Deamination of cytosine to uracil can occur in vivo at the level of nucleotide, in RNA and in DNA. Thus, enzyme-catalyzed deamination of cytidylic acid and deoxycytidine to dTMP and dUTP plays a key role in nucleotide metabolism, underpinning the pathway of de novo thymidylate synthesis (1). In the context of RNA, the major physiological example of programmed deamination is the tissue-specific editing of the terminal sequence context.

In Vivo Deamination of Cytosine to Uracil in Single-stranded DNA by Apolipoprotein B Editing Complex Catalytic Subunit 1 (APOBEC1)*

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Apolipoprotein B-editing complex catalytic subunit 1 (APOBEC1) is the catalytic component of an RNA-editing complex that deaminates C<sub>6666</sub> → U in apolipoprotein B RNA in gastrointestinal tissue, thereby generating a premature stop codon. Whereas RNA is the physiological substrate of APOBEC1, recent experiments have strongly indicated that, when expressed in bacteria, APOBEC1 and some of its homologues can deaminate cytosine in DNA. Indeed, genetic evidence derived from the study of the physiological function of activation-induced deaminase, a B lymphocyte-specific APOBEC1 homologue, is to perform targeted deamination of cytosine within the immunoglobulin locus, thereby triggering antibody gene diversification. However, biochemical evidence of in vivo DNA deamination by members of the APOBEC family is still needed. Here, we show that deamination of cytosine to uracil in DNA can be achieved in vivo using partially purified APOBEC1 from extracts of transformed Escherichia coli. Thus, APOBEC1 can deaminate cytosine in both RNA and DNA. Strikingly, its activity on DNA is specific for single-stranded DNA and exhibits dependence on local sequence context.

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2 The abbreviations used are: AID, activation-induced deaminase; APOBEC, apolipoprotein B-editing complex catalytic subunit 1; UDG, uracil-DNA glycosylase; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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phenol extraction and ethanol precipitation, the DNA was digested with (16). Using a phosphorimager. Chemical deamination of cytosine in DNA 20-fold before analysis. Sciences) in buffer H. Fractions (1 ml) were collected and concentrated S-200 high resolution 16/60 gel-filtration column (Amersham Biosciences) in buffer H. Fractions (1 ml) were collected and concentrated 20-fold before analysis.

**TLC-based Deaminase Assay—**Samples (2–4 μl) were incubated at 37 °C for 5 h in 20 μl of buffer R (40 mM Tris, pH 8, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% glycerol) containing 75,000 cpm of α-32P-dC-labeled single-stranded DNA (prepared by a 3 min heating to 95 °C of the products of asymmetric PCR amplification of the lacI region in pTrc99 used for PCR amplification of the lacI region in pTrc99 performed using [α-32P]dCTP (3000 Ci/mmol)). Following phenol extraction and ethanol precipitation, the DNA was digested with Penicillium citrinum P1 nuclease (Sigma) overnight at 37 °C (14), and the P1 digests then subjected to thin layer chromatography on polyethyleneimine-cellulose in either (i) 0.5 mM LiCl at 4 °C or (ii) at room temperature in 1 mM CH₃COOH until the buffer front had migrated 2.5 cm and then in 0.9 mM CH₃COOH:0.3 M LiCl (15). Products were detected using a phosphorimager. Chemical deamination of cytosine in DNA using bisulfite/hydrazine was performed as described previously (16).

**UDG-based Deaminase Assay—**Samples (1–2 μl) were incubated at 37 °C for 2 h in 10 μl of buffer R with 5′-biotinylated oligonucleotides that either were synthesized with fluorescein at their 3′-ends (3 pmol of oligonucleotide per reaction) or were 3′-labeled by ligation with [α-32P]dideoxyadenylate (100,000 cpm; 0.1 pmol) using terminal deoxynucleotidyltransferase. Reactions were terminated by heating to 90 °C for 3 min and oligonucleotides purified on streptavidin magnetic beads (Dynal), washing at 72 °C (except in Fig. 2a, where the streptavidin purification step was omitted). Deamination of cytosine in the oligonucleotides was monitored by incubating the bead-immobilized oligonucleotides at 37 °C for 30 min with excess uracil-DNA glycosylase (0.5 unit of UDG; enzyme and buffer from New England Biolabs) and then bringing the sample to 0.15 M in NaOH and incubating for a further 30 min. The oligonucleotides were then subjected to electrophoresis on 15% PAGE-urea gels which were developed by either fluorescence detection or phosphorimager analysis.

**Western Blotting—**Western blot detection of APOBEC1 following SDS-PAGE of samples that had been diluted 20–100-fold was performed using a goat-anti-APOBEC1 serum (Santa Cruz Biotechnology), developed with horseradish peroxidase-conjugated donkey anti-goat immunoglobulin antiserum (Binding Site, Birmingham, UK). Low molecular weight markers were from Bio-Rad.

**RESULTS**

**DNA Deamination Assay in Cell Extracts—**Since, of all the APOBEC family members tested, APOBEC1 displayed the most potent mutator activity in the E. coli mutation assay (13), we focused on APOBEC1-transformed E. coli in order to see whether we could detect DNA deamination activity in vitro using cell extracts. Initially, we tried using the UDG-based deaminase assay, working with an oligodeoxyribonucleotide substrate. However, no evidence of deamination was obtained using double-stranded oligonucleotide substrates whereas single-stranded oligonucleotides were rapidly degraded by both APOBEC1 and control extracts (data not shown).

We considered the possibility that the DNA deaminating activity might be specific for single-stranded substrates but that this activity might be masked by nonspecific nucleases. We therefore devised an assay that would be less sensitive to contaminating nucleases (Fig. 1A). The bacterial extracts were incubated with α-32P-dC-labeled single-stranded DNA which was then purified, digested with nuclease P1, and subjected to thin layer chromatography to test for the presence of [α-32P]dUMP. Clear evidence of dC deamination in this assay was detected using extracts of E. coli expressing two different APOBEC1 constructs but not from control extracts or from extracts made from E. coli cells carrying plasmids expressing mutant APOBEC1, APOBEC2, or dCTP deaminase (none of which function as DNA mutators in the bacterial assay (13)) (Fig. 1B). The DNA deaminase activity was also evident in APOBEC1 transformants of a mutant E. coli deficient in both dcd and cdd-encoded deaminases (Fig. 1B, iii). That the prod-
The presence of APOBEC1 polypeptide was detected by Western blot (panel ii). Deaminase activity was monitored by both TLC- and UDG-based assays (panels i and iii) in the total lysate (T), the flow-through (FT), and in the 800 and 1000 m M salt washes. Deaminase activity was monitored by both TLC- and UDG-based assays, which were performed on samples of the total clarified bacterial lysate (T) as well as on the eluate from the Mono Q column on Sephacryl S200. Fractions were analyzed by SDS-PAGE (i); bands were excised and analyzed by MALDI-TOF following in-gel trypsin digestion. The bands yielding peptide sequences derived from APOBEC1 and ribosomal proteins L1, L2, L6, and L9 and S4 are indicated. Molecular weight markers. ii, Western blotting for APOBEC1; iii, TLC-based; iv, UDG-based deaminase assays, which were performed on samples of the total clarified bacterial lysate (T) as well as on the eluate from the Mono Q. The UDG-based deaminase assay was performed using 3'-α-[32P]labeled SPM274; note that some of the 3'-label is removed during the incubation. The percentage of label associated with the 26-base product of the deamination/cleavage (as opposed to 40-base input oligonucleotide) is indicated.

Partial Purification—Pilot experiments revealed that ion-exchange chromatography could be used to obtain samples of APOBEC1 that contained diminished nonspecific nuclease activity. Thus, while only a proportion of the APOBEC1 polypeptide bound to the Mono Q column (around 10–20% based on ECL quantitation of the Western blot assay), elution of this bound fraction with >0.8 M Cl– yielded a sample that displayed cytosine-DNA deamination activity (as monitored using the TLC-based assay) but containing diminished nonspecific nuclease activity in the UDG-based assay (Fig. 2A). These fractions were then concentrated and subjected to gel filtration (Fig. 2B). The major APOBEC1 peak eluted in fractions 7–9 (corresponding to an M, of 95–140,000) co-eluting with peak DNA deaminating activity. Indeed, with these fractions from the gel filtration column, DNA deamination could now readily be detected by the UDG-based assay using a single-stranded oligonucleotide substrate (although the peak fractions also contained activity that removed the 3'-label from the oligonucleotide). Mass spectrometric analysis of proteins in fraction 9 following SDS-PAGE revealed the recombinant APOBEC1 migrating at the position marked by the asterisked in Fig. 2B, i, although the majority of the bands derived from ribosomal proteins.
Samples were incubated with a single-stranded oligodeoxynucleotide (with or without its complement), which contained internal dC residues and that was 5’-biotinylated as well as 3’-labeled. After purification on streptavidin, the oligonucleotide was treated with UDG (plus alkali), resulting in site-specific cleavage if the oligonucleotide had been subjected to dC→dU deamination. Then, deamination is read out by the appearance of the specific cleavage product following PAGE-urea analysis.

The partially purified wild type protein (but not the Glu63→Ala mutant) showed clear activity on a single-stranded oligonucleotide with the cleavage being dependent on the subsequent incubation with UDG (Fig. 3, B and C). The deaminating activity was not inhibited by trihydroxuridine (which inhibits cytidine deaminases (17)) or by Rnase (Fig. 3D). Strikingly (and consistent with our inability to detect deamination on double-stranded oligonucleotide substrates using crude extracts of bacterial transformants (see above)), the activity was blocked if a complementary (but not if an irrelevant) oligonucleotide was titrated into the assay (Fig. 3E). Examination of the cleavage products generated in the UDG-based assay suggests that not all dC residues are equally susceptible to APOBEC1-mediated deamination. While the data do not allow us yet to identify the ideal in vitro target for APOBEC1-mediated deamination, it is clear that in oligonucleotide SPM168 the third cytosine in the sequence TCCGG is much less favored than the other two (Fig. 3, B–E). Similarly, evidence of specificity comes from comparing various related oligonucleotides as substrates, where all the data taken together point to deamination being especially disfavored when a purine is located immediately 5’ of the cytosine (Fig. 3, F and G).

**DISCUSSION**

The results described here provide biochemical evidence that APOBEC1-mediated deamination of cytosine to uracil can occur on single-stranded DNA, is dependent on local sequence context, and is abolished by mutation of the APOBEC1 zinc coordination motif. Unlike AID (where genetic evidence indicates that the natural physiological substrate of deamination is DNA (11, 12)), the major physiological substrate of APOBEC1 is clearly apolipoprotein B RNA (4, 5). Nevertheless, the observation that misexpression of APOBEC1 in transgenic mice predisposes to cancer (18) suggests that APOBEC1-mediated deamination activity being especially disfavored when a purine is located immediately 5’ of the cytosine (Fig. 3, F and G).

The results reveal a clear sensitivity to the local sequence context of the APOBEC1-mediated dC deamination in the in vitro assay, most readily explained by a bias against a 5’-flanking purine residue. This would accord well with the in vivo data where a near-total restriction to mutation at dC residues with a 5’-flanking pyrimidine is seen at the rpoB locus (13) as well as a similar bias at another test locus in *E. coli* (2). Curiously, however, these findings contrast with the fact that the target cytosine of APOBEC1 in apolipoprotein B RNA is flanked by a 5’-A residue (2, 3). It will obviously be interesting to obtain more information about both the structural and kinetic features of the interaction and activity of recombinant APOBEC1 on different RNA and DNA substrates.

The in vitro assay also reveals that APOBEC1 deamination is targeted to single-stranded DNA and, indeed, was undetectable on double-stranded DNA. This specificity for single-stranded DNA is in accordance with the fact that the natural substrate of APOBEC1 is most likely single-stranded RNA (5), and presumably, the same active site in APOBEC1 is used for both types of polynucleotide. Furthermore, spontaneous deamination of cytosine is also much more rapid in single- (as opposed to double-) stranded DNA (19). If this in vitro preference of APOBEC1 for a single-stranded DNA substrate can be extrapolated to the in vivo situation as well as to other DNA-mutating APOBEC family members, it could go some way to explaining why AID-mediated DNA changes (somatic hypermutation and class switch recombination) correlate with transcription of the DNA target gene. Clearly, extending on the assays described here and applying them to other APOBEC family members should give valuable insight into the mechanism and specificity of APOBEC family-mediated DNA deamination.

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