Relative Importance of Incision and Polymerase Activities in Determining the Distribution of Damaged Sites That Are Mended in Xeroderma Pigmentosum Group C Cells

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ABSTRACT

Those pyrimidine dimers that are repaired in confluent xeroderma pigmentosum Group C cells are clustered together in the genome. Although the average level of repair in this complementation group is of the order of 25% of normal, this percentage represents normal levels of repair in one quarter of the genome and little repair in the remainder. The factors that regulate this clustering process have been investigated using inhibitors of the initial incision step of repair (novobiocin) and of the polymerization step (aphidicolin). Novobiocin at a concentration that permitted 30% of repair to continue reduced the clustering of mended sites only slightly. Aphidicolin, in contrast, at a concentration that permitted 30 to 60% of repair to continue caused the mended sites to be distributed randomly. The clustering of repair sites seen in xeroderma pigmentosum Group C cells, therefore, is produced by an excision repair mechanism in which an aphidicolin-sensitive DNA polymerase, presumably α, plays an important regulatory role in determining which damaged sites are mended.

INTRODUCTION

In one of the complementation groups of the human disease XP, repair of UV damage to DNA is reduced to about 20 to 30% of normal (1). Several previous studies have indicated that the residual repair in this group is highly clustered (2, 3), but that repair becomes randomized when cultures are stimulated to proliferate (4). Because DNA polymerase α is free to play a role as a major repair polymerase in confluent cells (5–8), but is simultaneously involved in DNA replication in proliferating cells, it seemed possible that the clustering of repair sites seen in XP Group C cells might be determined by DNA polymerase α.

To test this hypothesis, the effect of two inhibitors that act at different steps of the excision repair pathway on the clustering of repaired sites has been investigated. Novobiocin was used as an inhibitor of the initial stage of repair, possibly the ATP-dependent incision activity of a UV-specific endonuclease (7, 9–13). Aphidicolin was used as an inhibitor of the major repair polymerase activity for UV damage (5–9, 11). Although the precise biochemical target for the action of these two inhibitors is unknown, there is general agreement that novobiocin acts earlier in the excision pathway than does aphidicolin (7, 9, 10, 14), and this knowledge is used for analysis of the results of this study.

MATERIALS AND METHODS

Cell Culture. Normal human fibroblasts (HS27) and XP Group C fibroblasts (XP5098 and XP5126, from siblings, and XP17ME, from an unrelated patient) were grown in Eagle's minimal essential medium with 10% fetal calf serum. To obtain uniformly labeled DNA, cultures were grown in 0.01 μCi of [14C]dThd per ml (56 mCi/mmol) for at least 3 days. The medium was changed regularly until cultures were confluent and were then maintained in 1% serum for 2 or more days to ensure that cultures were not proliferating.

Irradiation and Labeling Procedure. To obtain repaired DNA labeled in repair sites, [14C]dThd-labeled cultures were first grown in BrdUrd (10 μM) and FdUrd (2 μM) for 60 min to begin synthesis of density-labeled DNA. They were then irradiated with UV light (254 nm, 1.3 J/m²) and incubated for 5 or 20 h with 10 μCi of [3H]dThd per ml (80 mCi/mmol) and BrdUrd (10 μM, FdUrd (2 μM), and hydroxyurea (2 mM) to improve resolution of repair synthesis. In some experiments, cultures were grown in novobiocin (0.5 to 1 mM) to inhibit the incision step of repair (9–13). In other experiments, cultures were grown in aphidicolin (5 to 20 μM) or dideoxethylene (50 to 100 μM) for 5 h to inhibit polymerases α and β, respectively (5–9, 11). At the end of the labeling period with aphidicolin, cultures were grown for at least 90 min to allow incomplete repair patches to become completely ligated (7, 8). Immediate analysis in alkaline sucrose gradients showed that, at this stage, the single-strand-break frequency was no more than 0.5 to 1 in 10⁶ daltons (15). Cultures were harvested by rinsing in phosphate-buffered saline, centrifuged, drained, rapidly frozen in a methanol/dry ice bath, and stored at −20°C for later use. DNA was isolated as previously described, and repaired DNA was purified by centrifugation in neutral isopyknic cesium chloride-cesium sulfate gradients (16). The average single-strand molecular weight (weight average) from isopyknic gradients was 1.50 ± 0.14 (SD) × 10⁶. In some experiments, the repaired DNA was used directly without prior isolation in isopyknic gradients because there were insignificant amounts of semiconservative replication in confluent cultures after 13 J/m² (4).

Enzymatic Analysis of Pyrimidine Dimers. DNA from normal-density regions of neutral isopyknic gradients was dialyzed against a buffer (20 mM Tris-2 mM EDTA-40 mM NaCI, pH 8.0) suitable for UV endonuclease treatment. Two hundred μl of DNA were incubated with 20 μl of crude UV endonuclease extract from Micrococcus luteus at 37°C for 45 min (3). At the end of digestion, DNA was centrifuged in alkaline sucrose gradients at 20,000 rpm in an SW28 rotor for 16 h, after which DNA radioactivity was determined, and molecular weights were calculated for [H]- and [14C]-labeled DNA (16). For DNA isolated from normal and XP cells immediately after a UV dose of 13 J/m², UV endonuclease treatment produced weight-average molecular weights of 4.68 ± 0.22 × 10⁶ and 4.35 ± 0.16 × 10⁶, respectively. These values represent the production of 62.0 ± 7.1 pyrimidine dimers per 2 × 10⁶ daltons by a dose of 13 J/m² in human fibroblasts. To facilitate comparisons between [H]- and [14C] distributions, profiles are displayed in terms of the percent-age of radioactivity in the whole gradient.

RESULTS

Repair replication in normal human and XP Group C cells was inhibited to about 50% by a 10 μM concentration of aphidicolin (Table 1). The sensitivity of normal cells was slightly greater than that of XP cells. Dideoxethylene, however, appeared to be ineffective even at the high concentration of 100 μM, as previously reported (17). Novobiocin inhibited repair to 10 to 30% at concentrations of 0.5 to 1 mM (Table 1). Inhibitor concentrations that still permitted about half of the repair to continue were chosen for subsequent analysis of the distribution of mended sites.
Table 1  Sensitivity of repair replication in normal (HS27) and XP Group C (XP5126 and XP17ME) cells to inhibition by aphidicolin or dideoxythymidine

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Normal cells</th>
<th>XP Group C cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HS27</td>
<td>XP5126</td>
</tr>
<tr>
<td>None</td>
<td>32.4 ± 2.1</td>
<td>1.34 ± 0.29</td>
</tr>
<tr>
<td>Aphidicolin (10 μM)</td>
<td>13.0 ± 0.8</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>Dideoxythymidine (100 μM)</td>
<td>35.6 ± 2.2</td>
<td>1.40 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>XP17ME</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.05 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Novobiocin (0.5 mM)</td>
<td>0.96 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Novobiocin (1.0 mM)</td>
<td>0.33 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE.

The repair replication in XP Group C cells compared to normal cannot be determined directly from the 3H/14C ratios because the two cell types do not incorporate 14C-dThd to exactly the same extent. Comparisons on an absolute basis showed that these XP cells carried out 10 to 30% of normal repair (14). Numbers in parentheses, percentage of repair seen in cells under the same growth conditions without inhibitor.

When the repaired DNA from XP cells was analyzed by first cleaving unrepaired pyrimidine dimers with UV endonuclease, 3H-labeled repair patches had a higher molecular weight than the bulk 14C-labeled DNA (Figs. 1 and 2). This result, which has been described previously for a large number of Group C cell lines (2-4), indicates that repaired sites are clustered together in the genome.

When repair was inhibited by novobiocin, the distribution of 3H-labeled repaired DNA still had a higher molecular weight than the bulk 14C-labeled DNA (Fig. 1). On quantitative analysis, the cluster size seemed to be slightly reduced, but still different from a purely random distribution (Table 2). When cells were grown in aphidicolin, however, the 3H-labeled repaired DNA now showed a slightly smaller molecular weight than the bulk 14C-labeled DNA (Fig. 1; Table 3). This is typical of a random distribution of DNA molecules, when considera...
tion is given to the way DNA is labeled with \(^3\)H and \(^{14}\)C. The \(^{14}\)C is distributed uniformly through the DNA, and therefore represents the weight of the DNA. The standard formulas for molecular weight calculation can therefore be used (16) and, since the pyrimidine dimers are randomly distributed, the \(^{14}\)C weight average molecular weight will be twice the number average molecular weight. The \(^3\)H, however, is in individual patches independent of the random distribution of DNA fragment sizes. The \(^3\)H, therefore, represents the actual distribution of the number of molecules; use of the standard formula for calculation of weight average molecular weight for \(^3\)H will therefore produce a value that actually approximates to the actual number average, about half of the weight average. Experimental evidence of this for end-labeled DNA has been shown (17).

The true ratio of molecular sizes for \(^3\)H- and \(^{14}\)C-labeled molecules can therefore be obtained by multiplying the molecular weight ratios in Tables 2 and 3 by 2. The cluster size in XP Group C therefore represents a molecular size of about 2.7 to 3 times the spacing of pyrimidine dimers, indicating a cluster size involving repair of no more than two adjacent pyrimidine dimers. In the presence of novobiocin, the cluster size was reduced slightly to 2.34, indicating that a significant number of adjacent dimers were still repaired even though the overall amount of repair was reduced to 30%. In the presence of aphidicolin, however, the cluster size was reduced to 1.4 to 1.66, indicating that repair under these conditions did not involve significant clustering because repair of individual dimers would be expected to produce a doubling of the ratio of \(^3\)H to \(^{14}\)C DNA sizes.

**DISCUSSION**

Those damaged sites that are repaired in XP Group C cells appear to be clustered together (2, 3), suggesting that the excision repair system acts processively in searching along DNA for adjacent damaged sites and mends several adjacent dimers in a coordinated process. Inhibition of the initial, incision step of repair to low levels (30%) by novobiocin still permitted the repair which continued to be in a clustered mode. Aphidicolin, however, at a concentration that reduced repair replication to about 50% in normal and XP cells, also caused those sites that were repaired in XP Group C cells to be randomly distributed. Therefore, a similar level of repair in the presence of novobiocin or aphidicolin has quite different distributions at the molecular level. Residual repair that occurs in the presence of aphidicolin may involve polymerase \(\beta\) but positive evidence for this is difficult to obtain because dideoxythymidine is an ineffective inhibitor of \(\beta\) in whole cells (17).

There is general agreement that novobiocin acts at an earlier step of the excision repair pathway than does aphidicolin (7, 9, 10, 14). Novobiocin appears to inhibit the incision step of repair, but it is unclear whether this is through its action on DNA topoisomerase (10, 11, 14, 18) or on other activities possibly associated with inhibition of ATP-dependent reactions (10, 11, 19, 20). Aphidicolin has generally been considered to act by blocking the deoxycytidine triphosphate binding site of DNA polymerase \(\alpha\) (6). An alternative polymerase, \(\delta\), has recently been described which is also sensitive to aphidicolin and which may also be involved in excision repair (12). Despite these uncertainties about the precise mode of action of these inhibitors, available evidence suggests that an appropriate working hypothesis is that novobiocin acts at the incision step of repair, whereas aphidicolin acts at the polymerase step.

Randomization of repair in Group C cells also occurs when the cells are stimulated to proliferate, when there is no change in the total amount of repair (4). In normal cells, aphidicolin reduces pyrimidine dimer excision but makes no detectable change in the distribution of repair sites (3, 22). The mechanism of repair in Group C cells that leads to a clustering of repaired sites therefore appears to involve a processive excision system more strongly influenced by DNA polymerase activity than by the incision activity that is sensitive to novobiocin. When the polymerase activity is inhibited, or when it is committed to semiconservative replication, damaged sites are mended at random. A similar processive mechanism has been reported for the T4 pyrimidine dimer glycosylase (23), but for this molecule the processivity occurs in the absence of polymerase, unlike the mechanism hypothesized here for human cells.

Polymerase activity, either of \(\alpha\) or \(\delta\), therefore appears to exercise more than a merely passive gap-filling role in repair. The polymerase enters actively into regulation of the amount and distribution of repaired sites. In normal cells, even though the polymerase would appear to act after the excision of pyrimidine dimers, inhibition by aphidicolin actually prevents excision (3, 22). This is best explained if the polymerase is involved with the excision enzymes in a reversible complex, which acts processively. Alternatively, the polymerase may be required to displace excision enzymes from DNA, although this action would not explain why repair would be clustered. When poisoned by aphidicolin, the polymerase may trap the excision enzymes or be unable to displace them and the excised fragment from the DNA. Under some conditions, aphidicolin also results in an increase in patch sizes (24, 25).

In XP Group C cells, the observation that clustered repair involves DNA polymerase \(\alpha\) suggests that this polymerase controls the diffusion of a repair complex along the DNA. If two or more repaired sites are adjacent and flanked by unrepaired pyrimidine dimers, then at a UV dose of 13 J/m\(^2\), the excision and polymerase complex must migrate along about 10\(^2\) daltons of DNA, or about 10\(^4\) base pairs. This represents an extensive distance of linear migration along the DNA. Alternatively, instead of linear migration the same result may be produced by restricted diffusion because of the high molecular weight of DNA polymerase \(\alpha\). Previous analysis has shown that, although Group C cells carry out repair, on average, at about 25% of normal, the sites mended are highly clustered and represent repair of 25% of the genome to a normal extent, the remaining 75% being unrepaired (2, 4). The clustered repair in XP Group C may be only an exaggerated version of what occurs in normal cells, because a certain degree of nonrandom repair has been observed in normal cells by a more sensitive technique (26). It will be necessary to examine the distribution of repair by this technique to determine if aphidicolin also randomizes repair in normal cells. From the results of the current analysis, randomization would be expected.

DNA polymerase \(\alpha\) or \(\delta\) therefore may play a major role, not only in resynthesis of excised regions, but also in regulating the activity and mobility of the excision enzymes. This role is flexible and may be the consequence of a reversible association between the polymerase and the excision enzymes, as has been observed for the repair enzyme uracil DNA glycosylase (27); it is possible that this association could be exploited in manipulating repair and in isolating and characterizing repair enzymes.

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REFERENCES

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