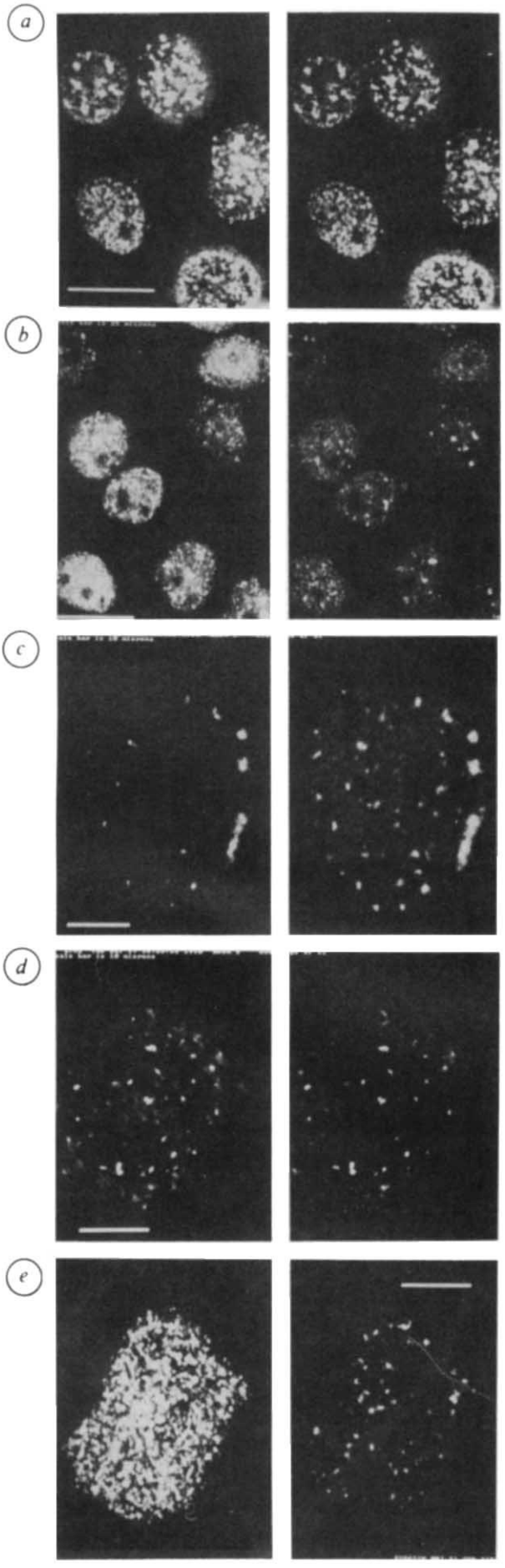

lead to a cell which permits permanent S phase, thus facilitating viral replication and tumour development. Dissection of the HSV-1 infection system may shed light on the functions and cellular targets of these two anti-oncogenes. □

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Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG

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OXIDATIVE damage to DNA, reflected in the formation of 8-oxo-7-hydrodeoxyguanosine (8-oxodG)[1,2], may be important in mutagenesis, carcinogenesis and the ageing process[3,4]. Kuchino *et al.* studied DNA synthesis on oligodeoxynucleotide templates containing 8-oxodG, concluding that the modified base lacked base pairing specificity and directed misreading of pyrimidine residues neighbouring the lesion[5]. Here we report different results, using an approach in which the several products of a DNA polymerase reaction can be measured. In contrast to the earlier report[5], we find that dCMP and dAMP are incorporated selectively opposite 8-oxodG with transient inhibition of chain extension occurring 3′ to the modified base. The potentially mutagenic insertion of dAMP is targeted exclusively to the site of the lesion. The ratio of dCMP to dAMP incorporated varies, depending on the DNA polymerase involved. Chain extension from the dA · 8-oxodG pair was efficiently catalysed by all polymerases tested.

```
5'CCTTCXCTACTTTCCTCT
          TGAAAGGAGA 5'
          ATGAAAGGAGA 5'
          GATGAAAGGAGA 5'
          N GATGAAAGGAGA 5'

X = dG or 8-oxodG
```

FIG. 1 Structure of 8-oxodG and sequence of templates and primers. Oligodeoxynucleotides, including those containing 8-oxodG, were prepared by solid-state synthesis on a DuPont Coder 300 automated DNA synthesizer as reported elsewhere[17], removed from the resin-support by treatment with concentrated ammonia containing 0.1 M 2-mercaptoethanol at 55 °C for 16 h, then purified by electrophoresis on 20% polyacrylamide gels (72 × 15 × 0.2 cm) in the presence of 7 M urea. Primers (100 pmol) were labelled with ^{32}P at the 5′ terminus with 5 μl of bacteriophage T4 polynucleotide kinase (10 units μl^{-1}) in the presence of 5 μl [γ-^{32}P]ATP (10 Ci μl^{-1})[18], heated at 100 °C for 3 min and concentrated on Centricon filters after adding 500 μl distilled water. Labelled primer (50 pmol) was added to 75 pmol template DNA, heated at 56 °C for 15 min then annealed by standing at room temperature for 1 h and overnight at 4° C.

Figure 1 lists nucleotide sequences of primers and template used in these experiments. In Fig. 2, 18-mers containing dC, dA, dG or dT at position 13 are distinguished by their relative electrophoretic mobility on 20% polyacrylamide gels (lanes 6–9). DNA synthesis on unmodified templates, catalysed by DNA polymerase α (pol α) (lane 1) and other polymerases (data not shown) led to the expected exclusive incorporation of dCMP at position 13. Using the DNA polymerases indicated in Fig. 2 and templates modified with 8-oxodG, 18-mers containing dA and dC were formed (lanes 2–5). The ratio of dC to dA for 18-mers synthesized by the Klenow fragment was 7:1; by intact pol I, 7:1 (data not shown), by pol β 4:1; by pol α 1:200; and by pol δ, 1:5 18-mers containing dG or dT were not detected. In experiments using the Klenow fragment or DNA pol α, the identity of dCMP and/or dAMP incorporated opposite the lesion was confirmed by Maxam–Gilbert sequence analysis[6].

Kinetic parameters of base insertion and extension, catalysed by Klenow fragment and polymerase α, were measured during translesional synthesis (Table 1). These reactions measure addition of a single dNMP to various 3′ termini under steady-state conditions. The Michaelis constant, K_m, and the maximum rate of the reaction, V_{max}, were determined experimentally; the frequency of dNMP insertion and extension opposite 8-oxodG was calculated relative to experiments using the unmodified template, as described by Mendelman *et al.*[7,8]. These kinetic constants are highly reproducible. Using the Klenow fragment, the frequency of insertion (F_{ins}) for dCMP opposite 8-oxodG (0.35) is seven times higher than for dAMP. The frequency of extension (F_{ext}) from the dC·8-oxodG base pair is, however, 12 times lower than from dA·8-oxodG. In reactions catalysed by calf thymus pol α, F_{ins} of dAMP opposite the lesion (0.13) is seven times higher than for dCMP; the dA·8-oxodG pair is efficiently extended. Similar results were obtained using human pol α (not shown). We conclude from these experiments that dCTP and dATP compete for insertion on DNA templates containing 8-oxodG. The polymerases tested vary greatly in their respective preference for inserting dA and dC opposite the lesion and extend preferentially the dA·8-oxodG base pair.

Kuchino *et al.*[5] reported misreading of pyrimidine residues located adjacent to 8-oxodG. Using the Klenow fragment and similar experimental conditions, we measured the relative insertion of dNMPs immediately 3′ to the lesion on a series of templates where the 5′ flanking base was dC, dG, dT or dA. In all cases, dG was inserted exclusively opposite the 3′ dC residue (not shown). Misincorporation of nucleotides opposite pyrimidines adjacent to the lesion was not observed even when sequences of the template used were identical to those reported by Kuchino *et al.*[5].

We also tested the proofreading ability of Klenow fragment (not shown) and intact DNA polymerase I to replace bases inserted opposite 8-oxodG at the 3′ terminus (Fig. 3). Although dA was readily excised and replaced by dC in dA·dG mismatches, neither dA nor dC was replaced when paired with 8-oxodG. Both base pairs were readily extended, however. Apparently, the 3′→5′ exonuclease proof-reading function of DNA polymerase I does not recognize dA·8-oxodG or dC·8-oxodG as a substrate.

Fidelity of DNA polymerases is primarily achieved through selective insertion of dNTPs opposite the lesion, proof-reading by 3′→5′ exonuclease and extension of the newly formed base pair[7–10]. This process is illustrated by our experiments with the Klenow fragment in which dAMP is inserted opposite 8-oxodG at a lower frequency than dCMP, forming a base pair that is more efficiently extended than dC·8-oxodG. In this case, proof-reading does not affect the composition of the final product of translesional synthesis, which is determined primarily by F_{ins}.

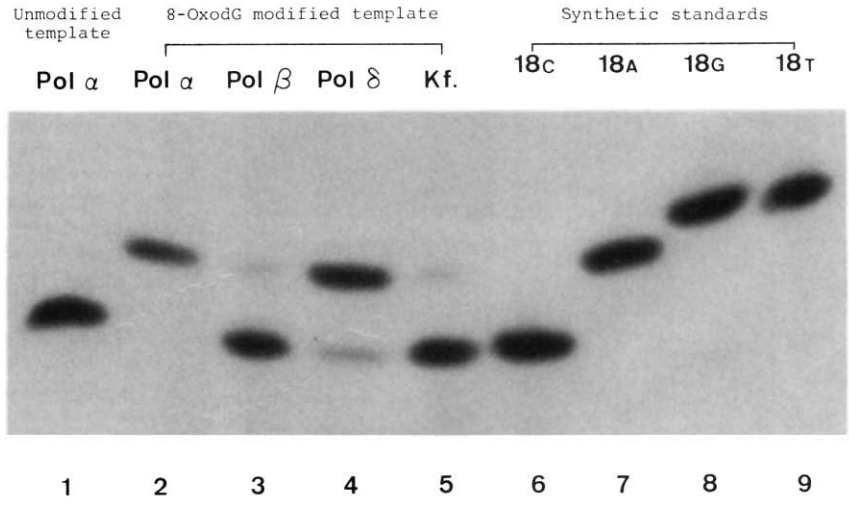

FIG. 2 Incorporation of nucleotides opposite 8-oxodG. Chain extension reactions were carried out in a solution (10 μl) containing DNA polymerase, buffer, salts, four dNTPs (100 μM each) and an unmodified 18-mer (lane 1) or 8-oxodG-modified 18-mer template primed with ^{32}P-labelled 10mer (0.05 μM), except in th case of DNA pol δ, in which a ^{32}P-labelled 12-mer was used. Sources of DNA polymerase are as follows: HeLa DNA pol α and pol β[19,20] (Dr S. Wilson), calf thymus pol δ[21,22] (Dr K. Downey), *Escherichia coli* pol I (Pharmacia) and the cloned Klenow fragment (Kf) (International Biotechnologies, Inc.) HeLa DNA pol α (1 unit) was incubated at 25° C for 1 h in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 20 mM ammonium sulphate, 2 mM dithiothreitol (DTT) and BSA (0.5 μg μl^{-1}). HeLa DNA pol β (1 unit) was incubated for 1 h at 25 °C in the same buffer used for pol α, omitting ammonium sulphate. Calf thymus DNA pol δ (0.7 units) was incubated at 30 °C for 2 h in 50 mM Tris-HCl buffer (pH 6.5) containing 10 mM $MgCl_2$, 2 mM DTT, BSA (0.04 μg μl^{-1}) and 100 ng human PCNA (proliferating cell nuclear antigen) cloned in *E. coli*, provided by Dr P. Fisher. The Klenow fragment (0.05 units) was incubated at 20 °C for 1 h in 50 mM Tris-HCl buffer (pH 8.0) containing 8 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. Reaction mixtures were subjected to 20% polyacrylamide gel electrophoresis. Mobilities of the 18-mer reaction products (lanes 1–5), are compared with those of synthetic oligodeoxynucleotides containing dC (lane 6), dA (lane 7), dG (lane 8) or dT (lane 9) at position 13.

TABLE 1 Kinetic parameters of insertion and extension reactions catalysed by Klenow fragment and DNA polymerase α

(a)	dNTP ↓G— 12-mer 5′-CCTTCXC— K_m (μM)	V_{max} (% min^{-1})	F_{ins}		dGTP ↓NG— 13-mer 5′-CCTTCXC— K_m (μM)	V_{max} (% min^{-1})	F_{ext}
C·G	2.04	14.5	1.00	G·C	0.63	24.8	1.00
A·G	ND	—	—	G·A	*ND*	—	—
C·G*	6.62	16.3	0.35	G·C	8.41	10.8	0.033
A·G*	28.3	9.5	0.05	G·A	1.00	19.4	0.50
(*b*)	K_m (μM)	V_{max} (% min^{-1})	F_{ins}		K_m (μM)	V_{max} (% min^{-1})	F_{ext}
C·G	5.54	4.2	1.00	G·C	11.7	4.9	1.00
A·G	1,410	0.17	1.56×10^{-4}	G·A	4,990	0.05	2.47×10^{-5}
C·G*	70.5	0.96	0.018	G·C*	1,160	0.46	9.45×10^{-4}
A·G*	42.3	4.1	0.13	G·A*	26.1	2.0	0.19

a, Kinetics of insertion (0.1 μM–10 mM dNTP) using the Klenow fragment (0.005 units) were determined at 20 °C; reaction mixtures were incubated for 90 s in 10 μl Tris-HCl buffer (pH 8.0) containing template DNA primed with ^{32}P-labelled 12-mer (0.05 μM), as described in the legend to Fig. 2. Experimental conditions are analogous to those reported by Mendelman *et al.*[7,8]; all reactions were linear with time and data reported are an average of three separate experiments. Kinetics of chain extension (0.05 μM–10 mM dGTP) were determined in reaction mixtures incubated at 20 °C for 90 s in 10 μl Tris-HCl buffer (pH 8.0) containing template DNA primed with ^{32}P labelled 13-mer containing dC or dA (0.05 μM). *b*, Kinetics of insertion (1.0 μM–10 mM dNTP) using calf thymus pol α (1 unit, Molecular Biology Resources Inc.) were measured at 25 °C in reactions incubated for 5 min (dC · dG pairs), 10 min (dA · 8-oxodG), 15 min (dC · 8-oxodG), and 30 min (dA · dG). Kinetics of extension (0.4 μM–10 mM dGTP) were determined in reactions conducted at 25 °C for 5 min (dC · dG), 10 min (dA · 8-oxodG), and 30 min (dC · 8-oxodG, dA · dG). Bands were cut from the gels and radioactivity was determined by liquid scintillation counting. Values for K_m and V_{max} were obtained from Hanes–Woolf plots. Insertion and extension frequencies (F) were determined relative to dC · dG according to equations described by Mendelman *et al.*[7,8] where $F=(V_{max}/K_m)$ [wrong pair]/(V_{max}/K_m) [right pair] with 'wrong pair' defined either as a mismatch or any pair involving 8-oxodG. G:X, dG; G*:X, 8-oxodG.

It seems that DNA polymerases differ significantly in their ability to incorporate specific dNMPs on the same DNA template. 8-oxodG occurs predominantly as the 6,8-diketo tautomer[11–13] and assumes the *syn* conformation as a nucleoside[13,14] or when located opposite dA in duplex DNA[15].

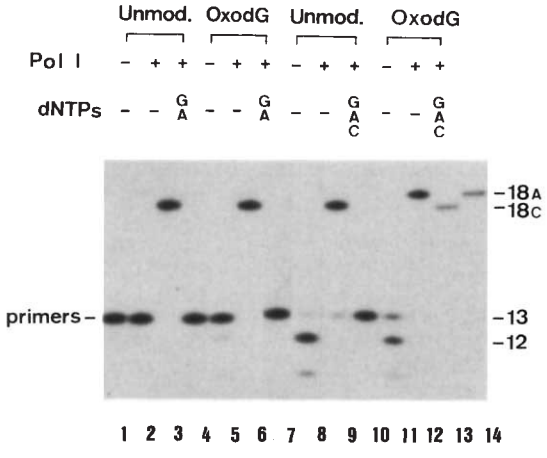

FIG. 3 Proof-reading of the 3′ terminus by DNA pol I. Unmodified and 8-oxodG-modified 18-mer templates primed with a 13-mer containing dC (lanes 1–6) or dA (lanes 7–12) opposite the modified base were incubated at 20 °C for 1 h in the presence (lanes 2, 5, 8, 11) or absence (lanes 1, 4, 7, 10) of DNA pol I (0.05 unit) or with pol I and 100 μM of each dNTP (lanes 3, 6, 9, 12) using described in the legend to Fig. 2. Lanes 13 and 14 contain synthetic 18-mers containing dC and dA, respectively, at position 13.

8-oxodG assumes the *anti* conformation when pairing with dC (E. Ohtsuka, personal communication). During DNA replication or repair, enzymes could encounter the lesion in either conformation.

Our results differ significantly from those described by Kuchino *et al.*[5] who reported that templates modified with 8-oxodG directed insertion of dA, dT, dC and dG opposite the lesion with almost equal frequency and caused misreading at neighbouring positions, an effect that depended on the sequence context. They reported also that the rate of DNA synthesis on 8-oxodG templates was not retarded. The discrepancy may be explained by the DNA polymerase used for dideoxynucleoside sequencing analysis by Kuchino *et al.*[5]. We found that Klenow fragment was not suitable for this particular procedure and used instead T7 DNA polymerase (Sequenase Version 2.0, USB), which lacks an exonuclease function. Under these conditions, only dA and dC were incorporated opposite the lesion; a result which was confirmed by direct Maxam–Gilbert sequencing analysis and by the relative electrophoretic migration of the reaction products. Targeted $G\rightarrow T$ transversions predicted by these *in vitro* experiments have emerged as the predominant base substitution induced *in vivo* by plasmids and phagemids modified with 8-oxodG, as studied by site-specific mutagenesis techniques[16,23]. □

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RNA editing by cytidine insertion in mitochondria of *Physarum polycephalum*

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A COROLLARY of the central dogma of molecular biology is that genetic information passes from DNA to RNA by the continuous synthesis of RNA on a DNA template. The demonstration of RNA editing[1] (the specific insertion, deletion or substitution of residues in RNA to create an RNA with a sequence different from its own template) raised the possibility that in some cases not all of the genetic information for a trait resides in the DNA template. Two different types of RNA editing have been identified in mitochondria: insertional editing represented by the extensive insertion (and occasional deletion) of uridine residues in mitochondrial RNAs of the kinetoplastid protozoa[2–4] and the substitutional editing represented by the cytidine to uridine substitutions in some plant mitochondria[5–7]. These editing types have not been shown to be present in the same organism and may have very different mechanisms. RNA editing of both types has been observed in non-mitochondrial systems[8–17] but is not as extensive and may involve still different mechanisms. Here we report the discovery of extensive insertional RNA editing in mitochondria from an organism other than a kinetoplastid protozoan. The mitochondrial RNA apparently encoding the α subunit of ATP synthetase in the acellular slime mould, *Physarum polycephalum*, is edited at 54 sites by cytidine insertion.

The mitochondrial DNA (mtDNA) of *P. polycephalum* is a circle of 60 kilobases (kb) and is extensively transcribed[18]. A 970-nucleotide complementary DNA with homology to *P. polycephalum* mtDNA was identified by screening a *Physarum* cDNA library with uniformly radiolabelled mtDNA. The location of the mtDNA homology was determined by radiolabelling the cDNA and hybridizing it with Southern blots of mtDNA digested with various restriction enzymes. The hybridization pattern indicated that the homology was located at a single site on the mtDNA map from strain M3 (ref. 18; Fig. 1*a*). Additional cDNAs with homology to this region of the mtDNA were produced and selectively amplified using the polymerase chain reaction (PCR)[19]. Sequence analysis of the cDNAs revealed a single reading frame of 528 codons which lacked all three of the classic termination codons. The amino-acid sequence deduced from the open reading frame using the classical genetic code had homology to the α subunit of ATP synthetase from a variety of organisms (Fig. 2).

The sequence of the region of the mtDNA corresponding to the cDNAs was determined. Although the mtDNA and cDNA sequences had a large degree of homology, there was no reading frame in the mtDNA sequence which could code for the ATP synthetase subunit and the regions of reading frame homology were separated by a number of −1 frameshifts. These sequence discrepancies were also reflected in restriction enzyme site differences between the cDNA and the mtDNA.

When the cDNA open reading frame was compared with the analogous sequence of the mtDNA, a single cytidine insertion was found at 54 sites in the cDNA relative to the mtDNA. These insertions generated the open reading frame in the cDNA by correcting the 54 frameshifts on the mtDNA (Fig. 3).

One possible explanation for the discrepancy between the sequence of the cDNA and the mtDNA is that the RNA with the open reading frame was encoded on a conventional DNA template located outside the mitochondrion. Southern blots of DNA from purified *Physarum* nuclei incubated with probes homologous to the cDNA sequence did not detect homologous sequences (data not shown). To screen for a conventional template present at levels below the limits of detection by Southern blotting, PCR was used to amplify DNA sequences with α subunit homology (Fig. 1*b*). This did not detect any DNA other than contaminating traces of mtDNA which lack the open

TABLE 1 Codons created by RNA editing*

TTT	Phe	22	TCT	Ser	16	TAT	Tyr	23	TGT	Cys	4
TTC	Phe	9 (2)	TCC	Ser	3 (1)	TAC	Tyr	1 (1)	TGC	Cys	1
TTA	Leu	31	TCA	Ser	2	TAA	End	0	TGA	End	0
TGG	Leu	2	TCG	Ser	0	TAG	End	0	TGG	Trp	0
CTT	Leu	13 (1)	CCT	Pro	7	CAT	His	5	CGT	Arg	13
CTC	Leu	2 (1)	CCC	Pro	0	CAC	His	0	CGC	Arg	1
CTA	Leu	5 (2)	CCA	Pro	10 (3)	CAA	Gln	24 (3)	CGA	Arg	2 (1)
CTG	Leu	0	CCG	Pro	2	CAG	Gln	1	CGG	Arg	0
ATT	Ile	31	ACT	Thr	16 (1)	AAT	Asn	23	AGT	Ser	4
ATC	Ile	15 (14)	ACC	Thr	8 (8)	AAC	Asn	2 (1)	AGC	Ser	1
ATA	Ile	1	ACA	Thr	8	AAA	Lys	23	AGA	Arg	7
ATG	Met	8	ACG	Thr	0	AAG	Lys	2	AGG	Arg	1
GTT	Val	15	GCT	Ala	28 (1)	GAT	Asp	34	GGT	Gly	31
GTC	Val	9 (8)	GCC	Ala	8 (5)	GAC	Asp	2 (1)	GGC	Gly	2
GTA	Val	6	GCA	Ala	4	Gaa	Glu	29	GGA	Gly	9
GTG	Val	1	GCG	AIA	0	GAG	Glu	1	GGG	Gly	0

* Cognate amino acids were assigned using the classical genetic code. Numbers directly to the right of the amino acid indicate the number of times the codon is used in the α subunit open reading frame. Numbers in parentheses refer to the number of codons created by C insertion.