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Action of intact AP (apurinic/apyrimidinic) sites and AP sites associated with breaks on the transcription of T7 coliphage DNA by *Escherichia coli* RNA polymerase

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The effect of apurinic/apyrimidinic (AP) sites in DNA on RNA and protein synthesis was studied *in vitro* using T7 coliphage DNA. Initiation of RNA synthesis by *Escherichia coli* RNA polymerase was synchronized and heparin was used to prevent reinitiation. When the T7 DNA contained AP sites, the rate of RNA synthesis was decreased but it remained higher than the values calculated on the assumption that an AP site in the transcribed strand is a complete block to the enzyme progression. Moreover, after the time taken by an unimpeded enzyme to go from promoter to terminator, the rate of RNA synthesis remained elevated and the number of complete RNA molecules (7000 nucleotides) continued to increase for some time. These results suggest that, if the *E. coli* RNA polymerase is stopped by an AP site, most often, after a pause, the enzyme resumes elongation of the RNA chain which is continuous over the AP site. Sometimes however, RNA synthesis is definitively interrupted during the pause; the probability of interruption has been estimated to be 0.3 in our experimental conditions. When a nick is placed 5′ to the AP site by an AP endonuclease, the results are similar: most often, the RNA chain is synthesized without interruption past the nick in the template strand. The pause of the *E. coli* RNA polymerase at this combined lesion appears to be shorter than when the AP site is intact. To investigate whether a nucleotide is placed in the RNA chain in front of the AP site in the template strand by *E. coli* RNA polymerase, RNA synthesis was taken to completion before using this RNA for protein synthesis and measuring the activity of gene-1 product, T7 RNA polymerase. The result suggests that, after pausing, the *E. coli* RNA polymerase places a nucleotide in the RNA chain when passing over an AP site. The mechanism of the delayed lethality of T7 coliphages treated with monofunctional alkylating agents, which is due to the appearance of AP sites, is discussed.

The major damage to cellular DNA is the loss of bases leading to AP sites. The loss may be spontaneous (Lindahl & Nyberg, 1972; Lindahl & Kalstrom, 1973); it is enhanced by chemicals like alkylating agents (Lawley & Brookes, 1963) or by physical causes like ionizing radiations (Dunlap & Cerutti, 1975); AP sites are also the result of the activity of DNA glycosylases which remove modified bases (Lindahl, 1976).

AP sites are toxic or lethal; the mechanism of this toxicity is completely unknown. The most suggestive work on this subject has been carried out on bacteriophages. Brakier & Verly (1970), studying the delayed inactivation of coliphage T7 by methyl methanesulphonate, made the suggestion that an AP site in DNA should lead to the synthesis of an altered RNA incapable of directing the synthesis of a functional protein.

The transcription of acid-depurinated T7 DNA by *Escherichia coli* RNA polymerase has been studied by Mamet-Bratley (1974). She found that AP sites decrease the rate of RNA synthesis, the most likely explanation being that the AP site blocks elongation. The AP sites are not used to

Abbreviations used: AP, apurinic or apyrimidinic; T7, coliphage T7.

initiate RNA synthesis, which remains totally asymmetric.

In the present work, we have found that most often the *E. coli* RNA polymerase is not definitively arrested at an AP site; usually, the AP site is only responsible for a pause, after which the enzyme resumes elongation of the RNA chain until it reaches the terminator: there is no interruption of the synthesized RNA. On the other hand, we present data suggesting that the RNA polymerase places a nucleotide in the RNA when it crosses the AP site.

Materials and methods

Preparation of untreated and depurinated T7 DNA

DNA from coliphage T7 labelled or not with ^3H in the thymine residues was prepared according to Verly *et al.* (1974); it was kept in 10mM-Tris/HCl, pH7.9, at a concentration of 0.6mg/ml. To depurinate this DNA while avoiding partial denaturation (partial denaturation dramatically stops RNA elongation so that RNA polymerase produces mostly small RNA fragments), to 1ml of DNA solution were added 50μl of 2M-NaCl and 25μl of 1M-sodium acetate, pH4.0. The incubation was carried out at 37°C; 30min were needed to produce 1 AP site/T7 DNA strand (39936 nucleotides). The depurination was stopped with 220μl of 1M-potassium phosphate, pH8.6, which brought the final pH to 6.6. The solution was then dialysed against 3vol. of 50mM-KCl/10mM-Tris/HCl, pH7.9.

The unlabelled T7 DNA was used in the transcription and translation experiments; the labelled T7 DNA, prepared in parallel, was used to count the number of strand breaks and AP sites.

Treatment with the AP endonuclease

The AP endonuclease (EC 3.1.25.2) was prepared from rat liver; the enzyme hydrolyses the phosphodiester bond 5′ to the AP site leaving 3′-OH and 5′-phosphate ends (César & Verly, 1983). The unit has been defined by Verly (1980). The preparation was devoid of non-specific endonuclease activities.

To 1ml of depurinated DNA (labelled or not) solution was added 50μl of AP endonuclease solution (30units) and the mixture was dialysed at 4°C against 0.15M-NaCl/15mM-sodium citrate/10mM-$MgCl_2$, pH7.0. The dialysis bag was then immersed in the same buffer kept at 37°C for 2h. The solution was finally dialysed at 4°C against 50mM-KCl/10mM-Tris/HCl, pH7.9.

The DNA concentration of the solutions was calculated from absorbance measurements at 260 and 280nm.

Determination of the number of strand breaks and AP sites

The determination of the number of single-strand breaks and intact AP sites (i.e. AP sites not associated with breaks) in T7 DNA strands has been described by Crine & Verly (1976). The [^3H]DNA is denatured by treatment with formamide which leaves the AP sites intact (Strauss & Robins, 1968), or by treatment with NaOH which places a break near each AP site (Tamm *et al.*, 1953) before layering the solution on top of a neutral 5–20% sucrose gradient. After centrifugation, the average number of breaks/T7 DNA strand is calculated with a computer from the sedimentation profile. The breaks found after formamide denaturation were already present in the native DNA. The breaks found after NaOH denaturation are the pre-existing breaks and those added near AP sites. The difference between the number of breaks determined after NaOH denaturation and after formamide denaturation equals the number of intact AP sites in the native T7 DNA.

E. coli RNA polymerase

Escherichia coli Q13 was chosen because it is deficient in RNAase I and has a decreased polynucleotide phosphorylase activity (Reiner, 1969). The RNA polymerase (EC 2.7.7.6) was prepared following the method of Burgess & Jendrisak (1975). Holoenzyme and core enzyme were separated by chromatography on phosphocellulose (Gonzalez *et al.*, 1977). The holoenzyme was pure as judged by poly-acrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate: Coomassie Blue revealed only the bands of the enzyme subunits. The σ subunit was present in 95% of the molecules in the holoenzyme preparation as shown by the challenge assay of Mangel & Chamberlin (1974). The RNA polymerase concentration was calculated from the A_{280} using $A^{1\%}_{1cm} = 6.5$ (Burgess, 1976), whereas the concentration of active molecules was deduced from the RNA molecules synthesized in the presence of an excess of T7 DNA and rifampicin to prevent reinitiation; the ratio of the two values indicated that 60% of the enzyme molecules were active.

The absence of RNAase from the holoenzyme preparation was shown in the following way. T7 mRNA was synthesized as described in the next section in the presence of rifampicin to prevent reinitiation, and the incubation was then continued for 2h more; there was no difference between the sedimentation profiles of the T7 RNA taken before and after this additional incubation. Neither could exoribonuclease activity be demonstrated; labelled T7 RNA incubated with the

holoenzyme preparation did not give radioactive molecules soluble in 5% trichloroacetic acid.

The holoenzyme was stored at −20°C in the elution buffer from the phosphocellulose chromatography [50mM-NaCl/10mM-Tris/HCl/10mM-$MgCl_2$/10mM-2-mercaptoethanol/0.1mM-EDTA/0.01% bovine serum albumin (pH7.9) containing 50% (v/v) glycerol]. Before use, it was diluted by steps with the same buffer containing 5% glycerol.

T7 RNA synthesis

When the *E. coli* RNA polymerase/T7 DNA ratio is not higher than 3, initiation of transcription occurs only at the three major promoters placed very close to one another at the left end of the T7 genome map (Stahl & Chamberlin, 1977). A termination signal is located at 19% of this map; transcription from the major promoters to the terminator gives RNA containing an average of 7000 nucleotides (Dunn & Studier, 1983). When the T7 DNA is preincubated with the RNA polymerase, initiation starts almost immediately on addition of the four nucleoside triphosphates and, under optimal conditions, the enzyme takes 7min at 30°C to travel from promoter to terminator. RNA appears to be synthesized at a constant rate although it is known that individual RNA polymerase molecules do not travel at a constant speed; slowing down, and even pauses, are observed at specific places along the genome (Kassavetis & Chamberlin, 1981), but some dyssynchrony in the progression of the enzyme molecules is sufficient to obscure the pausing phenomenon. The terminator is not 100% efficient; some RNA polymerase molecules do not stop and transcribe the DNA past the terminator (Chamberlin *et al.*, 1979).

Kinetic studies. The chosen conditions were adapted from those recommended by Herrlich & Schweiger (1974).

T7 DNA (0.96pmol) and *E. coli* RNA polymerase holoenzyme (2.15pmol) in 60μl of 40mM-Tris/HCl/8mM-$MgCl_2$/10mM 2-mercaptoethanol/50mM-KCl/0.2% bovine serum albumin, pH7.9 (preincubation buffer), were preincubated at 30°C for 10min to allow the formation of the preinitiation binary complex. The four nucleoside triphosphates (final concentrations 0.4mM for ATP, GTP and CTP, and 0.1mM for labelled UTP), heparin (final concentration 0.5mg/ml), spermidine (final concentration 8mM) and KCl (final concentration 200mM) in water were added bringing the total volume to 100μl. The UTP was labelled with ^3H in the uracil moiety (25μCi/mmol). Heparin was used to prevent reinitiation (Walter *et al.*, 1967). Spermidine is known to improve transcription (So *et al.*, 1967). The initial low ionic strength facilitated the formation of the DNA–RNA polymerase preinitiation binary complex (Strauss *et al.*, 1980) whereas the increased KCl concentration made the enzyme release at the terminator easier (Schäfer & Zillig, 1973).

The incubation was at 30°C to slow down the enzyme progression and determine more experimental points; at appropriate intervals, 5μl aliquots were taken, placed onto GF/C Whatman glass fibre filters which were immersed in ice-cold 5% (w/v) trichloroacetic acid containing 10mM-sodium pyrophosphate. After 10min, each filter was placed on a sintered glass funnel and rinsed 5 times with 5ml portions of 2% trichloroacetic acid/10mM-sodium pyrophosphate, and finally with 5ml of ethanol. The filters were dried at 80°C for 20min in an oven, then treated with 0.5ml of 1M-HCl for 1h also at 80°C to digest the nucleic acids. After addition of a scintillation solution, they were counted for radioactivity.

Preparation of T7 RNA. T7 DNA (1.4pmol) and RNA polymerase holoenzyme (3pmol) were preincubated before addition of the four nucleoside triphosphates together with heparin. The procedure was the same as above except that UTP was labelled with ^{32}P in the α position (125mCi/mmol) and that the incubation was carried out at 37°C for 10, 20 or 30min. The reaction was stopped by dipping the tubes in ice.

Analysis of the T7 RNA. The unincorporated nucleoside triphosphates were removed by Sephadex G-50 gel filtration. The nucleic acids were precipitated with ethanol, then dissolved in 200μl of 0.15M-NaCl/0.015M-EDTA, pH7.0.

Of this solution, 100μl was layered on top of a 5–20% sucrose gradient in 1M-NaCl/10mM-Tris/HCl/10mM-EDTA, pH7.0, in a 5ml tube. After 120min at 45000rev./min in the SW50.1 rotor of a Beckman L5 centrifuge at 20°C, the tubes were emptied from the top and the radioactivity of the collected 100μl fractions was determined.

Preparation of T7 early mRNA for the purpose of translation

T7 DNA [0.55pmol in 50μl of 10mM-Tris/HCl (pH7.9)/1mM-EDTA/50mM-KCl] and RNA polymerase holoenzyme [4.3pmol in 10μl of 10mM-Tris/HCl (pH7.9)/0.1mM-EDTA/50mM-NaCl/10mM-$MgCl_2$/10mM-2-mercaptoethanol/0.01% bovine serum albumin/5% (v/v) glycerol] were added at 0°C to 40μl of a stock solution of ribonucleoside triphosphates to give the following final concentrations: 0.4mM-ATP, 0.4mM-GTP, 0.4mM-CTP, 0.1mM-UTP, 40mM-Tris/HCl (pH7.9) 200mM-KCl, 8mM-$MgCl_2$, 10mM-2-mercaptoethanol, 8mM-spermidine and 0.2% bovine serum albumin. The tube containing this mixture was placed in a 37°C waterbath and incubated for 2min before addition of rifampicin

to 100 μg/ml; the incubation was continued for another 30 min before the tube was dipped into ice.

In this procedure, the molar ratio enzyme: T7 DNA is greater than 3. However, practically all RNA synthesis is initiated at the three major promoters. The same promoter can be used several times for initiation during the 2 min before addition of rifampicin (Stahl & Chamberlin, 1977). The synchrony of RNA synthesis is lost, but many more RNA molecules can be prepared for the translation step. Parallel experiments using [^{3}H]UTP have shown that, even with the depurinated DNA templates, RNA synthesis had completely ceased at 30 min.

Translation of T7 early RNA

The method of Herrlich & Schweiger (1974) was followed. Ribosomes and fraction S200 were prepared from *E. coli* Q13. Fraction S200 was purified on DEAE-cellulose to remove most of the nucleic acids and part of the nucleases. The concentrations of the ribosome and S200 preparations were determined by absorbance measurements at 260 and 280 nm. Portions of both preparations were frozen in liquid N_2 and stored at -20°C.

To 0, 5, 10, 15, 20 or 25 μl of incubated transcription mixture (see the preceding section) was added the volume of non-incubated transcription mixture without RNA polymerase necessary to reach 25 μl. Then was added 25 μl of translation mixture, pH 7.9, containing 80 mM-KCl, 50 mM-Tris/HCl, 3.2 mM-spermidine, 80 mM-$ZnCl_2$, 0.1 mM-tetrahydrofolic acid, 0.16 mM-GTP, 0.16 mM-CTP, 1.6 mM-ATP, 40 mM-phosphoenolpyruvate, 15 units of pyruvate kinase/ml, 30 μg of rifampicin/ml, 0.8 mg of bovine serum albumin/ml, 2 mM-dithiothreitol, 0.32 mM of each of the 20 amino acids, 50 A_{260} units of ribosomes/ml, 3.3 mg of S200 protein/ml, 0.5 mg of tRNA/ml, 8 mM-$MgCl_2$ {optimum concentration determined experimentally using poly(U) and [^{3}H]phenylalanine}. The six tubes containing these solutions were incubated at 37°C for 8 min.

Assay for T7 RNA polymerase

T7 RNA polymerase coded by gene 1 is one of the translation products of T7 early mRNA. This RNA polymerase, which is not inhibited by rifampicin, transcribes the T7 DNA late genes.

To each tube containing the translation mixture (see the preceding section) were added 50 μl of the following solution: 80 mM-phosphoenolpyruvate, 2 mM-dithiothreitol, 2 mg of bovine serum albumin/ml, 100 mM-$MgCl_2$, 50 μg of normal T7 DNA/ml and 20 μM-[^{3}H]UTP (20 mCi/ml). The tubes were incubated at 37°C for 10 min and the radioactive RNA synthesized was measured as described before.

The radioactivity of the synthesized RNA was plotted against the volume of transcription mixture used in the translation step. The slope of the best straight line passing through the experimental points was taken as a measure of the T7 RNA polymerase activity.

Effect of randomly distributed elongation blocks on the amount of RNA synthesized: a mathematical analysis

Initiation of RNA synthesis is synchronized and reinitiation is prevented.

If the average number of elongation blocks in the DNA segment from promoter to terminator (7000 nucleotide pairs) is n, the probability p that the RNA polymerase will be stopped at any nucleotide is $n/7000$. The ratio y of the amount of RNA synthesized in the presence of these blocks to that synthesized in their absence, as a function of the number x of nucleotides added to the growing RNA chain by an unimpeded enzyme molecule, is given by the following equation:

$$y = \frac{1}{x} \cdot \int_{m=0}^{m=x} e^{-pm} \cdot dm = \frac{1}{px} \cdot (1 - e^{-px})$$

Parameter x is readily converted into time units: if the unimpeded enzyme takes t min to travel from promoter to terminator, $tx/7000$ min are necessary to add x nucleotides to the growing RNA chain.

Experiments and results

Transcription of depurinated T7 DNA

Action of AP sites on the rate of RNA synthesis. T7 DNA (0.7 pmol) containing an average of 0, 1.28, 2.30 or 3.10 AP sites/strand between the major promoters and the terminator, and *E. coli* RNA polymerase holoenzyme (1.8 pmol) were preincubated for 10 min at 30°C. After addition of heparin and the nucleoside triphosphates containing labelled UTP, the incubation was continued at the same temperature and aliquots were taken at various intervals to measure the radioactivity incorporated in RNA (for details, see the Materials and methods section).

Fig. 1(*a*) shows that, with normal T7 DNA (0 AP sites, situation 1), the radioactivity incorporation into RNA was a linear function of time with a change of slope at 15 min; this indicates that the RNA polymerase took 15 min to travel from promoter to terminator and that about 15% of the enzyme molecules were not stopped at the terminator. When AP sites were present, the RNA synthesis was decreased and there was no abrupt change of slope at 15 min. The first experimental

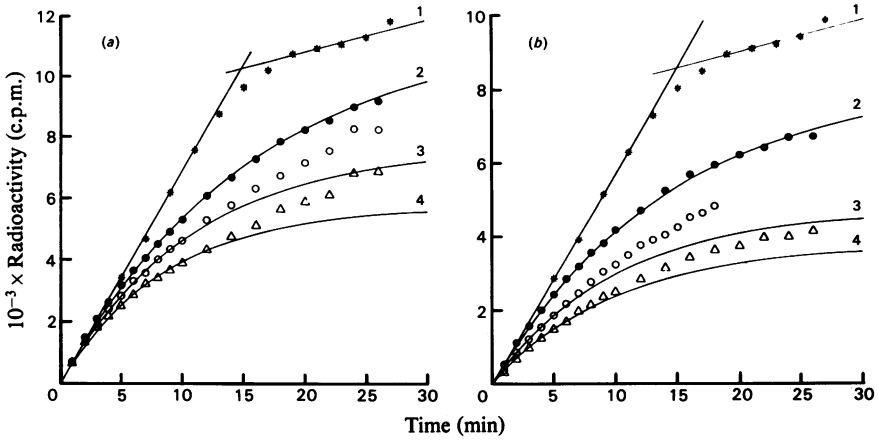

Fig. 1. *Action of intact AP sites and AP sites associated with breaks on T7 RNA synthesis*
(*a*) T7 DNA containing an average of 0 AP site (1), 1.28 AP sites (2), 2.30 AP sites (3) or 3.10 AP sites (4) per strand between the major promoters and the terminator were preincubated with *E. coli* RNA polymerase holoenzyme before addition of ATP, GTP, CTP and [^{3}H]UTP, together with heparin (0 min). (*b*) The conditions were the same as in (*a*), except that the depurinated T7 DNA has been incubated with rat-liver AP endonuclease to place a break near each AP site. Incubations were carried out at 30°C and samples taken at different times (min) to measure the radioactivity incorporated into RNA (c.p.m.). The theoretical curves were drawn for an average of 0.8, 1.2 and 1.5 complete stops [(*a*): 2, 3 and 4, respectively], and 0.9, 1.2 and 1.3 complete stops [(*b*): 2, 3 and 4, respectively] between the major promoters and the terminator, and a terminator of 0% efficiency (continuous lines).

points, which were not aligned linearly, could always be fitted with a theoretical curve calculated on the assumption that complete blocks prevented the progression of the polymerase. The average number of those randomly distributed hypothetical blocks per DNA segment from promoter to terminator is recorded in the legend of Fig. 1; it is lower than the average number of intact AP sites in one strand of the same segment for each situation. Fig. 1(*a*) shows that the fitting is very good for situation 2 even after 15 min if the model supposes that there is no terminator. For situations 3 and 4, the fitting is correct only for the first 10 min; thereafter the experimental points are above the theoretical curves which suppose that there is no terminator.

These results could be explained if the AP sites were not complete blocks to the progression of *E. coli* RNA polymerase molecules, but if the enzyme would rather make a pause at the AP sites before resuming the RNA chain elongation. This hypothesis is in agreement with the observation that the AP site frequency in DNA is higher than the frequency of hypothetical blocks used to draw the curves fitting the experimental points during the first minutes of RNA synthesis (see legend of Fig. 1). The hypothesis also explains why RNA synthesis remained so high after the time needed by the unimpeded enzyme molecules to reach the terminator: the RNA synthesis by the retarded enzyme molecules would add to the RNA synthesis by the molecules that did not meet an AP site but were not stopped at the termination signal.

The experiment was repeated after nicking the DNA near all AP sites with an AP endonuclease from rat liver. Fig. 1(*b*) shows that the results were essentially the same whether the AP sites were intact or associated with strand breaks.

Action of AP sites on the length of the synthesized RNA. Intact T7 DNA was incubated at 37°C with *E. coli* RNA polymerase holoenzyme and the RNA synthesized after 10 min was analysed by sedimentation on a 5–20% sucrose gradient (Fig. 2*a*). An incubation was carried out in parallel with T7 DNA containing 3.10 intact AP sites/strand in the promoter–terminator segment; the RNA synthesized after 10, 20 and 30 min was analysed in a similar way (Fig. 2*b*). The peak of complete RNA molecules (about 7000 nucleotides) is centred on fraction 34. There are a few longer molecules because the terminator is not 100% efficient.

If one makes the hypothesis that an AP site in the transcribed strand definitively stops the RNA polymerase, the fraction of complete RNA molecules (7000 nucleotides) should be only 4.5% (numbers) and the fraction of synthesized RNA in complete molecules 15% (mass). Fig. 2(*b*) shows that, after 30 min, the peak of complete RNA molecules is about 40% of the total (mass). Although the unimpeded enzyme takes only 5 min at 37°C to go from promoter to terminator, there is an increase of the fraction of complete RNA

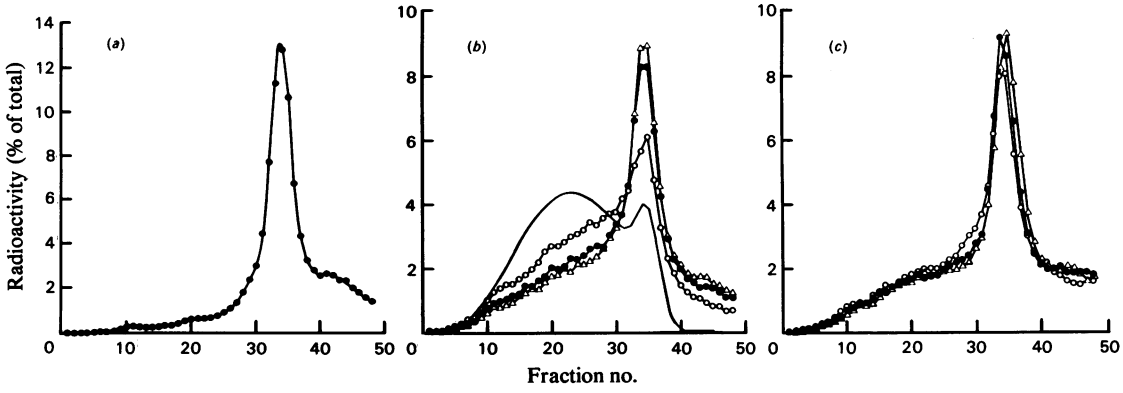

Fig. 2. *Length of RNA synthesized in the presence of intact AP sites or AP sites associated with single-strand breaks*
The RNAs were analysed by sedimentation on sucrose gradients and the radioactivity in the collected fractions (% of total) plotted against the sedimented distance (fraction number). (*a*) T7 DNA and RNA polymerase holoenzyme were preincubated before addition of ATP, GTP, CTP, [α-^{32}P]UTP, and heparin, followed by a 10min incubation at 37°C. (*b*) T7 DNA containing an average of 3.1 AP sites/strand in the promoter–terminator segment was preincubated with the RNA polymerase holoenzyme before addition of ATP, GTP, CTP, [α-^{32}P]UTP, and heparin. Samples were taken after 10min (○), 20min (●) and 30min (△) of incubation at 37°C. The line not joining experimental points represents the theoretical situation corresponding to an average of 3.5 complete stops in the promoter–terminator DNA segment. (*c*) Same as in (*b*) except that the depurinated T7 DNA had been submitted to rat-liver AP endonuclease to place a break near each AP site.

molecules between 10 and 30min and a decrease of the fraction of incomplete RNA molecules. All these observations are in agreement with the hypothesis that, in most cases, the RNA polymerase pauses for a time when it meets an AP site on the transcribed strand, but usually it can subsequently resume elongation to finally reach the terminator and give a complete, uninterrupted, RNA molecule of 7000 nucleotides.

Some initiated RNA molecules are however never completed. Even when T7 DNA does not contain AP sites, the sedimentation profile of the RNA synthesized for a time longer than the 5min needed at 37°C for the enzyme molecule to go from promoter to terminator shows a tail of unfinished RNA molecules (Fig. 2*a*); the experimental profile corresponds to the theoretical curve calculated for an average of 0.4 complete stops in the promoter–terminator segment. The tail of unfinished RNA molecules is more prominent when the T7 DNA contains AP sites (Fig. 2*b*) and it is stabilized after 20min when RNA synthesis is completely over. The tail after 30 min of RNA synthesis on a T7 DNA with an average of 3.1 AP sites between promoter and terminator is however much smaller than in the theoretical curve calculated for an average of 3.5 complete stops (0.4 for the background + 3.1 for the AP sites); it fits a theoretical curve calculated for an average of 1.4 complete stops in the promoter–terminator segment. Subtracting the background value, we come to the conclusion that 3.1 AP sites in the transcribed strand seem to be responsible for 1.0 stop, which means that the probability that *E. coli* RNA polymerase will interrupt elongation at an AP site is about 0.3 in our experimental conditions.

The sucrose-gradient analysis was also done on RNA synthesized on a depurinated T7 DNA template after nicking near the AP sites with an AP endonuclease. The results (Fig. 2*c*) are analogous to those obtained when the AP sites are intact (Fig. 2*b*), except that, after 10min, the fraction of complete RNA molecules is higher when there is a nick near each AP site, and that the difference between 10 and 30min is smaller. The conclusion is that the nick near the AP site does not prevent the RNA polymerase from continuing, without interruption, the synthesis of the RNA chain; moreover, it seems that the nick shortens the pause of the enzyme in front of an AP site.

Translation of the RNA synthesized on a T7 DNA template containing AP sites

Unlabelled RNA was synthesized by *E. coli* RNA polymerase during 30min at 37°C using T7 DNA containing AP sites as described in the Materials and methods section; rifampicin was used to prevent reinitiation (first transcription; stage 1). In a parallel experiment, [^{3}H]UTP was used to follow the synthesis of RNA; it had stopped at 30min.

Aliquots of 0, 5, 10, 15, 20 or 25μl of the preceding mixture containing unlabelled T7 early

Table 1. *AP sites in T7 DNA and activity of T7 RNA polymerase*

Four different T7 DNAs, containing 0, 1.28, 2.30 or 3.10 intact AP sites/strand in the promoter–terminator segment, were used for transcription by *E. coli* RNA polymerase followed by translation; the activity of T7 RNA polymerase was then assayed and the results are expressed as percentages of the activity observed when the T7 DNA was without AP sites. The theoretical values are calculated assuming that the probability that an AP site will block elongation is 0.3 and that an AP site in the transcribed strand of T7 gene 1 either is sufficient to have an inactive T7 RNA polymerase (*a*) or has no influence on the activity of the T7 RNA polymerase when transcription by *E. coli* RNA polymerase is not interrupted (*b*).

	AP sites/strand in promoter–terminator . . .	T7 RNA polymerase activity 1.28	2.30	3.10
Theoretical	(*a*)	55	34	24
	(*b*)	77	63	54
Measured		73	67	51

mRNA and all the factors necessary for translation (see the Materials and methods section) were incubated at 37°C for 8 min (translation; stage 2).

Intact T7 DNA was then added together with [^{3}H]UTP and the incubation was continued for another 10 min at 37°C (second transcription; stage 3) (for details, see the Materials and methods section). Because of the rifampicin, the only RNA synthesized during this third stage was T7 late mRNA made by T7 RNA polymerase coded by gene 1 of the T7 genome.

The radioactivity incorporated into RNA at stage 3 was measured and plotted as a function of the volume of stage 1 solution used at stage 2; the slope of the best straight line passing through the experimental points was taken as a measure of the T7 RNA polymerase activity. Table 1 gives the results of these experiments. Four different T7 DNAs were used containing respectively 0, 1.28, 2.30 and 3.10 AP sites/strand in the promoter–terminator segment. The results are given as percentages of the values obtained with T7 DNA not containing AP sites.

Discussion

AP sites in DNA do not usually stop RNA elongation by E. coli RNA polymerase

The results presented in this work show that an AP site in the transcribed DNA strand is usually not a final stop for the progression of *E. coli* RNA polymerase. The enzyme however pauses at an AP site. At the pause site, the probability that the RNA polymerase will interrupt the elongation of the synthesized RNA appears to be increased. But most enzyme molecules resume elongation, yielding an RNA that is continuous over the AP site, so that they have been only retarded. The pauses and the few unavoidable interruptions at AP sites are responsible for the decreased rate of RNA synthesis which was also observed by Mamet-Bratley (1974).

The results are not changed when a nick is placed 5′ to the AP sites with an AP endonuclease except that the pause in front of the AP site seems to be shorter.

When E. coli RNA polymerase crosses an AP site in the transcribed DNA strand, does it place a nucleotide in the RNA although the AP site is a non-coding lesion?

T7 DNA containing AP sites was transcribed by *E. coli* RNA polymerase for a time long enough so that the enzyme molecules retarded at AP sites could reach the terminator to give complete RNA molecules of 7000 nucleotides. Rifampicin was used to prevent reinitiation. As usual, the presence of AP sites decreased the amount of RNA synthesized (results not shown). We next studied the translation of gene-1 transcript present in these RNAs and measured the activity of the synthesized T7 RNA polymerase. The major promoters for *E. coli* RNA polymerase are clustered at 1.5% of the phage genome, but gene 1, which codes for the T7 RNA polymerase, starts only at 7.9% to end at 14.6%.

A first reason to have an inactive gene-1 product or no product at all is that *E. coli* RNA polymerase has interrupted transcription at an AP site in the transcribed T7 DNA strand before reaching the end of gene 1. The probability of interruption at an AP site was estimated to be about 0.3. When *E. coli* RNA polymerase is not prevented from reaching the end of gene 1, another reason to have an inactive T7 RNA polymerase is that an AP site within the transcribed strand of gene 1 may cause deletion or substitution of one nucleotide in the RNA transcript.

Making the hypothesis that the deletion and the resulting shift of the reading frame lead necessarily to an inactive protein, we have calculated the expected T7 RNA polymerase activity for various frequencies of AP sites in T7 DNA. The experimental results are far above the calculated values (Table 1). It is possible that the calculated values are too low since a frameshift leading to the change of only a few amino acids at the *C*-terminus of the protein might not suppress the T7 RNA polymerase activity; the enzyme might also have kept some

activity if only a few amino acids are missing because transcription did not proceed to the very end of gene 1. But the difference between experimental and calculated values seems too large to retain, as the only explanation, deletion of one nucleotide in the gene-1 transcript as the cause of the reduced enzyme activity.

On the other hand, the observed T7 RNA polymerase activity was about equal to the value calculated on the assumption that, when *E. coli* RNA polymerase transcribes the whole gene 1, the presence of an AP site in the transcribed strand of gene 1 does not decrease the T7 RNA polymerase activity. This result highly suggests that, after pausing, the *E. coli* RNA polymerase places a nucleotide in the RNA chain when passing over the AP site. If placing a nucleotide in front of the non-coding AP site sometimes leads to an error, it seems that the resulting base substitution has statistically little influence on the T7 RNA polymerase activity or, if it statistically decreases the enzyme activity, this just compensates for the shortened, but nevertheless active, protein molecules resulting from an incomplete gene 1 transcript.

It is worth comparing the behaviours of *E. coli* RNA polymerase and DNA polymerase I. Under stringent conditions, DNA polymerase I is stopped by an AP site in the template strand whereas, most often, the RNA polymerase makes only a pause before it resumes RNA chain elongation without interruption. Under relaxed conditions allowing the DNA polymerase to synthesize past an AP site in the template DNA strand, the enzyme places a nucleotide in front of the AP site, most often deoxyadenylic acid (Strauss *et al.*, 1982). It seems that RNA polymerase also places a nucleotide in the growing chain when passing an AP site, but we do not know whether it shows a preference for any particular one.

Are these results of any interest to understand better the mechanism of the delayed inactivation of T7 coliphage treated with monofunctional alkylating agents?

We recall that this delayed inactivation is due to depurination. Brakier & Verly (1970) found that, although the inactivation was a single-hit process, an average of 8 AP sites were needed to kill a phage particle. One interpretation is that the target in which the AP site must be placed is 1/8 of the phage genome. Brakier & Verly (1970) made the hypothesis that the target might be the transcribed strand of the genes coding for enzymes required for coliphage DNA replication. The idea was that an AP site was responsible for the synthesis of an abnormal RNA translated into a non-functional protein.

The experiments presented in this work show that blocking the transcription of T7 DNA early region by *E. coli* RNA polymerase cannot be the mechanism of AP site toxicity. Even if transcription is sometimes interrupted at an AP site, most often the *E. coli* enzyme resumes synthesis after a pause. There is thus only a decreased number of transcripts for each gene; this does not seem to be a cause of lethality.

Granting that there is a sufficient number of complete transcripts for each target gene, these transcripts might be abnormal so that translation leads to an inactive protein. But our results indicate that an AP site in gene 1 does not lead to an inactive T7 RNA polymerase. We must conclude that the hypothesis of Brakier & Verly (1970) does not explain the T7 coliphage delayed lethality.

It has always been a surprise that the logarithm of the number of surviving coliphages is a linear function of depurination without any shoulder indicative of DNA repair (Brakier & Verly, 1970), although we know that AP sites are repaired in *E. coli*. This would suggest that lethality depends on events that occur before the T7 DNA penetrates the host cell or on lesions that cannot be repaired. Karska-Wysocki *et al.* (1976) found that AP sites interfere with the injection of the T7 coliphage DNA into the *E. coli* cell, and they do not exclude the possibility that the injection defect might be the only cause of delayed inactivation. More recently, Mamet-Bratley *et al.* (1982) showed that it was the ejection of the DNA from the capsid which was incomplete; it is possible that the mechanism of delayed inactivation is mostly through the formation of protein–DNA cross-links within the virion. Another cause of AP site toxicity might be the formation of interstrand DNA crosslinks (Goffin & Verly, 1983) since the repair of these lesions seems to need recombination between two homologous DNA molecules (Yoakum & Cole, 1977).

A good understanding of the mechanisms of AP site toxicity for the T7 coliphage is important if one wishes to extrapolate some results to what happens in eukaryotic cells when nuclear or mitochondrial DNA loses bases.

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