University of Alberta

Examination of ADAR – RNA and ADAR – ADAR Interactions

by

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ABSTRACT

RNA within a cell is subject to multiple processing events prior to undergoing translation, including RNA editing through the chemical modification of RNA bases. An example of this is adenosine deamination to produce inosine, a reaction carried out in mammalian cells by the adenosine deaminases that act on RNA (ADARs). The experiments presented in this thesis examine both ADAR – RNA and ADAR – ADAR interactions using *in vitro* and *in vivo* techniques. Through the use of footprinting we were able to identify regions on a model substrate which were protected upon binding of an ADAR2 homodimer. Utilizing fluorescence microscopy, we were able to determine that ADAR1 and ADAR2 form both homodimers and heterodimers within the nucleoli of HeLa cells. This thesis also reports our endeavors at characterizing novel extra-nucleolar regions to which ADAR1 was discovered to localize. The results within suggest methods by which adenosine deamination is regulated within the cell.

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DEDICATION

I would like to dedicate this thesis to the three people without whom it would not have existed.

To my Mother: Thank you for being my best friend. You have always been there for me, good times and bad. Thank you for being a shoulder to cry on, a voice of reason, and a partner in my celebrations. I am truly blessed to have you in my life.

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CHAPTER 1

Introduction

Primary RNA transcripts can undergo a range of post-transcriptional modifications. These can include poly-adenylation, 5′ capping, splicing, cleavage in the RNAi pathway, or RNA editing. RNA editing involves the specific addition, deletion or chemical modification of nucleotides. Editing by chemical modification of a single nucleotide can occur in a variety of ways, one of which is adenosine to inosine deamination. Adenosine can be specifically deaminated at the C6 position, creating a non-standard nucleotide (Figure 1-1A). If the editing event occurs in a coding section of RNA, the resultant inosine is subsequently decoded as a guanosine by the translational machinery. This editing event can therefore result in a codon change in the mRNA transcript. Adenosine to inosine editing can also affect splice site formation, with the creation of new AI splice sites from an encoded AA in the transcript [1]. Additionally, since inosine pairs with cytosine, A to I editing can affect the structural stability of an RNA, changing a U:A Watson-Crick base pair to a U:I wobble pair. These editing events serve to fine-tune an RNA transcript, allowing for a greater diversity of proteins to be produced than is encoded in the genome.

The activity that results in adenosine to inosine modification in RNA was first discovered in *Xenopus laevis*. Antisense RNA injected into *Xenopus* eggs did not form stable RNA:RNA hybrids with complementary endogenous mRNA. It was found that there was a novel activity that unwound the RNA:RNA duplexes in *Xenopus* eggs and early embryos [2,3]. The intermediates of the unwinding reaction were analyzed and found to contain a large number of A to I conversions. These conversions resulted in the creation of less stable U:I wobble pairs from endogenous U:A Watson-Crick pairs, leading to the destabilization, or unwinding, of RNA:RNA duplexes [4,5]. The double-stranded RNA (dsRNA) unwinding and modifying activity was subsequently found to be localized to the nucleus and present in all cell and tissue types, regardless of the stage of the cell cycle [6]. This activity was originally named DRADA for double-stranded adenosine deaminase [7], but was later renamed ADAR for adenosine deaminases that act on

Figure 1-1

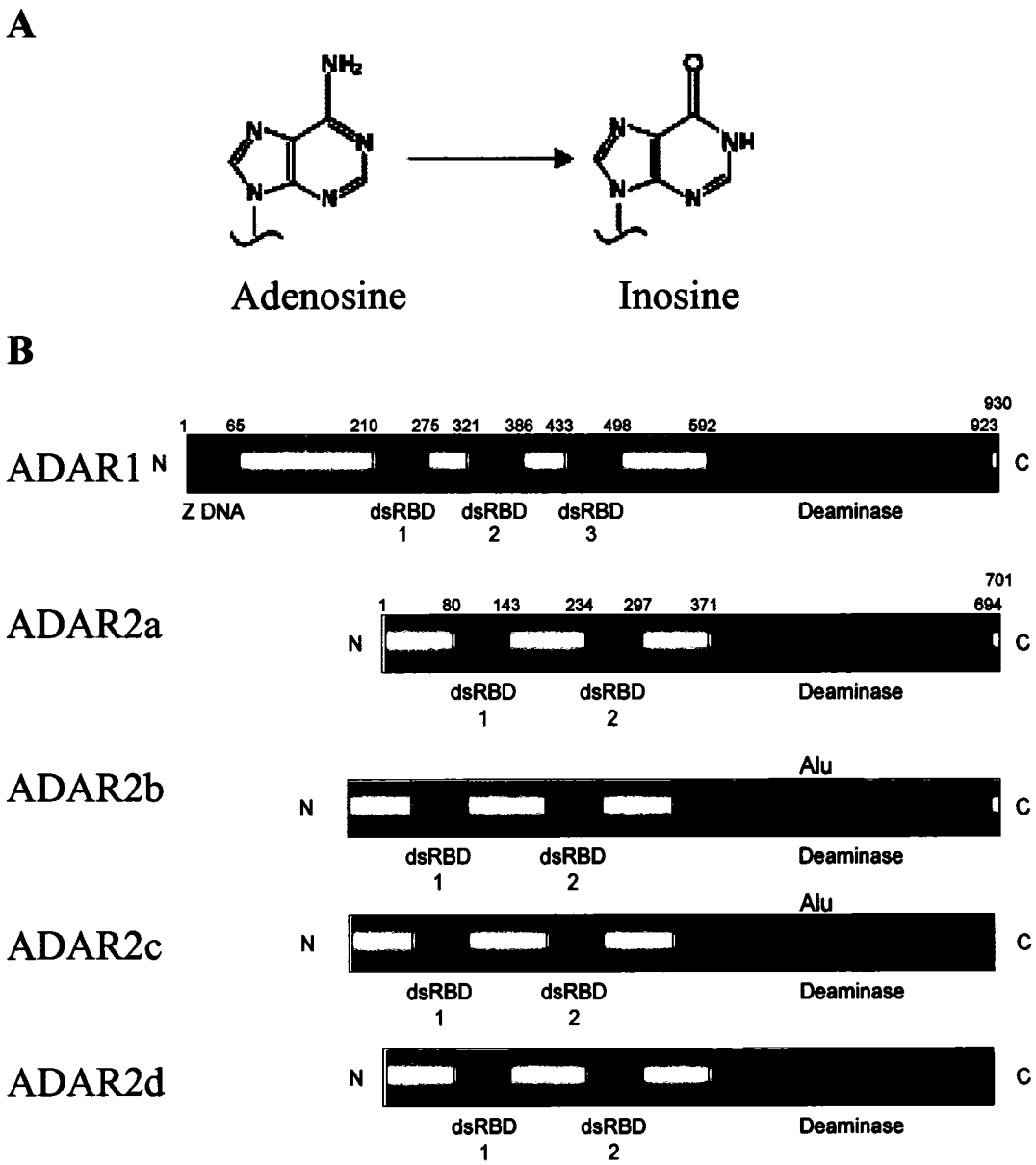

Figure 1-1. RNA editing by adenosine deaminases that act on RNA. (A) Adenosine deamination at the C6 position results in the production of inosine. (B) Schematic diagram of the domain structure of ADAR enzymes. The short, constitutively-expressed form of ADAR1 is shown.

RNA [8,9]. Three isoforms of ADAR were discovered in humans; ADAR1 [7,10-12], ADAR2 [13-16] and ADAR3 [17]. Thus far, homologues have been found in flies [18], worms [19], birds [20], fish [21,22] and frogs [23]. Human isoforms ADAR1 and ADAR2 are ubiquitously expressed although ADAR2 has increased expression in the brain and the heart [16]. ADAR3 is expressed solely in the brain, but has yet to be shown to be an active deaminase [17]. Human ADAR1 and ADAR2 are the most extensively studied isoforms, and it was with these two proteins that the experiments described in this thesis were performed.

Structure of the ADAR Enzymes

The ADAR family of enzymes has a general domain structure consisting of a highly conserved catalytic domain on the C-terminus and a varying number of double-stranded RNA binding domains (dsRBDs) on the N-terminus. The catalytic domain of ADARs is highly homologous to the catalytic domain of adenosine deaminases that act on tRNA (ADATs); these proteins differ from the ADARs in their lack of dsRBDs. Human ADAR1 and ADAR2 are very similar in sequence, but they also have some differences. Two major forms of ADAR1 exist in human cells; a 105 kDa form that is cytoplasmic and a 110 kDa form that is nuclear. The long, cytoplasmic, form of ADAR1 is expressed upon treatment with interferon [24] and contains two N-terminal Z-DNA binding domains, three dsRBDs and a C-terminal catalytic domain. The short, nuclear form of ADAR1 is constitutively expressed and is a result of a shift to a downstream start site and consequently has one less Z-DNA binding domain (Figure 1-1B). Human ADAR2 does not contain any Z-DNA binding domains; it consists of two dsRBDs and a C-terminal catalytic domain. Four major variants of ADAR2 exist, named ADAR2a through –d. Isoforms 2b and 2c differ from 2a in the addition of an Alu cassette – a highly conserved DNA motif which is recognized by the restriction enzyme Alu1 - within the catalytic domain; isoform 2c has a shorter C-terminal tail than 2b. ADAR2d does not contain an Alu cassette and has the

shorter C-terminus compared to 2a and 2b [14]. All experiments performed in this thesis were carried out with ADAR2a, henceforth called ADAR2 (Figure 1-1B).

Although the complete structures of ADAR1 or ADAR2 have not yet been solved, portions of their domains have been successfully crystallized. In 1998, Ryter and Schultz crystallized the second dsRBD of *Xenopus laevis* binding protein A (Xlrbpa) complexed with dsRNA and solved the structure to a resolution of 1.9 Å [25]. The sequence of dsRBDs is highly conserved across species and proteins and thus a great deal of information about ADAR dsRBDs can be obtained from this crystal structure. The Xlrbpa dsRBD shows an α-β-β-β-α which is characteristic of the domain [26,27]. It was shown that each dsRBD contacts dsRNA across two successive minor grooves and the intervening major groove on a single face of the double-stranded RNA (dsRNA) helix. Interactions with RNA are made through both backbone -OH and base mediated hydrogen-bonding [25].

Recently, the catalytic domain of ADAR2 was crystallized and the structure was solved to resolution of 1.7 Å. Bass and colleagues report a unique structure in which an inositol hexakisphosphate (IP_6) is bound at the core of the catalytic domain. The IP_6 appears to define and stabilize the protein fold, as it is buried in the interior of the domain. Located at the active site of ADAR2 are a catalytic zinc ion and a nucleophilic water molecule which are coordinated to the IP_6 through a network of hydrogen bonds. It is proposed that the IP_6 may modulate the catalytic activity of the enzyme through tuning the pKa of the nucleophilic water molecule at the active site. Interestingly, residues that coordinate IP_6 in ADAR2 are conserved in the ADAR1 catalytic domain as well as ADAR3 and some ADATs, indicating a similar folding and catalytic mechanism between family members [28].

In contrast to ADAR2, ADAR1 contains two Z-DNA binding domains. The most N-terminal Z-DNA domain is called Zα and is expressed only in the

interferon inducible form of ADAR1. The Zα domain of ADAR1 in complex with Z-DNA was crystallized and the structure resolved to 2.1 Å in 1999 and was shown to adopt a winged helix-turn-helix motif [29]. In addition to Z-DNA, the Zα domain of ADAR1 can also bind Z-RNA, and was shown to stabilize the formation of Z-form RNA from A-form RNA [30]. The Z-DNA binding domain present in the constituitively expressed form of ADAR1 is designated Zβ [31] and was successfully crystallized in 2005. The structure of Zβ is similar but distinct from Zα and does not interact with Z-DNA indicating different functional roles within the protein [32].

Taken together, the structures of the various domains of ADAR1 and ADAR2 can give us an idea of the structures of the entire proteins. Although it would be useful to see how the domains relate to each other in 3-dimensional space, both proteins are very large and thus a complete structure of either one may be in the distant future.

ADARs Edit Multiple Endogenous Substrates

The N-terminus of all ADAR family members contains a varying number of dsRBDs which are essential for the activity of the enzyme [33]. ADARs only edit double-stranded substrates and require a minimum of 15 base pairs, but are more effective on longer double-stranded substrates [34]. Long dsRNAs can originate in a cell in a variety of ways. Intermolecular base pairing can create long stretches of dsRNA, these base-paired regions are often imperfect in complementarity and thus have bulges and loops within the dsRNA. Editing within imperfect dsRNA duplexes is directed through the bulges and loops to target specific adenosines; the edited adenosine itself is normally unpaired [35,36]. Typically only a single editing event occurs within a stretch of imperfectly paired dsRNA; this is in contrast to promiscuously edited mRNAs. Aberrant transcription, viral transcription, or transcription from the anti-sense as well as sense strands of DNA can result in long, completely dsRNA. These

perfectly dsRNAs lack bulges and loops that target specific adenosines and are hyperedited at non-specific sites. Up to 50% of the adenosines in a long perfect duplex can be converted to inosines by the ADARs [37]. ADAR activity *in vivo* results in both site-selective and promiscuous editing, with drastically different cellular effects.

Selectively edited mRNAs have been identified in two ways; (1) when it is noticed that a genomically encoded A appears as a G in cDNA, a hallmark of ADAR editing of the mRNA for that gene, and (2) through an inosine specific cleavage assay that identifies inosine containing mRNAs [37]. To date, very few endogenous ADAR targets in humans have been identified. Those that have been identified are largely neural mRNAs, including sites in ionotropic glutamate receptors (GluRs) and serotonin receptors (5-HT2C isoforms). The GluR receptors allow passage of Na^+, K^+ and Ca^{2+} though the neuronal cell membrane upon activation with glutamate, resulting in propagation of excitatory neurotransmition. There are eight editing sites found in various subunits of the GluR channels which result in codon changes and thus altered amino acids in the translated product. Each site is named after the amino acid change the editing events induce, and include the Q/R sites in GluR-B, -5, and -6 (a glutamine C<u>A</u>G codon is changed to an arginine C<u>I</u>G codon) [38], the R/G sites in GluR-B, -C, and –D (an arginine <u>A</u>GA codon is changed to a glycine <u>I</u>GA codon) [39], the I/V site in GluR-6 (an isoleucine <u>A</u>TT codon is changed to a valine <u>I</u>TT codon) [40] and the Y/C site in GluR-6 (a tyrosine T<u>A</u>C codon is changed to a cysteine T<u>I</u>C codon) [40]. The result of these editing events is a modification of the function of the channel. For example, in the GluR-B subunit of the AMPA receptor, editing at the Q/R site severely decreases the channel's permeability to Ca^{2+} whereas editing at the R/G site results in a faster recovery after desensitization [41]. Although there are multiple sites within GluR transcripts that are targets for editing, ADAR1 and ADAR2 have different specificities for specific sites within the transcripts. ADAR1 has the ability to edit the R/G site of GluR-B and the Q/R

site of GluR-6 but is unable to edit the Q/R site of GluR-B; this is an activity of ADAR2 [42]. Editing of the Q/R site of GluR-B is the only site within the various GluR transcripts that is edited to nearly 100% *in vivo*. It has been shown that site-specific editing at this site is absolutely required for normal brain development and inhibition of editing at this site results in early death in mice [43]. Intriguingly, under-edited Q/R sites in GluR-B have been found in malignant gliomas [44] and in a brain region-specific manner in schizophrenia, in Alzheimer's disease and Huntington's Chorea [45]. The seeming necessity of editing at this site raises the question as to why editing even exists for the Q/R site; why not just genomically encode an arginine [9]? Although the answer to that question has yet to be discovered, it is obvious that editing at the Q/R site of the glutamate receptors is necessary for normal Ca^{2+} permeability. Editing at the remaining seven sites in the GluR transcripts is nonessential for viability and results in more subtle effects. It has been shown that editing at these sites is involved in receptor formation and composition [46], synaptic plasticity and seizure vulnerability [47], and, as mentioned before, recovery rates from desensitization [41].

Another human neuronal receptor that is selectively edited by the ADARs is the serotonin receptor 5-HT2C mRNA. This G-protein coupled receptor can be edited at up to five sites resulting in 24 different potential receptor versions from a single mRNA [48,49]. When edited, the basal activity of 5-HT2C is decreased and the efficiency of G-protein coupling is greatly reduced [50,51]. Intriguingly, a recent study showed that the editing of two sites in the 5-HT2C mRNA was regulated in a serotonin-dependant manner. Serotonin depletion resulted in decreased editing which produced receptors which were more sensitive to serotonin while treatment with a 5-HT2C agonist up-regulated the editing of the mRNA, resulting in less sensitive channels. This novel feed-back loop utilizes editing to change the activation properties of the serotonin receptor in response to prolonged changes in serotonin levels [52]. Altered levels of 5-HT2C editing

have been implicated in schizophrenia [53,54], but contradicting reports [55,56] leave the questions far from resolved. There is more conclusive evidence linking serotonin 5-HT2C receptor editing with severe depression. A recent study in a rat model of depression shows increased editing of 5-HT2C mRNA in affected rats; the level decreased when the rats were given anti-depressants [57]. This study confirmed previous studies done with rats and samples from human suicide victims with histories of severe depression [53,56] which showed altered levels of editing in 5-HT2C transcripts in the depressed brain. It is apparent that normal editing of neural channels and receptors is necessary for normal brain function, and without properly functioning ADARs serious mental disorders can arise.

To date, editing of the glutamate-gated ion channels and serotonin receptors remain the only identified endogenous mammalian ADAR substrates which result in a codon change. A remarkable fact of these substrates is the double stranded region surrounding the edited adenosine is created through base pairing of the exon with a downstream intron. The exon complementary sequence (ECS) in the downstream intron can be proximal to the editing site (50 nucleotides in the R/G sites of GluR-B, -C and –D) [58] or very distant (1900 nucleotides in the Q/R site of Glur5 and Glur6) [59]. The necessity of a downstream intronic sequence for editing implies that editing must occur prior to splicing. This suggests an intimate connection among ADARs, splicing machinery and transcriptional machinery given that splicing occurs co-transcriptionally. Correspondingly, ADARs have been found to co-localize with splicing factors in large nuclear ribonucleoprotein particles which are proposed to be responsible for splicing, 5′ capping and 3′ poly-adenylation [60].

Site-specific editing occurs not only at coding regions of mRNAs, but also in introns, 5′ and 3′ untranslated regions (UTRs) and non-coding RNAs [61]. Although editing at these sites does not alter the sequence of the resultant protein encoded in the transcript, it may have other important effects. Rat ADAR2, which is highly homologous to human ADAR2, was shown to selectively edit its

own transcript *in vivo*. Editing of its mRNA results in the creation of a 3′ acceptor splice site and causes the transcript to be alternatively spliced. This results in the inclusion of a 47 nucleotide cassette which abolishes the activity of the enzyme. It is proposed that this is an auto-regulation mechanism by which ADAR2 can check its own over-expression [1]. Interestingly, editing within the serotonin receptor 5-HT2C mRNA at the five sites previously mentioned has been shown to regulate alternative splicing. In this case, editing does not create donor or acceptor splice sites, but instead increases the frequency of the use of an alternative splice site downstream of the editing events [62]. The reason for site-selective editing within non-coding RNA remains a mystery; why alter a single nucleotide in a long stretch of nucleotides that are not used to create proteins? It may be that gratuitous ADAR editing sites occur within these regions, but do not affect the function of the cell and thus are not eliminated though evolution. However, since studies in this field have been limited in number, editing at these sites may prove to be important for some critical cellular function that has yet to be discovered.

In addition to site-selective editing, ADARs also have the ability to deaminate multiple adenosines in a dsRNA substrate. ADARs can promiscuously edit long, completely double-stranded stretches of RNA. These RNAs are normally created though aberrant or viral transcription, and therefore represent an unfavorable condition or problem for the cell. Editing of these RNAs disrupts a stable A:U base pair and creates a less stable I:U wobble pair. Promiscuous editing can disrupt base-pairing at many sites and destabilize long dsRNA – indeed, this is the exact activity by which the ADARs were discovered. In addition to destabilizing dsRNA, it has been shown that hypereditied RNAs are retained within the nucleus rendering them unavailable for transcription [63,64]. Within the nucleus, edited RNAs are bound by vigilin, a protein shown to complex with ADAR1 and proposed to be involved with heterochromatin formation [65]. Therefore, ADARs may help prevent abnormal or viral protein

production in two ways: (1) preventing the RNA from leaving the nucleus; and (2) directing heterochromatin formation on the aberrant gene.

Long dsRNA can have another fate in the cell if it exits the nucleus – it can initiate the RNA interference (RNAi) pathway. Long dsRNA can be cleaved by the cytosolic protein Dicer into small interfering RNAs (siRNAs) [66] which can proceed to induce cleavage of cognate mRNA [67] or stimulate production of heterochromatin [68]. Seeing that long dsRNAs are also substrates for ADARs, RNA editing may be antagonistic and intimately involved with RNAi through editing and destabilization of target mRNAs [69]. Indeed, it was shown that the ability of a dsRNA to trigger RNAi decreases with increased editing [70]. Therefore it seems plausible that editing may regulate the passage of RNAs into the RNAi pathway, providing a check-point on the way to altered gene regulation.

Genetic Knock-Out of ADARs

The ubiquitous expression pattern of ADAR1 and ADAR2 and the necessity of editing for normal neural function indicates the importance of normal ADAR expression for survival. Genetically modified organisms have been engineered that are either homozygous or heterozygous negative for ADAR alleles, with dramatic results. For example, *D. melanogaster* possess a single ADAR gene, therefore the removal of all A to I editing in fruit flies can result from the mutation of one gene. Interestingly, a null mutation in the dADAR gene results in viable progeny. However, the flies exhibit abnormal behavioral patterns including defects in locomotion, chemotaxis, flight and courtship. This lack of normal brain function is consistent with the fact that all known ADAR substrates in *Drosophila* are neural. Although dADAR mutants did not have shortened life spans, flies that lack dADAR were shown to be at a selective disadvantage when in a population with wild type animals [71]. Therefore, although it seems that ADARs are not necessary for viability in fruit flies, A to I editing is essential for normal neural function in these organisms. Similar to *D. melanogaster*, *C.*

elegans contain two genes that encode ADARs and the mutation of these two genes results in viable organisms with behavioral defects. These defects are subtle as single and double mutant worms have defects in chemotaxis, but otherwise appear normal [19]. Intriguingly, crossing ADAR deficient worms with RNAi deficient worms rescued the chemotaxis defect [72]. This strongly supports the hypothesis that editing and RNAi are interconnected dsRNA regulatory mechanisms in the cell.

The phenotypes displayed in mouse models that lack ADARs differ immensely from those of flies and worms. Creation of a chimeric mouse with a single null ADAR1 allele resulted in a heterozygous embryonic lethal phenotype. The embryos died prior to day 14.5 with defects in blood cell proliferation and differentiation [73]. In contrast to ADAR1 heterozygotes, disruption of a single allele of ADAR2 results in viable offspring with no apparent abnormal phenotypes. However, homozygous ADAR2 mutants die between P0 and P20, and had an increase in frequency of seizures after day P12. Death and seizures were attributed to under-editing at the Q/R site in the GluR-B as the phenotype reverted to normal when the edited adenosine was encoded in the genome as a guanosine [74]. It is apparent that ADAR1 and ADAR2 have different targets in the mammalian system as the presence of ADAR1 could not revert the ADAR2 mutant phenotype and vice versa. The difference in severity of the ADAR mutant phenotypes indicates that ADAR1 may have more of a global effect as compared to the minimal, albeit important, need for ADAR2.

The study of ADAR mutants in mammalian models has given us a better understanding of the vital role these proteins play in the human body. Further research is sure to uncover vital pathways in which the ADARs are indispensable, due to the knowledge that ADAR1 is required for survival. However, more work needs to be done to understand exactly how ADARs edit and identify substrates, and why those edited substrates are necessary. Previous studies have examined the role of surrounding nucleotides on the binding of the ADARs and selection of

a target nucleotide. Chapter 2 of this thesis will examine how human ADAR2 binds RNA. Specifically, we will examine the role of the edited adenosine in the binding of ADAR2 to its target RNA. In addition, footprinting studies will be presented which investigate how ADAR2 binds to one of its most physiologically important substrates, the R/G site of GluR-B. In addition to the *in vitro* work presented in chapter 2, this thesis will also examine the ADARs *in vivo*. Studies presented in chapter 3 investigate the highly debated theory that ADARs dimerize when active. Fluorescence microscopy techniques were used to study ADAR-ADAR interactions within human cells and reveal surprising information regarding ADAR dimerization. In addition, chapter 4 of this thesis examines ADAR localization within human cells. Through the examination of ADAR proteins both *in vitro* and *in vivo* valuable information regarding the mode of action of these extremely important proteins has been obtained.

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CHAPTER 2

Investigations of ADAR2 – RNA interactions *in vitro*

Introduction

The ADAR family of proteins modifies primary mRNA transcripts to increase the diversity of encoded genetic material. Through the conversion of adenosine to inosine, the primary sequence of a RNA can be altered, resulting in the potential to change the sequence of a protein. Within human cells, mRNA deamination is not limited to adenosine. Cytidine can also be deaminated to produce uridine by a related family of enzymes, the cytidine deaminases that act on RNA, or CDARs. The most well known of this family is the apolipoprotein B mRNA editing enzyme, APOBEC-1 (apoB editing catalytic subunit 1). APOBEC-1 converts a cytidine to a uridine within the apoB pre-mRNA, resulting in the creation of a premature stop codon [1]. Although the global sequence of the CDARs is divergent from that of the ADARs, the two families share homology within their catalytic domains. Both families share conserved residues that are essential for catalysis, including residues involved in zinc coordination and a catalytic glutamic acid [2]. In 1994, an enzyme closely related to APOBEC-1, *E. coli* cytidine deaminase, was crystallized to 2.3 Å. The structure showed that the enzyme, which deaminates free cytidine, forms a homodimer when trapped with a mimic of a reaction intermediate, with the active site being formed by portions of both proteins [3]. Subsequently, studies showed that APOBEC-1 also homodimerizes *in vitro* and was shown to function as a dimer *in vivo* [4]. Due to the similarity between the active site of APOBEC-1 and the ADARs, it was proposed that the ADARs may also dimerize when catalytically active. Primary studies on ADAR dimerization worked with purified enzyme from model systems. In 1994, Hough and Bass showed that partially purified *Xenopus* ADAR1 gave an apparent molecular mass of 210 kDa when passed over a gel filtration column. The molecular mass obtained from the partially purified enzyme is consistent with the protein forming a dimer. However, when the Xenopus ADAR1 was fully purified, it gave an apparent molecular mass of 90 kDa, consistent with being in monomer form. This suggested that the enzyme

may dimerize, but required the presence of other cellular factors [5]. Upon identification and purification of the third human ADAR family member, ADAR3, Nishikura and co-workers noted that the presence of the catalytically inactive ADAR3 inhibited the *in vitro* activity of both ADAR1 and ADAR2 [6]. This intriguing study introduced the possibility of heterodimerization between isoforms. Subsequently, an *in vitro* study by MacMillan and co-workers confirmed the hypothesis that ADARs homodimerize by showing that human ADAR2 formed a dimer upon the GluR-B mRNA substrate, and that the ternary complex was the catalytically active form of the enzyme [7]. In 2003, it was shown by O'Connell and co-workers that *Dropsophila* ADAR functions as a dimer, and that dimerization occurs following RNA binding [8]. Concurrently, Nishikura and colleagues performed tandem purification experiments on tagged ADAR proteins [9]. Their results showed that ADAR1 and ADAR2 homodimerize in both the presence and absence of RNA, in contrast to the work published by MacMillan and O'Connell. Nishikura and colleagues also established that purified ADAR3 was unable to form homodimers, and there was no observable heterodimerization between any ADAR isoforms [9]. This seeming disparity between different studies had led to many questions being asked as to whether the ADARs do or do not dimerize in their catalytically active form. The work presented in this thesis will attempt to answer this question in a compelling and definite manner. At this time, the further discussion of dimerization shall be deferred until additional data is presented.

It is known that catalytically active ADARs bind to, and edit, substrate RNAs. As previously mentioned, substrates for the ADARs consist of largely double-stranded mRNAs broken up by loops and bulges. One of the most extensively studied substrates is that of the GluR-B mRNA (Figure 2-1). The sequences surrounding the two sites that are edited within the GluR-B mRNA are often used as model substrates in editing reactions. Multiple studies have examined the sequences surrounding the edited adenosines in an attempt to

Figure 2-1

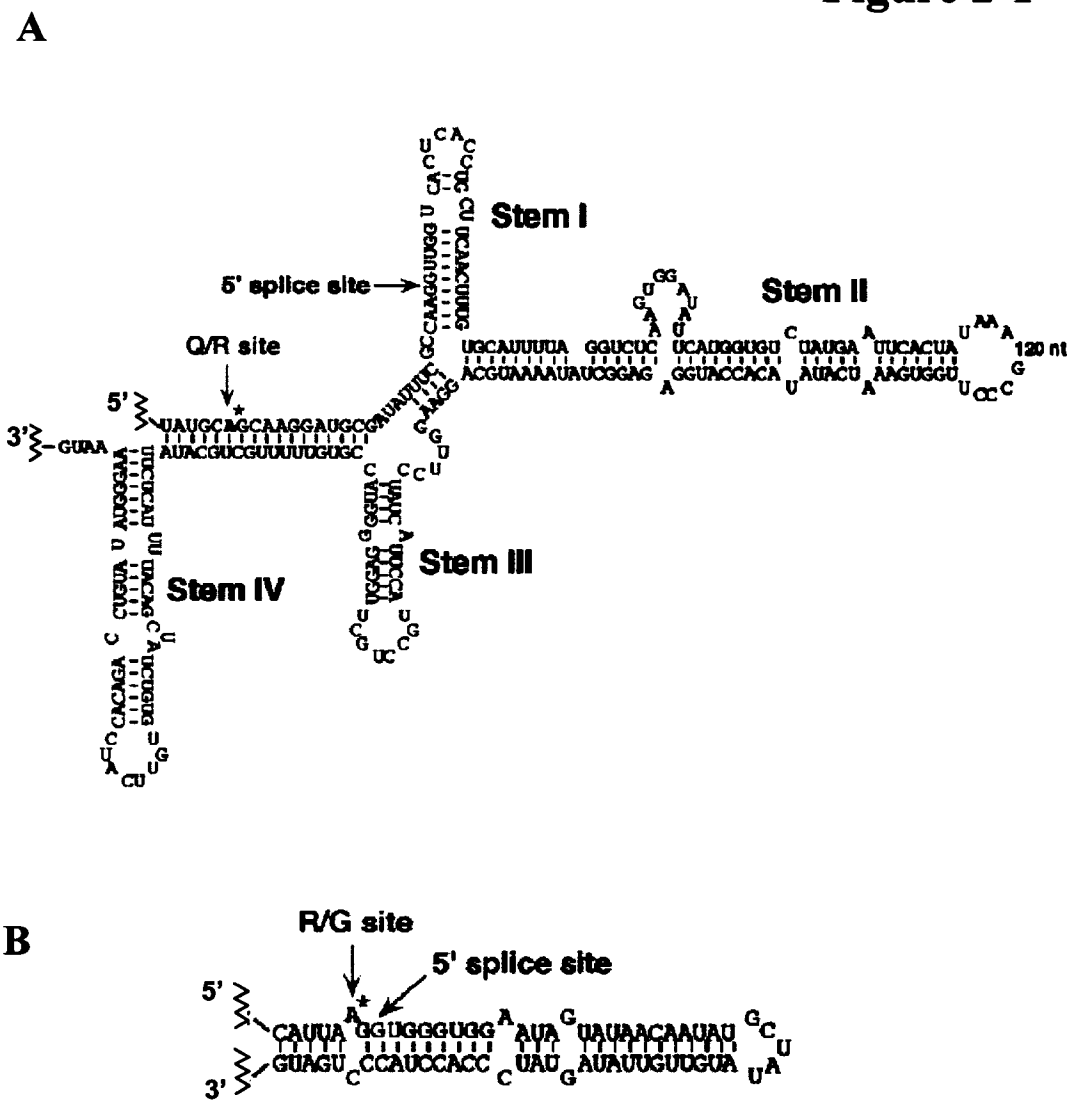


Figure 2-1. Predicted secondary structure of GluR-B mRNA. (A) Predicted secondary structure around the Q/R site of the GluR-B mRNA. The edited adenosine is shown in red. Downstream 5′ splice site is shown in blue. (B) Predicted secondary structure around the R/G site. The edited adenosine is shown in red. Downstream 5′ splice site is shown in blue. Double-stranded structure at both sites is formed through complementarity between the exon and downstream intron.

understand how the ADARs are targeted to a specific adenosine. Interestingly, in endogenous GluR-B transcripts, the edited adenosine at the Q/R site is proposed to be base paired with a uridine on the opposing strand, whereas at the R/G site the edited adenosine is located at an A:C mismatch. It was discovered that the frequency of editing at the Q/R site could be increased by creating mismatch, with an A:C mismatch having a greater effect than an A:A or A:G mismatch [10]. In addition to having a preference to certain nucleotides opposing the edited adenosine, ADARs also have a bias in regards to the neighbors of target adenosines. Both ADAR1 and ADAR2 have a 5′ neighbor preference (A ≈ U > C = G) whereas only ADAR2 has a 3′ neighbor preference (U = G > C = A). It was shown that both enzymes disfavor adenosines close to helix ends [11,12]. Furthermore, it was revealed that large internal loops (>6 nt) within a completely dsRNA function as helix ends to ADAR1 and therefore break up long dsRNAs into shorter substrates [13]. An interesting experiment using a synthetic, completely dsRNA substrate showed that both ADAR1 and ADAR2 deaminated the RNA to the same extent [12]. This was surprising as the two enzymes have different biological substrates and only share 41% identity, indicating the substrate, rather than the enzyme itself, imparts a great deal of selectivity in the editing reaction [12]. It is clear that loops, bulges, mismatches and primary sequence all serve to direct the ADARs to deaminate certain adenosines, but a formula for predicting which adenosine(s) will be edited in a given mRNA has yet to be determined.

The sequences surrounding the edited adenosine have been closely scrutinized to determine their effect on directing the binding of ADAR enzymes to a specific region of RNA. To date, no studies have examined the role of the edited adenosine itself in the binding of the ADAR enzymes. In this chapter, electromobility shift analysis is employed to examine the importance of the edited adenosine in the binding of human ADAR2 to dsRNA. Further studies in this chapter probe the nature of ADAR2-dsRNA interactions through the use of

footprinting techniques. Through the use of these techniques we have studied how ADARs interact with their substrates and elucidated possible ways in which ADARs may bind to target RNAs.

Experimental Methods

Oligonucleotide Synthesis

All oligonucleotides were purchased from the University of Alberta DNA Core Services Laboratory and contained a T7 RNA polymerase promoter as well as the coding sequence of interest.

PCR

The template for the 79 nucleotide substrate used in the footprinting reactions was produced through PCR reactions from a purified plasmid containing the endogenous R/G site using appropriate primers. Reactions were performed in a 200 μL reaction mix containing 10 mM Tris-HCL pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCL, 200 μM in each dNTP and 1 μM in each primer. Approximately 0.6 pmol of plasmid was used in each reaction. The thermal cycle used was as follows: (1) 3 min at 92°C, (2) 1 min at 92°C, (3) 1.5 min at 55°C, (4) 3 min at 72°C, (5) 1 min at 92°C, (6) 1.5 min at 54°C, (7) 3 min at 72°C, (8) 1 min at 92°C, (9) 1.5 min at 53°C, (10) 3 min at 72°C, (11) 1 min at 92°C, (12) 1.5 min at 52°C, (13) 3 min at 72°C, (14) 1 min at 92°C, (15) 1.5 min at 51°C, (16) 3 min at 72°C, (17) 1 min at 92°C, (18) 1.5 min at 50°C, (19) 3 min at 72°C, (20) 25 times to 17, (21) 10 min at 72°C, (22) 10 min at 25°C, (23) End cycle. To each reaction 40 μL 3 M NaOAc was added and the volume brought up to 400 μL with ddw. This was followed by extraction once with phenol/chloroform/isoamyl alcohol and once with chloroform, and ethanol precipitation.

RNA Synthesis

Gapped substrate and 79 mer footprinting substrate : 500 μL transcriptions were carried out in 40 mM Tris pH 7.5, 5 mM DTT, 1 μM spermidine, 3mM NTPs (Pharmacia), 0.01% Triton X-100, and 25 mM $MgCl_2$. The reactions contained 0.2 μM DNA template containing a double stranded T7 promoter, 10 μL T7 RNA polymerase and 20 U RNasin (Amersham). Transcriptions were carried out for 4 hours at 37°C. After the 4 hour incubation, reactions were lyophilized and resuspended in 400 μL 0.3M NaOAc followed by extraction with phenol/chloroform/isoamyl alcohol and then with chloroform, and ethanol precipitation. The final product was resuspended in deionized, distilled water (ddw) and quantified based on OD_{260} using a Hewlet Packard 8451A diode array spectrophotometer.

ApG Primed Substrate: 100 μL transcription was carried out in 40 mM Tris pH 7.5, 5 mM DTT, 1 μM spermidine, 2mM NTPs (Pharmacia), 0.01% Triton X-100, 25 mM $MgCl_2$ and 8 mM ApG. The reactions contained 0.2 μM DNA template containing a double stranded T7 promoter, 10 μL T7 RNA polymerase and 20 U RNasin. Transcriptions were carried out for 4 hours at 37°C, lyophilized and resuspended in 400 μL 0.3M NaOAc followed by extraction with phenol/chloroform/isoamyl alcohol and then with chloroform, and ethanol precipitation. The final product was resuspended in ddw and quantified based on OD_{260} using a Hewlet Packard 8451A diode array spectrophotometer.

5' End Labeling Reactions

Typical end labeling reactions were carried out in 20 μL reaction volumes containing 50 pmol RNA, 50 pmol [γ-^{32}P] ATP, 20 U RNasin, 2μL 10x T4 polynucleotide kinase buffer and 20 U T4 polynucleotide kinase (Invitrogen). Reactions were incubated at 37°C for 20 min, mixed with loading dye and loaded directly onto 8% (19:1) denaturing PAGE gels. The gels were run at room

temperature for 1 hour at 30 mA, bands visualized through exposure to film, excised, and extracted with 400 μL 0.3 M NaOAc and 40 μL phenol/chloroform/isoamyl alcohol with shaking overnight at 37°C. Following extraction, the polyacrylamide was removed by centerfugation through a paper-disk spin column (Perkin Elmer). The filtrate was then extracted once with phenol/chloroform/isoamyl alcohol and then with chloroform, and ethanol precipitated using 10 μg glycogen as a carrier.

Editing Assay

Editing reactions were carried out in 20 mM Hepes pH 8.0, 100 mM KCl, 0.5 mM DTT, 20% glycerol and 0.01% NP-40. A constant amount of 0.1 nM of 5' radiolabeled ApG primed substrate was used in each reaction. The substrate RNA was denatured at 100°C for 2 min and allowed to renature at room temperature to allow the formation of a uniformly double-stranded substrate just prior to its addition to the editing reactions. ADAR2 was added to the radiolabeled RNA to a final concentration of 1.1 μM. The editing reactions were incubated at 30°C for 2 hours then extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform, and then ethanol precipitated. Reactions were resuspended in 19 μL TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8) and digested with Nuclease P1 (1μg/reaction; Roche) for 3 hours at 55°C. The reaction volumes were concentrated to 2 μL and the products resolved using thin layer chromatography (saturated $(NH_4)_2SO_4$: 0.1M NaOAc : isopropanol (79:19:12)) using cellulose-PEI chromatography plates. TLC plates were exposed to a Molecular Dynamics Phosphor screen which was scanned using a Molecular Dynamics Storm 860 Phophorimager. Data was quantified using ImageQuant 5.0 software [7]

Native Gel Shifts

5' end labeled substrate was denatured at 100°C for 2 min and allowed to renature at room temperature just prior to binding with ADAR2. A constant

amount of 50 nmol radiolabeled RNA was used in all binding reactions, to which varying amounts of ADAR2 was added. Binding reactions were carried out in the same buffer as the editing reactions described above. ADAR2 was incubated with radiolabeled substrate on ice for 1 hour and loaded directly onto native 5% acrylamide (89:1) gels. The gels were run at room temperature in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.2 mM EDTA) for 3 hours at 150 volts. Gels were dried and exposed to Molecular Dynanics Phosphor screen which was scanned using a Molecular Dynamics Storm 860 Phophorimager. Data was analyzed using ImageQuant 5.0 software.

Fe-EDTA Footprinting

ADAR2 was dialyzed into 20 mM Hepes pH 8, 100 mM KCl, 0.5 mM DTT, 0.01% NP-40 buffer using Slide-A-Lyzer dialysis cassettes (Pierce) and stored at 4°C for a maximum of 5 days due to the lack of glycerol in the sample. All footprinting reactions were subsequently carried out in this buffer which was made up as a 10x stock for future use. Binding reactions and subsequent cleavages were carried out at 4°C. 5 pmol of 5′ radiolabeled 78 mer substrate was incubated with various concentrations of ADAR2 for 1 hour in a 20 μL reaction volume. To each cleavage reaction 2 μL Fe-EDTA (25 mM Fe and 25 mM EDTA pre-chelated for 10 min at room temperature) and 3.3 μL peroxide mix (0.5% H_2O_2, 50 mM ascorbate) was added and mixed through centerfugation. Reactions proceded for 10 min upon which quenching mix (40 μL 3 M NaOAc, 10 μg glycogen, 335 μL ddw) was added. Reactions were immediately extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform, and then ethanol precipitated.

T1 Ladder: 5 pmol of 5' radiolabeled substrate was suspended in 45 μL GSM buffer (20 mM citric acid pH 5, 1 mM Na_2-EDTA, 7 M urea, 0.15 mg/mL tRNA) and heated at 55°C for 5 min. 1 U of T1 RNase (Gibco) was added and allowed to cleave the substrate for 45 seconds, at which point quenching mix (40

μL 3 M NaOAc, 10 μg glycogen, 312 μL ddw) was added. The reaction was then immediately extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform, and ethanol precipitated.

All reactions were resuspended in 5 μL 50% loading dye and loaded onto 10% (19:1) denaturing polyacrylamide gels. Gels were run at room temperature for 1 hour 20 min at 75 watts. Gels were dried and exposed to Molecular Dynamics Phosphor screen which was scanned using a Molecular Dynamics Storm 860 Phophorimager. Data was analyzed using ImageQuant 5.0 software.

Results

Adenosine to Inosine Editing in Model R/G RNAs.

Recombinant human ADAR2a (hereafter ADAR2) was expressed in an *S. cerevisiae* expression system (a generous gift from Dr. Brenda Bass, University of Utah) and purified to homogeneity via conventional means [7,14]. To test the activity of the purified enzyme, a 71 nucleotide RNA substrate was created which was modeled after the GluR-B R/G editing site. This transcript was circularly permuted and therefore double-stranded in almost its entirety. The transcript was primed with adenylyl guanosine dinucleotide (ApG) to insert a target adenosine at a site homologous to the edited adenosine in the endogenous R/G transcript (Figure 2-2A). After 5' labeling the targeted adenosine, the efficiency of A to I conversion was measured. The radiolabeled substrate was incubated with ADAR2, purified, digested with nuclease P1 to mononucleotides, and analyzed using thin layer chromatography (TLC; Figure 2-2B). The purified ADAR2 was determined to have catalytic activity similar to previously studied expression systems [7] and was used for more detailed studies of ADAR2-RNA interactions.

Gel Mobility Analysis of ADAR2-RNA Interaction.

It has been shown previously that a 58 nucleotide RNA and a 54 nucleotide RNA that lack double-stranded structure 5' and 3' (respectively) to the

Figure 2-2

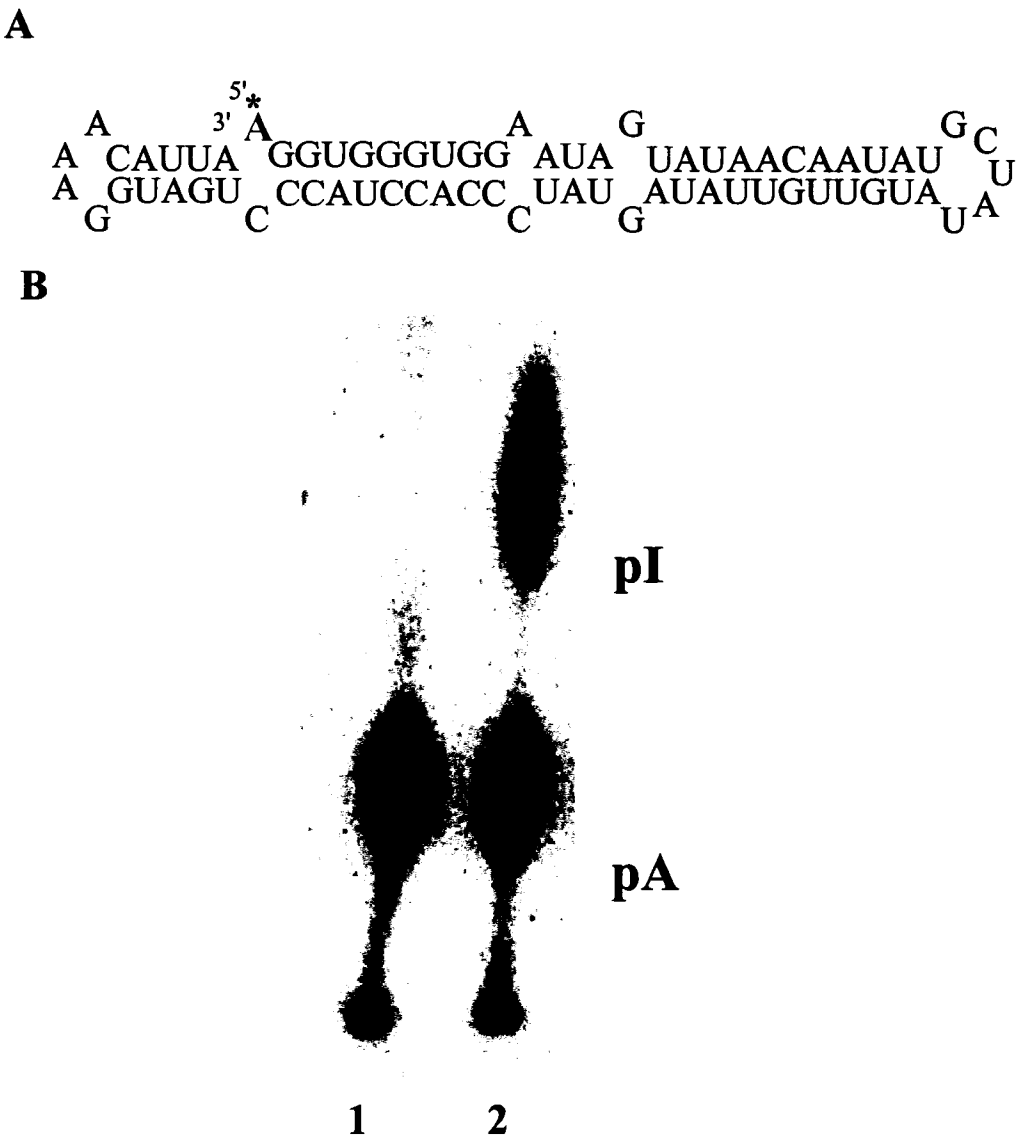


Figure 2-2. Editing of model ADAR substrate. (A) A 71 nucleotide substrate modeled after the GluR-B R/G site. The edited adenosine (shown in red) was 5′ radiolabeled with ^{32}P (asterisk). (B) Editing assay of model substrate. Substrate shown in (A) was incubated with hADAR2, purified, digested down to mononucleotides and analyzed using thin layer chromatography. *Lane 1*, unedited RNA, *Lane 2*, RNA in the presence of recombinant hADAR2.

target adenosine are edited inefficiently and bound approximately five-fold less tightly to ADAR2 [7]. This data suggests the necessity for double-stranded structure surrounding the editing site. It was our desire to determine if the target adenosine was involved in the binding as well as being the substrate for deamination.

In order to determine the role of the edited adenosine in binding, native gel mobility analysis was employed. Electromobility shift assays (EMSAs) allow the visualization of complexes between protein and RNA through the use of non-denaturing polyacrylamide gel electrophoresis. The resultant complexes are separated based on charge and mass and the interactions formed between protein and RNA are not disrupted. Two dsRNA substrates were developed for use in gel mobility analyses of ADAR2-RNA complexes. These constructs were identical in sequence except for the presence of the edited adenosine. The same DNA template was used to create both substrates, but for one substrate the transcription was primed with ApG to affix the adenosine that is edited by ADAR2. These substrates were 5' radiolabeled and incubated with increasing protein concentrations. The binding reactions were subjected to native gel electrophoresis.

The incubation of ADAR2 with ApG primed substrate led to the formation of primary and secondary complexes of ADAR2-RNA (Figure 2-3). It has been determined that the primary complex consists of one monomer of ADAR2 bound to each molecule of dsRNA whereas the secondary complex is generated through homodimerization of ADAR2 on its dsRNA substrate. The formation of homodimers results in the development of the catalytically active complex [7]. Quantification of the fractions of unbound RNA, primary complex, and secondary complex allowed us to determine dissociation constants based on equations previously utilized by our laboratory [7]. The K_d's of primary and secondary ADAR2 complex formation on ApG primed substrate are approximately 40 nM and 170 nM respectively.

Figure 2-3

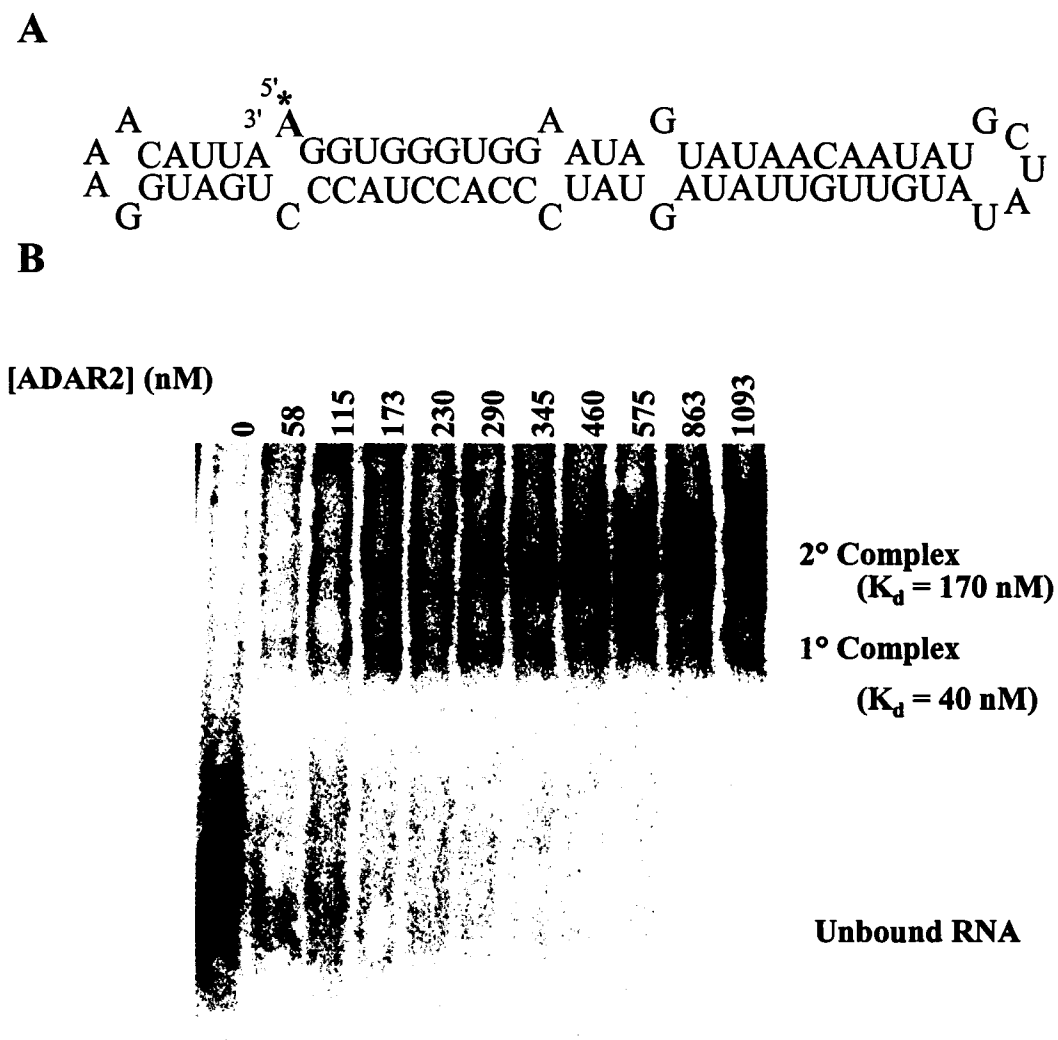


Figure 2-3. Gel shift analysis of model ADAR substrate. (A) The 71 nucleotide substrate modeled after the GluR-B R/G site used in gel shift analysis. The edited adenosine (shown in red) was 5′ radiolabeled with ^{32}P (asterisk). (B) Electromobility gel shift analysis of hADAR2 bound to 71 nucleotide substrate. *Lanes from left to right*, increasing concentrations of hADAR2 incubated with RNA. Indicated are unbound RNA and two complexes formed upon addition of ADAR2.

Upon incubation of ADAR2 with the second substrate, termed the gapped substrate due to the absence of the target adenosine, both primary and secondary complexes were formed (Figure 2-4). However, the affinity of ADAR2 for this substrate was dramatically decreased. The K_d's for both primary and secondary complex were shifted towards higher concentrations, resulting in a K_d of ~345 nM for the primary complex and a K_d of ~1095 nM for the secondary complex.

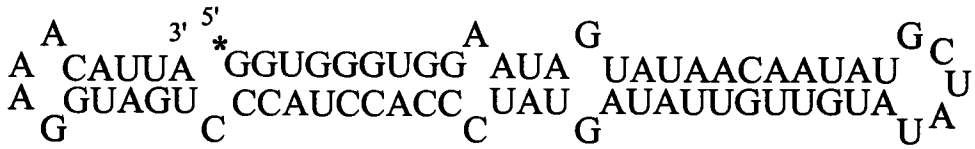
The presence of the target adenosine in the dsRNA has a dramatic effect on the binding affinity of ADAR2 for its substrate. In order to more extensively investigate the interactions between ADAR2 and its dsRNA substrates, a series of protection footprinting experiments were performed.

Fe-EDTA Induced Hydroxyl Radical Footprinting of ADAR2 on Model Substrates.

Through the use of protection footprinting it is possible to identify areas of polynucleotide substrates that make intimate contacts with bound protein molecules. Upon exposure to a non-specific cleavage agent and resolution on a polyacrylamide gel, radiolabeled nucleic acid produces a ladder of bands corresponding to cleavage at every nucleotide in the substrate. However, if a small molecule such as a protein is bound to the nucleic acid prior to cleavage, an area of decreased band intensity will be present upon running the cleavage products on a polyacrylamide gel. This area of decreased cleavage is termed a footprint and corresponds to the region of the substrate to which the protein is bound, as binding protects the nucleic acid from non-specific cleavage. Two different non-specific cleavage agents were used during protection footprinting determination of ADAR2-RNA interactions. Peroxynitrous acid and Fe-EDTA both generate hydroxyl radicals which primarily cleave RNA through hydrogen abstraction at the 4' carbon of the sugar backbone. In order to be confident that ADAR2 was forming the same primary and secondary complexes, it was necessary to perform footprinting reactions under similar conditions to those

Figure 2-4

A



B

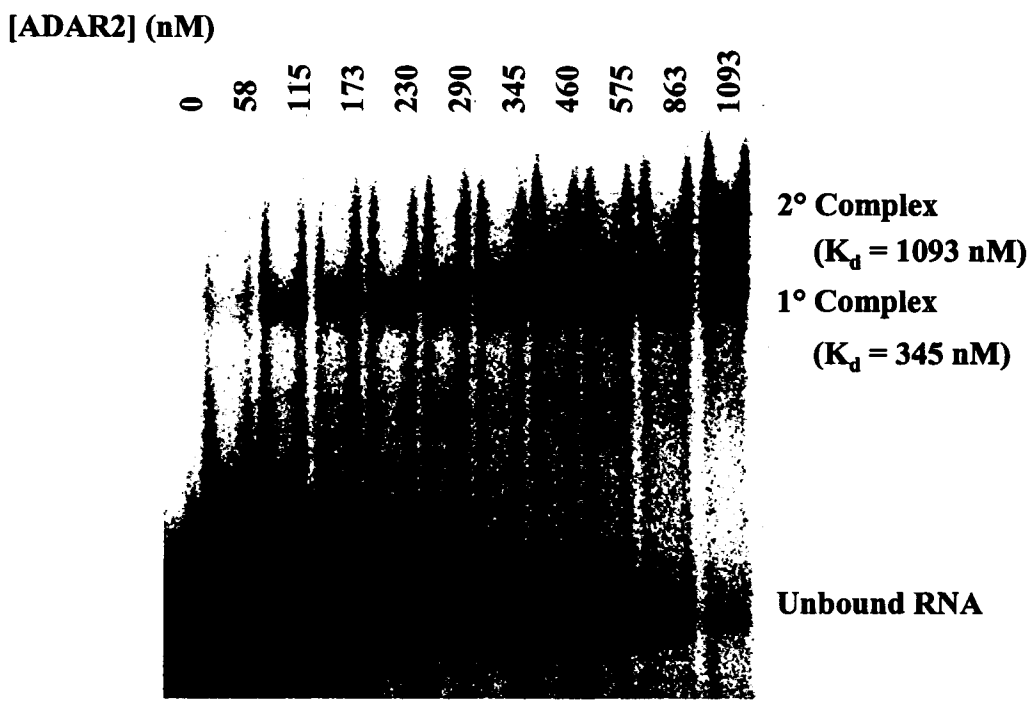


Figure 2-4. Gel shift analysis of model ADAR substrate. (A) A 70 nucleotide substrate modeled after the GluR-B R/G site but missing the edited adenosine. The RNA was 5′ radiolabeled with ^{32}P (asterisk). (B) Electromobility gel shift analysis of hADAR2 bound to 70 nucleotide substrate. *Lanes from left to right*, increasing concentrations of hADAR2 incubated with RNA. Indicated are unbound RNA and two complexes formed upon addition of ADAR2.

optimized for editing. Peroxynitrous acid did not give repeatable cleavage patterns under these conditions, but Fe-EDTA cleavage proved to be reliable under conditions very similar to those of editing.

A 79 nucleotide dsRNA substrate closely modeled on the R/G site of the GluR-B transcript was developed for Fe-EDTA footprinting with ADAR2 (Figure 2-5A). Prior to footprinting analyses, binding affinity of ADAR2 to this substrate was determined through EMSA techniques. The affinity of ADAR2 for this 79 nucleotide substrate is much higher than for the previously studied substrates indicated by a decrease in K_d for both the primary and secondary complexes (5 nM and 10 nM respectively).

After binding with various concentrations of ADAR2, the 79 nucleotide substrate was then subjected to Fe-EDTA induced hydroxyl radical cleavage. Three areas of marked decrease in cleavage are noted with increasing ADAR2 concentrations. The first footprint corresponds to an area on the 79 nucleotide substrate which overlaps the edited adenosine and spans 9 nucleotides. The second footprint corresponds with a region on the same strand as the target adenosine, but slightly 3' to the first footprint, and spans 10 nucleotides. The third footprint spans 18 nucleotides on the opposite strand of the dsRNA substrate (Figure 2-5B). These 37 nucleotides protected from non-specific cleavage produce a distinct footprint which suggests selective and specific binding of ADAR2 to it's substrate.

Discussion

We have examined the activity and binding of purified recombinant human ADAR2. Our data confirms that recombinant ADAR purified from an *S. cerevisiae* expression system produces an active deaminase. It has been determined through gel mobility shift analyses that the edited adenosine is necessary for efficient binding of ADAR2 to its dsRNA substrates. Removal of the edited adenosine results in a 7-fold decrease in formation of primary complex,

Figure 2-5

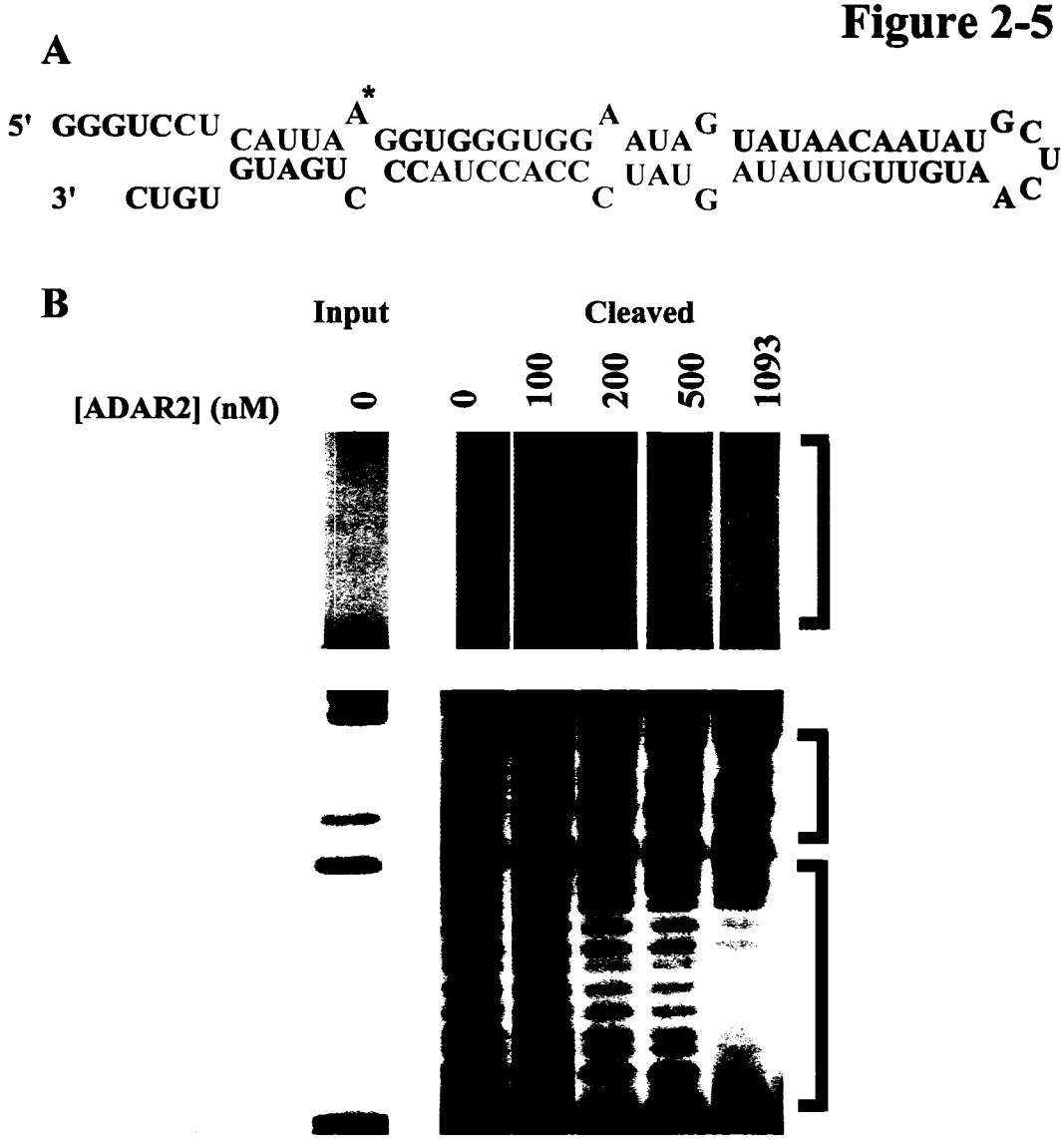


Figure 2-5. Protection foootprinting analysis of ADAR2 bound to RNA. (A) The 79 nucleotide substrate used in footprinting studies. The adenosine that is edited in endogenous GluR-B R/G site is noted with an asterisk. Regions shown in blue, red, and green correspond to protection footprints shown on the gel in the lower image. (B) Gel analysis of hydroxyl radical cleavage of 79 nucleotide RNA. RNA in the input lane has not been subjected to hydroxyl radicals. RNA in the cleaved lanes has been incubated with increasing concentrations of ADAR2 then subjected to hydroxyl radical cleavage. Colored areas correspond to regions on the above schematic that are protected through ADAR2 binding.

and a 5-fold decrease in formation of secondary, active complex. This suggests that the target adenosine is not only necessary for activity of ADAR2, but it is also involved in the binding of ADAR2 to its substrate. The exact role the adenosine plays in binding remains unclear. Structural studies of ADAR2 bound to a substrate could elucidate how and why the adenosine is necessary for binding.

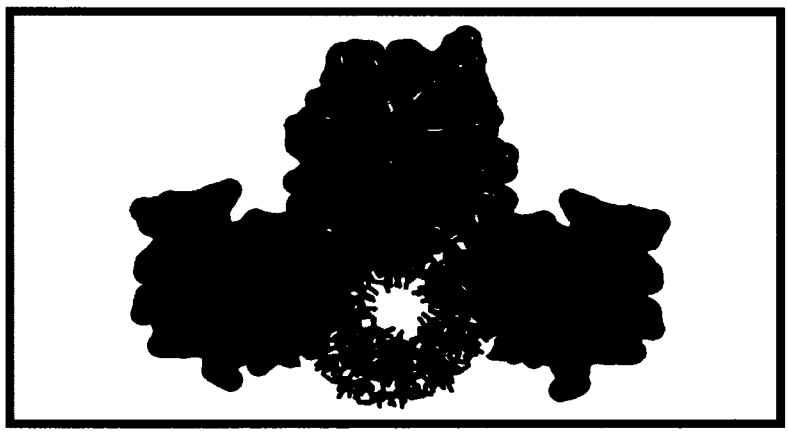
Protection footprinting analyses have shown three areas of a 79 nucleotide dsRNA closely modeled on the R/G site of the GluR-B transcript make intimate contacts with ADAR2. These areas collectively span approximately 40 nucleotides in the primary transcript. Taking into account that the footprinting studies were performed under conditions in which homodimerization of ADAR2 is expected, four dsRBDs (two from each ADAR2 monomer) are expected to be involved in binding the dsRNA. Structural studies of the second dsRBD of *Xenopus laevis* RNA-binding protein A (Xlrbpa) bound to RNA show that the dsRBD has a characteristic α-β-β-β-α fold and makes contact with 16 base pairs of dsRNA [15]. Interactions occur in two successive minor grooves and the central major groove. Interestingly, each asymmetric unit within the crystals contains two dsRBDs which bind to the dsRNA in an anti-parallel fashion and are rotated 90° around the long axis of the RNA relative to each other. The unit cell contains four dsRBDs. In our studies, each ADAR2 monomer contains two dsRBDs, and therefore each homodimer contains four; we can examine possible conclusions by drawing parallels between the structures.

In the crystal structure by Ryter *et al*, the four dsRBDs combine to make contacts with 22 nucleotides on one strand of the dsRNA, and 22 on the opposite stand (Figure 2-6A). Our footprinting studies show a footprint by ADAR2 homodimers of 19 nucleotides on one strand and 20 on the opposite strand. Additionally, if one dsRBD initiates contact with RNA at a minor groove on one side of the helix, the dsRBD making contacts on the opposite side of the helix will not begin to make contact until the minor groove reaches that side of the helix.

Figure 2-6

A

B



C

GGCGCGCGCCGGCGCGCGCCGGCGCGCGCC
CCGCGCGCGGCCGCGCGCGGCCGCGCGCGG

Figure 2-6. Crystal structure of Xlrbpa dsRBD bound to RNA. (A) Four dsRBDs (blue and teal) are present in the unit cell. The four dsRBDs make direct contact with 44 nucleotides in the RNA helix (green) while leaving 18 nucleotides unprotected (red). (B) A space-filling diagram of four dsRBDs of Xlrbpa bound to an RNA helix. All four dsRBDs contact 270° of the helix, leaving unprotected bases (red) on one side of the helix. (C) Linear representation of the RNA found in the crystal structure. Binding of the four dsRBDs in the manner shown leaves a characteristic footprint. Nucleotides making direct contact with the proteins are in green, while those left unprotected are in red.

This results in nucleotides that are not protected through dsRBD binding. These nucleotides would then show no protection from cleavage when exposed to a non-specific cleavage agent, resulting in an overhang in the cleavage footprinting pattern. This overhang would occur on both the 5' and 3' ends of the substrate but on opposite strands at either end. In the crystal structure, with binding of two dsRBDs rotated 90° to one another, there are two nucleotides unprotected by the dsRBDs. Our footprinting studies indicate a five nucleotide overhang on both the 3′ and 5′ ends, indicating a larger rotation between the dsRBD, likely closer to 180°. The structure of Xlrbpa bound to RNA shows that the four dsRBDs in the unit cell bind to 270° of the RNA helix, leaving one side of the helix bare and unprotected (Figure 2-6B). This results in two stretches of three to four base pairs within the helix unprotected by the dsRBDs, leaving a distinct footprinting pattern (Figure 2-6C). If the dsRBDs of ADAR2 have a wider angle between them, this will allow binding to almost the entire 360 ° of the RNA helix, resulting in a loss of the "gaps" in the footprint. Indeed, in our footprinting studies the footprint is continuous on one strand of the RNA and there is only one small three nucleotide gap on the opposite strand.

The experiments we have undertaken using footprinting studies of ADAR2 on a model R/G substrate are not the first of their kind. In 2001 Beal and colleagues reported footprinting studies of the RNA binding domain (RBD) consisting of both dsRBDs of ADAR2 bound to a substrate strongly resembling the one used in our studies [16]. The authors noted that the RBD of ADAR2 bound to RNA produced a 13 nucleotide footprint surrounding the editing site and a 10 nucleotide footprint on the opposite strand. Subsequently, the authors establish that the RBD of ADAR2 in absence of the catalytic domain is unable to flip the target adenosine out of the RNA helix; the mode by which the authors propose ADAR2 edits. Based on our knowledge that the RBD alone is catalytically inactive and that ADAR2 requires dimerization for activity, the size of the footprint that Beal and coworkers observed can be easily correlated with

our study. It is unlikely that the RBD alone dimerized upon the RNA substrate, instead it likely bound as a monomer. It is not surprising then, that the footprint in the paper by Beal and colleagues is almost exactly half the size of the footprint obtained in our studies [16]. If the RBD of ADAR2 dimerized upon the substrate we would likely see a footprint very similar to the one we observed with full length ADAR2.

The similarities and differences between the footprinting pattern obtained in our studies of ADAR2 and the dsRNA-dsRBD contacts shown in the crystal structure of Xlrbpa allow us to draw some conclusions about ADAR2 binding to dsRNA. First, we obtained a similar size footprint to the dsRNA-dsRBD contacts shown in crystal structure of Xlrbpa, indicating a similar method of binding to RNA. Second, the larger overhang and lack of gaps in the footprint of ADAR2 on dsRNA suggest a larger rotation between dsRBD monomers as compared to those in the crystal structure. Additional crosslinking or structural studies need to be performed to determine the exact orientation and location of the ADAR2 dsRBDs on dsRNA. However, these studies have shed some light on the method by which ADAR2 binds RNA.

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CHAPTER 3

FRET analysis of *in vivo* dimerization by RNA editing enzymes

- Adapted from Chillibeck *et al.*, JBC 2006, in press.

Introduction

Double-stranded RNAs (dsRNAs) in eukaryotes are subject to a variety of processing reactions including cleavage by the RNase III family members Drosha and Dicer in the miRNA and siRNA gene silencing pathways and editing by members of the ADAR (adenosone deaminase that acts on RNA) enzyme family [1,2]. This latter reaction involves the hydrolytic deamination of adenosine (A) to inosine (I) within the context of dsRNA. Editing events of this type have been demonstrated in both cellular and viral transcripts and have been shown to function in altering the coding properties of the edited RNAs. For example, the life-cycle of the Hepatitis δ virus is regulated by an editing event in the anti-genome in which a UAG stop codon is converted to a UIG tryptophan codon [3]. An A to I modification is involved in the regulation of function of a growing number of cellular factors. These include the tissue-specific editing of the serotonin 5-HT2C receptor which results in a reduction in response to serotonin agonists [4]. Transcripts for sub-units of the neural-specific AMPA class of glutamate-gated (GluR) ion channels undergo A to I modification at two positions, the Q/R and R/G editing sites, which affect the properties of the resulting channels [5,6]. In addition to the editing of these and other neuronal transcripts to effect codon changes, one deaminase family member, ADAR2, has been shown to auto-regulate its own expression by the creation of a 3' splice site (CAA to CAI) [7]. Despite the identification of these editing substrates, the global role of A to I modification in higher eukaryotes remains unclear. Measurement of inosine levels in RNA isolated from rat tissue suggests a greater level of editing than indicated by known RNA substrates [8]. As well, Morse and Bass, using a cloning protocol which depended upon an inosine specific cleavage of RNA, detected a large number of editing sites in non-coding regions of RNAs from *C. elegans* and humans which included sites in 5' and 3' UTRs and introns [9]. It has been suggested that A to I modification antagonizes RNA interference — editing could regulate endogenous miRNA mediated gene-silencing or limit RNAi resulting from non-specific anti-sense transcription [10-12].

One difficulty in identifying the biological targets and hence roles of A to I editing is that little is understood about the sequence determinants for editing of known substrates beyond the fact that editing sites are found within dsRNAs. The first RNA deaminase to be cloned, ADAR1 [13-16], was originally identified as an activity responsible for unwinding double-stranded RNA [17-19]; subsequently, this unwinding activity was correlated with deamination of A to I within these RNAs [20]. Constitutively expressed ADAR1 is a 110 kDa nuclear protein that contains three N-terminal double-stranded RNA binding domains (dsRBDs), a C-terminal deaminase domain, and one N-terminal Z-DNA binding domain (Figure 3-1). ADAR1 exhibited low deaminase activity with a number of specific substrates, including the Q/R site of the GluR-B pre-mRNA, but has been shown to efficiently edit the R/G site of GluR-B as well as the anti-genome of Hepatitis δ virus [3,21-23]. Screening of a rat hippocampal cDNA library with probes complementary to the deaminase domain of ADAR1 resulted in the cloning of ADAR2 deaminase [24]. The shorter of two isoforms, ADAR2a, is Approximately 80 kDa protein containing two N-terminal dsRBDs as well as a C-terminal deaminase domain (Figure 3-1); [25]. ADAR2a (henceforth ADAR2) has been shown to efficiently edit both the Q/R and R/G sites of the GluR-B transcript and in contrast to ADAR1 does not exhibit activity at a position within intron 11 of GluR-B [24]. ADAR1 and ADAR2 are both expressed ubiquitously although ADAR2 is enriched in the brain.

During a study of the *in vitro* editing of the GluR-B R/G site by ADAR2, we observed that efficient editing required dimerization of the enzyme on the RNA substrate [26]. This conclusion was based on a combination of kinetic and gel mobility shift analyses as well as RNA-dependent ADAR2•ADAR2 crosslinking. Subsequently, it was reported that recombinant, tagged human ADAR1 and ADAR2 could be purified as RNA-independent homodimers from Sf9 cells [27]. At the same time, O'Connell and coworkers found that the

Figure 3-1

Figure 3-1. Domain Structure of Nuclear Human dsRNA Deaminases. ADAR1 and ADAR2 contain three and two double-stranded RNA binding domains (dsRBD) respectively and a C-terminal deaminase domain. Also indicated is the Z-DNA binding region of ADAR1.

Drosophila ADAR (dADAR) dimerized in an RNA dependent fashion and that this self-association was required for editing [28].

Both ADAR1 and ADAR2 are known to be nuclear proteins. In 2003, Carmo-Fonseca and colleagues demonstrated through immunofluorescent staining that the short, constitutively expressed form of hADAR1 as well as hADAR2 are predominately localized to the nucleolus of HeLa cells [29]. The nucleolus is the largest and most well-defined nuclear compartment. Although not membrane bound, the nucleolus can be readily seen by differential interference contrast (DIC) optics due to its extremely dense structure. The nucleolus is composed of three sub-compartments: the fibrillar center (FC) which is surrounded by the dense fibrillar component (DFC) which is in turn surrounded by the granular component (GC) (reviewed in [30]). One of the major roles of the nucleolus is containing the primary site of ribosomal RNA (rRNA) transcription and processing, as well as ribosome assembly. Proteomic analysis has confirmed the importance of the nucleolus in ribosome biogenesis, with almost a third of the proteins existing in the nucleolus being involved in the pathway. However, many of the proteins identified that reside in the nucleolus have not yet been implicated in ribosomal synthesis, including DEAD box proteins, chaperones, nucleic acid binding proteins, and a large number of uncharacterized proteins [31]. The existence of proteins in the nucleolus that have not been linked to ribosome biogenesis suggests that the nucleus has other roles that have yet to be determined. Indeed, in recent years the nucleolus has been implicated in being involved in DNA damage repair [32], telomere metabolism [33], tRNA processing [34], and, most importantly for our work, RNA editing [29,35].

Corroborating the report published by Carmo-Fonseca, a separate study published by Emeson and colleagues in 2003 revealed that rat ADAR2 (rADAR2) was also localized to the nucleolus of NIH 3T3 fibroblasts. Additionally, the authors then reported that rADAR2 localization to the nucleolus is dependant upon the presence of rRNA, and nucleolar localization is lost upon inhibition of

rRNA synthesis. The ability of rADAr2 to bind RNA was revealed to be crucial to its nucleolar localization, as removal of both dsRBDs resulted in diffuse nuclear localization of the mutant rADAR2. Finally, the authors established that rADAR2 can shuttle rapidly between the nucleolus and the nucleoplasm, and nucleolar sequestration of rADAR2 limits the editing activity of the enzyme [35]. Subsequently, it was shown that hADAR2, but not hADAR1, was enzymatically active within the nucleolus [36]. Based on our previous findings that dimerization is required for enzymatic activity, we wanted to examine the dimerization state of the ADAR proteins within the nucleolus.

The fact that specific non-neural substrates have not been identified for either ADAR1 or ADAR2, despite the ubiquitous expression profile of these enzymes in all tissue types, coupled with a possible RNA dependence of their dimerization complicates study of ADAR self-association. We therefore decided to create fusion proteins of both ADAR family members with cyan and yellow fluorescent protein (CFP and YFP) and probe their association by fluorescence resonance energy transfer (FRET) in human cells [37]. Using the FRET approach, we were able to observe both homo- and heterodimerization of ADAR1 and ADAR2 in the nucleoli of HeLa cells. Mutational studies suggest that heterodimerization and homodimerization of ADAR1, but not necessarily ADAR2 are dependent on RNA binding suggesting fundamental differences in the activities of these proteins in the cell. Homo- and heterodimerization of ADAR family members, most likely in association with other cellular proteins, probably represent critical regulatory mechanisms governing A to I editing in mammalian cells.

Experimental Methods

Mammalian expression constructs

Human ADAR1 p110 was amplified by PCR from pJEL/hADAR1/H6 using primers containing *Hind*III and *Bam*HI restriction sites. Human ADAR2 was PCR amplified from a previously described template [26] using primers containing *Eco*RI and *Sal*I restriction sites. ADAR1 and ADAR2 were then inserted into pEYFP-C1, pEYFP-N1, pECFP-C1, and pECFP-N1 (Clonetech) using the appropriate restriction enzymes. Point mutations of both enzymes were created through PCR mutagenesis and confirmed by sequencing. Plasmids were transformed into chemically competent DH5α *E. coli*, amplified, and purified using a plasmid mini-prep kit (Sigma).

Cell Culture and Transfection

HeLa cells were cultured as monolayers in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C and 5% CO_2. Cells (2 x 10^5) were plated on 25 mm coverslips (Fisher Scientific) in six-well tissue culture dishes and were allowed to adhere for 24 hours. The cells were transiently transfected with 1-5 μg plasmid DNA using Perfectin (Gene Therapy Systems) according to the manufacturer's protocol and analyzed 20-24 hours post-transfection.

Fluorescence Microscopy

Transiently transfected cells on coverslips were rinsed 3 times in PBS and fixed for 15 minutes in freshly prepared 4% formaldehyde (Sigma) at room temperature. Coverslips were washed a further 3 times in PBS before being mounted onto slides using Vectasheild with DAPI (Vector Laboratories). Images were collected with a Zeiss Laser Scanning Confocal Microscope (LSM 510 NLO Meat) mounted on a Zeiss Axiovert 200M inverted microscope with a 40X F-fluar

lens (N.A. 1.3) equipped with 4 visible lasers with 5 laser lines and a spectral Meta detector. The 458 and 514 nm laser lines (emitted from a 25 mW argon laser) were used to image CFP and YFP. Band pass filters of 462-484 nm and 580-612 nm were used in collecting emission from CFP and YFP respectively.

Fluorescence Resonance Energy Transfer

FRET experiments were performed on fixed cells using the donor recovery after acceptor photobleach method [37]. First, images were obtained in the CFP and YFP channels and the intensity of the signals were calculated. YFP was then selectively photobleached to 20% of its original fluorescence at 514 nm in a defined region of the nucleus. A second set of images was then obtained using the same conditions as prior to photobleaching. FRET efficiency was calculated as follows:

$$\text{FRET efficiency} = \frac{(D_{post}-B_{post}) - (D_{pre} - B_{pre})}{(D_{post}-B_{post})}$$

where D is the donor channel intensity, B is the background intensity, pre and post indicate measurements before and after photobleaching. A non-bleached area of the same cell served as an internal control.

Results

Cellular Localization of ADAR1 and ADAR2 Fluorescent Fusion Proteins

In order to examine both cellular localization and associations of ADAR1 and ADAR2 by fluorescence, we cloned the cDNA for human ADAR1 and ADAR2 into the mammalian expression vectors ECFP and EYFP to produce ADAR fusion proteins with N-terminal CFP and YFP under the control of an SV40 promoter. We also prepared expression constructs with CFP C-terminal to the deaminase sequence in order to be able to probe for directionality in any observed association.

Previous studies have revealed a dynamic association of ADAR1 and ADAR2 with the nucleolus; ADAR2 shuttles between the nucleolus and the nucleoplasm while ADAR1 shuttles between the nucleolus, nucleoplasm, and cytoplasm (an interferon inducible form of ADAR1 is cytoplasmic) [38-40]. We determined the localization of the N-terminal CFP ADAR fusion proteins in transfected HeLa cells by confocal fluorescence microscopy observing, as expected, a predominantly nucleolar localization for these proteins (Figure 3-2). Identical results were achieved using either C-terminal CFP or N-terminal YFP expression systems (data not shown).

Homo and Heterodimerization of ADAR1 and ADAR2

Having established the nucleolar localization of the fluorescently tagged ADAR1 and ADAR2, we performed co-transfections of HeLa cells using expression vectors for N-terminal CFP/YFP ADAR1 together and CFP/YFP ADAR2 together as well as control transfections with plasmids expressing CFP and YFP alone. A FRET experiment in this system involves the direct or indirect measurement, using fluorescence, of energy transfer between CFP and YFP following specific excitation of the CFP fluorophore. In order to simplify analysis, FRET signals were quantified by measurement of donor recovery following photobleaching. Briefly, CFP fluorescence was measured at 475 nm, following excitation at 458 nm with a laser both before and after specific photobleaching of YFP. The increase in CFP fluorescence after YFP photobleaching corresponds to the original amount of energy transferred from CFP to YFP; thus the difference in CFP fluorescence prior to and after photobleaching corresponds to the level of FRET. Using this method, we were not able to measure any FRET between CFP and YFP alone. However, when N-terminal CFP ADAR1 was excited in the presence of N-terminal YFP ADAR1, we measured a FRET efficiency, between the fusion proteins localized to the nucleolus, of 18% ± 4% (Figure 3-3). For comparison, in a system expressing

Figure 3-2

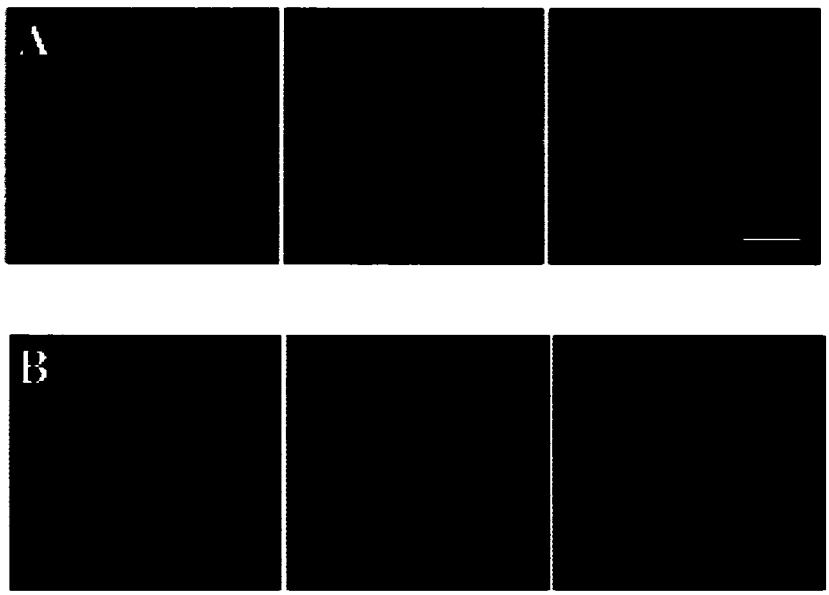

Figure 3-2. Localization of ADAR1 and ADAR2. (A) Confocal fluorescence imaging of HeLa cells transfected with ADAR1-CFP (left panel) showing DAPI imaging of the nucleus (middle panel). A merged image (right panel) shows ADAR1•CFP localizing to regions of faint DAPI fluorescence which correspond to nucleoli. (B) Confocal fluorescence imaging of HeLa cells transfected with ADAR2-CFP (left panel) showing DAPI imaging of the nucleus (middle panel). A merged image (right panel) shows ADAR2-CFP localizing to regions of faint DAPI fluorescence which correspond to nucleoli. Bar = 5 μm.

Figure 3-3

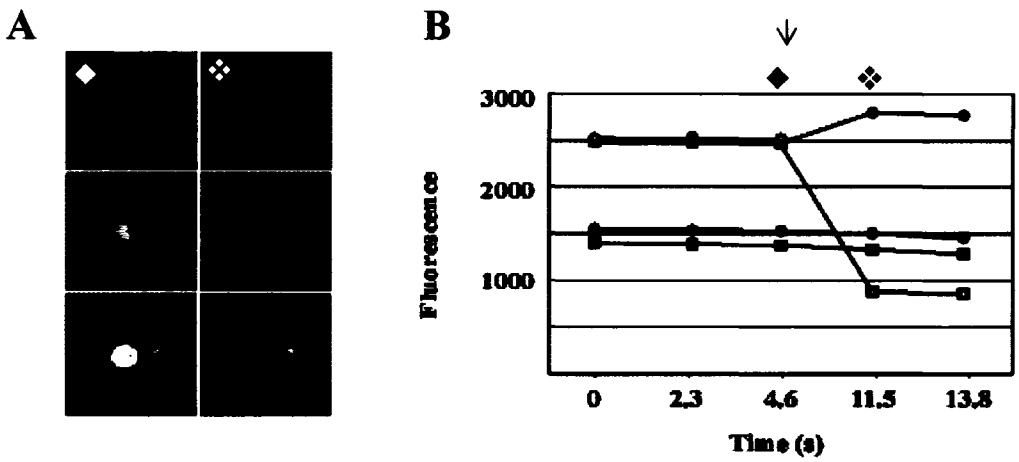

Figure 3-3. Homodimerization of ADAR1. (A) Confocal fluorescence imaging of HeLa cells co-transfected with CFP•ADAR1 and YFP•ADAR1. Direct excitation of CFP was performed using a 458 nm laser line. Shown are: emissions filtered at 462-484 nm (top panels; CFP), emissions filtered at 580-612 nm (middle panels; YFP), and merge (bottom panels). Images were taken before (left panels) and after (right panels) specifically photobleaching YFP within a nucleolus of the cell. Bar = 5 μm. (B) Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached nucleolus (open circles) and a non-bleached nucleolus (closed circles). The fluorescence of YFP is shown for the same photobleached nucleolus (open squares) and non-bleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ❖).

tandem CFP•YFP joined by a short ten amino acid linker, FRET efficiencies of 36% ± 1% were measured (data not shown). When we analyzed cells transfected with N-terminal CFP ADAR2 and N-terminal YFP ADAR2, we were able to measure FRET efficiencies of 19% ± 2% within the nucleolus (Figure 3-4). Thus, as assayed by FRET, both ADAR1 and ADAR2 form homodimers in the nucleolus.

We repeated co-transfection experiments with vectors expressing N-terminal CFP ADAR1 or ADAR2 as well as C-terminal ADAR1 or ADAR2. Because the FRET signal varies with r^6 [41] we reasoned that measurements made with the fluorophores in different orientations might yield information on the disposition of the ADAR monomers with respect to one another. However, FRET levels measured using these expression constructs were essentially the same as observed in our initial experiment (data not shown). Thus, the FRET experiment does not reveal the orientation of individual ADAR proteins with respect to one another in the dimer.

After determining that ADAR1 and ADAR2 form homodimers within the cell, we wanted to examine the possibility of heterodimerization between isoforms. We performed co-transfections in HeLa cells using N-terminal CFP ADAR2 and N-terminal YFP ADAR1. Both isoforms localized to identical nucleoli within the cells, and we were again able to measure a FRET signal between the two nucleolar localized fusion proteins (Figure 3-5). The amount of FRET observed between ADAR1 and ADAR2 was 11% ± 3% which is lower than that observed between either homodimer but is nevertheless consistent with the formation of ADAR heterodimers in the nucleolus. The lower FRET signal could arise from a weaker association within the heterodimers or a different spatial organization of the heterodimer with respect to the homodimer. Again, no difference in FRET signal was measured when co-transfecting N-terminal CFP ADAR2 and C-terminal YFP ADAR1 therefore an orientation of dimerization was impossible to determine.

Figure 3-4

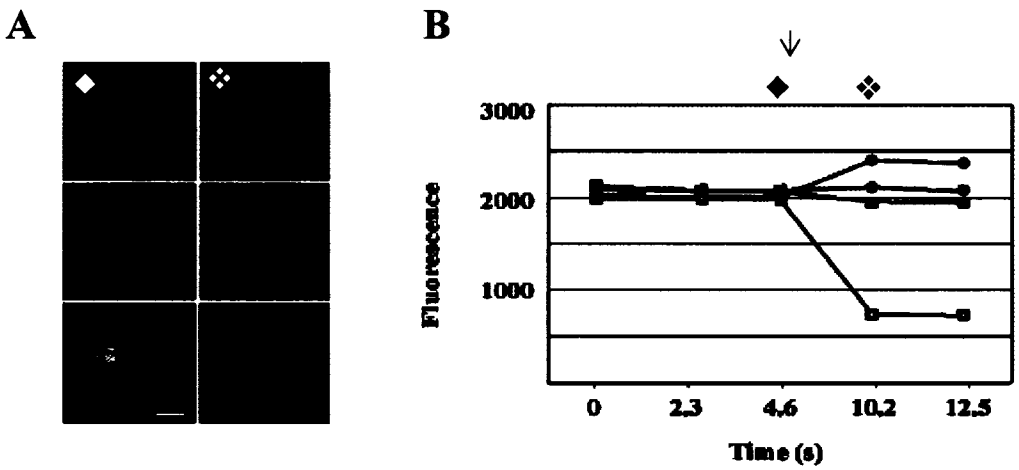

Figure 3-4. Homodimerization of ADAR2. (A) Confocal fluorescence imaging of HeLa cells co-transfected with CFP•ADAR2 and YFP•ADAR2. Direct excitation of CFP was performed using a 458 nm laser line. Shown are: emissions filtered at 462-484 nm (top panels; CFP), emissions filtered at 580-612 nm (middle panels; YFP), and merge (bottom panels). Images were taken before (left panels) and after (right panels) specifically photobleaching YFP within a nucleolus of the cell. Bar = 5 μm. (B) Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached nucleolus (open circles) and a non-bleached nucleolus (closed circles). The fluorescence of YFP is shown for the same photobleached nucleolus (open squares) and non-bleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ❖).

Figure 3-5

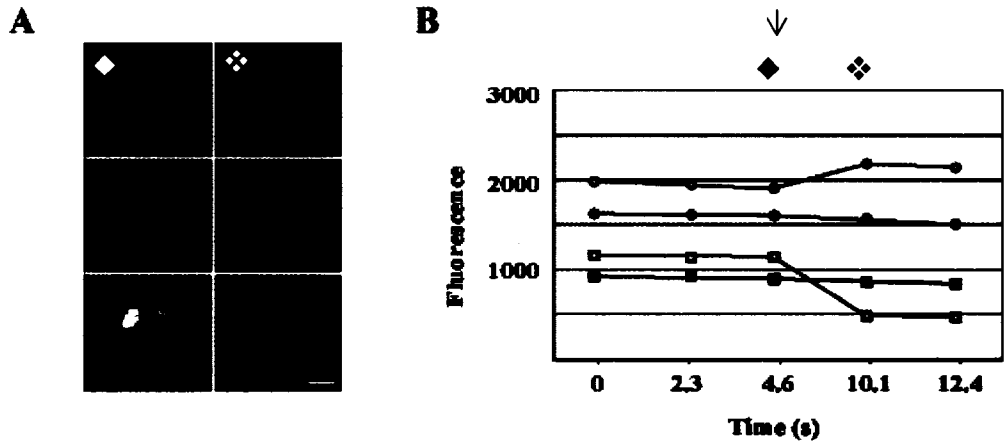

Figure 3-5. Heterodimerization of ADAR1 and ADAR2. (A) Confocal images obtained from HeLa cells co-transfected with ADAR2•CFP (top panels) and YFP•ADAR1 (middle panels). Direct excitation of CFP was performed using a 458 nm laser line. A merged image of the two top images is shown in the lower panels. Images were taken before (panels on left) and after (panels on right) specifically photobleaching YFP within a nucleolus of the cell. Bar = 5 μm. (B) Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached nucleolus (open circles) and a non-bleached nucleolus (closed circles). The fluorescence of YFP is shown for the same photobleached nucleolus (open squares) and non-bleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ❖).

RNA Binding and ADAR Dimerization

One of the reasons for examining ADAR dimerization in living cells is the possibility of a dependence on RNA or other proteins for dimerization: *in vitro* studies of ADAR association are in disagreement in this regard [42-44]. Indeed, studies of ADAR dimerization are complicated by the fact that the endogenous RNA substrates for ADAR1 and for ADAR2 in most cells are unknown. The results of our FRET experiments suggest that ADAR1 and ADAR2 form both homo- and heterodimers in the nucleolus — we also wished to examine whether disruption of RNA binding by ADAR1 or ADAR2 affected cellular localization and formation of homo- or heterodimers.

The N-terminus of ADAR family members contains two or more copies of the double-stranded RNA binding domain (dsRBD). Structural studies, including X-ray and NMR structures of dsRBDs bound to RNA have shown that dsRBDs have a characteristic α-β-β-β-α fold and that contacts to the RNA are largely mediated by amino-acid side chains of α1 and loops 2 and 4 between β1 and β2 and β3 and β4 respectively [45,46]. O'Connell and coworkers reported that mutation of a conserved alanine (A) to glutamic (E) acid in dsRBD1 of *Drosophila* ADAR abolished RNA binding and ADAR dimerization [28]. An inspection of high resolution structures shows that the A to E mutation likely causes misfolding of the dsRBD since the mutated residue is an internal one. Seeking a more conservative mutation which would be predicted to affect RNA binding, but not the overall structure of the protein, we noted that a histidine residue is conserved within the loop 2 of all ADAR1 and ADAR2 dsRBDs (Figure 3-6A). This residue is conserved in numerous other dsRBDS; in the X-ray structure of Staufen dsRBD1 bound to RNA the analogous histidine side chain contacts a 2'-OH of the sugar-phosphate backbone (Ryter and Schultz, 1998) and mutational analysis has shown that this residue is important for RNA binding (Krovat and Jantsch, 1996). We therefore prepared mutants in which the

Figure 3-6

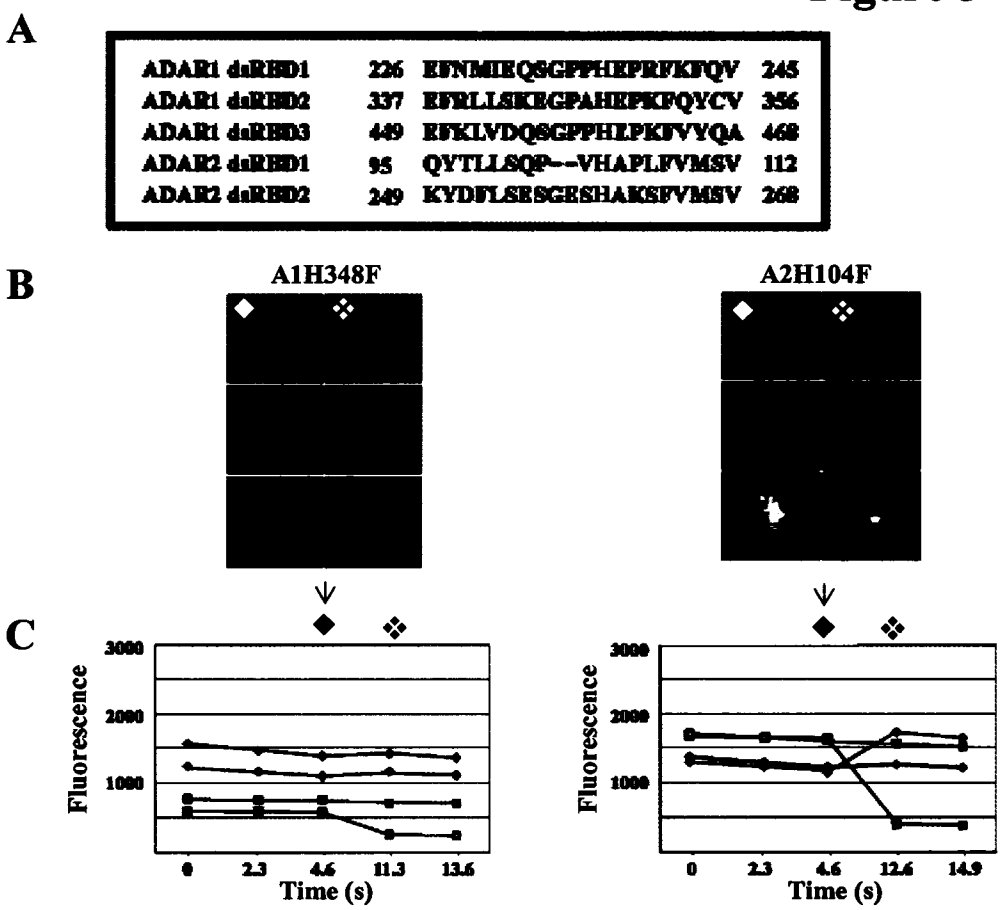

Figure 3-6. FRET of RNA Binding Mutant Heterodimerizaton. (A) Sequence alignment of dsRBDs from ADAR1 and ADAR2. Shown is a short sequence surrounding the conserved histidine (red) which was mutated in these studies. (B) Confocal images obtained from HeLa cells co-transfected with CFP-A1H348F (left, top panels) and YFP-A1H348F (left, middle panels) or CFP-A2H104F (right, top panels) and YFP-A2H104F (right, middle panels). A merged image of the two top images is shown in the lower panels. Images were taken before (panels on left) and after (panels on right) specifically photobleaching YFP within a region of the nucleus of the cell. Bar = 5 μm. (C) Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached region of the nucleus (open circles) and a non-bleached region of the nucleus (closed circles). The fluorescence of YFP is shown for the same photobleached region of the nucleus (open squares) and a non-bleached region of the nucleus (closed squares). Quantifications corresponding to images at left are indicated (◆, ❖).

appropriate histidine was changed to phenylalanine (ADAR1: A1H348F; ADAR2: A2H104F) within the context of N-terminal CFP and YFP fusion proteins of ADAR1 and ADAR2 and used these constructs in transfection of HeLa cells.

Our first observation, based on fluorescence microscopy of transfected cells, was that the A1H348F and A2H104F mutations strikingly effect the localization of the resulting proteins. Although both mutants are localized to the nucleus, neither is predominantly nucleolar and A2H104F appears to be excluded from the nucleolus (Figure 3-6B).

We performed FRET studies on cells transfected with CFP/YFP A1H348F, CFP/YFP A2H104F, or CFP A1H348F and YFP A2H104F (Figure 3-6, 3-7). These experiments show that the mutation abolished homodimerization of ADAR1 but not ADAR2 and that heterodimerization of the mutants does not occur. Thus, a mutation predicted to, at least partially, disrupt RNA binding not only changes the localization of ADAR1 and ADAR2 but abolishes ADAR1 self-association and heterodimerization. These results suggest significant differences in the mode of action of ADAR1 and ADAR2 including at least a partial RNA dependence for dimerization.

A recent report by Bass and coworkers reports the crystal structure of the deaminase domain of ADAR2 [47] and suggests that dimerization is not mediated through this domain. We expressed CFP and YFP fusion proteins of the same deaminase construct (ADAR2 299-701) used in the structural studies in HeLa cells, determined their localization, and looked for evidence of FRET between the fluorophores. The deaminase fusion proteins did not localize to the nucleolus — instead showing a diffuse expression throughout the cell — and did not undergo FRET (data not shown). This supports the conclusion that dimerization is not mediated by the deaminase domain and previous suggestions from work with *Drosophila* ADAR that ADAR self-association is mediated by N-terminal regions of the protein (Gallo, et al, 2003).

Figure 3-7

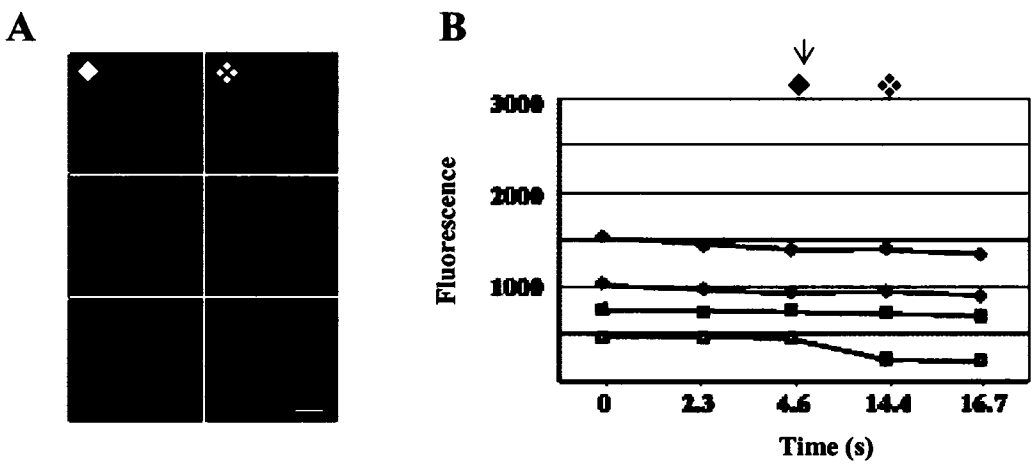

Figure 3-7. FRET of RNA Binding Mutant Homodimerization. (A) Confocal images obtained from HeLa cells co-transfected with CFP-A2H104F (top panels) and YFP-A1H348F (middle panels). Direct excitation of CFP was performed using a 458 nm laser line. A merged image of the two top images is shown in the lower panels. Images were taken before (panels on left in each pair) and after (panels on right in each pair) specifically photobleaching YFP within a region of the nucleus of the cell. Bar = 5 μm. (B) Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached region of the nucleus (open circles) and a non-bleached region of the nucleus (closed circles). The fluorescence of YFP is shown for the same photobleached region of the nucleus (open squares) and non-bleached region of the nucleus (closed squares). Quantifications corresponding to images at left are indicated (◆, ❖).

Discussion

We have examined the localization and association of human ADAR1 and ADAR2 in HeLa cell culture by transient expression of fluorescent fusion proteins followed by confocal fluorescence microscopy. Our observations are in agreement with the previously reported primary localization of both ADAR1 and ADAR2 to the nucleolus. The FRET studies reported here indicate that nucleolar ADAR1 and ADAR2 form both homo and heterodimers. The observation of ADAR homodimerization is consistent with previous *in vitro* studies; heterodimerization has not been previously observed. This suggests that heterodimerization may occur upon specific substrates which remain unidentified and that heterodimerization may serve to further modulate ADAR activity. In order to examine the RNA dependence of ADAR localization and dimerization, we mutated a conserved residue in the first dsRBD of ADAR2 and the second dsRBD of ADAR1 to disrupt association with RNA. This mutation had the effect of dramatically changing the localization of both proteins, which were now observed to have a diffuse nuclear localization. As well, this mutation abolished ADAR1 homodimerization and heterodimerization with ADAR2. The RNA dependence of at least heterodimerization explains the fact that heterodimers have not been observed in the *in vitro* studies.

The basis of nucleolar localization of ADAR1 and ADAR2 is unclear since there do not appear to be conserved nucleolar localization signals in proteins [48,49]. It appears to be, in part, mediated by the dsRNA binding by the ADAR dsRBDs; the human tRNA editing enzyme, which lacks a dsRBD, does not localize to the nucleolus and deletion of the first dsRBD of ADAR2 abolishes nucleolar localization [50]. Our observations with the A1H348F and A2H104F mutants are certainly consistent with this suggestion; it is notable that a dramatic change in nuclear localization results from loss of function of one of three and one of two dsRBDs of ADAR1 and ADAR2 respectively.

The functional significance of ADAR nucleolar localization is also unclear although is has been proposed that this might somehow modulate RNA editing by these enzymes [38]. The fact that the dsRNA structures which contain the best characterized cellular editing sites are formed by base-pairing between exon and intron sequences means that editing precedes splicing. When the editing competent portion of the GluR-B pre-mRNA was expressed in HeLa cells, this transcript accumulated in the nucleoplasm but not the nucleolus and also appeared to redirect localization of ADAR2 and ADAR1 to non-nucleolar sites containing the GluR-B transcript [50]. On the other hand, there are several pieces of evidence which suggest that RNA editing, of at least certain RNAs may take place in the nucleolus. In a recent study, Cavaillé and coworkers targeted specific editing substrates either to the nucleoplasm or nucleolus by placing their transcription under the control of a Pol II or Pol I promoter respectively and cotransfecting the corresponding minigenes with ADAR1 or ADAR2 expressing vectors [51]. Interestingly, the RNAs localized to the nucleolus were efficiently edited only at sites targeted by ADAR2 suggesting that, for these transcripts, ADAR2, but not ADAR1, is active in the nucleolus. A second, intriguing piece of evidence which may link editing to the nucleolus is the discovery in mice of a brain specific C/D small nucleolar RNA (snoRNA), MBII-52, which is predicted to target one of the A to I editing sites of the 5-HT2C receptor pre-RNA for 2'-O-methylation, a modification which has been shown to decrease editing efficiency [51]. Thus it is possible that transient nucleolar localization of the 5-HT2C RNA modulates editing.

Our observations both with respect to nuclear localization, dimerization, and dependence of both on RNA binding suggest that there are significant differences in the functional roles of ADAR1 and ADAR2 in the nucleus. The identification of previously uncharacterized ADAR heterodimers suggest a method by which the specificity of ADARs can be broadened to encompass the vast range of substrates suggested by inosine containing transcripts.

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CHAPTER 4

In vitro analysis of extra-nucleolar sites of ADAR1 localization

Introduction

Though advances in microscopy and fluorescent techniques, many individual sub-nuclear compartments in addition to the nucleolus have now been identified. These include Cajal bodies, promyelocytic leukemia (PML) bodies, the perinucleolar compartment (PNC), Sam68 nuclear bodies (SNBs), nuclear speckles, and paraspeckles (reviewed in [1]). Each of these sub-nuclear compartments can be distinguished based on appearance, composition, and function. In the previous chapter evidence for ADAR1 nucleolar localization was shown; however, ADAR1 was also found to localize in unique nuclear regions outside of the nucleoli. As a result, the experiments presented in this chapter were performed to identify these novel nuclear regions where ADAR1 was also located. Although several nuclear compartments were considered as candidates, only a limited number were studied and the reasoning for limiting the study is outlined below.

Additional sub-nuclear compartments

Cajal bodies were first discovered as bodies associated with the nucleolus in neurons in 1903 by Ramon J. Cajal. Research into these sub-nuclear bodies was limited until recent advances in microscopy. Fluorescent labeling of coilin, a component of the Cajal bodies, revealed small circular bodies between 0.2 μM and 2 μM, dependant on cell type. There are typically one to six Cajal bodies per cell, with number and size dependant upon the stage of the cell cycle. Cajal bodies have been implicated in cell cycle regulation, cellular aging, and the cellular stress response (reviewed in [2]) and have been shown to be involved in small nuclear ribonucleoprotein particle (snRNP) maturation. As there is no literature suggesting that components of these bodies participate in RNA editing, Cajal bodies were not investigated in this study.

Promyelocytic leukemia (PML) bodies are very similar to Cajal bodies in appearance, number and function. PML bodies range in size from 0.3 μm to 1 μm in diameter, with 5-30 PML bodies per cell, dependant upon cell type and stage of

the cell cycle. The main component of the PML body is the PML protein, but many other proteins are associated with this complex. These include proteins involved in gene regulation, apoptosis, DNA repair, and the anti-viral response (reviewed in [3]). Based on their association with similar potential pathways, it appears that PML bodies and Cajal bodies may be involved in some of the same cellular functions. PML bodies have not been associated with RNA modification pathways, so they were not examined as potential sites of ADAR1 localization.

The perinucleolar compartment (PNC) and Sam68 nuclear bodies (SNBs) share some common characteristics within the nucleus. Both compartments are irregularly shaped bodies localized to the periphery of the nucleoli. Both bodies range in size from 0.25 μm to 1 μm in diameter, are found in transformed cell types such as HeLa cells, and are absent in primary cell cultures. Despite their similarities in appearance and localization, fluorescent labeling of components of each body show that they are distinct and separate structures. The PNC has been shown to be involved in RNA metabolism although the exact activities occurring in the PNC have yet to be determined. In contrast, SNBs have been implicated in the transport of mRNA throughout the nucleus (reviewed in [4]). Interestingly, the integrity of both bodies appears to be dependant upon RNA polymerase I transcription, as inhibition of transcription by actinomycin D results in disassembly of both bodies. Although both bodies have been linked various mRNA modifying events, neither has yet to be shown to be involved in mRNA editing.

Within the nucleoplasm, there are abundant sub-nuclear regions called nuclear speckles. Nuclear speckles are also commonly referred to as interchromatin granule clusters (ICGs) or splicing factor compartments (SFCs). These speckled regions were discovered when performing immunofluorescence against certain splicing factors, including the SR (serine- and arginine-rich) family of splicing co-factors. Visualization of splicing factors reveals small irregularly shaped regions of variable size, with 25 to 50 speckles typically seen

per mammalian cell (reviewed in [5]). The presence of one SR protein, SC35, is typically used to identify nuclear speckles, and thus they are also referred to as "SC35 domains". In addition to splicing factors, other nuclear proteins are also concentrated in nuclear speckles. These include transcription factors [6], 3′-end RNA processing factors [7], and structural proteins [8]. Pools of poly A^+ mRNA are also localized to speckles, although it is unclear if this RNA encodes protein or is a non-coding population [9]. Speckles are not static structures; instead they are dynamic compartments that change in size, number and shape dependant on the transcriptional state and stage of the cell cycle. It has been proposed that nuclear speckles sequester mRNA processing factors from the soluble nuclear pool, releasing them to sites of active transcription in a regulated manner [5]. Therefore, nuclear speckles serve as storage compartments instead of active nuclear bodies. Although it has not been shown that the ADAR family of mRNA editing enzymes is concentrated in nuclear speckles [10], the highly dynamic nature of these bodies, along with their known concentration of mRNA, imparts a possibility of transient association of ADAR proteins in nuclear speckles and warranted further investigation.

The most recently identified and characterized sub-nuclear region is the paraspeckle domain. Paraspeckles are small punctate regions within the nucleoplasm, typically numbering 10 to 20 per mammalian nuclei. These regions bear a striking resemblance to nuclear speckles, but co-localization experiments have shown that they are distinct compartments and are often juxtaposed beside nuclear speckles, hence their name, paraspeckles [11]. Three components of paraspeckles have been identified, paraspeckle proteins 1 and 2 (PSP1 and PSP2) as well as p54/nrb, a multifunctional nuclear protein [11,12]. The function of these sub-nuclear domains has yet to be identified due to their relatively recent appearance.

The ADAR family of editing enzymes has been shown to localize to a specific nuclear structure, the nucleolus [13-15]. Our observations of

fluorescently tagged ADAR1 and ADAR2 confirmed their localization in the nucleolus and were discussed in the previous chapter of this thesis. Interestingly, we also noted a distinct localization of ADAR1 to regions outside the nucleolus. The current chapter of this thesis will discuss our characterization of these extra-nucleolar sites of ADAR1 localization. A review of the literature [1-15] suggested that ADAR1 was unlikely to be found within Cajal bodies, PML bodies, the PMC or SMCs as none of these nuclear compartments have been linked to RNA editing. As a result, nuclear speckles and paraspeckles were the most probable sites of ADAR1 localization outside of the nucleolus. In this chapter, studies of the dimerization state of ADAR1 within these two regions as well as our endeavors to understand the identity of these sub-nuclear domains are presented.

Experimental Methods

Cell Culture and Transfection

HeLa cells were cultured as monolayers in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin at 37°C and 5% CO_2. Cells (2 x 10^5) were plated on 25 mm coverslips (Fisher Scientific) in six-well tissue culture dishes and were allowed to adhere for 24 hours. The cells were transiently transfected with 1 to 5 μg plasmid DNA using Perfectin (Gene Therapy Systems) according to the manufacturer's protocol and analyzed 20 to 24 hours post-transfection.

Fluorescence Microscopy

Transiently transfected cells on coverslips were rinsed three times in PBS and fixed for 15 minutes in freshly prepared 4% formaldehyde (Sigma) at room temperature. Coverslips were washed an additional three times in PBS before being mounted onto slides using Vectasheild with DAPI (Vector Laboratories). Images were collected with a Zeiss Laser Scanning Confocal Microscope (LSM

510 NLO Meat) mounted on a Zeiss Axiovert 200M inverted microscope with a 40X F-fluar lens (N.A. 1.3) equipped with 4 visible lasers with 5 laser lines and a spectral Meta detector. The 458 and 514 nm laser lines (emitted from a 25 mW argon laser) were used to image CFP and YFP. Band pass filters of 462-484 nm and 580-612 nm were used in collecting emission from CFP and YFP, respectively.

Fluorescence Resonance Energy Transfer (FRET)

FRET experiments were performed on fixed cells using the donor recovery after acceptor photobleach method. First, images were obtained in the CFP and YFP channels and the intensity of the signals were calculated. YFP was then selectively photobleached at 514 nm in a defined region of the nucleus. A second set of images were then obtained using the same conditions as prior to photobleaching. FRET efficiency was calculated as follows:

$$\text{FRET efficiency} = \frac{(D_{post}-B_{post}) - (D_{pre} - B_{pre})}{(D_{post}-B_{post})}$$

where D is the donor channel intensity, B is the background intensity, pre and post indicate before and after photobleaching. A non-bleached area of the same cell served as an internal control.

Immunofluorescence

An immunofluorescence protocol was modified from previously descibed methods [16]. HeLa cells (2×10^5 cells) were plated on 25 mm coverslips in six-well tissue culture dishes and were allowed to adhere for 24 hours. The cells were then transiently transfected with 1 to 5 μg plasmid DNA using Perfectin according to the manufacturer's protocol. The cells were washed 20 to 24 hours post-transfection three times in PBS and then fixed for 15 minutes in freshly prepared 4% formaldehyde at room temperature. The cells were then washed three times for 10 minutes in PBS and permeabilized in 0.5% Triton X-100 plus

1% FBS for 5 minutes on ice. Following three 10 minute washes in PBS plus 1% FBS, cells were incubated with the appropriate concentration of primary antibody in a humidified chamber for 1 hour at room temperature. After three 10 minute washes in PBS plus 1% FBS, cells were incubated with 4.5 μg/mL Cy5 conjugated Goat anti-Mouse IgG (Jackson ImmunoReaserch, Catalogue #715-175-150) for 1 hour in a humidified chamber. The cells were then washed four times for 10 minutes in PBS and mounted using Vectashield with DAPI.

Results

ADAR1 Localizes to Nucleolar and Extra-Nucleolar Regions

Mammalian vectors containing human ADAR1 linked to CFP and YFP were created to examine cellular localization and dimerization of ADAR1, and were discussed in the previous chapter of this thesis. Upon transfection of HeLa cells with the vectors containing ADAR1 cDNA, a predominantly nucleolar localization of fluorescently tagged ADAR1 was observed. Interestingly, some ADAR1 fluorescence was localized to small regions within the nucleus, but distinct from the nucleoli. These regions appeared as tiny spots, and consistently appeared within transfected cells, but varied in number from a few to a few dozen per cell (Figure 4-1). When both CFP and YFP tagged ADAR1 were transfected into HeLa cells, it was determined that both proteins co-localized to the nucleoli and extra-nucleolar regions. We were able to examine the dimerization of ADAR1 within the extra-nucleolar regions using the FRET techniques previously described. Consistent with our discovery that ADAR1 forms dimers in the nucleus, ADAR1 also dimerizes within the extra-nucleolar regions. We were able to observe an increase in CFP-ADAR1 fluorescence after specifically photobleaching ADAR1-YFP, indicative of an energy transfer between CFP and YFP (Figure 4-1). The difference in CFP fluorescence before and after YFP photobleaching gave a FRET efficiency of 16% ± 2% within the extra-nucleolar

Figure 4-1

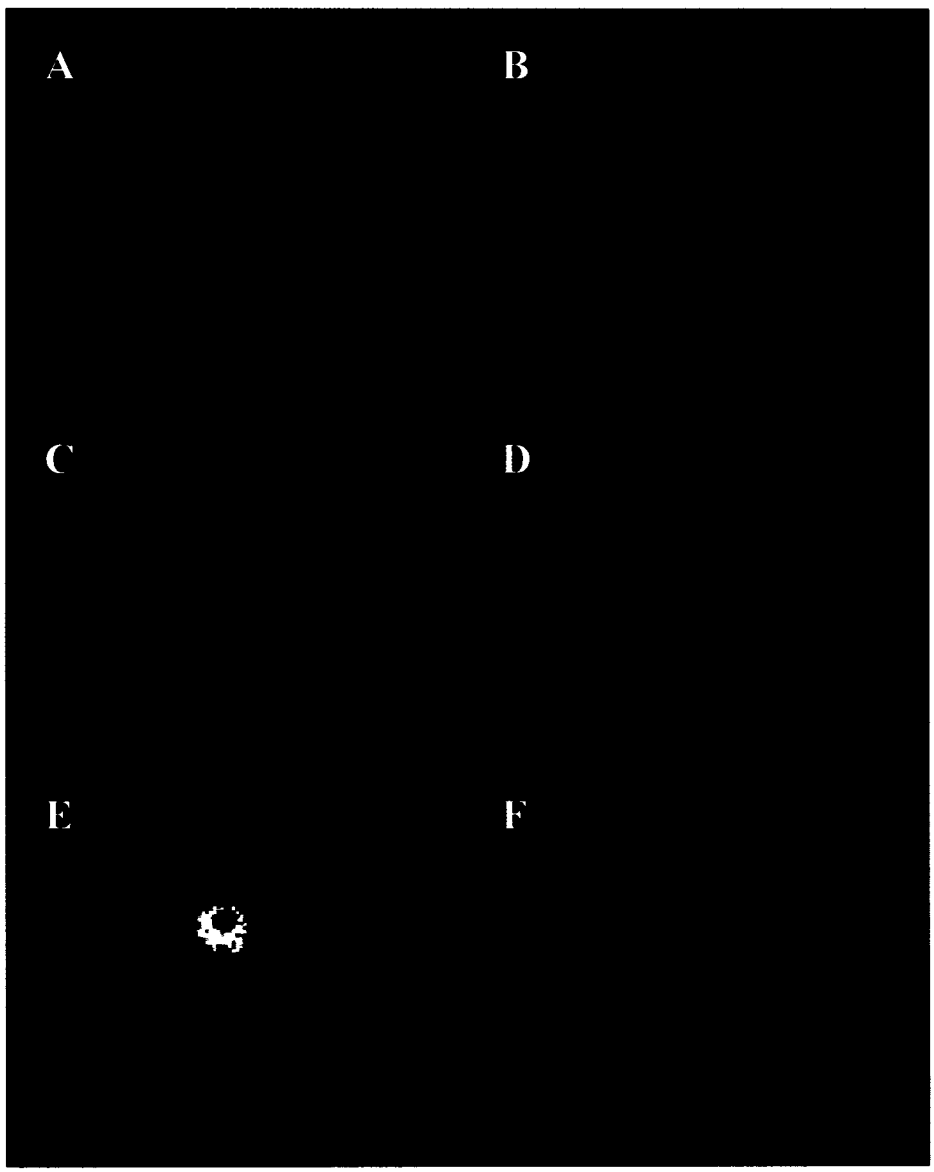

Figure 4-1. ADAR1 FRET in extra-nucleolar spots. Confocal images obtained from HeLa cells co-transfected with CFP-ADAR1 (A, B) and ADAR1-YFP (C, D). Direct excitation of CFP was performed using a 458 nm laser line. A merged image of the top two panels is shown in the lower panel (E, F). Images were taken before (images on left) and after (images on right) specifically photobleaching YFP within specific regions of the nucleus.

spots. The FRET efficiency observed stayed consistent despite changing the terminus of ADAR1 to which the CFP or YFP was attached (data not shown).

ADAR1 and ADAR2 Co-Localization in Extra-Nucleolar Spots

When ADAR2 alone was transfected into HeLa cells, we were unable to detect any fluorescence outside of the nucleolus. However, when ADAR2 was co-transfected with ADAR1, it was noted that both proteins co-localized to both the nucleolus and the extra-nucleolar regions. Again, we performed FRET on the extra-nucleolar regions that were present upon transfection with ADAR2-CFP and YFP-ADAR1. Intriguingly, we were only able to obtain a FRET efficiency for a subset of extra-nucleolar spots. Some extra-nucleolar spots, despite having both proteins present, gave a FRET efficiency of 0%. Even though there were spots that did not undergo FRET, there was a portion of the extra-nucleolar spots to which ADAR1 and ADAR2 co-localized that did have a FRET efficiency (Figure 4-2). The FRET efficiency measured in these extra-nucleolar spots was 12% ± 3%, which was significantly lower that that seen when ADAR1 alone was transfected. Therefore, as determined by FRET analysis, ADAR1 and ADAR2 heterodimerize within a small subset of extra-nucleolar spots, despite being co-localized in all extra-nucleolar regions assayed.

Extra-Nucleolar Regions and Sites of Active Transcription

After determining that ADAR1 forms homodimers in all extra-nucleolar spots assayed, but only forms heterodimers with ADAR2 in a small subset of spots, we wanted to identify the cellular processes occurring in the extra-nucleolar regions. As one of the main processes that occur in the nucleus is transcription, we wanted to examine if the extra-nucleolar regions corresponded to site of active transcription. Incorporation of a uridine analogue within a nascent transcript allows for its detection using an α-bromodeoxyuridine antibody, followed by a fluorescently labeled secondary antibody. When fluorouridine (FU) is incubated

Figure 4-2

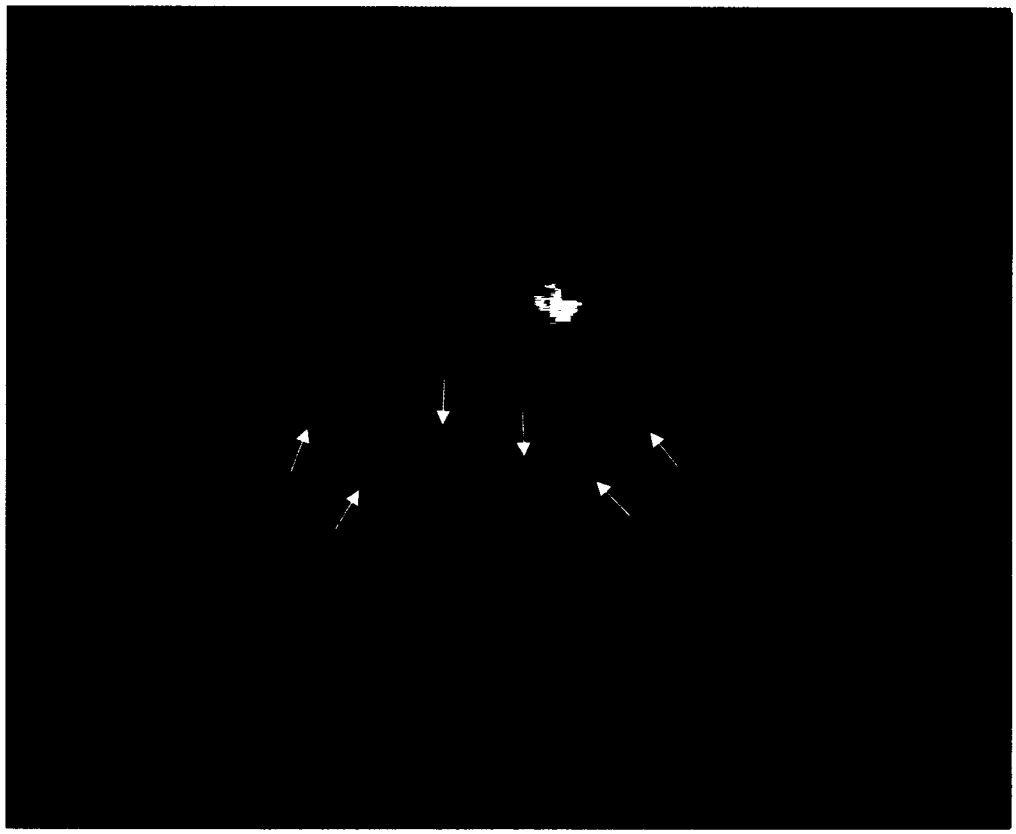

Figure 4-2. ADAR1/ADAR2 FRET in extra-nucleolar spots. Confocal image obtained from HeLa cells co-transfected with ADAR2-CFP and YFP-ADAR1. Direct excitation of CFP was performed using a 458 nm laser line. Both proteins co-localized to the same regions of the nucleus, as indicated by the yellow color (a merge of green and red channels) Arrows point to extra-nucleolar spots that had no FRET signal.

in culture medium, it is readily incorporated into the nucleolus and nascent RNA polymerase I transcripts. In contrast, bromouridine (BrU) is unable to penetrate the nucleolus and thus preferentially labels RNA polymerase II transcripts within the nucleoplasm [17]. We utilized both of these uridine analogues to examine the identity of the extra-nucleolar spots. Upon incubation of HeLa cells with 2 mM FU and performing immunofluorescence to detect the nucleotide, it was noted that the transcripts that contain the FU are located on the outer ring of the nucleoli (Figure 4-3). When BrU was incubated on HeLa cells and localized through immunofluorescence, a very different localization pattern was observed. Instead of being localized to the nucleolus, transcripts containing BrU were located throughout the nucleus, and were distinctly absent from the nucleoli (Figure 4-4). Clearly, BrU incorporation was preferentially being incorporated into RNA polymerase II transcripts while FU was being incorporated into RNA polymerase I transcripts. Although we were able to distinguish between nucleolar and non-nucleolar transcription, the resolution of the immunofluorescence images did not allow us to identify specific extra-nucleolar spots as sites of active transcription. Instead, we saw a diffuse staining throughout the nucleus when visualizing BrU containing transcripts. Due to mechanical limitations, we were unable to identify the extra-nucleolar regions as sites of active transcription using modified uridine incorporation and immunofluorescence.

In order to further probe the possibility that the extra-nucleolar regions correspond to sites of active transcription, we used the RNA polymerase II inhibitor, α-amanitin. Through specifically blocking transcription by RNA Polymerase II, α-amanitin effectively prevents transcription by this enzyme in the nucleus [18]. We used previously published data for α-amanitin in HeLa cells, giving us optimum concentrations and duration of incubation for the drug in HeLa cell media [19]. Upon incubation of 50 μg/mL α-amanitin for 5 hours on coverslips 70% confluent, we saw no change in the appearance of the extra-nucleolar regions containing ADAR1 (Figure 4-5). Due to their insensitivity to α-

Figure 4-3

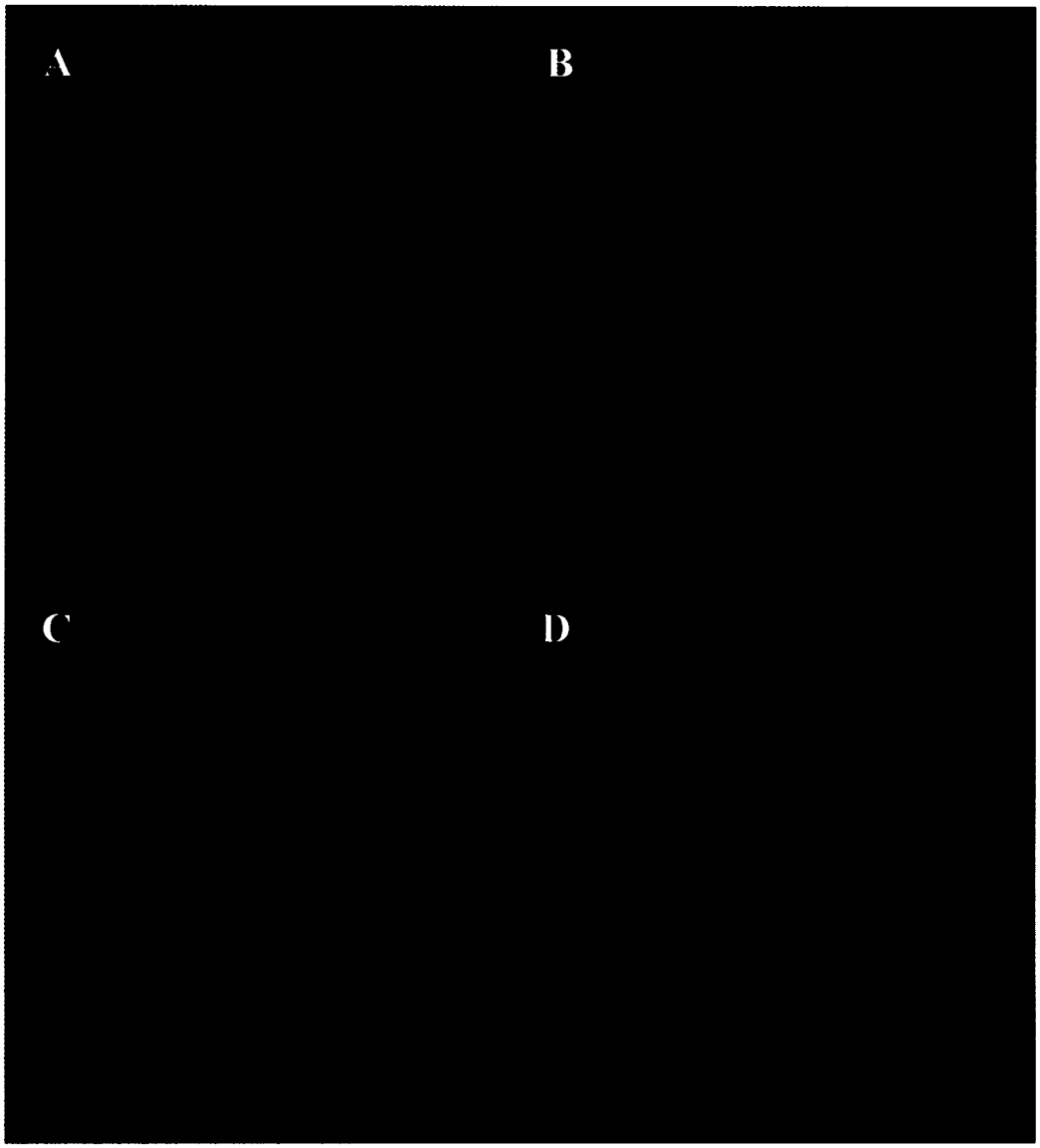

Figure 4-3. Fluorouridine labeling of HeLa cells. (A) 2 mM fluorouridine (FU) incubated in HeLa cell media for 1 hour, and detected using α-BrdUTP diluted 1:50. (B) Light microscope image showing nucleus (raised circular region) within HeLa cells. (C) DAPI staining of nucleus showing nucleoli (black regions within DAPI stained area). (D) Overlay of DAPI staining and FU fluorescence. Most of the FU florescence is located on the outer layer of the nucleoli.

Figure 4-4

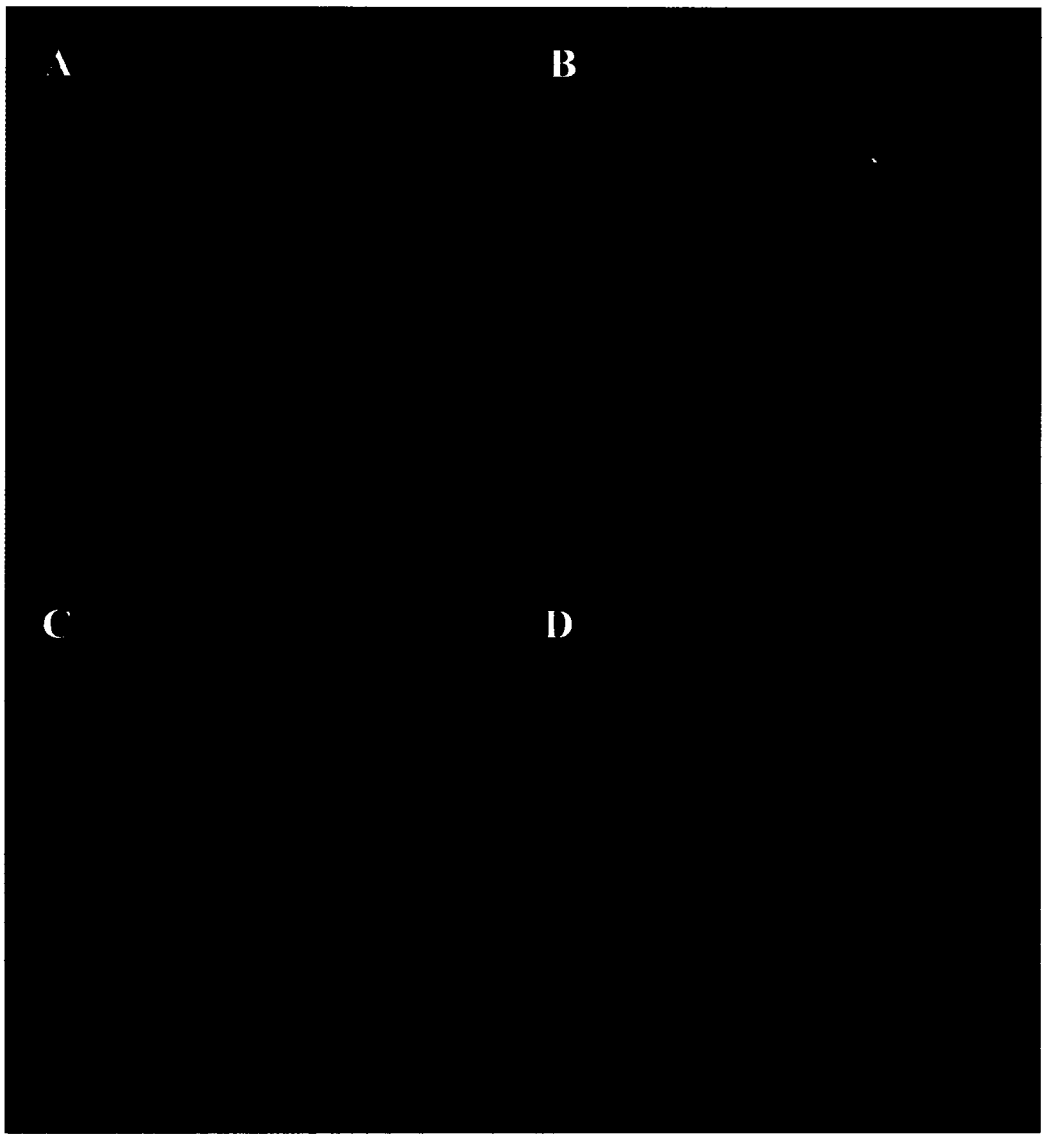

Figure 4-4. Bromouridine labeling of HeLa cells. (A) 5 mM fluorouridine (BrU) incubated in HeLa cell media for 1 hour, and detected using α-BrdUTP diluted 1:100. (B) Light microscope image showing nucleus (raised circular region) within HeLa cells. (C) DAPI staining of nucleus showing nucleoli (black regions within DAPI stained area). (D) Overlay of DAPI staining and BrU fluorescence. The BrU florescence located in the nucleus but is excluded from the nucleoli.

Figure 4-5

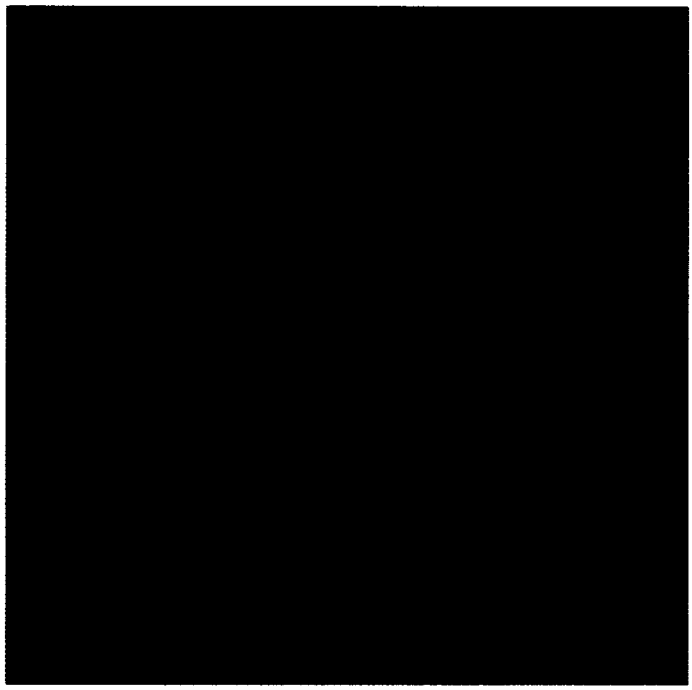

Figure 4-5. Extra-Nucleolar spots are not sensitive to α-amanitin. HeLa cells transfected with ADAR1-CFP and incubated with 50 μg/mL α-amanitin for 5 hours. The small spots seen surrounding the nucleoli do not appear to be sensitive to α-amanitin treatment.

amanitin treatment, it seems unlikely that the extra-nucleolar regions correspond to sites of active transcription.

Extra-Nucleolar Regions and Nuclear Speckles

A common sub-nuclear structure is the nuclear speckle domains, a term established to describe the intra-nuclear staining of intrachromatin granual clusters (ICGs). These domains are numerous within the nucleus and have been shown to be storage regions for splicing factors [20]. The number and appearance of speckles within HeLa cells was similar to the number and appearance of the extra-nucleolar regions we were observing, therefore we speculated that the extra-nucleolar regions might co-localize with speckle domains. A frequently used marker for speckle domains is the splicing factor SC35 [21]. Upon performing co-localization experiments using an antibody to SC35 and our fluorescently tagged ADAR1-CFP, we were able to see distinct SC35 foci within the nucleus. It was apparent that the speckles containing SC35 did not co-localize with the extra-nucleolar regions containing ADAR1-CFP (Figure 4-6). Therefore, we were able to show that the extra-nucleolar regions that ADAR1 localizes to are not nuclear speckles.

Paraspeckles and Extra-Nucleolar Regions

Having determined that the extra-nucleolar regions did not co-localize with nuclear speckles, we examined the possibility that they were localized to paraspeckles. Paraspeckles were discovered in 2002 though examining the localization of a protein identified through a mass spectrometry screen of nucleoli. Paraspeckle protein 1 (PSP1) was found to express in a punctate pattern similar to nuclear speckles. However, upon performing co-localization experiments, it was shown that PSP1 localized to a novel nuclear structure that existed near but did not overlap with nuclear speckles [11]. A generous gift of α-PSP1 antibody from the Lamond laboratory allowed us to investigate if the extra-nucleolar regions we

Figure 4-6

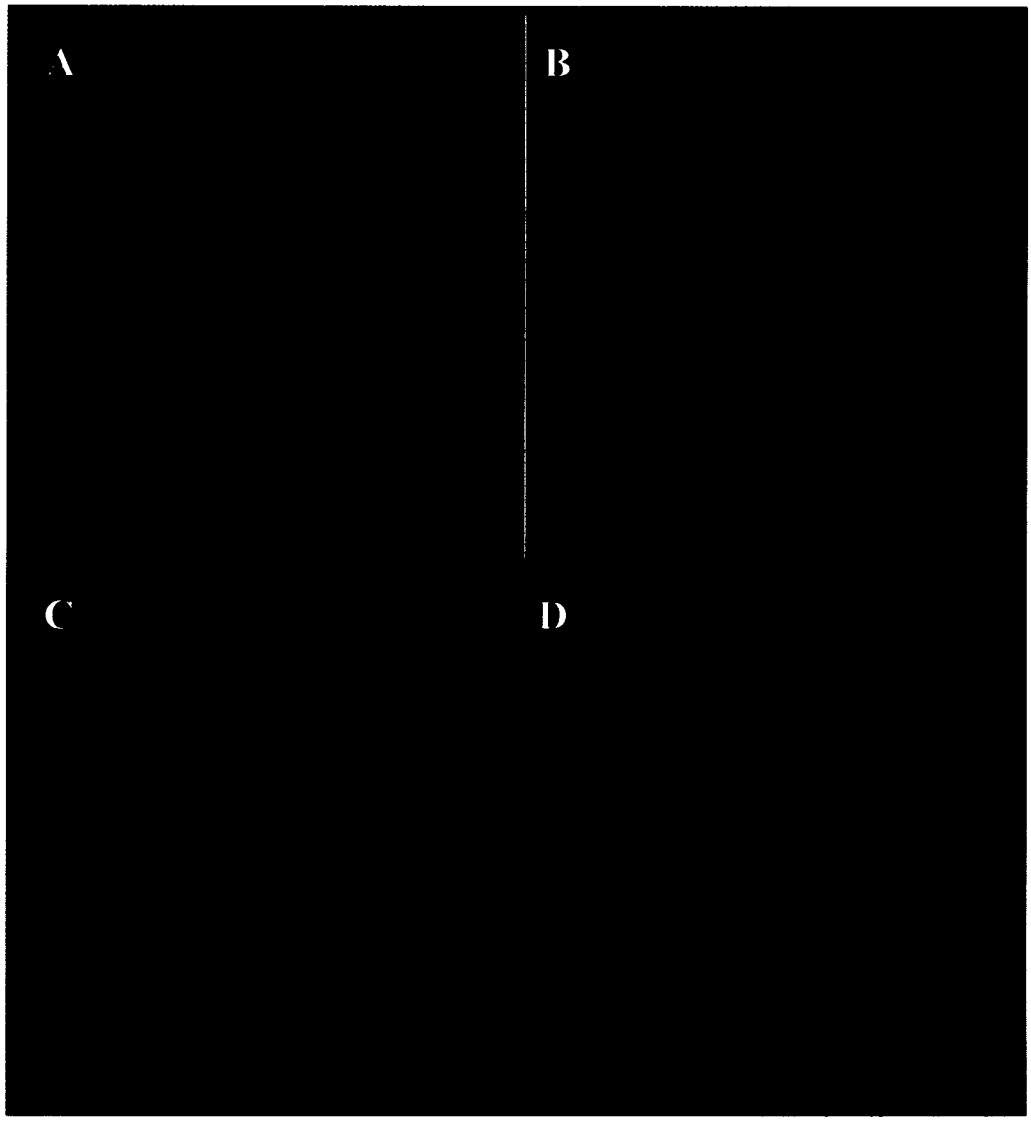

Figure 4-6. ADAR1-CFP and SC35 localization. (A) HeLa cells incubated with α-SC35 at a dilution of 1:200. (B) DAPI staining of nucleus. (C) ADAR1-CFP fluorescence showing nucleoli and small spots. (D) Overlay of DAPI , ADAR1-CFP and α-SC35 fluorescence. The α-SC35 fluorescence is in regions distinct from those of ADAR1-CFP.

were observing co-localized with nuclear paraspeckles. Using immunofluorescence, we were able to visualize the paraspeckles domains in our transfected HeLa cells. We observed no overlap in the location of PSP1 and ADAR1 in the extra-nucleolar regions (Figure 4-7). Thus, it seems that the extra-nucleolar regions present upon ADAR1 transfection are not nuclear paraspeckles domains.

Discussion

Upon transfection of fluorescently tagged ADAR1 into HeLa cells, ADAR1 was observed to be localized to both the nucleolus and small extra-nucleolar domains. Using FRET techniques we were able to determine that ADAR1 homodimerizes within the extra-nucleolar regions. These small, circular regions of extra-nucleolar fluorescence were also observed upon co-transfection of ADAR1 with ADAR2, but were absent when ADAR2 alone was transfected. Again, through the utilization of FRET techniques, we were able to determine that ADAR1 and ADAR2 heterodimerize only within a small subset of extra-nucleolar regions, despite being co-localized to all regions tested.

After determining the dimerization state of the ADAR proteins in the extra-nucleolar regions, it was our desire to identify these sub-nuclear domains. We examined the possibility that the small extra-nucleolar regions could correspond to sites of active transcription. Incorporation of bromouridine triphosphate (BrU) and subsequent detection by immunofluorescence allowed us to visualize nascent RNA polymerase II transcripts. Unfortunately, we only observed a diffuse staining throughout the nucleoplasm and were unable do detect discrete transcription foci with which to perform co-localization studies. Upon incubation of transfected cells with the RNA polymerase II specific inhibitor, α-amanatin, we observed no change in the appearance or frequency of the extra-nucleolar regions containing ADAR1. This indicated to us that the extra-

Figure 4-7

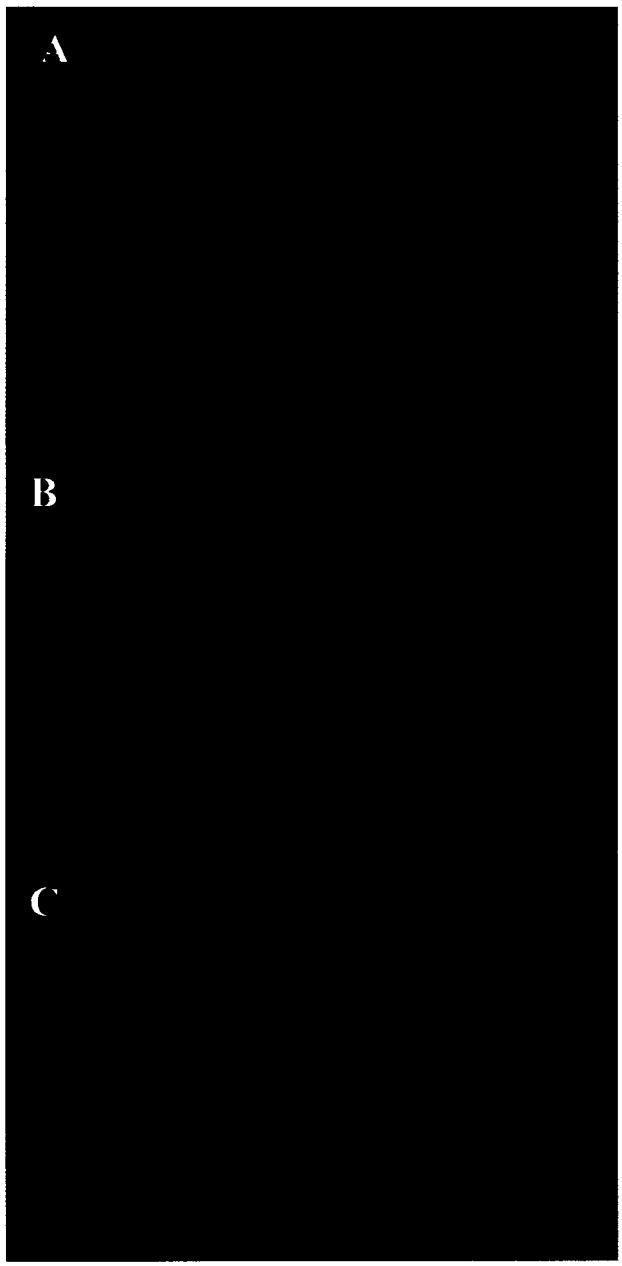

Figure 4-7. ADAR1-CFP and PSP1 localization. (A) HeLa cells incubated with α-PSP1 at a dilution of 1:200. (B) ADAR1-CFP fluorescence showing nucleoli and small spots. (D) Overlay of ADAR1-CFP and α-PSP1 fluorescence. The α-PSP1 fluorescence is in regions distinct from those of ADAR1-CFP.

nucleolar domains to which ADAR1 localized were not sites of active transcription.

As presented in the introduction, there are multiple sub-nuclear compartments within mammalian cell nuclei that could potentially co-localize with the extra-nucleolar regions we were observing upon transfection of ADAR1 into HeLa cells. We examined the two most likely candidates, nuclear speckles and paraspeckles. Fluorescently tagged ADAR1 was transfected into HeLa cells, and immunofluorescence techniques were used to visualize the specific sub-nucleolar compartments. Using an antibody against SC35, a splicing factor and a component of nuclear speckles, we were unable to detect any co-localization of ADAR1 and the speckle domains. An antibody against a component of paraspeckles, PSP1, revealed that the extra-nucleolar domains to which ADAR1 localized were not co-localized with paraspeckle domains. It was apparent that identity of the extra-nucleolar regions corresponded with neither of the two most promising candidates, speckles and paraspeckles.

There are other sub-nuclear compartments that may contain the extra-nucleolar regions to which ADAR1 localizes, but none with as much promise as speckles and paraspeckles. Cajal bodies are much less numerous in nuclei, with only a few per cell, whereas our observed extra-nucleolar regions numbered in the dozens. Unless transfection with ADAR1 changes the morphology of the nuclei and results in a great increase in the number of these bodies, it is unlikely that further experimentation will show that the extra-nucleolar regions co-localize with Cajal bodies. Similarly, PNCs and SNBs number only a few per cell, in contrast to our observed regions. In addition, PNCs and SNBs do not have a similar morphology, being larger and more irregularly shaped compared to the appearance of the extra-nucleolar regions we observed. PNCs and SNBs are predominantly located on the nucleolar periphery, whereas ADAR1 extra-nucleolar regions are located throughout the nucleoplasm. It seems then, that

none of these three sub-nuclear compartments is a strong candidate for the identity of the ADAR1 containing extra-nucleolar regions.

PML bodies have a similar shape and frequency to our observed extra-nucleolar regions. Dependant on cell type, there can be between 5 and 30 PML bodies per nucleus and when visualized they appear as small circular foci. The possibility that our observed ADAR1 containing extra-nucleolar regions may be PML bodies must be examined. Immunofluorescence with antibodies directed against a known PML protein would allow for co-localization experiments with fluorescently tagged ADAR1 to be performed. Further testing must be carried out to determine if the identify the ADAR1 containing extra-nucleolar regions corresponds to a specific sub-nuclear compartment. However, additional experimentation may reveal the ADAR1 containing extra-nucleolar regions do not co-localize with any known sub-nuclear regions and thus may be a novel sub-nuclear compartment that remains to be identified.

The appearance and frequency of the extra-nucleolar regions to which ADAR1 localizes is similar to the punctate appearance seen when antibodies are directed against actively transcribing RNA polymerase II [22]. Although we attempted to visualize nascent transcription through BrU incorporation, we were unable to identify transcription foci. Our results with α-amanitin suggest that the extra-nucleolar regions are not sites of active transcription. However, we did not perform titration experiments with α-amanitin. It may be that the concentrations of drug and incubation times used were ineffective at inhibiting RNA polymerase II in the HeLa cells transfected with ADAR1. To clarify if the ADAR1 containing extra-nucleolar regions are sites of active transcription, further experiments must be undertaken. Distinct transcription foci have been observed when using antibodies against the hyperphosphorylated, transcriptionally active form of RNA polymerase II [22]. Co-localization experiments examining the location of both ADAR1 and hyperphosphorylated RNA polymerase II may reveal if the extra-nucleolar regions are sites of active transcription. In addition,

more comprehensive studies involving a complete range of α-amanitin concentrations and incubation times would greatly support the immunofluorescence results. Both these sets of experiments must be undertaken to thoroughly explore the hypothesis that the extra-nucleolar regions may be sites of active transcription.

If the extra-nucleolar regions prove to be sites of active transcription, then a hypothesis about the state of dimerization of the ADARs at these sites can be formulated. This hypothesis is purely speculative and must be tested thoroughly. Based on immunofluorescence against transcriptionally active RNA polymerase II, there are many more sites of active transcription than my observed extra-nucleolar regions. It is possible that ADAR1 is targeted to nascent RNA in need of editing. However, edited RNAs are only a fraction of the total RNA being generated, resulting in ADAR1 only being recruited to a small percentage of the transcription foci. When ADAR1 is recruited to transcription foci, it may homodimerize upon the nascent RNA, potentially even when it is not actively editing, explaining our observations that ADAR1 homodimerizes in all extra-nucleolar regions tested. When ADAR1 was co-transfected with ADAR2, ADAR2 was also localized to the extra-nucleolar regions. Building on our hypothesis that these are sites of active transcription, this could be explained by ADAR2 being recruited to a nascent RNA substrate along with ADAR1. However, when ADAR2 was expressed in the absence of ADAR1, it was not localized to the extra-nucleolar regions. This could be due to the absence of ADAR2 targets in HeLa cells as all known ADAR2 substrates are neural in origin. HeLa cells are non-neuronal cell lines and thus likely do not transcribe ADAR2 substrate RNAs. Our previous work examining the dimerization state of ADAR2 and RNA binding deficient mutants, detailed in the previous chapter of this thesis, suggests that ADAR2 remains a homodimer even when not bound to RNA. Therefore, it may be that when ADAR2 is recruited to nascent RNAs, it is recruited as a homodimer. Recruitment of ADAR2 to nascent RNAs is dependant

on ADAR1, thus it may be that there are RNA substrates in HeLa cells that require heterodimerization of ADAR1 and ADAR2 to be edited. If this were the case, it could explain our observations that there is a FRET signal from only a subset of extra-nucleolar regions. If ADAR2 is recuited to the RNA as a homodimer it may remain homodimerized until loaded on the nascent RNA editing site with an ADAR1 monomer. Thus, in a portion of the extra-nucleolar regions where the ADAR2 is "waiting" to be active, it remains in a homodimerized form, giving no FRET signal. However, when ADAR2 interacts with an RNA with ADAR1, it is actively editing and heterodimerized, giving a FRET signal in a fraction of extra-nucleolar regions. This hypothesis is highly speculative and has been proposed to rationalize my observations; however it must be thoroughly tested before any conclusions can be made as to the nature of the extra-nucleolar regions.

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