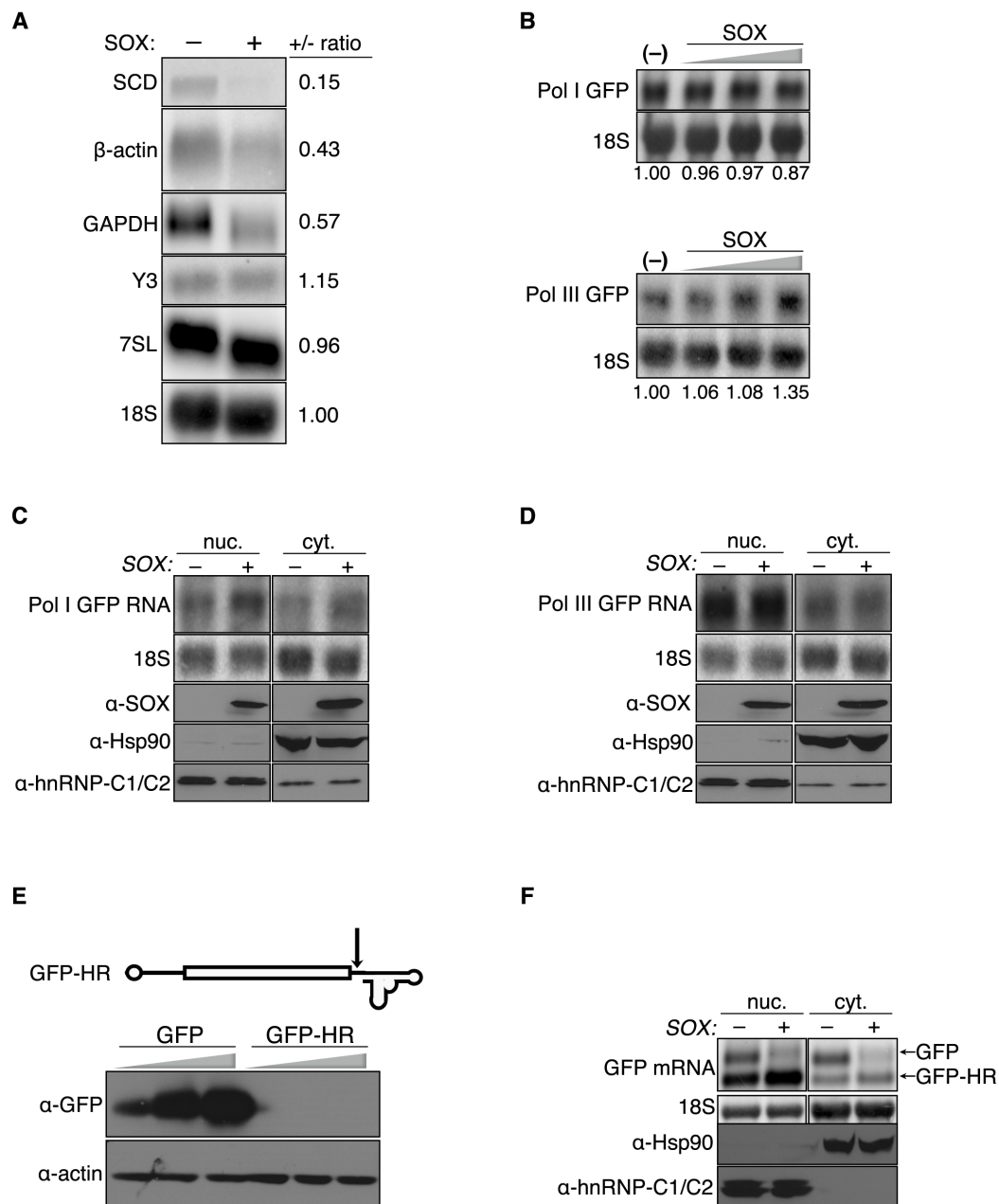


Figure 3.4 SOX specifically targets translationally-competent Pol II transcripts. (A) 293T cells were transfected with either GFP or GFP-SOX. Cells were then sorted for GFP fluorescence to generate a pure population of SOX-expressing and control cells prior to RNA extraction. RNA was Northern blotted with the indicated probes against endogenous Pol I (18S), Pol II (SCD, β -actin, GAPDH), and Pol III (Y3, 7SL) RNAs. (B) 293T cells were transfected with either Pol I-driven GFP or Pol III-driven GFP reporters with or without increasing amounts of SOX (200–600 ng). (C–D) 293T cells were transfected with either Pol I-driven GFP (C) or Pol III-driven GFP (D) reporter constructs in the absence or presence of SOX. Samples were fractionated into nuclear and cytoplasmic fractions and processed for RNA and protein as described in (B). (E) 293T cells were transfected with either normal GFP or a GFP reporter terminating in a hammerhead ribozyme, generating a transcript that is not polyadenylated

(GFP-HR; shown in diagram, arrow points at site of ribozyme cleavage). GFP protein expression was assessed by Western blot analysis. Actin was used as a loading control. (F) 293T cells were co-transfected with the normal GFP reporter and GFP-HR with or without SOX. Cells were divided into nuclear and cytoplasmic fractions from which total RNA and protein were extracted. RNA was Northern blotted with a probe annealing to the first 300 bp of the GFP coding region or an 18S probe (upper panels), and protein lysates were Western blotted with α -hnRNP-C1 and α -Hsp90 antibodies to assess the purity of nuclear/cytoplasmic fractions (lower panels).

Figure 3.4

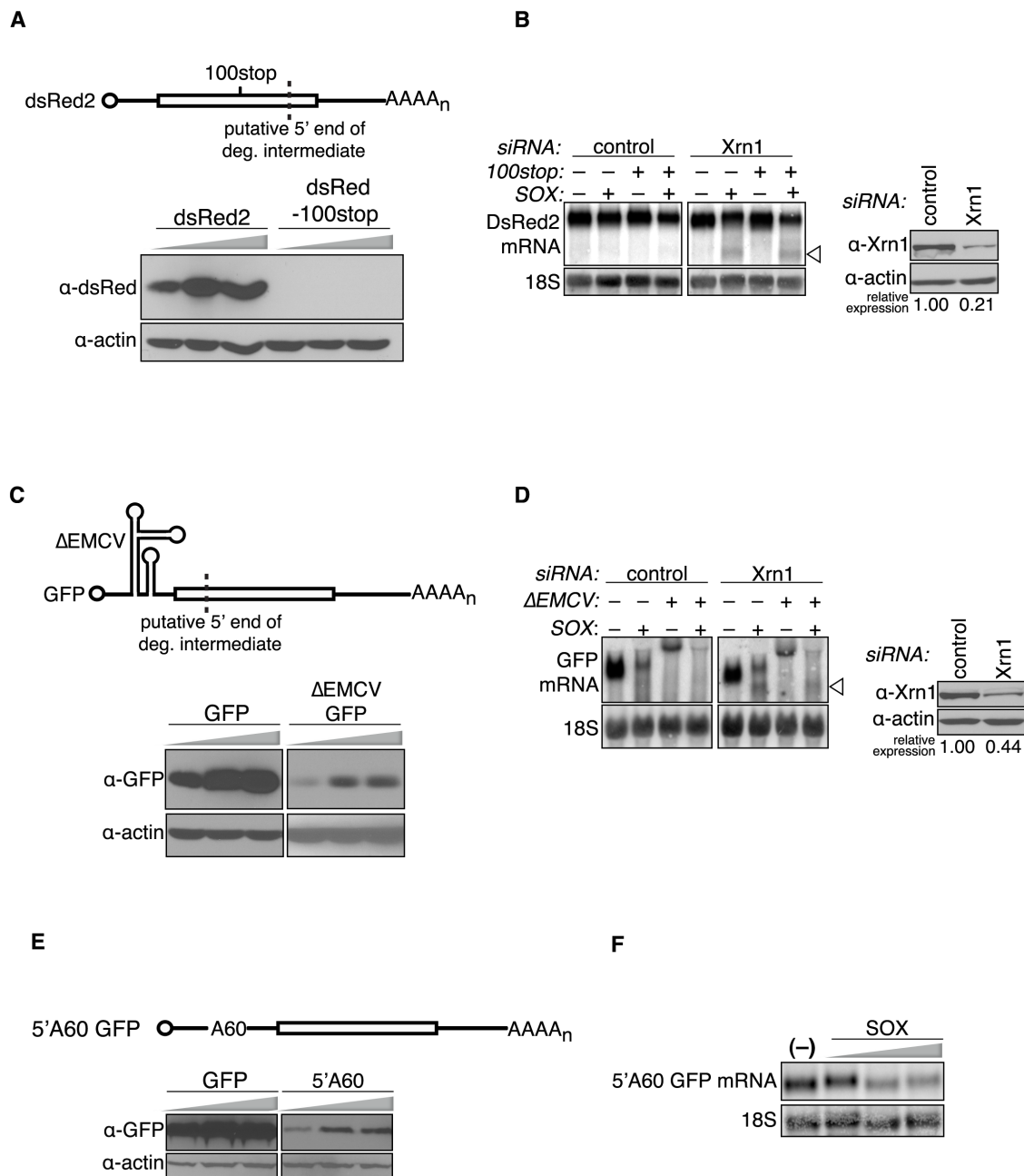


Figure 3.4 SOX targets mRNAs during early stages of translation.

(A) 293T cells were transfected with increasing amounts (100–300 ng) of dsRed2 or a dsRed2–100stop construct containing a premature termination codon upstream of the predicted cleavage site (dashed line), then Western blotted for dsRed protein. (B) 293T cells were transfected with control or Xrn1 shRNAs and subsequently with either the dsRed2 or dsRed2–100stop (100stop) reporter in the presence or absence of SOX. RNA was Northern blotted with 3' end dsRed2 or 18S probes. The arrowhead indicates the position of the SOX-induced cleavage product. (C) 293T cells were transfected with increasing amounts (100–300 ng) of GFP or a Δ EMCV-GFP construct containing the Δ EMCV IRES in the 5' UTR, then Western blotted for GFP protein. (D) 293T cells were transfected with control or Xrn1 shRNAs and then with either the GFP or Δ EMCV-GFP reporter in the presence or absence of SOX. (E) 293T cells were transfected with increasing amounts (100–300 ng) of GFP or a 5'A60-GFP construct containing a stretch of adenosines in the 5' UTR (depicted in diagram). Western blot demonstrates a significant reduction in translation of 5'A60-GFP. (F) HEK 293T cells were transfected with the 5'A60-GFP reporter with or without increasing amounts of SOX (100–300 ng). RNA was Northern blotted with GFP and 18S probes, and protein lysates were Western blotted with the indicated antibodies. The arrowhead indicates the position of the SOX-induced cleavage product. The Western blot shows level of Xrn1 depletion, and the actin loading control.

Figure 3.5

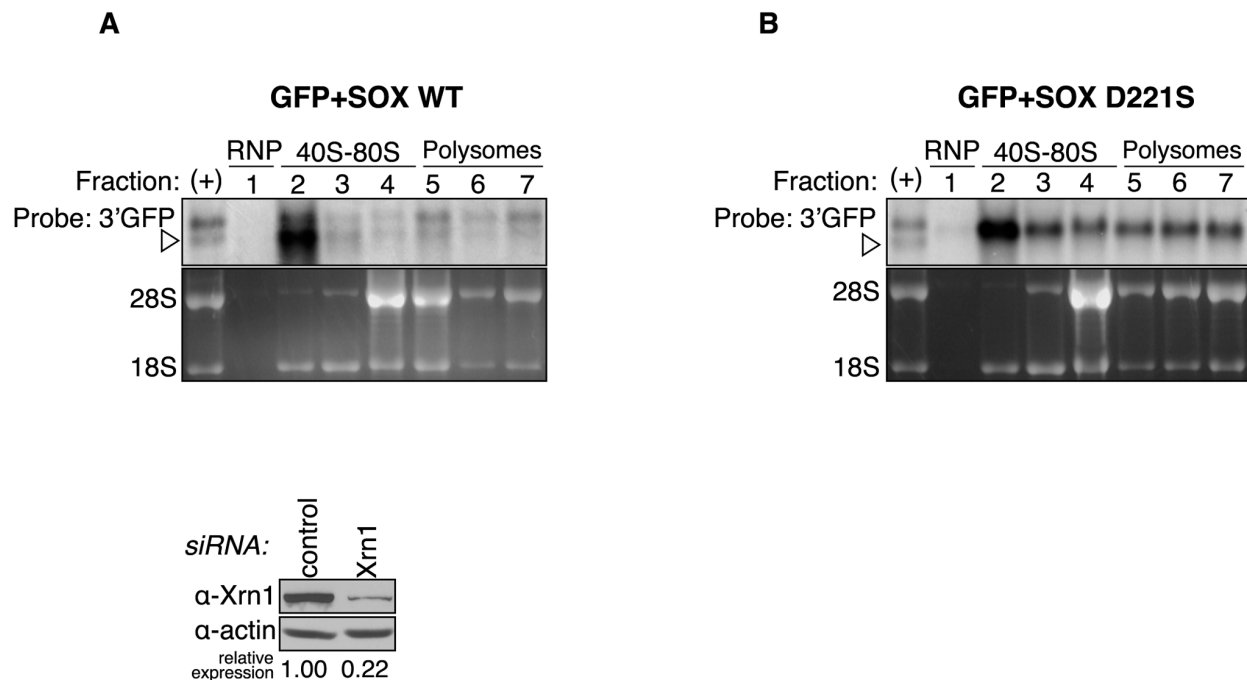


Figure 3.5 The degradation intermediate sediments predominantly with the 40S fractions.

293T cells were transfected with Xrn1 shRNA, followed by expression of GFP and either wild-type (WT) SOX (A) or the catalytically dead SOX D221S (B). They were then fractionated over sucrose gradients and RNA from each fraction was Northern blotted with a 3' GFP probe. Ribosomal RNA was visualized by ethidium bromide staining. In both panels the (+) lane shows the migration of full-length GFP and the degradation intermediate in unfractionated RNA from cells expressing wild-type SOX. It was used as a reference to identify the different RNA species in the fractionated RNA. Arrowheads point to degradation intermediates.

Chapter 4:

Translation-linked Mechanisms of Cellular mRNA Targeting by Other Host Shutoff-Causing Viruses

Background

Infection with a variety of viruses results in global reprogramming of host gene expression creating an environment that favors optimal viral progeny production. Similar to SOX, the SARS coronavirus (SCoV) and herpes simplex virus (HSV) both encode viral RNases that target messages in a translation-linked manner (51, 88). The SCoV non-structural protein 1 (nsp1) targets mRNA during early stages of translation by binding and inactivating the 40S initiation complex while at the same time promoting cleavage of the mRNA (51). Similarly, the HSV virion host shutoff (vhs) factor promotes extensive mRNA degradation by promoting cleavage at the 5' end of messages via its associations with translation factors 4H and 4B (88). SOX-mediated HS has been implicated to occur in a translation-linked manner (21), however no functional interactors have been identified that would explain the basis for specific targeting. In order to gain insight into the mechanism by which SOX selectively targets mRNAs, I compared turnover of various translationally incompetent RNAs by other shutoff factors including other gamma-HV homologs as well as SCoV nsp1 and HSV vhs. Here I demonstrate that all shutoff factors possess a remarkable specificity for Pol II mRNAs, similar to SOX. I show that the first step in translation initiation, 40S binding is required for the SCoV nsp1, but is dispensable for SOX and vhs shutoff factors. In addition, I use mass spectrometry analysis to identify potential SOX interactors. In this study, I provide additional molecular characterization of the specific stage of translation that is likely targeted by SOX. We anticipate these findings will be informative for predicting the specific factors associated with the mRNA at time that SOX initiates destruction of the message.

Results

Multiple host shutoff RNases specifically target translationally competent Pol II mRNAs

I have recently shown that SOX-mediated turnover in cells is executed in a translation-linked manner (21). In *in vitro* assays, both SCoV nsp1 and HSV vhs also target mRNA during early stages of translation initiation. Unlike SOX, both nsp1 and vhs have characterized interaction with specific cellular factors involved in translation initiation. Therefore, I sought to verify whether these shutoff factors possess the same specificity for mRNAs in cells, as is observed for SOX.

Transcription by Pol I and Pol III does not result in the addition of a cap and poly(A) tail. Rather, these mRNA modifications are associated with Pol II transcription, and are critical for

RNA export and translation initiation. I expressed the GFP reporter under the control of Pol I or Pol III promoters and found that in both cases they were not targeted by any shutoff factor (Figure 4.1A and B). As a positive control for shutoff activity, we used the Pol II Δ EMCV-GFP construct, which possesses a highly structured region that induces premature termination of 40S scanning (21); this mRNA was targeted by all shutoff factors, including SOX, confirming that all shutoff factors target mRNA during early stages of translation ((21), (Figure 4.1A and B)). The Δ EMCV-GFP construct was also used because it encodes a larger mRNA that can be easily resolved from the smaller Pol I or Pol III GFP RNAs. The inability to target the Pol I and Pol III RNAs was not due to their absence in the cytoplasm since I had previously confirmed using subcellular fractionation experiments that these RNAs were in fact exported to some degree (21). Collectively, these data indicate that RNAs must be Pol II transcribed to be targeted by all tested host shutoff factors.

40S binding is required for nsp1-mediated turnover, but not turnover by herpesvirus shutoff factors

In order to gain insight into the mechanism of SOX targeting, we created an mRNA that would be differentially targeted by nsp1 versus vhs and observe whether the turnover profile of SOX would mimic that of either nsp1 or vhs. It had previously been shown that the nsp1 requires 40S binding to mediate shutoff of the target RNA, which should be dispensable for vhs-mediated targeting (51, 88). In order to efficiently inhibit 40S binding, the first step of translation initiation, we placed a hairpin close to the cap (<5nt) (57) and found, as expected, no detectable protein levels (Figure 4.1C). The nsp1 protein was unable to target the hairpin-containing mRNA, which emphasizes its requirement for 40S binding (51) (Figure 4.1C). In contrast, I found that the hairpin did not block turnover by any HV shutoff factor, including the HSV vhs, known to bind various translation initiation factors (Figure 4.1C). My results suggest that SOX targeting of mRNA, similar to vhs, may occur prior to 40S binding or alternatively SOX targeting may not require 40S binding.

The cap and poly(A) tail are necessary, but not sufficient to allow efficient turnover by SOX in the cytoplasm

The cap and poly(A) tail of an mRNA have important roles in both RNA export and translation. We had previously shown that an mRNA with a truncated 3'UTR/poly(A) tail was not translated or exported efficiently, nor was it targeted by SOX (21, 66); It was unclear whether the inability to target this mRNA was due to either an export or a translation defect. I sought to determine whether SOX would be able to target an cytoplasmically-synthesized RNA that does not acquire a cap or poly(A) tail in order to determine whether these features are important once the RNA is in the cytoplasm. An inability to target this RNA would support a necessity for engagement of translation factors once the RNA is exported. We expressed T7-Polymerase (T7-Pol) for 24 hour to allow levels to accumulate in order to minimize depletion by SOX (Figure 4.2A, anti-myc T7-Pol). For negative controls, we transfected T7-driven GFP (lacking a cap and tail) with (control 2) or without (control 1) T7-Pol to demonstrate that the GFP RNA was in fact T7 transcribed (Figure 4.2A). For the indicated lanes, SOX or the catalytically dead mutant, D221S, were cotransfected into cells (Figure 4.2A). Interestingly, SOX was not able to efficiently turnover T7-GFP RNA supporting a role for translation once the RNA is in the cytoplasm (Figure 4.2A).

Given that the cap and tail are important features necessary for targeting, we reasoned that an in vitro transcribed (IVT) RNA, that is capped and polyadenylated, would be targeted by SOX when transfected into cells. We first transfected cells with either vector, SOX, D221S or nsp1-expressing DNA. Twenty-four hours later, cells were transfected with IVT firefly luciferase mRNA and bioluminescence levels were assessed as a read-out for shutoff (Figure 4.2B). Surprisingly, SOX was unable to target this mRNA for shutoff, in contrast to nsp1, which efficiently induced shutoff of this message (Figure 4.2B). These data confirm the importance of the cap and tail for SOX-targeting of RNA in the cytoplasm. Interestingly, the differential targeting of cytoplasmic IVT mRNA by SOX and nsp1 highlight another mechanistic difference in how these two shutoff factors target mRNA.

Identification of SOX interactors with potential role in its host shutoff activity

Although the mechanism of SOX-mediated host shutoff has been shown to involve a translation-linked process (21), no functional interactors have been identified that would explain this specific targeting. All host shutoff related activities require cytoplasmic SOX (22), suggesting that interaction in this compartment may be required for its activity. I sought to identify binding partners for SOX within the cytoplasmic fraction of SOX-transfected 293T cells. In order to ensure physiological levels of SOX expression I used the 3' Moloney murine leukemia virus long terminal repeat (LTR) promoter in the pBABE vector, which I found to yield SOX levels similar to viral infection ((76), unpublished observation). I performed anti-SOX pAb co-immunoprecipitation (coIP) from total cytoplasmic fractions and analyzed associated proteins by mass spectrometry (MS) analysis. Figure 4.3 lists potential SOX interactors that were reproducibly identified in three independent coIP/MS procedures. For simplicity, candidates were categorized into translation, splicing, and RNA binding based on their predicted function (Figure 4.3). I acknowledge that the functions of some factors may span all categories. The majority of factors identified were associated with RNA and had roles in translation, splicing, and/or RNA stability, suggesting that I was likely analyzing the appropriate pool of SOX. We eliminated factors found on the “common 293T contaminants list” (communicated by Nevan Krogan) compiled from over 200 coIP experiments and show the net list of candidates in Figure 4.4. Interesting, a variety of translation factors including eIF3 and eIF4E are listed, which may support target of translating mRNAs (Figure 4.4). In addition, a variety of RNA binding proteins including the regulator of nonsense transcripts 1 (UPF1) and RNA-binding protein 39 were observed on the list (Figure 4.4). We anticipate that one or more of factors listed could have a contributive role in SOX shutoff activity.

Discussion

Although SOX-mediated host shutoff is thought to occur in a translation-linked manner, no functional interactors have been identified that would shed light on the basis for this specificity. Similarly, the host shutoff factors nsp1 and vhs have been shown to target translating messages, however for these factors interactions with specific translation-associated cellular proteins have been demonstrated. In order to gain mechanistic insight into how SOX targets mRNAs, I compared turnover of various translationally-incompetent RNAs by different shutoff factors. I demonstrated that all host shutoff factors examined here possessed specificity for Pol II transcripts, which supports their link to translation. Furthermore, I dissected differences between the mechanisms of nsp1 and vhs, demonstrating that 40S binding is required for nsp1

targeting, but not for vhs or SOX induced turnover in cells. These observations have important implications for the specific mechanism of targeting used by SOX. Interestingly, I demonstrate that shutoff factors, known to interact with translation machinery, possess similar specificity for Pol II transcripts as observed for SOX. These findings strongly support the hypothesis that SOX likely binds to one or more cellular proteins involved in translation.

In order to search for potential SOX interactors that would explain the basis for SOX's specificity for mRNAs, we performed coIP/MS experiments and generated a list of potential interactors. As described in the previous paragraph, we have found evidence that suggests that the targeting mechanism of SOX may be similar to vhs. Interesting, we identified components of the translation initiation complex, 3 and 4E, as well as a variety of helicases with roles in translation. In addition, we identified a variety of RNA binding proteins including the regulator of nonsense transcripts (Upf1). Our efforts to verify interactions with specific candidates has resulting in the validation of weak interactions with DDX1, Upf1 and P(rC)BP, but no interactions were found for any of the translation initiation factors (Figure 4.5).

Materials and Methods

Cell Extracts, Western blots, Northern Blots and coimmunoprecipitations

Cytoplasmic fraction was isolated using the NE-PER fractionation kit (ThermoScientific). Affinity-purified SOX pAb was bound to protein A sepharose beads and coupled using dimethylpimelimidate (DMP) according to Harlow and Lane (43). Other methods are described in the materials and methods of chapter 3.

Plasmid Constructs

Plasmids pCDEF3-SOX (37), pCDEF3-HA-muSOX (22), pCDEF3-BGLF5-HA (86), pCDNA3.1-vhs (59), PCDEF-HA-nsp1 (59), pd2-ΔEMCV-GFP-N1(21), pHH21-GFP (Pol I) (21), pBSU6-GFP (Pol III) (21), pBABE-SOX (76) were described previously. Plasmid pd2eGFP-N1 was purchased from Clontech. A modified Quikchange Mutagenesis (Agilent) protocol was used to insert the hairpin7 (57) into 5'UTR of pd2eGFP-N1, with a predicted position of ~3nt from the cap.

Figures

Figure 4.1

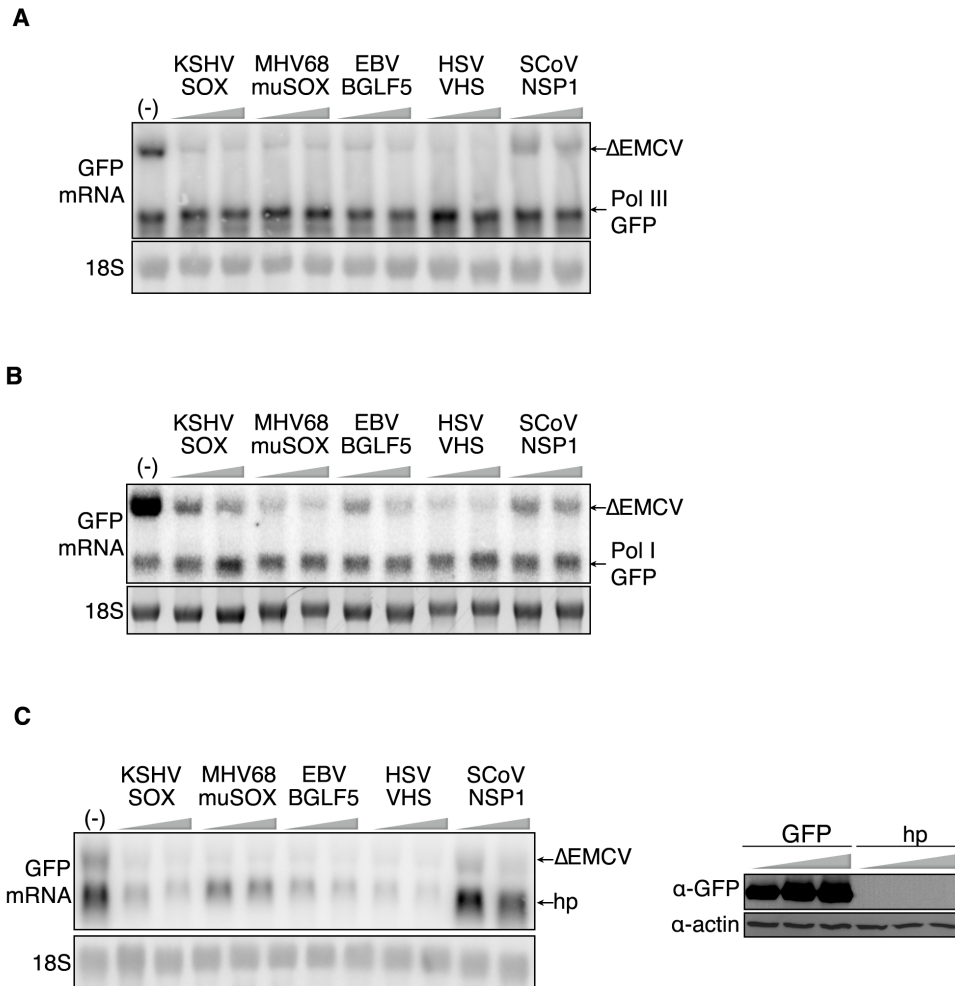
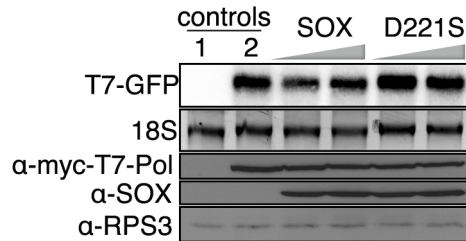


Figure 4.1 Assessment of host shutoff factor activity on various translationally-incompetent RNAs. (A-B) 293T cells were transfected with Δ EMCV-GFP and either Pol I-GFP or Pol III-GFP. In addition, cells were transfected with increasing amounts of either SOX(200–400 ng), muSOX (200–300 ng), BGLF5 (200–400 ng), vhs (100–200 ng) or nsp1 (200–300 ng). (C) 293T cells were transfected Δ EMCV-GFP and hp-GFP. Cells were also transfected with increasing amounts of various shutoff factor described in (A). For the western blot, 293T cells were transfected with increasing amounts (100–300 ng) of GFP or hp-GFP construct containing hairpin in the 5' UTR. RNA was Northern blotted with GFP and 18S probes, and protein lysates were Western blotted with the indicated antibodies.

Figure 4.2

A



B

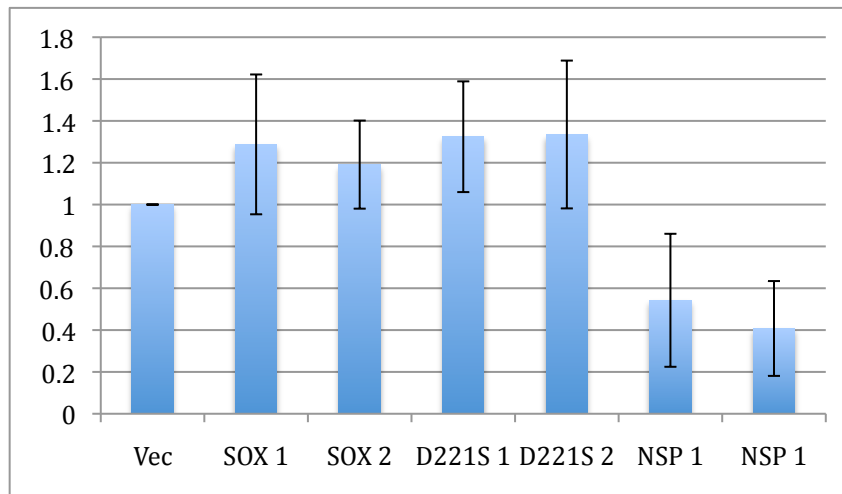


Figure 4.2 The cap and poly(A) tail are necessary, but not sufficient to allow efficient turnover by SOX. (A) 293T cells were transfected myc-T7-polymerase for 24h. Cells were then transfected with T7-GFP with or without increasing amounts of either SOX(200–400 ng) or D221S (200–400 ng). (B) 293T cells were transfected with either vector, SOX(100–200 ng), D221S (100–200 ng), or nsp1 (100-200ng). The next day cells were transfected with in vitro transcribed (IVT) (capped and polyadenylated) Firefly luciferase mRNA for 4hrs. Luciferase activities were determined using the luciferase assay system (Promega) and a bench-top luminometer according to manufacturer's protocol. The fold changes represent an average of triplicate samples.

Figure 4.3

CATEGORY	DESCRIPTION
Translation	28S ribosomal protein S27, mitochondrial
Translation	40S ribosomal protein S5, S7, S10, S13, S15, S16, S18, S20, S21, S21-like, S24,
Translation	60S acidic ribosomal protein P1, L3, L21, L31, L32
Translation	eukaryotic translation initiation factor 3 subunit A , subunit B
Translation	eukaryotic translation initiation factor 4E type 2
Translation	la-related protein 1 isoform 1
Translation	polypyrimidine tract-binding protein 1 isoform b
Translation	ATP-dependent RNA helicase DDX 1, DDX4, DDX36
Splicing	serine/arginine-rich splicing factor 3
Splicing	serine/arginine-rich splicing factor 7 isoform 1
Splicing	small nuclear ribonucleoprotein E, Sm D3, N, B
Splicing	U2 small nuclear ribonucleoprotein A'
Splicing	U5 small nuclear ribonucleoprotein 200 kDa helicase
RNA binding	5'-3' exoribonuclease 2
RNA binding	heterogeneous nuclear ribonucleoprotein F, H2, K, Q, R, U
RNA binding	nuclease-sensitive element-binding protein 1
RNA binding	nucleolar RNA helicase 2
RNA binding	nucleophosmin isoform 1
RNA binding	plasminogen activator inhibitor 1 RNA-binding protein isoform 1
RNA binding	poly(rC)-binding protein 1, 3
RNA binding	pre-mRNA-processing factor 8, 19
RNA binding	regulator of nonsense transcripts 1 (UPF1)
RNA binding	RNA-binding protein 39 isoform a
RNA binding	WD40 repeat-containing protein SMU1
RNA binding	regulator of differentiation 1 isoform 2
RNA binding	spermatogenesis-associated protein 5

Figure 4.3 Total cytoplasmic SOX interactors generated by coIP/MS analysis. Factors listed here reproducibly came down in three independent experiments. They are subdivided base on annotated function (PUBMED): Translation (purple), splicing (light green) and RNA binding (light blue).

Figure 4.4

CATEGORY	DESCRIPTION
Translation	eukaryotic translation initiation factor 3 subunit A , subunit B
Translation	eukaryotic translation initiation factor 4E type 2
Translation	polypyrimidine tract-binding protein 1 isoform b
Translation	ATP-dependent RNA helicase DDX 1, DDX4, DDX36
Splicing	serine/arginine-rich splicing factor 7 isoform 1
Splicing	small nuclear ribonucleoprotein E, Sm D3, N, B
Splicing	U2 small nuclear ribonucleoprotein A'
Splicing	U5 small nuclear ribonucleoprotein 200 kDa helicase
RNA binding	5'-3' exoribonuclease 2
RNA binding	plasminogen activator inhibitor 1 RNA-binding protein isoform 1
RNA binding	poly(rC)-binding protein 1*, 3
RNA binding	pre-mRNA-processing factor 8
RNA binding	regulator of nonsense transcripts 1 (Upf 1)
RNA binding	RNA-binding protein 39 isoform a
RNA binding	WD40 repeat-containing protein SMU1
RNA binding	regulator of differentiation 1 isoform 2
RNA binding	spermatogenesis-associated protein 5

Figure 4.4 Net cytoplasmic SOX interactors. We removed common 293T cell contaminants (communicated by Nevan Krogan) from the compiled list in Figure 4.2 and show net SOX interactors. (*) Only the poly(rC)-binding protein 1 was also found on a the HSV AE (host shutoff negative) interaction list (communicated by Sandy Weller).

Figure 4.5

candidate	interaction?	RNase sensitive?
DDX5	very weak	not tested
DDX1	very weak	not tested
eIF3j	no	not tested
eIF4B	no	not tested
eIF4E	no	not tested
eIF4H	no	not tested
P(rC)BP1	very weak	not tested
PABPC	no	not tested
RPS3	very weak	yes
Upf1	very weak	yes
Xrn1	very weak	yes

Figure 4.5 Verified SOX interactions. 293T cells were transfected with either vector or SOX. Total cell lysate was harvested in NETN buffer and coimmunoprecipitated using anti-SOX pAb. Coimmunoprecipitated endogenous interactors were detected by Western blot using their specific antibodies.

Chapter 5:

Perspectives

Many viruses have evolved finely tuned strategies to efficiently alter host gene expression in order to promote an optimal cellular environment that supports virus production. Regulation at the RNA stability level is an important contributor to cellular gene expression, and therefore it is not surprising that viruses have evolved mechanisms to directly interface with cellular RNA turnover machinery.

Lytic infection with KSHV results in drastic suppression of host protein synthesis mediated by destabilization of mRNAs. This phenomenon is caused by the conserved virally encoded multifunctional nuclease, SOX. Although SOX had been implicated as the dominant host shutoff factor, the exact mechanism of how it targets and destabilizes messages in cells had never been examined. Here I provided detailed mechanistic insight into how SOX specifically targets host mRNAs in cells. I showed that all host shutoff related activities are orchestrated by the cytoplasmic fraction of SOX. In the cytoplasm, SOX specifically targets conserved features on the 5' and 3' ends of Pol II-transcribed RNAs that normally ensure their efficient expression. Furthermore, I demonstrate that similar mechanisms of targeting are likely conserved among various other viruses. Identification of the factors targeted by SOX will likely give insight into important RNA-associated components involved in activating host RNA turnover pathways.

Is Host Shutoff Linked to Translation?

Several observations support a link between translation and SOX mediated turnover. First, we have observed that nonpolyadenylated transcripts lacking a 3' UTR were neither translated nor targeted by SOX for turnover (66). Second, SOX-induced nuclear relocalization of cytoplasmic poly(A) binding protein (PABPC), resulted in specific disruption in translation-related PABPC interactions(60). Third, I demonstrated that SOX specifically targets translationally competent Pol II transcripts, but not RNAs transcribed by Pol I or Pol III. Fourth, I observed that T7-transcribed cytoplasmic RNAs lacking a cap/tail are not efficiently targeted by SOX (Figure 4.2A). Therefore, the cap and poly(A) tail offer the most logical explanation for SOX's specificity for mRNAs. Unfortunately, we have found no interaction between SOX and either 4E or PABPC, suggesting that these factors are not directly targeted by SOX (Figure 4.5, G.R. Kumar, unpublished observations). However, it is possible that SOX may target other factors more peripherally-associated with the cap and poly(A) tail, such as other proteins that interact with 4E or PABPC.

Although several observations support a link between host shutoff and early stages of translation, it is unclear what specific stage(s) of translation initiation are involved. I assessed the necessity for first step of translation initiation, 40S binding, by placing a hairpin close to cap (<10nt), which prevents ribosomes from accessing the 5' end of the message (57). Surprisingly,

blocking 40S binding inhibited the shutoff activity of nsp1, while it appeared to be dispensable for targeting of mRNA by both SOX and vhs. My results suggest that the mechanism of targeting by SOX may mirror that of vhs. Interestingly, vhs has been shown to interact with translation helicases: 4H and 4B, components of the cap-associated translation initiation complex (30). Interaction with these helicases has been demonstrated to significantly enhance vhs activity and likely explains preferential cleavage at the 5' end of the RNA (25). I have assessed and detected no interaction between SOX and either 4H or 4B, indicating that SOX may require distinct cofactors (Figure 4.5, unpublished results). In contrast, nsp1 has been demonstrated to directly interact with the 40S component, ribosomal protein small subunit 6 (RPS6) (51). I demonstrated for the first time that nsp1-mediated shutoff requires 40S binding in cells, which is in line with its proposed *in vitro*-characterized mechanism of action (51).

The observation that SOX targeting of mRNA occurs prior to 40S binding provides an important clue to narrowing down the types of factors that we should expect to be targeted by SOX. It is anticipated that during this stage of pre-40S binding, the RNP will likely possess a significant amount of nuclear factors that have piggy-backed on the RNA into the cytoplasm.

Requirement for a Nuclear Life

Co-transcriptional nuclear processing events such as capping and splicing often result in the deposition of factors that can influence the fate of the RNA posttranscriptionally. Interestingly, I have found that protein expression from an *in vitro*-transcribed (IVT) mRNA (capped and polyadenylated) was not inhibited in SOX-transfected cells (Figure 4.2B). In contrast, protein expression from the IVT mRNA was significantly inhibited by nsp1 (Figure 4.2B). One possibility for SOX's inability to target IVT mRNA could be that it has never had a "nuclear life", and would therefore be lacking a variety of nuclear factors that are normally deposited co-transcriptionally. The capping reaction for example allows for the binding of the cap-binding complex (CBC), which is known to promote export via interactions with the transcription and export complex (TREX) (16). Additionally, the CBC can associate with translation initiation factor 4G to promote translation (32). Similarly, the splicing reaction results in deposition of the exon junction complex (EJC), which has been demonstrated to enhance translation and also plays a role in the recognition of premature stop codons to trigger non-sense mediated decay (NMD) (65). Given that SOX likely targets mRNA during early stages of translation initiation, it is likely that a variety of nuclear-deposited protein will still be associated with the message at this point. Why would SOX not target these nuclear factors in the nucleus? I hypothesized that transport of nuclear factor-bound mRNA into the cytoplasm may allow for their posttranslational modification (PTMs) or, alternatively, may allow for cytoplasmic interactions that may be important for the recruitment of SOX to the RNA. Similarly, the nuclear-deposited EJC activates NMD via specific cytoplasmic interactions. Additionally, differential phosphorylation of shuttling proteins has been shown to be dependent on their nuclear or cytoplasmic localization (87) and can affect their ability to interact with specific factors in each compartment.

The stability of an mRNA is traditionally thought to be influenced by its sequence in both coding and non-coding regions. Recent findings have proposed that the specific promoter from which a transcript is made from can directly influence its stability, even though no promoter sequences are not included in the mRNA (9, 101). The model proposes that promoter-specific transcription factors can facilitate co-transcriptional loading of certain RNA-binding

proteins that will accompany the transcript into the cytoplasm and influence its stability (9, 101). Interestingly, we have observed one example of an endogenously-expressed mRNA that escapes SOX mediated turnover. The resistance to SOX-mediated turnover is abrogated when this cDNA is expressed from an different promoter (CMV promoter), suggesting that its untranscribed sequences could be important in its stability (17). I have also observed that SOX specifically targets Pol II transcripts, while sparing Pol I and III RNAs. While the simplest explanation for these observations is that SOX somehow recognizes the cap/tail on the mRNA, it is feasible that transcription from a Pol II promoter also results in deposition of promoter-specific factors that may be targeted by SOX.

Evidence That SOX Targets Factors Associated with the 5' Cap

The translation-linked mechanism of SOX-targeting of mRNA predicts that both cap and poly(A) tail would be important for efficient targeting. In support of this we observed that nonpolyadenylated transcripts lacking a 3' UTR were neither translated nor targeted by SOX for turnover (66). However, addition of non-poly(A) 3'UTR sequences partially restored translation and SOX-mediated turnover suggesting that the poly(A) sequence is not necessarily important for SOX targeting. Additionally, we had previously observed that a mRNA possessing a histone 3' processed end, lacks a poly(A) tail, yet is efficiently turned over by SOX (66). I have also found that SOX can target Pol II-transcribed U1-terminating RNA (unpublished observations), which is 3'processed distinctly from messenger and histone RNAs and does not utilize common 3'processing factors. The common feature between all Pol II transcripts targeted by SOX is the 5' cap. Interestingly, vhs is known to target cap-associated factors and SOX appears to share similarities to vhs in its targeting of messages. However, we have failed to observe interactions with the various cap-associated proteins (Figure 4.5). In summary, the observation that SOX targets mRNAs that are drastically different at their 3' ends, suggests that the 5' cap is likely the main feature recognized and I predict that cap-associated factors may be the best candidates for explaining SOX's observed specificity.

Regulation of SOX by Differential Localization

SOX possesses two distinct functions, DNase and host shutoff activities, which are carried out in the nuclear and cytoplasmic cellular compartments respectively. It is presumed that the presence of SOX in each specific cellular compartment, should activate its corresponding function. However, it is unclear how or if SOX is regulated in the cell. I have observed that the expression of SOX is initiated at 8 hours post infection and is maintained for the duration of the lytic cycle (Figure 2.1B). Therefore, I presume that regulation of SOX host shutoff activity would likely occur posttranslationally. The HSV shutoff factor, vhs, is expressed during late stages of viral infection, yet it remains inactive due to its association with the viral protein, VP16, which binds and inactivates it allowing its packaging into the virion (3). Vhs is subsequently activated upon release into the cytosol during the next round of infection (3). It is possible that, similar to vhs, SOX may be regulated at the posttranslational level via interacting partners or other posttranslational modifications. Given that SOX host shutoff activity is carried out by the cytoplasmic fraction it is tempting to speculate that controlling localization could directly control host shutoff activity, as I have shown for NRS-muSOX (22). Interestingly, we have recently found that nuclear and cytoplasmic SOX are differentially phosphorylated (Z. Davis, unpublished observations), which could have important regulatory implications. Additionally, we have also observed that SOX appears to multimerize in the cell; mutant analysis

suggests that multimerization may be selectively required for its DNase activity, but not its host shutoff activity (Z. Davis, unpublished observations). It is therefore foreseeable that there will be one or more mechanisms of regulating both activities of SOX.

Concluding Remarks

In the past, studying how viruses modulate gene expression during a viral infection has led to fundamental discoveries in molecular biology. Given that viruses have to alter the host environment in such a drastic way, they often tap into cellular pathways as an efficient way to bring about large changes with minimal coding effort. Regulation at the RNA stability level is a major contributor to cellular gene expression and a variety of viruses have now been shown to directly interface with cellular RNA turnover machinery. I anticipate that these viruses will undoubtedly elucidate important regulatory aspects of RNA decay.

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Appendix: Generation and Characterization of SOX Monoclonal Antibodies.

Immunizations

Monoclonals were produced in BALB/c mice with the assistance of P. Robert Beatty and Fei Lin under Animal Use Protocol T044-1208 for the production of monoclonal antibodies as approved by the University of California, Berkeley Animal Care and Use Committee (ACUC) for use during Jan 1, 2008 - Dec 31, 2008. All handling of mice was done by PRB and FL. To generate SOX MAb, recombinant MBP-tagged SOX was purified with amylose resin and subsequently cleaved with FactorXa (New England Biolabs) to remove the MBP moiety. Immunizations of mice were done by trained personnel according to the following protocol. BALB/c mice were injected intraperitoneally with 10 µg of SOX in Ribi adjuvant at 8 weeks, 4 weeks, and 4 days prior to hybridoma fusion.

Hybridoma Fusions

Two mice were sacrificed and polyclonal sera were collected to confirm reactivity to SOX. Spleens of the SOX-immunized mice were harvested, and cells were isolated by mechanical disruption and straining. Cells were resuspended in 10 ml Dulbecco's Modified Eagle Medium containing 10 mM HEPES ("DMEM") and pelleted at 400 x g for 5 min. The cell pellet was resuspended in 8 ml of red blood cell lysis buffer and incubated for 2 min, followed by addition of 10 ml DMEM. Cells were pelleted and washed in 40 ml DMEM and resuspended in 10 ml DMEM. Mouse myeloma cell line P3X63-Ag8.653 was grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum (FBS), 50 µM 2-mercaptoethanol, 10 mM HEPES, 10 units/ml penicillin, 10 µg/ml streptomycin, 1 mM pyruvate, and 2 mM L-glutamine at 37°C / 5% CO₂. Cells were split 1:2 one day prior to fusion. Myeloma cells were pelleted and resuspended in 5 ml DMEM. The supernatant was saved as "myeloma-conditioned medium." To perform the fusion, 2 x 10⁷ viable myeloma cells were combined with 10⁸ viable splenocytes (viability determined by trypan blue exclusion), mixed and pelleted. The supernatant was decanted, and the pellet was mixed with the remaining medium. One ml of 50% polyethylene glycol 1500 was added dropwise while mixing at 37°C, and the cells were subsequently incubated at 37°C for 1 min. One ml DMEM was added dropwise, followed by incubation at 37°C for 1 min. Incubation was repeated for 2 min following the addition of 2 ml DMEM. Incubation was again repeated for 4 min following the addition of 4 ml DMEM. Six ml DMEM was added, and cells were pelleted at 200 x g for 10 min. Cells were resuspended in conditioned medium, consisting of 25 ml IMDM containing 20% FBS, 1X HAT (100 µM

sodium hypoxanthine, 400 nM aminopterin, 16 μ M thymidine), 10% BM Condimed H1 conditioned medium (CM; Roche Applied Science, Indianapolis, IN, cat# 11 088 947 001) plus 25 ml myeloma-conditioned medium. Cells were plated (100 μ l/well) in 96-well flat-bottom tissue culture plates and incubated at 37°C with 5% CO₂.

Selection and Cloning

At 2 days post-fusion, 100 μ l of IMDM medium containing 20% FBS, 1X HAT and 10% CM was added per well. At 7 days post-fusion, 100 μ l/well was removed and replaced with 100 μ l of IMDM medium containing 20% FBS, 1X HAT and 10% CM. At 12 days post-fusion, 100 μ l of supernatant was removed for screening by ELISA and replaced with 100 μ l of IMDM containing 20% FBS and 5% CM. Cells were fed at least once per week thereafter with IMDM/20% FBS. Hybridomas were cloned at 15 days post-fusion. Twelve wells were chosen for cloning based on ELISA reactivity and observed growth. Cells were removed from the master plate wells by pipetting and transferred to well A1 of a 96-well tissue culture plate. Cells were serially diluted 1:2 from wells A1 – H1 (rows 1 – 8). Cells were subsequently serially diluted 1:2 from column 1 to column 12 in each row. Cells were grown in IMDM containing 20% FBS and 10% CM. On day 4 post-cloning, an additional 100 μ l/well IMDM/20% FBS/10% CM was added. On day 8 post-cloning, 100 μ l/well of supernatant was discarded and replaced with 100 μ l/well IMDM/20% FBS/10% CM.

Screening of Hybridoma Supernatants

An ELISA assay was developed prior to screening. Primary screening utilized an anti-SOX ELISA with the following conditions:

1. Ninety-six-well plates were coated with 100 μ l/well with 200 ng/well of SOX-transfected lysate or vector-transfected lysate in carbonate coating buffer (100 mM NaHCO₃, 34 mM Na₂CO₃, pH 9.6) and incubated overnight at 4°C.
2. The coat was removed, and wells were blocked with a 100 μ l/well of Blocking buffer: 1XPBS, .05% Tween-20 (250 μ l 100% Tween), 2% BSA for 1 hour at room temperature (RT) (PBST).
3. The block was removed, and the plate was incubated overnight at 4°C with 50 μ l/well hybridoma supernatant. Anti-SOX Rabbit Ab was used as a positive control, while preimmune rabbit serum was used as a negative control.
4. Primary antibody was removed, and wells were washed 6 times in PBS-T. The plate incubated with 50 μ l/well biotinylated goat anti-mouse or goat anti-rabbit Ab diluted 1:1000 in blocking buffer.
5. Plates were washed 4 times with PBS-T and then incubated with 50 μ l/well streptavidin-conjugated alkaline phosphatase (SA-AP) diluted 1:1000 in blocking buffer for 30 – 45 mins at RT.
6. Plates were washed 6 times with PBS-T and developed with 100 μ l/well pnitrophenyl phosphate (pNPP). Read OD at 405nm.

Positive wells were rescreened using immunofluorescence assays (IF) and Western blotting, followed by two rounds of limited dilution cloning and retesting by ELISA, IF, and Western blotting. The 6FD8, 5HD9 and 5HF4 hybridomas were saved as liquid nitrogen stocks.

Thawing and Maintenance

Cells should be thawed in IMDM media supplemented with 10% fetal bovine serum (FBS), 50 μ M 2-mercaptoethanol, 10 mM HEPES, 10 units/ml penicillin, 10 μ g/ml streptomycin, 1 mM pyruvate, 2 mM L-glutamine and 10% CM. After a few days, viability should be assessed using trypan blue staining. If cell viability is >90%, CM can be reduced to 5%. Cells should be maintained at a concentration of 2×10^5 cells/ml. Once cells are stably growing, supernatant can be collected and tested or directly used for Western blots and IF.