

# The RNA-editing Enzyme ADAR1 Is Localized to the Nascent Ribonucleoprotein Matrix on *Xenopus* Lampbrush Chromosomes but Specifically Associates with an Atypical Loop

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**Abstract.** Double-stranded RNA adenosine deaminase (ADAR1, dsRAD, DRADA) converts adenosines to inosines in double-stranded RNAs. Few candidate substrates for ADAR1 editing are known at this point and it is not known how substrate recognition is achieved. In some cases editing sites are defined by basepaired regions formed between intronic and exonic sequences, suggesting that the enzyme might function cotranscriptionally. We have isolated two variants of *Xenopus laevis* ADAR1 for which no editing substrates are currently known. We demonstrate that both variants of the enzyme are associated with transcriptionally active chromosome loops suggesting that the enzyme acts cotranscriptionally. The widespread distribution of the protein along the entire chromosome indicates that

ADAR1 associates with the RNP matrix in a substrate-independent manner. Inhibition of splicing, another cotranscriptional process, does not affect the chromosomal localization of ADAR1. Furthermore, we can show that the enzyme is dramatically enriched on a special RNA-containing loop that seems transcriptionally silent. Detailed analysis of this loop suggests that it might represent a site of ADAR1 storage or a site where active RNA editing is taking place. Finally, mutational analysis of ADAR1 demonstrates that a putative Z-DNA binding domain present in ADAR1 is not required for chromosomal targeting of the protein.

**Key words:** RNA editing • chromosomal localization • RNA splicing • *Xenopus* oocytes • epitope tagging

DOUBLE-STRANDED RNA adenosine deaminase (ADAR1, dsRAD, DRADA)<sup>1</sup> is an RNA-editing enzyme that converts adenosines to inosines by hydrolytic deamination in double-stranded RNA (Polson et al., 1991). The enzymatic activity was first discovered in *Xenopus* embryos and was described as an unwinding and modifying activity that converts adenosines to inosines in double-stranded RNAs injected into embryos (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Since then, the enzymatic activity has been detected in all metazoan tissues tested (for review see Bass, 1997; O'Connell, 1997). Recently, cDNAs encoding ADAR1 have been cloned from several organisms, including human, rat, and *Xenopus* (Kim et al., 1994; O'Connell et al., 1995; Hough and Bass, 1997).

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1. *Abbreviations used in this paper:* AMD, actinomycin D; dsRNA, double-stranded RNA; dsRBD, double-stranded RNA-binding domain; GV, germinal vesicle; LBC, lampbrush chromosome; RNP, ribonucleoprotein particle; snRNA, small nuclear RNA; SLL, sequentially labeling loop.

The putative translation products of the cloned cDNAs from these organisms encode proteins in the range of 130–150 kD and share several characteristic features. In general, the COOH-terminal ends of ADAR1 proteins from these species are more homologous to each other than their NH<sub>2</sub>-terminal ends. Consistent with the high degree of conservation, a conserved catalytic domain required for deamination is found at the COOH-terminal end of all ADAR1 homologues (Kim et al., 1994; Hough and Bass, 1997). Three double-stranded RNA-binding domains (dsRBDs) are located in the central region while one or two putative nuclear localization signals (NLS) are located more NH<sub>2</sub>-terminally (St Johnston et al., 1992). Additionally, some ADAR1 proteins contain a variable number of a tandemly arranged oligopeptide motif at their NH<sub>2</sub>-terminal ends and a short protein motif that has been implemented in Z-DNA binding (Herbert et al., 1997).

The search for RNA adenosine deaminases has also led to the identification of related proteins like RED1 (for RNA-editing deaminase), which has recently been renamed to ADAR2 (Melcher et al., 1996; Bass et al., 1997). ADAR2 proteins are generally smaller than ADAR1 but are quite similar in their molecular architecture. The COOH-terminal regions contain conserved deamination

domains whereas the central region usually contains one or two dsRBDs. In contrast, the NH<sub>2</sub>-terminal region is shorter in ADAR2 and related proteins than in ADAR1 (for review see O'Connell, 1997).

A number of candidate substrates are currently known for editing by ADARs. These include the genomes of some RNA viruses, several subunits of the group of glutamate gated ion channels and the serotonin receptor 2C (Sommer et al., 1991; Lomeli et al., 1994; Hurst et al., 1995; Polson et al., 1996; Burns et al., 1997; reviewed by Bass, 1997). Editing by ADAR-like enzymes leads to conversion of an adenosine to an inosine. As inosines are interpreted as guanosines by the translational machinery, the editing event may lead to the alteration of a codon and thus change the coding potential of the edited RNA. Interestingly, RNA editing by ADARs can be quite different depending on the substrate. In the case of measles virus or the *Drosophila* 4f-rnp RNA, for instance, multiple editing events occur quite randomly (Cattaneo et al., 1988; Petschek et al., 1997). However, careful in vitro analysis revealed that ADAR1 exhibits a 5' next neighbor preference for RNA editing (Polson and Bass, 1994). On the other hand, editing of glutamate receptor subunits is quite specific. In GluR-B RNA, editing occurs preferentially at three different sites. Two of these sites are located in exons 11 and 13, respectively, and editing at these sites leads to a change of the coding potential of the two affected codons. Consistent with the observed codon change the two sites are termed Q/R and R/G sites, respectively. A third site, located in intron 11, does not affect the coding potential of the mRNA (Sommer et al., 1991; Lomeli et al., 1994).

Although it is not proven which enzyme is required for the editing of these three sites in vivo, in vitro studies show that ADAR2 (RED1) can edit both the Q/R and R/G sites efficiently while the cryptic intronic site is edited inefficiently (Melcher et al., 1996; O'Connell et al., 1997). In contrast, ADAR1 can edit the R/G site and the intronic site but shows little editing activity at the Q/R site (Hurst et al., 1995; Dabiri et al., 1996). Similar data has been obtained for the serotonin 2C receptor which is edited at a total of four sites. Three of these sites seem to be edited by ADAR1 while one site seems to be preferentially edited by ADAR2 (Burns et al., 1997). These data suggest that different ADARs might perform site-specific RNA editing in vivo.

In mRNAs encoding glutamate receptor subunits and serotonin receptor 2C, sites of editing are apparently defined by base-pairing between an exonic and an adjacent intronic sequence (Higuchi et al., 1993; Lomeli et al., 1994; Yang et al., 1995; Burns et al., 1997). The formed double-stranded regions are relatively short and it has been suggested that the degree of editing at these sites is limited by the instability of the base-paired region that increases, as editing progresses and base-pairing is inhibited by the presence of inosines (Bass, 1997). Nonetheless, the finding that intronic and exonic sequences are required, at least in these cases, suggests that editing might occur cotranscriptionally before introns are removed from the nascent transcript.

We have recently cloned two closely related variants of *Xenopus laevis* ADAR1 from an ovary cDNA library

(Brooks et al., 1998). The two cDNAs are, with the exception of a few exchanges, virtually identical to the two cDNAs isolated by Bass and coworkers which were first termed dsRAD-1 and dsRAD-2 but have since been renamed ADAR1.1 and ADAR1.2, respectively (Hough and Bass, 1997; Bass et al., 1997). Both proteins show a high degree of sequence identity in their central and COOH-terminal regions where they contain three dsRBDs and a conserved deamination domain, respectively. However, at the NH<sub>2</sub> terminus the two proteins show marked differences. ADAR1.1 contains 14 repeats of an 11-amino acids long sequence motif that is only present in one copy in ADAR1.2 (see Fig. 1 a). ADAR1.1 is a protein of 1,270 amino acids whereas no proper AUG start codon has been determined for ADAR1.2. However, Northern blots indicate that both proteins are well expressed and seem to encode proteins of similar molecular mass (Hough and Bass, 1997).

To this point, no substrate RNAs for ADAR have been identified in *Xenopus*, although bFGF mRNA might be edited by ADAR (Kimelman and Kirschner, 1989). However, the abundance of ADAR activity in *Xenopus* oocytes and embryos suggests the presence of several substrates for this enzyme. Furthermore, it has recently been shown that mRNA levels of ADAR1 correlate with the abundance of inosines present in cellular RNAs in various rat tissues, suggesting the existence of further, yet to be discovered ADAR1 substrates (Paul and Bass, 1998).

Therefore, we set out to study the intracellular localization of *Xenopus* ADAR1.1 and ADAR1.2 in *Xenopus* oocytes and on lampbrush chromosome spreads. We can show that both forms of ADAR1 associate with the nascent RNP matrix on transcriptionally active chromosome loops but also with a special loop.

## Materials and Methods

### Cloning of *Xenopus* ADAR1.1 and ADAR1.2

Part of a *Xenopus* ADAR1 cDNA was isolated from an expression screen of a lambda Zap cDNA library for RNA-binding proteins (Jantsch and Gall, 1992). Sequence analysis identified this cDNA as a homologue of human ADAR1 from which the 5' end was missing. Rescreening of a cDNA library and 5' RACE protocols resulted in the isolation of two variants of *Xenopus* ADAR1 that were identical to the previously published *Xenopus* ADAR1.1 and ADAR1.2 sequences. Whereas ADAR1.1 had a full-length open reading frame, ADAR1.2 had no AUG translational initiation codon. To allow expression of this clone, a self complementary oligonucleotide (5'-CTA GCG TGT AAT GCA TTA CAC G-3') was inserted in frame upstream of the cDNA in the SpeI restriction site of the pBluescript polylinker.

### Antibody Production

For antibody production a 564-bp long EcoRI fragment encoding amino acids 373–561 in ADAR1.1 was cloned into pGEX 1 vector (Pharmacia) from where the fragment was expressed as a glutathione S transferase (GST) fusion protein in *E. coli* BL21. The fusion protein was purified on glutathione Sepharose beads (Pharmacia) according to the manufacturer's protocol. Eluted fusion protein was at least 99% pure as judged on overloaded Coomassie stained gels. Fusion protein was dialyzed against 0.05% TFA in H<sub>2</sub>O and lyophilized. Two rabbits were immunized and boosted in 4-wk intervals. Test sera were taken 2 wk after each injection. After two booster injections antisera from both rabbits could recognize endogenous ADAR1 on Western blots at which time point animals were killed and all serum was collected. Antisera were produced by Eurogentec.

## Myc-tagging of ADAR1.1 and ADAR1.2

ADAR1.1 was tagged with six tandemly arranged myc epitopes at either the 5' end and the 3' end or at both ends. To do this the ~250 bp long region encoding the six myc tags was cloned in frame upstream, downstream or at both ends of the ADAR1.1 cDNA. ADAR1.2 cDNA was only tagged at its 3' end. After insertion of the AUG start codon at the 5' end of the cDNA the myc-encoding sequence was cloned in frame downstream of the ADAR1.2 cDNA sequence. Additionally, to stabilize in vitro synthesized RNAs when injected into oocytes, the 3' UTR including a poly(A)<sup>+</sup> tail of the *Xenopus* NO38 cDNA was cloned at the 3' end of all tagged ADAR variants (Peculis and Gall, 1992).

## Construction of ADAR1.1 Deletions

Construct ΔREP was made from a partial cDNA obtained from our original phage screen. This construct deletes the first 250 codons of the ADAR1.1 cDNA. ΔREP was myc tagged at its COOH terminus and the NO38 poly(A)<sup>+</sup> tail was added at its 3' end (Peculis and Gall, 1992). A self complementary oligonucleotide containing an AUG codon was introduced in-frame upstream of the cDNA in the SmaI site of the pBluescript polylinker (5'-GAT GCA TC-3').

Construct ΔZBD was made by digesting the ADAR1.1 clone containing a COOH-terminal myc-tag with NdeI and Aval (partial). After polishing the ends the DNA was religated. This construct deletes 363 codons from the 5' end of the ADAR1.1 cDNA. Translation starts at an internal Met at codon 364.

## Oocyte Injections

Myc-tagged ADAR1 variants were linearized at a unique restriction site downstream of the NO38 poly(A)<sup>+</sup> tail. Capped run off transcripts were synthesized in vitro from the linearized templates using T3 RNA polymerase. Aliquots of all RNAs were checked for integrity on RNA gels by ethidium bromide staining. Oocytes were injected with 50 ng RNA per oocyte and incubated at 16°C for 48–72 h to allow protein synthesis to occur.

25 to 50 ng of oligonucleotides anti-U2b (Pan and Prives, 1988) or unrelated oligonucleotides (M13-20 5'-GTAAAACGACGGCCAGT-3', MJ 183 5'-ACGGAGGATCCAATGAGTGAGTGAAGAGGAGCA-3') were injected into the cytoplasm of oocytes. Inhibition of transcription could be followed immediately by microscopic observation of lampbrush chromosome (LBC) preparations. Oocytes were subsequently incubated at 16°C for 24 h after which time transcription resumed. For double injection experiments with oligonucleotides and myc-tagged ADAR1.1 oocytes were injected with the oligonucleotide 6 h before injection of the RNA.

Destruction of U2 snRNA was monitored by Northern blotting as described (Tsvetkov et al., 1992).

## Inhibition of Transcription

Transcription was inhibited either by oligonucleotide injection (see above) or by incubation of oocytes in AMD or alpha-amanitin at concentrations of 50 μg/ml or 400 μg/ml, respectively, in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM Hepes, pH 7.8). Oocytes were typically held for 12–24 h in the presence of those inhibitors before they were used for chromosome preparations and immunofluorescence staining. In some cases incubation was extended for up to 5 d. Also, to ensure uptake of the drugs, some oocytes were injected with AMD or alpha-amanitin stock solutions. In all cases transcription seemed completely inhibited.

## LBC Preparations and Immunofluorescence Stainings

LBC preparations and immunofluorescence stainings were performed as described in Wu et al. (1991). For RNase treatment, preparations were digested after centrifugation but before fixation in paraformaldehyde with a mixture of RNase A (1 mg/ml) and RNase T1 (10,000 U/ml) at 37°C for 30 min. Subsequently, slides were washed in PBS and fixed for an additional hour in 2% paraformaldehyde in PBS before antibody staining. Antibodies used were Sat3 and Sat4 preimmune and immune sera, directed against *Xenopus* ADAR1; mAb Y12, directed against the Sm core proteins found on most splicing snRNPs (Lerner et al., 1981); mAb K121, directed against the trimethyl guanosine cap present on most splicing snRNAs (Krainer, 1988); mAb H14 and mAb CC3, directed against PolII (Kim et al., 1997; Vincent et al., 1996); affinity-purified CBP20 antiserum

(Visa et al., 1996); and mAb 9E10 directed against the myc tag (Evan et al., 1985). For single immunofluorescence labeling primary antibodies were detected with a secondary FITC-labeled antibody. For double labeling experiments the rabbit polyclonal sera were detected with a rhodamine-labeled secondary antibody while mouse mAbs were detected with a FITC-labeled antibody. Pictures were taken on a Zeiss fluorescence microscope equipped with DIC on Kodak Tmax 100 film which was pushed during development to 400 ASA.

## Western Blots

For Western blots, oocytes were hand enucleated and nuclei (GVs) and cytoplasms were collected separately. Typically five GV and five cytoplasms were loaded per lane on a 7% SDS-PAGE. Gels were blotted to Immobilon P membranes (Millipore, MA). Myc-tagged proteins were detected with mAb 9E10 and an alkaline phosphatase labeled goat anti-mouse antibody (Pierce) that was detected with the NBT-BCIP substrate. Endogenous proteins were detected with Sat3 or Sat4 antisera at 1:600 dilutions followed by detection with <sup>125</sup>I-labeled protein A (Amersham).

## Results

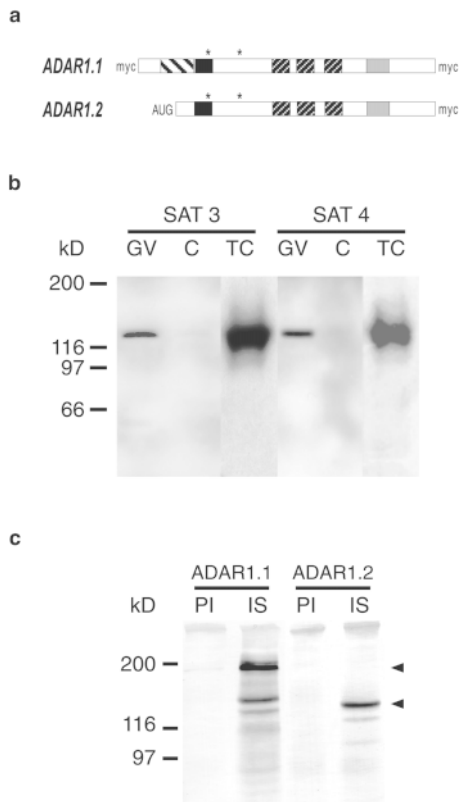
### Antibodies against ADAR1 Recognize both ADAR1.1 and ADAR1.2

To study the intracellular distribution of *Xenopus laevis* ADAR1 (xlADAR1), we generated antibodies against a 187-amino acid long fragment located in the NH<sub>2</sub>-terminal region of the protein. The region chosen showed only few amino acid exchanges between the conceptual translation products of both ADAR1.1 and ADAR1.2. It was thus assumed that both proteins would be recognized by antisera directed against the fusion protein.

Two antisera termed Sat3 and Sat4 recognized the GST fusion protein and were thus tested for their ability to detect the endogenous protein in oocyte extracts.

Oocytes were hand enucleated and nuclei (GVs) and cytoplasms were probed by Western blotting with both Sat3 and Sat4 antisera. Both antisera showed virtually identical results and detected a single band of ~125 kD in nuclei while no signal could be detected in cytoplasmic lanes. A single band of ~125 kD was also detected in XIA6 cells, a *Xenopus* fibroblast tissue culture cell line (Fig. 1 b). Both corresponding preimmune sera showed no signal (data not shown). The detected band of 125 kD is slightly smaller than the predicted molecular mass of 138 kD for xlADAR1.1. However, purified xlADAR1 also migrates with an apparent molecular mass of 120 kD (Hough and Bass, 1994). Similarly, ADAR1 proteins from other species also migrate faster than predicted from their amino acid composition (O'Connell et al., 1995). This anomalous migration has been attributed to proteolytic degradation at the NH<sub>2</sub>-terminal end of ADAR1 proteins, a phenomenon also observed for xlADAR1 (see below; Patterson and Samuel, 1995). The presence of ADAR1 in oocyte nuclei is in good agreement with previous findings that showed that the enzyme is confined to the nucleus until GV breakdown occurs (Bass and Weintraub, 1988).

To determine whether Sat3 and Sat4 antisera could detect both ADAR1.1 and ADAR1.2 proteins we performed immunoprecipitation experiments on oocytes that had been injected with mRNAs encoding either ADAR1.1 or ADAR1.2. Since ADAR1.2 lacks its own 5' AUG codon an oligonucleotide containing a suitable translational initiation codon was cloned in frame at the 5' end of the



**Figure 1.** *Xenopus* ADAR1.1 and ADAR1.2 are recognized by SAT antisera. (a) Schematic representation of myc-tagged *Xenopus* ADAR1.1 and ADAR1.2 proteins. ADAR1.1 contains an 11-amino acid long peptide motif that is repeated 14 times (left striped box). Both proteins contain a putative Z-DNA binding domain (black box), three dsRBDs (right striped boxes) and a catalytic deamination domain (light gray box). The positions of two putative NLSs is indicated by asterisks. ADAR1.1 was myc-tagged at the NH<sub>2</sub> terminus, the COOH terminus or at both ends while ADAR1.2 was only myc-tagged at its COOH terminus. An AUG codon was introduced at the 5' end of the ADAR1.2 cDNA that was missing from our original cDNA clone. (b) Western blots of oocyte nuclei (GV), cytoplasm (C), and XIA6 cells (TC) detected with Sat3 or Sat4 antisera directed against part of the ADAR1.1 protein. Both antisera recognize a nuclear protein of 125 kD that can also be detected in tissue culture cells. The detected band is smaller than the predicted molecular mass of ADAR1.1 but correlates well with the reported molecular mass of purified ADAR1.1 from *Xenopus*. 5 GVs and 5 cytoplasm were loaded per lane. (c) Sat antisera can precipitate ADAR1.1 and ADAR1.2. Myc-tagged ADAR1.1 or ADAR1.2 was expressed in *Xenopus* oocytes. Oocyte extracts expressing either ADAR protein were used for immunoprecipitation with Sat3 antiserum (IS), preimmune serum (PI), or Sat4 antiserum (not shown). The precipitated material was probed with mAb 9E10 for the presence of myc-tagged ADAR1. Both ADAR1.1 and ADAR1.2 could be precipitated by Sat antiserum (IS) but not by the corresponding preimmune serum (PI). Positions of myc-tagged ADAR1.1 and ADAR1.2 are indicated by arrowheads. myc-tagged ADAR1.1 can be detected in its full-length form (185 kD) and in a smaller version (150 kD) which is probably a proteolytic breakdown product.

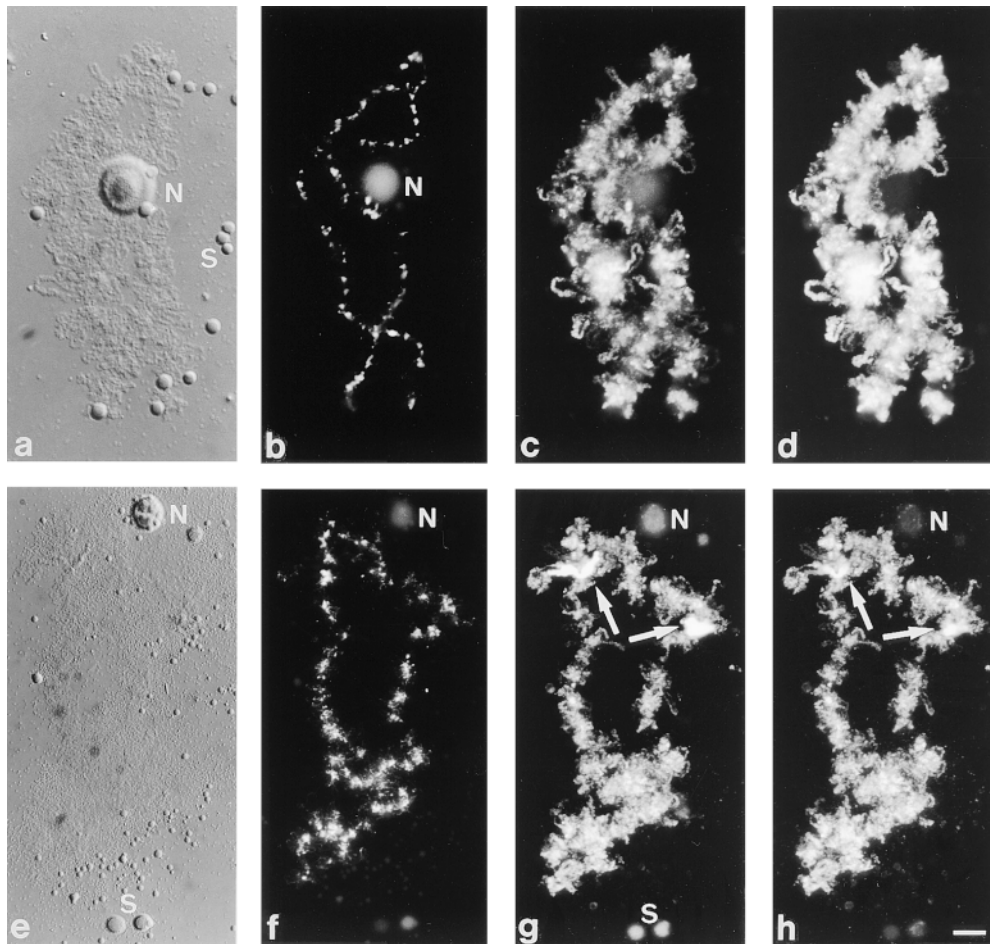
cDNA. Furthermore, to distinguish endogenous from injected ADAR1 proteins the cDNAs were fused in frame with a 6× myc-tag at both ends (ADAR1.1) or at the COOH terminus only (ADAR1.2). Capped mRNAs encoding the resulting fusion proteins were in vitro transcribed and injected into the cytoplasm of oocytes. After overnight incubation, nuclei were hand isolated and used for immunoprecipitation with Sat3 and Sat4 antibodies. The immunoprecipitated material was then tested for the presence of myc-tagged xlADAR1.1 or xlADAR1.2. Both antisera were capable of immunoprecipitating either protein, indicating that both proteins are recognized by both antisera (Fig. 1 c).

### *xlADAR1 Associates with the Nascent RNP Matrix and a Special Loop*

xlADAR1 is a nuclear protein. Furthermore, in some cases ADAR-mediated editing has been shown to require the presence of both exonic and intronic sequences indicating that this type of RNA editing might occur cotranscriptionally (Higuchi et al., 1993; Lomeli et al., 1994). ADAR acts exclusively on double-stranded RNAs. The interaction between ADAR and its double-stranded substrate RNAs is most likely mediated by the three double-stranded RNA-binding domains (dsRBDs) located in the central part of the protein. However, it is not known how substrate specificity is achieved as adenosines will be converted to inosines in virtually any synthetic double-stranded RNA when injected into oocytes (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). To test whether ADAR1 might indeed act cotranscriptionally and to determine whether ADAR1 might only associate with a specific subset of RNAs, we performed immunofluorescence stainings of spread GV contents with our ADAR1 antisera.

Spread germinal vesicles or LBC preparations allow the detailed observation of several nuclear structures at high resolution in the conventional light microscope. First, LBCs can be well observed with a nascent RNP matrix emerging from the transcriptionally active loops which themselves protrude from the condensed chromosomal axes of the two paired, homologous chromosomes, termed bivalents. Second, ~1,500 amplified nucleoli can be distinguished. Finally, two types of spherical structures can be distinguished in spread *Xenopus* GVs which, based on their association with snRNP components, have been termed B and C snurposomes (Wu et al., 1991).

Staining of LBCs with both Sat3 or Sat4 antisera showed an extraordinarily prominent signal on a single set of loops located on bivalent no. 3. In addition, there was moderate staining of all other loops. Preimmune sera, in contrast, showed no chromosomal staining indicating that the signals were specific for xlADAR1 (Fig. 2, and data not shown). Staining with Sat3 showed a weak background on C snurposomes that was also observed in the corresponding preimmune serum whereas staining with Sat4 showed a weak background on nucleoli also observable in the corresponding preimmune serum. Those weak background signals that were only observed at low dilutions of antisera were thus considered as nonspecific background signals. The staining of the brilliant loop was so intense that it



**Figure 2.** xlADAR1 localizes to the nascent RNP matrix and is specifically enriched on a special loop on bivalent no. 3. LBCs were prepared from oocytes expressing myc-tagged ADAR1.1. (a–d) Normal LBC and (e–f) bivalent no. 3. (a and e) DIC image, (b and f) DAPI staining, (c and g) localization of endogenous ADAR1 detected with SAT4 antiserum in the rhodamine channel and (d and h) localization of myc-tagged ADAR1.1 detected with mAb 9E10 in the fluorescein channel. Endogenous and ectopically expressed ADAR1 is localized to LBC loops. (g and h) A special loop on bivalent no. 3 is enriched for endogenous (g) and ectopically expressed ADAR1 (h). The special loop is marked by arrows. Faint background signals can be seen on nucleoli (N) or snurposomes (S) by staining with SAT4 antiserum that is not seen with myc-tagged ADAR1.1. Bar, 10  $\mu$ m.

could still be detected at antisera dilutions up to 1:3,000. In contrast, for the majority of all other loops staining was well visible at antisera dilutions of 1:500. Thus, it was hard to take photographic pictures with both the brilliant loop and the regular loops at good resolutions. Therefore, Fig. 2 shows images of bivalent no. 3 including the brilliantly labeling loop and images of other bivalents, showing the label observed on all other chromosome loops.

#### ***myc-E-tagged ADAR1 Mimics the Distribution of Endogenous xlADAR1***

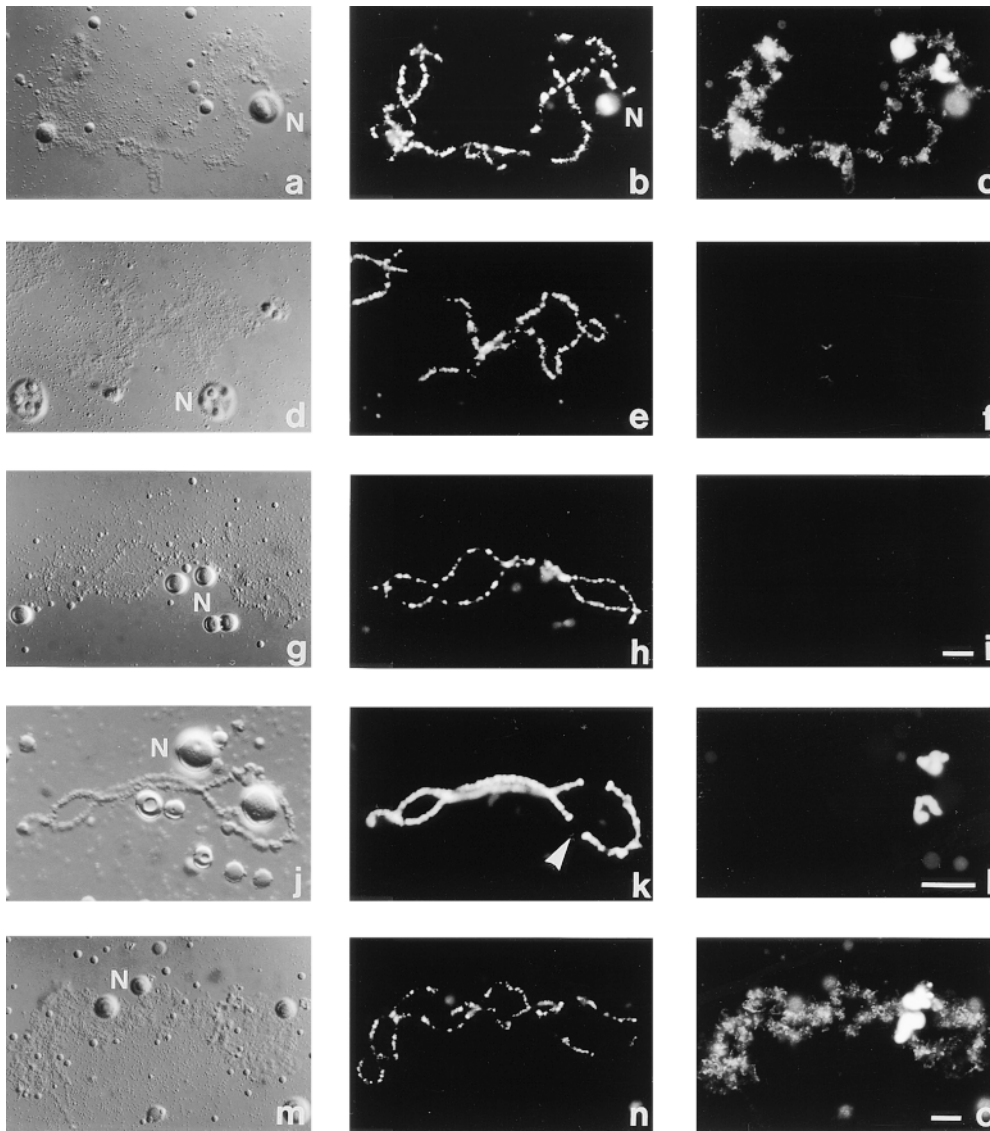
To determine whether the observed antibody staining was indeed specific for ADAR1, we wanted to determine the localization of myc-tagged ADAR1.1. To do this, myc-tagged ADAR1.1 was expressed in oocytes by mRNA injection. The protein was well detectable on Western blots 24 h after injection of the mRNA. To obtain good in situ staining, however, it was usually necessary to incubate oocytes for at least 48 h at 16°C. As protein synthesis did not increase dramatically after 24 h (as judged by Western blots) we believe that the prolonged incubation was required to displace endogenous protein by the epitope-tagged version. Staining with the mAb 9E10 directed against the myc-tag revealed that the tagged protein colocalized with endogenous ADAR1. The majority of the regular loops were clearly stained. In addition, the bril-

liantly labeling loop was also intensely decorated by the myc-tagged protein, at least in most cases (Fig. 2). This data indicates that ADAR1 indeed associates with the RNP matrix found on most loops and occurs in high concentrations on a loop on bivalent no. 3.

As a further control, to show that the chromosomal staining with both Sat3 and Sat4 antisera was specific for ADAR1, we blocked both antisera with the fusion proteins used to generate antibodies. This blocking eliminated chromosomal staining almost completely, leaving only a faint signal on the intensely labeling loops on bivalent no. 3. Thus, the observed signals reflect the localization of endogenous ADAR1 (Fig. 3).

#### ***The Intensely Labeling Loop on Chromosome no. 3 Has Several Specific Features***

The brilliantly labeling loop on bivalent no. 3 was not always labeled by the myc-tagged ADAR1 protein, even when the majority of all other loops showed clear labeling of the RNP matrix. Labeling of this special loop not only varied with the animal used for oocyte injection but also required more time to accumulate high amounts of myc-tagged protein, indicating that ADAR1 turnover at this particular loop is somewhat slower than on other loops. Furthermore, this set of loops is also morphologically outstanding as it represents the rare occurrence of a “double



**Figure 3.** Localization of ADAR1 to LBC loops is RNA dependent. Staining of bivalent no. 3 with SAT4 antiserum. (a, d, g, j, and m) DIC image, (b, e, h, k, and n) DAPI staining, and (c, f, i, l, and o) staining with Sat4 in the fluorescein channel. (a–c) ADAR1 is localized to LBC loops and is specifically enriched on a special loop. (d–f) Staining with Sat4 can be blocked by ADAR1 peptide. (f) SAT signal is almost completely diminished when the antiserum was blocked with ADAR1 peptide originally used for the immunization. (g–i) SAT staining is sensitive to RNase treatment. LBCs were digested with RNase before staining with Sat4 antiserum. (g) The loops appear “stripped” after RNase treatment, and (i) no signal can be observed in the fluorescein channel. (j–l) Treatment with actinomycin D inhibits transcription and diminishes staining of regular loops but not of the special loop on bivalent no. 3. (j) DIC image of bivalent no. 3 prepared from an oocyte after incubation in AMD. The chromosomal axes is condensed and shows no transcriptionally active loops. Also nucleoli (N) change their morphology. (k) The condensed, shortened chromosomal axes is well stained

with DAPI. The position of the special loop forming a “double loop bridge” is seen by the interrupted DAPI staining of the chromosomal axes on both homologues (arrowhead). (l) As transcription is inhibited no ADAR1 staining can be observed. Only the special loop is still brilliantly labeled by SAT4 antiserum. (m–o) Injection of an unrelated oligonucleotide temporarily inhibits transcription but does not affect ADAR1 localization on the special loop on bivalent no. 3. (m) DIC image of bivalent no. 3 prepared from an oocyte 24 h after injection of an oligonucleotide. The presence of loops on the chromosome indicates that transcription has already resumed. (n) DAPI image of the same region. (o) ADAR1 can be detected on most transcripts and on the special loop by staining with SAT4 antiserum. Note: Shortly after injection of the oligo transcription ceases and no ADAR1 staining can be detected on regular loops (not shown). However, staining of the brilliantly labeling loop is not affected by this treatment. Bars, 10  $\mu$ m.

loop bridge” where the chromosomal axis at the basis of the loop is interrupted, giving the impression of bridging two parts of a chromosomal axis by this loop. Therefore, we set out to analyze the brilliantly labeling loop and the regular loops showing ADAR1 staining in more detail.

First, we tested whether the observed labeling of all loops was RNA dependent. Therefore, we treated LBC preparations with RNase before staining with Sat3 or Sat4 antibodies. RNase treatment clearly abolished all chromosomal staining with anti-ADAR antibodies, indicating that ADAR1 was indeed directly or indirectly associated with RNA (Fig. 3). Next, we wanted to inhibit RNA synthesis with the transcriptional inhibitors actinomycin D

(AMD) and  $\alpha$ -amanitin. AMD is a DNA-binding drug that is a general inhibitor of transcription.  $\alpha$ -Amanitin, in contrast, shows dose-dependent inhibition of transcription. At low concentrations  $\alpha$ -amanitin is an efficient inhibitor of Pol-II while Pol-III is inhibited at higher concentrations. The two drugs were thus either added to the medium or injected into oocytes at concentrations high enough to inhibit all transcription. Oocytes treated this way were used to prepare LBCs which were subsequently stained with either Sat3 or Sat4 antibodies (Fig. 3).

An obvious sign for the inhibition of transcription is the lack of transcriptionally active chromosome loops. Staining of these preparations with DAPI shows the chromo-

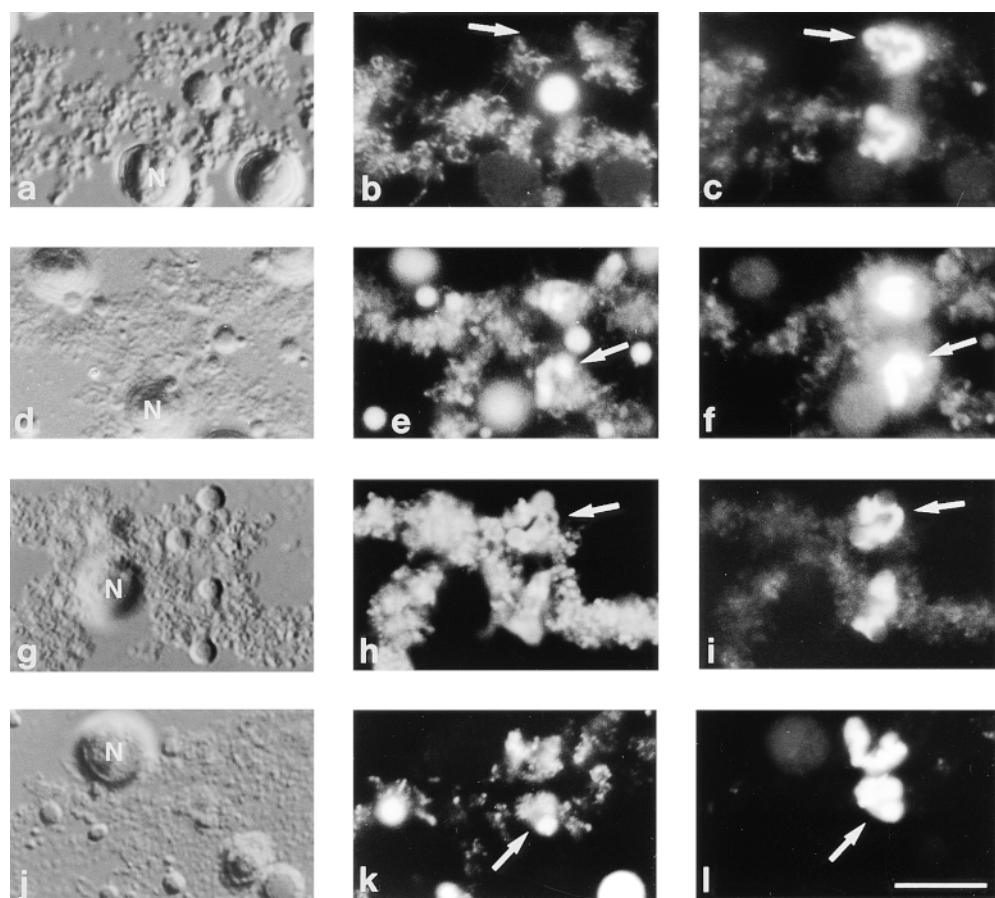
somes to be condensed and shortened. Furthermore, as AMD also inhibits Pol-I the nucleoli show a changed morphology and appear swollen. Consistent with the lack of transcriptionally active loops, no staining of regular loops was observed with either antiserum directed against ADAR1. However, the brilliantly labeling loops on bivalent no. 3 were still intensely labeled by Sat3 and Sat4 antisera (Fig. 3). Remarkably, this staining persisted even after 5 d of incubation in AMD or  $\alpha$ -amanitin.

Another possibility to inhibit transcription on LBCs is the injection of oligonucleotides into the nucleus or cytoplasm of oocytes. Shortly after injection of the oligonucleotide transcription stops (Tsvetkov et al., 1992). 12 to 24 h after injection of the oligo transcription resumes producing large, transcriptionally active loops. Therefore, we tested several unrelated oligonucleotides for their effect on transcription and ADAR1 localization on regular loops and on the brilliantly labeling loops. The outcome of these experiments was similar to the ones obtained with chemical inhibitors of transcription. As all loops disappeared no ADAR1 staining was observed on the regular chromosomal loops. However, the brilliantly labeling loop was still clearly recognizable, both morphologically and by staining with Sat3 or Sat4 antisera, indicating that the injected oligonucleotide did not affect this special loop (data not shown). After transcription resumed, normal staining of most loops and the special loop on chromosome no. 3 could be seen (Fig. 3). Taken together, these data suggest that whatever RNA is localized at this loop might not be

transcribed by a conventional polymerase, or, alternatively, is not synthesized at this location.

To test the former possibility we performed double immunofluorescence staining with mAbs H14 or CC3, both directed against Pol-II, and Sat4 antiserum (Vincent et al., 1996; Kim et al., 1997). This data showed the presence of Pol-II on all regular loops, as a faint line of signal could be seen throughout the axis of loops. The brilliantly labeling loops, in contrast, showed no detectable signal with either mAb directed against Pol-II, suggesting that no Pol-II-dependent transcription occurs at this particular loop (Fig. 4).

Most transcriptionally active loops are decorated with a multitude of snRNP and hnRNP components. Interestingly, all splicing components are present on the majority of loops where they are distributed almost homogeneously (Wu et al., 1991). However, several loops, so-called giant loops, have been observed that lack those components found on most other transcripts. Therefore, we tested whether the brilliantly labeling loop on bivalent no. 3 might in fact represent such a loop by staining LBCs with several antibodies directed against splicing and hnRNP components. Among these was antibody K121, directed against the 3mG cap present on most splicing snRNAs, mAb Y12, directed against the Sm core proteins, the anti-SR protein antibody SC35 and an antibody directed against the cap binding protein CBP20 (Lerner et al., 1981; Krainer, 1988; Fu and Maniatis, 1990; Visa et al., 1996). All those antibodies clearly labeled all regular loops but also the brilliantly labeling loop on bivalent no. 3 which was



**Figure 4.** Double staining of the special loop on bivalent no. 3 with various antibodies and SAT antiserum. (a, d, g, and j) DIC images, (b, e, h, and k) fluorescein channel, and (c, f, i, and l) staining with SAT4 antiserum in the rhodamine channel. Arrows mark the position of the special loop. (a–c) Staining with mAbH14 (b) shows the presence of RNA Pol-II on all regular loops as a fine signal seen in the center of each loop. However, Pol-II is absent from the special loop which is brilliantly labeled by SAT4 antiserum (c). (d–f) Staining with mAb K121 indicates the presence of 3mG snRNP cap structures on the special loop (e) which is also labeled with SAT4 antiserum (f). (g–i) mAb Y12 stains regular loops and the special loop indicating the presence of Sm proteins on the special loop (h). (j–l) The SR splicing factor SC35 can also be found on the special loop (k). Bar, 10  $\mu$ m.



identified by double staining the preparations with Sat3 antiserum (Fig. 4, and data not shown). This indicates that, despite the lack of detectable Pol-II on the special loop on bivalent no. 3, the RNP matrix present there is associated with all components typical for Pol-II transcripts.

#### ***Nuclear Splicing Is Not Required for ADAR1 Localization***

Editing sites in some mammalian substrate RNAs are defined by short basepaired regions formed between exonic and adjacent intronic sequences (Higuchi et al., 1993; Lomeli et al., 1994; Herb et al., 1996; Burns et al., 1997). This raises the possibility that RNA editing might be linked to splicing, at least in timing if not mechanistically. Thus, we tested whether inhibition of splicing influences the association of ADAR1 with the RNP matrix on chromosome loops. One would expect, for instance, if spliceosome formation is required for the association of ADAR with RNA that inhibition of splicing might lead to an increased or altered association of ADAR with the nascent RNP matrix.

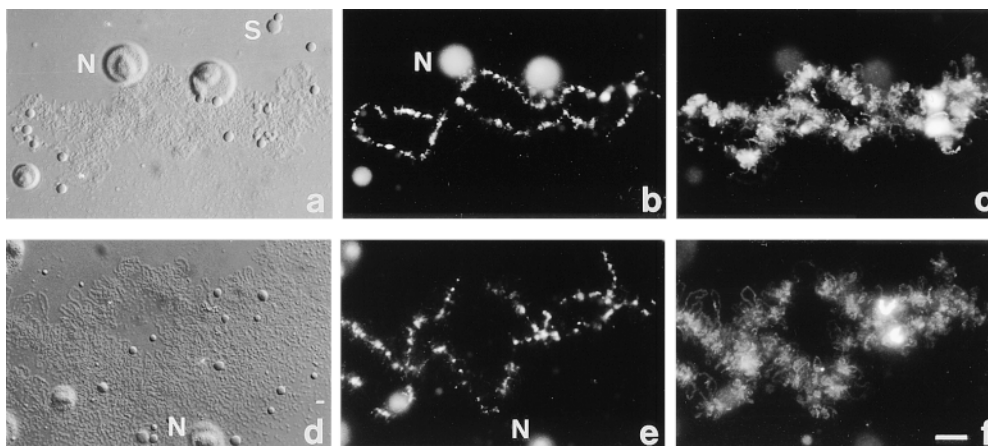
Therefore, an antisense oligonucleotide directed against U2 snRNA was injected into oocytes which leads to destruction of U2 snRNA via RNase H mediated cleavage (Pan and Prives, 1988). Oocytes depleted of U2 snRNA are defective in splicing. As mentioned, injection of any oligonucleotide temporarily inhibits transcription in a non-specific manner. However, after several hours normal transcription resumes with the exception that U2 is missing from the loop matrix (Tsvetkov et al., 1992).

Destruction of U2 was monitored by Northern blotting of RNAs isolated from individual GVs of injected and control oocytes (data not shown). After transcription resumed, LBCs were tested for the localization of ADAR1 by staining with Sat3 antiserum (Fig. 5). Interestingly, no difference in ADAR1 localization could be observed in U2 depleted oocytes, indicating that splicing and spliceosome formation is not required for the association of ADAR1 with the nascent RNP matrix.

#### ***The NH<sub>2</sub>-terminal Peptide Repeats and a Putative Z-DNA Binding Domain Are Not Required for Chromosomal Localization***

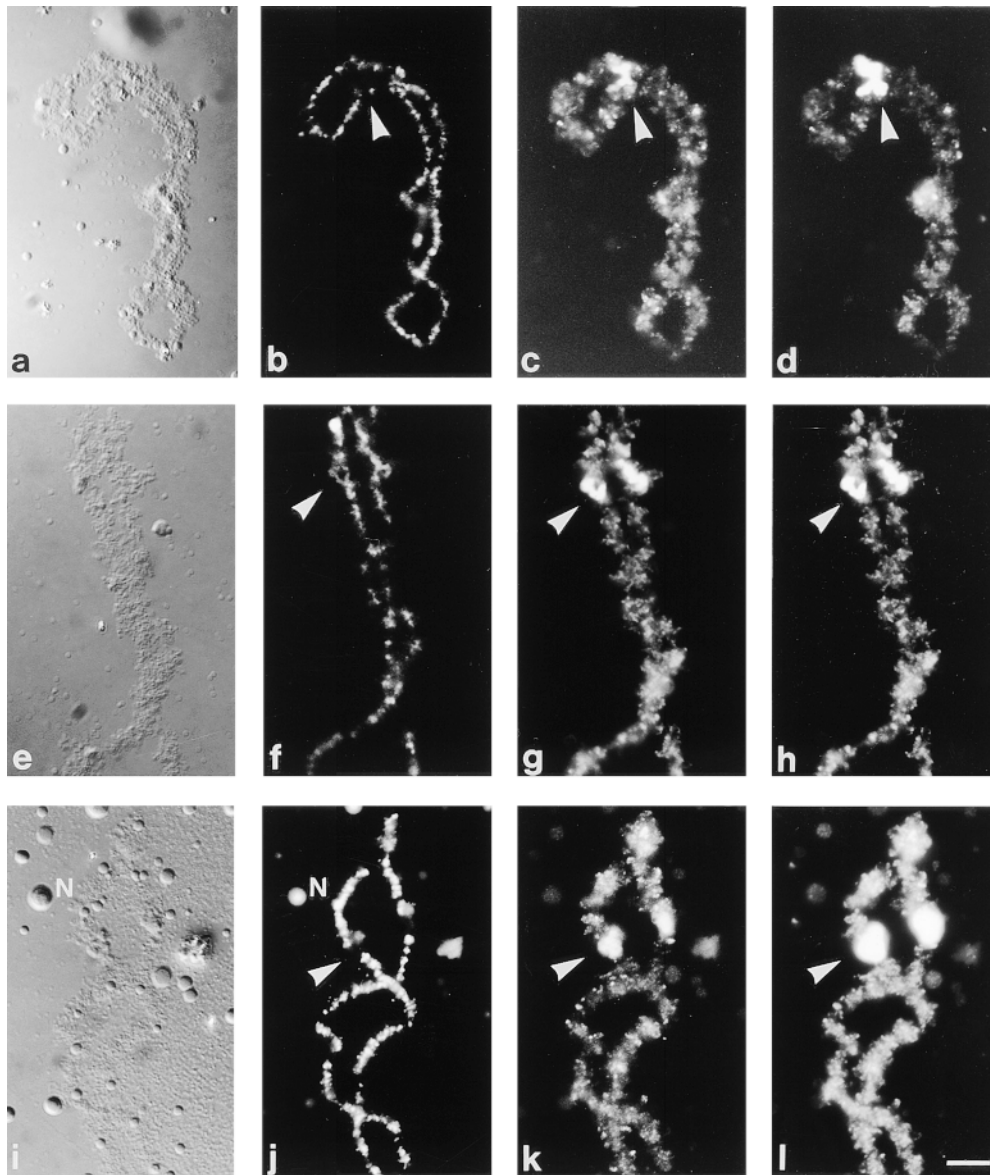
Although ADAR1.1 and ADAR1.2 show a high degree of sequence identity in their central region and at their COOH termini, their NH<sub>2</sub> termini differ considerably (Hough and Bass, 1997). Part of this difference can be attributed to the presence of an 11-amino acids long repeat that is present in 14 almost perfect tandemly arranged copies in ADAR1.1 but only in a single copy in ADAR1.2. To test whether these peptide repeats are required for proper association of ADAR1 with the RNP matrix we have analyzed the nuclear distribution of myc-tagged ADAR1.2 and ADAR1.1 from which the NH<sub>2</sub>-terminal end including the peptide repeats had been removed (construct  $\Delta$ REP, Fig. 7). When compared for their in situ localization on *Xenopus* LBCs, both clones showed an identical distribution: Strong labeling of the special loop was observed whereas moderate labeling was detectable on all other loops (Fig. 6). This indicates that the peptide repeats have no influence on the intranuclear association of ADAR1 with chromosome loops.

The 11-amino acid long peptide repeats are followed by a 70-amino acid long region that is highly conserved among all ADAR1 isoforms from various species including *Xenopus* ADAR1.1 and ADAR1.2. This sequence motif isolated from human ADAR1 has been shown to bind Z-DNA in vitro and has thus been suggested to be required for the localization of ADAR1 to transcriptionally active DNA that would facilitate the association of the protein with nascent transcripts (Herbert et al., 1997). To test this hypothesis, we constructed a myc-tagged version of *Xenopus* ADAR1 from which the entire NH<sub>2</sub>-terminal end including the putative Z-DNA binding region had been deleted (construct  $\Delta$ ZBD, Fig. 7). In situ, this deletion variant localized like wild-type ADAR1 indicating that the Z-DNA binding activity is not required for the recruitment of the protein to actively transcribing chromosome loops (Fig. 6).



**Figure 5.** Splicing is not required for ADAR1 localization. (a and d) DIC images, (b and e) DAPI staining, and (c and f) ADAR1 localization detected by staining with SAT4 antiserum in the fluorescein channel. (a–c) Staining of bivalent no. 3 from an untreated oocyte shows localization of ADAR1 on the RNP matrix of regular loops and on the special loop. (d–f) 24 h after injection of the U2b oligo transcription has resumed giving rise to prominent loops (d). (f) Staining with SAT4 antiserum shows the presence of ADAR1 on the loop matrix and on the special loop on bivalent no. 3. Bar, 10  $\mu$ m.





**Figure 6.** In situ localization of ADAR1.2 and ADAR1.1 deletions. (a, e, and i) DIC image, (b, f, and j) DAPI staining, (c, g, and k) localization of myc-tagged ADAR1 variants as seen in the fluorescein channel, and (d, h, l) localization of endogenous ADAR1 as seen after staining with SAT4 antiserum in the rhodamine channel. (a–d) myc-tagged ADAR1.2 localizes like ADAR1.1 to regular loops and to the special loop on bivalent no. 3. (c) the localization of myc-tagged ADAR1.2 and (d) endogenous protein is virtually identical. (e–h) The NH<sub>2</sub>-terminal peptide repeats found in ADAR1.1 are not required for localization of the protein. (g) myc-tagged  $\Delta$ REP construct localizes to nascent transcripts and to the special loop on bivalent no. 3. (i–l) Construct  $\Delta$ ZBD, deleting the NH<sub>2</sub>-terminal end of ADAR1.1 including a putative Z-DNA binding domain and one putative NLS, shows wild-type-like in situ localization. (k) myc-tagged  $\Delta$ ZBD construct (l) counterstaining with Sat4 antiserum. Bar, 10  $\mu$ m.

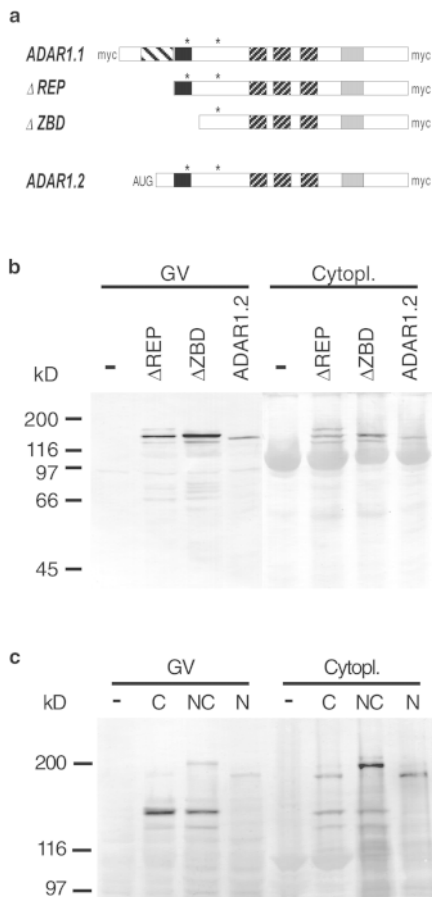
Interestingly, this deletion variant also removes one of two putative nuclear localization signals from the predicted protein sequence (Hough and Bass, 1997). However, Western blots of oocytes injected with this construct still showed clear nuclear accumulation of the protein (Fig. 7). As this deletion still localizes to chromosomes and enters the nucleus we can conclude that this first NLS sequence located between amino acids 291 and 307 in ADAR1 is not required for nuclear localization of the protein.

#### ***ADAR1 Undergoes NH<sub>2</sub>-terminal Proteolytic Cleavage***

ADAR1 protein purified from endogenous sources of various species is smaller than the calculated molecular mass predicted from the corresponding cloned cDNA sequences (Hough and Bass, 1994, 1997; Kim et al., 1994; Patterson and Samuel, 1995). This obvious discrepancy has been attributed to a proteolytic cleavage at the NH<sub>2</sub>-terminal end of the protein. It has to be noted, however,

that the smaller purified protein is enzymatically active indicating that the proposed cleavage does not influence enzyme activity (for review see Bass, 1997).

In the course of our experiments we have used ADAR1.1 variants that were epitope-tagged at their NH<sub>2</sub> terminus, COOH terminus or at both ends. Western blots of oocyte extracts that had been injected with any of those constructs confirmed the proposed cleavage of ADAR1.1 at its NH<sub>2</sub> terminus: oocytes injected with the NH<sub>2</sub>-terminally tagged construct showed a signal at 180 kD which was relatively faint. In contrast, oocytes injected with ADAR1.1 tagged at its COOH terminus or at both ends showed a much smaller signal of  $\sim$ 130 kD that was much stronger than the signal obtained from the NH<sub>2</sub>-terminally tagged protein. Additionally, the latter constructs showed faint bands of 180 and 185 kD, respectively, representing the full-length protein (Fig. 7). This finding is fully compatible with the proposed proteolytic cleavage in the NH<sub>2</sub>-terminal region of the protein. In the case of the NH<sub>2</sub>-ter-



**Figure 7.** (a) Schematic representation of deletion constructs used in this study. The peptide repeats (left striped box), the ZBD (black box), the dsRBDs (right striped box) and the deamination domain (gray box) are indicated.  $\Delta$ REP deletes the NH<sub>2</sub>-terminal peptide repeats from ADAR1.1 leaving a single, COOH-terminal myc tag.  $\Delta$ ZBD deletes a longer portion from the NH<sub>2</sub> terminus of ADAR1.1 including the putative Z-DNA binding domain and one of two putative NLSs. ADAR1.2 does not contain the peptide repeats and is only myc-tagged at its COOH terminus. An artificial AUG codon was introduced at the beginning of the cDNA. (b) Western blot of oocyte nuclei (GV) and cytoplasm (Cytopl.) of uninjected oocytes (-) and of oocytes expressing myc-tagged ADAR1 versions. All myc-tagged ADAR1 versions express well and accumulate in the nucleus. Cytoplasmic signals are seen 24 h after injection but diminish over time as the protein is transported to the nucleus. (c) ADAR1 undergoes NH<sub>2</sub>-terminal proteolytic cleavage. Western blot of oocyte nuclei (GV) and cytoplasm (Cytopl.) of uninjected (-) or injected oocytes expressing ADAR1.1 myc tagged at its COOH terminus (C), COOH and NH<sub>2</sub> terminus (NC) or NH<sub>2</sub> terminus only (N). In the cytoplasm full-length versions (180 to 190 kD) of all three constructs can be easily detected (upper two arrowheads). A smaller (150 kD) breakdown product can only be detected for the two versions carrying a COOH-terminal myc-tag (lower arrowhead). In the nucleus (GV) the breakdown product is the most prominent form found 24 h after injection. However, also the full-length versions can be detected in the nucleus. If the myc-tag is only present at the NH<sub>2</sub> terminus (N) only full-length protein can be detected, indicating that the NH<sub>2</sub> terminus undergoes proteolytic cleavage.

minally tagged construct we can only detect the full-length construct as the cleaved protein loses its tag. Since most of the protein is cleaved, the signal derived from this construct is relatively weak. The COOH-terminally tagged construct, in contrast, can be detected in its full-length and cleaved version, giving rise to the two bands detected in Western blots. As the majority of the protein is cleaved the larger band is much fainter than the lower band.

To confirm these results we have also performed Western blots of oocytes injected with the mentioned tagged constructs on high percentage gels. As expected, the small NH<sub>2</sub>-terminal cleavage product of ~25 kD can only be observed in oocytes injected with ADAR proteins tagged at their NH<sub>2</sub>-terminal end or at both ends (data not shown).

It should also be noted, that cleaved and uncleaved products could be detected both in the cytoplasm and the nucleus and that all tagged versions showed normal intranuclear distribution, indicating that the cleavage has no influence on the nuclear and intranuclear localization of ADAR1.1 (Fig. 7).

## Discussion

Editing by ADAR-like enzymes can be unspecific and affect many adenosine residues in a double-stranded region but can also be very specific as in the case of GluR-B mRNA where three adenosines are preferentially modified by ADAR1 or ADAR2 (Hurst et al., 1995; Dabiri et al., 1996; Melcher et al., 1996). Currently, it is not known how substrate specificity of ADAR1 is achieved. It could be, for instance, that the three dsRBDs of ADAR1 are solely responsible for substrate recognition. This view is supported by the finding that purified ADAR1 and ADAR2 can edit GluR-B pre-mRNA quite specifically without additional cofactors being required (Hurst et al., 1995; Dabiri et al., 1996; Melcher et al., 1996). Alternatively, substrate specificity could be mediated by the formation of a complex containing ADARs and other RNA-interacting proteins. However, since such complexes have not yet been reported this model is only speculative. Finally, the deaminase domain might discriminate among different substrates, a view supported by recent data on Tad1p a tRNA-specific adenosine deaminase lacking any dsRBDs (Gerber et al., 1998).

### ADAR1 Localization on Transcriptionally Active Chromosome Loops

We have shown that both endogenous and myc-tagged *Xenopus* ADAR1 are associated with the RNP matrix on LBC loops and specifically localizes to a special loop. Several conclusions can be drawn from this observation. First, ADAR1 mediated editing can, in principle, take place cotranscriptionally as the enzyme associates with the nascent RNP matrix before transcription is completed. This is particularly interesting when considering that intronic sequences define ADAR editing sites in GluR-B and serotonin 2C receptor mRNAs (Higuchi et al., 1993; Lomeli et al., 1994; Herb et al., 1996; Burns et al., 1997). Splicing is a cotranscriptional process as well (Beyer and Osheim, 1988; Baurén and Wieslander, 1994). In fact, the entire splicing machinery can be found associated with most

chromosome loops in *Xenopus* and *Notophthalmus* GVs showing a distribution similar to ADAR1 (Wu et al., 1991). Taken together, this leaves the possibility that editing substrates in *Xenopus* could also be defined by base-paired regions of intronic and exonic sequences. In fact, if editing takes place cotranscriptionally it could even be regulated by the rate of splicing.

However, oocytes depleted of their endogenous U2 snRNA showed a normal localization of endogenous and myc-tagged ADAR1, indicating that inhibition of splicing does not affect the enzyme's association with the RNP matrix. In this context, it is interesting to note that injection of anti-U2 oligonucleotides, just like injection of any other oligonucleotide, temporarily inhibits transcription leading to removal of the RNP matrix from the chromosome loops (with the exception of the special loop on bivalent no. 3, see below; Tsvetkov et al., 1992). After several hours, transcription resumes leading to well observable transcriptionally active chromosome loops. Therefore, the entire RNP matrix visible on those loops has to be assembled de novo, including ADAR1. We can thus conclude that ADAR1 assembly with hnRNAs does not require spliceosome formation.

The widespread distribution of ADAR1 on LBCs suggests that the enzyme's localization is not restricted to sites where substrate RNAs are being transcribed but indicates a substrate independent assembly of ADAR with the RNP matrix. No editing substrates for ADAR1 have been isolated from *Xenopus*. Nonetheless, as RNA editing is a rare process it is hard to imagine that all loops being associated with ADAR1 encode substrate RNAs. The general association of ADAR1 with chromosome loops therefore suggests that ADAR1-mediated editing is not regulated by the association of ADAR1 with the RNP matrix. Instead, other factors such as conformation or accessibility of the underlying RNA, or interaction with other protein components might regulate editing. However, in vitro editing reactions indicate that ADAR1 shows site-specific editing without the need for further cofactors (Hurst et al., 1995; Dabiri et al., 1996).

The mechanism by which ADAR1 associates with the RNP matrix is not yet clear. Not all underlying hnRNAs might contain sufficient double-stranded structures to provide binding sites for the dsRBDs. Therefore, ADAR1 might associate with the RNP matrix as part of a multiprotein complex. Such a situation has been found for Xlrpba, a protein exclusively consisting of three dsRBDs. Xlrpba is associated with hnRNAs as part of an hnRNP complex (Eckmann and Jantsch, 1997). Similarly, hnRNP proteins might facilitate the interaction of ADAR1 with the RNP matrix. Nonetheless, at this point an interaction of ADAR1 with hnRNP proteins remains to be determined. It should be noted, however, that deletion of an individual dsRBD from ADAR1 prevents chromosomal localization of the protein, underscoring the importance of the RNA binding domains for proper targeting of the protein (C. Eckmann and M. Jantsch, manuscript in preparation).

### ***The Special Loop on Bivalent no. 3***

ADAR1 was not only found on the majority of regular loops but was specifically enriched on a particular loop on bivalent no. 3. The concentration of ADAR1 on this loop

was several-fold higher than on other loops. This finding can be interpreted in several ways. On the one hand more RNAs might be present on this special loop than on any other loop. Alternatively, the RNAs on the special loop could contain more binding sites for ADAR1. A third possibility would be that ADAR1 is localized to that loop as pure protein or a multiprotein complex without RNA. Protein storage loops have been demonstrated on the LBCs of *Drosophila hydei* (Hulsebos et al., 1984). However, the sensitivity of the loops on bivalent no. 3 to RNase digestion clearly demonstrates that RNAs are present and required for the localization of ADAR1 to the special loops.

However, several features of the special loop are unusual. First, the loop represents the rare case of a so-called double loop bridge where the condensed chromosomal axes is disrupted only held together by the two loops formed from each chromatid on each of the two homologous chromosomes (Callan, 1986). Second, the special loops differ morphologically when compared with regular loops. They appear somewhat smoother in texture and are more diffracting when observed by phase contrast microscopy. Finally, and most important the special loops do not stain with antibodies directed against RNA Pol-II and are insensitive to treatment with inhibitors of known RNA polymerases including injection of oligonucleotides.

These data suggest that the RNAs present on the special loops might either be synthesized by a novel, unconventional RNA polymerase or, alternatively, might not be synthesized there but only stored or edited at that particular site.

While the first possibility appears rather unlikely, several arguments make us favor the latter hypothesis. First, our double staining experiments indicate that the composition of the RNP matrix on the special loops resembles that of other Pol-II derived hnRNPs as they contain Sm proteins, trimethyl guanosine caps, cap binding protein CBP20 and accessory splicing factors such as SC35. It is thus conceivable that a Pol-II transcript is made somewhere else and then transported to that particular loop where it is found associated with ADAR1, possibly to be edited. Such a scenario would also explain why ADAR1 localization to this loop is sensitive to RNase digestion but not to inhibition of transcription. The hypothesis is also consistent with our finding that localization of ADAR1 to this loop is not always observed and typically requires more time than localization to the normal loops. Transport of a transcript made on a regular loop to the special loop on bivalent no. 3 would certainly take more time than association of a primary transcript with myc-tagged ADAR1 would require. In this respect, the ADAR1-positive loops resemble the so-called sequentially labeling loops (SLL) described in urodeles. The hallmark of SLLs is their failure to incorporate <sup>3</sup>H-labeled ribonucleosides at a fast rate and the nonhomogeneous distribution of label along the loop (Callan, 1986). Regular loops incorporate label homogeneously over their entire length, within an hour or so, at all sites where transcription is taking place. SLLs, instead, incorporate <sup>3</sup>H-labeled ribonucleosides very slowly beginning from the thin end of the loop axes. Over a period of several days incorporation of label continuously progresses along the loop axes, being

pushed forward from the thin end always leaving a sharp margin between the labeled and unlabeled part of the loop (Callan, 1986). It has thus been suggested that SLLs do not represent sites of RNA synthesis but instead become labeled by associating with transcripts made at other sites (Callan, 1986). Therefore, it seems conceivable that the ADAR1 positive loop on bivalent no. 3 represents such a SLL which accumulates transcripts synthesized elsewhere, possibly for ADAR1 mediated editing. However, further time course experiments with both <sup>3</sup>H-labeled nucleosides and myc-tagged ADAR1 will be required to clarify this point.

### The Variable NH<sub>2</sub> Terminus of ADAR1

The NH<sub>2</sub>-terminal end of ADAR1.1 contains an 11-amino acid long peptide repeat followed by a 70-amino acid long region which, in human ADAR1, has been shown to bind Z-DNA in vitro (Herbert et al., 1997). It has thus been suggested that Z-DNA binding might be required to target the enzyme to sites of transcription where Z-DNA conformation is frequently observed. Targeting the enzyme to sites of transcription by this means could then facilitate the enzyme's association with nascent RNAs and thus help to target newly transcribed RNAs. Our comparison of myc-tagged ADAR1.1 and ADAR1.2, and of a deletion variant lacking the putative Z-DNA binding motif clearly showed that neither the 11-amino acid long peptide motif nor the Z-DNA binding region is required for targeting of the enzyme to chromosome loops. All three constructs localized to both regular loops and to the brilliantly labeling loop on bivalent no. 3. It thus seems as if the two naturally occurring variants of ADAR1 have similar if not equal functions. The repeated, 11-amino acid long peptide motif has so far only been found in ADAR1.1 of *Xenopus* and might thus represent a peculiar gene only found in this pseudotetraploid species.

However, while we could show that the Z-DNA binding domain is not required for ADAR1 association with the nascent RNP matrix we cannot exclude that this domain is required for enzyme function in vivo. Further studies to dissect the regions required for chromosomal localization and enzyme function of *Xenopus* ADAR1 are currently in progress.

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