

Fig. 2 Assembly of small subunit into whole RBPCase molecules. *a*, Separation of whole RBPCase molecules by transverse gradient non-denaturing gel electrophoresis. Wheat-germ products and a 3,200g stromal supernatant were prepared as in Table 1. Wheat-germ supernatant (50 µl) containing 1.25×10^6 c.p.m. of TCA-insoluble radioactivity was incubated with 200 µl of stromal extract, equivalent to 28.8 µg chlorophyll, for 60 min at 27 °C. A control incubation mixture consisted of 50 µl wheat-germ supernatant plus 200 µl intact plastid buffer. After incubation, samples were brought to 300 µl with a solution of sucrose and 2-mercaptoethanol with final concentrations of 10% (w/v) and 1.66% (v/v) respectively, for analysis by non-denaturing polyacrylamide gel electrophoresis. The gel system is based on that of Blair and Ellis³. A slab gel (17 × 17 cm) was cast with a linear gradient of 5–10% (w/v) acrylamide and 0.1–0.2% (w/v) bisacrylamide, stabilised with 0.2% (w/v) linear polyacrylamide. Once polymerised, the gel was turned through 90°, and a 5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide slot former was cast along one edge of the gradient gel. The gel was pre-electrophoresed for 3.5 h at 30 mA. Samples of incubation mixture containing 2×10^5 c.p.m. TCA-insoluble radioactivity were loaded onto all the slots. Electrophoresis was for 18 h at 27 mA after which the gel was stained with Coomassie brilliant blue R and destained². The photograph shows the stained gel. RBPCase is the most heavily stained band, and is marked with an arrow at the 5% end of the gel. Other, minor bands, change their mobility relative to RBPCase according to the gel concentration. Autoradiography for 100 h shows radioactivity co-migrating with the stained bands of RBPCase, but not at corresponding positions in tracks lacking stromal extract (not shown). *b*, Identification of labelled small subunit in whole RBPCase molecules. The transverse gradient non-denaturing polyacrylamide gel shown in (*a*) was rehydrated, and each RBPCase region was excised. Gel pieces were equilibrated twice with 5 ml of 62 mM Tris-HCl, 2% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol pH 6.8 for 15 min each, and then affixed to the top of an SDS slab gel with 1% (w/v) Agarose, 0.2% (w/v) SDS, 62 mM Tris-HCl, pH 6.8. Electrophoresis was as previously described². The photograph shows an autoradiograph of the SDS gel in the small subunit region; the arrow marks the small subunit marker. The autoradiograph shows that radioactivity co-migrating with small subunit is present in RBPCase molecules excised from across the entire gradient of the non-denaturing gel. Other radioactive polypeptides migrate above and below the small subunit. However, these polypeptides vary in distribution across the gradient, indicating that they do not co-migrate with RBPCase at all gel concentrations. Control incubations lacking stromal extract contain no radioactivity in the small subunit region (not shown).

non-denaturing gels shows that the large subunit of RBPCase synthesised by isolated pea chloroplasts does not enter pre-existing RBPCase molecules³, but accumulates as an aggregate¹. This observation has been interpreted as indicating that isolated pea chloroplasts lack a pool of small subunits³. It follows that supplying P20 to pea chloroplast fractions may result in the assembly of whole RBPCase molecules. When wheat-germ incubation mixture containing labelled P20 is incubated with a stromal extract, labelled small subunit co-migrates with whole RBPCase molecules over a wide range of polyacrylamide gel concentrations when electrophoresed in non-denaturing conditions (Fig. 2). Co-migration in such conditions is a good test for identity¹⁰. Control experiments where stromal extracts were omitted from the incubation show no label migrating with whole RBPCase molecules. These observations establish that the assembly of small subunit into RBPCase occurs in the chloroplast stroma as previously proposed², and not in the cytoplasmic compartment. Chua and Schmidt¹¹ have reported the processing of P20 and assembly of small subunit into RBPCase in intact pea and spinach chloroplasts, but the stromal location of the processing and assembly process was not established. Whether processing is an integral part of the assembly mechanism, or merely provides small subunit for that mechanism, can be resolved when the processing activity has been purified.

This work was supported by the SRC.

STEVEN M. SMITH

R. JOHN ELLIS

Department of Biological Sciences,
University of Warwick,
Coventry, UK

Received 18 January; accepted 13 March 1979.

1. Ellis, R. J. *Biochim. biophys. Acta* **463**, 185–215 (1977).
2. Highfield, P. E. & Ellis, R. J. *Nature* **271**, 420–424 (1978).
3. Blair, G. E. & Ellis, R. J. *Biochim. biophys. Acta* **319**, 223–234 (1973).
4. Kung, S. D. *Science* **191**, 429–434 (1976).
5. Ellis, R. J., Highfield, P. E. & Silverthorne, J. *Proc. Symp. Fourth Int. Congr. Photosynthesis* (eds Hall, D. O., Coombs, J. & Goodwin, T. W.) 497–506 (Biochemical Society, London, 1977).
6. Blobel, G. & Dobberstein, B. *J. cell. Biol.* **67**, 835–851 (1975).
7. Joy, K. W. & Ellis, R. J. *Biochim. biophys. Acta* **378**, 143–151 (1975).
8. Ellis, R. J. & Barracough, D. R. *Chloroplast Development* (ed. Akyounoglu, G.) 185–194 (North-Holland, Amsterdam, 1978).
9. Dobberstein, B., Blobel, G. & Chua, N.-H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1082–1085 (1977).
10. Hedrick, J. L. & Smith, A. J. *Archs. Biochem. Biophys.* **126**, 155–164 (1968).
11. Chua, N.-H. & Schmidt, G. W. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6110–6114 (1978).
12. Morgenstaler, J.-J., Marsden, M. P. F. & Price, C. A. *Archs Biochem. Biophys.* **168**, 289–301 (1975).
13. Arnon, D. I. *Pl. Physiol.* **24**, 1–15 (1949).

Sites of inhibition of *in vitro* DNA synthesis in carcinogen- and UV-treated $\Phi X 174$ DNA

THE availability of DNA molecules of known sequence and rapid methods for the determination of the sequence of particular DNA fragments^{1–3} make it possible to investigate the molecular action of mutagens and carcinogens more closely^{4,5}. For example, there is considerable evidence that some types of lesion in DNA such as UV light-induced pyrimidine dimers and various chemical adducts are blocks to DNA synthesis both *in vivo*^{6–9} and *in vitro*^{10,11}. The available data support the hypothesis that the block occurs at the level of the lesion on the template strand^{10,11}. We have used DNA containing such lesions on a template of known sequence for *in vitro* DNA synthesis by DNA polymerase 1 to investigate the site of the block at the level of the nucleotide sequence. This method allows us to

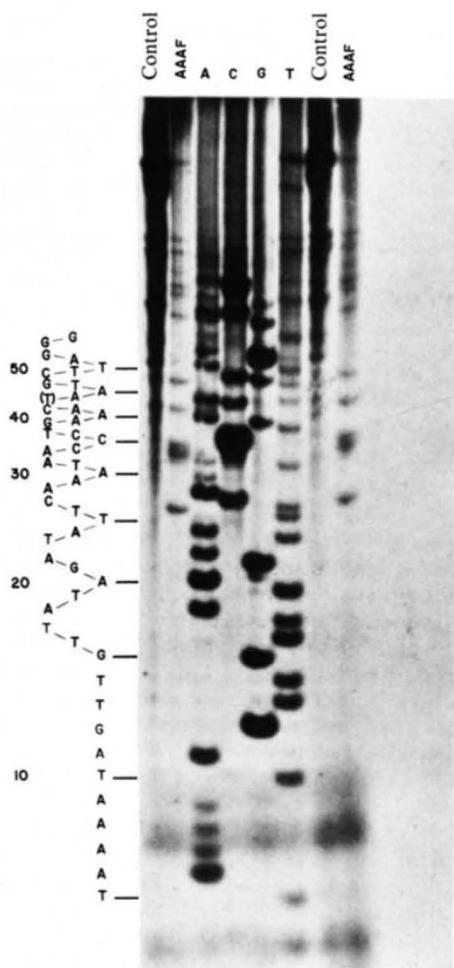


Fig. 1 Polyacrylamide gel analysis of the product synthesized by pol1 on a DNA template reacted with AAAF. Single-stranded phage Φ X174 DNA ($400 \mu\text{g ml}^{-1}$) was reacted with ^{14}C -AAAF ($50 \mu\text{g ml}^{-1}$) for 4 h at 30°C , extracted 4 times with amyl alcohol, precipitated with ethanol and resuspended in 10 mM Tris-HCl pH 7.5, 0.25 mM EDTA. The extent of reaction, determined from the ratio of ^{14}C c.p.m. to A_{260} was ~ 265 AAAF adducts per Φ X DNA molecule. Double-stranded RF Φ X DNA was digested with HaeII endonuclease (BRL) and individual restriction fragments were purified on agarose gels. Restriction fragment 2 (ref. 12) was heated at 100°C for 5 min and annealed to either AAAF-treated or unreacted DNA at 65°C for 90 min (molar ratio 1.2:1). Polymerase reactions were carried out in a volume of 20 μl and contained 50 mM Tris-HCl, pH 8.0, 8 mM MgCl₂, 5 mM dithiothreitol, dATP, dGTP and dCTP, 50 μM each, 1–2 μCi [α - ^{32}P]TTP (about 300 Ci mmol $^{-1}$), 0.1–0.2 μg DNA and 0.7 units DNA polymerase I (Klenow fragment, Boehringer). Incubation was at room temperature for 15 min and then 2 μl 0.5 mM TTP was added, incubation continued for a further 15 min and the reaction was stopped by heating at 65°C for 10 min. One unit of HaeII (BRL) was then added and incubated at 37°C for 60 min. For the sequence standards di-deoxyribonucleoside triphosphates were used as described by Sanger *et al.*³. The products were denatured and analysed on a 20% polyacrylamide gel². Lanes Control and AAAF are the products synthesized on untreated and AAAF-reacted templates respectively. Lanes A, C, G and T are the sequence standards produced with the di-deoxy chain terminating nucleotides. Numbering is from the position of the HaeII cleavage site.

determine both the site at which lesions capable of blocking synthesis by DNA polymerase occur and exactly where synthesis stops in relation to the lesion.

Φ X174 DNA was used in these experiments as it has already been completely sequenced^{12,13}. The Klenow fragment of *Escherichia coli* pol1 was used as the polymerase for most of our

experiments. The template consisted of single-stranded phage DNA to which individual restriction fragments, obtained by digestion of the double-stranded RF DNA, were annealed. We used fragments produced by the enzyme HaeII which cuts Φ X174 RF DNA at eight sites¹². These templates were used for the di-deoxynucleotide chain terminating method of Sanger and Coulson to provide a sequence standard. We found minor discrepancies with the preliminary sequence published by Sanger *et al.*¹²; however, in practically every case these were found to be in complete agreement with the revised sequence¹³. Single-stranded Φ X174 DNA was either UV-irradiated or reacted with *N*-acetoxy-2-acetyl aminofluorene (AAAF) or other carcinogens. The reacted template was then annealed with the priming restriction fragment and the polymerisation reaction carried out as for the sequencing reactions except that the di-deoxynucleotides were omitted.

In the reaction with AAAF-treated DNA a number of bands are seen which do not appear when untreated DNA is used (Fig. 1). These bands in the three cases in which identification is

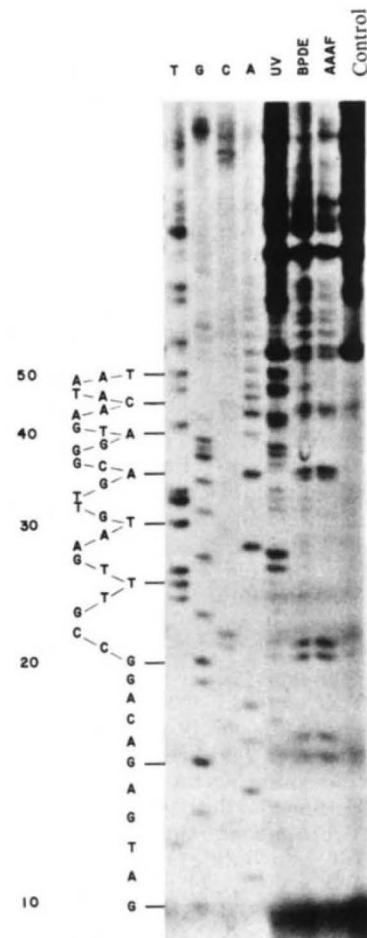


Fig. 2 Polyacrylamide gel analysis of the products synthesized by pol1 on DNA templates reacted with AAAF or anti-BPDE or irradiated with UV light. For the anti-BPDE template $46 \mu\text{g}$ Φ X DNA was incubated at 37°C with 30 nmol ^{14}C -anti-BPDE in 0.5 ml. At 60-min intervals a further three additions of ^{14}C -anti-BPDE were made and incubation continued for 5 h. The DNA was extracted four times with amyl alcohol and precipitated with ethanol. About 140 anti-BPDE adducts were added per Φ X DNA molecule. The DNA for the UV template was irradiated with 1000 J m^{-2} of UV at a DNA concentration of $100 \mu\text{g ml}^{-1}$. The primer used in this experiment was HaeII fragment 5. Reaction conditions were the same as for Fig. 1. Lanes Control, AAAF, BPDE and UV are the products synthesized by pol1 on untreated, AAAF or anti-BPDE-reacted and UV-irradiated DNA templates respectively. Lanes A, C, G and T are the sequence standards.

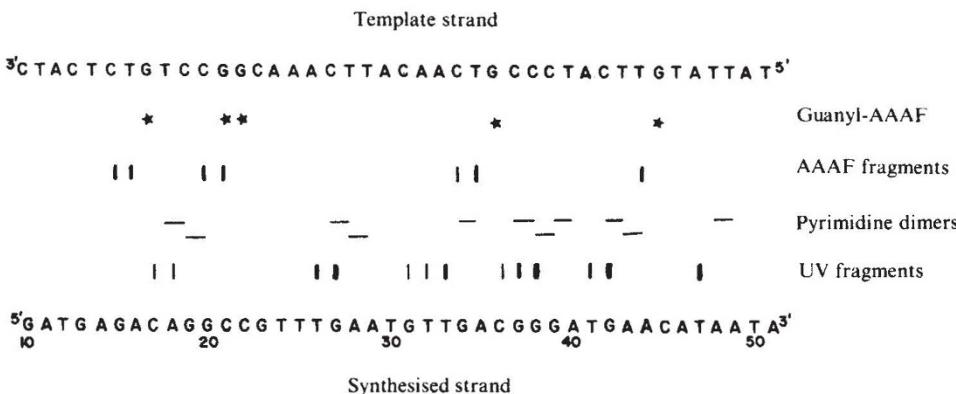


Fig. 3 The position of chain termination in relation to the sites of lesions in the DNA: diagrammatic representation of data shown in Fig. 2. Potential sites for the formation of *N*-acetyl-*N*-(guan-8-yl)-2-aminoindole (guanines) and of pyrimidine dimers (adjacent pairs of pyrimidines) are shown along side of the position of bands obtained with synthesis by polI on AAAF-reacted or UV-irradiated DNA templates.

possible occur one base before that of a cytosine residue in the sequence standards, that is, synthesis has stopped at the base before a guanine in the DNA template. AAAF has been shown to react predominantly with guanine in DNA to form *N*-acetyl-*N*-(guan-8-yl)-2-aminoindole^{14,15}. Our results both confirm the known major site of reaction of AAAF in DNA and demonstrate that this lesion is a block to DNA synthesis. Another conclusion that can be drawn from these experiments is that there seems to be no preferential reaction of AAAF with particular guanines in the section of tested DNA, at least in the conditions used here; that is, single-stranded DNA reacted *in vitro*. Bands can be detected corresponding to all the guanines in the region of the template for which the sequence can be resolved, and there is no obvious predominance of any of them. This may simply reflect the very high number of adducts in the DNA (about 1 per 5 guanines) although with a lower number of adducts (about 1 per 30 guanines) no bands at all were detected. There is no indication of lesions blocking synthesis at any position other than guanine. This conclusion was confirmed by an experiment using a different priming fragment (Fig. 2) which included templates either treated with (\pm)7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro benzo(a)pyrene (anti-BPDE) or irradiated with UV light. The pattern of bands obtained in this experiment is shown in Fig. 3. The bands produced with the AAAF-treated template are again consistent with those expected if synthesis terminated one nucleotide before an *N*-acetyl-*N*-(guan-8-yl)-2-aminoindole residue. A completely different pattern of bands is seen with the UV-irradiated template. Synthesis on such a template terminates one nucleotide before any pair of pyrimidine bases. All three types of pyrimidine dimer, cytosine-cytosine, cytosine-thymine and thymine-thymine, are blocks to DNA synthesis although there is some indication that the bands resulting from thymine-thymine dimers are more prominent, probably due to their faster rate of formation during irradiation¹⁶ and thus more frequent occurrence in the template.

The results with anti-BPDE are more difficult to interpret. Prominent bands occur at the same positions as found with AAAF treatment although many other bands can also be seen. This would suggest that guanine is a major site of reaction for anti-BPDE¹⁷ although reaction also occurs at other bases, notably adenine.

As synthesis stops at the base preceding a guanine with AAAF-treated template DNA and at the base before the first of the two pyrimidine bases with UV-treated template DNA, it is clear that termination occurs without a nucleotide present at the site(s) opposite the lesion. It is possible that DNA polymerase I does add nucleotides in this position but that they are rapidly excised by the 3'-5' exonuclease activity of the enzyme due to lack of base pairing¹¹; however, our observation (data not shown) that a DNA polymerase α isolated from human lymphoblastoid cells¹⁸ and having no 3'-5' exonuclease activity gives the same band pattern with UV-irradiated template DNA

as does polI suggests that synthesis terminates due to the inability of the enzyme to incorporate nucleotides opposite a non-pairing lesion. We occasionally found additional slightly lighter bands one or even two bases before the main termination bands, for example, in Figs 2 and 3 at positions 15 and 34 with AAAF and at positions 31 and 32 with UV. These may result from degradation by either the 3'-5' exonuclease or residual 5'-3' exonuclease that contaminates some commercial batches of polI (Klenow). Degradation by the 5'-3' exonuclease is considered unlikely as treatment at 65 °C before addition of the restriction enzyme completely eliminated spurious bands on the sequence standards caused by this activity but did not affect the extra bands on the AAAF-reacted or UV-irradiated templates.

These experiments are essentially qualitative and do not show that DNA polymerase stops at every lesion; one cannot determine by this method the amount of 'read through'. However, we expect that the method will be useful to distinguish between lesions that are blocks to DNA synthesis and those that are not as well as elucidating the behaviour of different DNA polymerases on encountering a lesion and possible ways in which DNA synthesis may be able to bypass lesions¹⁹.

We thank Dr Geoffrey Grigg for the conversation during which these experiments were devised. The work was supported by an ACS institutional grant (IN-41-R-30) and from NIH (GM 07816-20).

PETER MOORE

BERNARD S. STRAUSS

Department of Microbiology,
The University of Chicago,
Chicago, Illinois 60637

Received 29 December 1978; accepted 23 February 1979.

- Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).
- Sanger, F. & Coulson, A. R. *J. molec. Biol.* **94**, 441-448 (1975).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Hatayama, T., Goldberg, I. H., Takeshita, M. & Grollman, A. P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3603-3607 (1978).
- D'Andrea, A. D. & Haseltine, W. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3608-3612 (1978).
- Rupp, W. P. & Howard-Flanders, P. J. *J. molec. Biol.* **31**, 291-304 (1968).
- Caillet-Fauquet, P., Defais, M. & Radman, M. *J. molec. Biol.* **117**, 95-112 (1977).
- Lehmann, A. R. *J. molec. Biol.* **66**, 319-337 (1972).
- D'Ambrosio, S. M. & Setlow, R. B. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2396-2400 (1976).
- Hsu, W. T., Lin, E. J. S., Harvey, R. G. & Weiss, S. B. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3335-3339 (1977).
- Villani, G., Boiteux, S. & Radman, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3037-3041 (1978).
- Sanger, F. et al. *Nature* **265**, 687-695 (1977).
- Sanger, F. et al. *J. molec. Biol.* **125**, 225-246 (1978).
- Krieg, E., Miller, J. A., Juhl, U. & Miller, E. C. *Biochemistry* **6**, 177-182 (1967).
- Krieg, E. *Biochim. biophys. Acta* **355**, 177-203 (1974).
- Setlow, R. B. & Carrier, W. L. *J. molec. Biol.* **17**, 237-254 (1966).
- Meehan, T., Straub, K. & Calvin, M. *Nature* **269**, 725-727 (1977).
- Bose, K., Karran, P. & Strauss, B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 794-798 (1978).
- Higgins, N. P., Kato, K. & Strauss, B. *J. molec. Biol.* **101**, 417-425 (1976).