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Completion of excision repair patches in human cell preparations: identification of a probable mode of excision and resynthesis

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Excision repair of u.v. damage in human fibroblasts is more sensitive to inhibitors of DNA polymerase α (cytosine arabinoside, aphidicolin) than to an inhibitor of polymerase β (dideoxythymidine), which indicates a greater role in repair for polymerase α than for polymerase β. These inhibitors all generate shortened patches with free 3′ termini; the detailed structure of these patches was investigated in permeable cells or isolated nuclei by degradation of DNA with exonuclease III and by resynthesis with DNA polymerase I (Klenow fragment) and T4 DNA ligase. The structure of the shortened patches appears to be a short stretch of DNA synthesized in the 5′→3′ direction within a longer single-strand gap. The single-strand gap ahead of the 3′ terminus can be bridged only by the combined action of polymerase and ligase. This structure implies that excision must involve removal of an oligonucleotide or widening of a gap by 5′→3′ exonuclease action to produce a single-strand region wide enough to be a substrate for polymerase α. There is no evidence for structures generated by nick translation or strand displacement.

Introduction

The mechanism of excision repair of u.v. damage in mammalian cells is poorly understood. Specific u.v. repair enzymes have not been characterized from any mammalian source despite several efforts (1–3), and the kinetics of pyrimidine dimer excision vary widely according to the assay system used (4–7). In view of such uncertainties, a useful approach for understanding repair has been to analyze the structures produced in intact cells when repair has been interrupted by inhibitors (8–14).

Several possible structural alterations of DNA during excision of u.v. damage have been proposed (Figure 1): (i) transient triple-strand regions may result from strand displacement during the 'patch and cut' mechanism (15); (ii) single-strand gaps may arise through nick translation by a mechanism resembling that of *Escherichia coli pol*1 (16); and (iii) single-strand gaps may be left when excision of an oligonucleotide has preceded repair synthesis according to an early 'cut and patch' model (17). The newly synthesized repair patches and the adjoining regions of single- and double-stranded DNA have distinct differences in these possible models. They should be distinguishable experimentally by using various enzymes that degrade them or use them as substrates for synthesis (Figure 1).

To test these models, a large number of incomplete repair patches were accumulated in intact cells by incubation with inhibitors of DNA polymerases α and β (aphidicolin, cytosine arabinoside, and dideoxythymidine) (18–21). The resulting structures presumably resemble transient intermediates that occur during normal repair; these alternative structures were analyzed by determining the various combinations of enzymes (DNA polymerase and ligase) required to complete the repair patches in isolated nuclei and permeable cells. To the extent that the assumptions are correct about the three possible alternative structures for repair sites (Figure 1), and the mode of action of polymerase and ligase *in vitro*, the experiments provided data that allowed discrimination between the structures and support a gap-filling mechanism for repair.

Materials and methods

Cell culture and radiolabeling

Normal human fibroblasts (HF1 and ANA-1, established in our laboratory, and GM2060 and GM316, from the Institute of Medical Research, Camden, NJ) were grown in Eagle's minimal essential medium with 10% fetal calf serum. The results obtained with each cell line were indistinguishable and are therefore pooled in this report. To obtain uniformly labeled DNA, cultures were initially incubated for at least 3 days with 0.01 μCi/ml [^{14}C]thymidine ([^{14}C]dThd)* until they were confluent. Cultures were then rinsed and grown in unlabeled medium for 1 h or more to empty [^{14}C]dThd pools, followed by exposure to 2 mM hydroxyurea for 1 h to suppress any residual semiconservative replication.

Irradiation

Cultures were irradiated with 13 J/m² u.v. light (254 nm, 1.3 J/m²/s) and incubated with [^{3}H]dThd (20 μCi/ml, 80 Ci/mmol), 2 mM hydroxyurea, and either 10 μM aphidicolin, 25 μM cytosine arabinoside, or 10^{-4} M dideoxythymidine for 4 h. Previous studies have shown that, by this protocol, the number of repaired sites interrupted is optimal for each inhibitor, corresponding to ~85% of all repair events for aphidicolin and cytosine arabinoside (~5 single-strand breaks in 2 x 10^{8} daltons) (10). Hydroxyurea was used in combination with the other inhibitors because it increases the efficiency with which the others act to block a majority of the repair patches (8–11, 14, 19–21). At the end of the labeling period, some cultures were washed in phosphate-buffered saline, centrifuged, drained, and rapidly frozen in a methanol/dry ice bath and stored at −20°C until used for enzymatic studies. Other cultures were rinsed and grown in fresh medium for various periods before harvesting and freezing.

Preparation of permeable cells and nuclei

Two methods, one gentle and one vigorous, were used to prepare cells for enzymatic studies. Cells were first thawed rapidly in phosphate-buffered saline and recentrifuged, and either they were permeabilized with lysolecithin or the nuclei were isolated by hypotonic and detergent treatment. Cells were permeabilized by incubation in 80 mM KCl, 150 mM sucrose, and 35 mM Tris (pH 7.4) for 10 min in an ice bath. One-third volume of lysolecithin (1 mg/ml) in the same buffer was then mixed into the cell suspension, and after standing for 10 min, cells were centrifuged and promptly resuspended in the appropriate buffers for enzymatic treatment.

Nuclei were isolated by resuspending the cells in a hypotonic medium of 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris (pH 7.4) for 10 min on ice, followed by Dounce homogenization by 10 strokes of a tight-fitting pestle, then centrifugation and agitation with the same buffer containing 0.1% NP40, followed by one wash with the same buffer without detergent. After the final centrifugation, nuclei were resuspended in the appropriate buffer for enzymatic treatment. The DNA was isolated from some samples as previously described (8).

Enzymatic reaction conditions

Permeable cells or isolated nuclei were suspended at ~10^{5} cells/ml in a buffer containing all four deoxynucleotide triphosphates (100 μM), 2.5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris (pH 7.4), 10 mM dithiothreitol, and 10 mM β-

*Abbreviations: dThd, thymidine; *polI*, DNA polymerase I (Klenow fragment); *exo*III, exonuclease III.

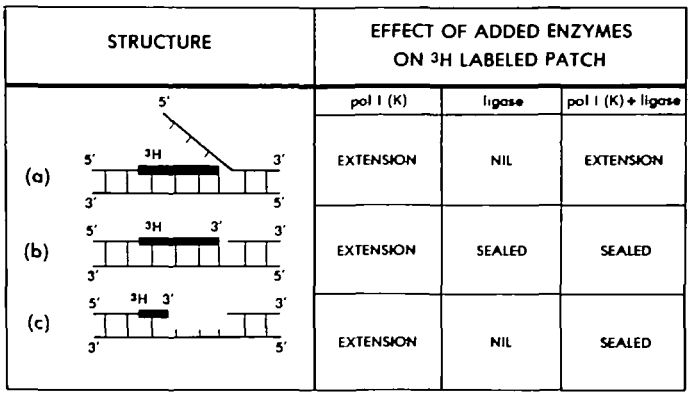

Fig. 1. Three possible intermediate structures for excision repair sites blocked by polymerase inhibitors, and the predicted consequences of *polI* and ligase action. Shaded block represents the [^{3}H]dThd incorporated during repair synthesis before inhibitors blocked further polymerization. (a) Repair is postulated to proceed by strand displacement producing a structure that cannot be sealed *in vitro* by either *polI*(K), ligase or a combination and repair ^{3}H will remain *exo*III sensitive. (b) Repair is postulated to proceed by nick translation producing a structure that has only a 3′-5′ single strand gap and which should be sealed by ligase or *polI*(K) plus ligase. (c) Repair is postulated to proceed by excision of an oligonucleotide to leave a gap that is subsequently filled in by a DNA polymerase. The gap will require both *polI*(K) and ligase to complete it and render the ^{3}H label resistant to *exo*III. Sizes of gaps, patches, etc. are shown schematically for simplicity and do not represent actual sizes.

mercaptoethanol, and dispersed in 100-μl aliquots. Depending on the experiment, aliquots were supplemented with either 1.4 units of DNA polymerase I (*polI*), or 2.5 units of T4 polynucleotide ligase, or both enzymes (Bethesda Research Laboratories, Gaithersberg, VA). The Klenow fragment of *polI* was chosen to restrict chain growth polymerization in single-strand regions and to avoid extensive nick translation. In some experiments nonradioactive TTP was replaced with [^{3}H]TTP (10 μCi/tube, 79 Ci/mmol). After incubation times of up to 30 min at 37°C, the cells were centrifuged; reactions wre stopped either by adding 1 ml 0.1 M NaOH followed by precipitation with 1 ml 0.1 M HCl, or by resuspending the nuclear pellet in 5 mM $MgCl_2$, 50 mM Tris (pH 7.4) with 25 units of exonuclease III (*exo*III) and incubating for 15 min at 37°C. Previous experiments have shown that under these conditions all the ^{3}H radioactivity at repaired sites that is sensitive to *exo*III has been completely solubilized (8–11). After *exo*III digestion, the fraction of ^{3}H radioactivity solubilized by *exo*III was determined as previously described (8).

Results

Accumulation of incomplete repair sites during growth with inhibitors

Polymerase inhibitors have been used extensively in studies of DNA repair because they prevent completion of repair patches and leave free 3′ termini that can be detected either as single-strand breaks or as sites sensitive to *exo*III (8). In the present studies, the ^{3}H label incorporated into DNA during repair of u.v. damage in the presence of inhibitors was rapidly digested. *Exo*III removed 60–70% of the ^{3}H label from both isolated nuclei and purified DNA within 10 min (Figure 2), without general breakdown of the bulk ^{14}C-labeled DNA. In subsequent experiments, therefore, a standard incubation time of 15 min was chosen for analysis of the amount of label associated with 3′ termini.

Both *exo*III sensitivity and single-strand break frequencies indicated that aphidicolin and cytosine arabinoside have similarly high efficiencies for blocking repair but that dideoxythymidine is relatively inefficient (Table I). The similarity in the two independent measures of inhibition of repair confirms that dideoxythymidine is truly inefficient at inhibiting repair, rather than blocking *exo*III digestion after being incorporated into DNA as a chain terminator that

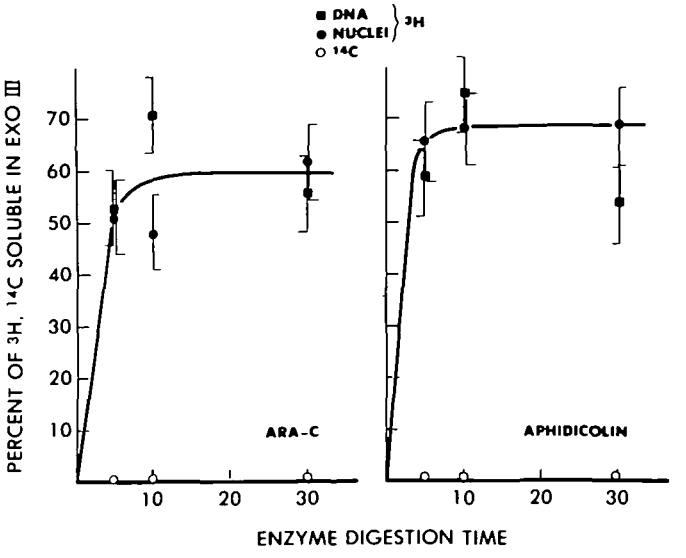

Fig. 2. Percentage of ^{3}H label in repair sites and uniformly distributed ^{14}C label rendered soluble by incubation with *exo*III. Purified DNA and nuclei were prepared from fibroblasts grown for 4 h after 13 J/m^2 u.v. light in 10 μCi/ml [^{3}H]dThd plus 2 mM hydroxyurea and 25 μM cytosine arabinoside (ARA-C) or 10 μM aphidicolin. ●, ^{3}H in nuclei; ■, ^{3}H in purified DNA; ○, ^{14}C label in nuclei. Mean and standard errors shown.

Table I. Relative frequencies of 3′ termini (*exo*III sensitive) and single-strand breaks in human fibroblasts incubated with various inhibitors for 4 h after 13 J/m^2 u.v. light

Inhibitor	*Exo*III-sensitive ^{3}H (%)	Single-strand breaks[a] (per control molecular weight of 1.6 x 10^8)
None	<3	Below resolution
Cytosine arabinoside	67.7 ± 0.9	5.46 ± 0.70
Aphidicolin	53.6 ± 4.3	6.45 ± 0.50
Dideoxythymidine	13.9 ± 1.5	2.26 ± 0.17

[a]From references 8,10.

presents a 3′H group instead of 3′OH.

Completion of repair sites in intact cells

The capacity of cells in culture to complete repair patches once inhibitors were removed was determined. Cells in which repair had been interrupted for 4 h by inhibitors were grown for various periods, and the fraction of ^{3}H incorporated into repair patches that remained at free 3′ termini was determined by *exo*III digestion. Repair patches interrupted by aphidicolin or cytosine arabinoside initially contained 60–70% *exo*III-sensitive ^{3}H label, whereas dideoxythymidine produced <20% *exo*III-sensitive label (Figure 3). Growth in fresh medium resulted in a rapid decrease in the fraction of [^{3}H]-dThd in repair sites that was sensitive to *exo*III, indicating completion and ligation of the repair patches. Interrupted patches accumulated by aphidicolin or dideoxythymidine were completed very rapidly, whereas those accumulated by cytosine arabinoside were completed more slowly. When expressed as a percentage of the initial amount of label associated with 3′ termini, data for aphidicolin and dideoxythymidine lay on the same curve whereas data for cytosine arabinoside indicated a much slower rate of sealing. It is known that aphidicolin acts as a competitive inhibitor of DNA polymerase α (18,20), whereas cytosine arabinoside blocks repair by actual incorporation into growing DNA

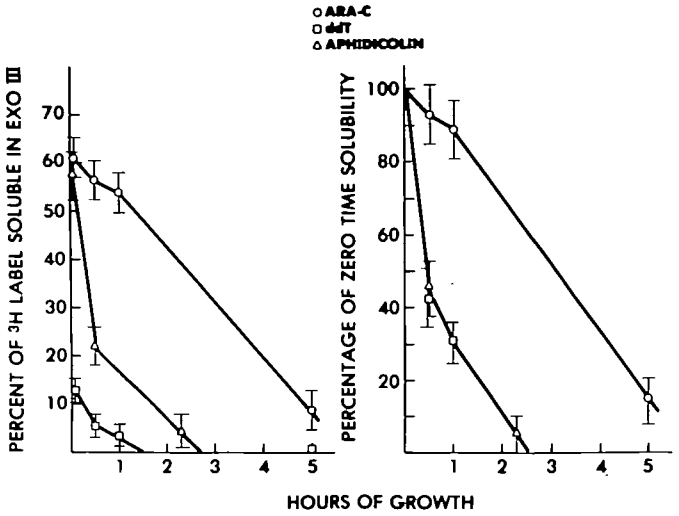

Fig. 3. Percentage of ^3H label rendered soluble in repair sites of nuclei by incubation with *exo*III. Cells were grown in fresh medium after first being irradiated with 13 J/m² u.v. light and incubated with inhibitors plus 2 mM hydroxyurea for 4 h. ○, 25 μM cytosine arabinoside; △, 10 μM aphidicolin; □, 100 μM dideoxythymidine. Left panel: actual *exo*III-soluble radioactivity as a percentage of the total ^3H incorporated into repair sites. Right panel: data normalized as a percentage of initial *exo*III-soluble fraction. Mean and standard error shown.

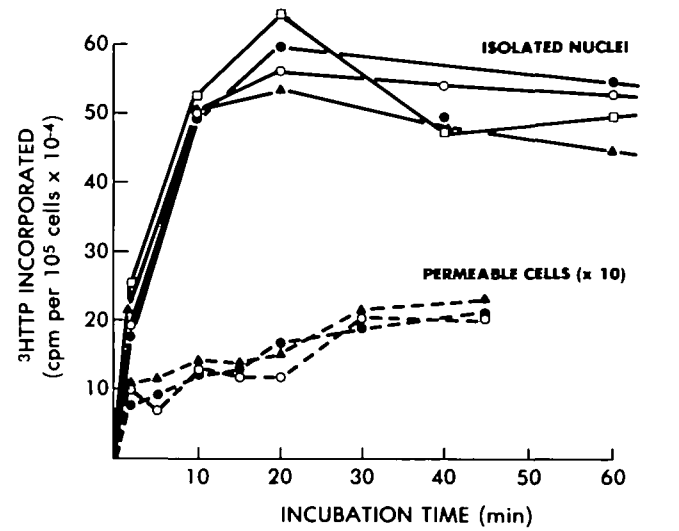

Fig. 4. ^3H radioactivity incorporated into isolated nuclei or permeable cells incubated with [^3H]TTP (100 μCi/ml, 79 Ci/mmol), 100 μM deoxynucleotide triphosphates, 1.4 units of DNA polymerase I (Klenow) and 2.5 mM ATP. ○, control; ●, 13 J/m² u.v. light and incubation for 4 h; ▲, 13 J/m² and incubation for 4 h with 25 μM cytosine arabinoside and 2 mM hydroxyurea; □, 13 J/m² and incubation for 4 h with 100 μM dideoxythymidine and 2 mM hydroxyurea. Solid lines shown for isolated nuclei; dashed lines shown for permeable cells, with counts x 10 for easier visualization.

chains (11,19,21). These kinetic data therefore suggest that dideoxythymidine may actually be acting as a rapidly reversible polymerase inhibitor similar to aphidicolin rather than being incorporated into the DNA as a chain terminator. Similar observations on the rate of reversal of inhibitor action based on single-strand break frequencies have been reported previously (9–11,19,21).

Primer sites of polI synthesis

Isolated nuclei and permeable cells were incubated with [^3H]-dTTP and *polI* to determine the extent of DNA synthesis under these experimental conditions and establish the time course of ^3H labeling. Nuclei showed a rapid initial phase of incorporation of radioactivity that reached a plateau after ~15–20 min; permeable cells showed much lower incorporation, by a factor of ~30. One set of measurements are shown and, in two repetitions of the experiment, incorporation reached a plateau at the same time although the absolute ^3H c.p.m./cell varied. No difference was observed between samples prepared from cells grown under various conditions after irradiation (Figure 4). Intact untreated cells would be expected to have very few single-strand gaps, in comparison to cells grown with inhibitors, that would be suitable substrates for *polI*. The observed incorporation therefore probably occurred at sites generated by the preparation procedures, which predominated over those generated before preparation. Several previous studies have shown that permeable and isolated nuclear systems accumulate single-strand breaks rapidly from mechanical forces and endogenous nuclease action, even though the absolute yield can be diminished with high ATP concentrations (22–24). These incorporation kinetics serve to define the time course of DNA synthesis in nuclei, but because of the extensive uptake at sites other than those of interest, the kinetics unfortunately cannot be used to estimate the number of bases incorporated at the interrupted repair sites. In subsequent experiments, attention was concentrated on the fate of ^3H label incorporated into repair patches before isolation of nuclei. Therefore, the presence of a large number of extraneous sites of *polI*(K) polymerization with unlabeled triphosphates will not affect polymerization at repair sites so long as enzymes are in excess.

Completion of repair sites in isolated nuclei

The conditions required for completion of interrupted repair patches in isolated nuclei were investigated by incubating nuclei with various enzymes and determining the extent to which the ^3H label that had been incorporated into the patches before isolation became resistant to *exo*III digestion. Incubation of nuclei without exogenous enzymes resulted in a slight decrease in *exo*III sensitivity for aphidicolin- and dideoxythymidine-treated nuclei, but no change for cytosine arabinoside-treated nuclei (Figures 5 and 6). Incubation with ligase or *polI* alone resulted in much smaller decreases in *exo*III sensitivity for all nuclei, although *polI* consistently caused the greater decrease (Figures 5 and 6).

The greatest decrease in *exo*III sensitivity – 60–70% reduction from the starting value – was achieved by combined incubation with *polI* and T4 ligase (Figures 5 and 6). The kinetic data are shown for individual experiments, and demonstrate that the activity of the various enzyme combinations in completing repair patches proceeds accordingly over the same time period at the [^3H]TTP incorporation with whole nuclei (Figure 4). To establish the precision of these results, a series of experiments were performed with nuclei incubated in *polI*, ligase, or both enzymes for a single time of 15 min at which the kinetic data (Figures 5 and 6) indicated the reactions to be complete (Table II). The results indicate that polymerase and ligase in concert, but not individually, were able to complete the majority of the repair patches that had been interrupted by aphidicolin or cytosine arabinoside before isolation of nuclei. It is difficult to be certain of the significance of the data for dideoxythymidine because the starting level of *exo*III sensitivity is so low, but with this inhibitor, also, both *polI* and ligase were needed for maximum completion of repair patches (Table II).

Low concentrations of ATP that were insufficient for DNA ligase resulted in diminished completion of repair patches (Table II). This indicates that the production of *exo*III-resistant ^{3}H-labeled repair sites is due to completion and ligation, not merely to extending the DNA chains by polymerase so that the ^{3}H label is far from the ends. Using longer *exo*III digestion times also did not increase the fraction of ^{3}H label digested.

The repair sites interrupted by cytosine arabinoside, aphidicolin, or dideoxythymidine were completed under essentially identical conditions in isolated nuclei, even though different relative kinetics were observed in intact cells in culture (Figure 3).

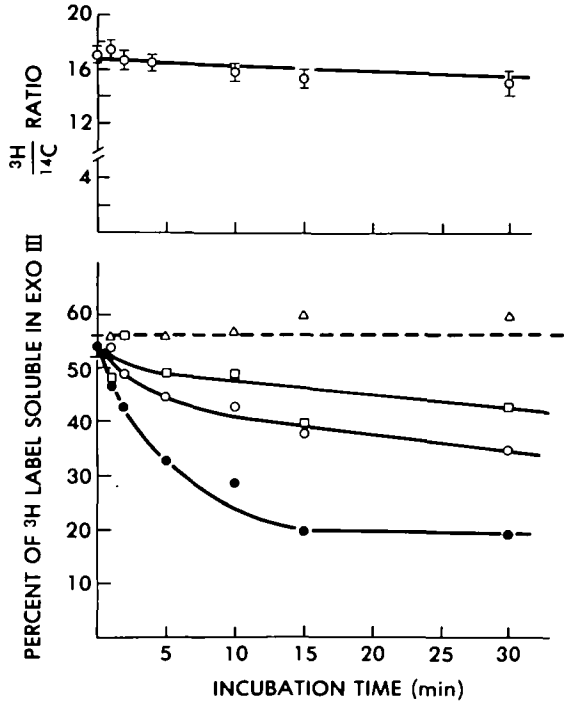

Fig. 5. Percentage of ^{3}H label rendered soluble in repair sites of nuclei by incubation with *exo*III as a function of the time of prior incubation with: ○, 1.4 units *pol*I; □, 10 units T4 ligase; ●, both enzymes; △, neither enzyme. Nuclei were prepared by growth for 4 h after 13 J/m^2 in 10 μCi/ml [^{3}H]dThd, 25 μM cytosine arabinoside, and 2 mM hydroxyurea. Top panel: specific activity (^{3}H/^{14}C) of DNA in nuclei as a function of prior incubation with or without *pol*I and ligase; values from all four conditions were pooled for mean and standard errors of mean.

Comparison of isolated nuclei and lysolecithin-permeabilized cells

Isolation of nuclei causes extensive disruption of cellular structure and loss of enzymes from nuclei. For comparison, therefore, a gentler method that made only the cell membranes permeable was used to prepare cells for incubation with *pol*I and ligase. This method gave results closely similar to those from isolated nuclei (Table III). Ligase was very ineffective at sealing the interrupted repair sites; polymerase and especially polymerase plus ligase were efficient (Table III).

Discussion

These experiments were designed to test which of three possible intermediates were produced when excision repair was blocked by polymerase inhibitors (Figure 1). Each of the

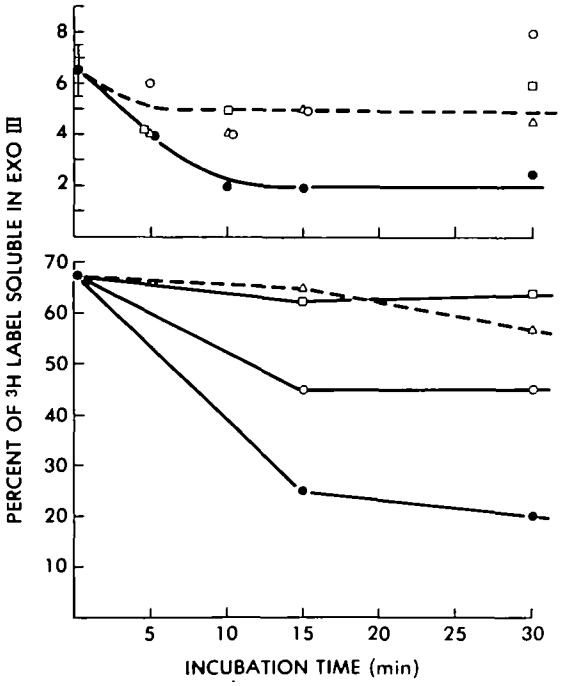

Fig. 6. Percentage of ^{3}H label rendered soluble in repair sites of nuclei by incubation with *exo*III as a function of the time of prior incubation with: ○, 1.4 units *pol*I; □, 10 units T4 ligase; ●, both enzymes; △, neither enzyme. Nuclei were prepared by growth for 4 h after 13 J/m^2 u.v. light, in 10 μCi/ml [^{3}H]dThd, and 2 mM hydroxyurea plus 100 μM dideoxythymidine (top) or 10 μM aphidicolin (bottom).

Table II. Completion of repair patches in isolated nuclei after digestion by *exo*III

Enzyme	Aphidicolin		Cytosine arabinoside		Dideoxythymidine	
	% ^{3}H soluble	% Repair sites completed	% ^{3}H soluble	% Repair sites completed	% ^{3}H soluble	% Repair sites completed
No incubation	67.7 ± 0.9		53.7 ± 4.3		13.9 ± 1.5	
None (37°C, 15 min)	65.0		65.3 ± 2.2		10.8 ± 1.3	
*Pol*I (Klenow)[a]	45.5	32	36.3 ± 5.3	40	8.0 ± 1.7	42
T4 ligase[a]	62.5	6	57.3 ± 1.6	5	6.7 ± 2.0	52
*Pol*I + T4 ligase[a]	25.5	62	24.8 ± 2.0	59	5.4 ± 2.1	61
*Pol*I + T4 ligase[b]	67.0	0	44.5	26	ND[c]	ND

[a]2.5 mM ATP.
[b]66 μM ATP.
[c]ND, not determined.

possible intermediates involves different mechanisms of excision, and the results may therefore shed light on how excision itself occurs. The results are most consistent with a gap-filling model; additional information can also be gleaned from the experimental results.

The following conclusions will be discussed individually: (i) many of the u.v. repair sites accumulated by growth with polymerase inhibitors have freely accessible 3′ termini, but the number is significantly less than the total number of repair sites (Figure 2); (ii) isolation of nuclei prepared with or without inhibitors generates substrates for *polI*, which were used up in ~20 min under our conditions (Figure 4); (iii) the intermediates accumulated in intact cells can be ligated by the cells once the inhibitors are removed, at rates characteristic of each inhibitor (Figure 3); and (iv) nuclei isolated from u.v.-irradiated cells grown with polymerase inhibitors contain repair intermediates that are poorly sealed with *polI* or ligase alone, but sealed extensively with both enzymes together (Figures 5 and 6).

Fraction of repaired sites containing free 3′ termini

*Exo*III, the main probe used throughout these studies, digests double-stranded DNA from 3′-OH termini in the 3′→5′ direction. *Exo*III also has endonucleolytic activity on apurinic sites, but by concentrating attention in these studies on the ^{3}H label incorporated at the ends of DNA strands by repair synthesis, endonucleolytic activity on occasional apurinic sites will be unimportant in contrast to the exonucleolytic activity on the ^{3}H labeled termini. The results showed that ~50–70% of ^{3}H label in repair patches blocked by cytosine arabinoside or aphidicolin was digested by *exo*III (Figure 2) whereas only 14% of the label from patches blocked by dideoxythymidine was digested (Table I). This difference could have been caused if the 3′ termini contained incorporated dideoxythymidine residues that would block *exo*III digestion. However, a similar low level of single-strand breaks from cells incubated with dideoxythymidine has been observed by means of alkaline sucrose gradients (8,10). It must be concluded that dideoxythymidine is a very poor inhibitor of repair either because of competition from endogenous TTP pools or because DNA polymerase β plays only a minor role in repair.

The reason that 100% of ^{3}H label in repaired sites cannot be rendered soluble by *exo*III has been discussed extensively (8–11) and is probably due to incomplete inhibition because of competing endogenous pools.

Substrates for polI in isolated nuclei and permeable cells

The nuclei and permeable cells used in these studies were unsuitable for discriminating between the number of primer sites generated for *polI* by the aborted repair sites and those generated by DNA breakage during preparation (Figure 4). The production of DNA damage during isolation has been reported before (24) and can also contribute importantly to incorporation by endogenous polymerases (22,23). Incorporation in the isolated nuclei and permeable cells proceeded to completion within ~20–30 min. This provided a time scale over which the effects of polymerase and ligase on the ^{3}H label incorporated into repair patches could be investigated.

Table III. Fraction of incomplete ^{3}H-labeled patches (accumulated by cytosine arabinoside) rendered *exo*III resistant by exogenous enzymes in 15 min, 37°C

Enzyme	Lysolecithin-permeabilized	Isolated nuclei
None	0	0
*Pol*I (Klenow)	0.53 ± 0.12	0.51 ± 0.18
T4 ligase	0.14 ± 0.05	0.13 ± 0.04
*Pol*I (Klenow) + T4 ligase	0.51 ± 0.09	0.67 ± 0.08

Completion of repair sites in intact cells

When cells in which ^{3}H label had been incorporated into repair patches in the presence of inhibitors were permitted to grow in fresh medium, the repair patches became sealed. This was observed as a decrease in the fraction of ^{3}H label remaining *exo*III sensitive (Figure 3). Repair sites accumulated with either aphidicolin or dideoxythymidine were sealed with half-times of ~30 min, but those accumulated with cytosine arabinoside had a half-time of several hours. Previous work has established that aphidicolin inhibits polymerase α competitively (18,20) and cytosine arabinoside causes chain termination through incorporation of several arabinose residues into DNA (19,25). The difference in the half-times observed during growth in fresh medium are therefore readily understood in terms of these two mechanisms. Rapid sealing occurs because aphidicolin is readily washed out of the cells, whereas sealing of cytosine arabinoside termini is slow because of inefficient addition of residues to arabinose termini and the lack of extensive 3′→5′ editing of the termini. Termini generated by dideoxythymidine and aphidicolin are sealed at a similar rapid rate, suggesting that, in intact cells, growth in dideoxythymidine may not inhibit repair by chain termination, as occurs during *in vitro* synthesis on purified DNA substrates (26). The small effect of dideoxythymidine, however, makes firm conclusions about its mechanism of action difficult to reach.

Completion of repair sites in isolated nuclei

The ^{3}H label incorporated into repair patches in intact cells during growth in inhibitors was, to a large extent, rendered resistant to *exo*III by completion of the patches with the combined action of *polI* and ligase (Figures 5 and 6). For aphidicolin and cytosine arabinoside, ~60% of the repair patches were completed when both enzymes were used, whereas only ~5–6% were completed with ligase alone and 30–40% with polymerase alone (Table II). *PolI* and T4 ligase appear insensitive to the arabinose in the termini that made the rate of sealing of cytosine arabinoside termini so low in intact cells. The 5′→3′ editing function which *polI* retains may permit removal of cytosine arabinoside-generated termini before rapid polymerization. For dideoxythymidine the individual enzymes were almost as efficient as both together, although the small fraction of repair sites actually available (i.e., the small starting fraction of *exo*III-sensitive ^{3}H) precludes detailed discussion of this inhibitor. These results imply that isolation of nuclei results in loss of most of the polymerase and ligase activity that seals the patches in intact cells (Figure 3). They are not completely absent, however, because limited repair polymerization and ligation occur in isolated nuclei (21).

The observation that *polI* and ligase individually seal a significant fraction of dideoxythymidine-generated repair patches (Figure 6) is probably due to there being few of these open patches, and small amounts of eukaryotic polymerase and ligase that remain in the nuclei cooperate with the added enzymes. The greater efficiency of *polI* over ligase on repair patches accumulated by cytosine arabinoside and aphidicolin (Table II) implies that a limited amount of endogenous

eukaryotic ligase may remain in nuclei and seal some patches after *pol*I action. Conversely, there must be much less endogenous eukaryotic polymerase remaining to fill in patches.

The structure of most of the repair patches accumulated by aphidicolin or cytosine arabinoside (i.e., at least 60% of them) must be of a form that can only be completed by both *pol*I and ligase (Figures 5 and 6). This structure is most likely, therefore, to be a limited patch inserted into a much longer, excised single-strand region (Figure 1c). Neither a strand displacement mode (Figure 1a) nor a nick translation mode (Figure 1b) satisfy the data adequately. Whether the failure of *pol*I plus ligase to seal all the patches is due to inefficiency of this cell preparation system, or to a significant fraction of patches having different structures, cannot be resolved at present. Some sites produced during growth in cytosine arabinoside are actually double-strand breaks from overlapping repair sites (27) and these would be irreparable by polymerase and ligase.

This proposed structure of repaired sites implies that the mechanism of excision is not the 'patch and cut' mode favored for so long because of the observed mechanism of *pol*I on u.v.-damaged DNA (15,16). Instead, excision repair in eukaryotic cells must involve either extensive exonuclease action to make a wide single-strand region before polymerization, or removal of an oligonucleotide considerably larger than the pyrimidine dimer, or a combination of both. The distinctive properties of eukaryotic polymerases (28,29) and the apparently greater role of polymerase α over β in repair (30,31; Table I) are consistent with this mechanism of excision. A gap resulting from excision would be a suitable substrate for DNA polymerase α, because this enzyme, unlike polymerase β, cannot function on nicked substrates (28,29). It would seem, therefore, that excision proceeds without intervention of DNA polymerase β until a large gap size of 10−20 bases is made in which polymerase α can begin polymerization. Once most of the gap is filled in by polymerase α, the patch may be completed by insertion of the final few bases with polymerase β and ligation. This sequential action of polymerases within an individual patch is consistent with their individual properties, and with observations based on the effect of combinations of inhibitors on repair (10,32).

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