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A minor fraction of basic fibroblast growth factor mRNA is deaminated in *Xenopus* stage VI and matured oocytes

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ABSTRACT

Adenosine deaminases that act on RNA (ADARs) convert adenosine to inosine in double-stranded regions of RNA. ADAR activity is in the nucleus in *Xenopus laevis* stage VI oocytes, and released into the cytoplasm at oocyte maturation. We previously demonstrated that a cytoplasmic double-stranded RNA (dsRNA) binding factor(s), cyto-dsRBP, protects microinjected dsRNA from the ADAR released at maturation. Here we describe experiments to determine whether an endogenous dsRNA, the duplex formed between sense and antisense transcripts of basic fibroblast growth factor (bFGF), is protected in a similar manner. Consistent with the presence of cyto-dsRBP, we observed that the majority of bFGF RNA was not deaminated, before or after maturation. However, a minor fraction of the bFGF RNA was deaminated whether the RNA was isolated from stage VI oocytes or matured oocytes. Since ADAR activity is in the nucleus in stage VI oocytes, our results suggest that a fraction of the bFGF RNAs are hybridized in the nucleus and are ADAR substrates. Adenosine deaminations result in A-to-G changes in cDNAs, so we quantified the fraction of modified molecules using restriction-enzyme assays of RT-PCR products. Caveats due to recombination during RT-PCR are discussed.

Keywords: ADAR; antisense RNA; deamination; double-stranded RNA

INTRODUCTION

Adenosine deaminases that act on RNA (ADARs) catalyze the conversion of adenosine to inosine within double-stranded regions of RNA. ADAR activity has been found in every metazoan tested and in all tissues assayed. Initially described as an unwinding/modifying activity from *Xenopus laevis*, ADAR1 (previously called dsRAD or DRADA) is one member of a growing family that together are responsible for the adenosine deaminase activity observed in metazoan tissues (reviewed in Bass, 1997).

ADARs have been implicated in two very different types of adenosine deamination in vivo: highly selective RNA editing and hypermutation. Highly selective RNA editing by ADARs is a process in which only one or very few adenosines within an RNA are deaminated. Conversely, hypermutation is a less selective process that typically leads to deamination of ~50% of the adenosines in a given RNA. Accessory factors that provide selectivity have not been identified. Rather, it has been suggested that selectivity is determined by the structure and stability of the RNA substrate (Bass, 1997). Consistent with this hypothesis, stable, perfectly base-paired duplexes of ≥50 nt show up to 50% deamination, while shorter, less stable dsRNA molecules are deaminated much more selectively (Nishikura et al., 1991; Polson & Bass, 1994).

Highly selective RNA editing by ADARs has been observed in the transcripts encoding certain glutamate receptor subunits (Maas et al., 1997) and the serotonin receptor subtype, 5-HT_{2c}R (Burns et al., 1997). In these RNAs, selective adenosine-to-inosine changes result in amino acid changes that have important biological consequences. Likewise, a single specific adenosine-to-inosine change in the antigenome of hepatitis delta virus results in the conversion of an amber stop codon to a tryptophan, allowing the virus to make two essential proteins from a single open reading frame (Polson et al., 1996).

Hypermutation of adenosines in vivo has been implicated in persistent infection of measles virus (Billeter

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et al., 1994; Cattaneo, 1994). In addition, extensive A-to-G changes have been observed in cDNAs derived from vesicular stomatitis virus (O'Hara et al., 1984), several other negative strand RNA viruses (Murphy et al., 1991; Rueda et al., 1994), and polyoma virus, a DNA virus (Kumar & Carmichael, 1997). A-to-G transitions are indicative of adenosine deamination because inosine, like guanosine, prefers to base-pair with cytidine. Consequently, after reverse transcription and second strand DNA synthesis, an inosine in an RNA appears as a guanosine in the cDNA.

Recently, a single cDNA of the 4f-rnp gene from *Drosophila* was also observed to contain extensive A-to-G changes (Petschek et al., 1996). This is the first report of a cellular RNA that appears to be deaminated by hypermutation, but certainly many others could exist. In fact, a number of eukaryotic genes have an overlapping open reading frame (ORF) on the antisense or noncoding DNA strand (reviewed in Dolnick, 1997). Transcription of opposite strands at the same genomic locus produces RNA transcripts containing an extended region of complementary sequence. If these RNAs hybridized, they would form an ideal substrate for hypermutation by ADARs.

One study suggests that a region of complementarity between the sense and antisense transcripts from the basic fibroblast growth factor (bFGF) gene are hypermutated according to the above scenario (Kimelman & Kirschner, 1989). In *X. laevis* the bFGF gene is transcribed bidirectionally to produce a 4.2-kb sense transcript and a 1.35-kb antisense transcript (Kimelman et al., 1988; Volk et al., 1989). A 900-base-pair overlap region exists between the mature sense and antisense transcripts, at each of their 3′ ends (see Fig. 1). Previously it was reported that, although the bFGF duplex was not deaminated in stage VI oocytes, it became deaminated after oocyte maturation, and was subsequently degraded (Kimelman & Kirschner, 1989). This report was consistent with our observation that ADAR activity is located in the nucleus in stage VI oocytes, and is released into the cytoplasm during oocyte maturation, when the nuclear membrane breaks down (germinal vesicle breakdown (GVBD); Saccomanno & Bass, 1994). That is, assuming the majority of the bFGF duplex is cytoplasmic, it should not be exposed to ADAR activity until after GVBD. However, further analysis of ADAR activity in our laboratory identified a factor(s) in the cytoplasm of stage VI oocytes, cytoplasmic dsRNA binding protein (cyto-dsRBP), that specifically protects dsRNA from ADAR activity released at oocyte maturation (Saccomanno & Bass, 1994). Although this factor was characterized by microinjection of an exogenous dsRNA, the presence of cyto-dsRBP was in conflict with the apparent deamination of endogenous bFGF RNA (Kimelman & Kirschner, 1989) and suggested the bFGF duplex should be protected from adenosine deamination in the stage VI oocyte and throughout

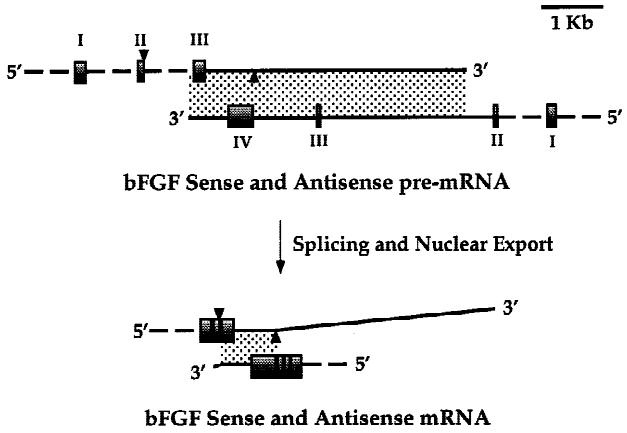

FIGURE 1. The basic fibroblast growth factor (bFGF) gene is transcribed bidirectionally to produce RNA transcripts that are complementary at their 3′ ends. Sense RNA is shown 5′ to 3′ from left to right, and the antisense RNA is shown in the opposite direction. Coding regions are represented with boxes and numbered. In the top half of the figure the sense and antisense transcripts are aligned to show a putative duplex region between the pre-mRNAs (shaded). Upon splicing, the sense and antisense mRNAs contain a 900-base-pair region of complementarity, shown as the shaded region in the bottom half of the figure. Annealing sites of PCR primers are shown as arrowheads on the sense pre-mRNA and sense mRNA. Black lines are introns and 3′ UTRs for which the genomic sequence is known. Hatched lines are introns and 5′ UTRs for which the genomic sequence is not yet known; consequently these are not drawn to scale.

oocyte maturation. Discussions between the two laboratories provided a possible solution to the apparent conflicts. In particular, although many laboratories induce maturation in vitro by incubating oocytes with progesterone, the protocols used by Kimelman and Kirschner involved microinjecting a crude preparation of maturation promoting factor (MPF) into stage VI oocytes. Experiments in our laboratory demonstrated that the crude preparation of MPF contained a significant concentration of ADAR activity (M. Paul & B. Bass, unpubl. data). Possibly, the injection of exogenous ADAR into the cytoplasm displaced the cyto-dsRBP bound to the bFGF duplex, leading to its aberrant deamination. Nonetheless, since ADARs require base-paired substrates, the work by Kimelman and Kirschner indicates that the bFGF sense and antisense RNAs are hybridized in the cytoplasm of stage VI oocytes.

Here we report experiments designed to directly determine whether bFGF transcripts are substrates for endogenous ADARs in stage VI oocytes or matured oocytes. To guard against possible artifacts, maturation was induced using conventional progesterone protocols. Consistent with the presence of cyto-dsRBP, we observed that the majority of bFGF mRNA was not deaminated before or after maturation. However, using three different assays, we showed that a small percentage of bFGF mRNA isolated from stage VI oocytes, as well as that derived from matured oocytes, contained sequence changes indicative of deamination. Since

ADAR activity is located in the nucleus in stage VI oocytes, the observation of deaminated bFGF RNA in stage VI oocytes indicates that a least a small fraction of the sense and antisense bFGF RNAs are hybridized in the nucleus. However, we surmise that the majority of bFGF sense and antisense RNAs escape deamination in the nucleus and reach the cytoplasm, where they are subsequently protected from ADARs by cyto-dsRBP.

RESULTS

The majority of the bFGF RNA population is not deaminated before or after oocyte maturation

To determine whether bFGF RNAs were deaminated in stage VI oocytes or matured oocytes, we sequenced cDNA clones derived from the 900-base-pair region of the sense mRNA that is complementary to the antisense mRNA. cDNA sequences were compared to the genomic sequence (Volk et al., 1989) to identify A-to-G changes. We chose to monitor changes in the bFGF sense RNA, rather than the antisense RNA, because the antisense RNA is present at 20-fold excess over the sense mRNA in oocytes and matured oocytes (Kimelman et al., 1988). Consequently, whereas almost all of the sense RNA would be predicted to be present in a duplex and serve as an ADAR substrate, most of the antisense mRNA would be single-stranded. Thus, the probability of finding a deaminated sense RNA would be much greater than the probability of finding a deaminated antisense RNA.

Total RNA was isolated from stage VI oocytes and matured oocytes, and cDNA was prepared using reverse transcriptase and random hexamers. (Matured oocytes were collected ~6 h after GVBD; see Materials and Methods). Primers for subsequent amplification by the PCR were designed to hybridize outside the predicted overlap region (see bottom half of Fig. 1), to guard against the possibility that a primer binding sequence would be altered by a deamination event. The approximate regions of primer binding sites are shown as arrowheads in Figure 1. Since the steady-state levels of mature bFGF mRNAs should be much greater than the levels of unspliced bFGF pre-mRNAs, we designed our primers based on the region of complementarity between the mature mRNAs. However, in theory, the bFGF sense and antisense pre-mRNA transcripts could hybridize before splicing, to form a much longer duplex (see top half of Fig. 1). In this case, our chosen primer binding sites would be predicted to be double-stranded and susceptible to deamination. Therefore, we also chose primer binding sites that had very few adenosines, and in addition, used degenerate primers containing both C and T at positions of adenosines in good context for deamination (Polson & Bass, 1994).

PCR products were cloned, and the sequences of cDNA clones from stage VI oocytes, as well as from matured oocytes, were compared to the genomic sequence (Volk et al., 1989). Although the majority of cDNAs did not contain A-to-G transitions, 5 of 32 cDNA clones sequenced from stage VI oocytes, and 2 of 27 cDNA clones sequenced from matured oocytes, contained A-to-G transitions characteristic of deamination by an ADAR. The genomic sequence of the overlap region, and the A-to-G changes observed in the five deaminated oocyte clones (1–5) and two deaminated matured oocyte clones (A, B) are shown in Figure 2. Almost all of the changes in the cDNA clones were A to G and ranged from a minimum of 10 to as many as 70 changes in a single clone. The A-to-G transitions did not occur at random positions, but were located according to the 5′ nearest-neighbor preferences previously reported for *Xenopus* ADAR1 (xADAR1; Polson & Bass, 1994); this supported the idea that the A-to-G changes were due to adenosine deamination by xADAR1. Furthermore, no A-to-G changes were observed outside the predicted overlap region, consistent with previous studies showing that a dsRNA structure is required for deamination by xADAR1.

Thus, our initial sequencing experiments indicated that the majority of the steady-state population of bFGF mRNA in stage VI oocytes did not contain A-to-G transitions, suggesting the molecules had not been deaminated by ADAR. Further, the bFGF mRNA sampled after maturation was also largely unmodified, even though ADAR is released into the cytoplasm at GVBD. This result was consistent with the idea that cytoplasmic bFGF duplex RNA was protected from deamination by cyto-dsRBP. In the bFGF mRNA analyzed before and after maturation, we did observe a small fraction of molecules with A-to-G transitions that appear to be derived from deamination by ADAR. Since ADAR activity is located in the nucleus in stage VI oocytes, the observation of deaminated molecules at this stage suggested that at least a fraction of the bFGF sense and antisense RNA hybridized in the nucleus and served as ADAR substrates. Although deaminated molecules were also observed in samples derived from matured oocytes, the total number of cDNAs sequenced was not large enough to determine the significance of the small differences in the number of deaminated molecules observed in oocytes versus matured oocytes.

Restriction-enzyme analyses of cDNA populations verify that only a minor fraction of the bFGF RNA population is deaminated

We wanted to determine more accurately the percentage of bFGF RNA molecules that were deaminated in the steady-state RNA populations from stage VI oocytes and matured oocytes. To this end, we examined large numbers of individual cDNA clones, as well as

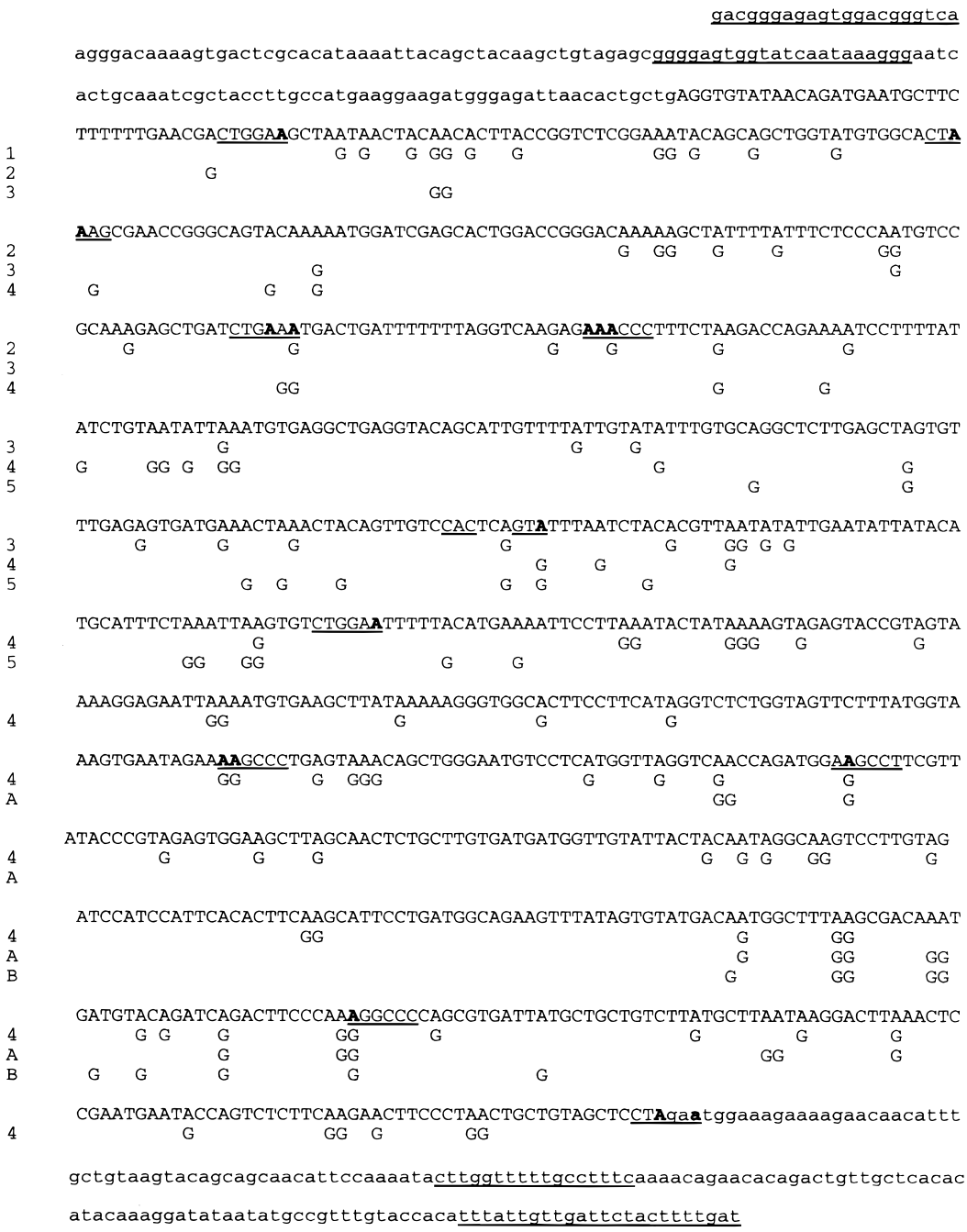

FIGURE 2. cDNAs derived from the bFGF sense RNA within the region of complementarity contain numerous adenosine-to-guanosine changes. The genomic sequence of the complementary region (top line; Genbank accession number X16627) is compared to the sequence of cDNA clones derived from the bFGF sense mRNA isolated from stage VI oocytes (1–5), and matured oocytes (A and B). Only the nucleotides which differ in the cDNA clones are shown below the genomic sequence. Nucleotides that lie outside of the 900-base-pair complementary region (defined by the region of complementarity between the mature RNAs) are written in lowercase; within this lowercase sequence underlined nucleotides show the primer binding sites for the first and second rounds of PCR. Adenosine-to-guanosine changes were not observed outside the overlap region. Underlined regions of uppercase sequence denote positions of new restriction sites that are created when adenosines in bold are changed to guanosine.

mixed populations of cDNAs, using a restriction-enzyme assay. After reverse transcription and the PCR, an inosine within an RNA appears as a guanosine in the corresponding cDNA. Thus, deamination events can be monitored using restriction sites that are not present in the wild-type bFGF sequence, but are created by specific A-to-G changes.

We chose three restriction enzymes, *Apa*I, *Dra*III, and *Stu*I, that together identified four of the seven deaminated cDNA clones derived from both the oocyte

and matured oocyte RNA populations (Fig. 2). *Bpm*I was also included based on its ability to identify deaminated cDNA clones sequenced during additional experiments not reported here. Together, the four restriction enzymes detect 69% of the total modified cDNA clones observed by sequencing. The underlined regions in the overlap sequence (uppercase) shown in Figure 2 denote the positions of the restriction sites. Adenosines in bold within the underlined regions (Fig. 2) show positions where deamination generates a new restriction site.

To analyze large numbers of individual cDNA clones for A-to-G changes, bFGF cDNA inserts within the plasmids of individual bacterial colonies from a single transformation were amplified using the PCR. Each PCR product was then incubated with a mixture of *Apa*I, *Dra*III, *Stu*I, and *Bpm*I, and the digestion products were separated by electrophoresis on a 1% agarose gel. Figure 3B shows a representative analysis of 15 cDNA clones. In this experiment, two clones (Fig. 3B, lanes 11 and 12) show the faster migrating bands that indicate cleavage by a restriction enzyme due to a deamination event. As tabulated in Figure 3A, we analyzed a total of 205 individual clones derived from the bFGF mRNA of stage VI oocytes, and 183 individual clones derived from bFGF mRNA from matured oocytes. Three percent of the cDNA clones analyzed from each of the stage VI oocyte and matured oocyte mRNA populations contained an A-to-G change at one of the restriction sites, suggesting they originated from a deaminated RNA molecule. Note that control reactions were performed in each experiment to confirm that the restriction enzymes were active during the incubation (data not shown; see Materials and Methods).

For analyses of mixed cDNA populations, total RNA from stage VI oocytes and matured oocytes was reverse transcribed and PCR amplified as described above, except the PCR product was labeled in the final cycle of the PCR, and assayed without cloning. After labeling, the PCR product was gel purified and then incubated with the restriction enzymes. Independent experiments from the same RNA sample are shown in Figure 4. In the experiment shown in Figure 4A, 4.5% of the cDNA molecules in the PCR population from stage VI oocytes (Fig. 4A, lane 2) and 9% of the cDNA molecules in the PCR population from matured oocytes (Fig. 4A, lane 4) were digested. In the experiment shown in Figure 4B, 20% of the cDNA molecules in the PCR population from stage VI oocytes (Fig. 4B, lane 2) and 33% of the cDNA molecules in the PCR population from matured oocytes (Fig. 4B, lane 4) were digested.

Experiments similar to those shown in Figure 4 were repeated multiple times and the large amount of variability was found to be an intrinsic characteristic of the experiments. We attributed the large deviation between experiments to inconstant amounts of recombination during reverse transcription and the PCR (detailed further in the Discussion section). To compensate for this variability, we performed multiple analyses and calculated mean values. In all, duplicate reverse transcription reactions were performed for each of three separate stage VI oocyte RNA samples and three separate matured oocyte RNA samples. For each of the reverse transcription reactions, duplicate PCR reactions were performed. The mean values calculated from a total of 12 PCR reactions of oocyte RNA indicate that 10 ± 5% of the bFGF mRNA is deaminated in VI oocytes; the mean value calculated from 12 PCR reactions of the matured oocyte sample indicate that 16 ± 10% of bFGF mRNA is deaminated after maturation. Of course, these

A

	Oocytes	Matured Oocytes
clones analyzed	205	183
clones digested	7	5
percent digested	3%	3%

B

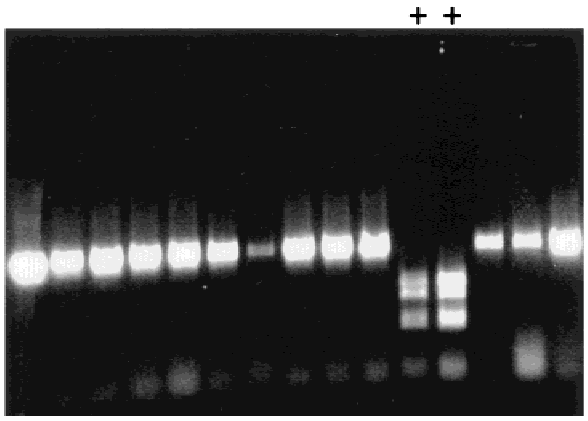

FIGURE 3. Restriction-enzyme assays of single cDNA clones. Inserts from individual cDNA clones were PCR amplified and incubated with *Apa*I, *Dra*III, *Stu*I, and *Bpm*I simultaneously. **A:** The data are tabulated for oocytes (stage VI) and matured oocytes according to the total number of cDNA clones analyzed, the number of cDNA clones digested, and the percentage of cDNA clones digested in the entire assayed population. **B:** PCR product from 15 different cDNA clones was incubated with *Apa*I, *Dra*III, *Stu*I, and *Bpm*I simultaneously and subsequently electrophoresed on a 1% agarose gel. Lanes 11 and 12 are PCR product from two different cDNA clones that were digested with at least one of the restriction enzymes and consequently show bands that electrophorese with a faster mobility. The PCR product of the cDNA clones in lanes 11 and 12 was digested into multiple bands, indicating that an A-to-G change was present at more than one site. Experiments performed to optimize the assay showed that PCR products derived from unedited cDNAs were never digested, and those derived from defined mixtures of unedited and edited sequences accurately reflected the composition of the sample. These experiments demonstrated that cleavage sites were not fortuitously created by PCR error. Further, the sizes of bands observed in digested samples correlated with cleavage at the restriction sites predicted to be created by A-to-G transitions (underlined sequences in Fig. 1).

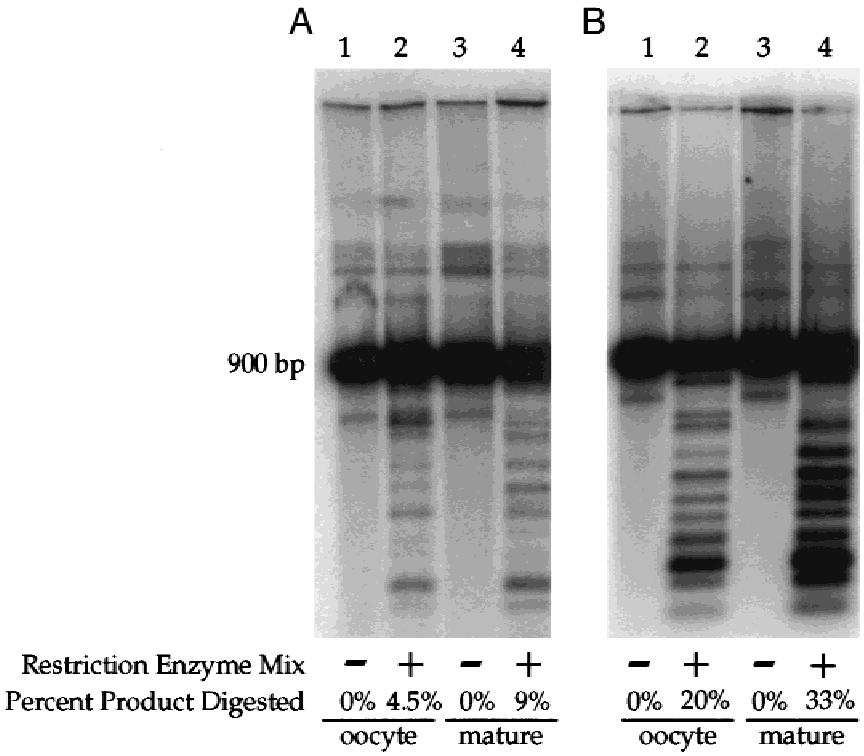

FIGURE 4. Restriction-enzyme assays of mixed cDNA populations. A PhosphorImager image of restriction digests electrophoresed on a 1% agarose gel is shown. **A** and **B** are two independent experiments. bFGF RNA was amplified by RT-PCR using RNA isolated from either oocytes (lanes 1 and 2) or from matured oocytes (lanes 3 and 4). The 900-base-pair PCR products, corresponding to the sense strand of the overlap region, were incubated in the absence (−) or presence (+) of restriction enzymes (*Dra*III, *Apa*I, *Stu*I, and *Bpm*I). The percent product digested was obtained by subtracting a background value obtained in the absence of restriction enzymes (−). Background values ranged from 0.6% to a maximum of 1.4%. The percent digestion was determined with a Molecular Dynamics PhosphorImager using ImageQuaNT software.

numbers could be slightly higher if some deaminated molecules were not detected with the restriction enzymes used in our assay.

The percentage of deaminated bFGF molecules determined in the analysis of a large number of individual cDNA clones (e.g., oocytes, 3%) differed somewhat from that determined in the mixed cDNA analyses (e.g., oocytes, 10%). However, we note that the former value was derived from an analysis of a single PCR reaction, whereas the latter value was averaged from multiple PCR reactions. Because of variable amounts of recombination between different PCR reactions (see Discussion), we consider the value obtained in the mixed cDNA analyses to be the more accurate number. We surmise that if the single cDNA analysis were repeated for multiple PCR reactions, the average fraction of deaminated molecules would be close to the average determined from the mixed cDNA analyses.

The data obtained from analyzing individual cDNA clones and mixed cDNA populations with restriction enzymes confirms our sequencing results: the majority of bFGF mRNA molecules were not deaminated before or after maturation. Further, as in the previous sequence analysis, a small percentage of deaminated bFGF molecules were observed in stage VI oocytes as well as matured oocytes. Within the sensitivity limits of our experiments, we observed no significant difference in the fraction of deaminated molecules observed before or after maturation. Thus, in contrast to previous reports (Kimelman & Kirschner, 1989), our data indicate that deaminated molecules are not degraded even 6 h after maturation.

DISCUSSION

Using three different assays we observed that the majority of bFGF mature mRNA was not deaminated in *Xenopus* stage VI oocytes. We also found that the bFGF mRNA population isolated after oocyte maturation remained predominantly unmodified, despite its exposure to the ADAR released into the cytoplasm following GVBD. The latter observation agrees with our earlier report that predicted that the factor cyto-dsRBP would protect cytoplasmically located dsRNA from deamination (Saccomanno & Bass, 1994). With all three assays we detected A-to-G transitions in a small fraction of the bFGF mRNA, whether the RNA was isolated from stage

VI oocytes or matured oocytes; this suggested that at least a small fraction of the bFGF mRNA from both stages was deaminated by ADAR.

Since ADAR activity is located in the nucleus in stage VI oocytes, our observation of a low level of deaminated bFGF mRNA at this stage indicates that a fraction of the bFGF sense and antisense RNAs are hybridized in the nucleus and acted on by ADARs. As shown at the top of Figure 1, the bFGF pre-mRNA transcripts can form a much longer duplex prior to splicing that might serve as an ADAR substrate in the nucleus. However, we did not find any A-to-G changes in the sequence outside the region of complementarity between the mature RNAs, suggesting adenosine deamination occurred on the mature, spliced mRNA, rather than the pre-mRNA (see Fig. 2 legend). Of course, further analysis will be required to prove this definitively.

Within the sensitivity limits of our experiments, the percentage of deaminated bFGF RNA molecules observed before and after oocyte maturation was similar. We speculate that the small percentage of deaminated bFGF RNA molecules detected after oocyte maturation were deaminated in the oocyte nucleus and persisted after maturation. However, previous studies indicate that a significant population of the bFGF sense and antisense mRNAs are hybridized in the oocyte cytoplasm (Kimelman & Kirschner, 1989). Therefore, we cannot rule out the possibility that a low level of cytoplasmically located bFGF duplex molecules escape protection from cyto-dsRBP and are deaminated by ADAR released at oocyte maturation.

In previous studies hypermutated bFGF RNA transcripts were not observed in the RNA isolated from stage IV or stage VI oocytes, or mixed oocyte populations from total ovary (Kimelman & Kirschner, 1989). However, our current data indicate that the percentage of deaminated bFGF transcripts in the RNA population from stage VI oocytes is low, and suggest that analysis of many cDNA clones would be required to identify those which are deaminated. We believe that we identified deaminated cDNA clones derived from the stage VI oocyte RNA because we analyzed large populations of cDNA molecules utilizing three different methods.

Can RT-PCR be used to quantify amounts of deaminated molecules?

The amount of deaminated bFGF mRNA determined by restriction digest analyses of large numbers of individual cDNA clones differed slightly from that determined with mixed cDNA populations, and the latter was quite variable from experiment to experiment. We attribute the variability in our data to the variable amounts of recombination which can occur during the reverse transcription reaction (Luo & Taylor, 1990; Ouhammouch & Brody, 1992) and the PCR (Saiki et al., 1988; Meyerhans et al., 1990; Odelberg et al., 1995). Interestingly, evidence of recombination can be observed in our sequence analysis (Fig. 2). In all but one of the cDNA clones (clone 4 from the oocyte population), A-to-G changes were clustered in the 5′ or 3′ half of the sequence, as if a fully modified molecule had recombined with an unmodified molecule.

Recombination during reverse transcription and the PCR will artificially combine parts of two different sequences into a single cDNA molecule. Deaminated regions of a single RNA can be represented in many cDNA molecules, depending on the position and frequency of the crossover events in each successive cycle of the PCR. The amount of recombination generated during a single PCR cycle, presumably by template switching, can vary 3- to 10-fold (Odelberg et al., 1995), consistent with the variability observed in our experiments. Because our method of simultaneously digesting with a mixture of restriction enzymes detects all of the cDNA molecules that obtain a new restriction site, the percentage of deaminated cDNA molecules will vary, and increase with the frequency of recombination. However, the frequency of any single site should not change. Therefore, a more accurate estimate of the percentage of deaminated molecules might be determined by including in each assay only a single restriction enzyme. Of course, this method has its problems as well, and the values determined from single-enzyme digests might be lower than the actual number, since a given adenosine may not be deaminated in every hypermutated molecule.

To explore the possible use of single-enzyme digests, additional experiments were performed using two enzymes separately (*Dra*III and *Stu*I) to analyze mixed cDNA populations derived from oocyte RNA (data not shown). Two independent reverse transcription reactions were performed for an oocyte total RNA population, followed by either duplicate or triplicate PCR experiments. The mean value calculated from five PCR reactions of the oocyte cDNAs was 14% according to the *Stu*I digestion (standard deviation $\pm$ 3%), and the mean value according to the *Dra*III digestion was 10% (standard deviation $\pm$ 3%); thus, using single enzymes we observed an average of 12% deamination. The standard deviation of single-enzyme digests was lower than that of the multiple enzyme digests, suggesting that the former method is less influenced by recombination.

The data from multiple-enzyme digests on the mixed cDNA were variable, yet the mean value was similar to that determined using two different restriction enzymes singly; this suggests that errors due to recombination averaged out over the large number of PCR reactions we analyzed. Restriction-enzyme analyses of mixed cDNA populations with single (12%) or multiple enzymes (10%) suggest the percentage of deaminated molecules in the bFGF RNA from oocytes is $\sim$11%. We observed a similar percentage of deaminated bFGF RNA molecules after oocyte maturation.

Our current study demonstrates that caution should be exercised when utilizing RT-PCR and cloning to analyze deaminated or heterogeneous RNA populations. It is postulated that recombination during the PCR occurs only at high DNA concentrations, like that which exists in the last few cycles of the PCR (Meyerhans et al., 1990). We used a PCR scheme to produce high concentrations of cDNA so as to be able to detect a very small percentage of potentially deaminated molecules. Unfortunately, by such a scheme we likely encouraged recombination. Nevertheless, the occurrence of recombination during RT-PCR does not alter our conclusion that the majority of the bFGF mRNA was not deaminated before or after oocyte maturation.

The significance of the identification of deaminated bFGF mRNA

As mentioned (see Introduction), two types of deamination, hypermutation and selective, have been observed among ADAR substrates. The cellular substrates discovered so far are largely of the selective type, and only a single hypermutated cellular RNA, the *Drosophila* 4f-rnp transcript, has been reported. Our studies indicate that bFGF mRNA provides a second example of a cellular RNA that is hypermutated by an ADAR. By analyzing RNA populations we determined that the deaminated bFGF mRNA molecules comprise a minor population of the steady-state bFGF mRNA. The results of our analyses emphasize that the identification of a single hypermutated cDNA does not mean that all RNAs of a given sequence are ADAR substrates. Since only a single cDNA has been analyzed in the case of 4f-rnp (Petschek et al., 1996), analyses of populations of 4f-rnp cDNAs would be informative.

Our studies say nothing about the function of the inosines within bFGF mRNAs. It has been hypothesized that inosine could mark an RNA for degradation and previous reports correlated the deamination of bFGF mRNA during oocyte maturation with its subsequent degradation (Kimelman & Kirschner, 1989). In our studies the fraction of deaminated molecules observed before and after oocyte maturation was almost identical, suggesting that inosine-containing molecules are stable and persist after maturation. Still other studies in our laboratory involving the microinjection of synthetic dsRNA indicate that hypermutation does not lead to degradation during oocyte maturation (data not shown). Regardless, the recent discovery of a mammalian ribonuclease (I-RNase) that is specific for inosine-containing single-stranded RNA (Scadden & Smith, 1997) suggests that degradation mechanisms are still worthy of further consideration. This is particularly true since the bFGF antisense transcript has also been implicated in regulating the stability of the sense RNA in other organisms, including rat, chicken, and human (reviewed in Knee & Murphy, 1997).

Although we observed that only a minor fraction of bFGF mRNA was deaminated, it remains possible that the deamination of this minor fraction causes a significant alteration of bFGF expression. Inosine is translated as guanosine (Basilio et al., 1962), and thus, the multiple deamination events we observed within bFGF mRNAs would be predicted to drastically alter the amino acid sequence of the encoded protein. In cells infected with polyoma virus, complementary viral transcripts are deaminated, and the resulting hypermutated viral RNAs are retained in the nucleus (Kumar & Carmichael, 1997). Hypermutated cellular RNAs such as bFGF might also be retained in the nucleus, thus reducing the level of transcript available for translation. Further studies are clearly required to determine if the ADARs play a role in the posttranscriptional regulation of bFGF expression. Even if the deamination of bFGF mRNA does not play a role in stage VI oocytes, it could play a role at other times in *Xenopus* development.

Of course, it is possible that the deamination of bFGF serves no biological role, but merely exemplifies a background reaction that will occur to a minor extent anytime complementary RNAs hybridize. In this regard, our observation of deaminated bFGF molecules, combined with the previous observation (Kimelman & Kirschner, 1989), emphasizes that the complementary region of the sense and antisense transcripts are hybridized in vivo. Possibly the hybridization of these RNAs, even in the absence of deamination, is biologically important. Increasing evidence suggests that naturally occurring antisense RNAs can regulate gene expression by inhibiting translation, nuclear export of the mature mRNA, transcription, or splicing of the pre-mRNA transcript (Knee & Murphy, 1997), similar to the mechanisms by which artificial antisense techniques are thought to act (reviewed in Denhardt, 1992).

There are many studies that suggest the bFGF antisense RNA does have an important biological function. For example, the bFGF gene is transcribed bidirectionally in all species that have been examined, including human (Murphy & Knee, 1994), rat (Grothe & Meisinger, 1995), chicken (Zuniga et al., 1993), and frog (Kimelman et al., 1988; Volk et al., 1989). The antisense sequence is highly conserved among vertebrates, and in frog and human**,** the gene organization of the sense and antisense transcription units are conserved as well (Murphy & Knee, 1994). The antisense mRNA contains an ORF that encodes a putative 24 kDa protein in frog (Kimelman et al., 1988; Volk et al., 1989) and a 35 kDa protein in rat (Knee et al., 1997) that shows a high degree of similarity to the MutT-related family of DNA repair enzymes (Li et al., 1996). Recently it was shown that the antisense ORF is translated in vivo (Li et al., 1996; Knee et al., 1997). Possibly the antisense sequence is important only for the protein it encodes, but future experiments will be required to prove this.

MATERIALS AND METHODS

Preparation of stage VI oocytes and matured oocytes

Stage VI oocytes and matured oocytes were isolated and prepared as described previously (Saccomanno & Bass, 1994), except that individual oocytes were removed from follicle cells with MBS (10 mM HEPES, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.7 mM $CaCl_2$, and 50 μg/mL of gentamicin sulfate) that contained 1 mg/mL of collagenase D. Stage VI oocytes were matured by incubating in MBS containing 10 μg of progesterone per milliliter. Matured oocytes were collected 6 h after ~70% of the oocytes had undergone GVBD as determined visually by the appearance of a white spot at the center of the animal hemisphere. GVBD occurred within 3–4 h after progesterone addition.

Total RNA isolation

Total RNA was isolated as previously described for sources enriched in ribonuclease (Chirgwin et al., 1979), except that 15–25 mL of stage VI oocytes or 15–25 mL of matured oocytes were homogenized using a glass dounce on ice in 10× volume (relative to oocyte volume) of 4 M guanidinium thiocyanate containing 0.5% sodium *N*-lauroylsarcosine, 25 mM sodium citrate, pH 7.0, and 0.1 M 2-mercaptoethanol. The RNA samples were then treated with DNase by incubating for 20 min at 37 °C in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, and 3 U RQ1 RNase free DNase (1 unit/μL, Promega). A final extraction was performed with an equal volume of phenol:chloroform (1:1, v/v), and the RNA was precipitated with ethanol. A sample of the RNA was electrophoresed in a 1% formaldehyde gel and visualized by ethidium bromide to confirm that the RNA was intact.

Reverse transcription, PCR, and cloning

For reverse transcription reactions, 20 μg total RNA (from stage VI oocytes or matured oocytes), 1 μg random primer oligonucleotide (3 μg/μL, Life Technologies, Inc., mostly hexamers), and 1 mM each of the four deoxyribonucleotide triphosphates were incubated together at 65 °C for 5 min in a 47-μL reaction containing 50 mM Tris-HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, and 1 mM DTT. The reactions were then snap-cooled on ice for 10 min at which time 50 U AMV reverse transcriptase (25 U/μL, Boehringer) and 40 U RNasin (40 U/μL, Promega) were added. The reactions were incubated at 42 °C for 1 h. To degrade any remaining RNA, 0.5 μg DNase free RNase A (10 μg/μL) were added to the reactions and incubated at 37 °C for 5 min. Following the incubation period, 220 μL of 0.4 M NaCl were added to each reaction. The samples were extracted with phenol:chloroform (1:1, v/v) and the cDNA was ethanol precipitated and redissolved in the initial reaction volume of 50 μL in ddH_2O.

The PCR was performed in two rounds using nested primers. Each round was 35 cycles with a denaturation of 45 s at 94 °C, annealing of 45 s at 49 °C, and elongation of 1.5 min at 72 °C, followed by a final cycle of 72 °C for 5 min. Reactions were 50 μL containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (5 U/μL, Life Technologies, Inc.), 200 μM each of the four deoxyribonucleotides and 25 pmol each primer. First round PCR reactions included 5 μL of the reverse transcription reaction. The first round 5′ primer sequence was 5′-GACGGGAGAGTGG ACGGGTCA-3′ and 3′ primer sequence was 5′-GAAAGG CAAAAACCAAG-3′. Second round PCR reactions included 1 μL of the first round PCR reaction and primers internal to those used in the first round. The second round 5′ primer sequence was 5′-GGGAGTGGTATCAATAAAGGG-3′ and the 3′ primer sequence was 5′-AYCAAAAGTAGAAYCAACA ATAAA-3′ where Y represents both C and T. The 3′ primer was degenerate at positions that are complementary to adenosines in good context for deamination (Y; Polson & Bass, 1994). Control reactions were performed without template to ensure the reaction samples were not contaminated.

Prior to cloning, PCR reactions were extracted with phenol:chloroform (1:1, v/v) and ethanol precipitated. cDNA was then cloned using the Invitrogen TA Cloning Kit according to the manufacturer's instructions. cDNAs were sequenced by the University of Utah Health Sciences Sequencing Facility.

Restriction-enzyme assays

Restriction digest assay of individual cDNA clones

The PCR of individual cDNA clones was performed by inoculating individual bacterial colonies containing plasmid DNA into a 50 μL reaction containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Life Technologies, Inc., 5 U/μL), 200 μM each of the four deoxyribonucleotides and 12.5 pmol each primer. The PCR included a single denaturation step of 1 min at 94 °C and 35 cycles of a denaturation at 94 °C for 45 s, annealing at 49 °C for 45 s, and elongation at 72 °C for 1 min. Five microliters of the PCR reaction were then added directly into a restriction digest reaction containing 20 mM Tris-HCl, pH 7.5, 70 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 0.05 mM Spermine, 12.5 μM Spermidine, 0.1% digitonin, 10 mM DTT, and 0.5 μL *Apa*I (10 U/μL, NEB) in a final volume of 20 μL, and incubated at 25 °C for 2 h. One-half microliter each of *Apa*I (10 U/μL, NEB), *Bpm*I (3 U/μL, NEB), *Dra*III (3 U/μL, NEB), and *Stu*I (10 U/μL, NEB), were then added to the reactions and incubated at 37 °C for 2 h. (In control experiments *Apa*I was active at 37 °C, although optimal activity was at 25 °C). A second 0.5-μL aliquot of *Bpm*I, *Dra*III, and *Stu*I were added and the reactions were continued for an additional 2 h at 37 °C. Unlabeled PCR product of four different sequences, each containing one of the four restriction sites, were incubated as for the restriction digests, but in a separate reaction, to confirm that the restriction enzymes were active during the digests (data not shown). Five microliters of the restriction digest reaction was loaded directly onto a 1% agarose gel, and the products were visualized by ethidium bromide.

Restriction digest assay of PCR populations

Reverse transcription and the PCR were performed as above except that the PCR product was labeled by the addition of 10 μCi [α-^{32}P]dCTP (10 mCi/mL, Amersham) at the final cycle of the second round of the PCR. The entire 50 μL PCR was electrophoresed on a 1% agarose gel, and a band of 900 base pairs was excised and purified using the Qiagen QIAquick Gel Extraction Kit following the manufacturer's instructions.

Half of the purified PCR product (12 μL) was then added to the restriction digest reaction in a final volume of 20 μL, and incubated as described above for restriction digests on PCR product of individual cDNA clones. Instead of performing control digestions in separate reactions, unlabeled PCR product of four sequences (unrelated to bFGF sense RNA sequence) each containing one of the four restriction sites was added directly to each reaction to monitor that the digests were complete (data not shown). For undigested controls, the second half (12 μL) of the labeled PCR product was incubated in a final volume of 20 μL in the restriction digest reaction conditions as above, but in the absence of the restriction enzymes. The entire reaction mixture was loaded onto a 1% agarose gel. After electrophoresis the unlabeled PCR product was visualized by ethidium bromide to determine if the digestion was complete. The gel was then dried onto Whatman DE-81 cellulose paper to prevent the loss of small fragments. The radioactivity of the digested and undigested cDNA population was quantitated with a Molecular Dynamics PhosphorImager using ImageQuaNT software.

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REFERENCES

Basilio C, Wahba AJ, Lengyel P, Speyer JF, Ochoa S. 1962. Synthetic polynucleotides and the amino acid code, V. *Proc Natl Acad Sci USA 48*:613–616.

Bass BL. 1997. RNA editing and hypermutation by adenosine deamination. *Trends Biochem Sci 22*:157–162.

Billeter MA, Cattaneo R, Spielhofer P, Kaelin K, Huber M, Schmid A, Baczko K, ter Meulen V. 1994. Generation and properties of measles virus mutations typically associated with subacute sclerosing panencephalitis. *Ann NY Acad Sci 724*:367–377.

Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, Emeson RB. 1997. Regulation of serotonin-2C receptor G-Protein coupling by RNA editing. *Nature 387*:303–308.

Cattaneo R. 1994. Biased (A $\rightarrow$ I) hypermutation of animal RNA virus genomes. *Curr Opin Genet Devel 4*:895–900.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry 18*:5294–5299.

Denhardt DT. 1992. Mechanisms of action of antisense RNA. Sometime inhibition of transcription, processing, transport, or translation. *Ann NY Acad Sci 660*:70–76.

Dolnick BJ. 1997. Naturally occurring antisense RNA. *Pharmacol Ther 75*:179–184.

Grothe C, Meisinger C. 1995. Fibroblast growth factor (FGF)-2 sense and antisense mRNA and FGF receptor type 1 mRNA are present in the embryonic and adult rat nervous system: Specific detection by nuclease protection assay. *Neurosci Lett 197*:175–178.

Kimelman D, Abraham JA, Haaparanta T, Palisi TM, Kirschner MW. 1988. The presence of fibroblast growth factor in the frog egg: Its role as a natural mesoderm inducer. *Science 242*:1053–1056.

Kimelman D, Kirschner MW. 1989. An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. *Cell 59*:687–696.

Knee R, Li AW, Murphy PR. 1997. Characterization and tissue-specific expression of the rat basic fibroblast growth factor antisense mRNA and protein. *Proc Natl Acad Sci USA 94*:4943–4947.

Knee R, Murphy PR. 1997. Regulation of gene expression by natural antisense RNA transcripts. *Neurochem Int 31*:379–392.

Kumar M, Carmichael GG. 1997. Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. *Proc Natl Acad Sci USA 94*:3542–3547.

Li AW, Too CK, Murphy PR. 1996. The basic fibroblast growth factor (FGF-2) antisense RNA (GFG) is translated into a MutT-related protein in vivo. *Biochem Biophys Res Commun 223*:19–23.

Luo GX, Taylor J. 1990. Template switching by reverse transcriptase during DNA synthesis. *J Virol 64*:4321–4328.

Maas S, Melcher T, Seeburg PH. 1997. Mammalian RNA-dependent deaminases and edited mRNAs. *Curr Opin Cell Biol 9*:343–349.

Meyerhans A, Vartanian JP, Wain-Hobson S. 1990. DNA recombination during PCR. *Nucleic Acids Res 18*:1687–1691.

Murphy DG, Dimock K, Kang CY. 1991. Numerous transitions in human parainfluenza virus 3 RNA recovered from persistently infected cells. *Virology 181*:760–763.

Murphy PR, Knee RS. 1994. Identification and characterization of an antisense RNA transcript (gfg) from the human basic fibroblast growth factor gene. *Mol Endocrinol 8*:852–859.

Nishikura K, Yoo C, Kim U, Murray JM, Estes PA, Cash FE, Liebhaber SA. 1991. Substrate specificity of the dsRNA unwinding/modifying activity. *EMBO J 10*:3523–3532.

O'Hara PJ, Nichol ST, Horodyski FM, Holland JJ. 1984. Vesicular stomatitis virus defective interfering particles can contain extensive genomic sequence rearrangements and base substitutions. *Cell 36*:915–924.

Odelberg SJ, Weiss RB, Hata A, White R. 1995. Template-switching during DNA synthesis by *Thermus aquaticus* DNA polymerase I. *Nucleic Acids Res 23*:2049–2057.

Ouhammouch M, Brody EN. 1992. Temperature-dependent template switching during in vitro cDNA synthesis by the AMV-reverse transcriptase. *Nucleic Acids Res 20*:5443–5450.

Petschek JP, Mermer MJ, Scheckelhoff MR, Simone AA, Vaughn JC. 1996. RNA editing in *Drosophila* 4f-rnp gene nuclear transcripts by multiple A-to-G conversions. *J Mol Biol 259*:885–890.

Polson AG, Bass BL. 1994. Preferential selection of adenosines for modification by double-stranded RNA adenosine deaminase. *EMBO J 13*:5701–5711.

Polson AG, Bass BL, Casey JL. 1996. RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature 380*:454–456.

Rueda P, Garcia-Barreno B, Melero JA. 1994. Loss of conserved cysteine residues in the attachment (G) glycoprotein of two human respiratory syncytial virus escape mutants that contain multiple A-G substitutions (hypermutations). *Virology 198*:653–662.

Saccomanno L, Bass BL. 1994. The cytoplasm of *Xenopus* oocytes contains a factor that protects double-stranded RNA from adenosine-to-inosine modification. *Mol Cell Biol 14*:5425–5432.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science 239*:487–491.

Scadden AD, Smith CW. 1997. A ribonuclease specific for inosine-containing RNA: A potential role in antiviral defense? *EMBO J 16*:2140–2149.

Volk R, Koster M, Poting A, Hartmann L, Knochel W. 1989. An antisense transcript from the *Xenopus laevis* bFGF gene coding for an evolutionarily conserved 24 kDa protein. *EMBO J 8*:2983–2988.

Zuniga A, Borja M, Meijers C, Zeller R. 1993. Expression of alternatively spliced bFGF first coding exons and antisense mRNAs during chicken embryogenesis. *Dev Biol 157*:110–118.