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Nucleoside Modifications Suppress RNA Activation of Cytoplasmic RNA Sensors

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Abstract
Multiple innate defense pathways exist to recognize and defend against foreign nucleic acids. Unlike innate immune receptors that recognize structures specific for pathogens that are not shared by mammalian hosts — for example, toll-like receptor (TLR)4-lipopolysaccharide, TLR5-flagellin, NOD1 and 2-peptidoglycan — all nucleic acids are made from four components that are identical from bacteria to man. Nucleoside modifications are prevalent in nature but vary greatly in their distribution and frequency, and therefore could serve as patterns for recognition of pathogenic nucleic acids. The presence of modified nucleosides in RNA reduces the activation of RNA-sensing TLRs and retinoic acid inducible gene I (RIG-I), which initiate signaling cascades following activation and result in transcription of pro-inflammatory genes. Unexpectedly, translation of *in vitro* transcribed mRNA is enhanced by incorporation of modified nucleosides, but the mechanism responsible for this enhanced translation has not been identified. To identify the pathways responsible for enhanced translation of modified nucleoside-containing mRNA, we studied two cytoplasmic RNA-sensing innate defense mechanisms known to influence translation, the RNA-dependent protein kinase (PKR) pathway and the 2-5A system (oligoadenylate synthetase [OAS] and RNase L). Using purified protein *in vitro*, cell culture, and *in vivo* mouse studies, we show that unmodified *in vitro* transcribed mRNA activates PKR and OAS and is rapidly cleaved by RNase L. However, we show that incorporation of modified nucleosides into *in vitro* transcribed mRNA reduces each of these pathways. Furthermore, we demonstrate that these pathways are necessary for enhanced translation of mRNA containing modified nucleosides. Additionally, we demonstrate that the presence of pseudouridine in *in vitro* transcripts increases mRNA half-life following delivery. From these data, we conclude that unmodified *in vitro* transcribed mRNA is stimulatory to the cytoplasmic RNA sensors PKR and OAS. This stimulation is reduced by the presence of modified nucleosides. The enhanced translation of mRNA containing modified nucleosides results from reduced PKR and OAS activation. These data support a larger interpretation that the absence or reduction in frequency of modified nucleosides in RNA is a common pattern for recognition of pathogenic RNA by numerous innate defense systems.

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Dedication

First and foremost this work is a tribute to my wonderful wife, Katie Anderson. She cheerfully and tirelessly provided the patient encouragement and loving support which were critical to completing this effort. She believes in me even when I do not believe in myself, and helps me be who she believes I am. The numberless hours she spent listening to presentations and editing my writing count double, knowing that science itself holds little interest and her only goal has been my success. I cannot express how much her sustaining assistance means to me.

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and HIV-1 p24 monoclonal antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly.

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ABSTRACT

NUCLEOSIDE MODIFICATIONS SUPPRESS RNA ACTIVATION OF CYTOPLASMIC RNA SENSORS

Bart R. Anderson

Dissertation Supervisor: Drew Weissman, M.D., Ph.D.

Multiple innate defense pathways exist to recognize and defend against foreign nucleic acids. Unlike innate immune receptors that recognize structures specific for pathogens that are not shared by mammalian hosts — for example, toll-like receptor (TLR)4-lipopolysaccharide, TLR5-flagellin, NOD1 and 2-peptidoglycan — all nucleic acids are made from four components that are identical from bacteria to man. Nucleoside modifications are prevalent in nature but vary greatly in their distribution and frequency, and therefore could serve as patterns for recognition of pathogenic nucleic acids. The presence of modified nucleosides in RNA reduces the activation of RNA-sensing TLRs and retinoic acid inducible gene I (RIG-I), which initiate signaling cascades following activation and result in transcription of pro-inflammatory genes. Unexpectedly, translation of *in vitro* transcribed mRNA is enhanced by incorporation of modified nucleosides, but the mechanism responsible for this enhanced translation has not been identified. To identify the pathways responsible for enhanced translation of modified nucleoside-containing mRNA, we studied two cytoplasmic RNA-sensing innate defense

mechanisms known to influence translation, the RNA-dependent protein kinase (PKR) pathway and the 2-5A system (oligoadenylate synthetase [OAS] and RNase L). Using purified protein *in vitro*, cell culture, and *in vivo* mouse studies, we show that unmodified *in vitro* transcribed mRNA activates PKR and OAS and is rapidly cleaved by RNase L. However, we show that incorporation of modified nucleosides into *in vitro* transcribed mRNA reduces each of these pathways. Furthermore, we demonstrate that these pathways are necessary for enhanced translation of mRNA containing modified nucleosides. Additionally, we demonstrate that the presence of pseudouridine in *in vitro* transcripts increases mRNA half-life following delivery. From these data, we conclude that unmodified *in vitro* transcribed mRNA is stimulatory to the cytoplasmic RNA sensors PKR and OAS. This stimulation is reduced by the presence of modified nucleosides. The enhanced translation of mRNA containing modified nucleosides results from reduced PKR and OAS activation. These data support a larger interpretation that the absence or reduction in frequency of modified nucleosides in RNA is a common pattern for recognition of pathogenic RNA by numerous innate defense systems.

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CHAPTER 1

Introduction

1.1 Innate nucleic acid sensing

Human cells contain extensive systems to recognize the presence of pathogens and cell damage as signs of danger to the cell or tissue and to limit their spread. Innate recognition of these dangers relies on pattern recognition of danger-associated molecular patterns (DAMPs) by host defense proteins known as pattern-recognition receptors (PRR). All pathogens contain nucleic acids encoding their genome and nucleic acids are released during necrotic cell death [52], therefore exogenous nucleic acids are associated with pathogenicity and can serve as DAMPs.

Numerous nucleic acid-sensing PRR exist, and the presence of additional receptors, which have not yet been characterized, is indicated by nucleic-acid signaling that occurs independently of known pathways [240]. All of these receptors must perform the task of identifying nucleic acids associated with danger, which must be distinguished from normal cellular DNA and RNA. As has been proposed, protein-coating of cellular nucleic acids and compartmentalization of PRR away from cellular nucleic acid ligands likely contribute to differential recognition of danger-associated nucleic acids [53], but do not fully account for nucleic-acid DAMP identification. Molecular features of the nucleic acids themselves are also important determinants. For example, long, perfectly double-stranded (ds)RNA is produced during replication of some viruses, but is not otherwise present in substantial amounts in cells and activates several PRR [30]. Other examples for

molecular determinants of nucleic acid recognition include the presence of 5'-triphosphate on cytoplasmic RNA [115] and unmethylated cytidine in CpG motifs of DNA [112]. Other distinguishing features of danger-associated nucleic acids remain unidentified.

The immunogenicity of RNA is well established, having been demonstrated using multiple approaches [13, 129, 140, 184, 207]. One surrogate measure of RNA immunogenicity is cytokine release by dendritic cells (DC) following exogenous delivery of RNA. This approach has been used to compare the immunogenicity of RNA from various sources. It was demonstrated that bacterial RNA is immunostimulatory, but less so if only the tRNA fraction is delivered. Mammalian RNA is much less immunostimulatory, and again with variable potency depending on the RNA fraction tested. Mitochondrial RNA, which is very similar to bacterial RNA, was responsible for the majority of immunostimulation by mammalian RNA. In contrast to mammalian mRNA, *in vitro* transcribed mRNA is highly immunostimulatory [130]. There is an inverse relationship between the immunogenicity of these RNA fractions and the frequency of nucleoside modifications they contain, which suggests nucleoside modification as a determinant of RNA immunogenicity [132].

1.2 RNA modification

Although fundamentally consisting of four nucleosides – adenosine (A), cytidine (C), guanosine (G), and uridine (U) – RNA in nature is rife with variations. In addition to damage-induced modifications, there are over 100 different nucleoside modifications that are formed in RNA during normal maturation [212]. These modifications are found in all domains of life, although the number and types of modification vary greatly between

species [173]. In general, both the types of modifications found and the number of modified nucleosides present increase when moving up the evolutionary ladder [42].

The most common modification, both in terms of frequency and species distribution, is pseudouridine (Ψ; also known as 5-ribosyluridine), which has been found in nearly all species studied to date. Pseudouridine is formed by isomerization of uridine. The N^1–$C^{1'}$ glycosyl linkage between uracil and ribose is broken, uracil is rotated 180° around its N^3–C^6 axis, and is then reattached with a C^5–$C^{1'}$ uracil-ribose linkage (Figure 1-1). This confirmation leaves the imino nitrogen on pseudouridine free to form an additional hydrogen bond that is not present in uridine, which contributes to its unique properties. The presence of pseudouridine in RNA facilitates base-stacking interactions, increases rigidity in both single-stranded (ss) and double-stranded (ds)RNA, and stabilizes RNA secondary structures. Importantly, base-pairing between pseudouridine and adenosine remains intact [43].

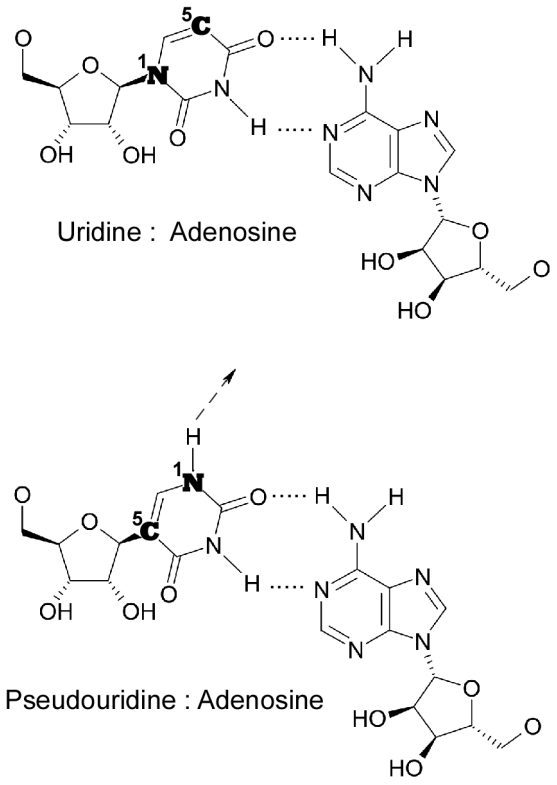


Figure 1-1. Structures of uridine and pseudouridine and base-pairing to adenosine
In pseudouridine, uracil is linked to ribose via C5 instead of the N1 linkage found in uridine (C5 and N1 are indicated in bold type). Hydrogen bonds between adenosine and uridine or pseudouridine are indicated by dotted lines. Additional hydrogen bonding potential of pseudouridine is indicated by dashed arrow.

Another common modification is the addition of a methyl group, including 2'-*O*-methylation of ribose (Nm) and base methylation, such as N^6-methyladenosine (m^6A), 5-methylcytidine (m^5C), and 5-methyluridine (m^5U; also known as ribothymidine). Other modifications include addition of thiol groups, such as 2-thiouridine (s^2U), hydroxyl groups, amino acids derivatives, and others, as well as combinations of modifications on the same nucleoside [212].

Maturation-associated RNA modifications are formed post-transcriptionally in a site-specific manner, either through the use of specific enzymes or by enzymes directed

by guide RNAs [273]. In eukaryotic organisms, RNA modification occurs primarily in the nucleus, and therefore mitochondrial RNA (mtRNA) contains the fewest modified nucleosides of any mammalian RNA fraction [157]. By far, the most heavily modified fraction of cellular RNA is tRNA, where in mammals up to 25% of the nucleosides contain modification [157]. Modifications are also common in rRNA, which accounts for ~80% of RNA in cells, with approximately 250 RNA modification sites occurring in human rRNA [145]. Multiple methylation variants occur for a uniquely 5'–5' triphosphate-linked N^7-methylguanosine (m^7G) cap that is found on RNAs transcribed by RNA polymerase II, including mRNA, snRNA, and pri-miRNA [27]. Internal modification of mRNA is primarily m^6A, averaging 3–5 m^6A per mRNA, although m^6A is absent in some mRNAs. Additionally, there have been limited reports of m^5C in mRNA [27, 179]. RNA modifications are also found in most other fractions of cellular RNA including snRNA, snoRNA, and miRNA.

The function of modified nucleosides in RNA is poorly understood. In many cases blocking RNA modifications produces no obvious impact *in vitro*, leaving the question of their biological roles unresolved [145]. However, the importance of RNA modification is demonstrated by the evolutionary conservation of both RNA modification in general as well as specific modification sites. Furthermore, genes encoding RNA-modifying enzymes are essential, as demonstrated in yeast [145]. RNA modification is best studied in tRNA, where RNA modifications have been shown to have roles in stabilizing critical tRNA structures and in fine-tuning decoding in translation [97]. Highly thermophillic organisms have increased tRNA modifications, adding support for their role in stabilizing RNA structures. Modified nucleosides in rRNA have also been closely

examined, where a limited number of specific modifications are required for maximal translation [145, 255]. In the absence of data showing specific roles for other modification sites, it has been suggested that their role is to stabilize rRNA structure [145]. The m^7G cap found on mRNA facilitates nuclear export, protects the RNA from exonuclease attack, and has a well-established role in enhancing the translation of mRNA through cap-binding proteins [27]. The major naturally-occurring internal nucleoside modification in mRNA is m^6A. Inhibition of m^6A methylation does not change RNA stability [35], and instead is thought to play a role in pre-mRNA splicing and transport [27], although this is disputed by a recent study of m^6A in unspliced yeast mRNA [26].

In addition to the naturally-occurring nucleoside modifications discussed above, a plethora of chemically synthesized modifications and nucleoside analogs have been developed. Nucleoside analogs are used as investigational and FDA-approved antiviral and chemotherapeutic agents [79]. Chemically-synthesized modified nucleosides have been incorporated into nucleic acids, often with the intent to increase nuclease resistance [45]. While beneficial in proper circumstances, these modified nucleosides also present the dangers of re-entering the cellular NTP pool where they may interfere with RNA or DNA synthesis [146, 148]. In contrast, it has been demonstrated that naturally-occurring modified nucleosides do not re-enter the NTP pool, and cellular pathways exist for their controlled removal [24]. A goal of the laboratory is to develop modified RNA for therapeutic use, including transient gene therapy and vaccination. For this reason, only nucleoside modifications that occur naturally during RNA maturation will be examined and discussed in this dissertation.

1.3 Influence of modified nucleosides on RNA immunogenicity

Following the observation that high levels of nucleoside modification correlate with low RNA immunostimulation, the affect that the presence of modified nucleosides have on RNA immunogenicity was tested by measuring immunostimulation of DC by *in vitro* transcribed RNA. The immunogenicity of *in vitro* transcribed mRNA is evidenced by cytokine release from DC following mRNA transfection. To generate *in vitro* transcribed RNA, one or more NTPs were replaced with a corresponding modified NTP in phage polymerase transcription reactions. This led to the complete replacement of one nucleoside with a modified nucleoside (Figure 1-2). mRNA containing modified nucleosides stimulated less cytokine production by DC. In monocyte-derived DC (MDDC), this impact was observed for RNA containing m^5C, m^6A, Ψ, and s^2U. In primary DC, only U modifications — Ψ, s^2U, and m^5U — reduced RNA immunogenicity, whereas m^6A and m^5C did not (Figure 1-3). Similarly, modified nucleosides reduced the RNA-induced activation of MDDC, as measured by upregulation of maturation marker CD83 on the cell surface (Figure 1-4) [130].

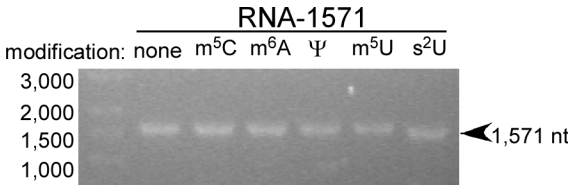


Figure 1-2. Incorporation of modified nucleosides into mRNA during *in vitro* transcription

Aliquots (1 μg) of *in vitro*-transcribed RNA-1571 without (none) or with m^5C, m^6A, Ψ, m^5U, or s^2U nucleoside modifications were analyzed on denaturing agarose gel followed by ethidium bromide-staining and UV illumination. Reprinted with adaptations from Immunity, 23(2), Katalin Karikó, Michael Buckstein, Houping Ni, and Drew Weissman, Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA, 165–175, Copyright 2005, with permission from Elsevier.

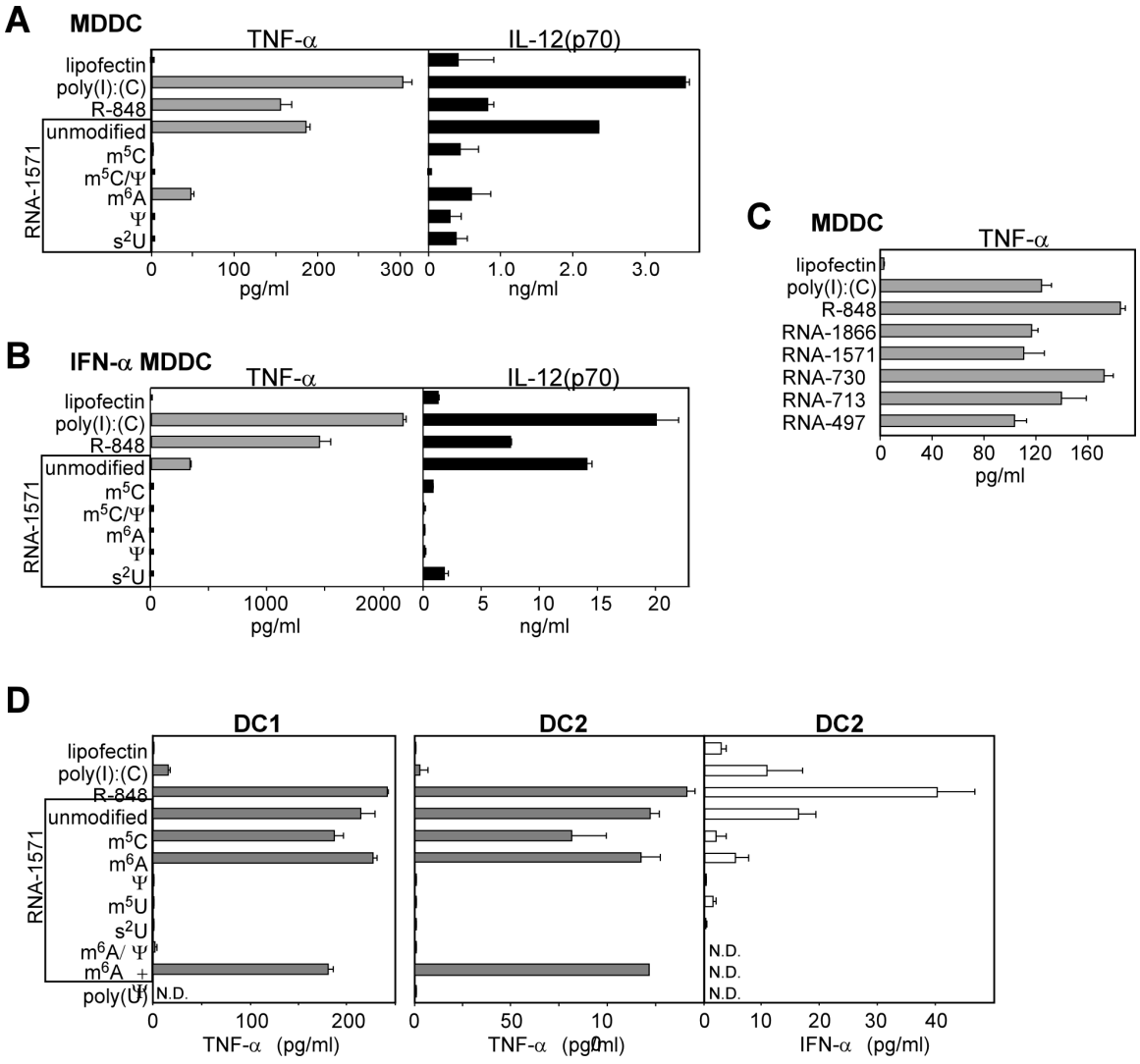
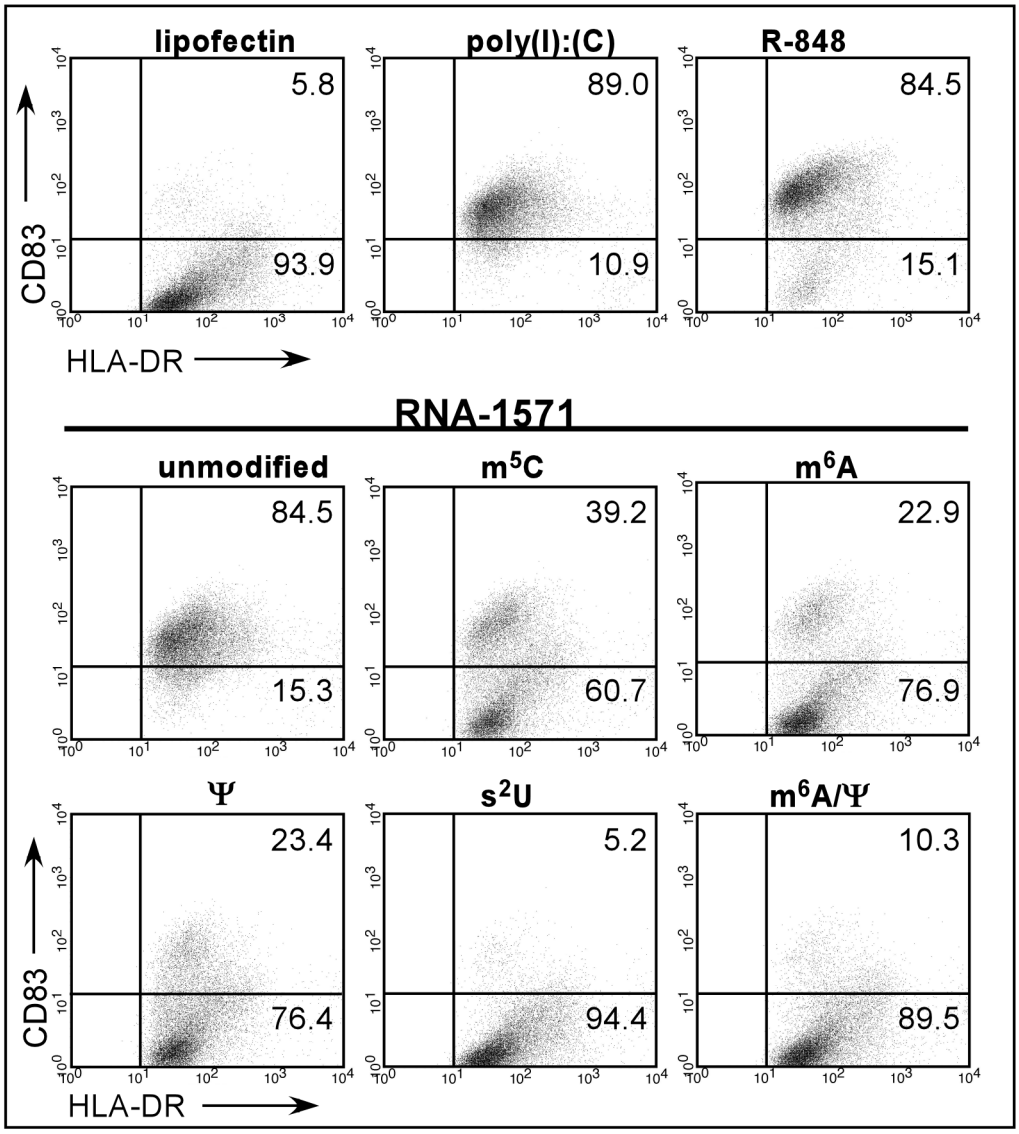


Figure 1-3. Cytokine production by RNA transfected DCs
MDDC (**A** and **C**), IFN-α MDDCs (**B**), and primary DC1 and DC2 (**D**) were treated for 8–16 hr with lipofectin alone or complexed with R-848 (1 μg/ml) or the indicated RNA (5 μg/ml). Modified nucleosides present in RNA-1571 are noted. TNF-α, IL-12(p70), and IFN-α were measured in the supernatant by ELISA. Mean values ± SEM are shown. The results are representative of ten (A and C), four (B), and six (D) independent experiments. N.D., not determined. Reprinted with adaptations from Immunity, 23(2), Katalin Karikó, Michael Buckstein, Houping Ni, and Drew Weissman, Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA, 165–175, Copyright 2005, with permission from Elsevier.

A



B

	TNF-α*	CD80	CD86
	pg/ml	mean fluorescence	
lipofectin	0	7.6	55.3
poly(I):(C)	45.6	59.4	257.4
R848	48.3	55.2	235.4
RNA-1866			
unmodified	26.7	52.7	246.4
m^5C	0	16.4	108.6
m^6A	0	12.4	78.4
Ψ	0	12.0	87.5
s^2U	0	8.0	62.7
$m^6A/Ψ$	0	8.6	68.4

Figure 1-4. Activation of DCs by RNA
MDDCs were treated for 20 hr with lipofectin alone or complexed with R-848 (1 μg/ml) or the indicated RNA (5 μg/ml). Modified nucleosides present in RNA-1571 are indicated. (**A**) CD83 and HLA-DR staining is shown. (**B**) TNF-α was measured in the supernatants by ELISA (the asterisk represents cells that were cultured in 30-fold larger than usual volume of medium for flow cytometry). Mean fluorescence of CD80 and CD86 was determined by flow cytometry. Data are representative of four independent experiments. Reprinted with adaptations from Immunity, 23(2), Katalin Karikó, Michael Buckstein, Houping Ni, and Drew Weissman, Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA, 165–175, Copyright 2005, with permission from Elsevier.

Studies in other systems also observed reduced immunostimulation by RNA containing modified nucleosides. The reduced immunogenicity of RNA containing m^5C was confirmed in peripheral blood mononuclear cells (PBMC) [236]. The dsRNA polyinosinic:polycytidylic acid (poly(I:C)) is a well established immunostimulatory RNA. However, the presence of 2'-*O*-methylated inosine (I) or C in poly(I:C) reduces the stimulation of type I interferon (IFN) production by primary human fibroblasts [95]. Subsequently, multiple studies found that 2'-*O*-methylation (Nm) reduces RNA immunostimulation of human PBMC, which has become a popular strategy for reducing the immunogenicity of siRNAs [123, 171, 231].

1.4 Influence of modified nucleosides in RNA on pro-inflammatory RNA receptors

The observation that incorporation of modified nucleosides reduced RNA immunogenicity promoted interest in understanding how modified nucleosides influence activation of RNA receptors that initiate pro-inflammatory signaling pathways. The toll-like receptor (TLR) family of receptors contains 10 members in humans, which respond to diverse stimuli [135]. Three endosomally-located TLRs respond to RNA: TLR3, 7, and 8.

TLR3 is activated by dsRNA [1]. Both TLR7 and TLR8 are activated by ssRNA in humans [66, 111, 130], although the agonists of TLR8 are less well characterized, in part because its functional relevance in mice is debated [124, 156]. It has been reported that poly-U RNA activates TLR7 [66, 67, 111], but this has not been replicated in all studies [130]. Activation of TLRs initiates nuclear factor-κB (NF-κB) and interferon regulatory factor (IRF) signaling pathways, resulting in production of pro-inflammatory cytokines [195]. Additionally, closely related TLR9 is stimulated by unmethylated DNA, but not by DNA containing 5-methyl-deoxycytidine in CpG motifs [112], establishing a precedent that nucleoside modification can alter TLR activation. Activation of individual TLRs by *in vitro* transcribed RNA was tested in stably transformed 293T cell lines, each transfected with a single RNA-responsive TLR. Unmodified *in vitro* transcribed RNA was stimulatory to all three RNA-responsive TLRs. However, RNA containing modified nucleosides m^5C, Ψ, m^6A, m^5U, or s^2U did not stimulate TLR7 and TLR8. In cells expressing TLR3, m^5U-containing RNA was as stimulatory as unmodified RNA, and the presence of Ψ or m^5C only modestly decreased stimulation. In contrast, incorporation of m^6A or s^2U into RNA eliminated stimulation of TLR3-transformed cells [130]. In another report, Am also reduced TLR7-mediated IFN production in plasmacytoid DC [219].

The cytoplasmic RIG-I-like receptor (RLR) family was discovered more recently, consisting of retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LPG-2). The archetype of the family, RIG-I, is best studied and is thought to be activated by RNA containing 5'-triphosphates (5'ppp) [115], although ongoing debate continues [253]. It has also been suggested that the uridine content of RNA plays an important role in RIG-I activation [215].

MDA5 is thought to be activated by longer dsRNA or branched RNA molecules [196, 270]. The role of LPG-2 is much less clear, but because it lacks a key protein-interacting domain, it has been proposed to serve in regulatory roles [210, 218, 256, 270]. In human monocytes and plasmacytoid DC, 5'ppp-bearing RNA stimulated IFN-α production, but not if the RNA contained Ψ, s^2U, or Um, suggesting that RIG-I is not activated by RNA containing modified U [115]. For Ψ, this was later confirmed through comparison of IFN induction in wild-type (WT) and RIG-$I^{-/-}$ murine embryonic fibroblast (MEF) cell lines [131]. Subsequently, it was demonstrated that RIG-I binds RNA containing Ψ but is not activated, and therefore Ψ-modified RNA functions as a competitive inhibitor of RIG-I activation [253]. Additionally, the modified nucleoside inosine (I) can be formed by the deamination of adenosine, and cytoplasmic dsRNA containing I:U base pairs inhibits IRF3 activation, suggesting that IU-dsRNA inhibits RLR activation [258]. No studies directly examining the influence of modified nucleosides on MDA5 or LPG-2 have been reported.

Activation of RNA signaling receptors promotes increases in both innate and adaptive immune responses [29]. The combined data examining TLR and RLR indicate a trend toward reduced activation of RNA signaling receptors by RNA that contains modified nucleosides. Furthermore, there are indications that modification of uridine may be especially significant and, therefore, additional studies of U-modifications are warranted.

1.5 Influence of modified nucleosides on RNA sensors PKR and OAS

In addition to RNA receptors that initiate signaling cascades leading to new transcription of pro-inflammatory proteins, there exist RNA sensing pathways that result in more immediate effector functions without requiring new transcription. The best characterized of these effector-type RNA sensors are RNA-activated protein kinase (PKR) and oligoadenylate synthetase (OAS).

PKR was classically characterized as an anti-viral protein that is activated by binding to long, perfectly double-stranded RNA. Binding to dsRNA allows activation of PKR by dimerization and autophosphorylation. The primary substrate of activated PKR is the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). Phosphorylation of eIF2α reduces functional translation initiation complexes and, therefore, globally inhibits translation in an affected cell [88]. In addition to long, perfect dsRNA, subsequent studies have demonstrated activation of PKR by numerous RNA ligands, provided that they contain some feature with RNA secondary structure [18, 21, 57, 121, 188, 244, 280]. Studies using poly(I:C) demonstrated that the presence of Im or Cm in dsRNA reduced PKR activation [247]. When PKR was activated by short RNAs containing modified nucleosides, the effects were different in ssRNA than in dsRNA. PKR activation by short ssRNA required 5'ppp and the presence of nucleoside modifications eliminated PKR activation. In comparison, PKR activation by short dsRNA was much higher and was increased if RNA contained m^5U or m^6A, reduced by the incorporation of Ψ, and eliminated by the presence of s^2U or s^4U [177].

OAS is not a single protein, but instead represents a small family of 8–10 related proteins arising from gene duplication and alternative splicing. Similar to PKR, OAS was

originally characterized as an anti-viral protein activated by long dsRNA, but additional RNA activators have since been identified [63, 109, 167-169, 225]. Upon activation, OAS uses ATP to form unique, small 2'–5' linked oligoadenylate molecules, which are collectively called 2-5A. These 2-5A in turn activate a latent cytoplasmic endoribonuclease named RNase L. Activated RNase L cleaves ssRNA with limited specificity, including exposed loops on rRNA, cellular RNA, and exogenous RNAs, such as pathogenic or transfected RNAs [23]. Activation of OAS by poly(I:C) is reduced by the presence of Im or Cm [247]. Effects of other nucleoside modifications on activation of OAS have not been reported.

1.6 Additional RNA sensors for which the influence of modified nucleosides has not been tested

Multiple additional innate RNA-sensing pathways exist upon which the influence of modified nucleosides have not been studied. Only a brief overview of these pathways will be presented here.

General control non-derepressible-2 (GCN2) is closely related to PKR, and similarly functions to inhibit translation through phosphorylation of eIF2α. Activation of GCN2 can be induced by a wide variety of cell stresses, including nutrient deprivation and certain viral RNAs. Regardless of the initiating stress, GCN2 activation is thought to function through sensing of uncharged tRNAs. The mechanism by which GCN2 recognizes uncharged tRNAs is not well understood [64]. Because tRNA contains more modified nucleosides than any other RNA fraction, it would be interesting to study how modified nucleosides affect activation of GCN2.

In addition to TLR and RLR, a third family of signaling receptors that result in pro-inflammatory cytokine production are the nucleotide-binding domain (NBD)– and leucine-rich-region (LRR)–containing receptors (NLRs). Among these, it was recently demonstrated that in addition to other ligands, viral and ssRNA activate nucleotide-binding oligomerization domain 2 (Nod2), resulting in IFN production [214]. Similarly, bacterial RNA was discovered as an activating ligand for NACHT, leucine rich repeat and PYD containing 3 (Nalp3) [128].

RNA-specific adenosine deaminase (ADAR) converts adenosine to inosine in dsRNA. This can be an anti-viral response, permitting RNA cleavage by I-RNase. However, RNA editing by ADAR also has important cellular functions [248]. Similarly, the apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC) family are RNA editing proteins which deaminate C to U in RNA and DNA in response to various cellular and viral stimuli [201].

It was recently discovered that leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) is activated by both dsRNA and dsDNA, resulting in IFN-β production through IRF3. This activity allows LRRFIP1 to contribute to IFN-β production by macrophages in response to vesicular stomatitis virus (VSV) and the intracellular bacterial pathogen *Listeria monocytogenes* [269].

The high-mobility group box (HMGB) proteins have also been demonstrated to possess RNA- and DNA-binding characteristics. Rather than directly initiating a signaling pathway themselves, it is proposed that they facilitate activation of other RNA receptors, including TLR and possibly RLR [268].

1.7 Translation of mRNA containing modified nucleosides

With the exception of 5'-cap methylation, the most commonly found modification in mRNA is m^6A [27], although there have been limited reports of mRNAs containing m^5C [72, 73, 234] and a unique mRNA that contains Ψ [278]. Modified nucleosides can be experimentally incorporated into mRNA during *in vitro* transcription, and this approach has been used to study how translation is influenced by modified nucleosides in mRNA. In cell culture, no translation of the transfected mRNA occurred if mRNA contained complete replacement of U with s^2U or of A with m^6A, although if mRNA contained 5% m^6A, which is similar to the naturally-occurring rate, translation was equivalent to unmodified mRNA. In contrast, translation was enhanced if mRNA contained Ψ or m^5C. Enhanced translation of Ψ-containing RNA was also observed following RNA delivery to mice. When translation of Ψ-containing mRNA was examined in lysate systems, enhanced translation was observed in rabbit reticulocyte lysate, but translation was reduced in wheat-germ extract and eliminated in *E. coli* lysate (Figure 1-5) [131]. These data suggest that the mechanism for enhanced translation of Ψ-containing mRNA requires factors found in higher eukaryotes but absent in plants and prokaryotes.

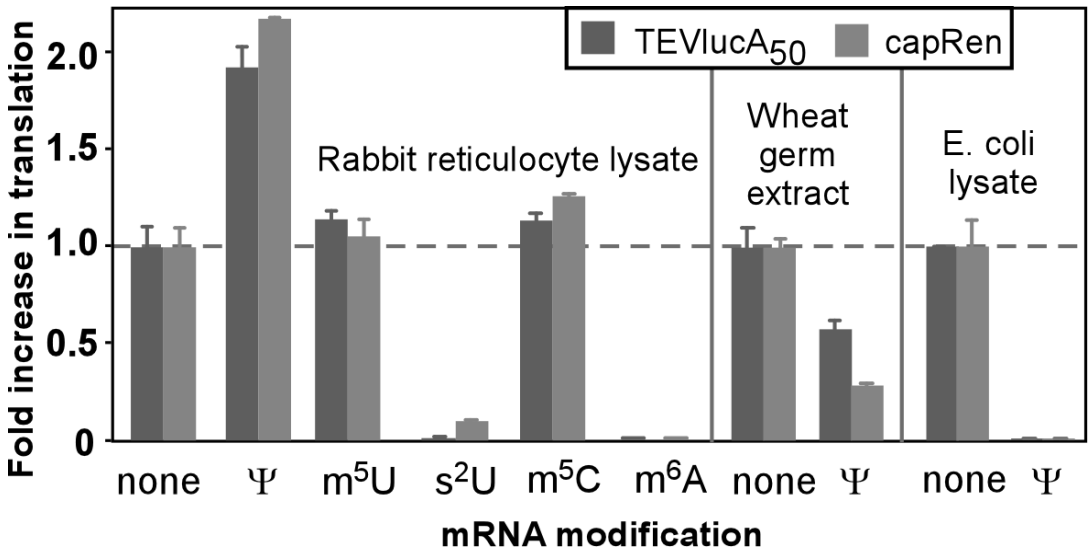


Figure 1-5. *In vitro* translation of nucleoside-modified mRNAs
Rabbit reticulocyte lysate, wheat germ extract, and *Escherichia coli* S30 lysate were incubated in the presence of 50 ng/µl mRNA encoding firefly luciferase ($TEVlucA_{50}$) or *Renilla* luciferase (capRen). The mRNAs contained the indicated nucleoside modifications. Fold increase in translation was calculated by normalizing the measured relative light units to those obtained with non-modified mRNA. Error bars indicate SEM (n = 4), and the dotted line represents the relative value obtained with unmodified mRNA in each of the lysates. Adapted by permission from Macmillan Publishers Ltd: Molecular Therapy 2008 Nov;16(11):1833-40, copyright 2008.

The direct influence of U-modifications on the translation apparatus has been examined by assessing scanning-dependent translation initiation and elongation in rabbit reticulocyte lysates. The presence of Ψ reduced the efficiency of both initiation complex formation and processive translation elongation. Initiation was reduced by m^5U, but elongation was unaffected. The presence of s^4U, in contrast, substantially increased initiation but was not permissive for elongation. Additionally, s^4U was permissive for leaky scanning and initiation, while Ψ was not [7].

The only RNA modification tested in both studies was Ψ, which despite being used less efficiency by the translational apparatus resulted in enhanced net translation of the encoded reporter protein. These data therefore suggest that RNA containing Ψ has additional effects on translation that do not result from direct impacts on the translational apparatus.

1.8 Nuclease resistance of RNA containing modified nucleosides

In the human genome, hundreds of genes encode products involved in nuclease digestion of RNA. Evolutionary studies suggest that the eight vertebrate-specific RNases may have evolved from a host-defense RNase [235]. Indeed, numerous RNases have primary or secondary defense functions, including I-RNase, RNase L, RNases 1–8, and dicer. Together, these facts point to evolutionary pressure to control exogenous RNA as a self-defense mechanism. Additionally, multiple pathways exist for degradation of mRNA, which is important to control the quality, quantity, and timing of gene expression [16].

Altered endonuclease cleavage has been reported for RNA containing nucleoside modifications. RNA containing Nm are more stable in serum [15], suggesting that they are resistant to cleavage by serum nucleases, which are predominantly A-type RNases [235]. Although pancreatic diesterase and snake venom phosphodiesterase do cleave Ψ-containing RNA, there is some indication that they may do so with reduced efficiency [180]. However, the presence of Ψ has not been shown to prevent cleavage by the nucleases RNase A, RNase H [279], RNase T1, RNase T2, or nuclease P1. RNA containing Um is not bound or cleaved by RNase L [250].

1.9 Aims and organization of dissertation

In this dissertation, I seek to identify how nucleoside modifications alter the activity of RNA sensors and effectors, specifically PKR, OAS, and RNase L. These studies will further our understanding of the specificity of recognition and activity of these receptors/enzymes and the mechanisms used by the host to identify pathogenic RNA and differentiate it from self RNA. In doing so, these studies will also contribute to a deeper understanding of how nucleoside modifications alter the translation and stability of exogenously delivered *in vitro* transcribed mRNA. **The primary hypothesis of this dissertation is that nucleoside modifications inhibit activation of intracellular RNA sensors PKR and OAS and the effector function of RNase L. A secondary hypothesis is that modification of RNA can be employed to reduce immune activation and increase the translation and stability of exogenously delivered mRNA.**

The results of experiments testing these hypotheses are presented in three chapters. Each experimentation chapter contains the individually relevant background, detailed methods, results, and conclusions. Following the results chapters, the final chapter of this dissertation will discuss the overall conclusions and implications of this dissertation, as well as future directions and applications of this work.

The first results chapter, Chapter 2, focuses on PKR. Nucleoside modifications have been shown to influence the translation of *in vitro* transcribed mRNA and also the activation of RNA sensors, including TLR and RLR. Therefore, we postulated that nucleoside modifications influence translation through PKR, a RNA sensor that inhibits translation following activation. We hypothesized that unmodified *in vitro* transcribed mRNA activates PKR, resulting in global inhibition of translation, but that mRNA

containing modified nucleosides does not activate PKR, and therefore translation continues uninhibited. We tested the *in vitro* activation of purified PKR by mRNA, as well as examining PKR and eIF2α phosphorylation induced in cell culture following mRNA transfection. Translation of modified and unmodified mRNA was assessed in the absence of PKR activity using PKR inhibitors and $PKR^{-/-}$ MEF cells. *In vitro* PKR activation and immunoprecipitation from cell lysates were used to investigate the mechanism by which modified RNA influences PKR activation. The results of these experiments have been published [3], and the information contained in Chapter 2 is an adaptation of that publication.

Chapter 3 presents experiments addressing OAS and RNase L, which comprise the protein components of the 2-5A system. Activation of the 2-5A system can influence both translation and stability of RNA. Because both OAS and RNase L are RNA interacting proteins, nucleoside modifications in RNA could influence the 2-5A system in multiple ways. We hypothesized that unmodified *in vitro* transcribed mRNA activates OAS and is cleaved by activated RNase L, but that nucleoside modifications in RNA reduce OAS activation and RNase L mediated RNA cleavage. Purified proteins were used for *in vitro* assays to examine OAS activation by mRNA and mRNA cleavage by RNase L. Using northern blotting, the half-life of mRNA was assessed following incubation in RRL, transfection in cell culture, and injection into mice. Luciferase enzyme activity and northern blots were used to assess translation and retention of reporter mRNA in RNase $L^{-/-}$ MEF cell culture and RNase $L^{-/-}$ mice. The degradation of rRNA following mRNA transfection was evaluated to address the mechanism by which the 2-5A system affects translation following exogenous delivery of mRNA. Chapter 3 is

based upon a manuscript that has been prepared for publication and will be submitted for publication following review by all co-authors.

The experiments in Chapter 4 study the impact of nucleofection on translation. Although commonly used, lipid-based transfection reagents vary in effectiveness depending on cell type and can be toxic to cells. Nucleofection is a popular and effective alternative delivery method based on electroporation using cell type-specific buffers and electrical parameters. While using nucleofection for mRNA delivery, we observed that nucleofection induced phosphorylation of eIF2α. Using western blotting, we examined nucleofection-induced eIF2α phosphorylation in cells deficient for individual eIF2α kinases PKR, general control non-derepressible 2 (GCN2), and PKR-like endoplasmic reticulum kinase (PERK). The impact of other transfection reagents on eIF2α phosphorylation is also presented.

Prior to investigating the influence of modified nucleosides on cytoplasmic RNA sensors, I performed studies on a conditionally-replicating RNA vaccine strategy based on transduction of dendritic cells with a lentiviral vector. Because these experiments are outside of the scope of this dissertation, they will not be discussed further in the body of this dissertation. This work was published [263] and is included herein as Appendix A.

CHAPTER 2

Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation

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2.1 Introduction

In vitro transcribed mRNA has many advantages as a vehicle for gene delivery. Transfection of mRNA is very efficient [262] and rapid expression of the encoded protein can be achieved. In addition, unlike viral vectors or plasmid DNA, cell-delivered mRNA does not introduce the risk of insertional mutagenesis [70, 185]. Previous studies have shown that mRNA can activate a number of innate immune receptors, including TLR3, TLR7, TLR8, and RIG-I. However, activation of these receptors can be avoided by incorporating modified nucleosides, e.g., Ψ, s^2U, and others into the RNA [115, 130].

PKR is a ubiquitous mammalian enzyme with a variety of cellular functions, including regulation of translation during conditions of cell stress. During viral infection, PKR binds viral dsRNA, autophosphorylates, and subsequently phosphorylates eIF2α, thus repressing translation [77, 120]. Originally, potent activation of PKR was thought to require >30 base pair-long dsRNA [167]. It has subsequently been shown that PKR can be activated by a variety of RNA structures that include ssRNA containing hairpins structures [21, 121], imperfect dsRNA containing mismatches [21], short dsRNA with single-stranded tails [280], stem-loop structures with 5'-triphosphates [56, 178], and unique elements present in interferon gamma and TNF-alpha mRNAs [125]. Viral [76, 225] and cellular RNAs [18, 57, 188, 244] transcribed as single-stranded RNA but containing secondary structure can also be potent PKR activators. PKR activation by short dsRNA, such as siRNA, has also been demonstrated [5, 93, 203, 222, 232, 277]. These reports indicate that a wide variety of RNA structures can activate PKR, provided they contain some dsRNA element. Modified nucleosides present in homopolymeric RNAs [49, 166, 246, 247] or in short transcripts [177, 202, 203] can influence activation of PKR. However, it has not been investigated whether

modified nucleosides present in long, protein-encoding mRNAs impact activation of PKR.

Previously, we demonstrated that *in vitro* transcribed mRNAs containing Ψ and m^5C are translated at significantly higher levels than those containing unmodified nucleosides [131]. However, the molecular mechanism underlying this enhancement has not been identified. Here, we show that one cause of this translational difference is that Ψ and m^5C-containing mRNA activates PKR less efficiently than uridine-containing mRNA. This reduced PKR activation also mitigates general translational inhibition of cellular proteins that is induced when unmodified *in vitro* transcribed mRNAs are delivered to cells.

2.2 Materials and methods

Cells and reagents

Human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), and 10% fetal calf serum (HyClone). Immortalized wild-type (WT) and PKR knockout ($PKR^{-/-}$) mouse embryonic fibroblasts (MEFs) were generously provided by Robert Silverman (Cleveland Clinic Foundation) and were maintained in RPMI medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum. Polyinosinic:polycytidylic acid (poly(I:C)), yeast tRNA, and human poly$(A)^+$ RNA were purchased from Sigma and polydeoxycytidylic acid (poly(dC)) was purchased from Midland Certified Reagent Co.

mRNA synthesis

Reporter plasmids encoding firefly luciferase (pT7TS-fLuc and pTEVluc) or *Renilla* luciferase (pT7TS-Ren) were linearized with SalI/BamHI to generate templates. Transcriptions were performed at 37°C for 3 hours using T7 RNA polymerase and nucleotide triphosphates at 7.5 mmol/l final concentration (MEGAscript kit; Ambion). Except where otherwise specified, capped mRNA was generated by performing transcription in the presence of 6 mmol/L cap analog 3'-O-Me-m7G(5')ppp(5')G (New England Biolabs) and lowering the concentration of guanosine triphosphate (3.75 mmol/l). All mRNAs were transcribed to contain 30 or 50 nt-long 3' poly(A) tails. Selected mRNAs were further poly(A)-tailed in a reaction of ~1.5 µg/µl RNA, 5 mmol/l

adenosine triphosphate, and 60 U/µl yeast poly(A) polymerase (USB) and incubated at 30°C for 3 hours according to the manufacturer's instructions. The length of poly(A) tails were estimated to be ~200-nt long and is indicated with A_n. Triphosphate-derivatives of Ψ, s^2U, m^5C, m^6A, and m^5U (TriLink) were used in place of their cognate unmodified NTP to generate modified nucleoside-containing RNA. Following transcription, the template plasmids were digested with Turbo DNase and RNAs were precipitated with 2.5 M lithium chloride at –20°C for 4 h. RNAs were pelleted by centrifugation, washed with 75% ethanol and then reconstituted in nuclease-free water. The concentration of RNA was determined by measuring the optical density at 260 nm. All RNA samples were analyzed by denaturing agarose gel electrophoresis for quality assurance. Each RNA type was synthesized in 4–10 independently performed transcription experiments and all experiments were performed with at least two different batches of mRNA. Enzymatic capping was performed using ScriptCap m^7G capping kit (Epicentre) on mRNA transcribed with guanosine 5'-[γ-^{32}P]-triphosphate (GE Healthcare). Efficiency of capping was verified by monitoring the elimination of γ-^{32}P from the mRNA. Biotinylated mRNA was transcribed with the addition of 1:5 biotinylated CTP (Roche Applied Sciences) in the transcription reaction.

Detection of reporter proteins in RNA-transfected cells

Cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well one day prior to transfection. RNA was complexed with lipofectin (Invitrogen) according to the method of [130], as follows. Potassium phosphate buffer was prepared and supplemented 1 µg/µl bovine serum albumin (BSA; Sigma). RNA was diluted in cold serum-free DMEM

to 0.07 μg/μl. Stock phosphate buffer was added to give final concentrations of 20 mM potassium phosphate, pH 6.4, and 100 ng/ml BSA. For 3 wells, lipofectin complexed RNA was prepared in the following ratios: 2.4 μl (2.4 μg) was added to 21.3 μl diluted phosphate buffer, and then incubated at room temperature for 10 minutes. Then 9.9 μl nucleic acid (0.69 μg) was added and the mixture was incubated for 10 additional minutes at room temperature. Lastly, 116.4 μl serum-free DMEM was added to bring up the final volume to 150 μl. The mixture was vortexed and 48 μl of it (0.25 μg RNA/well) was added directly cells plated in 96-well plates. Transfected cells were incubated for 1 h at 37ºC in a 5% CO_2 incubator. The lipofectin-RNA mixture was removed and replaced with 200 μl pre-warmed DMEM containing 10% FCS, and cells were further cultured at 37ºC until lysis. Cells were lysed in 25 μl firefly, *Renilla*, or dual-luciferase specific lysis reagents (Promega). Aliquots of 2 μl were assayed with the corresponding enzyme substrates and a LUMAT LB 950 luminometer (Berthold) at a 10-second measuring time.

Assessment of total protein synthesis

HEK293T cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well with 1000 U/mL interferon-αA/D (Sigma) one day prior to transfection. Cells were incubated in methionine/cysteine-free medium (Invitrogen) for 1 hour, then pulsed with complete medium supplemented with ^{35}S-methionine/cysteine (140 mCi/mL) (PerkinElmer) for 1–3 hours. Cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma). Lysate was diluted in 0.1% BSA, and macromolecules were precipitated by the addition of trichloroacetic acid (TCA) and 30

minutes incubation on ice. Precipitates were filtered onto glass microfiber filters (Whatman) and washed with 10% TCA and 100% ethanol. Incorporated ^{35}S-methionine/cysteine was quantified using Ecolite(+) scintillation cocktail (MP Biomedicals) and a Beckman LS 6000IC scintillation counter.

PKR activation *in vitro*

Recombinant human PKR containing a $(His)_6$ tag [280] was expressed in *E. coli* strain BL21(DE3) grown in LB media. PKR was purified from *E. coli* lysate by passing lysate over a Ni-NTA-agarose FPLC column. Immediately prior to use, purified PKR was dephosphorylated by incubating 5.33 μM PKR with 1280 units lambda protein phosphatase (New England Biolabs) for 60 minutes at 30ºC, then stopping phosphatase activity by the addition of 1 mM sodium orthovanadate (MP Biomedicals). Final concentrations of 0.75 μM dephosphorylated PKR, 0.1 mM ATP, and 0.15 μCi/μL adenosine 5'-[γ-^{32}P]-triphosphate (γ-^{32}P-ATP) (PerkinElmer) were mixed with the indicated concentration of RNA for 10 minutes at 30°C in a buffer consisting of 4 mM $MgCl_2$, 100 mM KCl, and 20 mM HEPES, pH 7.5. The reaction was stopped by the addition of NuPage LDS sample buffer and reducing agent (Invitrogen) and heating for 10 minutes at 70°C. Unincorporated γ-^{32}P-ATP was separated from radiolabeled PKR by running samples on a 12% SDS-PAGE gel. Phosphorylated PKR was imaged in dried gels using a phosphor storage screen (Molecular Dynamics) and detected using Storm or Typhoon Phosphorimagers (GE Healthcare). Band densities were quantified using ImageQuant software (GE Healthcare).

Western blotting

HEK293T cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well, with 1000 U/mL interferon-αA/D one day prior to transfection. At the indicated time following RNA transfection, cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail and HALT phosphatase inhibitor (Pierce). Equal mass of protein (10–30 µg per sample) was loaded onto a 12% SDS-PAGE gel. Proteins were subsequently transferred to a Hybond-P PVDF membrane (GE Amersham), blocked with 2.5% non-fat milk in TBS containing 0.05% Tween 20, and probed with antibodies for PKR-pT446 and PKR (Epitomics), eIF2α-pS51 and eIF2α (Cell Signaling Technologies), or PABP (Abcam). Membranes were stripped by agitating gently in a buffer of 2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris pH 6.7 for 30 minutes at 50°C, then subsequently re-blocked and re-probed. Image was captured using the Fujifilm LAS1000 digital imaging system. Linear brightness and contrast were adjusted using GIMP 2.6 software.

Biotinylated RNA pull down

HEK293T cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well one day prior to transfection. Where indicated, cells were incubated in methionine/cysteine-free medium (Invitrogen) for 30 minutes, then pulsed with complete medium supplemented with ^{35}S-methionine/cysteine (140 mCi/mL) (PerkinElmer) for 3.5 hours prior to lysis. HEK293T cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail and RNase inhibitor (RNasin, Promega). Biotinylated mRNA

(0.5–2 µg) was added to 25 µL lysate and incubated on icœ for 2 hours. Subsequently, 50 µL of streptavidin-agarose bead 50% slurry (Invitrogen) was added and incubated on ice for 1 hour. Beads with bound RNA and proteins were centrifuged and washed, and proteins were released from RNA by heating samples at 70°C for 10 minutes in the presence of NuPage LDS sample buffer and reducing agent. Samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. PKR and poly(A)-binding protein (PABP) were detected by western blotting.

Statistical analysis

All data are reported as mean ± standard error of the mean (SEM). Statistical differences between treatment groups were calculated by the Student's *t*-test using Microsoft Excel. For all statistical testing, a P-value <0.05 was considered significant.

2.3 Results

Conventional *in vitro* transcribed mRNA induces translational repression

We previously observed that mRNA transcribed *in vitro* containing Ψ in place of uridine or m^5C in place of cytidine is translated more efficiently than mRNA containing unmodified nucleosides [131]. In order to determine whether the translational enhancement exerted by Ψ incorporated into RNA is restricted to the modified transcript or also extends to unmodified transcripts, we performed co-transfection experiments delivering equal amounts of *Renilla* and firefly luciferase-encoding mRNAs to cells. As expected, the mRNAs were translated much more efficiently when both contained Ψ as compared to when both were unmodified (Figure 2-1A). However, when only one of the mRNAs contained Ψ modification, the translation level of the Ψ-containing RNA decreased (~50%) relative to the level measured when both contained Ψ. One explanation for these findings could be that unmodified RNA inhibits the translation of the co-delivered RNA, while Ψ-containing RNA has no such inhibitory effect. To explore whether translation of endogenous cellular mRNAs are similarly influenced by exogenously delivered *in vitro* transcribed mRNAs, total cellular protein synthesis was monitored in cells transfected with mRNA containing Ψ modification or no modification. Both types of mRNA reduced cellular protein translation; however, the suppression of protein synthesis was greater with unmodified RNA than with Ψ-containing RNA (Figure 2-1B). PKR-activating poly(I:C) and non-activating poly(dC) were used as controls. Mock transfected cells were treated with the transfection reagent (lipofectin) only, without nucleic acid.

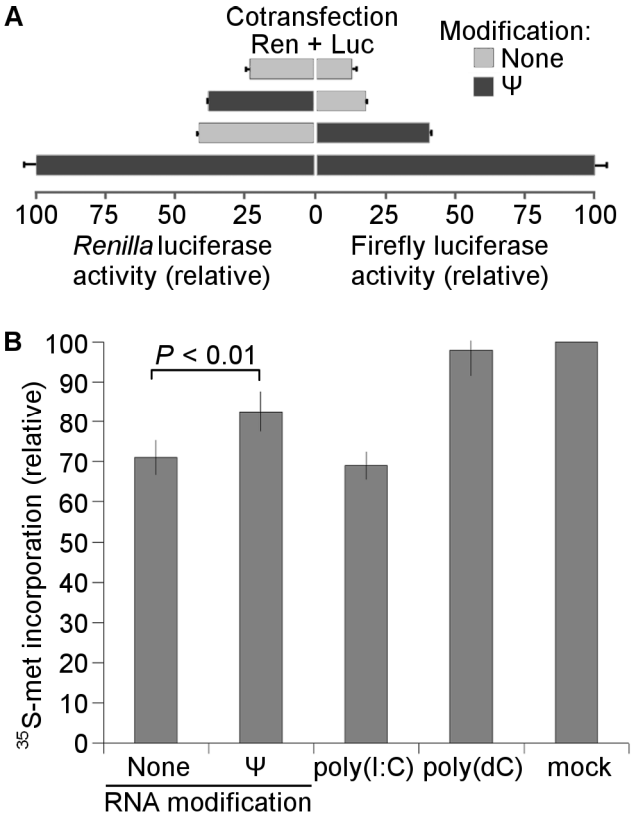

Figure 2-1. Translational inhibition by unmodified *in vitro* transcribed mRNA
(**A**) *In vitro* transcribed mRNAs encoding *Renilla* luciferase (Ren) and firefly luciferase (Luc) were synthesized with and without Ψ modifications then mixed (1:1 mass ratio) as indicated. The mixed mRNA was complexed with lipofectin and added to HEK293T cells seeded in 96-well plates (0.25 µg RNA/well). Cells were lysed 4 h after transfection and dual luciferase measurements were performed in aliquots (1/20th) of the lysates. Values presented are normalized to cells transfected with Ren and Luc mRNAs when both contained Ψ modifications. Error bars indicate the standard error of $n = 3$ samples. (**B**) Unmodified or Ψ-containing RNA was complexed with lipofectin and delivered to HEK293T cells. Cells were subsequently incubated with ^{35}S-methionine/cysteine supplemented medium, lysed, and proteins were TCA precipitated. Data are presented as percentage of counts obtained from mock transfected cells. Data shown are mean values from three independent experiments ± SEM.

Conventional *in vitro* transcribed mRNA activates PKR

To determine whether the inhibition of translation by unmodified mRNA is mediated by PKR, *in vitro* transcribed mRNAs were first analyzed in a cell-free system

using purified PKR. Four different mRNAs were tested: unmodified and Ψ-modified mRNA, each with either a cap or a triphosphate at their 5' end (5'ppp). *In vitro* transcribed mRNA with 5'ppp and containing uridines activated PKR to a greater extent than those containing Ψ (Figure 2-2A). Neither UTP nor ΨTP alone activated PKR (Figure 2-2B). This reduced activation of PKR by Ψ-containing transcripts is consistent with the previously observed enhancement of *in vitro* translation from Ψ-containing RNA in rabbit reticulocyte lysates [131]. Since the presence of 5'ppp on short RNAs has previously been shown to enhance the activation of PKR [56, 178], it was important to determine whether the 5'ppp present on long mRNAs also contributed to PKR activation. To remove 5'ppp, *in vitro* transcripts were capped enzymatically (Figure 2-2C), which completely removed the 5'ppp, and then tested. As Figure 2-2A demonstrates, the presence or absence of 5'ppp on unmodified and Ψ-modified transcripts did not significantly alter their ability to activate PKR. It has been shown that a variety of nucleoside modifications in RNA can influence the activation of RNA sensors [115, 130, 177]; therefore, the effect of incorporating the modified nucleosides s^2U, m^5C, m^6A, or m^5U into mRNA was also analyzed. The mRNA containing s^2U, m^5C, or m^6A activated PKR to a lesser extent than unmodified RNA, while RNA with m^5U activated PKR to the greatest extent (Figure 2-2D and E). For comparison, PKR activation by natural RNAs was also tested. Like *in vitro* transcribed mRNA, natural mRNA activated PKR, and this activation was higher than PKR activation induced by Ψ-containing *in vitro* transcribed mRNA. In contrast, natural tRNA did not activate PKR (Figure 2-2F).

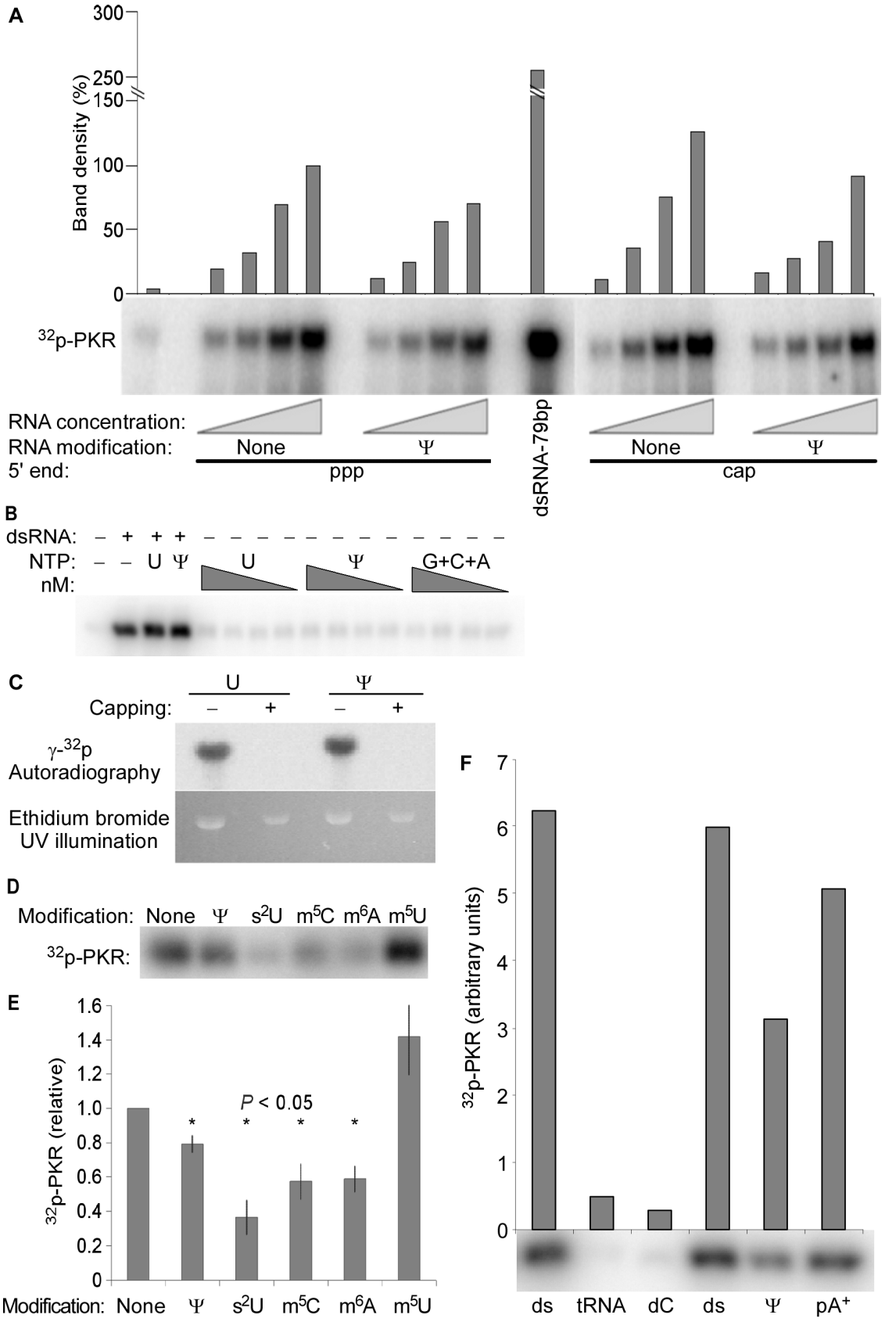
A
Band density (%)
300
250
150
100
50
0
32p-PKR
RNA concentration:
RNA modification:
5' end:
None
Ψ
ppp
dsRNA-79bp
None
Ψ
cap
B
dsRNA:
NTP:
nM:
U
Ψ
G+C+A
C
U
Ψ
Capping:
γ-32p
Autoradiography
Ethidium bromide
UV illumination
D
Modification: None Ψ s2U m5C m6A m5U
32p-PKR:
E
32p-PKR (relative)
P < 0.05
Modification: None Ψ s2U m5C m6A m5U
F
32p-PKR (arbitrary units)
ds
tRNA
dC
ds
Ψ
pA+

Figure 2-2. Activation of purified PKR by *in vitro* transcribed RNA
Purified PKR was incubated with γ-^{32}P-ATP and *in vitro* transcribed mRNA for 10 min. Reaction products were separated by SDS-PAGE and imaged using phosphor storage radiography. (**A**) Unmodified or Ψ-containing mRNAs encoding firefly luciferase contained triphosphates (ppp) or cap at their 5' ends were analyzed. Complete capping of RNA was achieved post-transcriptionally using vaccinia capping enzyme. Concentration of mRNA in reactions was 3.1, 6.2, 12.5, and 25 μg/mL. No RNA (–) and 79 bp dsRNA were used as negative and positive controls. Quantified phosphorylation is presented as a bar graph above each band. Values were normalized to those obtained with 25 μg/mL uncapped, unmodified RNA. (**B**) Purified PKR was mixed with γ-^{32}P-ATP and the indicated nucleotide triphosphates at 200, 20, 2, and 0.2 nM concentrations. Reaction products were separated by SDS-PAGE and imaged using phosphor storage radiography. No RNA and 200bp dsRNA were used as negative and positive controls, respectively. (**C**) RNA transcribed in the presence of γ-^{32}P-GTP was capped enzymatically, separated on a denaturing agarose gel, stained with ethidium bromide and UV illuminated. RNA was then transferred to a nylon membrane and exposed to film. (**D**) Purified PKR was mixed with γ-^{32}P-ATP and *in vitro* transcribed firefly luciferase mRNA that contained the indicated modified nucleosides. Reaction products were separated by SDS-PAGE and imaged using phosphor storage radiography. A representative of three independent experiments is shown. (**E**) Quantification of PKR activation by mRNA containing modified nucleosides. Data represented as mean value ± SEM from five independent experiments using 5–25 ng/μL RNA, normalized to PKR activation by unmodified RNA. Asterisks indicate P-values <0.05 calculated by two-tailed Student's *t*-test. (**F**) Purified PKR was activated using 125 μg/mL yeast tRNA, 25 μg/mL Ψ-containing *in vitro* transcribed mRNA, or 25 μg/mL human poly(A)$^+$ RNA. Poly(dC) and 200 bp dsRNA (ds) were used as negative and positive controls. Quantified phosphorylation is presented as a bar graph above each band.

Pseudouridine-containing mRNA does not activate PKR in cells

Next, we investigated the impact of Ψ-containing mRNA on PKR activation in the complex cellular environment. Following control studies demonstrating that RNAs with or without nucleoside modification can be delivered to cells with the same efficiency (data not shown), unmodified or Ψ-containing mRNA was complexed with lipofectin and delivered into HEK293T cells. PKR activation was assessed by western blot using an antibody specific for PKR phosphorylated on Thr446, a site at which phosphorylation is requisite for PKR activation [209]. Consistent with the results observed

using purified PKR, transfection of unmodified transcript induced PKR phosphorylation, which was dramatically reduced if the transfected RNA contained Ψ (Figure 2-3A). Similarly, incorporation of s^2U or m^5C into RNA reduced the level of PKR phosphorylation relative to that induced by unmodified RNA, while m^5U incorporation into RNA enhanced PKR phosphorylation (Figure 2-3B). Incorporation of m^6A into RNA also enhanced PKR phosphorylation in cells, despite reducing PKR activation *in vitro*.

Phosphorylation of eIF2α, a substrate of PKR, was induced in HEK293T cells by transfection with unmodified RNA but not with Ψ-containing RNA (Figure 2-3C). Incorporation of modified nucleosides other than Ψ into mRNA altered the phosphorylation of eIF2α in direct parallel to their alterations of PKR phosphorylation (Figure 2-3D). To exclude the possibility that ΨTP either free or complexed with lipofectin induced PKR or eIF2α phosphorylation, cells were treated with UTP or ΨTP and western blotted. Neither UTP nor ΨTP induced PKR or eIF2α phosphorylation in cells (Figure 2-3E).

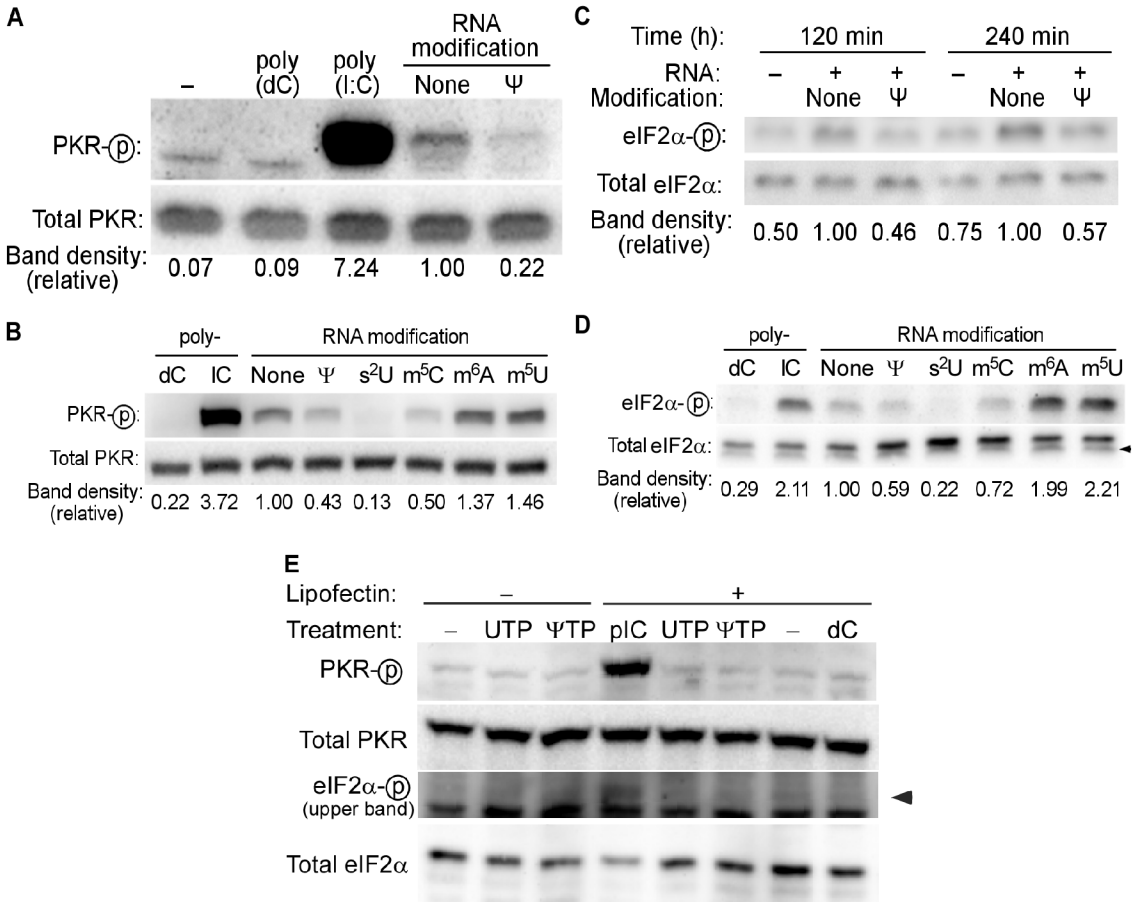

Figure 2-3. PKR activation in cells by *in vitro* transcribed mRNA
In vitro transcribed firefly luciferase mRNA incorporating the indicated modified nucleoside (A–D) or UTP and ΨTP (E) were complexed with lipofectin and delivered to HEK293T cells. Following cell lysis at 4 hours after transfection, proteins were separated by SDS-PAGE, and phosphorylation of PKR (**A, B** and **E**) and eIF2α (**C, D** and **E**) was assessed by western blotting. No RNA (–), poly(dC), and poly(I:C) were used as controls. Relative phosphorylation is indicated below each gel lane, calculated as phosphorylated band density divided by total band density and then normalized to the phosphorylation induced by unmodified RNA. Arrowhead in (D) indicates the eIF2α band below a heavier non-specific band and arrowhead in (E) indicates the phospho-eIF2α band above a heavier non-specific band. Representative images of at least three independent experiments are shown.

Translation of unmodified mRNA is enhanced upon inhibiting or eliminating PKR

Viral proteins C8L of swinepox and K3L of vaccinia are inhibitors of PKR and have been shown to reverse PKR-mediated inhibition of translation in mammalian cells

[133]. Thus, to confirm the role of PKR in the translational differences observed between uridine- and Ψ-containing transcripts, we utilized C8L, K3L, and two K3L mutants: hyperactive K3L-H47R and inactive K3L-Y76A [133, 134]. Based on the premise that PKR is activated by *in vitro* transcribed mRNAs that contain uridine but not by those with Ψ, inhibition of PKR would be expected to increase the translation of unmodified mRNA but have no effect on the translation of Ψ-containing RNA. Indeed, in the presence of PKR inhibitors, the amount of translation increased from unmodified transcripts but not from Ψ-modified transcripts (Figure 2-4A).

Further evidence confirming the role of PKR in suppressing translation of unmodified mRNAs was obtained using mouse embryonic fibroblasts (MEFs) derived from PKR-knockout animals. In wild-type MEFs, translation of Ψ-containing transcripts was 4–5-fold greater than that of unmodified transcripts (Figure 2-4B). In PKR-deficient MEFs, the amount of translation of Ψ-modified mRNA was not different from that of unmodified mRNA. Notably, the equivalent translation of unmodified and Ψ-containing mRNA was not due to reduced translation of Ψ-modified mRNA. In fact, when comparing raw RLU data, the translation of both unmodified and Ψ-containing mRNAs increased in $PKR^{-/-}$ cells relative to WT cells; however, the increase in translation of unmodified mRNA was disproportionately larger than the increase in translation of Ψ-modified mRNA (Figure 2-4C). Additionally, RNA transfection does not induce phosphorylation of eIF2α in PKR-deficient MEFs, as it does in WT cells (Figure 2-4D). These results demonstrate that the activity of PKR is necessary for the decreased translation of unmodified transcripts relative to Ψ-containing transcripts.

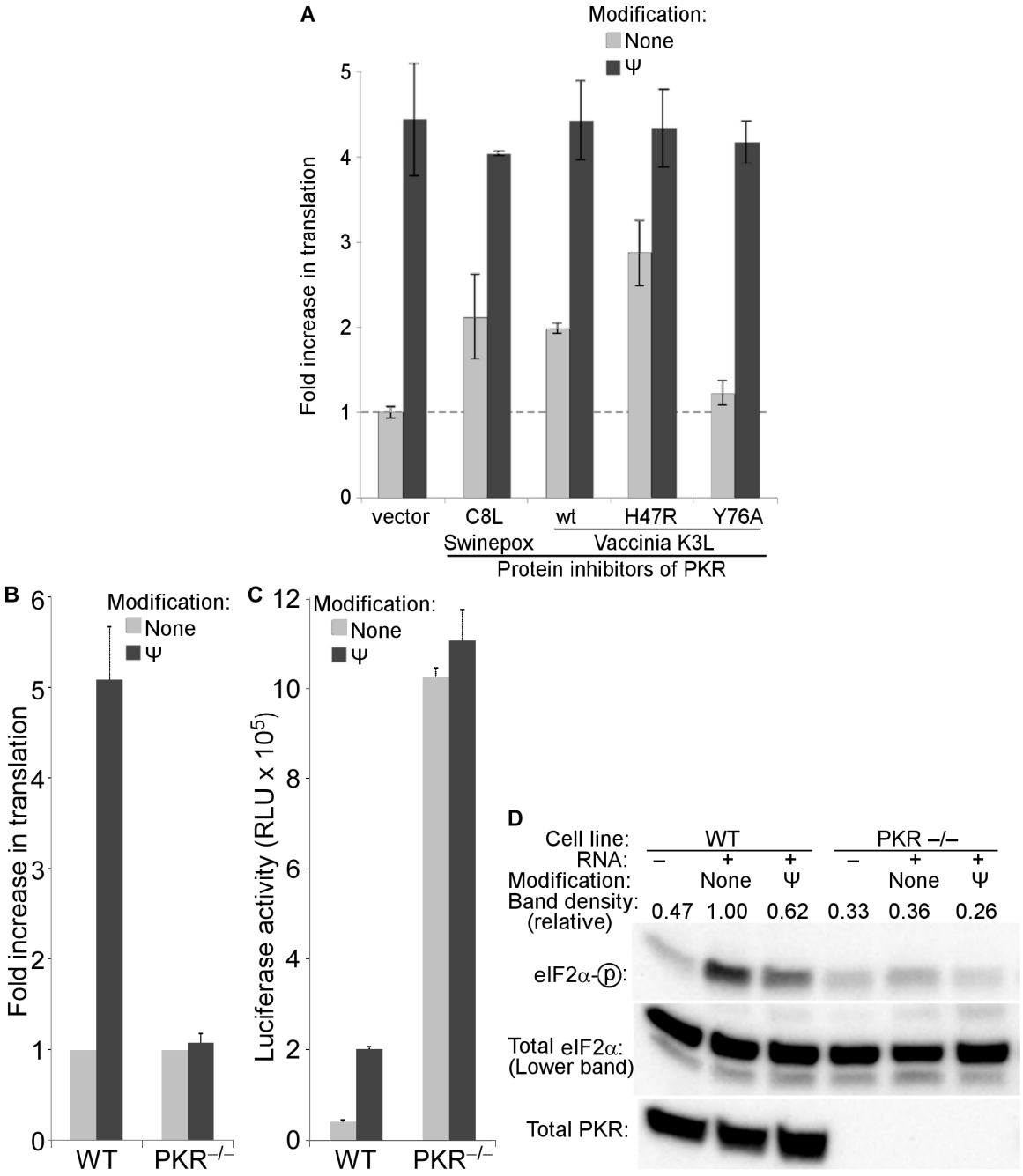

Figure 2-4. Translation of *in vitro* transcribed mRNA in the absence of PKR activity
(**A**) HEK293T cells were transfected with plasmids encoding protein inhibitors of PKR: swinepox C8L protein, wt vaccinia K3L, hyperactive K3L-H47R, inactive K3L-Y76A, or pG5 empty vector. Twenty-four hours later, unmodified or Ψ-modified *in vitro* transcribed mRNAs encoding firefly luciferase were complexed with lipofectin and delivered to cells and luciferase activity was measured 4 hours later. Data were normalized to values obtained when cells were first transfected with empty vector then

with unmodified RNA. Presented data are mean values from three replicates ± SEM. (**B** and **C**) MEF cell lines derived from wild-type (WT) or transgenic mice that do not express functional PKR ($PKR^{-/-}$) were transfected with unmodified or Ψ-containing *in vitro* transcribed mRNAs encoding firefly luciferase. Data were normalized to values obtained when cells were transfected with unmodified RNA and expressed as fold increase in translation of Ψ-containing mRNA over unmodified RNA (B) or displayed without normalization (C). Values are from three replicate wells ± SEM and are representative of at least three independently performed experiments. (**D**) WT and $PKR^{-/-}$ MEF cells were transfected with unmodified or Ψ-containing *in vitro* transcribed mRNAs encoding firefly luciferase or mock transfected with no RNA (–). Cells were lysed 2 hours following RNA transfection; proteins were then separated by SDS-PAGE and assayed for eIF2α phosphorylation by western blotting. Relative phosphorylation is indicated above each gel lane, calculated as phosphorylated band density divided by total band density and then normalized to the phosphorylation induced by unmodified RNA in wild-type cells. Absence of PKR was also confirmed by western blotting.

Pseudouridine-containing mRNA is not bound by PKR

To test whether Ψ-modified mRNA is a competitive inhibitor of PKR, a 200 bp dsRNA known to activate PKR was mixed with a 5–125-fold mass excess of Ψ-modified RNA. All concentrations of Ψ-modified RNA tested failed to inhibit the activation of PKR by the 200 bp dsRNA (Figure 2-5A). Similarly, a 125-fold mass excess of mRNA containing s^2U, m^5C, or m^6A did not inhibit PKR activation by dsRNA (Figure 2-5B). The results were the same using lower mass excess, equal mass, or equal molar mixes (data not shown), demonstrating that RNAs containing modified nucleosides are not competitive inhibitors of PKR. The lack of PKR inhibition by transcripts containing modified nucleosides suggests a lack of binding between PKR and modified RNAs. To directly test this, biotinylated transcripts having 30 nt-long poly(A) tails and containing either Ψ or uridine were mixed with HEK293T cell lysates and complexes were then precipitated using streptavidin-agarose beads. Unmodified mRNA pulled down substantially more total protein than Ψ–modified mRNA, including multiple bands that

bound to unmodified mRNA but not to Ψ-containing mRNA (Figure 2-6A). Western blots of the precipitates indicated that PKR bound to unmodified, but bound poorly to Ψ-modified RNA (Figure 2-6B), consistent with reduced activation of PKR by Ψ-containing RNA. By contrast, equal amounts of PABP were pulled down by both RNAs. These results indicate that unmodified RNA, but not Ψ-modified RNA, is bound by PKR.

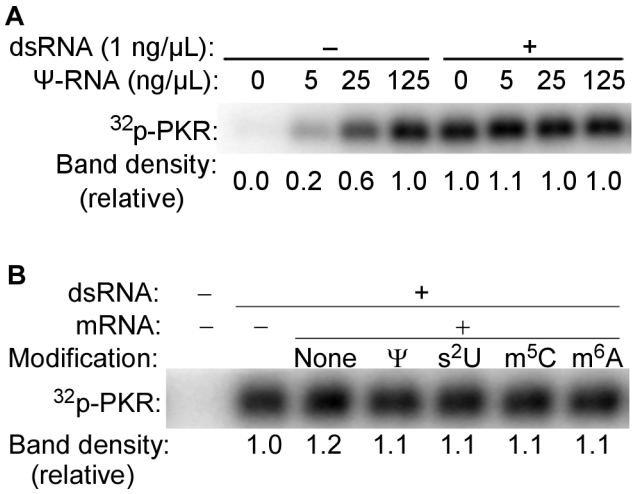

Figure 2-5. mRNA containing modified nucleosides does not inhibit PKR activation
An activating 200 bp dsRNA was mixed with a 5–125-fold mass excess of *in vitro* transcribed firefly luciferase mRNA containing Ψ (**A**) or a 125-fold mass excess of mRNA containing the indicated modified nucleoside (**B**) prior to incubation with purified PKR. Reaction products were separated by SDS-PAGE. Relative band densities are presented below each gel lane and normalized to dsRNA only. Data shown are representative of three independent experiments.

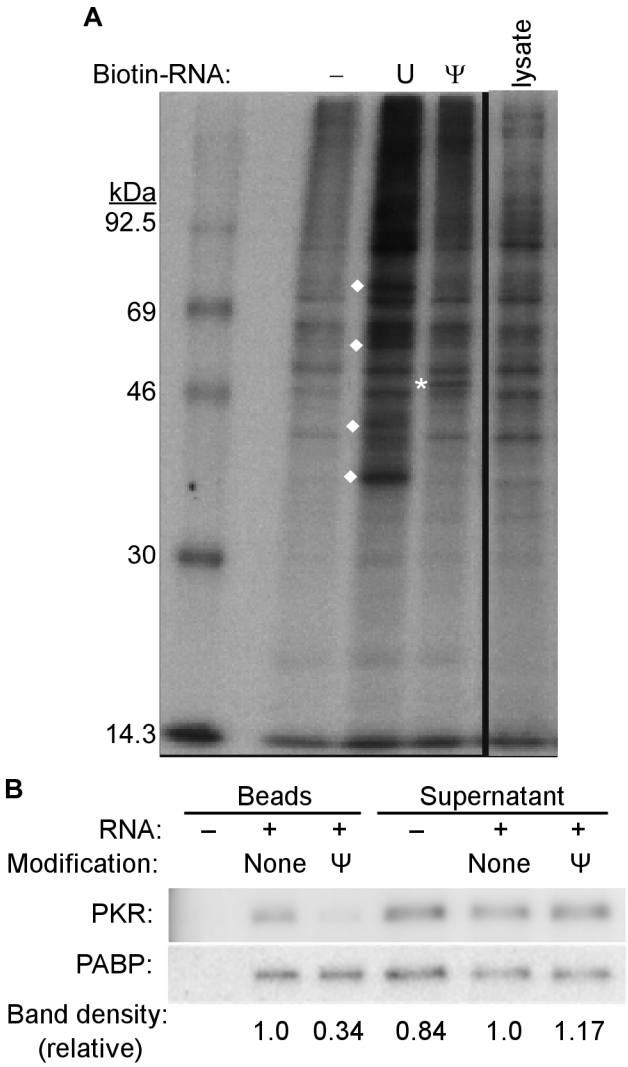

Figure 2-6. Ψ-containing mRNA does not pull down PKR
Biotinylated *in vitro* transcribed unmodified or Ψ-containing RNAs were incubated with HEK293T cell lysates for 2 hours. The RNA and bound proteins were pulled down using streptavidin-agarose beads. An aliquot of lysate that was incubated only with beads but without RNA (–) was also processed. Aliquots of pull down proteins as well as the supernatants were separated by SDS-PAGE. (**A**) HEK293T cells were pulsed with ^{35}S-methionine/cysteine for 4 hours prior to lysis. Following pull down and gel separation, ^{35}S was visualized by radiofluorography. Diamonds indicate bands specifically pulled-down by unmodified RNA. Star indicates band specifically pulled down by Ψ-modified RNA. (**B**) Pull down of PKR and PABP was detected by western blotting. Relative band densities of PKR divided by PABP compared to unmodified RNA are presented below each gel lane.

2.4 Discussion

We demonstrate that modified nucleosides in mRNA reduce PKR activation and identify a mechanism by which nucleoside modification in mRNA enhances translation of the encoded protein. Our data show that conventional *in vitro* transcribed RNA inhibits translation of reporter and cellular mRNAs, in part through the activation of PKR. However, this inhibitory activity is not induced by Ψ or m^5C-containing mRNA. Using multiple lines of investigation, our studies demonstrate that unmodified *in vitro* transcribed mRNA activates PKR, resulting in phosphorylation of eIF2α and inhibition of translation. Replacement of 5'ppp with 5'cap structure on the mRNA does not substantially alter this PKR activation. Examining translation in the context of PKR inhibitors and in PKR-deficient cells confirmed that enhanced translation of Ψ-containing mRNA is a consequence of diminished PKR activation. Mechanistically, modified nucleoside incorporation reduces RNA recognition by PKR. This is supported by data demonstrating that RNAs containing modified nucleosides do not inhibit PKR activation by dsRNA and that PKR binds poorly to Ψ-containing RNA.

PKR activation by unmodified RNA has a more pronounced impact on translation of the transfected reporter mRNA than on total cellular translation (Figure 2-1). A similar local translation effect has been observed with PKR activation by IFN-γ mRNA [18, 48]. The pronounced local inhibition is likely due to the kinetics of phosphorylation and dephosphorylation of PKR. Activated PKR most dramatically inhibits local translation because rapid dephosphorylation of PKR limits the impact on more distant translation. Therefore, translation of a PKR-activating mRNA is more severely impacted than total cellular translation. Furthermore, the observation that Ψ-containing RNA also causes

some reduction in total protein synthesis suggests that there are additional effects on cellular translation, which are not mediated by PKR.

Ψ-containing RNA activates PKR more effectively *in vitro* as compared to *in vivo* (Figs. 2-2 and 2-3). One possible reason for this difference is that PKR activation *in vivo* occurs in the presence of competing factors such as phosphatases, components of the translational system and other proteins affecting the structure and accessibility of the RNA to PKR. In contrast, *in vitro* assays lack such competing factors that would limit or reverse PKR phosphorylation.

Although mRNA is normally transcribed without a complementary antisense transcript or long stretches of self-complementarity, it contains many short double-stranded regions and other intramolecular secondary structures (Figure 2-7). In addition to long perfectly double-stranded RNA, PKR is activated by RNA that contains either hairpins [121], bulges, mismatched base-pairing [21], short internal dsRNA regions [280], or unique structures naturally present in selected cellular mRNAs [18, 57, 188, 244]. As previously demonstrated for TLR3 [132], it is likely that the activation of PKR by *in vitro* transcribed mRNA is due to the formation of intra- and intermolecular secondary structures. PKR is then activated upon binding to these structures, similar to the classical dsRNA-mediated mechanism of PKR activation. Nucleoside modifications influence base pairing and secondary structure formation [43, 136, 144, 155, 176, 239, 282, 283], which likely contribute to their effects on PKR activation. Alterations to the shape of the helix formed and interruptions to the minor groove, which is presumed to be the principal location of PKR interaction with RNA [20, 177, 213], are also likely to play significant roles in determining how each modified nucleoside will impact RNA-mediated PKR activation.

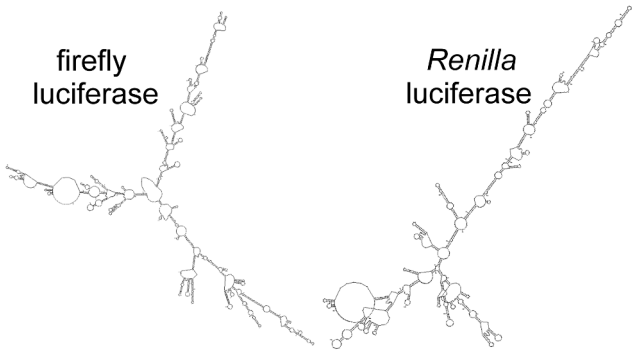

Figure 2-7. Double-stranded characteristics of mRNA
mFold web server prediction of firefly and *Renilla* luciferase mRNA secondary structures.

Unlike short ssRNAs [178], PKR activation by long *in vitro* transcribed mRNA is not dependent on the presence of a 5'-triphosphate, as mRNA containing complete replacement of 5'ppp with cap structure also activates PKR (Figure 2-2A and C). The difference between these findings might reflect the amount of 5'ppp in the RNAs being compared. Forty-seven nt-long ssRNA induced 100-fold more PKR activation when the 5'-end contained triphosphates [178], while our data did not show any significant effect of removing the 5'ppp from 1976 nt-long mRNA, which contains ~40-fold less 5'ppp. Our finding is more consistent with the result reported for 47 bp-long dsRNA, wherein PKR activation did not depend on 5'ppp [178].

Previous reports indicate that PKR activation is altered by the presence of modified nucleosides in homopolymeric RNA [166, 246, 247] and short ssRNA and dsRNA [177]. Our data extends these findings by demonstrating that incorporation of modified nucleosides into long *in vitro* transcribed mRNA also alters activation of PKR and subsequent translation of the RNA. We observe substantial PKR activation by *in vitro*

transcribed mRNA, which is reduced by incorporation of Ψ. Additionally, our studies show reduced PKR activation by mRNA that contains m^5C, enhanced PKR activation by mRNA containing m^5U, and elimination of PKR activation by s^2U-containing mRNAs. These results vary from those obtained when testing PKR activation by short 47 nt ssRNA: a low level of PKR activation by unmodified RNA, which was dependent on the presence of a 5'-triphosphate, and near complete elimination of PKR activation by incorporation of modified nucleosides [177]. However, when testing short 47 bp dsRNA, the effects observed were similar to those reported here: PKR activation by unmodified RNA, which is reduced by Ψ incorporation, increased by m^5U incorporation, and eliminated by s^2U incorporation. This similarity to short dsRNA, and dissimilarity to ssRNA, supports our model that PKR activation by long *in vitro* transcribed mRNA, where 5'ppp is limited, is due to regions of secondary structure formed within the RNA.

Unlike the other nucleoside modifications tested, the presence of m^6A in mRNA impacted PKR activation differently *in vivo* compared to *in vitro*. *In vitro*, mRNA containing m^6A activated PKR only moderately (Figure 2-2D and E) whereas *in vivo*, m^6A-containing mRNA activated PKR more potently than unmodified RNA (Figure 2-3B). Although the significance of this observation is not fully understood, the discrepancy may be explained by the presence of additional factors in cells that facilitate increased double-stranded formation in m^6A-containing mRNA *in vivo*.

Nucleic acids containing modified nucleosides can act as antagonists of nucleic acid-sensing TLRs [103, 208, 260, 272]. Therefore, we asked whether mRNAs containing modified nucleosides inhibit activation of PKR by its cognate ligand, dsRNA. PKR is still activated by dsRNA in the presence of a 125-fold excess of mRNA containing Ψ or

other modified nucleosides (s^2U, m^5C, or m^6A), indicating that mRNAs containing modified nucleosides are not inhibitors of PKR (Figure 2-5). This extends previous data demonstrating that short ssRNAs containing modified nucleosides do not inhibit PKR [177]. Furthermore, in cell lysates, RNA containing Ψ pulls down less PKR than RNA containing uridine (Figure 2-6B). This reduction in PKR binding is consistent with prior *in vitro* data demonstrating small reductions in PKR binding to short dsRNA and ssRNA that contain modified nucleosides [177]. From these data, we conclude that the mechanism of reduced PKR activation is reduced recognition and binding to RNA containing modified nucleosides.

It is possible that mRNAs with different nucleoside modifications have different optimal concentration for activating PKR. Figures 2-2 and 2-3 indicate that none of the modified nucleosides tested, with the exception of s^2U, completely eliminate PKR activation. Rather, each modified nucleoside might alter the ability of RNA to bind and activate PKR (Figure 2-6B).

PKR plays an integral part in the cellular response to viral RNA. However, mechanisms to avoid PKR activation by cellular RNAs are required, as constitutive PKR activation and translational inhibition would obstruct normal cellular function. Here, our data show that PKR activation is reduced when RNAs contain nucleoside modifications that are naturally present in many cellular RNAs, including piRNA [138], snRNA, tRNA, mRNA, and rRNA [212]. Activation of TLRs [130] and RIG-I [115] is also influenced by modified nucleosides in RNA and most commonly RNA modifications decrease the immunogenicity of RNA. Together, these data support a general interpretation that modified nucleosides supply a pattern for differential recognition by RNA binding

proteins. One purpose of common natural modifications may be avoiding activation of PKR and other RNA sensors by self RNA.

Using mRNA for gene delivery has the benefits of efficient transfection and rapid protein expression without the risk of insertional mutagenesis. The potential of mRNA as a delivery vehicle is enhanced further by incorporating modified nucleosides that reduce host defense responses initiated by PKR, TLRs, and RIG-I [115, 130, 177]. We observed the additional benefit of increased translation from Ψ and m^5C-containing mRNA [131]. *In vitro* transcribed mRNA is regularly delivered to cells in a research setting and has entered clinical trials as a cancer vaccine. As the interest in non-coding RNA continues, the delivery of RNA is likely to continue expanding. In most cases, activating PKR is an unwanted side-effect. High translation and low immunogenicity make mRNA containing Ψ or m^5C applicable to express therapeutic proteins, whereas s^2U-modified RNA is best suited for applications where avoiding non-specific immunogenicity is desirable but where translation is unnecessary [131], such as delivering antisense RNA [200] or stimulating RNA interference.

CHAPTER 3

Nucleoside modifications in RNA reduce OAS activation and ability to be cleaved by RNase L

3.1 Introduction

The antiviral 2-5A system is initiated when double-stranded (ds)RNA is bound by 2'-5'-oligoadenylate synthetases (OAS), of which 8–10 isoforms exist due to gene duplication and alternative splicing. These isoforms have divergent cellular localization [44], are differentially induced by IFNs [117], produce different size 2-5A molecules [117], and each possess unique activation parameters and catalytic profiles [160]. However, the specific roles for each OAS variant are not well understood. In addition to 2-5A production, the alternatively spliced 9-2 isozyme of OAS1 can act as a pro-apoptotic mediator independent of 2-5A production [90]. Recently extracellular OAS1 was demonstrated to have antiviral activity independent of 2-5A production [142]. OAS2 binds to NOD2, which enhances its antiviral effects and suggests an involvement with antibacterial defenses as well [74].

OAS proteins lack any homology to known RNA-binding motifs and instead rely on a positively charged groove for RNA binding [108]. In addition to dsRNA, OAS can be activated by specific viral RNAs [63, 169, 225, 226], ssRNA aptamers [109], and even certain cellular mRNAs [168]. A recent report indicated that interaction with the minor groove of the consensus sequence NNWWNNNNNNNNNWGN is required for OAS1 activation by short dsRNA [139].

Activated OAS links ATP into unique, short 2'–5'-linked oligomers called 2-5A [$p_x5'A(2'p5'A)_n$; $x = 1–3$; $n \geq 2$]. The primary function of 2-5A is activation of the latent endoribonuclease RNase L. Binding of RNase L monomers by 2-5A allows RNase L dimerization and exposes the nuclease domain. Activated RNase L cleaves ssRNA preferentially following UU or UA dinucleotides (reviewed in [229]).

Certain OAS proteins produce distinct profiles of 2-5A sizes. For instance, OAS3 predominantly produces 2-5A dimers, which do not activate RNase L [206]. Therefore, it has been proposed that 2-5A might also have other cellular functions. Consistent with this proposal, a recent report indicated that in addition to RNase L activation, 2-5A_4 also serves as a TLR4 ligand [68].

The 2-5A system is a conserved host defense pathway, with evidence of the 2-5A system found down to marine sponges, the lowest metazoa [223]. In response, pathogens have evolved strategies to circumvent the 2-5A system, such as inhibition of OAS activation by HSV1 Us11 protein [216] and inhibition of RNase L activity by a conserved structure in poliovirus RNA [104].

Here, we report that the presence of modified nucleosides in RNA has multiple effects on the 2-5A pathway. Unmodified *in vitro* transcribed mRNA activates OAS, resulting in rRNA cleavage and reduced translation. Additionally, unmodified mRNA is more rapidly cleaved by activated RNase L. In contrast, all of these effects are reduced when RNA contains modified nucleosides. Unmodified RNA is therefore identified as a distinguishing pattern for 2-5A system activity.

3.2 Materials and methods

Cells and reagents

Immortalized wild-type (WT) and RNase $L^{-/-}$ mouse embryonic fibroblasts (MEFs) were maintained in RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum. RNA oligos $C_{11}U_2C_7$ and $C_{11}\Psi_2C_7$ were custom synthesized (Dharmacon) and were 5'end-labeled using adenosine 5'-[γ-^{32}P]-triphosphate (γ-^{32}P-ATP) (PerkinElmer) and T4 polynucleotide kinase (New England Biolabs).

Immunoprecipitation

HEK293T cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well one day prior to transfection. Cells were exposed to 50 µl DMEM containing lipofectin-complexed RNA (0.25 µg) for 1 hour, which was then replaced with complete medium and further cultured. Cells were incubated in methionine/cysteine-free medium (Invitrogen) for 1 hour, then pulsed with complete medium supplemented with ^{35}S-methionine/cysteine (140 mCi/mL) (PerkinElmer) for 3–5 hours prior to lysis in 50 µl RIPA buffer supplemented with protease inhibitor cocktail (Sigma). *Renilla* luciferase was immunoprecipitated from lysates using an anti-*Renilla* luciferase antibody (MBL) and protein G-coated dynabeads (Invitrogen) and separated by 15% polyacrylamide gel electrophoresis. Gels containing the labeled samples were treated with 1 M sodium salicylate, dried, and a fluorogram was generated by exposure to BioMax MS film (Kodak).

RNA stability in rabbit reticulocyte lysate

Equal mass (25 ng/μl) or equal molar (40 μM) firefly and *Renilla* mRNAs were incubated in 15 μl rabbit reticulocyte lysate (RRL, Promega) at 30°C. At the indicated time a 2 μl aliquot was removed and the RNA was recovered using Trizol (Invitrogen) for subsequent detection by northern blotting.

RNA stability in cell culture

HEK293T, WT MEF, or RNase $L^{-/-}$ MEF cells were nucleofected with 5 μg mRNA using nucleofector program T-020 and nucleofector V kit (Lonza). After 15 minutes recovery in RPMI, cells were plated in complete media and incubated at 37°C. At the indicated time, RNA was recovered from cells using Trizol (Invitrogen) for subsequent detection by northern blotting.

Northern blotting

RNA was isolated from RRL or cells using Trizol (Invitrogen). To enhance the RNA yield, 70 μg of glycogen (Roche Diagnostics) was added as carrier, and the precipitation was performed in siliconized tubes at −20°C overnight. RNA samples were denatured then separated in denaturing, 1.4% agarose, 0.22 M formaldehyde gel submerged into MESA buffer (Sigma) supplemented with 0.22 M formaldehyde. RNA was transferred to NYTRAN Nylon(+) membrane (Schleicher & Schuell) and UV cross-linked. The membranes were prehybridized at 68°C for 1 h in MiracleHyb (Stratagene). To probe the northern blots, 50 ng of DNA was labeled using [α-^{32}P]dCTP (Amersham Biosciences) with a random prime labeling kit (Roche Applied Science). Probes were

derived from plasmids and were specific for the coding region of firefly or *Renilla* luciferase, or for a β-globin 5' UTR and 3' UTR sequence present in both mRNAs. The membranes were hybridized at 68°C for 20 hours with MiracleHyb containing the labeled and denatured probe. The membranes were washed and exposed to Kodak MS film using an MS intensifier screen at −70°C for 2–72 hours or exposed to a phosphor-storage screen and imaged using a Typhoon phosphorimager (GE Healthcare).

In vitro OAS activation

Recombinant human OAS1 p42 [108], recombinant human RNase L [243], and $p_3[2'p5'A]_2A$ (2-5A_3) [242] were prepared as described previously. Dual-labeled fluorescent probe 6-FAM-UUA UCA AAU UCU UAU UUG CCC CAU UUU UUU GGU UUA-BHQ-1 was custom synthesized by Integrated DNA technologies. *In vitro* OAS1 activation was performed as previously described [243]. Briefly, 20 μg/ml OAS1 was activated with 2 μg/ml RNA for the indicated time in buffer consisting of 20 mM HEPES pH 7.5, 20 mM MgOAc, 20 mM KCl, 1 mM EDTA, and 10 mM ATP. Reactions were stopped by heating to 95°C for 3 minutes. Microcon YM-3 centrifugal filters (Millipore) were used to separate 2-5A, and the 2-5A produced was then measured by mixing with 8 μg/ml RNase L and 0.1 μM fluorescent probe in RNase L cleavage buffer (consisting of 25 mM Tris-HCl pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 50 mM ATP, and 7 mM β-mercaptoethanol) and compared to the linear range of a standard curve generated using 0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 1, 3, 5 and 10 nM of purified 2-5A_3. Fluorescent intensity is proportional to 2-5A concentration.

***In vitro* RNA cleavage by RNase L**

Recombinant human RNase L [243], and $p_3[2'p5'A]_2A$ (2-5A_3) [242] were prepared as described previously. For RNA oligos, 12.5 nM RNase L was activated on ice with 100 nM 2-5A_3 for 30 minutes in RNase L cleavage buffer (consisting of 25 mM Tris-HCl pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 50 mM ATP, and 7 mM β-mercaptoethanol). Then 100 nM 5' end-labeled RNA oligo [^{32}P]-$C_{11}U_2C_7$ or [^{32}P]-$C_{11}\Psi_2C_7$ (Dharmacon) was added and reactions were incubated at 30°C. At the indicated time, reactions were stopped by the addition of urea-TBE loading buffer (BioRad) and heating to 95°C for 3 minutes. Aliquots were separated by 15% polyacrylamide gel electrophoresis, gels were dried, and samples were imaged using a phosphor storage screen and detected using a Typhoon phosphorimager. Cleavage of mRNA was performed similarly, using 10 nM RNase L, 10 nM 2-5A_3, and 100 nM mRNA. Reactions were stopped by heating to 95°C for 5 minutes. The mRNA was recovered by phenol:chloroform extraction and detected by northern blotting.

Translation of mRNA in mice

All mice were cared for according to institutional guidelines at the University of Pennsylvania under a protocol approved by the Institutional Animal Care and Use Committee. Wild-type C57Bl/6 (NCI) and C57Bl/6 backcrossed RNase $L^{-/-}$ mice at 9–16 weeks of age received tail-vein injections of 1 μg RNA complexed with lipofectin (Invitrogen) in 60 μl DMEM. At the indicated time, mice were sacrificed and spleens were isolated. Each spleen was bisected and spleen fragments were homogenized in 200 μl cell culture lysis reagent (Promega). Luciferase activity was detected in a 40 μl aliquot

of cell culture lysis reagent using 200 µl luciferase assay substrate (Promega) and a LUMAT LB 950 luminometer (Berthold) at a 10-second measuring time.

rRNA cleavage

One day prior to transfection, WT or RNase $L^{-/-}$ MEF cells were seeded into 96-well plates at a density of 5.0×10^4 cells/well and treated with 1000 U/ml interferon-αA/D (Sigma). Poly(I:C) or mRNAs were complexed with lipofectin (Invitrogen) as described previously [130]. Cells were exposed to 50 µl DMEM containing lipofectin-complexed RNA (2.5 µg) for 1 hour, which was then replaced with complete medium and further cultured. At 3 hours post-transfection, total RNA was recovered from cells using Trizol (Invitrogen). RNA was separated by agarose gel electrophoresis, stained with SybrGold reagent (Invitrogen), and detected using UV fluorescence and a GelDoc 2000 imager (BioRad).

3.3 Results

mRNA containing nucleoside modifications activates OAS less than unmodified mRNA

We first compared the activation of purified human OAS1 by unmodified or modified nucleoside-containing mRNA with identical sequence. Activation of OAS1 was assessed by recovering the 2-5A produced, which was then quantified using a fluorescence quenching assay. Unmodified mRNA activated OAS1, which was significantly ($p<0.05$) reduced when the mRNA contained pseudouridine (Figure 3-1A). Similar results were obtained when OAS activation was tested using a panel of mRNAs containing the modified nucleosides m^6A, m^5C, or s^2U (Figure 3-1B). Subsequent experiments focused on the comparison of unmodified RNA to Ψ-containing mRNA with identical sequence.

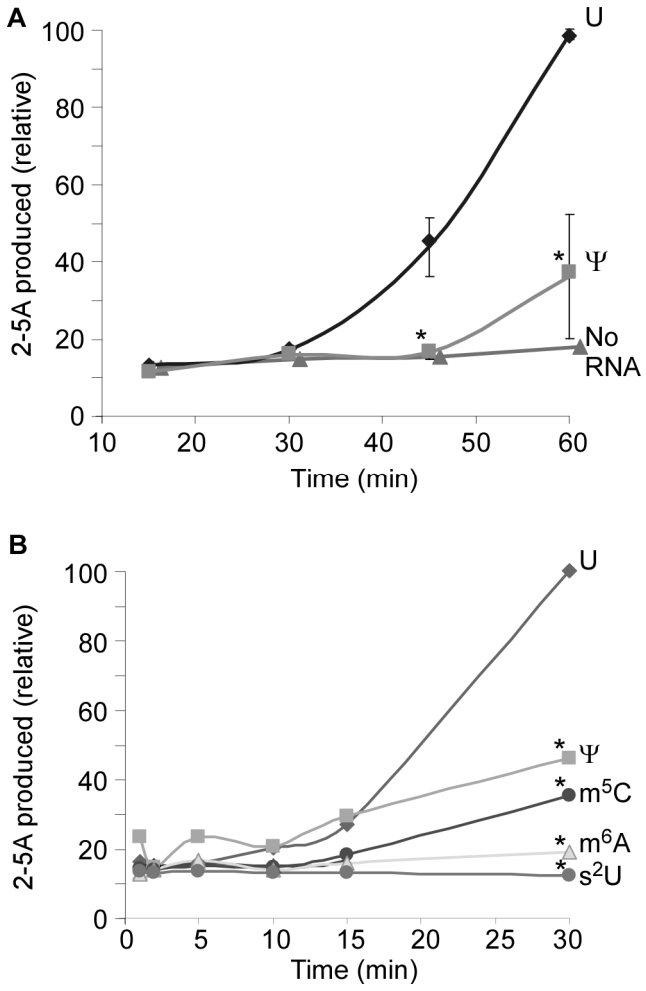

Figure 3-1. OAS activation by mRNA containing modified nucleosides
Purified human OAS1 p42 was activated by mixing with (**A**) unmodified (U) or Ψ-containing mRNA or (**B**) a panel of mRNAs containing the indicated nucleoside modifications. Reactions were stopped at indicated times by heating at 95°C. Spin columns were used to isolate the 2-5A produced in each reaction, and 2-5A levels were assessed from an aliquot of each reaction using a fluorescence quenching assay. Asterisks indicate P-value <0.05 compared to U-mRNA. Data shown are mean ±SEM of three replicates in one experiment and are representative of three independent experiments. In (B) SEM error bars were omitted for clarity.

Pseudouridine-containing mRNA induces less rRNA cleavage than unmodified mRNA

OAS activation by pathogenic RNA leads to activation of RNase L, which mediates the effector function of the 2-5A system by cleaving ssRNA. RNase L-mediated cleavage at exposed loops of rRNAs in intact ribosomes results in well-defined cleavage patterns in rRNA [264]. Therefore, the integrity of rRNA following mRNA transfection was examined. Lipofectin-complexed mRNA was transfected to WT and RNase $L^{-/-}$ MEF cells, and total RNA was subsequently recovered and examined by agarose gel electrophoresis and UV imaging. No RNA and the dsRNA analog poly(I:C) were included as negative and positive controls, respectively. In WT cells, delivery of unmodified *in vitro* transcribed mRNA induced cleavage of rRNA but rRNA cleavage was reduced if the mRNA contained Ψ. In contrast, this rRNA degradation was not observed in cells lacking RNase L (Figure 3-2).

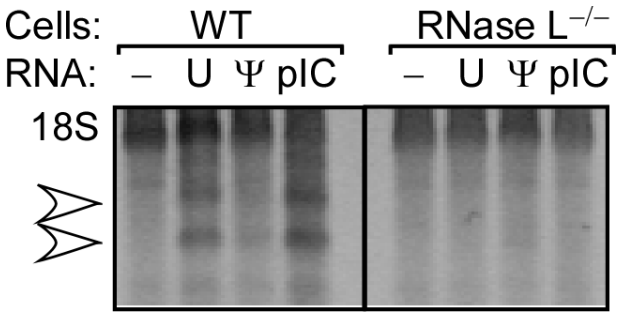

Figure 3-2. Induction of rRNA cleavage by *in vitro* transcribed mRNA
Unmodified (U) or Ψ-containing mRNA encoding firefly luciferase was complexed to lipofectin and delivered to WT or RNase $L^{-/-}$ MEF cells, as were no RNA (–) and poly(I:C) (+) controls. Three hours following transfection, total RNA was recovered from cells. RNA aliquots were separated in an agarose gel and visualized by UV fluorescence. Arrowheads indicate rRNA cleavage products. Representative data from one of three independent experiments is shown.

RNase L cleaves uridine-containing RNA more readily than Ψ-containing RNA

Activated RNase L cleaves preferentially following UpNp in ssRNA. Therefore, we next compared the ability of RNase L to cleave Ψ-containing RNA. Purified recombinant human RNase L was activated with 2-$5A_3$ and mixed with a 5'-[^{32}P] end-labeled RNA oligo containing a single RNase L cleavage site ($C_{11}U_2C_7$ or $C_{11}\Psi_2C_7$). Reaction products were gel separated to visualize RNA cleavage. The RNA oligo containing unmodified uridine was rapidly cleaved, but there was no significant cleavage of RNA oligo containing Ψ (Figure 3-3A and B). Full-length mRNA was then analyzed for cleavage by RNase L. Both unmodified and Ψ-mRNA could be cleaved by RNase L. However, consistent with the results obtained with RNA oligos, Ψ-containing mRNA was cleaved at a slow rate by RNase L (Figure 3-3C).

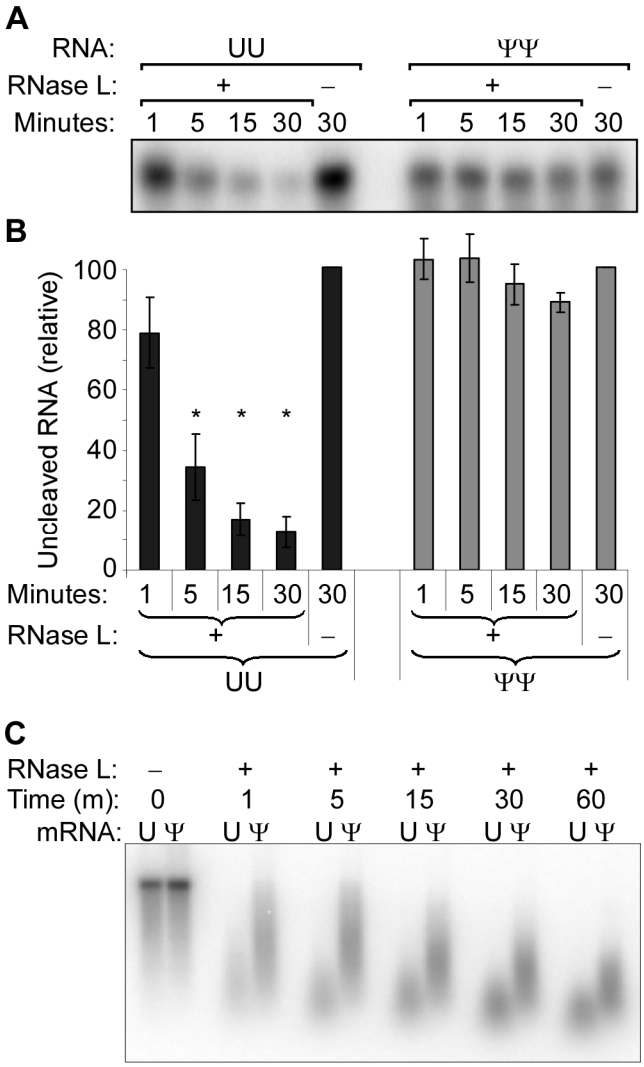

Figure 3-3. Cleavage of Ψ-containing RNA by RNase L
Purified RNase L was activated on ice by 2-5A_3 prior to mixing with RNA substrates. (**A**) Cleavage of RNA oligos [^{32}P]-$C_{11}U_2C_7$ (UU) or [^{32}P]-$C_{11}\Psi_2C_7$ (ΨΨ) by RNase L. Reactions were stopped at the indicated time by addition of loading buffer, and reactions were separated by PAGE and visualized by phosphor storage radiography. Representative data from one of three independent experiments is shown. (**B**) Quantification of phosphor storage intensities. Values were normalized to the values obtained in 30 minutes reactions not containing RNase L. Data represents average ±SEM of *n*=3 experiments. Asterisks indicate P-values <0.05 comparing UU to ΨΨ. (**C**) Cleavage of unmodified (U) or Ψ-containing mRNAs with identical sequence by RNase L. Reactions were stopped at indicated times by heating to 95°C. Following phenol:chloroform extraction and precipitation, aliquots from each reaction were assessed by northern blotting. Representative data from one of three independent experiments is shown.

RNase L facilitates enhanced translation of Ψ-containing mRNA in cells and after *in vivo* administration

Because Ψ-modification of mRNA reduced activation of OAS1, rRNA degradation, and mRNA cleavage by RNase L, we asked how the absence of RNase L influences translation of unmodified and Ψ-containing mRNA. To do so, mRNAs encoding luciferase were transfected into wild-type (WT) or RNase $L^{-/-}$ MEF cell lines and translation was assessed by measuring luciferase activity. In WT cells, Ψ-containing mRNA is translated to a greater level than unmodified RNA. However, in RNase $L^{-/-}$ cells, the translational advantage of Ψ-mRNA over unmodified mRNA is dramatically reduced (Figure 3-4A).

Similar patterns of translation occur in the spleens of mice following injection of mRNA. Either WT C57Bl/6 or RNase $L^{-/-}$ mice were given lipofectin-complexed luciferase mRNA by tail-vein injection. At sacrifice, luciferase activity was assessed in spleen lysate. In WT mice, Ψ-containing mRNA is translated to higher levels than unmodified RNA. In RNase $L^{-/-}$ mice, translation of Ψ-containing mRNA reached the same level as observed in WT mice, but translation of unmodified mRNA is increased relative to WT ($p<0.05$), (Figure 3-4B).

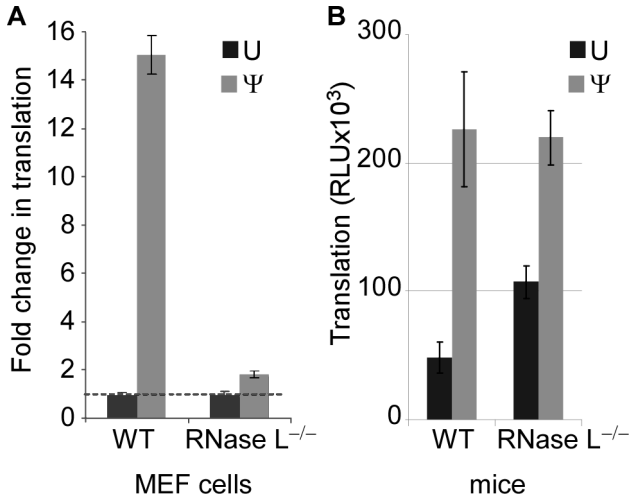

Figure 3-4. Translation of unmodified and Ψ-containing mRNA in wild-type and RNase L$^{-/-}$ cells and mice

Unmodified (U) or Ψ-containing *in vitro* transcribed mRNA encoding firefly luciferase was complexed to lipofectin and delivered to wild-type (WT) and RNase L$^{-/-}$ (RL$^{-/-}$) MEF cells or mice. Luciferase activity was assessed in aliquots of cell lysate. (**A**) MEF cells lysed at 5 hrs following transfection. Luciferase relative light unit (RLU) values were normalized to the RLU obtained with unmodified mRNA (indicated by dashed line) and expressed as fold increase. Error bars indicate SEM of quadruplicate wells from one representative of at least six independent experiments. (**B**) Lipofectin-complexed mRNA was delivered by tail-vein injection into mice. At 4 hours following delivery, mice were sacrificed and spleens were lysed. Values presented are luciferase relative light units (RLU) in 1/5 of the total 200 µl lysate. Error bars represent SEM of n = 3 mice.

Pseudouridine-containing mRNA is actively translated longer than unmodified mRNA

We next compared translation over time, to determine how modified nucleosides influence the duration of translation. RNA was complexed to lipofectin and delivered to cells, which were then pulsed with ^{35}S-methionine/cysteine at different times after transfection, and translation of the mRNA was assessed by immunoprecipitation of the encoded *Renilla* luciferase protein. As previously observed, there was a higher level of translation of Ψ-containing mRNA at each time point (Figure 3-5A). In addition, the

translation of Ψ-containing mRNA continued after measurable translation of unmodified mRNA ceased, persisting at 48 hours (Figure 3-5B).

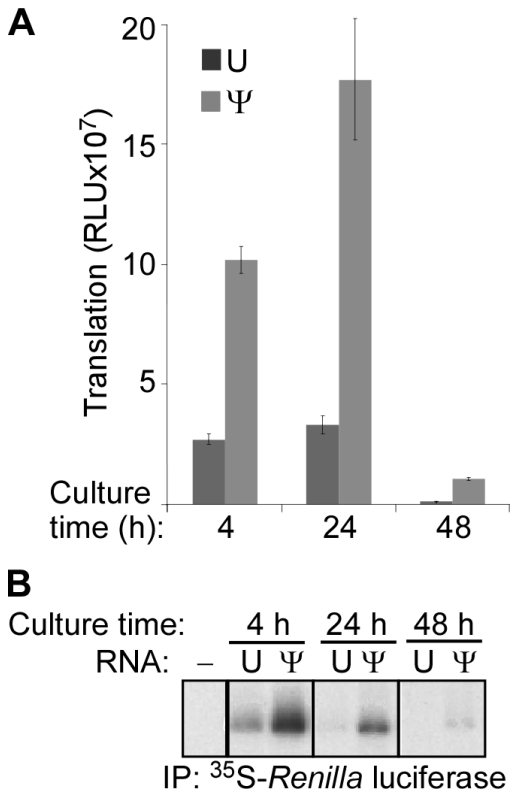

Figure 3-5. Translation of Ψ-containing mRNA in cell culture
Unmodified (U) or Ψ-containing mRNA was complexed to lipofectin and delivered to HEK293T cells. Cells were subsequently pulsed with ^{35}S-methionine/cysteine prior to lysis. (**A**) *Renilla* luciferase activity was assessed in aliquots of cell lysate. Data displayed is mean ± SEM from four replicates, each performed in duplicate. (**B**) *Renilla* luciferase protein was immunoprecipitated from cell lysates and PAGE separated prior to radio-fluorography. Data shown is one of four replicates and is representative of three independent experiments.

Nucleoside-modified mRNA has an increased half-life

The extended translation of Ψ-modified RNA demonstrated that the RNA persisted in a functional state for an extended period of time. Accordingly, we examined

the stability of unmodified and Ψ-containing mRNA by northern blot analysis. Both unmodified and Ψ-mRNA were equally stable at room temperature through experimental time-courses and indefinitely in storage at –20°C (data not shown). Unmodified and Ψ-mRNAs were added to rabbit reticulocyte lysate (RRL) or transfected to HEK293T cells. When transfecting cells using cationic lipids, a portion of RNA complexed with transfection reagents persists as an extracellular, nuclease-protected fraction. Therefore, for these experiments, we used nucleofection to deliver naked mRNA and confirmed the rapid degradation of extracellular RNA by serum nucleases in the culture media (data not shown). Total RNA was subsequently re-isolated, and aliquots were examined by northern blot to compare degradation rates of the reporter mRNAs. Two reporter mRNAs were studied simultaneously, to ensure that stability differences were not a result of differences in coding sequence. As shown in figure 3-6, Ψ-modified mRNAs had longer half-lives than unmodified mRNAs both in RRL (Figure 3-6A) and in HEK293T cells (Figure 3-6B).

Subsequently, the influence of RNase L on the stability of unmodified and Ψ-containing mRNA was also compared using RNase $L^{-/-}$ MEF cells. As in HEK293T cells, the mRNA was delivered by nucleofection. Total RNA was recovered from cell culture and firefly and *Renilla* luciferase mRNA were assessed by northern blot. In both WT and RNase $L^{-/-}$ MEF cells, Ψ-modified mRNA had an increased half-life compared to unmodified RNA (Figure 3-6C).

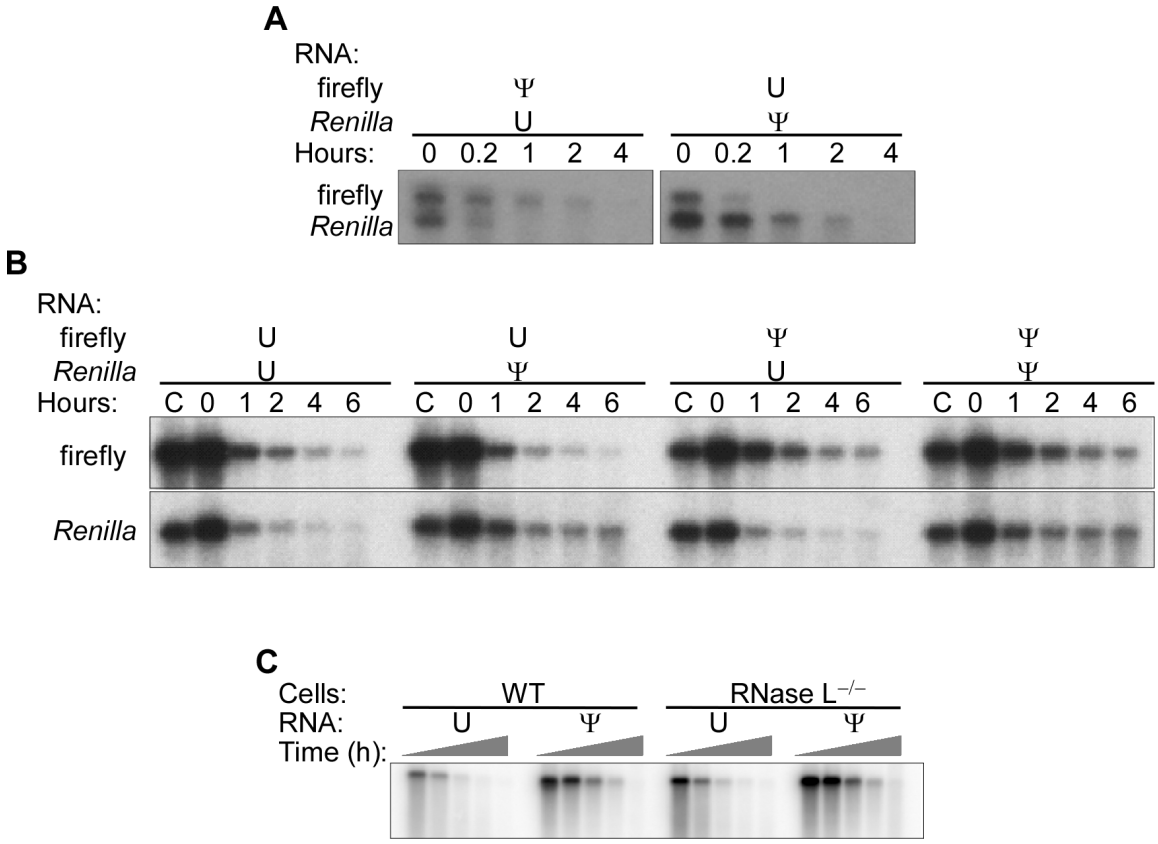

Figure 3-6. Half-life of Ψ-containing mRNA

(**A** and **B**) Unmodified (U) or Ψ-containing mRNAs encoding firefly or *Renilla* luciferase proteins were mixed 1:1 and either added to RRL (A) or nucleofected to HEK293T cells (B). At the indicated time points, RNA was recovered using Trizol reagent. RNA was subsequently detected in aliquots of the recovered RNA by northern blotting. Data shown is representative of at least five independent experiments. (**C**) Unmodified (U) or Ψ-containing *in vitro* transcribed mRNA encoding firefly luciferase was delivered to wild-type (WT) or RNase $L^{-/-}$ MEF cells by nucleofection. Cell were lysed at 0.2, 1, 3, 6, or 24 hrs following transfection, total RNA was recovered, and luciferase mRNA was assessed by northern blotting. Representative data is shown from one of three independent experiments.

3.4 Discussion

Here, we identify novel impacts of nucleoside modifications in RNA on the 2-5A pathway. Our data show that conventional *in vitro* transcribed mRNA activates OAS1, but this activation is reduced when mRNA contains modified nucleosides. OAS activation by unmodified RNA leads to RNase L-mediated rRNA cleavage in cells, which is not induced by Ψ-mRNA. Furthermore, we demonstrate reduced RNase L cleavage of Ψ-containing RNA. Experiments using RNase $L^{-/-}$ MEF cell culture and following injection in RNase $L^{-/-}$ mice demonstrate that translation of unmodified mRNA is decreased in the presence of the intact 2-5 system, but the translation level of Ψ-mRNA is largely independent of the 2-5A system. In addition, we demonstrate that incorporation of Ψ increases the half-life of *in vitro* transcribed mRNA in cells and lysates. Finally, Ψ-containing mRNA is translated for a longer duration than unmodified *in vitro* transcribed mRNA.

RNA sensing in the 2-5A pathway is performed by the OAS family of proteins. OAS was originally characterized as requiring >8 base-pairs (bp) of uninterrupted helix in >30 bp-long dsRNA [167], but activation has also been demonstrated by a variety of ssRNAs, including aptamers [109], viral RNAs [63, 169, 226, 247], and some cellular RNAs [168, 187]. RNAs containing 2'-*O*-methylation [166, 217] or 5-methyluridine [9] do not activate OAS. Here, we report that unmodified *in vitro* transcribed RNA activated OAS1 to produce 2-5A, but this was substantially reduced when RNA contained Ψ, m^6A, m^5C, or s^2U. Recently, the consensus sequence nnWWnnnnnnnnnWGn (W = U or A) was demonstrated to be important for OAS1 activation by dsRNA, and this interaction is dependent on the minor groove and free OH groups on the critical base pairs [139]. The requirement that three out of

the four critical base-pairs in this sequence be U:A highlights the importance of uridine for OAS1 activation. However, pseudouridine does not disrupt base-pairing to adenosine and the imino group of Ψ is oriented toward the major groove [101], so how Ψ disrupts OAS1 activation remains unclear. The presence of Ψ stabilizes secondary structure and adds rigidity to both ss and dsRNA (reviewed in [43]). In this capacity, Ψ could affect OAS activation by altering the equilibrium structure of the RNA, rather than directly affecting OAS binding.

Production of 2-5A by activated OAS results in activation of the latent endoribonuclease RNase L, which is the effector protein of the 2-5A pathway. Activated RNase L cleaves various ssRNA including accessible sites in rRNA, resulting in specific cleavage products visible by gel electrophoresis [264]. Therefore, we examined the rRNA cleavage induced in cells by transfection of *in vitro* transcribed mRNA. In WT MEF, unmodified mRNA induced rRNA cleavage, which was reduced if mRNA contained Ψ. However, no RNA caused rRNA cleavage in RNase $L^{-/-}$ cells, confirming that the 2-5A system is required for RNA-induced rRNA cleavage. High levels of 2-5A result in global rRNA cleavage by RNase L [149], and when sustained, ultimately lead to apoptosis [40, 281]. In comparison, the level of rRNA cleavage induced here by transfection of *in vitro* transcribed mRNA is relatively small, and may not be expected to induce high levels of apoptosis. On the other hand, this level of rRNA cleavage is sufficient to have a profound impact on translation of the reporter mRNA. We propose that unmodified RNA induces local OAS and RNase L activation, as demonstrated with viral RNAs and ssRNA covalently linked to dsRNA [8, 186]. Accordingly, locally-activated RNase L cleavage likely

reduces translation of unmodified RNA through local cleavage of rRNA without inducing global rRNA cleavage and apoptosis.

The presence of Ψ has been shown to enhance the stability of RNA secondary structures, but has not previously been demonstrated to cause resistance to nucleases. The presence of Ψ did not inhibit cleavage by the nucleases RNase A, RNase H [279], RNase T1, RNase T2, nuclease P1, and snake venom phosphodiesterase, although there is some indication that pancreatic diesterase and snake venom phosphodiesterase may cleave with reduced efficiency[180]. A previous report based on cleavage of a $C_{11}N_2C_7$ RNA oligo showed that RNA containing 2'-deoxy-2'-α-fluorouridine was bound by RNase L but cleaved slowly, whereas RNA containing 2'-*O*-methyluridine was not bound by RNase L [250]. Here, we used a similar approach and demonstrated that purified RNase L readily cleaved the ssRNA oligo $C_{11}U_2C_7$ but not when the cleavage site contained Ψ. We also extended those findings to the examination of long *in vitro* transcribed mRNA and showed that unmodified mRNA was cleaved by purified RNase L, but cleavage of Ψ-mRNA proceeded more slowly. The slow cleavage of Ψ-mRNA despite inactivity toward $C_{11}Ψ_2C_7$ is not surprising given RNase L's loose sequence specificity. Although RNase L cleaves preferentially following UpNp, with highest activity following UU and UA, it is also capable of cleaving following AU, AA, AC, and CA but not CC [38, 84, 265].

As seen in previous reports [3, 131], in wild-type cells there was dramatically higher translation of Ψ-mRNA than unmodified mRNA. By contrast, in RNase $L^{-/-}$ MEF cells, the enhanced translation of Ψ-mRNA was modest. Similarly, the translational advantage of Ψ-mRNA was reduced in RNase $L^{-/-}$ mice. Notably, however, the absolute translation

level of Ψ-mRNA remained equal in WT and RNase $L^{-/-}$ mice, while the translation of unmodified mRNA increased in RNase $L^{-/-}$ mice. This indicates that neither the presence of RNase L nor Ψ-mRNA alone significantly affects translation, but rather that unmodified RNA causes translational inhibition through RNase L activation. Moreover, these results are consistent with the *in vitro* activation of OAS1 by unmodified RNA that we observed.

We also examined the effect of Ψ-modification on the stability of *in vitro* transcribed mRNA. In RRL and in cell culture, Ψ-mRNA was degraded more slowly than unmodified mRNA. Previous experiments also suggested that Ψ-mRNA is retained longer following injection in mice [131]. Despite the rapid cleavage of unmodified RNA by RNase L *in vitro*, the half-life of unmodified RNA did not increase to the level of Ψ-mRNA in RNase $L^{-/-}$ cells. This suggests that in addition to RNase L, other intracellular nucleases also cleave unmodified RNA more efficiently than Ψ-containing RNA.

The RNase L gene is genetically linked to prostate cancer and individuals homozygous for the R462Q mutant of RNase L, which exhibits reduced catalytic activity, are at increased risk for developing prostate cancer [39]. The established role of RNase L as an antiviral effector lead to speculation regarding a viral etiology in prostate cancer. Subsequently, a novel gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), was discovered in prostate tumors from RNase L-R462Q homozygous patients [252]. RNase L dysfunction has also been reported in association with chronic fatigue syndrome (CFS) [237], and CFS has also recently been linked to XMRV [150]. IFN-β inhibits XMRV replication, and this action is mediated in part by RNase L activity [69]. Nucleoside

modifications are found in viral mRNAs, including reports of hyper-modification [179]. However, the presence of modified nucleosides in XMRV viral RNA (vRNA) has not been investigated. Because RNA modifications reduce 2-5A system activity, nucleoside modifications in XMRV vRNA could contribute to the development or progression of prostate cancer and CFS.

Nucleases play a central role in host defense through destruction of pathogenic nucleic acids. The 2-5A system functions to detect and degrade danger-associated intracellular RNAs. Activation of RNase L also leads to reduced translation due to rRNA cleavage and when sustained, results in apoptosis, providing further limitations to pathogen replication. Here, we identify unmodified RNA as a molecular pattern recognized by OAS and RNase L. However, 2-5A system activity is decreased when RNA contains nucleoside modifications, which reduce both OAS activation and cleavage by RNase L. We propose that modified nucleosides are a general pattern, which facilitate recognition of danger-associated RNA as distinct from endogenous cellular RNA, as part of the extensive system of innate host defense against pathogenic RNA.

CHAPTER 4

Nucleofection induces eIF2α phosphorylation mediated by GCN2 and PERK

4.1 Introduction

Nucleofection is an advanced electroporation technique which varies electrical parameters and buffers to optimize delivery for specific cell types with high efficiency and reproducibility [96]. A major advantage of nucleofection is its versatility in transfecting a wide variety of primary and non-dividing cell types [96, 102, 107]. Nucleofection can be used to deliver a variety of nucleic acids including mRNA [51, 254], siRNA [96, 220], miRNA [199, 259], cDNA [25], and DNA plasmids [102, 211].

An increasingly common use of nucleofection is delivery of mRNA. Gene transfer based on mRNA is safe, because unlike DNA-based and viral vector approaches [100], mRNA-based gene transfer does not bear the risks of chromosomal integration. Protein expression is fast, beginning immediately upon mRNA reaching the cytoplasm. High transfection efficiency can be obtained, in part because there is no requirement for mRNA to reach the nucleus. Unlike other gene delivery strategies, no additional transcripts are made following transfection, so translation rates following delivery are a key consideration for mRNA-based gene delivery applications.

We recently reported enhanced translation of *in vitro* transcribed mRNA containing Ψ [131], which results in part from reduced phosphorylation of eukaryotic initiation factor 2-alpha (eIF2α) [3]. Phosphorylation of eIF2α reduces functional translation initiation complexes, resulting in a general decrease in translation initiation events and therefore a global decrease in translation (see [114] for review). There are four known eIF2α kinases in mammalian cells, each responding to different forms of cellular stress: RNA-dependent protein kinase (PKR), PKR-like ER kinase (PERK), general control non-derepressible-2 (GCN2), and heme-regulated inhibitor (HRI). Activation of

PKR occurs upon binding to double-stranded (ds)RNA and in this capacity is primarily characterized as an anti-viral sensor, although it also plays additional roles [3, 88]. HRI is active primarily in erythroid cells during heme deprivation. PERK is activated under conditions of ER stress as part of the unfolded protein response. GCN2 is stimulated by a variety of stresses, including amino acid starvation, proteosome inhibition, and UV irradiation [64].

Here, we show that nucleofection alone induces phosphorylation of eIF2α. We identify GCN2 as an eIF2α kinase contributing to this phosphorylation. Preliminary data also suggests that the involvement of PERK in this nucleofection-induced phosphorylation. Further studies are ongoing to confirm the involvement of PERK, and to measure translational repression following nucleofection. Developing approaches to overcome nucleofection-induced eIF2α phosphorylation will enhance the continued progression of mRNA-based therapies.

4.2 Materials and methods

Cells and reagents

Immortalized mouse embryonic fibroblasts (MEFs) were generously provided by David Ron (WT, GCN2$^{-/-}$, and PERK$^{-/-}$), Robert Silverman (PKR$^{-/-}$), and Alan Diehl [with permission from Douglas Cavener] (PERK/GCN2$^{-/-}$). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and 10% fetal calf serum (HyClone), MEM non-essential amino acids (Invitrogen), and 55 µM β-mercaptoethanol (BioRad).

Nucleofection

MEF cells were nucleofected using program T-020 and nucleofector V kit (Lonza). After 15 minutes recovery in RPMI, cells were plated in complete media and incubated at 37° C. At the indicated time following nucleofection, cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma) for western blotting.

Lipid and polymer transfections

Cells were seeded into 48-well plates at a density of 1.0×10^5 cells/well one day prior to transfection. Cells were exposed to 50 µl DMEM containing lipid-based lipofectin (Invitrogen), 50 µl DMEM medium alone, or 200 µl complete DMEM medium containing polymer/lipid-based TransIT-mRNA (Mirus) for 1 hour, which was then replaced with complete medium and further cultured. At the indicated time following

RNA transfection, cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma) for western blotting.

4.3 Results

Nucleofection induces eIF2α phosphorylation in WT MEF cells

The impact of nucleofection on translation was first studied in a MEF cell line derived from wild-type (WT) C57Bl/6 mice. Nucleofection conditions were optimized according to manufacturer guidelines, and it was determined that program T-020 provided the best cell survival with high transfection efficiency (data not shown). WT MEF were then nucleofected without including any nucleic acid in the transfection mix. As a negative control, cells were mock treated by subjecting them to the same manipulation and buffers but without electric shock. Following nucleofection, eIF2α phosphorylation was assessed by western blotting using an antibody specific for eIF2α phosphorylated at serine 51. As shown in Figure 4-1, nucleofection induced phosphorylation of eIF2α four-fold over the baseline level present in mock-treated cells.

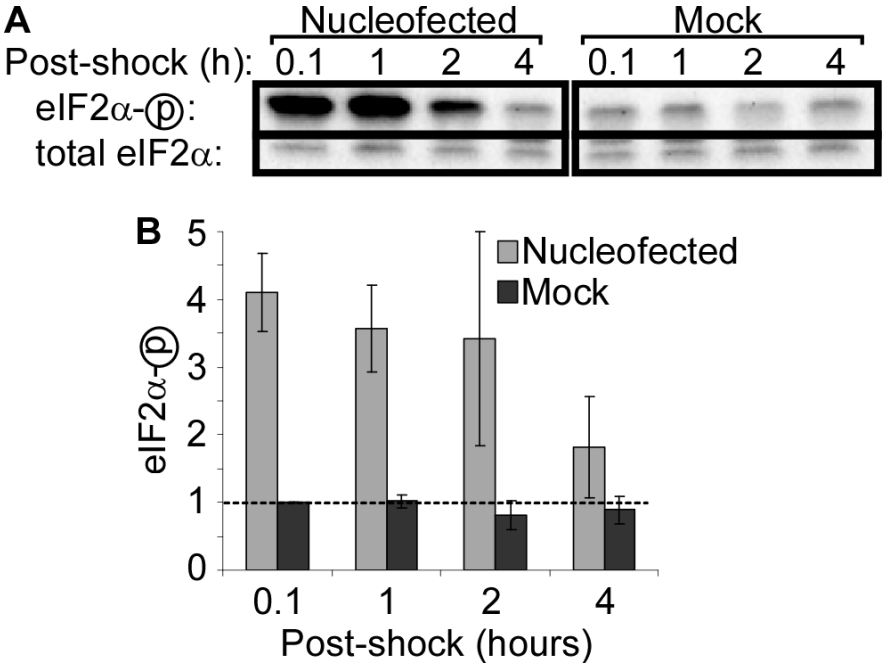

Figure 4-1. Phosphorylation of eIF2α in wild-type cells following nucleofection
Wild-type MEF cells were nucleofected or mock-treated, then lysed at the indicated times. (**A**) Phosphorylation of eIF2α was assessed in aliquots of lysate by western blotting with antibody specific for phosphorylated eIF2α (eIF2α–(P)) and then re-probed for total eIF2α. Representative data from one of three independent experiments is shown. (**B**) Quantification of western blot band densities. Values were calculated as the ratio of phosphorylated to total eIF2α and normalized to the values obtained in mock-treated cells at 0.1 hours post-shock. Data displayed is mean ± SEM of $n = 3$ experiments.

Lipid and polymer transfection reagents do not induce eIF2α phosphorylation in WT MEF cells

To determine if eIF2α phosphorylation is a common feature of transfection, eIF2α phosphorylation was measured following treatment with lipid and polymer transfection reagents. Reagents were prepared and delivered according to manufacturer instructions, but without including nucleic acid in the transfection mixes. Neither lipid-

based nor polymer-based transfection protocols induced phosphorylation of eIF2α (Figure 4-2).

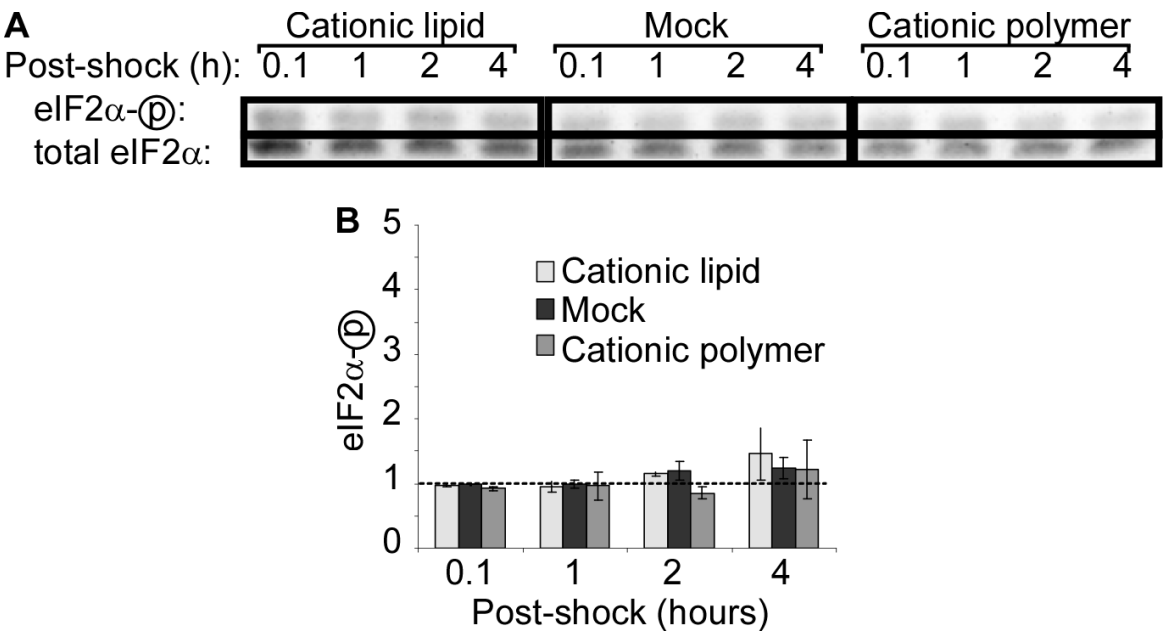

Figure 4-2. Phosphorylation of eIF2α in wild-type cells following transfection
Wild-type MEF cells were transfected with the indicated transfection reagent or mock-treated and lysed at the indicated time points. (**A**) Phosphorylation of eIF2α was assessed in aliquots of lysate by western blotting. Representative data from one of two independent experiments is shown. (**B**) Quantification of western blot band densities. Values were calculated as the ratio of phosphorylated to total eIF2α and normalized to the values obtained in mock-treated cells at 0.1 hours post-shock. Data displayed is mean ± SEM of $n = 2$ experiments.

GCN2$^{-/-}$ delays and reduces nucleofection-induced eIF2α phosphorylation

Of the four mammalian eIF2α kinases, three — PKR, PERK, and GCN2 — are widely distributed, whereas HRI is reported to function primarily in erythroid cells [151]. Therefore, to identify the eIF2α kinase responsible for the nucleofection-induced eIF2α phosphorylation, we took advantage of MEF cell lines created from mice deficient in

PKR, PERK, and GCN2. As was done with WT MEF, cells were nucleofected and eIF2α phosphorylation was assessed by western blotting. Nucleofection of $PKR^{-/-}$ MEF cells resulted in eIF2α phosphorylation comparable to that induced in WT MEF cells (Figure 4-3A), as did nucleofection of $PERK^{-/-}$ MEF cells (Figure 4-3B). Nucleofection also induced eIF2α phosphorylation in $GCN2^{-/-}$ MEF cells, but the peak level of phosphorylation was reduced and occurred later than in other cell lines (Figure 4-3C). Mock treatment did not induce eIF2α phosphorylation in any cell line (Figure 4-3).

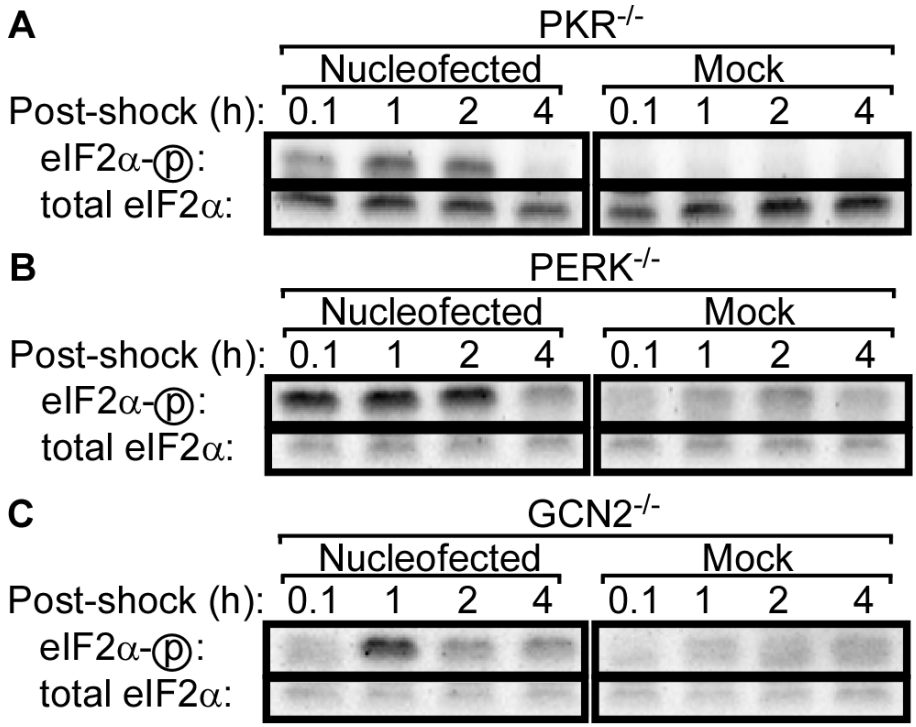

Figure 4-3. Phosphorylation of eIF2α in kinase-deficient cells following nucleofection
MEF cells deficient in eIF2α kinases, PKR$^{-/-}$ (**A**), PERK$^{-/-}$ (**B**), or GCN2$^{-/-}$ (**C**) were nucleofected or mock-treated, then lysed at the indicated time points. Phosphorylation of eIF2α was assessed in aliquots of lysate by western blotting. Representative data of 2–4 independent experiments is shown.

GCN2 and PERK are responsible for nucleofection-induced eIF2α phosphorylation

The removal of single eIF2α kinases did not completely prevent nucleofection-induced eIF2α phosphorylation, suggesting the possibility that multiple kinases are involved. Therefore, we assessed phosphorylation of eIF2α following nucleofection of MEF cells derived from a dual-knockout GCN2$^{-/-}$/PERK$^{-/-}$ mouse. No eIF2α phosphorylation was visible in dual-knockout GCN2$^{-/-}$/PERK$^{-/-}$ MEF cells (Figure 4-4A). Of interest, a dramatically reduced baseline level of eIF2α phosphorylation was

observed in these cells. The dual knockout cells responded to poly(I:C) demonstrating functional PKR and the ability of their eIF2α to be phosphorylated (Figure 4-4B). These data demonstrate that both GCN2 and PERK are responsible for nucleofection-induced phosphorylation of eIF2α.

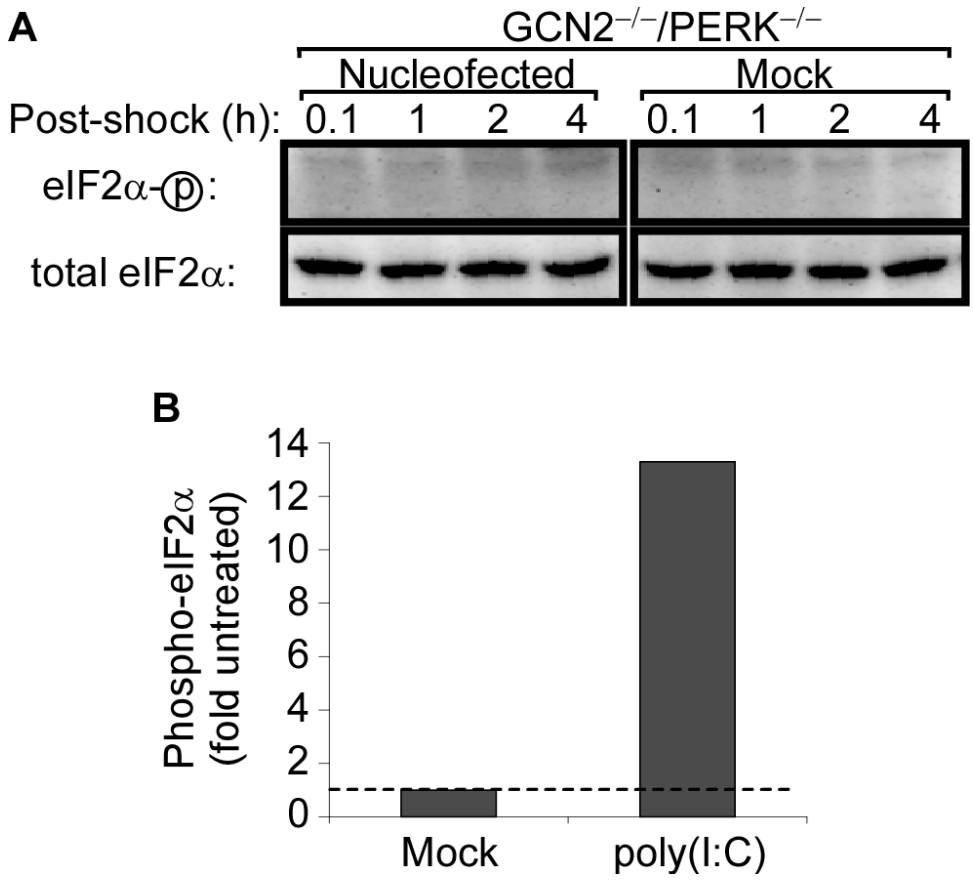

Figure 4-4. Phosphorylation of eIF2α in dual-knockout cells following nucleofection (**A**) GCN2$^{-/-}$/PERK$^{-/-}$ MEF cells were nucleofected or mock-treated, then lysed at indicated time. Phosphorylation of eIF2α was assessed in aliquots of lysate by western blotting. (**B**) GCN2$^{-/-}$/PERK$^{-/-}$ MEF cells were treated with poly(I:C) or mock treated and then lysed four hours following transfection. Phosphorylation of eIF2α was assessed in aliquots of lysate by western blotting and quantified. Band intensities are represented as the ratio of phosphorylated to total eIF2α, expressed as fold increase over eIF2α phosphorylation in mock treated cells (indicated by the dashed line).

4.4 Discussion

We demonstrate that nucleofection induces phosphorylation of eIF2α. In studies using knockout cell lines, we identify GCN2 and PERK as the kinases responsible for the remaining nucleofection-induced phosphorylation of eIF2α. The experiments in dual-knockout cells will be repeated to confirm this finding. We identify that the electrical shock component of nucleofection leads to the activation of GCN2 and PERK and subsequent phosphorylation of eIF2α. This is demonstrated by the lack of induced eIF2α phosphorylation in mock-treated cells, which were subjected to the same handling and buffers as nucleofected cells. This effect of electrical shock has not been previously reported and could be a common feature of electroporation or may be a specific effect of nucleofection. Underhill *et al.* [251] examined eIF2α phosphorylation following electroporation, but did not observe an increase unless DNA was also included in the electroporation. Similarly, data reported by Tesfay *et al.* did not find substantial phosphorylation of eIF2α following electroporation [241]. However, it is likely that the timing and level of eIF2α phosphorylation induced by electrical shock will vary with the electrical parameters.

In single-knockout cell lines, the absence of GCN2 had a pronounced effect on nucleofection-induced eIF2α phosphorylation, while no effect was observed with the absence of PERK alone. Additionally, the kinetics of eIF2α phosphorylation were unique in GCN2$^{-/-}$ cells, where there was little initial phosphorylation of eIF2α, which then increased to peak at 2 hours following nucleofection. In PERK$^{-/-}$ cells, on the other hand, the pattern of eIF2α phosphorylation was similar to that observed in WT cells.

Elimination of nucleofection-induced eIF2α phosphorylation required the absence of both GCN2 and PERK. Together these data suggest that the initial phosphorylation of eIF2α is mediated through GCN2, which is subsequently supplemented by increasing PERK activity.

GCN2 is activated by a range of stresses, including nutrient limitation, proteosome inhibition, oxidizing conditions, high salinity, and UV irradiation. In all cases, it is thought that GCN2 activation requires the binding of uncharged tRNA to GCN2 (see [64]). In contrast to WT cells, in GCN2$^{-/-}$ cells, we observed only low level eIF2α phosphorylation at the earliest time point following nucleofection, suggesting that nucleofection has an immediate impact on the availability of charged tRNAs.

PERK is an ER-associated transmembrane protein that normally exists inactive as a heterodimer with the chaperone BiP. ER stresses, such as excess misfolded protein, cause dissociation of BiP from PERK, allowing PERK homodimerization and activation [19]. The absence of nucleofection-induced eIF2α phosphorylation in GCN2$^{-/-}$/PERK$^{-/-}$ cells suggests that the phosphorylation of eIF2α seen in GCN2$^{-/-}$ cells results from PERK activation and occurs later than GCN2 activation following nucleofection. Nucleofection may directly cause ER stress leading to PERK activation. Alternatively, nucleofection-induced PERK activation may occur only in the absence of GCN2. In this scenario, GCN2 activation following nucleofection results in translational repression. However, in the absence of GCN2, unrepressed translation leads to ER stress and PERK activation. Examining GCN2 and PERK phosphorylation in WT cells and comparing PERK phosphorylation in WT and GCN2$^{-/-}$ cells would clarify if PERK activation is a primary consequence of nucleofection or a secondary consequence of GCN2-knockout.

In MEF cells, the level of nucleofection-induced eIF2α phosphorylation typically returned to baseline levels by four hours following nucleofection, although in some cases extended through 24 hours (data not shown). Given this timeframe, transgene expression following delivery of mRNA is likely to be affected most dramatically, although early expression following plasmid transfection would be impacted as well. Numerous therapeutic approaches using mRNA delivery are under exploration, including transfection of dendritic cells (DCs) with mRNA encoding tumor antigens [92], delivery of mRNA encoding vaccine antigens [192], cancer immunotherapy through transfection of T cells with mRNA encoding chimeric antigen receptors (CAR) [22], stem cell research [198], and induced pluripotent stem (iPS) cell generation [4, 126, 238, 261, 266]. Notably, nucleofection delivery has now translated into ongoing clinical trials.

In addition to reducing translation of a transfected gene, eIF2α phosphorylation has a general impact by repressing global translation in cells. This is of particular concern for nucleofection of primary cells, which are often more fragile, and where minimal disturbance is requisite. Therefore, my ongoing studies are examining nucleofection-induced eIF2α phosphorylation in human PBMC, DC, and $CD4^+$ T cells. Multiple current trials employ nucleofection of patient's cells for re-administration and minimizing cellular stress to maximize cell survival is important to these approaches.

To achieve maximum benefit from nucleofection, it will be valuable to design methods to obviate nucleofection-induced eIF2α phosphorylation. When serine 51 of eIF2α is mutated to alanine (eIF2α-S51A), eIF2α cannot be phosphorylated, and therefore is unaffected by eIF2α kinase activity, allowing ongoing translation despite eIF2α kinase activation [221, 276]. Co-delivery of mRNA encoding eIF2α-S51A with an

mRNA transgene of interest would generate an increasing pool of non-phosphorylated eIF2α which could offset nucleofection-induced eIF2α phosphorylation. A similar approach was seen to enhance translation following delivery of plasmid DNA [251]. Due to the long half-life of eIF2α, estimated as 10 days [75], this approach would be expected to have a long-lasting impact in transfected cells. Similarly, mRNA encoding inhibitors of eIF2α kinases, such as the vaccinia K3L protein [134], could be co-transfected with an mRNA of interest to limit eIF2α phosphorylation. Alternatively, mRNA transcripts could be designed to take advantage of eIF2α phosphorylation rather than attempting to prevent it. In contrast to the majority of transcripts, translation of select cellular mRNAs is upregulated following eIF2α phosphorylation, including GCN4 [174] and ATF4 [106], and this property is dependent on the 5' UTR of these transcripts. Producing *in vitro* transcribed mRNAs containing the GCN4 5'UTR might therefore allow selective translation through the duration of eIF2α phosphorylation following nucleofection.

In summary, we demonstrate that nucleofection of cells stimulates transient phosphorylation of the translation initiation factor eIF2α, mediated by GCN2 and PERK. Phosphorylation of eIF2α, in general, results in inhibition of translation, limiting translation of transfected mRNA and causing cell stress. These consequences have important implications in the design and delivery of nucleic acid-based therapies.

CHAPTER 5

Conclusions, implications, and future directions

5.1 Summary of results

Cells possess multiple pathways to detect and defend themselves from the danger of exogenous RNA. Here, we establish that exogenously delivered *in vitro* transcribed mRNA activates two of these systems, PKR and the 2-5A system. We further demonstrate that the presence of modified nucleosides in RNA reduces the activation of these RNA sensors, as well as the ability of RNase L to cleave modified RNA. The activation of these systems results in an inhibition of translation of the unmodified *in vitro* transcribed mRNA. In contrast, translation of mRNA containing modified nucleosides remains high as a consequence of reduced PKR and OAS activation. In addition, incorporation of modified nucleosides increases the *in vivo* half-life of mRNA. Prompted by studies of mRNA transfection methods, we also show that nucleofection induces phosphorylation of eIF2α. This effect is independent of mRNA delivery and is mediated through activation of GCN2 and PERK.

5.2 RNA danger recognition

The presence of modified nucleosides in RNA reduces the activity of PKR, OAS, and RNase L compared to unmodified RNA. Previous reports have indicated similar results for other RNA sensors, TLR3, TLR7, TLR8 and RIG-I. Together, these data support an interpretation that the absence of modified nucleosides in RNA is a common

pattern recognized by RNA-responsive host defense sensors. Furthermore, this is consistent with the precedent set by the identification of non-methylated CpG DNA as the ligand for activation of TLR9. A conceptually similar but mechanistically distinct approach is also used by some bacteria, which methylate portions of their own genome. This protects their DNA, whereas external, unmethylated DNA is recognized and degraded by restriction enzymes.

The frequency and types of nucleoside modifications found in RNA increase in parallel with evolutionary complexity. Accordingly, mammalian RNA contains many more modified nucleosides than bacterial RNA. Consequently, the paucity of modified nucleosides in bacterial RNA serves as a molecular pattern for recognition by mammalian RNA sensors [130]. Similar to bacterial RNA, mtRNA contains few modified nucleosides. During apoptosis mtRNA is degraded, but is not degraded in necrotic cell death. RNA from necrotic cells activates DC more than RNA from apoptotic cells. Again, in this scenario the presence of unmodified RNA may function as a danger signal recognized by innate RNA sensors.

Uridine has been reported to be especially important for immunostimulation by RNA [231] and has been identified as an important contributor to activation of TLR7 and RIG-I [66, 67, 111, 116, 253], and immunostimulation is reduced by modification of uridine [115, 130, 131, 219, 231]. Consistent with these reports, we saw that the uridine modification Ψ had the largest impact on mRNA translation, and that RNA containing the uridine modification s^2U was the least activating to PKR and OAS.

The functions of nucleoside modifications in RNA are not well understood. Although some modification sites play important structural roles in tRNA and rRNA, no

specific function has been identified for most sites of nucleoside modification. A contributing factor in the evolutionary development and maintenance of nucleoside modifications in RNA may be the ability to mark self-RNA and distinguish it from pathogenic RNA. Consistent with this proposal, previous work in our lab demonstrated that mammalian tRNA, which is highly modified, does not induce tumor necrosis factor (TNF)α secretion by DC. Here, we additionally demonstrate that mammalian tRNA does not activate PKR. In contrast, mammalian poly(A) purified mRNA induced little TNFα secretion by DC but did activate PKR *in vitro*. Mammalian mRNA contains relatively few modified nucleosides and, therefore, other mechanisms may be needed to prevent its recognition by PKR and other RNA sensors. From this standpoint, nucleoside modification is likely to function in concert with RNA capping, protein-coating of RNA, compartmentalization of RNA sensors, and RNase-mediated control.

5.3 Roles of modified nucleosides in pathophysiology

Hyper-modification of viral RNAs has been reported, with viral RNAs containing 1–15 m^6A compared to 3–5 in mammalian mRNAs [27, 179]. Adenosine m^6A modification has been reported for adenovirus, avian sarcoma virus, reovirus, herpes simplex virus (HSV)1, influenza, Rous sarcoma virus, and simian virus 40 [36, 127, 143, 172, 234]; and m^5C was reported in the mRNA from Sindbis virus and adenovirus [72, 234]. Uniquely, turnip yellow mosaic virus RNA contains Ψ and m^5U [14]. Methylation of viral RNA occurs during the nuclear phase of the viral life cycle, and thus has not been reported for viruses that replicate exclusively in the cytoplasm. Increased nucleoside modification would benefit viruses by preventing immune activation by viral RNAs, and, therefore, may

represent an immune evasion strategy. In contrast to viruses, the level of nucleoside modification in bacterial RNA is vastly reduced as compared to mammalian RNA. In the studies leading up to this dissertation, it was shown that the presence of Ψ in mRNA prevented translation in *E. coli* lysate [131]. Therefore, bacteria may be unable to use the strategy of increased nucleoside modification due to other functional limitations, such as intolerance of nucleoside modifications by the bacterial translation apparatus. To address this possibility, it would be valuable to perform studies examining bacterial translation of mRNA containing m^6A and m^5C, which are naturally-occurring components of eukaryotic mRNA. However, bacteria have taken advantage of nucleoside modifications to develop resistance to antibiotics. Bacteria introduce additional sites of nucleoside modification, primarily methylation, into functionally relevant rRNA sites. These modifications sterically block drug binding and thereby prevent drug activity [71].

RNA modification and RNA-modifying enzymes also contribute to genetic and autoimmune diseases. Dyskeratosis congenita, mitochondrial myopathy, and sideroblastic anemia each result from mutations in pseudouridine synthases [34, 271]. The expression level of TLR7 is linked to the development of systemic lupus erythematosus (SLE) [197, 228]. Fibrallarin, a 2'-*O*-methylating enzyme, is an autoantigen in autoimmune rheumatoid arthritis, SLE, and systemic sclerosis (reviewed in [132]), although the role of RNA methylation has not been explored. With these conditions as precedents, RNA sensors and RNA-modifying enzymes may be involved in other autoimmune conditions as well. In light of the role that nucleoside modifications play in limiting RNA sensor activation, defects in RNA-modifying enzymes should be investigated as risk factors or disease modulators in the development and progression of autoimmune diseases.

5.4 Implications for therapeutic RNA delivery

Gene therapy approaches based on mRNA delivery have advantages over plasmid and viral-vector based delivery [28, 192, 204, 267]. The safety profile for mRNA delivery is excellent, as there is no danger of replication or recombination. RNA cannot integrate into the host genome, which is an inherent risk for plasmid and viral vectors. Having no protein component, mRNA-based delivery avoids the problems of adaptive immunity that limit viral-based delivery. Manufacturing of mRNA is simple and easily scalable. Because mRNA does not need to reach the nucleus, unlike plasmids and some viral vectors, the transfection efficiency of mRNA is high even in primary and non-dividing cells. Protein expression from mRNA is very rapid, beginning within minutes of transfection. Translation from mRNA is directly proportional to the amount of mRNA delivered, allowing dose-dependent control of transgene expression. Gene size restrictions are prohibitive for some vector strategies, but are not an issue for mRNA. The transient nature of mRNA – typical intracellular mRNA half-lives are only hours to days – can be beneficial as well, allowing temporal control of gene expression and cessation of treatment in the event of an adverse reaction. This would be especially beneficial for applications such as iPS cell generation, where temporary expression is required but long-term expression and retention of the vector are undesirable [4, 261, 266].

Use of mRNA containing modified nucleosides retains these valuable features of mRNA-based therapy and also benefits from additional advantages. Delivery of mRNA containing modified nucleosides would permit a reduced dose, due to enhanced protein expression from each transcript. Modified mRNA persists and is translated longer, due to

enhanced stability, which would decrease the frequency of administration needed for long-term replacement. Activation of RNA sensors is reduced by nucleoside modifications, and therefore modified RNA avoids unwanted side effects including inhibition of cellular translation, rRNA cleavage, and pro-inflammatory signaling. Additionally, decreased activation of RNA sensors also reduces the risk of developing adaptive immune responses against the encoded protein(s).

When mRNA is used to deliver a vaccine antigen, it has been proposed that immunostimulation by the RNA could contribute to developing the desired adoptive immune response. Because nucleoside modifications reduce RNA immunogenicity, if modified mRNA were used for vaccine delivery this potential benefit might be compromised. This drawback might be mitigated through careful selection of the nucleoside modification used. For example, incorporation of m^5C in mRNA enhances translation but m^5C-containing mRNA still activates primary DC. Alternatively, a vaccine based on modified mRNA may need to be supplemented with an adjuvant. This would enhance immunostimulation, while still retaining the other advantageous properties of nucleoside-modified mRNA. In addition, the lack of PKR and OAS activation in the DC would allow greater production of important cytokines and costimulatory molecules needed in the generation of an effective adaptive immune response.

For *ex vivo* delivery of mRNA, nucleofection is an efficient delivery approach but, as we demonstrate, also induces phosphorylation of eIF2α. Phosphorylation of eIF2α concurrent with mRNA delivery will limit translation during the critical window when

the concentration of transfected mRNA is at its peak. Strategies to circumvent this caveat are needed to maximize the utility of this transfection method.

Translation of both unmodified and Ψ-containing mRNA was increased in $PKR^{-/-}$ MEF cells compared to WT cells. This result is intriguing, but extrapolation of data obtained through direct comparison must be taken with caution given the clonal nature of the cells used in this study. However, if this result is repeatable in primary cells from $PKR^{-/-}$ mice, it suggests that further translational enhancement of modified RNA can be obtained in the absence of PKR activity. Co-delivery of PKR inhibitors or siRNA with therapeutic mRNA could permit additional translational enhancement.

Importantly, the modifications tested herein all occur naturally in cells and do not run the potential risks associated with chemically synthesized modified nucleosides that do not occur naturally in RNA. Of the modifications tested, U-modifications most consistently reduced RNA sensor activation and Ψ-modification produced the largest increase in translation. Therefore, we propose that of the tested modifications, Ψ-containing mRNA has the largest potential therapeutic benefit. Studies of additional U modifications may yet identify additional modified mRNAs with even better properties offering still greater therapeutic benefits.

5.5 Future directions and applications

Although RNase L cleaves unmodified RNA more efficiently than Ψ-containing mRNA *in vitro*, the half-life of Ψ-modified mRNA is longer than that of unmodified mRNA in both WT and RNase $L^{-/-}$ MEF. Therefore, the factor(s) responsible for the

enhanced retention of Ψ-mRNA remain unknown at this time. The enhanced persistence of Ψ-mRNA may result from reduced nuclease cleavage at Ψ residues. Nucleases that could be responsible for the enhanced stability of Ψ-mRNA include Xrn1 or the exosome, which comprise the predominant RNA degradation pathways [16], or ISG20, an IFN-inducible ssRNA-specific ribonuclease [183]. A related possibility is that the presence of Ψ alters RNA binding by non-RNase proteins which facilitate RNase activity, such as zinc-finger antiviral protein (ZAP) or exosome components [98]. Alternatively, the presence of Ψ has also been shown to increase the thermal stability of RNA duplexes, and in this way could reduce RNA accessibility to ssRNA-specific nucleases.

Additional experiments are needed to expand our understanding of nucleofection-induced eIF2α phosphorylation. In GCN2$^{-/-}$ MEF the timing of eIF2α phosphorylation was delayed and the extent was reduced. This indicates that GCN2 contributes to nucleofection-induced phosphorylation but may not be solely responsible. An initial study in GCN2$^{-/-}$/PERK$^{-/-}$ double-knockout cells suggested that nucleofection does not induce eIF2α phosphorylation if both PERK and GCN2 are absent. Examining GCN2 and PERK phosphorylation in WT and GCN2$^{-/-}$ cells will verify GCN2 and PERK activation following nucleofection. In addition, although inhibition of translation is the expected result of eIF2α phosphorylation, this should be experimentally confirmed and its effect on translation of transfected mRNA should be demonstrated. Finally, the extent and duration of nucleofection-induced eIF2α phosphorylation should be examined in clinically relevant cells such as human T-cells.

The effect of modified nucleosides on other RNA sensors needs to be investigated, including NLRs, ADAR, and HMGBs. We propose that nucleoside modifications will reduce activation of these sensors. In addition to PKR, other unidentified proteins were pulled down from cell lysates by unmodified mRNA by not by Ψ-mRNA, and we hypothesize that some of these proteins may yet be identified as other RNA sensors. It will also be of interest to identify the protein that was pulled down specifically by Ψ-mRNA.

The conceptual value of modified mRNA must also be tested in translational therapeutic models. Delivery of mRNA containing modified nucleosides could readily be tested for treatment of conditions that are currently treated by injection of purified proteins, such as erythropoietin for treatment of anemia, clotting factors VIII and IV for hemophilia, and IFN for cancer and anti-viral therapies. Abundant clinical and pre-clinical trials are investigating the mRNA transfection of DCs for anti-tumor immunotherapy, and these as well as other vaccination strategies could benefit from the use of modified mRNA. The ability to generate iPS cells through transfection of a limited number of transcription factors holds great promise for the development of cell therapies. However, the clinical application of iPS cells may be limited by the risks associated with integration of the retroviral vectors used for transduction. Therefore, delivery of transcription factors encoded on modified mRNA may provide a safer alternative method for iPS cell generation.

APPENDIX A

Induction of HIV-specific T and B cell responses with a replicating and conditionally infectious lentiviral vaccine

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A.1 Abstract

The development of an HIV vaccine that induces broad and potent immunity is critically needed. Viruses, including lentiviruses, have been used as vectors for *ex vivo* transduction of antigens into dendritic cells (DC). We hypothesized that DC transduced with a vector that allows selective infection of DC could induce potent immunity by continually priming DC. A lentiviral vector encoding HIV gag-pol without env would form viral cores in transduced DC, but would release non-infectious particles by budding into endosomes and releasing apoptotic bodies or exosomes containing viral cores. DC function by endocytosing DC-derived apoptotic bodies, and they are specialized in their ability to move endocytic contents into the cytoplasm. We postulated that endocytosis of vector cores could lead to transduction of a second round of DC. In this report, we demonstrate accumulation of viral cores inside transduced DC and show second-round transduction of immature DC that endocytose transduced DC *in vitro*. The effectiveness of immunization of mice with transduced DC to induce specific lymphocyte activation was assessed. Mice developed antigen-specific T cell responses and specific antibodies after immunization. Transduction of DC with a replication-competent but conditionally infectious lentivirus could be a novel vaccine strategy for HIV.

A.2 Introduction

A safe and effective vaccine for HIV is critically needed to combat the worldwide scourge of AIDS. While the correlates of immune protection have yet to be clearly defined, either for protective or therapeutic vaccines, it is widely believed that both $CD4^+$ and $CD8^+$ T cell as well as humoral immunity are important. How sufficiently broad, potent, and sustained responses can be elicited has yet to be determined, and this represents a critical gap in our understanding of how to generate an effective vaccine such that protective or therapeutic immunity can be achieved.

Selection and activation of lymphocytes is a function of antigen presentation by dendritic cells (DC), making DC a logical vehicle for immunotherapy [11]. Early studies showed that DC loaded with tumor extracts [6, 182] or antigenic peptides [6, 41, 164, 182] induced anti-tumor immunity in both laboratory mice [6, 41, 164] and human melanoma patients [182]. More recently, viruses have been used as gene transfer vectors for the *ex vivo* transduction of DC with selected antigens. Lentiviral vectors have a number of advantages over adenoviruses, adeno-associated viruses, poxviruses, and alphaviruses. Transduction is stable due to chromosomal integration and non-dividing cells are efficiently transduced [153]. Lentivirally transduced DC have been studied for their ability to induce anti-tumor immunity. Breckpot *et al.* [31] demonstrated that murine DC transduced with a lentivirus encoding a truncated variant of ovalbumin (OVA) protected against tumor challenge with OVA-expressing cells. He *et al.* [110] recently described the transduction of murine DC with a lentiviral vector encoding OVA, and showed, by direct comparison, superiority to peptide/protein-pulsed DC for the stimulation of T cell responses. B cell responses to lentiviral vector-transduced DC are typically absent.

Optimal antigen presentation depends on the form in which the antigen is delivered as well as DC activation (reviewed in [12]), which is induced *in vivo* by the interaction of pathogen-associated molecular patterns with Toll-like receptors (TLR) on DC, as well as other inflammatory mediators and receptors. Most DC vaccination strategies employ *ex vivo* maturation through one of several agents, such as TLR4-recognized lipopolysaccharide [31, 275] or tumor necrosis factor-α (TNF-α) [165]. Mature DC, after activating T cells, undergo apoptosis [58]. Therefore, regardless of the stability of DC transduction, optimal antigen presentation is limited to the time during which the transduced DC are mature but non-apoptotic. The added complication to induction of immunity against transformed cells and possibly also chronic viral infections is that in the absence of a continued danger signal, the immune response may diminish. This has led to the hypothesis that repeated immunization with cancer vaccines are required [32]. The weak immunogenicity of many important antigens led us to consider whether we could overcome the apoptosis limitation and develop an approach that continues to prime until a strong antigen-specific effector response is achieved.

Lentiviruses have three main genes: gag, pol, and env. The gag and pol genes encode proteins required to replicate nucleic acid and assemble and process the virus, but env is required for infectivity. It has been demonstrated that a vector containing gag and pol but not env makes and releases viral cores [83, 94], but these cores are not infectious. In lymphoid organs, mature DC apoptose after presenting their antigens to T and B cells. The resulting apoptotic bodies are taken up by neighboring DC leading to a process of antigen sharing [2]. DC are highly specialized in moving endocytic contents into their cytoplasm [61, 86, 158, 163, 190, 227], and we hypothesized that uptake of free viral cores or

apoptotic bodies or exosomes containing viral cores could lead to transduction of the engulfing DC. As this would lead to continued priming through multiple cycles of engulfment, vaccination with DC transduced with such a vector would induce strong, specific T and B cell responses against vector constituents.

A.3 Materials and methods

Mice

Female BALB/c mice aged 6 weeks were purchased from Charles River Laboratories (Wilmington, MA) and cared for according to institutional guidelines at the University of Pennsylvania under a protocol approved by the Institutional Animal Care and Use Committee.

DC generation

Murine DC were generated as previously described [122, 154] in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 10 mM HEPES buffer solution (RPMI/10% FBS; Invitrogen) and 20 ng/mL recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN). Media with GM-CSF were replaced every 2–3 days and DC were harvested on day 7.

Lentiviral vector production and determination of copy number

Vectors were created by VIRxSYS Corporation. Their genomic structure and production has been previously described [152]. Briefly, a two-plasmid system packages vector genome, as well as reverse transcriptase and integrase. Viruses were pseudo-typed with vesicular stomatitis virus-G envelope, and copy number was determined by TaqMan quantitative PCR. Vector VRX418 encodes full-length, functional gag-pol, while the otherwise identical control vector, VRX494, does not. Both vectors express eGFP.

Transduction of DC with lentiviral vector

Concentrated vector solution (~10^9 transducing units/mL) was added to DC at a MOI of 50 and incubated overnight. Cells were extensively washed, TNF-α (16 ng/mL; R&D Systems) was added, and cells were incubated overnight. Negative control DC were treated identically except for vector addition. In certain experiments, spinoculation was used (2 hours at 1200 × *g*), where a non-significant increase in transduction efficiency was noted [189].

Second-round transduction was performed by freeze-thawing 1–2 × 10^5 transduced, with either VRX418 or VRX494 (range of transduction efficiencies from 25 to 55%), DC three times using dry ice and a 37º C water bath and adding them to tenfold more (based on the number of transduced cells) autologous DC. The second set of DC were prepared 1 week after the first set. One and 4 days after addition of freeze-thawed DC to new DC, cells were analyzed by flow cytometry.

Real-time PCR

Cells (2.5 × 10^3) were lysed (100 mM KCl, 0.1% Nonidet P-40, 20 mM Tris pH 8.0, 500 μg/mL proteinase K (Sigma-Aldrich, St. Louis, MO) at 60º C overnight, then at 90º C for 25 minutes. Real-time PCR was performed in an Applied Biosystems Prism 7700 Sequence Detector (Foster City, CA) using AmpliTaq Gold enzyme with buffer II per manufacturer's instructions (Applied Biosystems), including 50 mM $MgCl_2$ and 2.5 mM of each dNTP (Promega, Madison, WI). Gag DNA was detected using the following primers and probe: 5'-CAGAATGGGATAGATTGCATCCA-3', 5'-ATCCTATTTGTTCCTGAAGGGTACTAGTA-3', and 5'-[FAM]-

CTATTGCACCAGGCCAGATGAGAGAACC-[TAMRA]-3' (Sigma-Genosys, The Woodlands, TX). Copy number was quantified against a dilution series from lysed ACH-2 cells (NIH AIDS Research and Reference Reagent Program; original source Dr. T. Folks [47, 85]). Quantification of the abortive forms, 2-LTR circles [33], used the following primers and probe: 5'-GCTAACTAGGGAACCCACTGCTTA-3', 5'-TCAGGGAAGTAGCCTTGTGT-
3', and 5'-[FAM]-GTCACACAACAGACGGGCACACACTACT-[TAMRA]-3'.

p24 ELISA on DC culture supernatants

Transduced DC supernatants were analyzed for p24 gag protein content by ELISA (Beckman Coulter, Fullerton, CA).

Electron microscopy with anti-p55 staining

DC were analyzed according to standard EM protocols by the University of Pennsylvania Biomedical Imaging Core and stained using a monoclonal antibody to HIV-1 p55 (ARP313; Centralized Facility for AIDS Reagents, Herts, UK; original source Drs. R. B. Ferns and R. S. Tedder [80, 81] . ARP313 was visualized using anti-mouse IgG labeled with electron-dense gold particles [181].

Vaccination and serum collection

Mature DC were resuspended in PBS at 5×10^6 cells/mL. Mice were injected with 1×10^5 VRX418-transduced DC in a hind footpad. Control mice were injected with 1×10^5 untransduced, matured DC. Immunizations were separated by 2 weeks for two to

three injections. Animals were sacrificed 7–12 days after the final injection, with all animals in a given group analyzed simultaneously. Prior to injections, serum was collected from each mouse using a Goldenrod Animal Lancet (MEDIpoint, Mineola, NY).

Flow cytometry

Transduced DC expressing eGFP were analyzed with the addition of propidium iodide (PI) to exclude dead cells. For intracellular p24 analyses, DC were fixed in 2% paraformaldehyde (Sigma-Aldrich), which quenches eGFP, and permeabilized in PBS/1% FBS/0.1% saponin (Sigma-Aldrich). Samples were stained with anti-p24-FITC antibody (KC57) (Beckman Coulter) for 30 minutes at room temperature, washed, and analyzed. Samples for the above experiments were acquired on FACScan and FACSCalibur flow cytometers (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

For Tetramer analyses, splenocytes were incubated with Fc block (anti-mouse CD16/CD32; BD Pharmingen), and then stained with MHC class I tetramer H-2K^{d}/AMQMLKETI-streptavidin-PE (NIAID MHC Tetramer Facility, Atlanta, GA; peptide from New England Peptide, Gardner, MA) [161]. Samples were then stained with anti-mouse antibodies CD3e-FITC, CD11b-PerCPCy5.5, and CD8a-PE-Cy7; or CD62L-FITC, CD8a-PerCP, and CD11b-allophycocyanin (BD Pharmingen). For certain samples, prior to running, TO-PRO-3 (Invitrogen) was added.

For intracellular cytokine analyses, splenocytes (5×10^6 cells/mL) were stimulated with a gag peptide pool (15-mer peptides spanning the protein with 11 amino acid overlaps, each peptide at 0.25 μg/mL; NIH AIDS Research and Reference Reagent

Program, No. 8117), or a pool of irrelevant peptides (equally concentrated env clade C 15-mer peptides; NIH AIDS Research and Reference Reagent Program, No. 9499). As a positive control, splenocytes were treated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin calcium salt (Sigma-Aldrich). Human recombinant IL-2 (50 U/μL) was added to enhance IFN-γ production [194]. After 1 h, 10 μg/mL brefeldin A (Sigma-Aldrich) was added. After four more hours, cells were treated with PBS/0.5 mM EDTA for 10 minutes and then stained with the anti-mouse antibodies CD44-FITC, CD4-PerCP, CD8a-PE-Cy7, and CD3eallophycoyanin-Cy7 (BD Pharmingen), fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin, and stained for IFN-γ-PE and IL-4-allophycocyanin (BD Pharmingen). For tetramer and intracellular cytokine studies, 1×10^6 events were acquired on a FACSCanto (BD Biosciences) and analyzed using FlowJo software.

p24 antibody ELISA of serum samples

Serum samples were added in triplicate at 1:50 and 1:500 dilutions to 96-well plates coated with HIV gag p24 protein (Immunodiagnostics, Woburn, MA). A standard curve was constructed using a purified anti-HIV-1 p24 monoclonal antibody (183-H12–5C; NIH AIDS Research and Reference Reagent Program; original source Dr. Bruce Chesebro and Kathy Wehrly [46, 245]). Detection antibody (anti-mouse IgG-peroxidase antibody; Sigma-Aldrich) was added followed by a 50/50 solution of 3,3',5,5'-tetramethylbenzidine and H_2O_2 (KPL, Gaithersburg, MD), and absorbance was measured at 450 nm on a Dynex Technologies MRX microplate reader.

Statistics

Statistics were performed using Microsoft Excel. Standard errors of the mean were calculated from triplicate measurements of a sample. Standard deviation and Students *t*-test (two-tailed) were used for comparing populations.

A.4 Results

Lentiviral transduction of murine DC

We first determined the efficiency of transduction of the HIV vector containing functional gag-pol, VRX418, and the otherwise similar vector VRX494, which lacks a functional gag-pol (Fig. A-1). During preliminary experiments, we established a protocol in which bone marrow-derived murine DC were transduced on day 7 at an MOI of 50. Enhanced GFP (eGFP) expression gave a typical transduction of 20–35% clearly positive cells after 1 day, which was still present 8 days later (Fig. A-2A and B). These data show that VRX418 and VRX494 efficiently transduced cultured bone marrow-derived murine DC. DC were also tested for intracellular p24 content. Fig. A-2C and D show that over 50% of VRX418-transduced cells stained positive after 1 day, which could be argued to be input virus except that high-level p24 staining remained through 8 days of culture, while VRX494-transduced DC had baseline p24 staining. The difference in transduction efficiencies as measured by eGFP and p24 could be explained by the pattern of eGFP staining where a single shift in expression is observed, but not all cells express enough eGFP to move beyond the brightest of the unstained population.

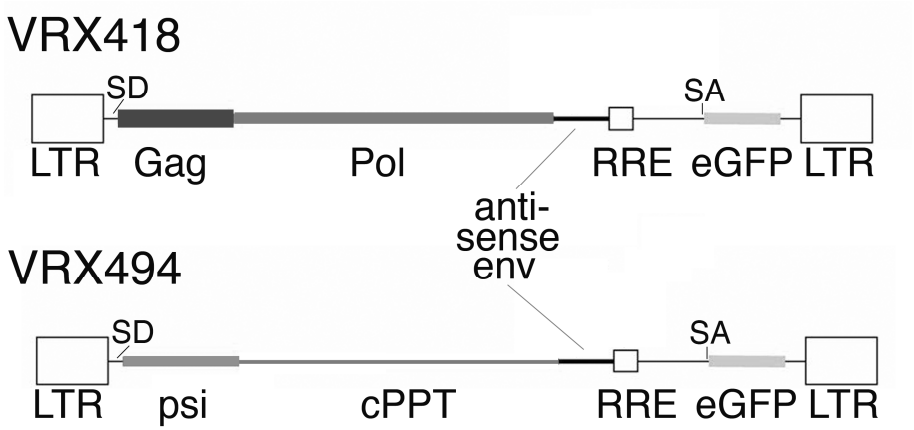

Figure A-1. Map of the VRX418 and VRX494 lentiviral vector genomes
VRX418 and VRX494 are derived from the NL4-3 molecular clone of HIV-1. VRX418 contains 5'- and 3'-LTR, full-length and functional gag and pol, splice donor (SD) and splice acceptor (SA) sites, an anti-sense envelope payload, the Rev response element (RRE), and eGFP. VRX494 differs from VRX418 in that the gag-pol gene is replaced with the genome packaging site (psi) and the central polypurine tract (cPPT).

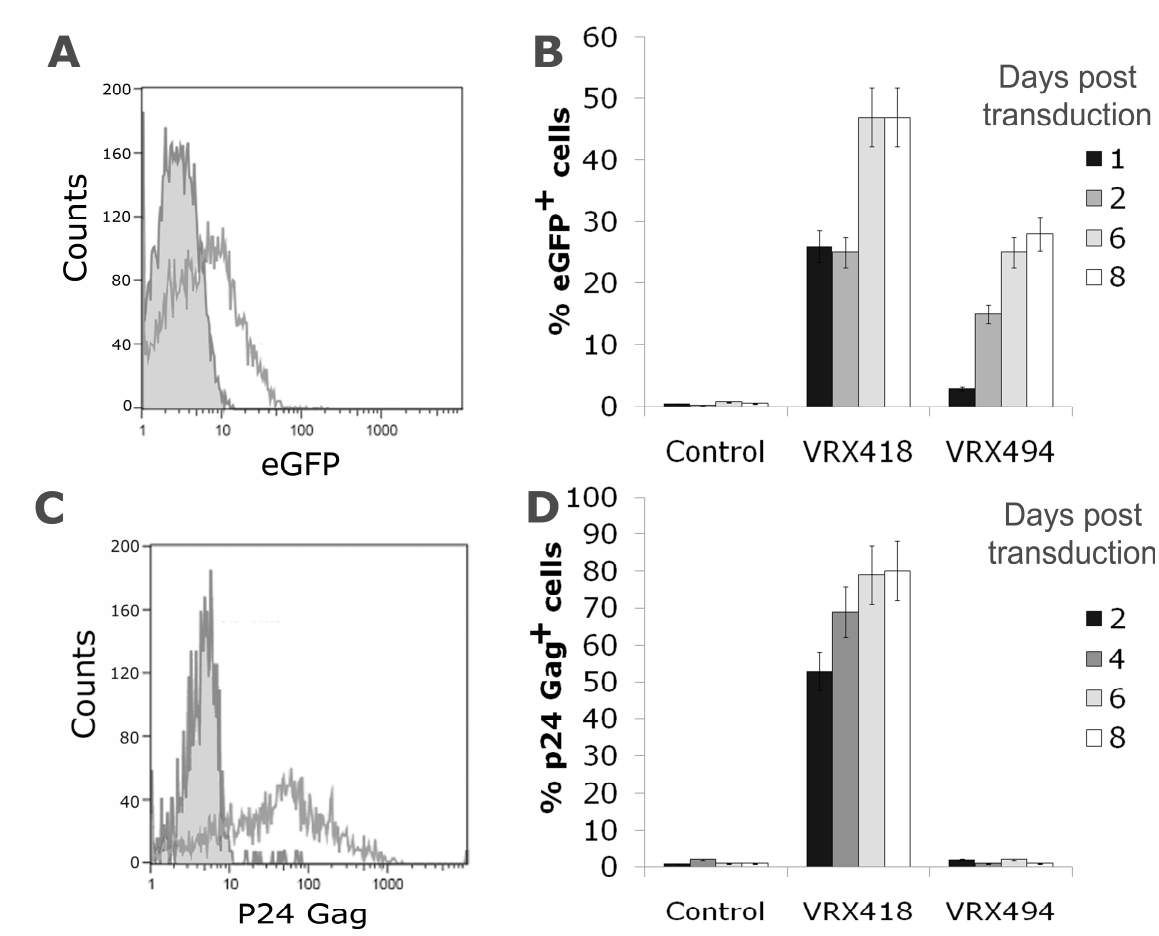

Figure A-2. eGFP and p24 gag protein expression in transduced DC
Bone marrow-derived DC were transduced with VRX418 or VRX494 at an MOI of 50. Flow cytometric analysis was carried out to determine eGFP or intracellular p24 gag expression. (**A**) Day-1 plot of eGFP expression in live cells in VRX418- (empty) versus untransduced (shaded) DC. (**B**) Continued eGFP expression in VRX418- and VRX494-transduced DC through day 8. (**C**) Day-1 plot of intracellular p24 gag staining in VRX418-transduced DC (empty) versus untransduced (shaded) DC. (**D**) Intracellular p24 gag content remained elevated through day 8 post-transduction. Error bars are standard error of the mean for triplicate measurements. Data are representative of three experiments.

The high level of intracellular p24 by day 1 shows that gag is produced quickly in transduced cells, but this does not clarify whether similar levels of continued production occur. We quantified the amount of p24 in transduced cell culture supernatants over days

1 through 8. To rule out the possibility that input gag from the vector contributed significantly to the measured values, we employed the control vector VRX494, which included a similar amount of input gag, as measured by p24 gag content of viral stocks, but does not encode functional gag protein. On day 1 post-transduction, cells were washed three times to remove input virus, and supernatants were collected after 4.5 hours and then on following days. As shown in Fig. A-3, p24 levels were high (>30 ng/mL) and remained high over all days of analysis in the VRX418 sample but were baseline in the VRX494 culture.

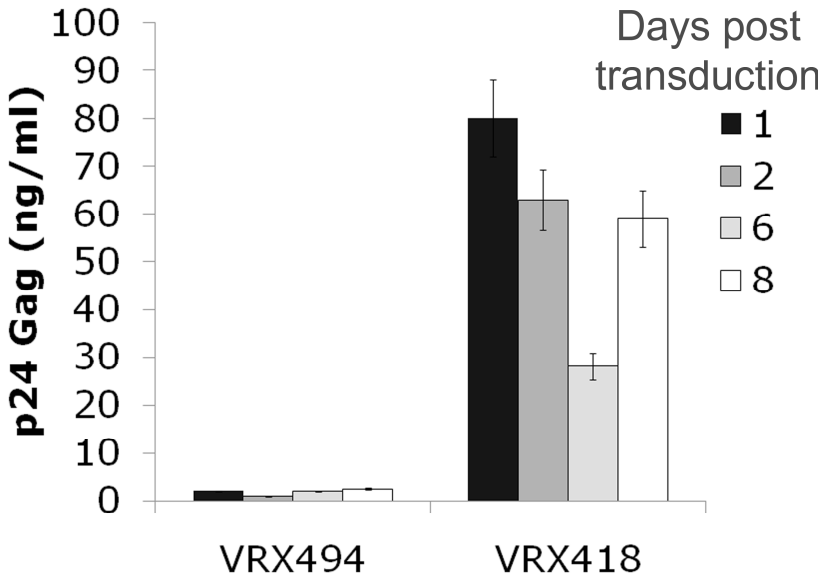

Figure A-3. Extracellular p24 gag production by transduced DC
Bone marrow-derived DC were transduced with VRX418 or VRX494 at an MOI of 50. Transduced cells were washed three times the following day to remove input virus, and supernatants were collected on the indicated days. Supernatants were analyzed for p24 gag content by ELISA. The day-6 level of gag protein is likely artificially low as fresh medium was added to the cells prior to the removal of supernatant for analysis. Error bars are standard error of the mean for triplicate measurements. Data are representative of three experiments.

Having proven strong and continued transgene expression, we next sought to investigate the formation and integration of vector DNA in transduced cells with real-time PCR. The number of gag DNA copies exceeded the number of cells in culture (data not shown), suggesting either that each cell underwent multiple integrations or that abortive, non-integrating DNA forms were present. We assayed for 2-LTR circles (and 1-LTR circles by implication) [33], and we were able to show their presence, demonstrating that abortive integrations accounted for a portion of the vector DNA in DC.

Formation of viral cores in transduced cells

The inclusion of full-length gag-pol within the coding region of the vector would lead to viral core formation in transduced cells. These would not be expected to form infectious particles, however, since there is no envelope present, but they could still bud [83, 94] into endosomal compartments and be released (reviewed in [141]) or be released within exosomes (reviewed in [60]) or apoptotic bodies. To investigate this phenomenon, transduced DC were analyzed by electron microscopy (EM) with and without staining for HIV-1 p55. Illustrative photomicrographs from triplicate sample preparations are shown (Fig. A-4).

In unstained samples of transduced DC (Fig. A-4A), small round cores (arrows) demonstrated the formation of vector cores. p55 staining confirmed consistent, significant p55 presence and clumping within transduced cells (Fig. A-4B) with absence of staining in untransduced samples (Fig. A-4C). Close grouping of p55 molecules in transduced samples confirmed viral core formation. The p55 monoclonal antibody specifically stains immature gag. As an additional control, viral stocks were stained for p55 and fewer than 1% of the viruses had measurable staining. This demonstrated that the viral stock contained mature virions that did not stain. The identification of stained viral cores within DC demonstrated that these were newly formed virions and not trapped input virus. One day after pulsing, a time when exogenously delivered virus is cleared from endosomes [249], viral cores were observed that had budded into endosomal-like compartments (Fig. A-4D). In fact, one of the two virions in the endosome in Fig. A-4D has the morphology of a matured virion with a well-defined core region. These results correspond closely with

the antibody staining shown previously [181], and the photomicrographs displayed here show particles similar to the HIV-1 cores seen in the EM studies of other groups [170, 233].

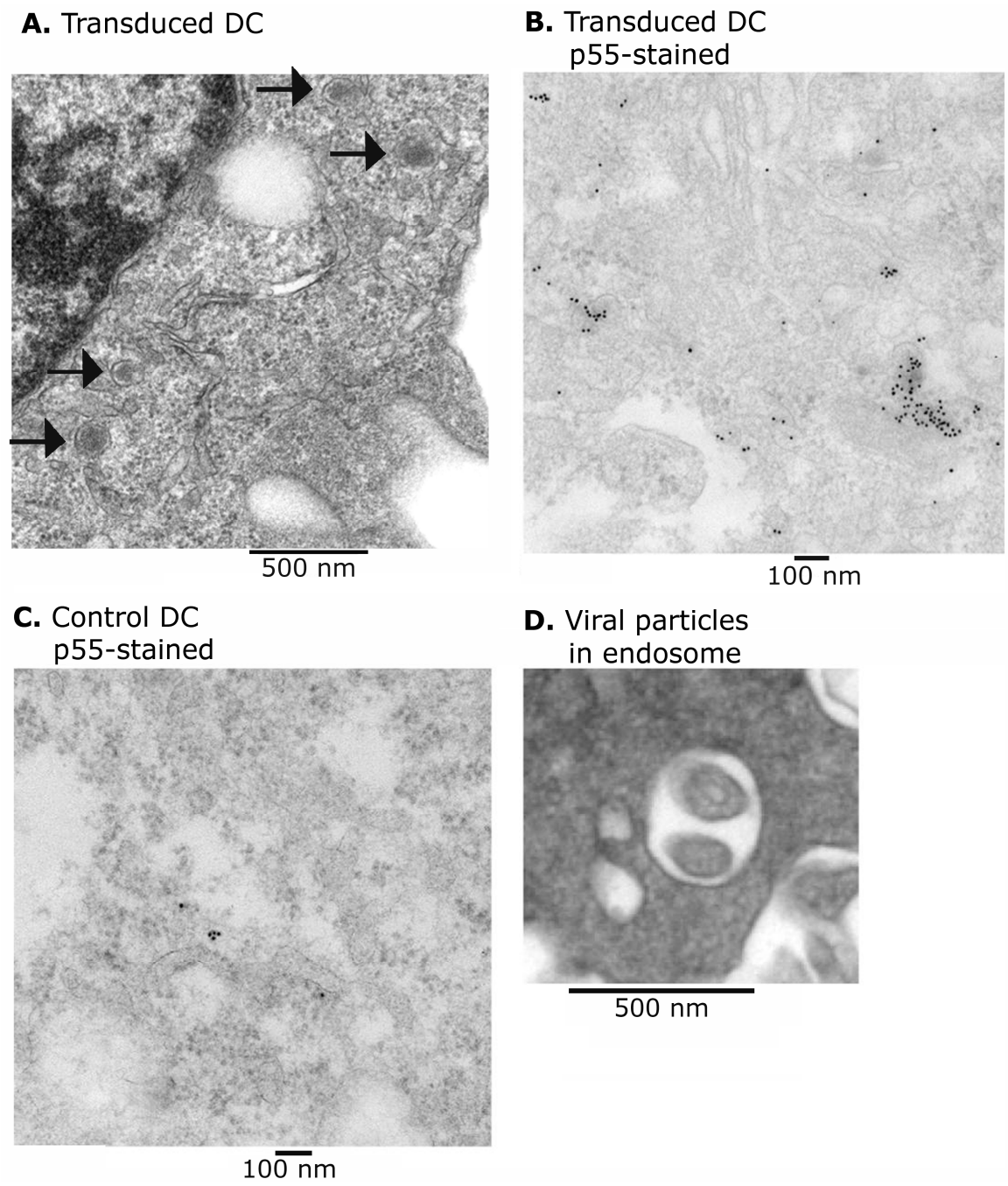

Figure A-4. EM of bone marrow-derived DC transduced with VRX418
Cells were transduced at an MOI of 50 and placed in culture for 24 hours. Certain samples were stained with a monoclonal antibody to HIV-1 p55; the antibody was visualized using a secondary antibody with attached electron-dense gold particles. (**A**) High-power view showing viral cores (arrows) accumulating in the cytoplasm of transduced DC. (**B, C**) Gag p55 antibody-stained transduced (B) and untransduced (C)

DC. Transduced sample (B) shows a clumping distribution of p55 overlying viral cores. (**D**) High-power view showing two viral particles in an endosome-like compartment. One and possibly both of these particles demonstrate the classic appearance of mature particles with a central core. Scale bars are 100 or 500 nm. Data are representative of three preparations of transduced DC.

"Second-round" transduction of DC

We next tested whether immature DC could be transduced by uptake of viral cores from previously transduced cells. This ability would demonstrate that multiple rounds of transduction are possible, as each generation of DC is transduced by uptake of viral cores from the previous generation. DC were transduced with either VRX418 or its non-replicating counterpart, VRX494, and washed extensively to remove unbound virus. After a 24-hour incubation, cells were washed again, analyzed for eGFP expression, and equal numbers of transduced DC (5×10^4), set to 1 (Fig. A-5), were freeze-thawed three times to ensure complete cell death. New, immature DC were then added to each sample at a 10:1 ratio (5×10^5) to the transduced DC.

eGFP analysis completed 1 and 4 days later showed that added cells in the VRX418 sample had been transduced as noted by an increase in the number of transduced cells to 4.5-fold greater than the input number of killed transduced DC, i.e. an increase from 5×10^4 transduced killed cells to 2.26×10^5 newly transduced added cells (Fig. A-5). VRX494-transduced cells that were pulsed with equal numbers of infectious units of vector demonstrated no transduction of second-round DC, indicating that initially added, trapped vector was not responsible for the second-round transduction.

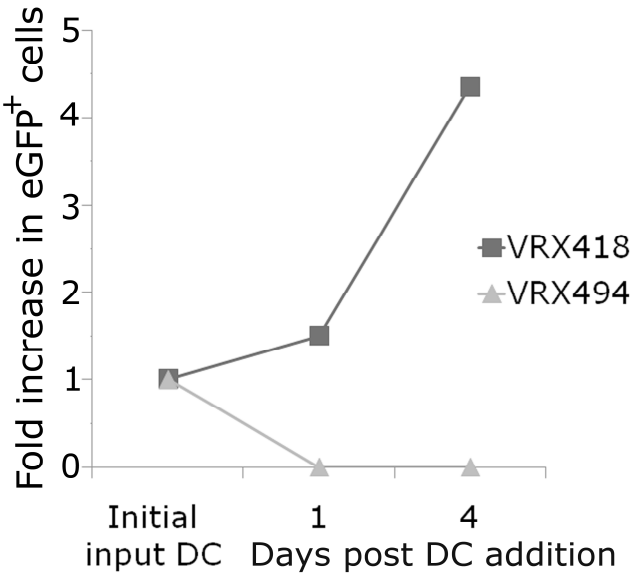

Figure A-5. Second-round transduction
Bone marrow-derived DC were transduced with either VRX418 or VRX494. After overnight incubation and extensive washing, flow cytometric analysis was performed, and the number of transduced cells in each sample was normalized to allow the addition of equal numbers of cells that were transduced (set to 1) to tenfold more untransduced immature DC. The first-round DC were freeze-thawed three times to ensure complete cell death, and immature second-round DC were then added. After 1 and 4 days, flow cytometric analysis was performed, which showed increasing transduction in the second-round DC in the VRX418 sample but not in the non-replicating control (VRX494). Data are representative of three experiments.

T and B cell responses to vaccination

We next sought to test the immunogenicity of vector-transduced DC in a small animal model. Lentiviral vectors, especially ones based on HIV, that are used in mice have mammalian promoters to produced tumor-associated antigens [110, 165, 191, 274, 275]. The commonly used Balb/c mouse model should be deficient in the ability of our vector, which uses HIV's promoter system, to replicate and undergo second-round transduction, as a post-integration block to HIV replication in mice has been described (reviewed in [50]), although the above data showing p24 gag protein and viral cores suggests that murine DC

are capable of some level of protein production and virion formation.

Initial experiments looking for subsequent rounds of DC transduction *in vivo* in mice found a rapid extinction of vector-derived PCR signal in lymphoid tissue, suggesting that in the mouse, this vector could not efficiently undergo multiple rounds of transduction. Thus, repeated immunizations of vector-transduced DC were given to Balb/c mice. We delivered DC by subcutaneous injection, a route proven to lead to DC trafficking to draining lymph nodes [205] and induction of immune responses (reviewed in [91, 92]). DC were transduced on day 7 of culture, TNF-α was added on day 8, and 1×10^5 cells, either transduced with VRX418 or untransduced controls, were injected on day 9, with two or three injections of freshly transduced DC separated by 2 weeks. Following the final immunization, mice were sacrificed after 7–12 days.

MHC class I tetramer H-2K^d/AMQMLKETI staining demonstrated significant (p=0.0004) expansion of gag-specific CD8$^+$ T cells. Data for a single experiment (Fig. A-6A) and cumulative data for all immunized mice are shown (Fig. A-6B). Splenocyte populations were assayed for the intracellular production of IFN-γ and IL-4 in response to stimulation by either a gag peptide pool or a pool of irrelevant peptides. Mice developed significant CD8 responses as measured by IFN-γ expression (data not shown). CD4 responses were also monitored, and VRX418 DC-immunized mice developed significant (p=0.00012) CD4$^+$ T cell IFN-γ responses. Data for a single experiment (Fig. A-6C) and cumulative data for all immunized mice are shown (Fig. A-6D). No mouse developed a population of IL-4$^+$ CD4$^+$ T cells, arguing that a Th1 response was induced.

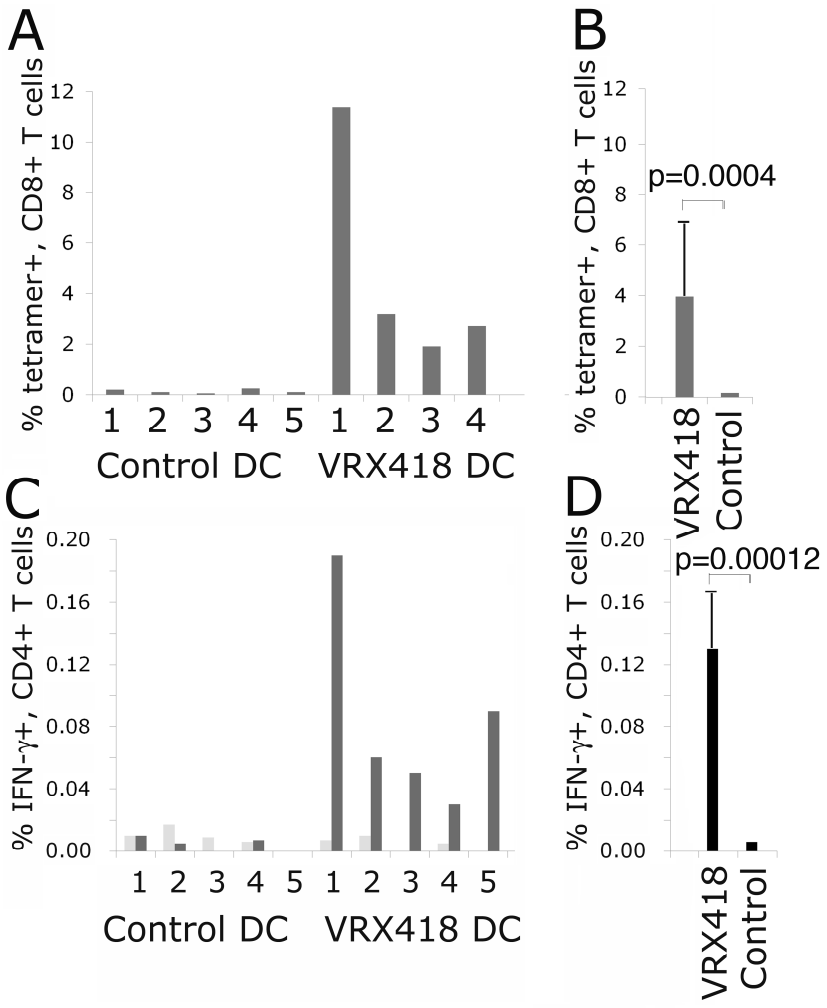

Figure A-6. T cell responses to vaccination with VRX418-transduced DC
Bone marrow-derived DC were transduced with VRX418 at an MOI of 50. (**A**) TNF-α-treated, transduced (VRX418) or untransduced (control) DC (1×10^5) were injected into the footpads of mice. Mice were sacrificed 7 days after a boost injection. Splenocytes were analyzed for tetramer (MHC class I tetramer H-2K^d/AMQMLKETI) expression on CD8$^+$ T cells. One vector mouse did not receive the second immunization and was removed from this analysis. (**B**) Cumulative data for all immunized mice (14 VRX418 and 15 control mice) are shown. Averaged tetramer staining level for each mouse is shown with standard deviations. (**C**) Transduced (VRX418) or untransduced (control) DC were injected into the footpads of mice every 2-weeks for three immunizations. Mice were sacrificed 11 days after the final injection. Splenocytes were analyzed for antigen-specific IFN-γ production by CD4$^+$ T cells. Data shown are the percentages of CD4$^+$ T cells that expressed high levels of intracellular IFN-γ. (**D**) Cumulative data for all immunized mice are shown. The percent CD4$^+$ T cells expressing IFN-γ were corrected by subtracting the level of staining from the irrelevant peptide pool averaged. Three

separate experiments with groups of five mice (represented by individual numbers) were immunized and analyzed, and representative (A, C) and all (B, D) data are shown.

Serum was collected from each of the animals before each injection (days 0, 14, and 28) and at sacrifice (days 35–40). A p24-binding antibody ELISA was performed and the results are shown in Fig. A-7. A purified gag p24-specific monoclonal antibody was used to generate a standard curve in order to quantify the amount of gag-specific antibodies in serum. By the third immunization, every mouse immunized with VRX418-transduced DC developed high antibody levels (10–120 μg/mL).

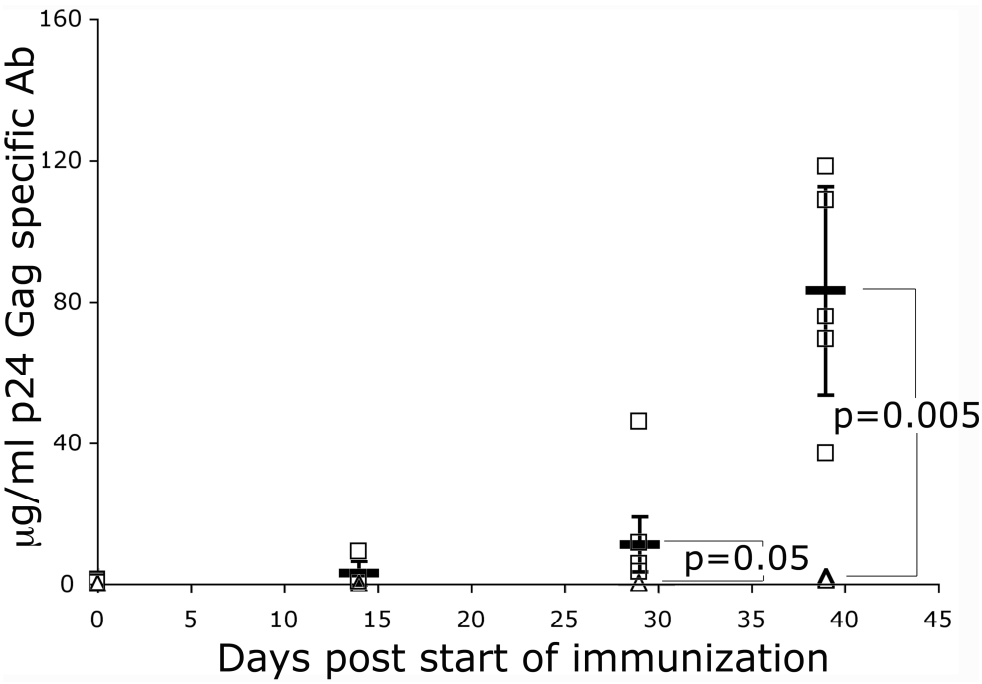

Figure A-7. Antibody responses to VRX418-transduced DC vaccination
TNF-α treated, VRX418-transduced (squares) or untransduced (triangles) DC (1×10^5) were injected into the footpads of mice. Two boost injections were performed at 2-week intervals. Serum was collected before each injection (days 0, 14, and 28) and at sacrifice (day 39). Serum samples were analyzed in a direct binding ELISA against a standard curve constructed with an HIV-1 p24 monoclonal antibody. Data are representative of three experiments. Mean (horizontal bar) and standard deviation (error bars) are shown. *p*-values compare VRX418-transduced DC to control DC-immunized mice.

A.5 Discussion

The purpose of this study was to investigate whether a conditionally infecting lentivirus could infect a second round of DC by the release of viral cores, either free or as part of apoptotic bodies or exosomes that are taken up by new DC leading to infection through the endosomal route. We observed second-round transduction of DC exposed to killed transduced DC, *in vitro*. Upon vaccination with these transduced DC, we observed potent anti-gag T and B cell activation. Unlike most studies that have induced T cell responses against diverse antigens through a variety of lentiviral approaches [78, 110, 165, 191, 274], the potent B cell response is novel.

While we were able to demonstrate second-round infection of DC *in vitro*, we could not demonstrate this *in vivo* and needed to reimmunize mice to obtain potent responses. This is likely due to the post-integration block to HIV replication in mice [50] that makes second-round transduction an inefficient process. The likely mechanism for the potent CD4, CD8, and B cell response observed with VRX418 was the continuous production of gag-pol protein that was released as free protein or viral-like particles. The role of second-round infection/transduction in immune response development, *in vivo*, could not be measured, but remains the focus of future studies.

VRX418 efficiently transduced bone marrow-derived murine DC, as evidenced by eGFP expression (Fig. A-2A and B), intracellular and extracellular p24 gag protein (Fig. A-2C, D and A-3), and the presence of gag DNA in transduced cells (data not shown). EM studies demonstrated approximately 100-nm viral cores in the cytoplasm and endosomes and a clumping distribution of anti-p55 antibody in association with cores within the cytoplasm of transduced cells (Fig. A-4). We hypothesized that these viral

cores would become accessible to immature DC for uptake by phagocytosis of apoptotic vesicles or exosomes. In addition, we observed mature virions in endocytic vesicles (Fig. A-4D) that could be released from transduced DC. Uptake by immature DC would then make possible a "second round" of transduction if vector cores could escape from the endosome to transduce the engulfing cell. This has been demonstrated to be a pathway for HIV infection in multiple cell types [54, 82, 105, 113, 193, 257].

Figure A-8 illustrates this hypothesis in the context of our experiments. We designed *in vitro* investigations where we killed transduced cells that contained no original infectious vector and then added fresh, immature DC. When we analyzed these added DC by flow cytometry 1 and 4 days later, we found an increasing level of eGFP expression (Fig. A-5), indicating that these newly added, immature cells had been transduced by the viral cores present in the apoptotic DC. To exclude the possibility that the new DC were actually infected by free infectious virions from the initial transduction, we (i) waited a length of time shown in other studies to degrade endocytosed virus in DC and (ii) used DC infected with the same MOI of VRX494, a vector incapable of replication, and observed no increase in eGFP expression in exposed DC (Fig. A-5). This demonstrates that our "second-round" hypothesis occurs *in vitro*.

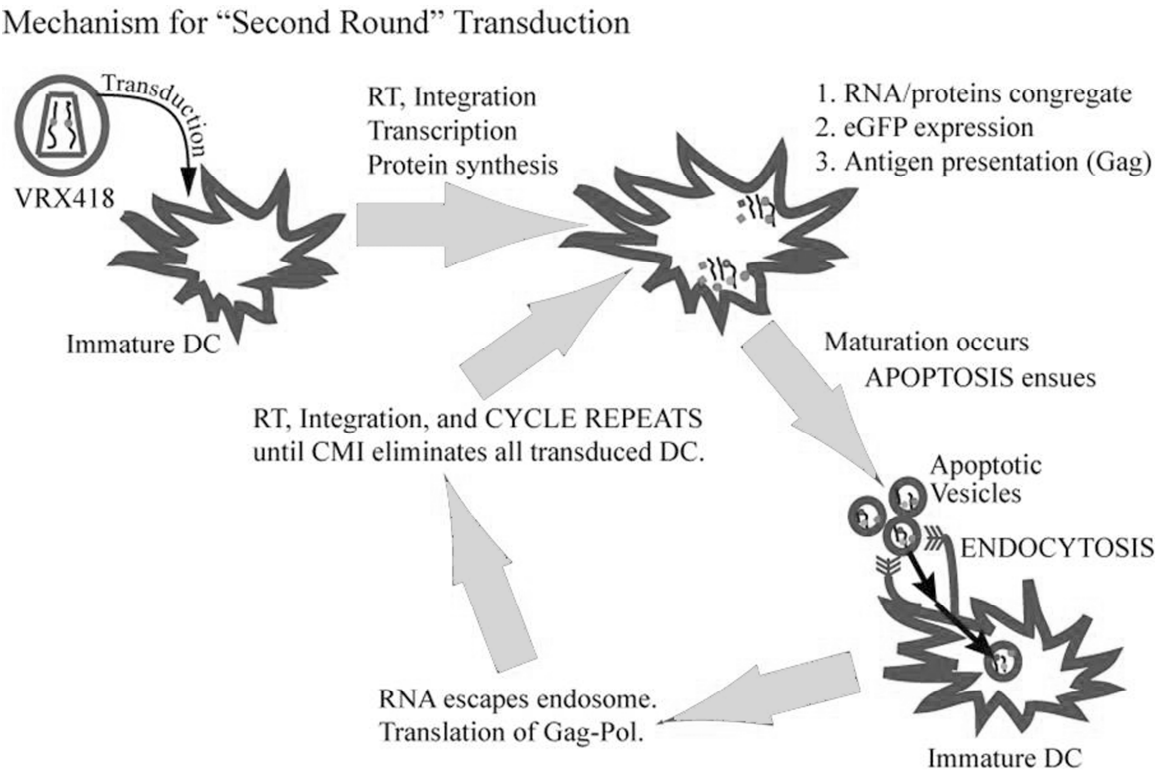

Figure A-8. Illustration of mechanism for second-round transduction
Viral cores formed in the cytoplasm of DC transduced with the self-replicating, conditionally infectious lentivirus VRX418 are released by budding into endocytic vesicles followed by fusing of the vesicles with the plasma membrane, release of apoptotic bodies containing viral cores (pictured), and release of exosomes with viral cores. The viral cores are accessible to immature DC by endocytosis. Viral cores gain entrance to the DC cytoplasm and begin a second round of transduction. The cycle continues, with each round of transduced DC presenting antigen to T and B cells and activating specific immunity against the vector-derived antigens. The continued presentation would only end when cell-mediated immunity was able to eliminate transduced DC. Thus priming continues until a potent response develops.

When mice were immunized with transduced DC, we observed no significant evidence of second-round transduction in limited experimentation, and we had to reimmunize mice to generate a potent response. It is understood that virions need to bud before efficient maturation occurs, and this would be required for second-round transduction. We believe that apoptotic blebbing of viral cores, or endocytic release of

viral cores as was observed by EM, led to virion maturation, but second-round transduction was a relatively inefficient process *in vitro* and likely a more inefficient process *in vivo* in the mouse model system where a post-integration block to viral replication has been observed [50]. Our *in vitro* studies demonstrate that vector/viral replication in murine cells is not completely blocked. This is supported by other studies (reviewed in [65]). It has been observed that by simply codon optimizing viral sequences, the production of infectious virions from murine cell lines could occur [65]. Further studies are needed to optimize second-round transduction *in vivo* in models where HIV- or SIV-based vectors replicate efficiently.

Although antigen transfer in lymph nodes has been studied [17, 37], the mechanism in our system differs from the native activity of circulating and resident DC in that replication-competent viral cores are the antigen transfer vehicle. Studies of cross-presentation have demonstrated that breakdown of endosomes and release of their contents occurs [61, 86, 158, 163, 190, 227], especially after certain types of DC activation including those induced by TLR3 and TLR9 ligands [55, 224]. Proteins made within a cell are processed and presented via the MHC class I pathway, whereas exogenous proteins are presented by MHC class II. Although DC cross-presentation of exogenously obtained peptides by MHC class I is often discussed [59, 89], MHC class II presentation of endogenous antigens is a more novel concept. Typically, antigens produced within a DC to which a CD4 response is desired are linked to an endosomal protein such as lysosome-associated membrane protein 1 [175]. A likely explanation for the strong CD4 response in our study is that DC secreted and then endocytosed gag protein or whole viral cores, thus enabling processing via the MHC class II pathway. Our laboratory has previously

demonstrated this phenomenon after gag mRNA transfection of PBMC-derived human DC [262]. The potent antibody response directed against gag was likely induced by a similar mechanism of release of gag protein or whole virions and activation of B cells.

Our work may be initially applicable as an HIV vaccine strategy, although future uses could include immunization against a variety of transgene-encoded antigens. The antibody responses to gag are not useful against HIV and do not constitute a viable vaccine strategy. However, given the strong and broad immune responses that the mice developed, a reasonable strategy could be modifying the vector to encode a defective env that induces neutralizing antibodies, or even to use a membrane-based protein that contains neutralizing antibody-inducing epitopes [62, 87, 119, 137, 284]. Such a strategy could generate potent T cell immunity against gag-pol and potentially env as well as a neutralizing antibody response to envelope.

We observed potent $CD4^+$ and $CD8^+$ T cell and antibody responses. In a direct comparison of vaccines, a *Listeria monocytogenes* recombinant vaccine expressing HIV-gag [162] arm was included in our studies. We observed that there was a similar gag-specific tetramer and $CD4^+$, IFN-γ^+ response comparing *Listeria* to our lentiviral approach (data not shown). No antibody response was observed with the *Listeria*-gag vaccine. The response induced by a vaccine is dependent on its mode of delivery of antigen to the APC. Adjuvanted protein typically induces $CD4^+$ T cell and antibody responses but is deficient in $CD8^+$ T cell responses because no cytosolic protein production occurs (reviewed in [147]). Attenuated viruses with limited ability to replicate in cells induce $CD4^+$ and $CD8^+$ T cell and antibody responses are typically used as boosters to other priming vaccines (protein and DNA). The amount of gag-specific antibody in our

study appears typical of the level observed in these studies (reviewed in [147]).

An interesting facet of this work is the efficiency with which the transduced DC produced gag protein (Fig. A-2C, D and A-3) in the absence of Rev and Tat. The vector is designed such that a 5'-splice donor site exists between the 5'-LTR and the gag sequence, and a 3'-splice acceptor site is present downstream of the Rev response element and 5' to eGFP. The presence of Rev and Tat enable production of full-length genomes, a fact that is employed during production of the vector for clinical use [152]. In the absence of Rev and Tat, however, minimal promoter activity and splicing of any transcripts produced should occur, yielding predominantly eGFP mRNA. Our results, however, indicate that murine DC are able to bypass these mechanisms and express large quantities of gag in a Rev- and Tat-independent manner, although an explanation for this phenomenon is not readily apparent. It is possible that transcription occurs due to the high levels of NF-κB members in DC (reviewed in [10]), which are augmented by TNF-α activation. Nonetheless, the extremely high level of gag production in the absence of Rev remains surprising.

Our lentiviral construct contains the necessary components to form viral cores but cannot produce infectious virus since no envelope is present. Insertional mutagenesis is a chief concern with any lentiviral approach, as recent adverse events from retroviral therapy have made clear [99]. This concern is lessened, however, when lentiviruses are used to transduce only terminally differentiated cells, as in this *ex vivo* DC system. The specific vector employed, VRX418, has been constructed by VIRxSYS Corporation (Gaithersburg, MD) in a manner analogous to the first lentiviral vector approved for use in a clinical trial, VRX496 [118, 152, 159]. Production safety mechanisms are therefore already

in place to allow promising vectors to enter clinical trials expeditiously, and issues pertaining to scaled manufacturing have been addressed [152]. Additional safety and production issues for lentiviral vector work have been recently reviewed [230].

Antigen-specific immune responses form the basis of host defense against most infectious and neoplastic processes. The development of an effective vaccine against HIV that either alters the course of infection or induces sterilizing immunity is critically needed. We have demonstrated strong and broad immune responses in mice vaccinated with DC transduced with the replicating conditionally infectious lentiviral vector VRX418. This study identifies two future directions: (i) to study in a more appropriate model and improve the *in vivo* second-round transduction that will allow a single injection of transduced DC to induce a potent and broad immune response, and (ii) to understand and develop the current vector to induce antibodies with neutralizing activity against HIV.

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