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Gene Repair: Depletion of PADPRP by Antisense RNA Expression

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ABSTRACT

To define the role of poly(ADP-ribose) polymerase (PADPRP) in DNA repair, we have developed cell lines from human HeLa cells, that express abundant PADPRP antisense transcripts under the control of MMTV promoter. The depletion of endogenous PADPRP by induced antisense RNA expression was established by: (1) a progressive synthesis of antisense transcripts in cells as assessed by Northern analysis; (2) an 80% decrease in activity of the enzyme; and (3) a greater than 90% reduction in the cellular content of PADPRP protein, as demonstrated by both immunoblotting and immunohistochemical analysis in intact cells.

In cells expressing antisense RNA of PADPRP the ability to repair MMS-induced DNA single-strand break is totally inhibited at 20 minutes, partially recovered after 45 minutes, and fully recovered after 90 minutes indicating that the low amount of PADPRP enzyme delayed the DNA single-strand break repair.

In order to examine the role of PADPRP in preferential gene repair, we have measured the nitrogen mustard (HN2) and UV light repair in the active gene,

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dihydrofolate reductase gene (DHFR). Results indicate that the antisense cells show no HN2 repair within 1-3h, 10% repair at 8 h and only 29% repair at 24 h. However, cells in the control group showed 13-26% repair at 1-3 h, 67% repair at 8 h and almost total repair at 24 h. No difference in UV repair was observed between antisense and control cells, indicating PADPRP plays an important and complicated role in DNA repair with different DNA damaging agents.

Introduction

Poly(ADP-ribose) polymerase (PADPRP) is a nuclear protein thought to participate in DNA repair and replication as well as other cellular processes, including differentiation, transformation, sister chromatid exchange, gene rearrangements, and transposition. However, a majority of past experiments supporting a function for PADPRP have been indirect, because they have depended predominantly on the use of chemical inhibitors, most of which lack specificity. A stably transfected Hela cell line which inducibly expresses PADPRP antisense mRNA was established to gain further insight into the biological roles of the poly(ADP-ribosyl)lation modification of nuclear protein in DNA gene repair.

Results

Vector, transfection and selection of antisense-positive clones by PADPRP activity assay. A full-length human cDNA encoding PADPRP was subcloned into the expression vector pMAM-neo under the control of the MMTV promoter (Fig. 1). pMAM-As or pMAM-neo vector alone (as a control) were transfected separately into HeLa cells, which were then selected in G-418 (400μg/ml) for four weeks (2). Eight G-418-resistant clones were then purified and expanded in large cultures. As an initial screen for identification of clones expressing the inducible PADPRP antisense RNA, PADPRP activity was measured in sonicated extracts of the selected G-418 resistant clones. After induction with 1μM dexamethasone (Dex) for 48 h, clone #7 (antisense cells) showed the highest level of inhibition (50%) and was therefore chosen for further characterization.

Expression of PADPRP antisense RNA in transfected cells. We investigated whether PADPRP antisense transcripts accumulated in antisense cells in response to Dex. A riboprobe that specifically hybridizes to PADPRP antisense RNA was prepared by cloning a 940 bp fragment of PADPRP cDNA into pGEM-4z, downstream of the bacterial T7 promoter. Because of the long half-life of the enzyme (about 24 h), induction of PADPRP antisense RNA was quantitated over 72 h. Total RNA was isolated at time points between 0 to 72 h and analyzed by Northern hybridization (Fig. 2). Dex did not induce a hybridizable transcript in control cells (Fig. 2 lanes 7 to 11). Antisense RNA was detectable within 2 h of Dex treatment in antisense cells, and the amount remained relatively constant for at least 48 h. Partial or total degradation of antisense transcripts after 48 h induction was observed (Fig. 2 lane 5). This finding is consistent with several observations

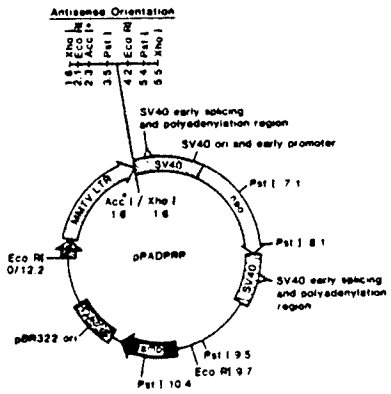


FIG. 1. **Structure of pMAMneo and restriction sites of PADPRP antisense cDNA.**

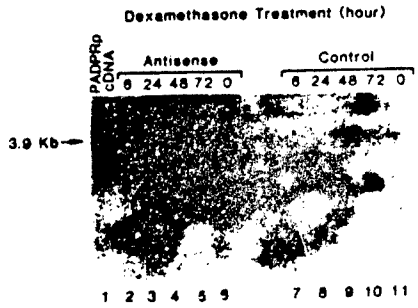


Figure 2. Expression and stability of PADPRP antisense transcripts in antisense and control cells after induction.

Incubation time	PADPRP activity		Inhibition
	−Dexamethasone	+Dexamethasone	
	pmol min⁻¹ mg⁻¹ protein		%
Control cells (Vector alone)			
48 h	440 ± 50	430 ± 26	
72 h	460 ± 38	450 ± 23	
Antisense cells (PADPRP-as[7])			
48 h	430 ± 43	190 ± 9	57
72 h	390 ± 30	70 ± 10	83

Table I. Effect of antisense inhibition on PADPRP Activity.

showing that antisense transcripts may be unstable and that hybrid sense-antisense duplex RNAs have short half-lives.

Effect of antisense RNA induction on PADPRP activity. We measured PADPRP activity in antisense and control cells after incubation in the presence or absence of Dex for 48 or 72 h (Table I). The specific activity of PADPRP remained constant in the control cells during 72 h of culture, and Dex had no effect on PADPRP activity. In contrast, Dex caused a significant reduction in PADPRP-specific activity in antisense cells. The specific activity in antisense cells was reduced by 57% and 83% after 48 and 72 h induction, respectively. The similarities between the time courses of the reduction in enzyme specific activity and of the concentration of PADPRP antisense RNA (Fig. 2) are consistent with the induction of antisense RNA causing a reduction in translation of endogenous PADPRP mRNA, which in turn leads to a decrease in the amount of enzyme protein and activity.

Effect of antisense RNA induction on PADPRP protein. Total cellular protein, extracted from both antisense and control cells incubated with Dex for various periods of time, was subjected to immunoblot analysis with antibodies to human PADPRP. Fig. 3A shows a Coomassie blue-stained duplicate of the gel used for immunoblotting (Fig. 3B) and reveals equivalent protein loading for all samples. In control cells (Fig. 3B, lanes 4 and 5), incubation with Dex for 72 h caused no apparent change in the amount of PADPRP. The amount of PADPRP in induced antisense cells was approximately the same as that in control cells (Fig. 3B, lanes 1 and 4). In contrast, the amount of enzyme was markedly reduced by induction of PADPRP antisense RNA. After 48 h of induction (Fig. 3B, lane 2), a small amount of immunologically reactive PADPRP was detected upon inspection of the freshly developed membrane, and by 72 h, essentially no PADPRP band was visible (Fig. 3B, lane 3).

Phenotypic characteristics of cells expressing PADPRP antisense RNA. We performed immunohistochemical staining with the antibodies to human PADPRP on antisense cells before (Fig. 4A) and after (Fig. 4B) four days of incubation with Dex. In the absence of hormone, the nuclei showed dark red uniform staining of PADPRP (which appear as darkly stained regions in Fig. 4). In contrast, the same cells incubated with Dex for 4 days showed negligible nuclear staining for PADPRP. The cells also exhibited an altered morphology. In general, the hormone-treated antisense cells appeared to be more spindle-shaped and displayed a mosaic structure not usually observed for HeLa cell growth; more than 90% of the cells were viable according to staining with trypan blue. The most prominent morphological change was a tendency of some cells to exist as multinuclear aggregates (Fig. 4B, arrows).

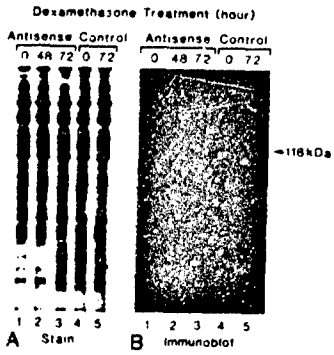


Figure 3A and 3B. Effect of dexamethasone induction of antisense RNA on cellular PADPRP content as determined by immunoblotting.

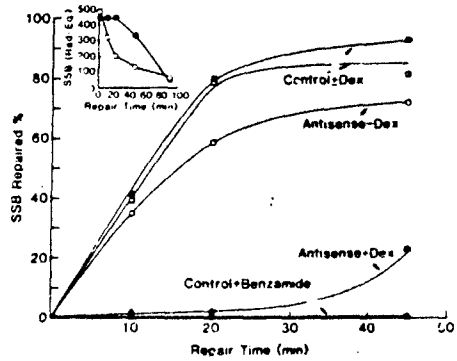


Figure 5. The repair of MMS-induced SSBs in control and antisense cells.

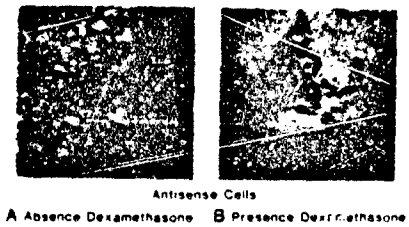


Figure 4A and 4B. Effect of induction of antisense RNA on nuclear content of PADPRP as detected by immunochemical staining.

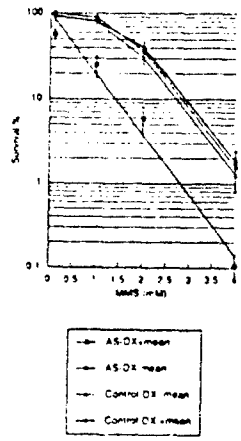


Figure 6. The cell survival curve of antisense and control cells treated with MMS.

Effect of antisense RNA expression on poly(ADP-ribosyl)ation and DNA strand break resolution. In order to determine whether poly(ADP-ribosyl)ation participates in DNA single-strand break (SSB) rejoining, we first established the concentration range in which the alkylating agent, methyl methanesulfonate (MMS), caused sufficient DNA strand breaks as detected by the alkaline elution method. With 2 mM MMS for 1 h, DNA damage representing 500-600 rad-equivalents was observed in both control and antisense cells.

Despite a significant reduction in PADPRP content of the induced antisense cells, the alkaline elution curves after 5 h of repair were the same, showing approximately 95% DNA SSB repair for both cell lines in the absence or presence of hormone. Accordingly, repair capacity was analyzed at relatively early repair periods (10 - 90 min). After 90 min, 90% of DNA repair was again complete; therefore, no difference in the *extent* of SSB repair was apparent in antisense cells, whether induced or uninduced (Fig. 5, insert). Thus, minute nuclear amounts of PADPRP appear to be sufficient to allow SSB repair. However, significant differences were noted in the capacity for SSB rejoining at the early time point. In control cells, the SSB rejoining at 10, 20 and 45 min was the same in the absence and presence of Dex, representing 34%, 58% and 82% of SSB rejoining (Fig. 5). Similarly, for the uninduced antisense cells the rejoining rate displayed approximately the same pattern. Control cells treated with or without Dex as well as uninduced antisense cells, showed essentially the same alkaline elution curves either during short time repair (10 - 45 min) or long time repair (5 h) (Fig. 5) indicating the absence of the effect of Dex, *per se*, on SSB repair. In contrast, the induced antisense cells showed a significant reduction in the SSB repair rate (Fig. 5, insert). After 10 and 20 min, no SSB rejoining had occurred. Even after 45 min of post-damage incubation, only 25% of SSB rejoined. However, repair resumed very rapidly from 45 to 90 min. At 90 min approximately 90% of SSB rejoining had occurred, which was equivalent to that of uninduced antisense cells (Fig. 5, insert). In agreement with the results of others, when endogenous PADPRP activity was completely inhibited by benzamide (5 mM), no SSB rejoining was observed for up to 45 min.

Taken together, our data suggest that the PADPRP concentration in HeLa cells is not limiting for DNA repair. However, initial rates of DNA SSB rejoining are markedly inhibited by depletion of this enzyme.

Effect of Reduced Nuclear PADPRP Content on Cell Survival. To further extend these observations on MMS-induced strand break rejoining, the survival of control and antisense HeLa cells after exposure to various concentration of MMS and their ability to form colonies was determined (Fig. 6). The cells were incubated in the presence or absence of Dex (1 μM) for 72 h. Subsequently, Dex was removed from cultures and colony survival curves were obtained for 60 min exposures to 0.1 - 4.0 mM of MMS. The data indicated that at a dose of 4 mM MMS, the control cells and uninduced antisense cells showed one log of kill. But, induced antisense

cells showed a 10-fold sensitivity to the same dose of MMS. Also, it was of interest that the disappearance of the shoulder of the cell survival curve of antisense cells further indicated the enhanced toxicity to induced antisense cells. This biological assay further confirms earlier data that reduced PADPRP inhibits DNA repair.

Repair of alkylation adducts in DHFR gene caused by HN2 treatment. It has become evident that the DNA damage is preferentially repaired in active genes as compared to inactive genes and noncoding regions. It is now also apparent that nitrogen mustards are a group of agents which react with native DNA in a sequence-specific manner. These agents predominantly cause the alkylation of guanines in the N-7 position or with GC-rich regions to form DNA interstrand cross-links. Their biological effects may depend on preferential reaction at certain genomic locations (2). In order to better understand the role of PADPRP in gene-specific repair, especially in preferential repair of active gene damage introduced by HN2, we analyzed the repair of the active gene for DHFR gene repair in antisense cells. As indicated in Fig. 7, we incubated antisense and control cells with medium in the presence or absence of Dex for 3 days. On the last day of Dex treatment, the cells were treated with 50 μM HN2 for 1 h and then allowed to repair DNA damage for 0 to 24 h. The parental DNA was isolated and purified as described (Fig 7). The repair kinetics were determined in the DHFR gene (an active gene). In the 20-kb Hind III fragment of the DHFR gene, we found no difference in DHFR gene repair either in short repair (1-4 h) or long repair (8-24 h) in control cells (Figs. 8A and 9A) indicating that Dex alone has no effect on gene repair. However, in the antisense cells induced by Dex, DHFR gene repair was much slower than that in the antisense cells with no Dex treatment. This effect was especially apparent in long repair (8-24 h repair). Figures 8B and 9B show that induced antisense cells only removed about 20% and 30% of alkylation adducts within 8 and 24 h , respectively. However, the HN2-induced lesions were removed more efficiently within a 24 h period in the antisense cells with no Dex treatment. Thus, about 50% and 70 % of repair occurred within 8 h and 24 h, respectively. This apparent distinction between repair patterns of induced and non-induced antisense cells indicates that an 80% reduction of PADPRP inhibits the repair of HN2-induced DNA lesions in the active DHFR gene. An equally interesting observation noted was that the reduction of PADPRP had no effect on the UV light repair in antisense cells (Fig. 10). Antisense cells that were either induced with Dex or not induced with Dex showed the same repair kinetics: about 25% of UV-dimer was removed within 4 h and more than 80% of repair occurred within 24 h. It is evident that UV light-induced damage has been shown to be homogenous over the genome and cyclobutane pyrimidine dimers and photoproducts are excised rapidly from actively transcribed genes, but persist longer in the noncoding regions of the genome in CHO cells (1). The different repair pattern for DHFR gene damage induced by HN2 or UV light in the antisense cells strongly suggests that PADPRP plays a specialized role in active gene repair.

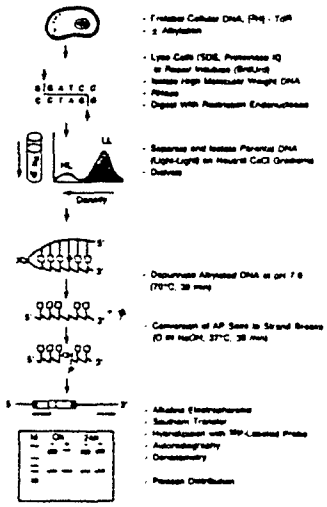
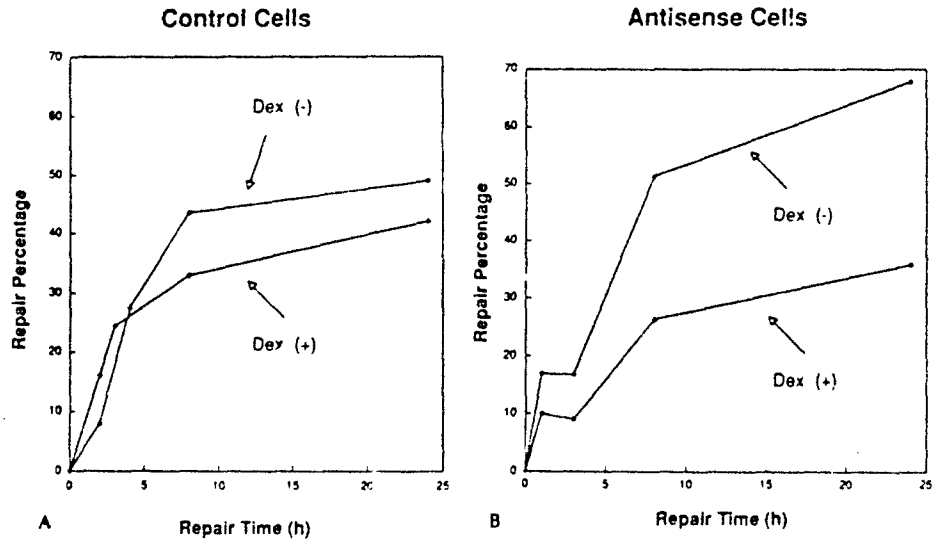
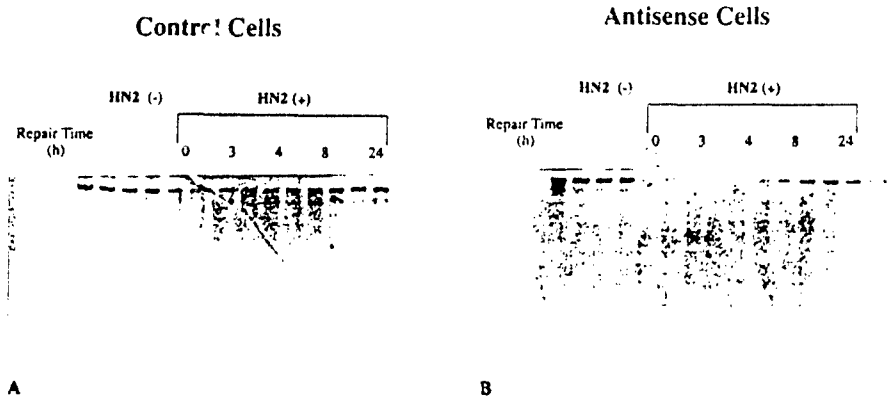


Figure 7. Flow chart describing the experiment procedures for preferential gene repair (taken from Bohr ref. 1).



Figures 8A and 8B. The repair curve of HN2-induced lesion in DHFR gene in antisense and control cells.



Figures 9A and 9B. The autoradiogram of the repair of HN2-induced lesion in DHFR gene in antisense and control cells.

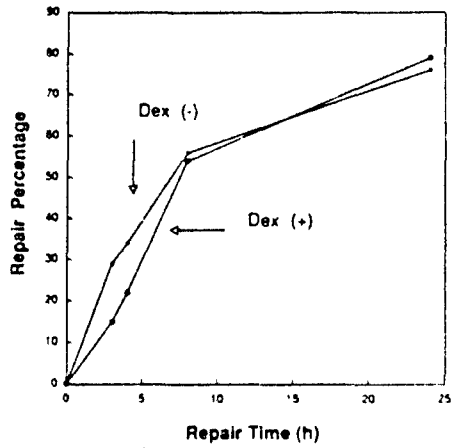


Figure 10. The repair of UV-induced lesion in DHFR gene in antisense cells.

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