Depletion of Poly(ADP-ribose) Polymerase by Antisense RNA Expression Results in a Delay in DNA Strand Break Rejoining*

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The effects of inducible expression of poly(ADP-ribose) polymerase (PADPRP) antisense RNA in HeLa cells were determined in order to gain further insight into the biological roles of the poly(ADP-ribose)ylation modification of nuclear proteins. A recombinant expression plasmid was prepared with the mouse mammary tumor virus (MMTV) promoter upstream of the antisense-oriented PADPRP cDNA. Expression of the antisense RNA was under strict control, with negligible effects on cell growth being apparent in the absence of inducer. Consistent with the previously described stability of PADPRP (half-life of at least 2 days, in vivo), 48–72 h were required after induction of antisense RNA expression by dexamethasone for the abundant concentration of PADPRP, normally present in HeLa cells, to be reduced by greater than 80%. The depletion of endogenous PADPRP as mediated by induced antisense RNA expression was established by: (i) a progressive synthesis of antisense transcripts in cells as assessed by Northern analysis; (ii) an 80% decrease in activity of the enzyme; and (iii) a greater than 90% reduction in the cellular content of PADPRP protein, as demonstrated by both immunoblotting and immunohistochemical analysis in intact cells. Several biological parameters were monitored in cells depleted of PADPRP. The chromatin of PADPRP-depleted cells was shown to have an altered structure as assessed by deoxyribonuclease I susceptibility. Cell morphology was also altered, with multinucleated aggregates being evident 72 h after induction of antisense RNA expression. Cells depleted of PADPRP were not able to commence DNA strand break joining of damaged DNA. However, DNA repair capacity was re-established at later time periods, indicating that PADPRP may contribute to alterations in chromatin structure that occur initially in DNA strand break joining and that the concentration of the enzyme in nuclei exceeds the requirement for DNA repair/replication.

Poly(ADP-ribose) polymerase (PADPRP)1 is a nuclear, chromatin-bound enzyme that has an absolute requirement for DNA for activity and is proportionally activated by the presence of strand breaks in the DNA (1–4). PADPRP is thought to participate, along with other enzymes, in DNA repair and replication and in other cellular processes, including differentiation, transformation, sister chromatid exchange, and gene rearrangements and transpositions, in which cleavage and rejoining of DNA segments may be required (5–7). In this regard, it is of interest that Yamagoe et al. (8) have recently shown, by the use of PADPRP inhibitors, that poly(ADP-ribose)ylation may mediate UV-induced HIV-1 gene expression (8). PADPRP uses nuclear NAD as a substrate to catalyze the poly(ADP-ribose)ylation of a subclass of nuclear acceptor proteins, which include histones, topoisomerases, high mobility group chromosomal proteins, and SV40 large T antigen (9, 10). Human and mouse PADPRP cDNAs have been cloned and sequenced and subsequently overexpressed in COS cells by transient transfection in an attempt to analyze the role of the enzyme in DNA strand break repair after exposure to x-radiation (11). Poly(ADP-ribose)ylation has frequently been inferred to play an important role in some unspecified step in DNA strand break resolution, yet the majority of experiments supporting this hypothesis have been indirect and have depended on the use of chemical inhibitors of PADPRP (9, 10, 12). Many of these inhibitors (for example, 3-aminobenzamide and thymidine) have been shown to be useful but may lack specificity (13) in elucidating the biological roles of PADPRP.

Stable integration and expression of human PADPRP cDNA in mouse cells were recently achieved in an attempt to help clarify the mechanisms by which the mRNA for this enzyme is regulated (14). Stably transfected cell lines capable of expressing antisense transcripts in response to a specific inducing agent have helped to reveal the biological roles of several enzymes that are important for cell growth and differentiation and DNA repair (15–20). We therefore attempted to deplete intact cells of PADPRP with the use of an antisense expression vector in order to help define the cellular functions of this enzyme. The fact that this method reduces the amount of enzyme protein rather than simply inhibiting activity is particularly advantageous for elucidating the biological roles of this protein that may have essential structural as well as enzymatic functions in chromatin (21–23). We established a cell line from human HeLa-S3 cells in which the expression of PADPRP antisense transcripts is controlled by a MMTV 1 The abbreviations used are: PADPRP, polyadenosine diphosphate ribose polymerase; MMTV, mouse mammary tumor virus; MMS, methyl methanesulfonate; SSB, single strand breaks; kb, kilobase(s); bp, base pair(s); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; LTR, long terminal repeat; HIV, tumor immunodeficiency virus.
long terminal repeat (LTR) promoter and is induced by dexamethasone. Despite the fact that the biological half-life of PADPRP in cells is relatively long (48–72 h), we found that induction of PADPRP antisense transcripts could be adjusted to cause a progressive decrease in both PADPRP activity and protein in the nucleus.

The development of this stably transfected cell line provides a system in which to test directly whether poly(ADP-ribose)ylation participates in, and perhaps is rate-limiting in, the recovery of cells after DNA strand breaks and probably during replication (21–23). Because poly(ADP-ribose)ylation is one of the earliest cellular responses to DNA strand breakage or exposure to mutagens (3, 4, 12) and because the genes for several key nuclear proteins (for example, c-fos and c-jun) as well as the HIV-1 LTR are transcriptionally activated by DNA strand breaks (24–26), perhaps via poly(ADP-ribose)ylation (8), this cell line may help to determine the role of nuclear protein modifications in growth and gene expression.

**MATERIALS AND METHODS**

Vector and Probes—A recombinant plasmid capable of generating RNA molecules was constructed complementary to the PADPRP cDNA as outlined below and expressed in the vector pMAMneo (Clontech). A 3.9-kb XhoI fragment containing the entire coding region of PADPRP (27, 28) was placed into a multiple cloning site adjacent to the MMTV LTR. The antisense orientation was differentially expressed from the sense orientation by restriction enzyme mapping (Fig. 1) utilizing AccI, EcoRI, and XhoI endonucleases and Southern hybridization with 32P-labeled full-length PADPRP prepared by random primer extension (>106 cpm/μg of DNA). To demonstrate antisense PADPRP transcripts using Northern blot analysis, single-strand RNA probe was prepared from a bacterial T7 promoter containing plasmid, pGEM-4z (Promega Corp.), which contains a 940-bp BamHI insert from the 5′ end of the PADPRP cDNA. The plasmid was linearized with SmaI, and the 32P-labeled sense orientation of RNA probe complementary to antisense PADPRP transcripts was synthesized with T7 RNA polymerase.

Cell Culture and Transfection—HeLa-S3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and 100 μg/ml streptomycin (all from Gibco Laboratories). For transfection, 5 × 105 cells on 100-mm plates were exposed overnight to 20 μg/dish of calcium phosphate-precipitated DNA (Stratagene, La Jolla, CA). After 24 h, transfectants were selected by culturing in medium which contained 0.2 to 0.4 mg/ml G-418 sulfate (Gibco Laboratories), until colonies appeared (approximately 100 colonies per plate). Eight surviving clones were selected and grown to mass culture under continued G-418 selection. The remaining clones were pooled and frozen in liquid nitrogen.

Growth Curve—Cells (5 × 105/plate) were seeded on 100-mm plates and, after 1 day, incubated in the presence or absence of dexamethasone. Samples were collected at day 1, 2, 3, 5, and 7, and the cell number and viability were determined by cell counting in the presence of trypan blue. The doubling time was calculated during the linear portions of cell growth.

DNA and RNA Analysis—Genomic DNA from 5–6 × 105 cells was isolated for each cell clone according to Sambrook et al. (29). DNA (20 μg) was digested with PstI, XhoI, and EcoRI restriction endonuclease and fractionated by 1% agarose gel, and Southern blot transfer and hybridization were performed. Total RNA from 1 × 106 cells was obtained for each cell clone by the guanidium/phenol method (30) and analyzed by Northern blot hybridization with 32P-labeled RNA probes (5–6 × 106 cpm/μg of template DNA).

Western Blot—Polyacrylamide gel electrophoresis and Western transfer were performed by standard procedures. The immobilized protein on the nitrocellulose paper was stained with 0.1% Ponceau S to confirm equal transfer. Polyclonal rabbit anti-human PADPRP (27) was used for the immunoblot; the color was developed using anti-rabbit IgG second antibody conjugated with alkaline phosphatase (Promega).

PADPRP Activity Assay—Cells in log phase (approximately 105 cells) were harvested by scraping, washed three times with ice-cold phosphate-buffered saline (PBS), and sonicated two times for 10 s each to break cells and introduce an excess of DNA strand breaks (required for PADPRP activity). The initial velocity of [32P]NAD incorporation into acid-insoluble acceptors was measured at 25°C for 1 min, according to the method described by Jacobson et al. (31). The specific activity of enzyme was expressed in picomoles of NAD incorporated per mg of cellular acceptor protein per min.

**Immunohistological Staining**—Cells were grown on ethanol-treated cover-slips and subjected to dexamethasone induction. The slips were washed in ice-cold PBS, fixed in cold paraformaldehyde for 5 min, and allowed to air dry. The primary antibody against human PADPRP was applied to the slips, followed by addition of biotinylated second antibody, which served as a linker between the primary antibody and the alkaline phosphatase-streptavidin conjugate. The signal-generating reagent, streptavidin-alkaline phosphatase, was then added to bind to the biotin residues on the linking antibody. The presence of enzyme was revealed by the addition of a mixture of substrate-chronogen solution (Zymed Laboratory Inc., Ostaside, CA).

**Results**

**Vector and Transfection**—A full length human cDNA encoding PADPRP, which was previously shown to express active enzyme in COS cells (11, 27), was subcloned into the expression vector pMAMneo under the control of the MMTV promoter as described in Fig. 1. The antisense orientation of

2 N. A. Berger, unpublished data.
As an initial screen for identification of clones required for PADPRP activity (31), activity in the G-418-resistant clones after induction by dexamethasone. The antisense insertion.

The entire cloned plasmid is 12.2 kb. Several restriction sites as well as mapping distances relative to the EcoRI site upstream of the PADPRP cDNA insert (27).

7.4 kb XhoI fragment was noted which is also specific for the antisense orientation. Accordingly, pMAM-as and pMAMneo (as a control) were transfected separately into HeLa cells, which were then selected in G-418 in a stepwise fashion with concentrations ranging from 200 to 400 μg/ml for isolation of transfectants containing a high copy number of integrated plasmids (35). The transfection efficiency was approximately the same (1 in 10⁴ cells) for cells transfected with either the control or antisense plasmid. After selection in G-418 for 4 weeks, eight G-418-resistant colonies were purified, expanded in large culture, and preserved by freezing. A large number of single cell colonies were also combined as a “pooled sample” and preserved by freezing.

Selection of Antisense-positive Clones by PADPRP Activity Assays—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 by dexamethasone. The sonication conditions had previously been established to maximize the number of endogenous cellular DNA strand breaks, which are required for PADPRP activity (31). Activity in the assay is proportional to the number of PADPRP molecules in a given extract, because automodified PADPRP is the major product of the reaction under these conditions (31). After induction with 1 μM dexamethasone for 48 h, PADPRP activity was compared in the eight isolated G-418-resistant cell lines. No reduction of PADPRP activity was noted in five of the G-418-resistant clones, whereas 25 to 50% inhibition was observed in three of the strains; clone PADPRP-as[7] showed the highest level of inhibition (50%) and was therefore chosen for further characterization (Table I). This concentration of dexamethasone (1 μM) was utilized in the current work based upon several studies with both human and murine cell lines which used this level of inducer (17, 36, 37). Additionally, we tested the effect of dexamethasone on cell viability by a trypan blue assay. Both antisense and control cells were treated with 1 μM dexamethasone for 1, 2, 3, 5, and 7 days. There was essentially no effect on dye exclusion by this concentration of inducer. Also, no effect on DNA repair by 1 μM dexamethasone was observed in control cells (see Fig. 8 below).

Genomic DNA Analysis—Chromosomal DNA prepared from the cell line PADPRP-as[7] and cells transfected with the vector pMAMneo were analyzed for the integration of antisense PADPRP sequences by restriction enzyme digestion with EcoRI, PstI, and XhoI, followed by Southern hybridization with a full length human cDNA probe for PADPRP (data not shown). In brief, digestion of the plasmid with AccI produced 11.5-kb and 0.7-kb fragments. A 5.5-kb EcoRI fragment was noted which is also specific for the antisense orientation. Accordingly, pMAM-as and pMAMneo (as a control) were transfected separately into HeLa cells, which were then selected in G-418 in a stepwise fashion with concentrations ranging from 200 to 400 μg/ml for isolation of transfectants containing a high copy number of integrated plasmids (35). The transfection efficiency was approximately the same (1 in 10⁴ cells) for cells transfected with either the control or antisense plasmid. After selection in G-418 for 4 weeks, eight G-418-resistant colonies were purified, expanded in large culture, and preserved by freezing. A large number of single cell colonies were also combined as a “pooled sample” and preserved by freezing.

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Genomic DNA Analysis—Chromosomal DNA prepared from the cell line PADPRP-as[7] and cells transfected with the vector pMAMneo were analyzed for the integration of antisense PADPRP sequences by restriction enzyme digestion with EcoRI, PstI, and XhoI, followed by Southern hybridization with the human PADPRP cDNA probe (Fig. 2). Genomic DNA from PADPRP-as[7] cells that had been digested with XhoI contained a 3.9-kb band corresponding to the expected size of full length PADPRP cDNA (Fig. 2, lane 7). Large amounts of radioactivity in bands corresponding to the size

TABLE I
Identification of antisense PADPRP transfectants: relative effects of antisense mRNA induction on PADPRP in vitro activity of G-418-resistant clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PADPRP activity</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>HeLa S₀ cells</td>
<td>570 ± 47</td>
<td>%</td>
</tr>
<tr>
<td>PADPRP-as[2]</td>
<td>540 ± 19</td>
<td>5</td>
</tr>
<tr>
<td>PADPRP-as[3]</td>
<td>520 ± 35</td>
<td>9</td>
</tr>
<tr>
<td>PADPRP-as[4]</td>
<td>540 ± 37</td>
<td>5</td>
</tr>
<tr>
<td>PADPRP-as[6]</td>
<td>340 ± 15</td>
<td>40</td>
</tr>
<tr>
<td>PADPRP-as[7]</td>
<td>280 ± 4</td>
<td>51</td>
</tr>
<tr>
<td>PADPRP-as[9]</td>
<td>680 ± 58</td>
<td>0</td>
</tr>
<tr>
<td>PADPRP-as[10]</td>
<td>590 ± 20</td>
<td>0</td>
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of PADPRP cDNA (27, 28) excised by either EcoRI or PstI (2.1 kb and 1.9 kb, respectively), were also noted in the same blot (Fig. 2, lanes 3 and 5, respectively) indicating that pMAM-as was incorporated intact into genomic sites with a high copy number. The large number of bands, other than those mentioned above, in each digestion also suggested that multiple integration of the exogenous gene into genomic DNA had occurred. Control cells (transfected with pMAMneo) showed the expected endogenous genomic PADPRP hybridization bands, but only after longer exposure times (Fig. 2, lanes 2, 4, and 6).

Expression of PADPRP Antisense RNA in Transfected Cells—Because PADPRP-as[7] cells contained multicity, integrated inverse PADPRP sequences (Fig. 2), and enzyme activity appeared to be significantly reduced in these cells after hormone induction, we investigated whether PADPRP antisense transcripts accumulated in PADPRP-as[7] cells in response to dexamethasone. A riboprobe that specifically hybridizes to PADPRP antisense RNA was prepared by cloning a 940-bp BamHI fragment representing nucleotide positions 2611 to 3551 from the AGT start site of PADPRP cDNA (28) into pGEM-4z, in an orientation such that the 5' BamHI region of the PADPRP cDNA was downstream of the bacterial T7 promoter. Induction of PADPRP antisense RNA was quantitated over a broad range of incubation periods, because of the long half-life of the enzyme and also because the regulation of endogenous PADPRP transcripts is complex and involves both mRNA stabilization and destabilization mechanisms (14). PADPRP-as[7] and control cells were incubated with dexamethasone, and total RNA was isolated at time points between 0 and 72 h and analyzed by Northern hybridization (Fig. 3). Dexamethasone did not induce a hybridizable transcript in control cells (Fig. 3, lanes 7 to 11). No antisense transcripts could be detected in PADPRP-as[7] cells in the absence of dexamethasone (Fig. 3, lane 6), indicating that the MMTV promoter is under tight control, a matter of concern given that many eukaryotic inducible expression systems have been reported to be "leaky." Antisense RNA was detectable within 2 h of dexamethasone treatment in PADPRP-as[7] 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. 3, lane 5). In several experiments (not shown), where cDNA rather than single strand probe was used, extensive degradation of PADPRP antisense and sense mRNA was observed, as evidenced by a hybridization smear, similar to the degradation noted in Fig. 3. This finding is consistent with several observations showing that antisense transcripts may be unstable and that hybrid sense-antisense duplex RNAs have short half-lives (38). Both PADPRP protein (Fig. 4, A and B) and activity (Table II) was markedly reduced by 48 h, and hence endogenous PADPRP mRNA was unavailable for translation by this time period. Current work, utilizing an RNAase protection assay, is directed at measuring the kinetics of the reappearance of both PADPRP mRNA and protein subsequent to the removal of dexamethasone.

**Effect of Antisense RNA Induction on PADPRP Activity**—The half-life of PADPRP is approximately 48 to 72 h in intact

![Fig. 3](image1.png)

**Fig. 3. Expression and stability of PADPRP antisense transcripts in PADPRP-as[7] and control cell lines after induction by dexamethasone.** PADPRP-as[7] and control cells (transfected with vector alone) (5 × 10^6) were treated in the presence or absence (time 0) of 1 μM dexamethasone. Total RNA was isolated after various time intervals, as indicated. That the amount of total RNA loaded in each lane (20 μg) was the same was verified by ethidium bromide staining. Samples were analyzed by Northern transfer and hybridization to a 32P-labeled riboprobe that detects PADPRP antisense RNA (see "Materials and Methods" and "Results"). Lane 1 represents hybridization to PADPRP cDNA (3.9 kb), which was used as a size marker for the expected antisense transcript.

![Fig. 4](image2.png)

**Fig. 4. Effect of dexamethasone induction of antisense RNA on cellular PADPRP content as determined by immunoblotting.** Control and PADPRP-as[7] cells were grown in the presence or absence of dexamethasone (1 μM). At the times indicated, cells were collected and washed with phosphate-buffered saline, and their protein concentration was determined. Equal amounts of total cellular protein (20 μg) were subjected to electrophoresis on duplicate SDS-polyacrylamide gels. A, Coomassie Blue staining. B, enzyme-linked immunoblotting with rabbit antibodies to human PADPRP as described under "Materials and Methods." The 116-kDa arrow indicates the position of human PADPRP.

### TABLE II

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>PADPRP activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[32P]NAD incorporation</td>
<td>%</td>
</tr>
<tr>
<td><strong>Control cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Vector alone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>440 ± 50</td>
<td>430 ± 26</td>
</tr>
<tr>
<td>72 h</td>
<td>460 ± 38</td>
<td>450 ± 23</td>
</tr>
<tr>
<td><strong>Antisense cells</strong></td>
<td>(PADPRP-as[7])</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>430 ± 43</td>
<td>190 ± 9</td>
</tr>
<tr>
<td>72 h</td>
<td>390 ± 50</td>
<td>70 ± 10</td>
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Cells (2 × 10^6 to 3 × 10^6/plate) were incubated in the presence or absence of dexamethasone (1 μM) for the indicated times. Subsequently, cells were washed, detached, sonicated in 0.25 M sucrose buffer containing 50 mM Tris (pH 8.0), 2 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride as described under "Materials and Methods." Initial velocity (1 min) assays of [32P]NAD incorporation were performed in triplicate as previously described (31). The data are expressed as the average ± S.D.

![Antisense to Poly(ADP-ribose) Polymerase and DNA Breaks](image3.png)
cells. It was therefore anticipated that a relatively long induction period of antisense transcription would be required to achieve a significant reduction in the cellular content of PADPRP. We measured PADPRP activity in PADPRP-as[7] and control cells after incubation in the presence or absence of dexamethasone for 48 or 72 h (Table II). The specific activity of PADPRP remained constant in the control cells during 72 h of culture, and dexamethasone had no effect on PADPRP activity. There was no marked change in PADPRP activity in PADPRP-as[7] cells incubated in the absence of dexamethasone for 72 h. Moreover, the specific activity of PADPRP in PADPRP-as[7] cells in the absence of hormone was similar to that in control cells, which is consistent with the results of the Northern analysis (Fig. 3) showing that the MMTV promoter was not leaky. In contrast, dexamethasone caused a significant reduction in PADPRP specific activity in cells transfected with the antisense construct. The specific activity in PADPRP-as[7] cells was reduced by 57% and 83% after 48- and 72-h incubations with dexamethasone, respectively, relative to the values for cells incubated without hormone. The similarities between the time courses of the reduction in enzyme specific activity and of the concentration of PADPRP antisense RNA (Fig. 3) 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. To obtain further evidence for this sequence of events, we performed immunoblot and immunohistochemical analyses.

Effect of Antisense RNA Induction on PADPRP Protein—Total cellular protein extracted from both PADPRP-as[7] and control cells incubated with dexamethasone for various periods of time was subjected to immunoblot analysis with rabbit antibodies to human PADPRP (27). Fig. 4A shows a Coomassie blue-stained duplicate of the gel used for immunoblotting (Fig. 4B) and reveals equivalent protein loading for all samples. Although the immunostaining experiment was not designed to be quantitatively proportional to the content of the enzyme, in control cells (Fig. 4B, lanes 4 and 5), incubation with dexamethasone for 72 h caused some apparent change in the amount of PADPRP. The amount of PADPRP in PADPRP-as[7] cells in the absence of induction was approximately the same as that in control cells (Fig. 4B, 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. 4B, 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. 4B, lane 3), which is consistent with the significant reduction in enzyme activity observed at this time (Table II).

Phenotypic Characteristics of Cells Expressing PADPRP Antisense RNA—To confirm the immunoblot results, we performed immunohistochemical staining with the rabbit antibodies to human PADPRP on PADPRP-as[7] cells before (Fig. 5A) and after (Fig. 5B) 4 days of incubation with dexamethasone. In the absence of hormone, nuclei showed dark red (which is represented as dark in Fig. 5) uniform staining of PADPRP. In contrast, the same cells incubated with dexamethasone for 4 days showed negligible nuclear staining for PADPRP, and the cells exhibited an altered morphology. Although these morphological changes may be attributable in part to the loss of PADPRP, they may also reflect cellular effects of the hormone per se, because small changes in morphology were also noted in control cells in the presence of dexamethasone. They may also be due to general cellular stress. In general, the hormone-treated PADPRP-as[7] cells appeared to be more spindle-shaped than normal 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. 5B, arrows).

Dexamethasone also caused a 28% increase in the doubling time of control cells. Associated with reduced PADPRP expression and the morphological changes in PADPRP-as[7] cells after dexamethasone treatment was an additional 9% increase in the doubling time of the cells. The morphological changes seemed to be reversible after removal of dexamethasone after 72 h and incubation of cells with fresh medium; normal cell morphology was partially restored by 48 h, although cell death of the altered cells could not be ruled out. Effect of Antisense RNA Induction on Chromatin Structure—We have proposed a structural, as well as catalytic role for PADPRP in chromatin (21–23). On the basis of the high abundance of PADPRP in nuclei (39) and data suggesting that the polymerase might be bound in chromatin with a periodicity of approximately one molecule per 8 to 10 nucleosomes (21, 22), PADPRP may fulfill a structural as well as functional role at each turn of the 300-Å chromatin fiber. We were thus interested in determining the effect of a decrease in PADPRP activity and protein on chromatin structure, as detectable by nuclease digestion. In the experiment shown in Fig. 6, PADPRP-as[7] or control cells were incubated with dexamethasone for 72 h, the last 24 of which [H]thymidine was also present. Nuclei were prepared and their sensitivity to DNase I digestion was determined. Treatment of control cells with dexamethasone had negligible effects on chromatin structure (Fig. 6). However, a marked difference in DNase I sensitivity was observed between nuclear DNA derived from control cells and that from cells depleted of PADPRP. During the linear portion of digestion (2 min), 30% of the DNA from PADPRP-depleted cell nuclei was digested compared to 10% of the DNA from control nuclei. The data were confirmed by measuring the release of nucleotides at 260 nm rather than by [H]thymidine labeling (not shown). These observations suggest that chromatin of the two cell lines may be organized differently.

Effect of Antisense RNA Expression on Poly(ADP-ribose) Polymerase and DNA Breaks—In order to determine whether poly(ADP-ribose)lation participates in, or is rate-limiting in, DNA single strand break (SSB) rejoining, which might also suggest a role for this nuclear protein...
modification in replication and recombination, we first established the concentration range in which the alkylating agent, methyl methanesulfonate (MMS) caused sufficient DNA strand breaks to be detectable by the poly(ADP-ribose)ylation response in PADPRP-as[7] and control cells. It has been consistently observed that NAD is rapidly depleted as a result of the activation of poly(ADP-ribose)ylation by the introduction of DNA strand breaks in cells either by radiation, alkylating agents, or H$_2$O$_2$ (9, 10, 32). Incubation of control cells with 1 mM MMS for 1 h caused a small depletion of NAD, whereas 5 mM MMS reduced the amount of NAD by greater than 90%. In agreement with the NAD data, a progressive increase in DNA strand breaks with increasing concentrations of MMS was observed as detected by the alkaline elution method (Fig. 7). With 2 mM MMS for 1 h, DNA damage representing 500–600 rad-equivalents was observed in both control and antisense cells.

To avoid nonuniform thymidine labeling that might result from the differential effects of dexamethasone on the growth of control and PADPRP-as[7] cells and which might result in errors in the alkaline elution assay, we labeled cells (5 × 10$^5$/plate) with [14C]thymidine prior to hormone induction. After labeling, the two cell lines were incubated in the presence or absence of dexamethasone for 72 h, treated with 2 mM MMS for 1 h, and allowed to carry out DNA repair for 5 h. Samples of cells corresponding to 10,000 to 20,000 cpm of [14C]thymidine were subsequently applied to polycarbonate filters as described (11, 34). To provide an internal standard, samples of cells corresponding to 10,000 to 20,000 cpm of [3H]thymidine-labeled L1210 cells that had been irradiated with 2000 rads to the samples just before the cells were lysed on the filter. Despite a significant reduction in PADPRP content of the PADPRP-as[7] cells treated with dexamethasone, the alkaline elution curves after 5 h of repair were the same, showing approximately 95% DNA SSB repair for both cell lines and in the absence or presence of hormone (data not shown). Accordingly, in subsequent experiments, 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 PADPRP-as[7] cells, whether induced or uninduced (Fig. 8, inset). Thus, minute nuclear amounts of dexamethasone were sufficient to allow SSB repair. However, significant differences were noted in the capacity for SSB rejoining at 10, 20, and 45 min. MMS-induced SSBs were quantitated by alkaline elution. The ability of control cells (not incubated with dexamethasone) to repair SSB in the presence of 5 mM benzamide is also shown (closed squares). The inset shows the SSB rad-equivalents of the DNA damage due to MMS treatment and its change during repair in the antisense cells, either induced or uninduced by dexamethasone.
ing (Fig. 8). Similarly, for the PADPRP-as[7] cell line, in the absence of dexamethasone the rejoining rate displayed the approximate same pattern. Control cells treated with or without dexamethasone as well as antisense cells without dexamethasone showed essentially the same alkaline elution curves either during short time repair (10-45 min) or long repair (5 h) (Fig. 8) indicating the absence of an indirect effect of dexamethasone, per se, on SSB repair. In contrast, the induced PADPRP-as[7] cells showed a significant reduction in the SSB repair rate (Fig. 8, inset). After 10 and 20 min, about 460 rad-equivalents of SSB remained, equal to the initial damage, indicating that no SSB rejoining had occurred during this time period. Even after 45 min of post-damage incubation, 330 rad-equivalents of breaks remained in the antisense cells, representing only 25% of SSB rejoining. However, repair resumed very rapidly from 45 to 90 min. At 90 min, only about 60 rad-equivalents of SSB were detected indicating that approximately 90% of SSB rejoining had occurred, which was equivalent to that of antisense cells, not induced by dexamethasone (Fig. 8, inset). 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 (40).

Taken together, our results suggest that PADPRP makes an important contribution to early stages of DNA strand break resolution. Our data also 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. The results indicate that under appropriate conditions complementary RNA can be induced in cells and can greatly lower both the amount and activity of PADPRP. This system thus provides a means for analyzing the influence of PADPRP on the reorganization of chromatin structure that occurs subsequent to DNA damage (41) or perhaps during DNA replication and such experiments are underway.

DISCUSSION

PADPRP has been suggested to have functional roles in several biological reactions, yet none of these roles has been firmly established. The cloning and sequencing of the cDNA for PADPRP (27, 28) led us to develop the strategy of selective expression of PADPRP antisense RNA for the analysis of that under appropriate conditions complementary RNA can break resolution. Our data also suggest that the PADPRP antisense expression may be mediated via which Northern blots were probed with PADPRP cDNA to constant for at least 48 h (Fig. 3). In several experiments in which Northern blots were probed with PADPRP cDNA to detect both sense and antisense RNAs, considerable degradation of hybridizable regions was apparent, suggesting that the effects of antisense RNA expression may be mediated via hybrid-induced degradation of endogenous PADPRP mRNA. It has been shown previously that the steady state concentration of endogenous PADPRP mRNA is relatively low and that the changes in PADPRP mRNA accumulation that occur during replicative phases of the cell cycle result from post-transcriptional stabilization and destabilization mechanisms (14). In this regard, by performing indirect mutagenesis, Berger and colleagues obtained V<sub>α</sub> Chinese hamster lung cell lines with reduced PADPRP activity and a 5-fold increase in doubling time compared to parental cells (42). The half-life of PADPRP, as assessed by [35S]methionine labeling of cells followed by a nonradioactive methionine chase, has been estimated to be quite long (48 to 72 h); we were therefore concerned that such a protein might not be susceptible to experimental manipulation with antisense constructs. However, the generation of PADPRP antisense transcripts in the current study was accompanied by a progressive decrease in the nuclear content of PADPRP protein, such that by 72 h of antisense RNA induction, enzyme protein could not be detected by immunoblot analysis (Fig. 4), although approximately 15% of the enzyme molecules remained in the cells as detected by a sensitive enzyme assay (Table II).

Our results indicate that depletion of PADPRP results in hypersensitivity of chromatin to DNase digestion (Fig. 6). It has been demonstrated by a variety of experiments that chromatin regions corresponding to newly synthesized DNA repair patches also show a heightened and transient hypersensitivity to nuclease digestion (41). This hypersensitivity may be due to localized changes in the nuclear organization of the 300-Å fiber of chromatin, which may in turn be a result of those nucleosomes that are undergoing DNA repair possessing a relaxed or open DNA structure. Experiments performed in a number of laboratories have shown that poly(ADP-ribosyl)ation of chromosomal proteins may somehow influence the generation of DNA domain changes in chromosomes to allow for DNA replication and perhaps other reactions (9, 10, 42, 43). In this regard, Mathis and Althaus (44) have shown an influence of chromatin structure on DNA repair while studying the inhibition of DNA repair by chemical antagonists of PADPRP. An opening of chromatin structure became apparent during bulky adduct repair. The DNA adducts were not able to be excised and tended to accumulate in presumably non-nucleosomal domains of chromatin when poly(ADP-ribosyl)ation was interrupted. In an earlier study, we estimated that there are approximately 8 x 10<sup>5</sup> molecules of PADPRP per HeLa cell nucleus (45), a number that proved consistent with later estimates (46). Assuming uniform distribution, the enzyme might be bound at a domain frequency of approximately every 70–100 nucleosomes in chromatin. As noted earlier, we observed that the activity and content of the enzyme per unit of DNA chromatin changes with polynucleosome chain size and reached a maximum in polynucleosomes of a specific periodicity (21, 22) suggesting a potential relationship between PADPRP distribution and activity and chromatin organization similar to that suggested for topoisomerase II (47); this can now be addressed by the antisense approach.

The most interesting of our results is the observation that PADPRP-depleted cells performed very limited DNA repair during early time periods of recovery from MMS-induced DNA strand breaking. Repair of the damage induced by this type of alkylating agent is accompanied by a stimulation of poly(ADP-ribosyl) synthesis that is induced by strand breakage. PADPRP activity has been shown to increase in cells treated with agents, such as MMS, methylisothiourea, ionizing radiation, endonucleases, and numerous DNA-alkylating drugs, that induce SSBs (2, 4, 7, 16). During the initial period of DNA repair, the NAD concentration of PADPRP-depleted cells was not significantly reduced; in contrast to the situation with control cells. Substantial data exist supporting the view that a major role for poly(ADP-ribosyl)ation is the covalent modification of proteins in domains of chromatin that are actively engaged in DNA repair or recovery from DNA strand breaks (9, 10). In fact, fractionation of chromatin on an affinity column prepared from antibody to poly(ADP-ribosyl) verified the presence of significant levels of SSBs adjacent to
chromatin units engaged in poly(ADP-ribose)ylation (48, 49). Purified PADPRP appears to possess a high affinity for DNA containing SSBs (27, 45), and sequence analysis of PADPRP cDNAs has revealed that the human and mouse enzymes contain DNA binding domains with two potential zinc fingers (28).

Although the initial rate of DNA repair was greatly reduced in PADPRP-depleted cells, at later time points (90 min) the extent of DNA repair in these cells did not differ from that in control cells. These experiments suggest that even minute quantities of PADPRP are sufficient to allow DNA repair. This conclusion is consistent with earlier studies showing that DNA damage or strand breaks caused by physical or chemical agents did not cause an induction in the transcription of the PADPRP gene (11). Thus, the poly(ADP-ribose)ylation reactions that occur in cells immediately after DNA damage do not appear to require an increase in PADPRP mRNA or protein. Also, overexpression of PADPRP by transient transfection (11) did not significantly alter the extent of DNA repair, although the rate of repair was slightly enhanced. Cell survival studies coupled with DNA damage in the process of being performed; however, the design of such experiments has been thus far difficult because of the short repair window observed with the antisense cells. Initial results, however, indicate a 10-fold increased sensitivity to MMS when induced antisense cells were compared with noninduced cells. With respect to the data of Fig. 5, it should be noted that MMS is a simple alkylating agent which mainly methylates DNA and forms DNA adducts. At high doses of MMS, such as used here, it may also introduce DNA strand breaks. The alkaline elution assay measures DNA strand breaks either directly caused by MMS treatment or those formed during the DNA repair process to remove adducts. The disappearance of DNA strand breaks by 90 min probably represents DNA repair of the breaks caused directly by MMS and may not reflect total DNA adduct repair. Also, while the extent of DNA ligation appears to eventually be similar to controls in antisense cells, one must also take into account the re-establishment of chromatin structure which is required after DNA repair (41). PADPRP plays a prominent role in chromatin morphology, and the data of Fig. 6 clearly indicate that induced antisense cells have an altered chromatin structure. Accordingly, nucleosome reformation, per se, may not be complete in antisense cells by 90-min repair, although DNA ligation is. Experiments are currently directed at measuring this parameter, subsequent to DNA damage.

The establishment of the PADPRP-as[7] cell line has provided a new experimental system for elucidating the role of PADPRP and its mechanism of action in chromatin repair, replication, and recombination reactions, and in transcriptional activation involving DNA strand breaks (25, 26). Using HeLa cells transfected with the HIV-1 long terminal repeat driving the CAT gene, Yamagoe et al. (8) recently observed that chemical inhibitors of PADPRP suppress UV-induced HIV-1 gene expression but not tat-mediated expression. The inhibition appears to occur at the post-transcriptional level of expression and the mechanism of how the poly(ADP-ribose)ylation modification functions in this DNA strand break mediated gene activation could be further clarified by the use of the PADPRP-as[7] cell line, in the absence of inhibitors, which may have alternative effects (13).

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