Ongoing Activity of RNA Polymerase II Confers Preferential Repair of Nitrogen Mustard-induced N-Alkylpurines in the Hamster Dihydrofolate Reductase Gene

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ABSTRACT

Recently, it has been demonstrated that nitrogen mustard-induced N-alkylpurines are excised rapidly from actively transcribing genes, while they persist longer in noncoding regions and in the genome overall. It was suggested that transcriptional activity is implicated as a regulatory element in the efficient removal of lesions. By treating cells or not with the transcription inhibitor α-amanitin, we have explored whether ongoing activity of RNA polymerase II was coordinately related to proficient repair of nitrogen mustard-induced alkylation products in the actively transcribed dihydrofolate reductase gene in the Chinese hamster ovary (CHO) cells. Nuclear run-off transcription analysis verified that α-amanitin completely and selectively inhibited transcription by RNA polymerase II. At the drug exposure examined, nitrogen mustard induced DNA damage capable of a complete transcription termination in the RNA polymerase II-transcribed dihydrofolate reductase gene and reduced 28S rDNA transcription by a factor of 7.9. The transcription activity did partially recover following reincubation in drug-free medium; this recovery was about 34 and 76% of ribosomal 28S gene transcripts and dihydrofolate reductase gene transcripts, respectively, after 6 h of repair incubation. α-amanitin significantly inhibited the removal of nitrogen mustard-induced N-alkylpurines in the 5'-half of the essential, constitutively active dihydrofolate reductase gene, while no effect of α-amanitin was observed on the lesion removal from a noncoding region 3'-flanking to the gene and from the genome overall. In the actively transcribed gene region, about 77% of N-alkylpurines were removed 21 h following drug exposure of cells not treated with α-amanitin and about 47% in 21 h in α-amanitin treated cells. The global semiconservative replication seemed unaffected by the α-amanitin treatment. From these results we suggest that gene-specific repair of nitrogen mustard-induced N-alkylpurines is dependent on ongoing activity of the transcribing RNA polymerase II. The findings are discussed in terms of the current ideas about the mechanism of preferential DNA repair.

INTRODUCTION

Recent development of various techniques to examine the formation and the repair of various DNA lesions in individual genes has advanced our knowledge of the DNA repair processes in a wide array of organisms (see Ref. 1 for review). Especially in the case of UV light-induced cyclobutane pyrimidine dimers, ample evidence has been presented that repair of this lesion occurs preferentially in transcriptionally active genes (2–5). Also, the efficiency of DNA repair may be affected by the nature of the lesions, its location within the genome, the transcriptional activity, and the conformation of the chromatin structure of the damaged DNA regions.

DNA base damage is known to undergo a complex excision repair mechanism involving several specific enzymes (6). Considerable differences in the mode of repair exist among different monofunctional alkylating agents and even among agents which cause similar types of DNA lesions. DNA base methylation introduced with dimethyl sulfate seem to be randomly removed from the genome independently of its location within the genome (7, 8), while a correlation between transcriptional activity and availability to lesion removal of methylnitrosourea-induced DNA methylation has been demonstrated in the same hamster cells (9), a rat insulinoma cell line (10), and cultured human cells (11). Although alkylation by Sβ2-alkylating agents (e.g., methylnitrosourea) has greater sequence selectivity than Sβ2-alkylating agents (e.g., dimethyl sulfate) in vitro (12), it is not clear how this would result in differences in the mode of repair. It is likely that the observed differences in repair of methylpurines may depend on the degree of chromatin distortion invoked by these monofunctional alkylating agents and thereby the recognition enzymes involved. Alternatively, it may reflect the degree of transcription-terminating lesions induced by the DNA adducts.

A particularly intriguing problem is to establish the relationship, if any, between DNA repair and transcription. HN2 is a particularly intriguing problem is to establish the relationship, if any, between DNA repair and transcription. HN2 is a highly electrophilic anticancer agent which alkylates DNA bases mainly at N7-guanine and to a much lesser extent at other positions (13–15). HN2 bears two alkylating groups per molecule which together allow the formation of cross-links between paired DNA strands, between guanines in the same strand, or between DNA and protein (16–20). The biological effect of HN2 may depend on preferential reaction at certain genomic locations. Recently, we described a general approach to analyze gene-specific damage and repair of N-alkylpurines; the methodology is especially suitable for measuring N7-guanine alkylation upon exposure in vivo and measures the production of both DNA monoadducts and cross-links (8). We have shown that HN2-mediated N-alkylpurines are excised rapidly from actively transcribing genes but persist longer in an inactive gene region and a noncoding region of the hamster genome (8, 21). Also, the much less frequent HN2-mediated DNA interstrand cross-links have recently been reported to be preferentially repaired (22, 23). Finally, by using the same general technique for the measurements of N-alkylpurines (8), but probing the membranes with single-stranded DNA probes (riboprobes), we observed a slight bias toward repair in the transcribed strand after exposure to HN2. Thus, the phenomenon of preferential repair of HN2-induced N-alkylpurines resembles that seen for the removal of UV light-induced cyclobutane pyrimidine dimers, although some quantitative differences may occur (1). The biological advantage of preferential repair is evident, but the mechanism by which it occurs is still largely unknown. The observation that repair of HN2-induced alkylation products occurs nonrandomly in the genome suggests that the transcription complex could be actively involved in the recognition and removal of HN2-induced N-alkylpurines. The lesions generated by HN2 capable of blocking transcription at the N7-guanine alkylation sites in DNA (24) may be an important signal: stalled RNA polymerase could direct repair enzymes to the transcription-terminating lesion. To determine whether ongoing transcription is required for the preferential repair of HN2-induced DNA lesions, we have used α-amanitin to inhibit transcription by RNA polymerase II in HN2-treated CHO cells. α-Amanitin, the bi-
cyclic octapeptide from the poisonous mushroom, *Amanita phalloides*, binds to RNA polymerase II, thereby specifically blocking the elongation of mRNA transcripts without affecting the binding of the polymerase to the DNA template (25, 26). We examined the rate and extent of removal of HN2-induced N-alkylpurines from the actively transcribed DHFR gene, a noncoding region 3′-flanking to the DHFR gene, and from total cellular DNA in cells treated or not with α-amanitin. The effectiveness of transcription inhibition by α-amanitin and HN2 was confirmed by nuclear run-off transcription analysis.

**MATERIALS AND METHODS**

**Isotopes, Drugs, Enzymes, and Hybridization Probes.** [methyl-3H]-Thymidine (85 Ci/mmol), [α-32P]dCTP (3000 Ci/mmol), [5,6-3H]-uridine (49 Ci/mmol), and [α-32P]UTP (800 Ci/mmol) were purchased from Amersham Denmark. α-Amanitin was purchased from Boehringer Mannheim (Ercopharm, Denmark). HN2 was obtained through the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and was kept as a 0.1 M stock solution in 0.1 M HCl at −20°C. Restriction endonucleases (KpnI and HindIII) were purchased from New England Biolabs, Inc. All probes for the DHFR gene (pMB5) and the downstream, noncoding region (cs-14DO) were kindly provided by V. A. Bohr (National Institute on Aging, National Institutes of Health, Baltimore, MD) and have been described (27). The pMB5 probe was used to detect a 14-kilobase KpnI fragment of the 5′-half CHO DHFR, and a subclone of cs-14 (cs-14DO) was used to detect the downstream, noncoding 14-kilobase KpnI fragment of the DHFR gene (Fig. 1). The cs-14DO probe was generated by selecting for fragments which contained a minimum of repetitive sequences.4 The plasmid pAbb was kindly provided by P. C. Hanawalt (Stanford University, Stanford, CA). The plasmid contains a 1.4-kilobase insert from human 28S ribosomal DNA sequence (28) homologous to the hamster sequence containing the 5.8S and 28S rDNA sequences in CHO cells (Fig. 1).

**Cell Culture.** CHO-B11 cells (29) carrying the amplified DHFR gene were grown in Ham’s F-12 medium without glutamine, glycine, hypoxanthine, and thymidine (Gibco Laboratories) supplemented with penicillin and streptomycin, 500 μg/ml methotrexate, and 10% dialyzed fetal calf serum (Gibco) in humidified 5% CO2, 95% air at 37°C. Cell cultures for all experiments in this study were in exponential growth phase at the time of DNA damage.

**RNA Synthesis.** Cells were incubated with 10 μg/ml α-amanitin for 0, 3, 4, 5, 6, and 10 h at 37°C. Aliquots of cells were incubated in triplicate with [5,6-3H]uridine (0.05 μCi/ml) during the last hour of treatment with α-amanitin. Incorporation of [3H]uridine into RNA was stopped by aspirating the radioactive medium followed by addition of ice-cold phosphate-buffered saline. After trypsinization cells were resuspended in ice-cold 10% trichloroacetic acid and 2% (w/v) sodium PPi in distilled water. The resulting acid-insoluble material was collected on Millipore GS 0.22-μm filter discs. The filters were washed once with 5 ml of the trichloroacetic acid/sodium PPi solution and twice with 5 ml of 95% ethanol and were then air dried. Filters were placed in scintillation vials and 1.5 ml of 10% Tris, pH 9.1, 1 mM EDTA was added. Capped vials were incubated at 65°C for 1 h, 10 ml of Uniscent BD (National Diagnostics) was added, and the radioactivity was determined using a Beckman LS 1801 liquid scintillation system.

**Nuclear Run-off Transcription.** The procedure for transcription of nuclei, subsequent RNA isolation, blotting, and hybridization were performed as previously described (30, 31). Briefly, nuclei were prepared from CHO-B11 cells: the control treatment consisted of nuclei treated with 10 μg/ml α-amanitin, with 150 μM HN2, or with both 10 μg/ml α-amanitin and 150 μM HN2 and as in the gene repair analysis (see below). Nuclei were isolated by harvesting cells in ice-cold phosphate-buffered saline with a rubber policeman from Petri dishes placed on ice, pelleting the cells, vortexing the cells in Nonidet P-40 lysis buffer (10 mM Tris-CI, pH 7.4-10 mM NaCl-3 mM MgCl2-0.5% Nonidet P-40), resuspending at 200 μl/ml of the lysis buffer with a 100 μl/min pipette, and stored in liquid nitrogen. Nuclear run-off transcription reactions were carried out with 200 μl thawed nuclei (105 nuclei per sample), 200 μl of 2X reaction buffer (10 mM Tris-CI, pH 8.0-5 mM MgCl2-0.3 μM KCl), 2.5 mM dithiothreitol, 100 μCi [α-32P]UTP, and 0.5 mM ATP, CTP, and GTP. Transcripts were isolated as described. The entire transcription products ([32P]UTP) were hybridized at 65°C for 36 h to plasmid DNA: pMB5 to detect DHFR transcripts and pAbb to detect ribosomal gene transcripts. Linearized, denatured plasmid (4 μg/slot) was immobilized on a nylon membrane (Amersham Hybond-N%) with a slot blot apparatus (Hoefer Scientific Instruments). The membranes were prehybridized (QuickHyb hybridization solution, Stratagen) as for a Southern blot. After hybridization with the transcription products, membranes were washed twice with 2X standard saline citrate (1× is 150 mM NaCl-15 mM trisodium citrate, pH 7.0) for 1 h each at 65°C, incubated with 0.01 mg/ml RNase for 30 min at 37°C, washed again once in 2X standard saline citrate for 1 h at 37°C, and then autoradiographed.

**Gene Repair Analysis.** Cellular DNA was prelabeled by allowing cells to grow for 64 h at 37°C in [3H]thymidine (0.3 μCi/ml) and 10 μM "cold" thymidine. Following this procedure, the cells were subcultured and incubated in label-free medium for 24 h. The steps involved in the measurement of DNA damage and repair in specific genomic regions have been described in detail (8). Briefly, the prelabeled cells were treated with 10 μg/ml α-amanitin for 6 h in fresh medium prior to an exposure to 150 μM HN2 for 30 min at 37°C in fresh medium supplemented with 1% fetal calf serum. At the end of drug exposure, cells were washed with phosphate-buffered saline and either lysed (16 h at 37°C, pH 9.0) immediately or after repair incubation. For repair, the α-amanitin-containing medium was returned to the culture, which contained bromodeoxyuridine and fluorodeoxyuridine, to density label DNA replicated during the repair incubation. Control cultures received no α-amanitin. High molecular weight DNA was isolated by high-concentration salt extraction (32) and then treated with the appropriate restriction endonuclease. The parental DNA was then separated from that which had been replicated during repair incubation by neutral CsCl density gradient centrifugation (Fig. 2).

**Analysis.** Cellular DNA was prelabeled by allowing cells to grow for 64 h at 37°C in [3H]thymidine (0.3 μCi/ml) and 10 μM "cold" thymidine. Following this procedure, the cells were subcultured and incubated in label-free medium for 24 h. The steps involved in the measurement of DNA damage and repair in specific genomic regions have been described in detail (8). Briefly, the prelabeled cells were treated with 10 μg/ml α-amanitin for 6 h in fresh medium prior to an exposure to 150 μM HN2 for 30 min at 37°C in fresh medium supplemented with 1% fetal calf serum. At the end of drug exposure, cells were washed with phosphate-buffered saline and then lysed (16 h at 37°C, pH 9.0) immediately or after repair incubation. For repair, the α-amanitin-containing medium was returned to the culture, which contained bromodeoxyuridine and fluorodeoxyuridine, to density label DNA replicated during the repair incubation. Control cultures received no α-amanitin. High molecular weight DNA was isolated by high-concentration salt extraction (32) and then treated with the appropriate restriction endonuclease. The parental DNA was then separated from that which had been replicated during repair incubation by neutral CsCl density gradient centrifugation (Fig. 2).

**Overall Genome Repair.** Repair of alkylation damage in total cellular DNA was determined by a gel electrophoresis method using densitometer scan of photographic negatives of ethidium bromide-stained agarose gels. Detailed methodology can be found elsewhere (8, 33). Samples of total parental cellular DNA were prepared as described above in order to convert drug-induced
cells were prelabeled with $[^3]H$thymidine and incubated with 10 $\mu$g/ml a-amanitin or not 6 h before and after 150 $\mu$M HN2 exposure for 30 min. For repair, cells were incubated in medium containing 10 $\mu$M bromodeoxyuridine and 1 $\mu$M fluorodeoxyuridine. Total cellular Kpn1-restricted DNA was sedimented in neutral CsCl gradients and fractionated. Graphs represent the total radioactivity in each fraction containing parental and density-labeled replicated DNA. A, DNA from cells 0 h after HN2 exposure; B, DNA from cells in medium containing 10 $\mu$g/ml a-amanitin and 150 $\mu$M HN2.

RESULTS

Effect of a-Amanitin and Nitrogen Mustard on Ongoing Transcription. Cell cultures were pretreated with a-amanitin to induce complete blockage of transcription by the time of drug treatment and subsequent repair incubation. To determine the optimal exposure time of this pretreatment we began measuring the time-dependent effect of a-amanitin on total RNA synthesis. Within 6 h, 10 $\mu$g/ml a-amanitin reduced precursor incorporation into RNA to about 50% of that observed in untreated control cells (Fig. 3). Because the level of $[^3]H$ activity of acid-insoluble material did not further decrease upon longer exposure to a-amanitin, we chose 6 h as the time of pretreatment to ensure maximal inhibition of RNA synthesis. The residual activity of RNA synthesis reflects rRNA transcription since RNA polymerase I is resistant to a-amanitin (34).

To examine whether a-amanitin selectively induced stalled RNA polymerase II-mediated transcription complexes, we performed a nuclear run-off transcription assay. In this in vitro assay, new RNA transcripts are not initiated, but transcripts which are already initiated are faithfully elongated, giving a reasonably accurate measure of the level of transcription at any time chosen and from any gene of interest. Since a-amanitin induced a 50% reduction of total RNA synthesis, we could not assume that the overall level of RNA synthesis was constant and independent of the function of cell state (31). Therefore, as we examined the effect of both a-amanitin and HN2 on transcription, we hybridized the entire run-off product from the same number of nuclei in all samples.

The results from the nuclear run-off analysis are shown in Fig. 4. The several hundred copies of the ribosomal 28S genes resulted in very high transcription levels as compared to the RNA polymerase II-transcribed DHFR gene (Fig. 4A). Exposure to a-amanitin confirmed that RNA polymerase II is highly sensitive, whereas RNA polymerase I is resistant to this inhibitor (Fig. 4B). Yet, complete inhibition of DHFR transcription by a-amanitin obtained after 6 h of exposure to 10 $\mu$g/ml partially reversed upon removal and reincubation for 6 h in a-amanitin-free medium (Fig. 4C); this recovery was calculated to be 20%. Treatment of cells with 150 $\mu$M HN2 for 30 min caused a severe block of DHFR transcription and significantly reduced ribosomal gene transcription by a factor of 7.9 (Fig. 4D); this was revealed by densitometry of an autoradiogram. It appeared that 6 h of reincubation of HN2-treated cells in a-amanitin- and drug-free medium caused a partial recovery of both ribosomal gene and DHFR gene transcription (Fig. 4E); this recovery was calculated to 34.4 and 75.9% for the ribosomal gene and DHFR gene, respectively. The regain of RNA polymerase II-mediated transcription was sensitive to a-amanitin (Fig. 4F).

Effect of a-Amanitin on Removal of N-Alkylpurines in the DHFR Gene and in Its 3'-Flanking Noncoding Region. HN2-induced N-alkylpurines are removed preferentially from the actively transcribed DHFR gene in various CHO cells (8, 21). To determine whether this preferential repair is dependent on ongoing transcription, we compared the rate of removal of HN2-induced N-alkylpurines from specific genomic regions in the CHO-B11 cells by use of the established procedure described by Wassermann et al. (8). After treatment of cells with 150 $\mu$M HN2 alone or of cells preincubated with 10 $\mu$g/ml a-amanitin, repair incubation for 0, 6, and 21 h, isolation, and purification of parental DNA as described, the repair kinetics were determined in the 5'-half of the DHFR gene and in the 3'-flanking noncoding region of the DHFR gene. In order to secure that heating of untreated purified DNA did not cause nonspecific depuration,
duplicate samples of nonheated DNA from untreated cells were either loaded directly into the alkaline agarose gel or subjected to neutral depurination (heating at 70°C for 30 min at pH 7.0), followed by alkaline hydrolysis (0.1 N NaOH for 30 min at 37°C) prior to electrophoresis (Fig. 5). A decrease in the amount of full-length restriction fragment in the heated control compared to the nonheated sample represents nonspecific depurination. From repeated experiments, this nonspecific cleavage was estimated to be <10%.

Quantitation of the autoradiograms revealed that α-amanitin did not interfere significantly with the initial lesion frequency in either the DHFR gene or the noncoding region (0.05 < P < 0.1, t test) (Fig. 5, Table 1). Although small differences were seen in the formation of N-alkylpurines, one should interpret these differences with caution since there was considerable variation among experiments; this was mainly due to variation in the precise measurements of the low band intensities. For the DHFR gene, efficient repair of N-alkylpurines appeared by 6 h in cells not treated with α-amanitin (Fig. 5, Table 1). The removal of HN2-induced lesions in the DHFR region from α-amanitin-treated cells, however, was significantly less (Fig. 5, Table 1). Thus, in the 14-kilobase 5'-half DHFR region about 57 and 77% of N-alkylpurines were repaired in 6 and 21 h, respectively, in cells not treated with α-amanitin and 23 and 47% in 6 and 21 h, respectively, in α-amanitin-treated cells (Fig. 5, Table 1). The removal of alkali-labile lesions from the nontranscribing 3'-flanking region of the DHFR gene confirmed that HN2-induced N-alkylpurines are preferentially repaired because they were considerably less efficiently removed than in the DHFR gene (Fig. 5, Table 1). The repair of the noncoding region was not affected by treatment of the cells with α-amanitin (Fig. 5, Table 1).

Effect of α-Amanitin on the Removal of N-Alkylpurines from Total Cellular DNA. The overall genome repair was determined in order to define whether α-amanitin grossly affected the repair system active in the removal of HN2-induced N-alkylpurines in addition to its demonstrated inhibition of RNA polymerase II. By measuring the overall genome repair from the agarose gels, which also was used for the gene analysis, we could compare overall genome- and gene-specific repair on the same biological sample. Electrophoresis of DNAs on agarose gels disperses DNA according to molecular length. A typical photographic negative of an alkaline agarose gel used for overall DNA repair by the alkaline gel electrophoresis method is shown in Fig. 6. Lane L contained the molecular length marker (HindIII-digested λ-phage DNA) as a standard used for the calculations of the average molecular weight and repair percentages of the

**Fig. 4.** Effect of α-amanitin and HN2 on ongoing transcription. CHO-Bll cells were exposed to different conditions: A, untreated control; B, 10 µg/ml α-amanitin for 12 h at 37°C; C, 10 µg/ml α-amanitin for 6 h at 37°C followed by 6 h at 37°C in α-amanitin-free medium; D, 150 µM HN2 for 30 min at 37°C; E, 150 µM HN2 for 30 min at 37°C followed by reincubation for 6 h in drug-free at 37°C; F, 10 µg/ml α-amanitin for 12 h and 150 µM HN2 for 30 min at 37°C, where nuclei were isolated 6 h after exposure to HN2. Top, 32P-labeled nuclear run-off transcription products ([32P]nRNA) were hybridized to nylon-bound plasmid DNA: pAbb to detect ribosomal 28S gene transcripts and pMB5 to detect 5'-half of DHFR transcripts. Bottom, data derived from densitometry analysis of autoradiograms of nuclear run-off transcription products. ■, ribosomal 28S gene; □, 5'-half of the DHFR gene.

**Fig. 5.** Effect of α-amanitin on repair of HN2-induced N-alkylpurines in the CHO DHFR gene. DNA from CHO-Bll cells that had been treated with 10 µg/ml α-amanitin or not prior to and after exposure to 150 µM HN2 for 30 min at 37°C were digested with KpnI. Autoradiography of Southern blots detected hybridization of 32P-labeled genomic probes made from pMB5 to the 14-kilobase KpnI fragment of the 5'-half of the DHFR gene and cs-14DO to the 14-kilobase KpnI fragment of the 3'-flanking noncoding region. Lanes A and B, duplicate samples of DNA from nonheated (nondepurinated) control and heated (depurinated) control, respectively. Lanes C–J, duplicate samples of DNA from cells treated with: 150 µM HN2 for 30 min at 37°C and repair incubated for 0 h (C), 6 h (D), and 21 h (H); 10 µg/ml α-amanitin 6 h prior to a treatment with 150 µM HN2 for 30 min at 37°C and during repair incubation for 0 h (E), 6 h (F), and 21 h (I); 10 µg/ml α-amanitin for 12 h (G) and 27 h (J) as a 6- and 21-h α-amanitin repair control, respectively.
individual DNA samples. The mobility of DNA from cells treated with 150 μM HN2 was increased and was distributed toward the lower part of the gel indicating alkylation, which had been converted to strand breaks with a subsequent lower average molecular weight (lanes C and D). As cells were allowed to repair, their DNA distribution profiles accordingly indicated an increase in the average length of DNA. This was evident as a slower migration in the electrophoresis (lanes E, F, H, and I). Within 6 h of repair incubation, 19 and 21% of the alklylation adducts had been removed from cells treated with HN2 and D). As cells were allowed to repair, their DNA distribution profiles considerably differed from noncoding regions and from the genome overall (8, 21–23). It was suggested that transcriptional activity is preferential removal of HN2-induced N-alkylpurines from the actively transcribed DHFR locus. The recent experiments by May et al. (43) showed a slight strand bias toward repair of HN2-induced N-alkylpurines in the transcribed strand of the hamster DHFR gene. Although these studies attempted to examine a role for transcription in preferential repair, they did not reveal any direct role of participating transcription factors. While the observed difference of the repair rate between the template and complementary strand, however, was less than the α-amanitin-sensitive fraction in this study, both studies point to a role for transcription in gene-specific repair.

**DISCUSSION**

Recent evidence suggests that N-alkylpurines produced by HN2 are processed in a nonrandom fashion in the mammalian genome and that these alklylation lesions are more efficiently removed from actively transcribing genes than from noncoding regions and from the genome overall (8, 21–23). It was suggested that transcriptional activity is implicated as a regulatory element in the availability of efficient lesion removal.

By treating cells or not with the transcription inhibitor α-amanitin we have explored whether RNA polymerase II activity was coordinately related to proficient repair of HN2 alkylation products in an actively transcribed gene. The findings presented in this report show that α-amanitin markedly inhibited the removal of HN2-induced N-alkylpurines in the 5'-half of the essential, constitutively active DHFR gene, while no effect of α-amanitin was observed on the lesion removal from a noncoding region 3'-flanking to the DHFR gene and the genome overall. Nuclear run-off transcription reactions verified that α-amanitin completely and selectively inhibited transcription by RNA polymerase II.

The nuclear run-off experiments revealed a profound HN2-mediated inhibition of transcription of both the DHFR gene and rDNA. The hamster DHFR gene has several G-rich GC boxes proximal to the major DHFR transcription start site and several GC boxes of the opposite orientation (C-rich) in a distal region upstream (35). The first exons lie within a region marked by an exceptionally high G+C content and lack of DNA methylation, which correlates with transcriptional activity (35). At 150 μM HN2, we detected less than one alkali-labile lesion per 10 kilobases in the DHFR probe region. Since regions of the genome rich in contiguous guanines are considered preferential target sites for HN2, one would expect the 5' portion of the gene to contain a large fraction of this damage at or near the promoter sequence. Although the exact sequence of the sites of lesions are unknown, other studies have shown that HN2 produces transcription-terminating lesions at or near guanine pairs in vitro (24) and that the DNA sequence selectivity of N7-guanine alkylation by HN2 is preserved in intact cells (15). Also, ongoing transcription of the ribosomal probe sequence was significantly reduced immediately following exposure to HN2. Since ribosomal genes of mammalian cells are known to be significantly enriched in G+C content with several large segments containing >80% G+C (36, 37), the transcription of these genes impeded was as expected by HN2 alkylation. These observations further add to other reports demonstrating that transcribing polymerases stall at bulky lesions on the DNA template (38–42).

The α-amanitin experiments showed obliteration of preferential repair of HN2-induced N-alkylpurines in the actively transcribed DHFR gene. α-Amanitin had no effect on repair in the noncoding region. The combined results suggest that the transcriptional complex can direct repair enzymes to the DNA lesions possibly because these act as a termination site of RNA polymerase or at least stall its progress. Alternatively, the observed differences in repair could be related to a unique or enhanced alteration in the DNA secondary structure, which targets the repair enzyme to the site of lesion. Moreover, HN2 and other bulky adducts, which cause termination of transcription, may provoke a buildup of truncated transcripts or stalled transcription complexes further which could distort the DNA and act as a signal for the repair enzymes. Either scenario would explain the preferential removal of HN2-induced N-alkylpurines from the actively transcribed DHFR locus. The recent experiments by May et al. (43) showed a slight strand bias toward repair of HN2-induced N-alkylpurines in the transcribed strand of the hamster DHFR gene. Although these studies attempted to examine a role for transcription in preferential repair, they did not reveal any direct role of participating transcription factors. While the observed difference of the repair rate between the template and complementary strand, however, was less than the α-amanitin-sensitive fraction in this study, both studies point to a role for transcription in gene-specific repair.

Although provocative, the use of the elongation-specific inhibitor α-amanitin may also leave open an alternative model: preferential repair requires the continuous expression (i.e., transcription translation) of a very labile or unstable "coupling" factor. Such a drastically different model may be supported by the fact that α-amanitin is not selective for the inhibition of RNA polymerase II transcribing the DHFR gene but may inhibit the elongation step mediated by any RNA polymerase II. Thus, the inhibition of gene-specific repair may alternatively, or additionally, result from inhibition of transcription of this coupling factor rather than inhibition of DHFR transcription per se. Within this context, it is interesting that an intriguing transcription-coupling factor that conferred gene- and strand-specific repair of UV, psoralen, and cisplatin-induced damage recently was partially purified (44). This important finding further supports the notion that a protein couples transcription and repair; yet the actual candidate(s), remains to be identified.

The treatment of cells with α-amanitin had no discernible effect on the removal of N-alkylpurines from the genome overall. This suggests, that genes which are actively transcribed by RNA polymerase II must represent a small component of the total cellular genome. Furthermore, the neutral CsCl gradient profiles revealed that the global semi-
strand is mediated by enzymes associated with the transcription machinery. While we cannot completely rule out an indirect effect of the α-amanitin-mediated transcription block on chromatin structure, the simplest interpretation of our results is also a transcription-mediated direction of repair toward lesions in the DNA. Yet, a combination of cellular factors that help repair complexes to find and correct the lesions may exist. Nonetheless, as HN2-treated cells were allowed to incubate in drug- and α-amanitin-free medium, the RNA polymerase II resumed to a remarkable level (75.9% of control), which allowed the active gene region to be efficiently repaired.

Examining transcription activity by nuclear run-off analysis provides information concerning the ongoing transcription and may, in turn, afford valuable information regarding the processing of DNA lesions introduced into the gene fragment of interest. As such, the run-off analysis invites speculation that HN2-induced N-alkylpurines may remain in ribosomal genes, in contrast to in the 5′-half of the DHFR gene, because only a minor recovery of transcription products was observed upon repair incubation (Fig. 4). It is important to consider when interpreting data derived from nuclear run-off analysis, however, that inhibition of transcription run-off depends on the distance between the promoter and RNA probe as well as the lesion frequency within this gene segment. Such factors are important for comparison of transcription inhibition of different genes and should be further explored. Yet, our findings correlate with previous reports showing inefficient removal of psoralen photoadducts (47), pyrimidine dimers, and HN2 alkylation (48) from rRNA genes.

Recently, other studies have shown that α-amanitin reduces preferential repair of UV-induced pyrimidine dimers in mammalian cells (45, 49, 50); yet, only in the study by Christians and Hanawalt (45) did the authors verify the selective effect of α-amanitin on RNA polymerase II. In the case of HN2-induced N-alkylpurines we have shown that ongoing activity of RNA polymerase II confers preferential repair in the hamster DHFR gene, but questions still remain regarding how the transcriptional complex directs repair to the lesion.

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TRANSCRIPTION-DEPENDENT GENE REPAIR OF HN2 ALKYLYATION


Ongoing Activity of RNA Polymerase II Confers Preferential Repair of Nitrogen Mustard-induced N-Alkylpurines in the Hamster Dihydrofolate Reductase Gene

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