Intragenomal Distribution of DNA Repair Synthesis: Repair in Satellite and Mainband DNA in Cultured Mouse Cells

(N-acetoxy-2-acetylaminofluorene/Ag⁺–Cs₂SO₄ gradients/carcinogenesis/mutagenesis/ultraviolet radiation)

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Abstract DNA repair synthesis was examined in mouse satellite and mainband DNA derived from confluent Balb/c 3T3 cells damaged with ultraviolet radiation or N-acetoxy-2-acetylaminofluorene. Two different approaches were used: (i) Contact-inhibited cells were treated with hydroxyurea to reduce replicative synthesis to low levels; and (ii) bromodeoxyuridine was used to label newly replicated DNA in cells that had escaped contact inhibition. DNA was separated into mainband and satellite fractions in Ag⁺–Cs₂SO₄ gradients. After treatment with either ultraviolet radiation or N-acetoxy-2-acetylaminofluorene, repair synthesis occurred to the same extent in mainband and satellite DNA. Repair synthesis increased over an ultraviolet radiation dose range of 30–200 erg/mm², and the extent of repair in the two DNA species was similar at each dose level. An analysis of the separated strands of satellite DNA from ultraviolet-irradiated cells indicated that the extent of repair is closely correlated with the availability of pyrimidines for cyclobutyl dimer formation and provided evidence that repair synthesis occurs at the site of damage. Within the precision of our experiments the results suggest that at least one group of highly repetitive, nontranslated DNA sequences is repaired to about the same extent as the rest of the genome.

DNA repair in response to both radiation and chemical damage has been studied in various cultured mammalian cells (1–7) and tissues (8–10). To date, interest has focused primarily upon differences in the responsiveness of various cell lines (1, 5, 11, 12), evaluation of several repair deficient lines (2, 3, 11, 13, 14), and classification of response patterns to various agents (7, 15–18). One important aspect of this problem that has not been extensively studied is the distribution of repair within the genome. Are all types of DNA sequences repairable? Such a question has important functional implications: differences in repairability of intragenomal segments might be related to the now well-documented differential rates of genetic drift of different DNA sequences (19–22) or to different mutation rates at different loci. Processes such as regulation, transcription, and differentiation might be affected by the rate and extent of repair of damage. While no comprehensive theory of carcinogenesis exists at present, many recent formulations have suggested that mutational events and/or altered states of differentiation may be involved (e.g., refs. 18 and 23). Therefore, it would be of interest to know the extent to which different DNA sequences are repaired.

We have been utilizing a number of approaches to analyze this problem. Because mouse satellite DNA is an unusually well-studied and highly specialized set of DNA sequences (for reviews see refs. 21 and 24), our initial studies have focused on an analysis of repair of satellite DNA relative to the remainder of the genome (mainband DNA). Satellite DNA is thought to be a highly reiterated set of tandemly repeated sequences located primarily in pericentromeric regions of chromatin during mitosis and in heterochromatic and perinucleolar regions during interphase (21, 24). Furthermore, it is probably not transcribed and undergoes rapid genetic drift (21, 24). As a result, we have been able to utilize two well-characterized mutagens and carcinogens, ultraviolet radiation (254 nm) and N-acetoxy-2-acetylaminofluorene (NA-AAF) (25), both of which are believed to result in DNA damage that is repaired by a process resulting in the removal of about 100 nucleotides (3, 11, 12, 15, 26, 27).

Materials and Methods

Balb/c 3T3 cells were grown to confluence in Dulbecco–Vogt modified Eagle’s medium with 10% fetal-calf serum and 73.5 mg/liter of neomycin at 37°C in an atmosphere containing 5% CO₂. This system was used to limit replicative synthesis in these cells and to make repair events more easily observed (27). Because the great majority of cells are in interphase, one may study repair in satellite DNA confined to its heterochromatic location (21, 28, 29). This type of analysis seemed easier than attempting to use asynchronous cultures in which the physiological state of satellite DNA might vary extensively with the cell cycle. Mycoplasma sp. assays were routinely negative.

Experiments were done in which bromodeoxyuridine and/or hydroxyurea were used to label the small amount of replicating DNA present (from cells that had escaped contact inhibition), or to suppress such synthesis, or both. In experiments with bromodeoxyuridine, 5 μg/ml was added to confluent cells for 10 hr before the cells were treated with UV radiation or NA-AAF (provided by the Standard Reference Compound Bank of the Carcinogenesis Program, National Cancer Institute) (27); BrdU was present in the medium for the remainder of the experiment (about 5 hr). Treatment was followed immediately (UV radiation) or 20 min later (NA-AAF) by addition of [methyl-¹H]thymidine (50 Ci/mmol; New England Nuclear Corp.) (27). The use of unlabeled BrdU + [¹H]dIT has been shown to be equivalent to the use

Abbreviations: NA-AAF, N-acetoxy-2-acetylaminofluorene; H strand and L strand, heavy and light strands of mouse satellite DNA, respectively.

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allowed to stand for 30-40 min, followed by either no treatment (control, left), 200 erg/mm² UV radiation (center) or 10 μM NA-AAF (right). Fifteen minutes later the cells received 5 μCi/ml of [3H]dT and were incubated for 5 hr before they were harvested. Samples (500 μg) were centrifuged in 10 ml Ag⁺-Cs₂SO₄ gradients at r₉ = 0.3 for 18 hr at 45,000 rpm followed by 48 hr at 33,000 rpm in a 50 Ti Beckman rotor at 25°C. Processing was as described under Methods. Fraction 1 represents the bottom of the gradient. Left-hand ordinates represent absorbance at 260 nm after dilution with 0.5 ml of H₂O (O—O). Right-hand ordinates represent cpm in 0.1-ml aliquots (•—•). Note change in scale; values have been multiplied by 10⁻¹, as indicated.

of [3H]BrdU (5, 30). All experiments were carried out in reduced light. The protocol for hydroxyurea experiments was as described (27): sufficient hydroxyurea was added to give a final concentration of 10 mM, and the treated cells were allowed to stand for 30-40 min before [3H]dT was added.

Five hours later cells were harvested and crude nuclei were prepared by detergent lysis (27). DNA was prepared by a modified phenol method (see ref. 31) and dialyzed into 10 mM sodium borate buffer, pH 8.8. Separation of satellite DNA from mainband DNA was achieved by centrifugation in Ag⁺-Cs₂SO₄ gradients (r₉ = Ag⁺/DNA-P = 0.3) as described (31). Three-drop fractions (0.12 ml) were collected from the bottom of the tube and diluted with various amounts of water, depending on the amount of DNA added. Absorbances at 260 nm were read, and aliquots were counted in either Aquasol (New England Nuclear Corp.) or Instagel (Packard). Specific activities were calculated as described (27). Counting efficiency was about 90%, as calculated from internal standards. Centrifugation of mouse satellite DNA in alkaline CsCl was adapted with minor modifications from the work of Flamm et al. (32).

The nature of the radioactivity incorporated into replicated and repaired DNA after [3H]dT labeling was investigated by paper chromatography of DNA digests as described (ref. 27, system 2). With label incorporated during either replicative synthesis (asynchronous cultures, 5-hr labeling time) or repair synthesis in the presence of hydroxyurea after damage with UV radiation or NA-AAF, 96.5-99.5% of the radioactivity was present as dT.

RESULTS

Repair Synthesis (Unscheduled DNA Synthesis) in Hydroxyurea-Suppressed Cells. The results of an experiment in which repair synthesis was studied after suppression of residual replicative synthesis with hydroxyurea are presented in Fig. 1. In the presence of hydroxyurea alone (control), only a small amount of incorporation is seen. Irradiation with 200 erg/mm² of UV radiation or treatment with 10 μM NA-AAF produces a large increase in DNA synthesis in these non-dividing cells. Such synthesis is generally believed to represent repair synthesis (e.g., refs. 1, 3-6, 10, 27). Note that after exposure to both agents, label is incorporated into mouse satellite DNA (fractions 50-60) as well as into mainband DNA. On a specific activity basis, the extent of incorporation into satellite and mainband DNA is approximately the same in each case. We have not investigated small differences that

fig. 1. Separation of mouse satellite and mainband DNA in hydroxyurea-suppressed cells. Cells were treated with hydroxyurea for 15 min, followed by either no treatment (control, left), 200 erg/mm² UV radiation (center) or 10 μM NA-AAF (right). Fifteen minutes later the cells received 5 μCi/ml of [3H]dT and were incubated for 5 hr before they were harvested. Samples (500 μg) were centrifuged in 10 ml Ag⁺-Cs₂SO₄ gradients at r₉ = 0.3 for 18 hr at 45,000 rpm followed by 48 hr at 33,000 rpm in a 50 Ti Beckman rotor at 25°C. Processing was as described under Methods. Fraction 1 represents the bottom of the gradient. Left-hand ordinates represent absorbance at 260 nm after dilution with 0.5 ml of H₂O (O—O). Right-hand ordinates represent cpm in 0.1-ml aliquots (•—•). Note change in scale; values have been multiplied by 10⁻¹, as indicated.

Fig. 2. Separation of newly replicated DNA from nonreplicated mouse mainband and satellite DNAs in cesium salt gradients. Cells were treated with BrdU as described in Methods; during the last 5 hr, 10 μCi/ml of [3H]dT was added. Samples were then centrifuged in Cs₂SO₄ alone (left, 250 μg of DNA), in Ag⁺-Cs₂SO₄ at r₉ = 0.3 (center, 250 μg of DNA), or in CsCl (right, 225 μg of DNA). Centrifugation was as described in legend of Fig. 1; initial densities were adjusted to band DNA peaks in the center of the gradient (see ref. 31). Fractions were diluted with 0.2 ml of H₂O before A₆0₀ was measured, and 0.2 ml of each was counted. Left-hand ordinate represents absorbance of diluted sample at 260 nm. Right-hand ordinate represents cpm (•—•) in each 0.2-ml aliquot.
might arise from differences in base composition of mainband DNA (40% G + C) compared to satellite (35% G + C) (32, 33) or the specificity of the damaging agent (UV radiation produces primarily thymine dimers while NA-AAF produces primarily guanine adducts).

Good contact inhibition is needed to demonstrate repair (unscheduled DNA synthesis) in cultured mouse cells with the hydroxyurea method. Periodically it was necessary to go back to frozen stocks, since, in our hands, escape from contact inhibition, which occurred on frequent passage without re冷链ing, obscured the phenomenon. Similar problems occurred when mouse primary cultures were used.

Centrifugation of BrdU-Labeled DNA in Ag + -Cs2SO4 Gradients. Because it was desirable to confirm our findings with hydroxyurea-suppressed cells by an independent method, a bromodeoxyuridine technique was devised to separate newly replicated DNA from nonreplicated mainband and mouse satellite DNA. Cells were treated with BrdU (5 µg/ml) for 15 hr; during the last 5 hr [3H]dT (10 µCi/ml; 50 Ci/m mole) was added as a marker for newly replicated DNA. DNA was then centrifuged in Cs2SO4 (Fig. 2, left), Ag + -Cs2SO4 at r = 0.3 (Fig. 2, center), or CsCl (Fig. 2, right). As expected, centrifugation in Cs2SO4 alone gave sharp bands, little separation of the two bands, and no separation of satellite DNA from mainband DNA (31, 34, 35). Centrifugation in CsCl gave good separation from nonreplicated DNA, but, under the conditions used (see legend to Fig. 2), there was little resolution of satellite DNA. However, centrifugation in Ag + -Cs2SO4 allowed good resolution of three peaks in the same gradient. Under these conditions, a fourth peak composed of newly replicated satellite DNA was not resolved; perhaps the increased BrdU content of newly replicated satellite compared to that of newly replicated mainband caused them to coband.

Repair of Nonreplicating DNA in Conjunction with BrdU Labeling of Replicating DNA. Repair synthesis was easily demonstrable after bromodeoxyuridine density labeling of replicating DNA and its separation from nonreplicated DNA in Ag + -Cs2SO4 gradients (Fig. 3). The result is similar to that presented in Fig. 1, namely, that, after damage by either 200 erg/mm² UV radiation or 10 µM NA-AAF, substantial incorporation is seen in nonreplicating DNA (Fig. 3, center and right). Again, there is approximately equal incorporation into mainband and satellite DNA. With both the hydroxyurea method and the BrdU method, at the doses used, NA-AAF stimulates about 1/3 as much repair synthesis as UV radiation.

Effect of Dose on Repair of Satellite and Mainband DNA. The effect of dose on the extent of repair synthesis in satellite and mainband DNA was analyzed after treatment of cultures with 30–200 erg/mm² of UV radiation. In these experiments cells were pretreated with both BrdU and hydroxyurea, as indicated in Methods, and then irradiated. Gradients containing 500 µg of DNA were centrifuged, and the specific activities of the mainband and satellite DNAs were calculated from the dpm and absorbances (Fig. 4). Repair is easily detectable with doses as low as 30 erg/mm². It is apparent that, over the dose range examined, satellite and mainband DNA incorporated about equal amounts of label during repair (see Discussion).

Fig. 3. Repair of mouse satellite and mainband DNA after density labeling of newly replicated DNA with BrdU. Cells were labeled with BrdU as described in Methods. After treatment with either 200 erg/mm² of UV radiation or 10 µM NA-AAF, cells were incubated for 5 hr with 10 µCi/ml of [3H]dT. Control (left); UV radiation (center); NA-AAF (right). Each gradient contained 230 µg of DNA and was centrifuged as described in legend of Fig. 1. Sample was diluted with 0.2 ml of H2O and 0.2-ml samples were counted. Left-hand ordinate represents absorbance of diluted samples at 260 nm (Ο – O). Right-hand ordinate represents cpm (Ο——Ο) per 0.2-ml aliquot. Note change in scale.

Fig. 4. DNA repair in satellite and mainband DNA as a function of dose of UV radiation. Cells were treated with both BrdU and hydroxyurea as described in the text. After irradiation with 0–200 erg/mm² of UV radiation, cells were incubated with 20 µCi/ml of [3H]dT. Samples (500 µg) were centrifuged, and the specific activities of the mainband (Ο) and the satellite peaks (Ο) were determined. Over the dose range examined, recovery of DNA from cultures was approximately equal.
Fig. 5. Incorporation of [3H]dT into heavy and light strands of satellite DNA during repair synthesis. Satellite DNA was prepared as described in legend of Fig. 1 and the text. About 25 μg of satellite DNA was placed on a 4.7-ml alkaline CsCl gradient as described by Flamm et al. (32). Centrifugation was at 42,000 rpm for 48 hr in a 50 T (Beckman). Four-drop samples were diluted with 0.1 ml of H2O and read at 260 nm; 0.2-ml aliquots were counted. Left-hand ordinate, A260 of diluted sample (O − − −O); right-hand ordinate, cpm of 0.2-ml aliquots (●●●●●●); inscrbed scale, relative specific activity of the H and L strands (Δ). The L strand specific activity of 890 dpm/μg of DNA was set to 1.0.

Strand-specific Repair of Satellite DNA Damaged by UV Radiation. In the past, there has been little attempt to demonstrate that repair (as measured by new base insertion) occurs at the site of damage. This problem may be analyzed by taking advantage of the fact that the heavy (H) strand of mouse satellite DNA is both thymine- and pyrimidine-rich (32, 33) compared to the light (L) strand and, thus, might reasonably be expected to sustain substantially more damage and subsequently to undergo more repair synthesis. One may make a rough estimate of the expected ratio of repair synthesis between the two strands from the base composition of the strands (32); a nearest-neighbor calculation for pairs of pyrimidines (ref. 36; Table 4) with the assumption of a random distribution; an estimate of the relative efficiency of dimer formation (ref. 36; Table 5) for pyrimidine pairs; and the fact that at 200 erg/mm² the frequency of dimers will be such (36) that a repair patch of a hundred nucleotides (12) will result in the insertion of 2.2 times the number of thymines in the H strand as in the L strand. The actual value for such an estimate is that during repair synthesis about eight times as much dT should be inserted in the H strand as in the L strand. Cells were damaged with 200 erg/mm² UV radiation and labeled with [3H]dT in the presence of hydroxyurea. After separation of DNA into satellite and mainband (similar to Fig. 1, center), the satellite strands were separated in alkaline CsCl, and the distribution of the [3H]dT incorporated during repair was analyzed (Fig. 5). As expected, during repair synthesis most of the label appears in the heavy strand; the ratio of specific activities of the H and L strands is about 3.6 to 1. The calculated and observed ratios are in reasonable agreement with one another, considering the approximation necessary to make the calculations and limitations in the accuracy measurements. They probably are influenced by a large number of factors, including the assumption of randomness of nearest neighbors in the absence of data (24), the assumption of random damage (37), lack of good data on dimer formation at 200 erg/mm² and 254 nm (36), the cross-contamination of H and L strands with each other, and possible contamination with a small amount of mainband DNA. As little as 10–15% contamination of satellite DNA with mainband DNA would raise the specific activity of the L strand sufficiently to halve the ratio of observed specific activities and result in the findings similar to those presented in Fig. 5. In theory, it is also possible to examine repair synthesis in separated strands of satellite DNA after induction of guanine damage with NA-AAF.

DISCUSSION

DNA repair synthesis in cultured mouse cells (1, 11, 12) and mouse tissues (8, 10) has been demonstrated after treatment with UV radiation and NA-AAF. Though some investigations were unable to demonstrate dimer removal in mouse cells (38, 39), recent reports (12, 40) suggest that at least two mouse lines (Balb/c 3T3 and L5178Y) are able to excise dimers, but do so to a lesser extent than human lines.

We have analyzed DNA repair synthesis in confluent monolayers treated either with hydroxyurea to suppress residual, semiconservative DNA synthesis or with BrdU to label replicating DNA from remaining S-phase cells. Both methods of analysis indicate that repair occurs to about the same extent in satellite and mainband DNA. It is of interest that this finding holds for two agents that produce very different types of DNA damage (3, 11, 25, 26), but that are presumed to be repaired by a process involving the excision of about 100 nucleotides. Implicit in this study has been the assumption that these agents, like others (41, 42), damage satellite and mainband DNA to about the same extent. We have not investigated agents that in mouse cells may be repaired by insertion of only a few bases (43). Equal repair in satellite and mainband DNA is probably not the result of saturation of repair mechanisms, since even at low doses of UV radiation (Fig. 4) both DNA species show about the same extent of repair synthesis. Some variation occurs in determining specific activity from gradients in which one peak comprises 91–92% of the DNA and the other 8–9% (31). This effect arises primarily from the presence of a small amount of background absorbance and low counts in the satellite peak, especially at low doses of UV radiation. In addition, the precise molar extinction coefficients for satellite and mainband DNA are unknown (see ref. 31). Thus, there is about a 10–15% variation in specific activity measurements (Fig. 4) which precludes investigation of possible small differences such as those that might be due to differences in the G + C content between satellite and mainband DNA.

The analysis of repair synthesis in separated strands of satellite DNA from UV-irradiated cells has proved interesting. Much more repair occurs in the H strand than in the L strand (Fig. 5), in keeping with the increased availability of pyrimidines for cyclobutyl dimer formation. This demonstration
suggests that insertion of new bases occurs in regions where damage has been removed and provides support for present models of excision repair (e.g., refs. 3 and 44).

Assessment of the cellular consequences of similar degrees of repair in satellite and mainband DNA is difficult. Although the physiologic role of satellite DNA is not clear, there is general agreement on several points (for reviews see refs. 21 and 24): (1) Satellite DNA is primarily, though possibly not exclusively, pericentromeric. (2) During interphase it is located in the heterochromatic and perinuclear portions of the nucleus in a number of different cell types. (3) Satellite sequences are tandemly arranged and are probably not transcribed or translated. (4) Satellite DNA may be undergoing rapid genetic drift.

These facts, in conjunction with the above data, suggest the following conclusions: (1) Repair enzymes in mouse cells appear to have access to damaged satellite sequences within heterochromatin, although it has generally been believed that access to heterochromatic DNA sequences for transcription is highly restricted (presumably by bound chromosomal proteins). Whether this finding turns out to be the rule for all heterochromatic sequences in all species remains to be investigated. (2) Repair is not confined to unique or translatable DNA sequences (genes), implying that some preservation of sequence fidelity is important even in highly repetitive DNAs. Because of the centromeric position of mouse satellite DNA, its repair may be essential since either breakage or malfunction would mean loss of not only local structure but also much or all of the chromosome during mitosis, in contrast to loss of a unique sequence in which only one allele of a single gene might be altered. In this context, it may be that an important aspect of repair of satellite DNA is elimination of strand breaks whether introduced spontaneously, by physical or chemical agents, or by nucleases during repair. (3) Within the precision of these experiments and to the extent that one may extrapolate from data with these agents to a situation in which "spontaneous" mutations arise (see ref. 44 for discussion), the "rapid drift" of mouse satellite sequences is probably not related to the differential repairability of them compared to the rest of the genome (mainband DNA).

In addition to the present work on the repair of mouse satellite DNA, work by Meltz and Painter on UV radiation-damaged HeLa cells (45) and by Lieberman and Poirier on human, diploid fibroblasts damaged with UV radiation and NA-AAF (46) indicates that many types of repetitive and unique sequences are repairable or partially repairable. None of these results conflicts with the possibility that some DNA sequences are selectively repaired while others are refractory to repair processes.

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