The Expression of Poly(ADP-ribose) Polymerase during Differentiation-Linked DNA Replication Reveals That It Is a Component of the Multiprotein DNA Replication Complex†

Cynthia M. Simbulan-Rosenthal,‡ Dean S. Rosenthal,‡ Helmuth Hilz,§ Robert Hickey,|| Linda Malkas,⊥ Nancy Applegren,⊥ Yan Wu,⊥ George Bers,# and Mark E. Smulson*,‡

Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, NW, Washington, DC 20007, Institut fur Physiologie Chemie, Universitats-Krankenhaus, Eppendorf, 2000 Hamburg 20, West Germany, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, and Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201, and Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California 94547

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ABSTRACT: 3T3-L1 preadipocytes have been shown to exhibit a transient increase in poly(ADP-ribose) polymerase (PARP) protein and activity, as well as an association of PARP with DNA polymerase α, within 12−24 h of exposure to inducers of differentiation, whereas 3T3-L1 cells expressing PARP antisense RNA showed no increase in PARP and are unable to complete the round of DNA replication required for differentiation into adipocytes. The role of PARP in differentiation-linked DNA replication has now been further clarified at both the cellular and enzymological levels. Flow cytometric analysis revealed that control 3T3-L1 cells progressed through one round of DNA replication prior to the onset of terminal differentiation, whereas cells expressing PARP antisense RNA were blocked at the G_0/G_1 phase of the cell cycle. Confocal microscope image analysis of control S phase cells demonstrated that PARP was localized within distinct intranuclear granular foci associated with DNA replication centers. On the basis of these results, purified replicative complexes from other cell types that had been characterized for their ability to catalyze viral DNA replication *in vitro* were analyzed for the presence of PARP. PARP exclusively copurified through a series of centrifugation and chromatography steps with core proteins of an 18−21S multiprotein replication complex (MRC) from human HeLa cells, as well as with the corresponding mouse MRC from FM3A cells. The MRC were shown to contain DNA polymerases α and δ, DNA primase, DNA helicase, DNA ligase, and topoisomerases I and II, as well as accessory proteins such as PCNA, RF-C, and RP-A. Finally, immunoblot analysis of MRCs from both cell types with monoclonal antibodies to poly(ADP-ribose) revealed the presence of approximately 15 poly(ADP-ribosyl)ated proteins, some of which were further confirmed to be DNA polymerase α, DNA topoisomerase I, and PCNA by immunoprecipitation experiments. These results suggest that PARP may play a regulatory role within the replicative apparatus as a molecular nick sensor controlling the progression of the replication fork or modulates component replicative enzymes or factors in the complex by directly associating with them or by catalyzing their poly(ADP-ribosyl)ation.

Poly(ADP-ribose) polymerase (PARP) modulates the structure and function of several nuclear histone and non-histone proteins that contribute to either chromatin architecture or DNA metabolism by catalyzing their poly(ADP-ribosyl)ation. This posttranslational modification plays an auxiliary role in nuclear processes requiring cleavage and rejoining of DNA, such as DNA repair (Satoh & Lindahl, 1992; Satoh et al., 1993; Molinete et al., 1993; Ding et al., 1993; Smulson et al., 1994), DNA replication (Burzio & Koide, 1972; Lehman et al., 1974; Anachkova et al., 1989; de Murcia et al., 1983; Stone & Shall, 1975; Cesarone et al., 1990; Simbulan et al., 1993), recombination (Ferro & Olivera, 1984), and cellular differentiation (Farzaneh et al., 1987; Bhatia et al., 1990; Suzuki et al., 1989; Caplan & Rosenberg, 1975). PARP also plays a structural role in chromatin organization (Jump et al., 1979; Butt & Smulson, 1980), particularly since it has been shown to complex with and modify histone H1, which plays a role in the maintenance of higher order chromatin structure (Wong & Smulson, 1984; Malik & Smulson 1984, Wong et al., 1986). PARP undergoes extensive automodification, which has been implicated in a cycling mechanism by which the enzyme reversibly binds to DNA strand breaks (Satoh & Lindahl, 1992; Smulson et al., 1994). An ICE (interleukin-1β-converting enzyme)-like protease, apopain, has also recently been identified that specifically cleaves PARP to render it

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* To whom correspondence and reprint requests should be addressed at Georgetown University School of Medicine, Basic Science Building, Rm 351, 3900 Reservoir Road, NW, Washington, DC 20007. Phone: 202/687-1718. Fax: 202/687-7186.

‡ Georgetown University School of Medicine

§ Universitats-Krankenhaus.

|| University of Maryland School of Pharmacy.

⊥ University of Maryland School of Medicine.

Bio-Rad Laboratories.

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inactive during the "DNA ladder" stage of apoptosis, and inhibition of apopain by peptide inhibitors blocks apoptosis (Nicholson et al., 1995).

Past studies on the role of PARP in nuclear processes have relied largely on the use of chemical inhibitors of PARP, which have been useful but are limited because of a lack of specificity and their potentially unrelated effects on other biological processes (Hunting et al., 1985; Milam et al., 1986; Cleaver et al., 1985). Adopting a more specific approach, we have established and characterized several mammalian cell lines, including HeLa cells (Ding et al., 1993), keratinocytes (Rosenthal et al., 1995), and 3T3-L1 preadipocytes (Smulson et al., 1995), that are stably transfected with PARP antisense cDNA under the control of an inducible promoter. Induction of PARP antisense RNA in these cells has consistently shown significant depletion of endogenous PARP protein and activity. Using PARP-depleted cells, it was shown that PARP and poly(ADP-ribosyl)ation facilitate the initial rate of DNA repair in HeLa cells (Ding et al., 1993) and keratinocytes (Rosenthal et al., 1995). Survival of cells exposed to DNA damage due to mutagenic agents is reduced, chromatin structure is altered, and gene amplification is enhanced (Ding & Smulson, 1994). On the other hand, Wang et al. (1995) recently showed that although knock-out mice lacking PARP protein and activity are viable and fertile, PARP-deficient mutant fibroblasts and thymocytes derived from these mice exhibited proliferation deficiencies following DNA damage. Apparently, isolated cell systems may show more subtle effects due to the lack of PARP, which is not evident in whole animals.

Previous studies have suggested a role for PARP in terminal differentiation. Potent inhibitors of PARP, such as nicotinamide and benzamide, markedly inhibit differentiation of 3T3-L1 cells into adipocytes, apparently by preventing a transient increase in PARP activity that appears essential for entering the differentiation program (Lewis et al., 1982). PARP mRNA levels also increase 12−24 h after induction of HL-60 cells with retinoic acid or dimethyl sulfoxide (DMSO) (Bhatia et al., 1990), and a corresponding decrease in PARP mRNA occurs on the terminal differentiation of these cells into granulocytes (Suzuki et al., 1989). Stably transfected 3T3-L1 cells expressing PARP antisense RNA do not show the increase in PARP protein and activity normally apparent 24 h after exposure to insulin, dexamethasone, and methylisobutylxanthine, and they fail to differentiate into adipocytes (Smulson et al., 1995). The inability of the antisense cells to synthesize PARP during the critical period after exposure to inducers of differentiation appears to be related to their inability to undergo the round of DNA replication characteristic of the initial stages of differentiation. This induced DNA replication in postconfluent control cells may require PARP, in part, because it has been shown to form a complex with DNA polymerase α during differentiation-linked DNA replication in control cells (such a complex was not detected in PARP-depleted antisense cells) (Smulson et al., 1995).

Although a definitive functional role for PARP in DNA replication has yet to be clarified, the observations that PARP activity is markedly enhanced in proliferating cells (Burzio & Koide, 1972; Lehman et al., 1974), in newly replicated chromatin (Anachkova et al., 1989), and in DNA fragments enriched in replication forks (de Murcia et al., 1983) support a role for the enzyme in this process. Furthermore, PARP mRNA concentrations have been observed to be stable only during S and S/G_2 phases of the cell cycle (Bhatia et al., 1990); steady-state levels of poly(ADP-ribose) polymer are also highest during these phases of the cell cycle (Kidwell & Mage, 1976).

We have now performed cell cycle analysis of 3T3-L1 control and PARP antisense cells at critical early time points after induction of differentiation to better clarify the role of PARP in differentiation-linked DNA replication. In addition, we examined the localization of PARP in S phase nuclei by confocal microscopy and further explored the significance of the association of PARP with DNA polymerase α. These observations stimulated an examination whether PARP might be an active component of purified and well-characterized multiprotein DNA replication complexes (MRCs) from mouse FM3A and HeLa cells (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995).

MATERIALS AND METHODS

Cell Culture, Vectors, and Transfection. Monolayer cultures of control and PARP antisense 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin−streptomycin and subcultured every 4 days. The PARP antisense cell lines were obtained as previously described (Smulson et al., 1995) by transfection of 3T3-L1 preadipocytes with 20 μg of calcium phosphate precipitated pMAM-As DNA, a 1.1 kb *Xho*I fragment of murine PARP cDNA subcloned in the antisense orientation into the expression vector pMAM-neo (Clontech) under control of the mouse mammary tumor virus long terminal repeat (MMTV LTR). This was followed by selection of transfectants in medium with 400 μg/mL G-418. Expression of PARP antisense RNA was verified by RNA, DNA, and immunoblot analysis of control and stably transfected antisense cell lines after incubation with dexamethasone for various times.

Induction of Differentiation. Control and PARP antisense 3T3-L1 preadipocyte cells were grown to confluence in DMEM with 10% FBS and maintained for an additional 2 days postconfluence, after which differentiation was induced by addition of 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. This medium was replaced with DMEM with 1.7 μM insulin and 10% FBS after 48 h and placed back in the regular medium after another 48 h. Terminal differentiation of the 3T3-L1 preadipocytes was monitored by washing the cells in phosphate-buffered saline (PBS), followed by fixation in 3.7% formaldehyde/PBS for 10 min, staining with 0.3% Oil-Red-O dye for triglyceride droplets for 1 h, and observation under a phase contrast microscope.

Assays on PARP Activity and in Vivo DNA Replication Assay. At indicated time intervals after exposure to inducers of differentiation, control and PARP antisense cells were harvested by scraping and washed with ice-cold PBS, after which duplicate aliquots were kept for either PARP activity assay or for an *in vivo* DNA replication assay by [^{3}H]-thymidine incorporation as previously described (Smulson et al., 1995). For the PARP activity assay, cells were sonicated for 20 s (three times) to lyse cells and introduce DNA strand breaks required for PARP activity, followed by measurement of [^{32}P]NAD incorporation into acid-insoluble acceptors at 25 °C for 1 min as described by Cherney et al. (1985). For [^{3}H]thymidine incorporation, 10^6 cells were

aliquoted per cell culture well, allowed to stabilize in a 37 °C incubator for 1 h, pulsed for 15 min with [^{3}H]thymidine (40 Ci/mmol, 0.2 μCi/mL), harvested, washed with PBS, and lysed with 0.1% SDS and 1 mM EDTA. Incorporation of [^{3}H]thymidine into acid-insoluble DNA was monitored by scintillation spectroscopy.

Preparation of Nuclei for Flow Cytometric DNA/Cell Cycle Analysis. Cells were harvested at various times after induction of differentiation, and nuclei were prepared by a detergent−trypsin method for flow cytometric analysis as previously described (Vindelov et al., 1983). Briefly, the cells were trypsinized to obtain a single cell suspension, and after the trypsin was neutralized by serum, the cells were aliquoted to 10^6 cells per tube and centrifuged. The cells were then resuspended in 100 μL of citrate/DMSO buffer (pH 7.6), containing 250 mM sucrose, 40 mM citrate, and 5% DMSO, and cell lysis was done by incubation for 10 min with a detergent−trypsin mixture containing 30 μg/mL trypsin in a stock solution with 3.4 mM citrate, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris-HCl (pH 7.6). After incubation for a further 10 min with trypsin inhibitor and ribonuclease A (0.1 mg/mL) in the stock solution, nuclei were stained with propidium iodide (0.42 mg/mL) and spermine tetrahydrochloride (1.16 mg/mL) in stock solution for at least 15 min, filtered through a 37 μm nylon mesh, and run in a dual laser flow cytometer/cell sorter (FACS).

Immunocytochemical Staining and Confocal Microscope Image Analysis. Cells were pulsed for 1 h with 10 μM bromodeoxyuridine and washed with ice-cold PBS. The cells were then fixed with cold 95% ethanol for 30 min at room temperature and genomic DNA denatured with 0.07 M NaOH in 70% ethanol for 2 min, after which they were washed with PBS again and incubated in a humid chamber for 4 h with both mouse monoclonal anti-BrdU (Becton Dickinson, 1:10) and a polyclonal anti-PARP antibody (1:1000) in PBS with 12% BSA. The next incubation was done in the dark with a mixture of two secondary antibodies, rhodamine-labeled anti-mouse (Tago, 1:10) and goat fluorescence-labeled anti-rabbit (1:40), and washed with PBS and water. Finally, the plates were cut, mounted with Vectashield (Vector), and observed under a Zeiss immunofluorescence microscope, as well as under a Bio-Rad MRC 600 laser scanning confocal microscope.

Purification of Mouse and Human Multiprotein Replication Complex (MRC) from FM3A and HeLa Cells. Fractionation of FM3A or HeLa cells and purification of the MRCs were performed in a series of centrifugation steps and passage through two chromatography columns as previously described (Malkas et al., 1990; Wu et al., 1994). The experiments in this paper used protein fractions in various stages of purification designated as PEG NE/S3, P4, S4, DE-52 high salt, DE-52 low salt, Q-Sepharose peak, and Q-Sepharose flow-through (FT) fractions, in the case of the mouse FM3A MRC purification, and P4, S4, Q-Sepharose peak, Q-Sepharose flow-through (FT), and sucrose gradient peak fractions (SG), in the case of the HeLa MRC. The replication-competent form of the mouse FM3A MRC has been shown to exclusively reside in the P4, DE-52 high salt, and Q-Sepharose peak fractions (Wu et al., 1994), while the replication-competent HeLa MRC partitions exclusively with the P4, Q-Sepharose peak, and sucrose gradient peak fractions (Malkas et al., 1990; Applegren et al., 1995).

Immunodetection of PARP, Poly(ADP-ribose) Polymer, and DNA Polymerase δ, DNA Ligase I, Topoisomerases I and II, RFC, RPA, and PCNA. SDS−polyacrylamide gel electrophoresis and Western transfer to nitrocellulose membrane were performed according to standard procedures, with 20 μg of protein loaded per lane, using a Bio-Rad Mini-Protean II trans-blot apparatus. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. The blots were then blocked, incubated with either a polyclonal rabbit antibody which we have shown to bind murine PARP (1:2000; Ludwig et al., 1988) or a polyclonal rabbit anti-human PARP antibody (1:5000). The blots were then probed with peroxidase-labeled anti-rabbit IgG (1:3000) and detected by enhanced chemiluminescence (ECL, Amersham). To detect poly(ADP-ribose) polymer bound to the separated proteins in the same blots, the blots were stripped, with a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50 °C, blocked again, and reprobed with either a monoclonal antibody against poly(ADP-ribose) polymer (provided by Drs. M. Miwa and T. Sugimura, Japan; 1:250; Kawamitsu et al., 1984) or a polyclonal rabbit antibody to the polymer produced in our laboratory. Immunodetection of the blots with the same MRC fractions was also performed for various DNA replication proteins by sequentially stripping and reprobing the blots with various antibodies to the DNA replication proteins, as previously described (Applegren et al., 1995). The monoclonal antibody to DNA polymerase δ was a kind gift from Dr. Marietta Lee; the anti-DNA ligase I antibody was a gift from Dr. Alan Tomkinson; and the anti-RFC and anti-RPA monoclonal antibodies were kindly provided by Dr. Bruce Stillman. The antibodies to topoisomerases I and II and the PCNA were purchased from TopoGEN, Inc., and Boehringer-Mannheim, respectively.

Immunoprecipitation Protocols. Immunoprecipitation of the modified proteins of the MRC was performed using a polyclonal anti-polymer antibody according to procedures described previously for purified proteins (Simbulan et al., 1993). Briefly, equal amounts (10 μg) of purified MRC (SG fraction) were added with 200 μL of EBC buffer (50 mM Tris-HCl, pH 8, 120 mM NaCl, 0.5% NP-40, and 0.1 TIU of aprotinin), precleared overnight with 10 μL/sample of protein A−Sepharose at 4 °C, and centrifuged. The supernatants were then rocked for 1 h with 0.5 mL of NET-N buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing a polyclonal anti-polymer (1−3 μL of antibody solution per sample), followed by another incubation for 20 min with 20 μL of protein A−Sepharose in Tris-buffered saline with 10% BSA (1:1). After extensive washing of the beads (up to five times) with NET-N buffer, the immunoprecipitated proteins bound to the beads were then separated by SDS−polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunodetected sequentially with antibodies to different replication proteins as described above.

RESULTS

Time Courses of PARP Induction and Differentiation-Linked DNA Replication in 3T3-L1 Cells. Postconfluent cultures of 3T3-L1 preadipocytes induced to differentiate by exposure to insulin, dexamethasone, and methylisobutylxanthine exhibit marked increases in PARP protein and activity, effects that are inhibited by PARP antisense RNA expression. Cells expressing PARP antisense RNA are

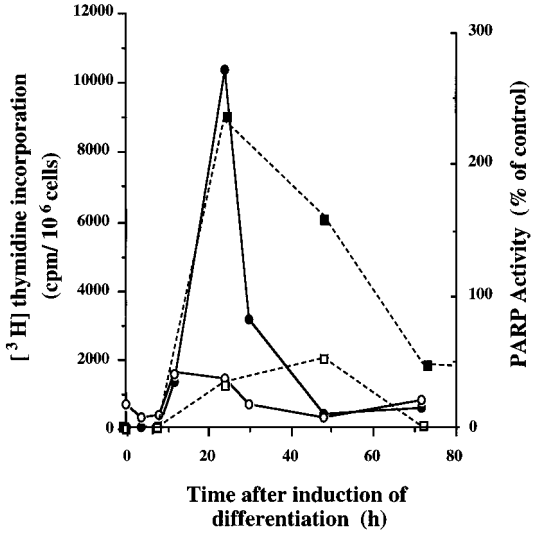

FIGURE 1: Time courses of PARP activity and *in vivo* DNA replication in 3T3-L1 control and PARP antisense cells after exposure to inducers of differentiation. Control (solid symbols) and antisense (open symbols) cells were exposed to inducers of differentiation and harvested at the indicated times. Duplicate cell pellets were assayed for either PARP activity (dotted lines) or *in vivo* DNA replication (solid lines) as described in Materials and Methods. Cells were assayed for PARP activity (squares) by measuring [^{32}P]NAD incorporation into acid-insoluble acceptors at 25 °C for 1 min, with 20 μg of protein per determination and triplicate determinations per time point. [^{32}P]NAD incorporation of uninduced control 3T3-L1 cells was taken as baseline, and PARP activity values were calculated and plotted on the basis of this control value. *In vivo* DNA replication activity (circles) was determined by measuring [^{3}H]TdR incorporation into acid-insoluble DNA after a 15 min pulse as described in Materials and Methods. Values are means of duplicate determinations, and essentially identical results were obtained in two independent experiments.

unable to differentiate, as confirmed by their inability to synthesize and accumulate cytoplasmic triglyceride (Smulson et al., 1995). The time courses of PARP activity and DNA replication after exposure to inducers of differentiation have now been compared in 3T3-L1 control cells and cells expressing antisense PARP (Figure 1). The peak of PARP activity coincided with the peak of DNA replication during the first 24 h; both PARP and DNA polymerase α have also been previously shown to coimmunoprecipitate from extracts derived from cells at 24 h (Smulson et al., 1995). In contrast, the antisense cells did not show an increase in PARP activity and did not undergo DNA replication in response to inducers of differentiation.

Effects of PARP Antisense RNA Expression on the Cell Cycle Distribution of 3T3-L1 Preadipocytes after Exposure to Inducers of Differentiation. To further investigate the inhibition of differentiation-linked DNA replication in PARP antisense preadipocyte cells, we performed FACS analysis on control and antisense cells at various times after exposure to inducers of differentiation (Figure 2). For comparison, the data have been quantitated in Figure 2C. Prior to induction of differentiation (zero time), 70−80% of post-confluent control (A) and antisense cells (B) were in growth arrest induced by contact inhibition, as evidenced by a predominant G_0/G_1 content of DNA. More than 95% of the control cells synchronously entered the S phase by 24 h (Figure 2A, arrow; Figure 2C), consistent with the peak of [^{3}H]thymidine incorporation observed 24 h after induction of differentiation (Figure 1). In contrast, >60% of the antisense cells remained in G_0/G_1 after 24 h. The control cells progressed through one round of replication prior to

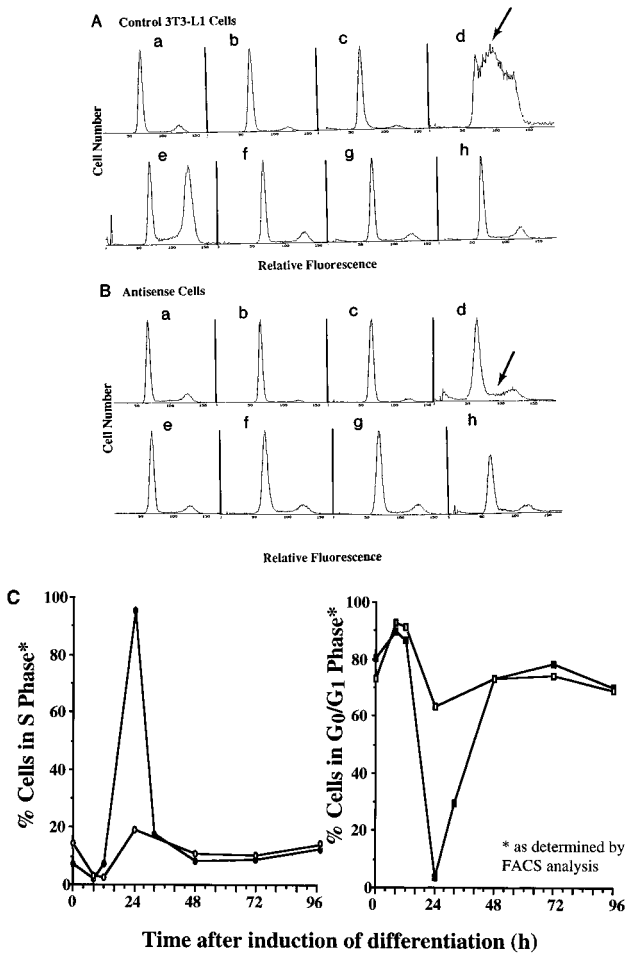

FIGURE 2: Flow cytometric analysis of 3T3-L1 control and antisense cells at various time intervals after induction of differentiation. After postconfluent control and PARP antisense cells were induced to differentiate and harvested at 0 (a), 8 (b), 12 (c), 24 (d), 32 (e), 48 (f), 72 (g), and 96 (h) h after induction, nuclei were prepared by a detergent−trypsin method and stained with propidium iodide (0.42 mg/mL) for flow cytometric analysis as described in Materials and Methods. (A) DNA histograms of control 3T3-L1 cells at various times after induction. Panels a−c and f−h show one main peak of nuclei in G_0/G_1; panel d shows a major peak of nuclei in S phase; and panel e shows two major peaks at G_0/G_1 and G_2/M phases of the cell cycle. (B). DNA histograms from PARP antisense cells at various times after induction of differentiation. All panels (a−h) show one major peak of nuclei at G_0/G_1 phase of the cell cycle. (C) FACS summary figure quantifying cell cycle phase distribution and showing percent of control (closed symbols) and PARP antisense (open symbols) cells in S phase (left panel) or in G_0/G_1 (right panel) phases of the cell cycle.

the onset of terminal differentiation, whereas the antisense cells appeared to be blocked at G_0/G_1 and did not enter the S phase (Figure 2B, C). FACS analysis and determination of [^{3}H]thymidine incorporation through the 7−8 days of differentiation showed that the control cells go through only one cell cycle and one peak of DNA replication during the initial 12−48 h period, maintaining a G_0/G_1 content of DNA thereafter, thus indicating that only one round of replication occurs prior to the onset of terminal differentiation (Figure 2).

Colocalization of PARP with DNA Replication Foci in 3T3-L1 Preadipocyte Nuclei. With double indirect immunofluorescence staining, we previously observed the simultaneous accumulation of newly synthesized PARP and BrdU incorporation into DNA within intranuclear granular foci 24 h after induction of differentiation of control 3T3-L1 cells (S phase cells); negligible PARP and BrdU staining was

apparent in antisense cells exposed to inducers of differentiation or in uninduced control cells (Smulson et al., 1995). A 1 h pulse period was chosen according to procedures used in similar immunofluorescence studies which generally used a BrdU pulse-labeling period of 30−120 min for colocalization of replication proteins, such as PCNA (Kill et al., 1991), DNA polymerase α (Hutchinson & Kill, 1989), and RPA (Adachi & Laemmli, 1992), to discrete replication centers. Kill et al. (1991) showed that pulse labeling for 30 min results in PCNA-positive but DNA replication-negative cells, and pulse-labeling of nuclei in the early S phase for 5 min periods at 5 min intervals did not show any replication-positive cells until later than 15 min after addition of the label, presumably due to a delay of more than 15 min between the time when some replication proteins are assembled at a specific site and the time when they are actively involved in DNA synthesis. Consistent with the results of other studies, instead of a uniform general fluorescence, numerous discrete bright foci of BrdU incorporation 24 h after induction were observed in most control cells, indicative of clustered replication forks or replication centers (RCs) in the early or mid-S phase (Nakayasu & Berezney, 1989; Cox & Laskey, 1991; Cook, 1991). This pattern was very similar to the punctate pattern of PARP protein localization apparent at the same time point. A few control nuclei also showed a predominantly perinuclear pattern of BrdU incorporation, indicative of mid-S to late S phase (Fox et al., 1991); however, no PARP foci were noted in cells showing this perinuclear pattern. These observations suggested that PARP may be localized in RCs during the early and late S phase.

Although a temporal relation was noted between PARP expression and DNA replication at 24 h after induction of differentiation, we were unable to ascertain by simple immunofluorescence microscopy alone whether nascent DNA synthesis actually occurs at sites of PARP localization. Accordingly, we now used laser scanning confocal microscopy (LSCM) (Figure 3) to precisely establish whether an intranuclear spatial relationship exists between foci of newly expressed PARP (middle panel) and sites of nascent DNA synthesis (left panel). For each nucleus, the sites of BrdU incorporation detected by anti-BrdU/ rhodamine generate a red signal (left panel), while PARP localization detected by anti-PARP/fluorescein displays a green signal (middle panel). Figure 3 shows confocal images of nuclei in the S phase where PARP and BrdU incorporation have coincident distributions and appeared to colocalize within the same granular foci (note the similar red and green patterns). Colocalization of the newly expressed PARP with sites of nascent DNA synthesis is evident in merged color micrographs (right panel), where coincident spots appear in yellow as a result of the exact superimposition of the red BrdU signal with the green PARP signal.

Colocalization of replication proteins such as PCNA (Kill et al., 1991; Wilcock & Lane, 1991), RPA (Adachi & Laemmli), and DNA polymerase α (Hutchinson & Kill, 1989) to the discrete replication centers has likewise been reported previously in confocal image analyses of double-immunostained nuclei. In our immunofluorescence studies, more than 90% of the nuclei exhibited a regular distribution of granular foci of PARP and BrdU incorporation throughout the nucleus, such as that shown in panel A, while a few (less than 5%) had nuclei in which the distribution of both PARP and BrdU incorporation is in a small number of very large granules (panels B and C). Consistently, the distribution of

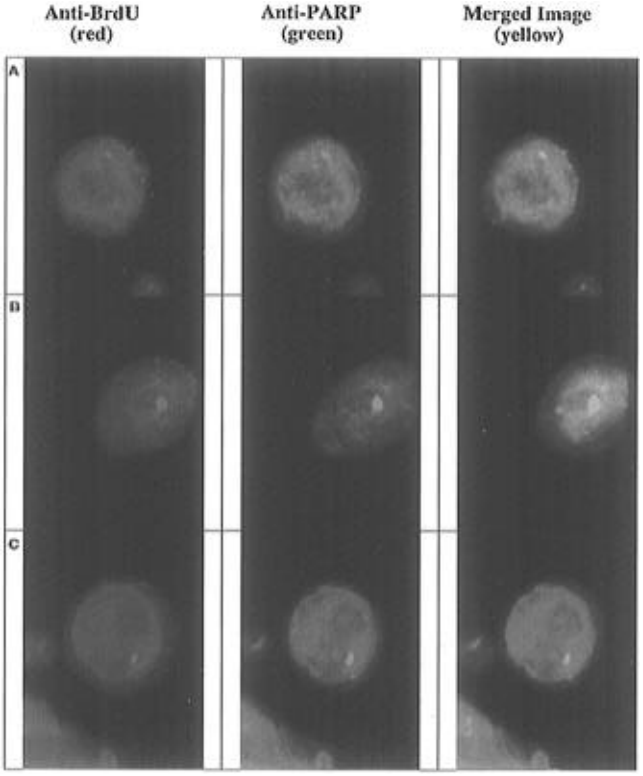

FIGURE 3: Colocalization of PARP protein and BrdU in 3T3-L1 control cells at 24 h after induction of differentiation by immunocytochemical staining and confocal microscope image analysis. Immunocytochemical staining of control cells at 24 h after induction (S phase) was done after a pulse for 1 h with BrdU, using mouse monoclonal anti-BrdU and polyclonal anti-PARP antibodies. After being probed with rhodamine-labeled anti-mouse IgG and fluorescein-labeled anti-rabbit IgG, fluorescence was visualized under a Zeiss immunofluorescence microscope and a Bio-Rad laser confocal microscope, as described in Materials and Methods. Panels A−C represent three different confocal microscope images of control nuclei showing colocalization of BrdU labeling and PARP protein within the same intranuclear granular foci. For each nucleus, the site of BrdU incorporation detected by anti-BrdU/rhodamine generates a red signal (left panel); PARP localization, detected by anti-PARP/fluorescein, displays a green signal (middle panel); and merged images show coincident spots which appear as yellow as a result of the exact superimposition of the red BrdU signal with the green PARP signal (right panel).

PCNA and DNA replication foci has been shown to exhibit very similar staining patterns which correlate with a temporal sequence of replication, i.e., a regular granular pattern throughout the nucleus (type I) indicative of cells in the early S phase, a punctated but predominantly perinuclear pattern (type II) typical of the mid-S to late S phase, and distribution in a few large densely staining granules (type III) which appear to be granules of types I and II clustered in ring or horseshoe-like arrays indicative of cells in the late S phase (Kill et al., 1991; Nakayasu & Berezney, 1989). Apparently, PARP can be colocalized to these granular replication centers during early as well as late S phase. DNA polymerase α, DNA ligase, PCNA, and RPA, which have all been shown to exist in 17−21 S multiprotein replication complexes (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995), have also been shown to be localized in discrete granular foci, whereas DNA polymerase β is not associated with the complex (Applegren et al., 1995) and is diffusely distributed in the nucleus (Li et al., 1993).

DNA polymerase α, a DNA replication enzyme involved in lagging strand DNA synthesis, interacts with and binds to PARP both *in vitro* (Simbulan et al., 1993) and *in vivo*

(Smulson et al., 1995). Additionally, the colocalization of PARP and DNA replication centers, coupled with the observation that a portion of PARP is present in a 400 kDa−700 kDa complex containing DNA polymerase α (Simbulan et al., 1993), suggests that PARP may exist and play a biological role as part of multiprotein DNA replication complexes (MRC), which have recently been purified and characterized (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995).

PARP Is a Component of the Multiprotein Replication Complex in Mouse FM3A and Human HeLa Cells. Since a functional role for PARP in the MRC would help to explain its immediate enhanced expression early in differentiation, this area was pursued further. Protein fractions and derived Western blots containing various purification fractions of well-characterized, extensively purified, DNA replication-competent MRCs from human HeLa cells (Figure 4) as well as mouse FM3A cells (Figure 5) were analyzed for immunologically detectable PARP using polyclonal anti-PARP antibodies.

PARP was found to exclusively copurify with the human MRC purified from HeLa cells (Figure 4A), as determined by immunoblot analysis using a polyclonal antibody against the full-length human PARP protein (116 kDa). The purified 18−21 S human MRC is fully competent to support origin-specific and large T-antigen-dependent SV40 DNA replication *in vitro* (Malkas et al., 1990; Applegren et al., 1995). The 30 polypeptides copurifying with the human complex include DNA polymerase α, DNA primase, RNase H, and a DNA-dependent ATPase (Malkas et al., 1990), as well as DNA polymerase δ, DNA ligase I, RF-C protein, and DNA helicase, comprising the "core" proteins in the complex; and PCNA, topoisomerase I, and RP-A protein, which appeared to be loosely associated with the core (Applegren et al., 1995). Like the core proteins of the human MRC, PARP was found to partition exclusively with the replication-competent P4, Q-Sepharose (QS) peak, and sucrose gradient (SG) peak fractions (Figure 4A, lanes 1, 3, and 5). In contrast, PARP was not observed in the replication-poor S4 and Q-Sepharose flow-through (FT) fractions (lanes 2 and 4).

Western analysis of the component proteins from the same fraction of the MRC (the SG fraction) was performed using different antibodies (Applegren et al., 1995) to sequentially probe for the known replication proteins on the same membranes (Figure 4B). The presence of the other known DNA replication proteins copurifying with PARP and the human MRC was confirmed by their exact Western signals and includes the replication enzymes DNA pol δ (125 kDa), ligase I (110 kDa), and topoisomerase II (180 kDa), as well as the accessory proteins RFC (140 kDa), RPA (34 kDa), and PCNA (35 kDa) (Figure 4B, lanes 2−7, respectively).

PARP was found to also copurify with core proteins in the mouse FM3A MRC, as indicated by the coincidental presence of the 116 kDa PARP in exactly the same distribution as the other core MRC proteins, exclusively in the replication-competent P4, DE-52 high salt, and Q-Sepharose peak activity fractions (Figure 5A, lanes 2, 4, and 6). In contrast, no detectable PARP was found in the replication-deficient S4, DE-52 low salt, and Q-Sepharose flow-through fractions (lanes 3, 5, and 7). The polyclonal antibody to porcine PARP used here has previously been shown to react with a 116 kDa protein, corresponding to full-length PARP, and a smaller truncated PARP around 100 kDa observed only in murine cell extracts (Ludwig et al., 1988). Since PARP apparently follows the DNA replication activity associated with the complex, it would appear to be tightly associated with the core proteins in the complex.

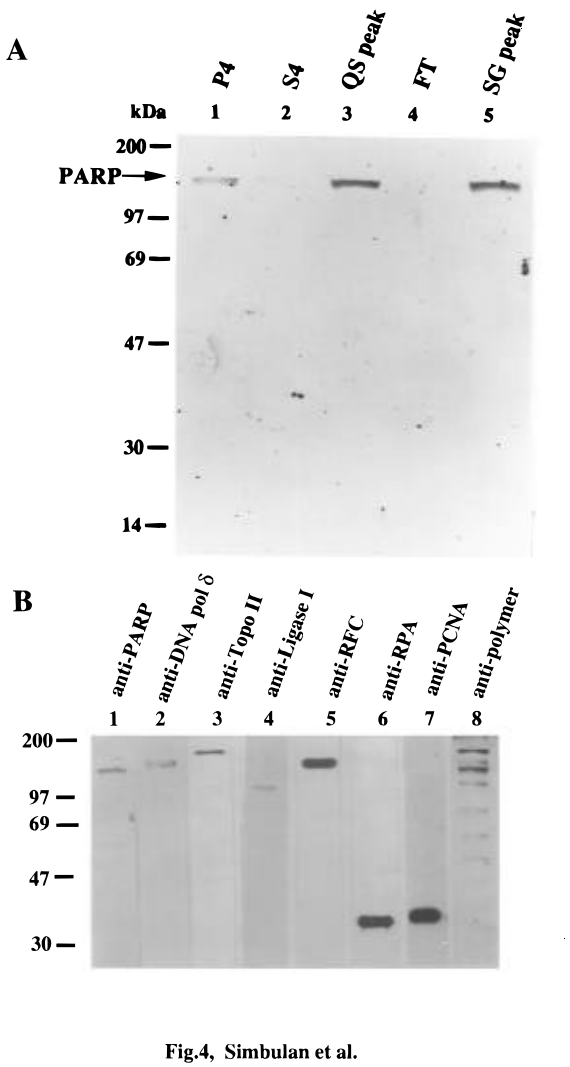

Fig.4, Simbulan et al.

FIGURE 4: Immunoblot analysis for the presence of PARP protein in fractions from purification of the human MRC. Twenty micrograms of different protein fractions from human HeLa MRC purification, starting with a high-speed supernatant (PEG/NE-S3) and culminating with eluted protein samples with peak replication activity purified by passage through Q-sepharose columns (QS peak activity) as well as sucrose gradient centrifugation (SG peak), was subjected to SDS−polyacrylamide gel electrophoresis and Western transfer to nitrocellulose membranes according to standard procedures. (A) The blots were incubated with a polyclonal anti-human PARP antibody (1:5000) and then with peroxidase-labeled anti-rabbit IgG (1:3000), after which the immobilized proteins were visualized by enhanced chemiluminescence (ECL, Amersham). The positions of the molecular mass markers are shown on the left side; the left arrow indicates the location of 116 kDa full-length human PARP. (B) Immunodetection of the blots with the same MRC fractions was also done for various DNA replication proteins by repeatedly stripping the membranes with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50 °C, blocking with 5% milk in PBS−Tween, and reprobing with various antibodies to the DNA replication proteins, i.e., lane 2, anti-DNA pol δ; lane 3, anti-topoisomerase II; lane 4, anti-ligase I; lane 5, anti-RFC; lane 6, anti-RPA; and lane 7, anti-PCNA, as well as with anti-PARP (lane 1) and anti-polymer (lane 8) antibodies.

Immunodetectable protein and enzymatic activities associated with this mouse MRC have been shown to include DNA polymerases α and δ, DNA primase, DNA ligase I,

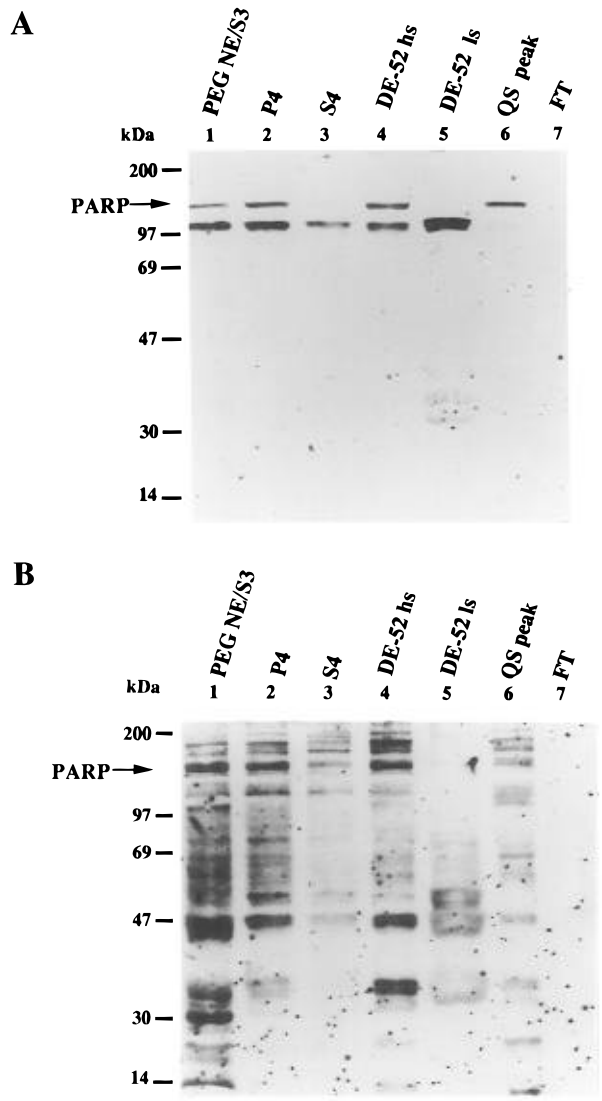

FIGURE 5: Immunoblot analysis for the presence of PARP protein in fractions from purification of the mouse MRC. (A) Different protein fractions (20 μg/fraction) from mouse FM3A MRC purification were subjected to SDS−polyacrylamide gel electrophoresis and the separated proteins transferred to nitrocellulose membrane. The blots were then blocked and incubated with polyclonal antibodies which specifically bind murine PARP (1:2000) Ludwig et al., 1988), followed by peroxidase-labeled anti-rabbit IgG (1:3,000), and the immobilized proteins were then visualized by enhanced chemiluminescence (ECL, Amersham). The positions of the molecular mass markers are shown on the left side; the left arrow indicates the location of 116 kDa full-length PARP. (B) The same immunoblot in (A) containing protein samples from various stages of mouse MRC purification was stripped as described in Figure 4 and reprobed with monoclonal anti-(ADP-ribose) polymer antibodies which specifically bind to linear poly(ADP-ribose) polymers (1:250). After incubation with peroxidase-labeled anti-mouse IgG (1:3000), the immobilized proteins were visualized by ECL (Amersham). The positions of the molecular mass markers are shown on the left side; the left arrow indicates the location of 116 kDa full-length mouse PARP.

DNA helicase, and DNA topoisomerase II (Wu et al., 1994). The complex is fully capable of supporting *in vitro* polyomavirus DNA replication. On the basis of their sedimentation and chromatographic profiles, these core proteins were likewise found to be exclusively present in the P4 fraction, while PCNA and topoisomerase I were also associated with the S4 fraction, suggesting that the latter proteins are loosely associated with the core proteins of the complex (Wu et al., 1994). The specific tight association between members of the mouse and human MRC has been further verified by their resistance to detergent, salt, RNase, and DNase treatments (Applegren et al., 1995).

Table 1: Relative Enzyme Activities of DNA Pol α, Primase, and PARP in Various Mouse FM3A MRC Fractions

purification step (MRC fraction)	total units of enzyme activity[a]			specific activity[b]		
	DNA pol α	primase	PARP	DNA pol α	primase	PARP
PEG/NE/S3[c]	209	48	55	0.40	0.09	0.10
S4[d]	9	1	19	0.05	0.01	0.11
P4[c]	186	25	91	2.35	0.31	1.15
DE52 hs[c]	22	3	29	0.76	0.23	1.99
DE52 ls[d]	14	1	4	0.96	0.10	0.25
QS (peak)[c]	10	nd[e]	28	1.75	nd	4.70

[a] One unit of DNA pol α activity is defined as 1 nmol of [^{3}H]TTP incorporated into DNA per hour at 35 °C; one unit of primase activity is equivalent to 1 nmol of [^{3}H]ATP incorporated into a poly(dT) template per hour at 35 °C; and one unit of PARP activity is equivalent to 1 pmol of [^{32}P]NAD incorporated into acceptor proteins for 1 min at 25 °C. [b] Specific activity = total units of enzyme activity/total protein (mg) for each purification step. [c] *In vitro* viral DNA replication-competent fractions. [d] DNA replication-incompetent fractions. [e] nd = not determined.

A comparison of the fractionation and chromatographic profile of PARP with representative replication proteins such as DNA pol α and primase is summarized in Table 1, where the specific activities and units of PARP are shown relative to DNA pol α and primase during various stages of purification of the mouse FM3A MRC. The *in vitro* assay for PARP is complex due to the variety of potential nuclear acceptors for the enzyme and the relationship of DNA strand breaks with activity. Nevertheless, within the limits of quantitation of this assay, it is of interest that about 50% of the total units of PARP activity is associated with the MRC (in the most purified replication-competent DE52 high salt and QS peak fractions) relative to the total units of PARP present in crude combined nuclear/cytosolic extract (PEG/E/S3 fraction). Consistent with the results of Western analysis, PARP activity fractionated almost exclusively with the replication-competent, MRC-enriched P4, DE52 high salt, and QS peak fractions, in exactly the same pattern as fractionation of DNA pol α and primase activities (Wu et al., 1994). It is also interesting to note that, in the crude PEG/NE/S3 fraction, the DNA pol α activity was about 4-fold higher than PARP or primase, whereas in the more purified fractions such as the DE52 high salt fraction, or the purified QS peak fraction, total units and specific activity of PARP were approximately two to three times more than DNA pol α activity. Whereas 95% of the DNA pol α was found to be tightly bound to the other core MRC proteins in the less purified P4 fraction, 83% of PARP was tightly bound and 17% of activity was loosely or not associated with the core MRC proteins (in the S4 fraction).

Poly(ADP-ribosyl)ation of Component Proteins of the MRC. To investigate whether, as a part of the complex, PARP may catalyze the poly(ADP-ribosyl)ation of MRC component proteins, we subjected immunoblots containing protein samples from various stages of MRC purification that had been probed with PARP antibodies to further analysis with antibodies that specifically recognize linear ADP-ribose polymers bound to nuclear proteins (Kawamitsu et al., 1984). Approximately 15 component proteins of purified MRCs were extensively poly(ADP-ribosyl)ated (Figure 5B). The same pattern of poly(ADP-ribosyl)ated proteins was observed consistently with several independent

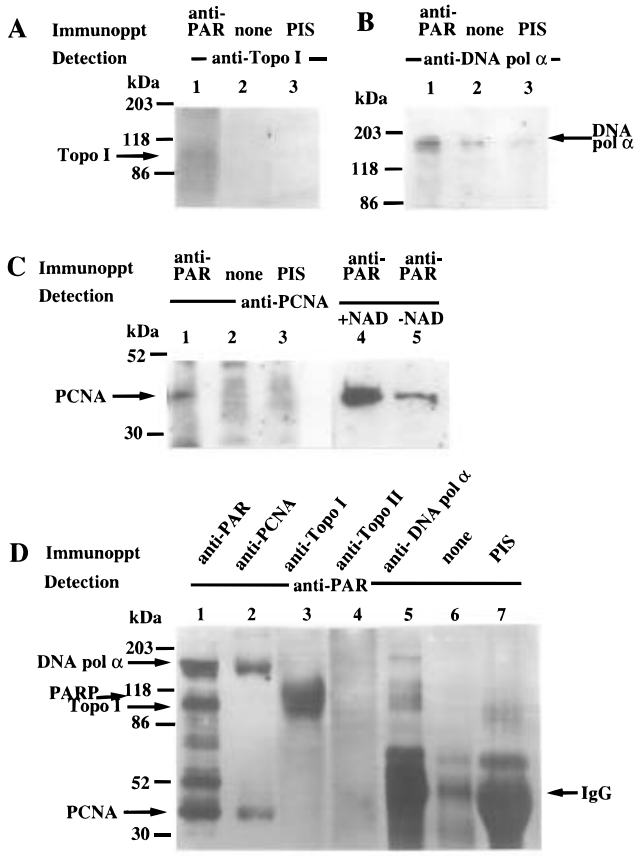

FIGURE 6: Immunoprecipitation of modified replication proteins in the MRC with anti-polymer antibody and with antibodies to different MRC replication proteins. (A−C) HeLa MRC (SG fraction) were immunoprecipitated using a polyclonal anti-poly(ADP-ribose) polymer antibody (anti-PAR), as described in Materials and Methods. The immunoprecipitated proteins were then separated by gel electrophoresis, transferred to nitrocellulose, and then probed with the relevant antibodies to the different replication proteins [A−C, lane 1, detected with anti-topoisomerase I antibody (A), anti-DNA pol α (B), and anti-PCNA (C), respectively]; as control, immunoprecipitations were also performed with protein A−Sepharose alone (none, lane 2) or with preimmune serum (PIS, lane 3). Control HeLa cell extracts were treated with (lane 4) and without NAD (lane 5), immunoprecipitated with the anti-PAR antibody, and probed with anti-PCNA antibody. (D) Conversely, MRC fractions were also immunoprecipitated with antibodies to PCNA (lane 2), topo I (lane 3), topo II (lane 4), and DNA pol α (lane 5) or with protein A−Sepharose alone (lane 6) or preimmune serum (PIS, lane 7) as control. The immunoprecipitates were then separated, transferred, and probed with a monoclonal anti-polymer antibody.

blots of both mouse and human MRCs (Figure 4B, lane 8) and also with polyclonal antibodies to ADP-ribose polymer recently developed in our laboratory.

Poly(ADP-ribose) chains may be cleaved from proteins by incubation of immunoblots with phosphodiesterase (Adamietz, 1987). When a control HeLa cell extract was incubated *in vitro* in the presence (Figure 7D, lane 1) or absence (lane 2) of NAD, various poly(ADP-ribosyl)ated proteins were specifically detected in the NAD-treated extract using the monoclonal anti-polymer antibodies. No immunoreactivity was detected when duplicate lanes of the membrane (lanes 3 and 4, respectively) were incubated with phosphodiesterase and subsequently reprobed with anti-polymer antibodies, verifying the specificity of the treatment to characterize authentically ADP-ribosylated proteins. To rule out the possibility that proteinase in the phosphodiesterase sample could have degraded the proteins, the blot

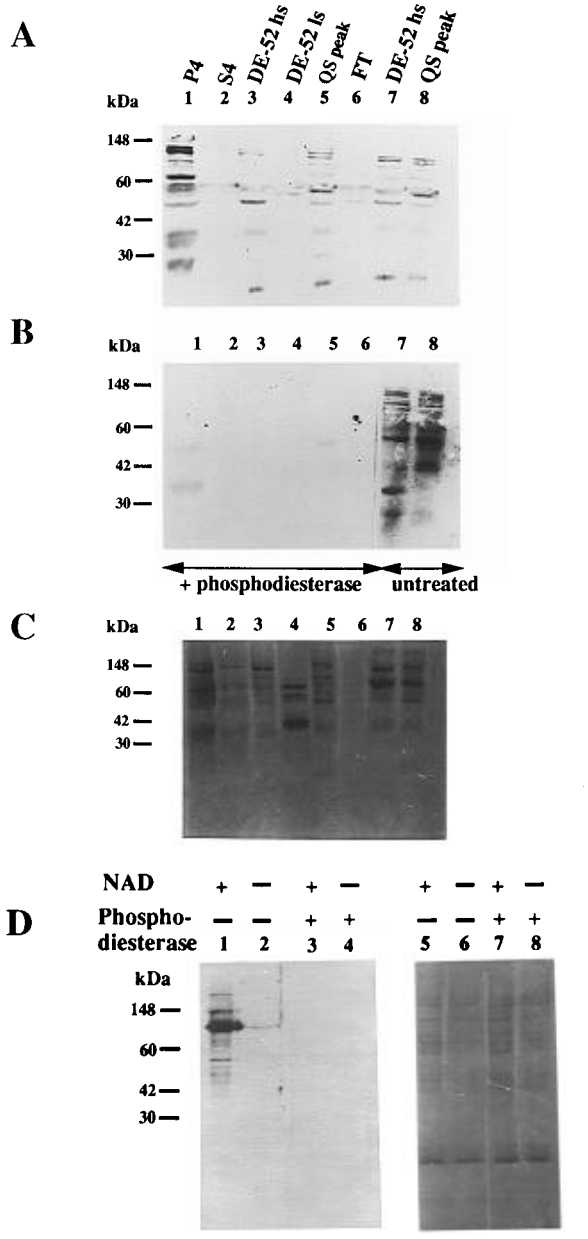

FIGURE 7: Immunoblot analysis of mouse MRC with antibodies to poly(ADP-ribose) and cleavage of the polymer from proteins by phosphodiesterase treatment. (A) Immunoblot analysis of protein samples from various stages of mouse MRC purification with the monoclonal antibodies to poly(ADP-ribose) polymer (1:250) and peroxidase-labeled antibodies to mouse IgG (1:3000), immunodetected by ECL (Amersham). Lanes 7 and 8 are duplicates of lanes 3 and 5, respectively. (B) The blot in (A) was stripped of antibodies, free protein binding sites were saturated with 5% BSA in PBS−Tween, and lanes 1−6 were incubated with snake venom phosphodiesterase I (50 μg/mL) for 1 h at 22 °C in a solution containing 50 mM Tris-HCl (pH 7.8) and 0.5 mM PMSF; a control, lanes 7 and 8, was incubated in the same buffer without phosphodiesterase I. The blot was then reprobed with anti-polymer antibodies. (C) The blot was subsequently stripped again of antibodies and then stained with Ponceau S for the presence of proteins. (D) HeLa cell extracts (50 μg of protein) were incubated for 10 min at 37 °C in a reaction mixture (200 μL) containing 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0), 20 mM DTT, and 2 mM $MgCl_2$, in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 125 μM NAD. The samples were then subjected to SDS−PAGE and immunoblot analysis with the monoclonal anti-polymer antibody. Lanes 3 and 4 were stripped, blocked with BSA, treated with phosphodiesterase as in (B), and then incubated again with the anti-polymer antibodies. The blot was subsequently stripped again and stained with Ponceau S to confirm presence of the proteins after cleavage of the polymer (lanes 5−8).

was subsequently stripped and stained with Ponceau S to confirm the presence of the proteins after cleavage of the polymer (Figure 7D, lanes 5−8). The same phosphodiesterase treatment was performed with the MRC fractions showing various levels of poly(ADP-ribosyl)ation (Figure 7A). Again, phosphodiesterase cleaved poly(ADP-ribose) from the proteins and rendered them unreactive with the anti-polymer antibodies (Figure 7B, lanes 1−6); proteins in a part of the blot treated identically except in the absence of phosphodiesterase were still capable of reacting with the anti-polymer antibodies (lanes 7 and 8). The observation that certain component proteins of the replication complex are poly(ADP-ribosyl)ated suggests that this specific post-translational modification may have a role in regulating their activities within the complex.

Several of the modified proteins were initially tentatively identified on the basis of their relative mobilities on SDS gels (Figure 4B, 5B, and 6) and immunoblot analysis on identical membranes, i.e., topoisomerase II (180 kDa), PARP (116 kDa), topoisomerase I (100 kDa), DNA pol α (140 kDa), ligase I (110 kDa), RPA (34 kDa), and PCNA (35 kDa). The identities of several of these modified replication proteins were further confirmed by a series of immunoprecipitation experiments wherein purified fractions of the MRC were immunoprecipitated with a polyclonal anti-polymer antibody, and the immunoprecipitated proteins were separated by SDS−PAGE, transferred to membranes, and subsequently probed with the relevant antibodies obtainable with good specificities to different replication proteins.

Results in Figure 6A−C demonstrate that topoisomerase I (100 kDa), DNA pol α (180 kDa), and PCNA (35 kDa), indicated by arrows, were specifically immunoprecipitated from the complex by the anti-poly(ADP-ribose) polymer antibody (anti-PAR) and are shown to react with their respective antibodies on the Western blots (lane 1, panels A−C)). On the other hand, no nonspecific binding of these proteins occurred with protein A-Sepharose beads (lane 2, panels A−C)) alone or with preimmune serum (PIS, lane 3, panels A−C). When control HeLa cell extracts treated with (lane 4) and without NAD (lane 5) were immunoprecipitated with the anti-PAR antibody and the blots probed with anti-PCNA antibody, much more modified PCNA was shown to be in the NAD-treated extracts, confirming that immunoprecipitation with this antibody specifically selects poly(ADP-ribosyl)ated proteins.

Conversely, identification of PCNA, topoisomerase I, and DNA pol α, as modified MRC proteins, was further confirmed when the same fractions of the MRC were immunoprecipitated with antibodies to PCNA, topo I, and DNA pol α and subsequently detected with the anti-PAR antibody (Figure 6D, lanes 2, 3, and 5, respectively). On the other hand, topoisomerase II was apparently unmodified; thus it did not react with the anti-PAR antibody (lane 4). Consistent with our previous results on the binding of PARP and DNA pol α *in vitro* and *in vivo*, immunoprecipitation with anti-DNA pol α (lane 5) coimmunoprecipitated both DNA pol α and PARP (which shows a denser band because it is expectedly more ribosylated than DNA pol α). Interestingly, PCNA also appears to physically associate in the complex with DNA pol α, as shown by their coimmunoprecipitation using the anti-PCNA antibody (lane 2).

Effects of NAD and 3-Aminobenzamide on the in Vitro Viral DNA Replication Ability of HeLa MRC. On the basis of the initial new observations on a putative role of poly(ADP-ribosyl)ation in the replication complex, which stemmed directly from the earlier differentiation-linked DNA replication studies, preliminary studies have been directed at assessing the effects of poly(ADP-ribsosyl)ation, NAD, and PARP inhibitors, such as 3-aminobenzamide, on the overall replicative ability of the MRC. As shown in Figure 8A, inhibition of PARP activity by 3-aminobenzamide diminished the ability of human HeLa MRC to undergo SV40 DNA replication by about 50% in a dose-dependent manner. However, this inhibition was reversed by addition of more NAD into the reaction mixture (Figure 8B). This suggests that PARP and/or poly(ADP-ribosyl)ation plays a role and can modulate the overall replicative competence of the MRC. Studies are now underway to investigate the effect of NAD and 3-aminobenzamide on the activities of the component enzymes in the complex, i.e., DNA pol α activity. Thus far, we have not observed any significant effect of addition of more NAD by itself on the overall replicative ability of the complex (data not shown), perhaps due to the already extensively modified state of the component proteins *in vivo*.

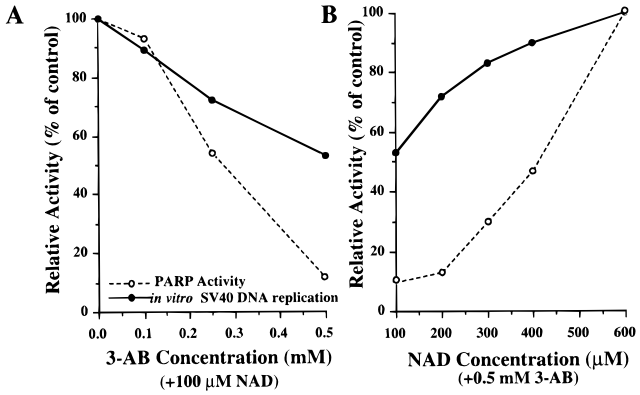

FIGURE 8: Effects of varying concentrations of NAD and 3-aminobenzamide on SV40 replication of the HeLa MRC (QS fraction). The effects of poly(ADP-ribsosyl)ation, NAD, and a PARP inhibitor, 3-aminobenzamide (3-AB) on the overall *in vitro* SV40 replicative ability of the HeLa MRC was studied. *In vitro* SV40 DNA replication assays were performed as previously described (Applegren et al., 1995); PARP activity was measured as described in Materials and Methods. The relative PARP or DNA replication activities were then calculated as percent of the control (without 3-aminobenzamide treatment, +100 μM NAD); 100% activity corresponds to 2907 pmol of NAD incorporated into acceptor proteins mg^{-1} min^{-1}. (A). Dose-dependent inhibition of PARP activity and SV40 DNA replication by indicated concentrations of 3-aminobenzamide. (B) Reversal of inhibition by 3-aminobenzamide by addition of increasing concentrations of NAD into the reaction mixture.

DISCUSSION

Using an antisense strategy, we recently initiated studies on the effects of PARP antisense RNA expression and consequent PARP depletion on the differentiation of 3T3-L1 preadipocytes, with special regard to differentiation-linked DNA replication (Smulson et al., 1995). Various models, all of which involve chromatin restructuring, have been proposed to explain the requirement for DNA replication prior to differentiation. DNA replication is thought to be the primary decision point during initiation of differentiation because it results in reconfiguration of chromatin and thereby sets and changes committed patterns of gene expression (Villareal, 1991). Poly(ADP-ribosyl)ation may contribute to the repositioning of nucleosomes, resulting in the activa-

tion or inhibition of specific genes, and has been shown to alter nucleosomal structure adjacent to replicating chromatin regions (Butt & Smulson, 1980; Thibeault et al., 1975). Chromatin structure was also previously shown to be markedly changed with respect to susceptibility to nuclease digestion in HeLa cells depleted of PARP by expression of PARP antisense RNA (Ding & Smulson, 1994). Thus, the inability of the PARP-depleted antisense 3T3-L1 cells, induced to differentiate, to undergo sufficient replication-associated reconfiguration of chromatin could result in their failure to terminally differentiate into adipocytes.

It has also been suggested that poly(ADP-ribose) may act to ensure the fidelity of differentiation by resulting in a suicidal metabolic response (by NAD depletion) if the chromatin alterations do not conform to the correct completion of the differentiation process (Caplan, 1989). Consistently, antisense 3T3-L1 cells that do exhibit some degree of replication, but only about half of that of the control cells, float and eventually die (data not shown), consistent with a putative role for PARP in apoptosis (Nosserrie et al., 1994; Kaufmann et al., 1994). Additionally, it has recently been shown that, during spontaneous apoptosis in a human osteosarcoma cell line, PARP is cleaved by a highly specific ICE-like protease (Nicholson et al., 1995).

Purified PARP specifically binds to DNA polymerase α *in vitro*, markedly stimulating its activity in a dose-dependent manner; this effect is lost when PARP is automodified (Simbulan et al., 1993). This physical interaction between PARP and DNA polymerase α also occurs *in vivo* in control 3T3-L1 cells during the initial stages of differentiation but not in PARP-depleted antisense cells (Smulson et al., 1995). Thus, PARP may participate in DNA replication in the initial stages of differentiation by associating with DNA polymerase α, and the inability of PARP-depleted antisense cells to form such a complex may explain their failure to undergo differentiation-linked DNA replication (Figure 1), and consequently their inability to enter the S phase of the cell cycle (Figure 2).

Earlier observations on the interaction between PARP and DNA polymerase α led us to speculate that PARP may exist within nuclear replicative complexes. We have now shown for the first time that PARP exclusively copurifies with core proteins in the well-characterized MRCs purified from HeLa cells (Figure 4) or from mouse FM3A cells (Figure 5). Because PARP closely followed the DNA replication activity associated with these complexes, it appears to be tightly associated with the core proteins in the complex. The MRC has previously been shown to contain replicative enzymes important in leading- and lagging-strand DNA synthesis, including DNA polymerases α and *δ*, DNA primase, DNA ligase, DNA helicase, and topoisomerases I and II, as well as accessory proteins such as PCNA, RF-C, and RP-A (Wu et al., 1994; Malkas et al., 1990; Applegren et al., 1995). The identities of some of these component MRC proteins were confirmed by immunoblot analyses using different antibodies to sequentially probe for the known replication proteins on the same membranes (Applegren et al., 1995) and shown to include DNA pol *δ*, topoisomerase II, ligase I, RFC, RPA, and PCNA (Figure 4B). These purified MRCs are fully capable of supporting viral DNA replication *in vitro*.

With double immunofluorescence staining and confocal microscope image analysis, we have also demonstrated the colocalization of PARP and distinct intranuclear DNA replication foci in 3T3-L1 cells at the S phase. Similar to the punctate pattern of PARP protein localization, discrete bright foci of BrdU incorporation indicative of clustered replication forks or RCs in the early or mid-S phase were observed in most induced control cells. The absence of a perinuclear pattern of PARP distribution similar to the mid-S to late S phase pattern of BrdU incorporation seen in a few nuclei suggests that PARP may be localized in RCs during the early S phase. Confocal images of nuclei in the early S phase revealed coincident distributions of PARP and BrdU incorporation; colocalization of newly expressed PARP with sites of nascent DNA synthesis was evident in merged images showing yellow spots indicating superimposition of the red BrdU signal with the green PARP signal (Figure 3).

Since PARP has been shown in several cases to be capable of modulating the catalytic activity of various replicative enzymes or factors either by directly associating with them (i.e., DNA polymerase α; Simbulan et al., 1995) or by catalyzing their poly(ADP-ribosyl)ation [i.e., DNA polymerase α (Yoshihara et al., 1985), DNA topoisomerases I and II (Kasid et al., 1989; Ferro & Olivera, 1984; Darby et al., 1985), and RP-A (Eki & Hurwitz, 1991)], PARP may play a regulatory role within the replicative complexes. Testing this hypothesis will constitute the aims of the next stage in these studies. In most cases, poly(ADP-ribosyl)-ation causes a reduction in their enzyme activities. For example, purified PARP has been demonstrated to markedly inhibit the catalytic activities of purified preparations of DNA topoisomerases I and II (Kasid et al., 1989; Ferro & Olivera, 1984; Darby et al., 1985) and DNA polymerase α (Yoshihara et al., 1985). These inhibitions have been attributed to significant decreases in the DNA-binding affinities of the modified enzymes due to the electrostatic repulsion between DNA and the ADP-ribose polymer covalently linked to the enzymes by PARP. Replication factors such as RP-A (Eki & Hurwitz, 1991) and SV40 large T-antigen (Baksi et al., 1987; Goldman et al., 1981) are also modified by poly(ADP-ribosyl)ation *in vivo*. Interestingly, in the case of topoisomerase I, one of the major acceptors of this chromatin-dependent posttranslational modification (Kasid et al., 1989), a portion of the total DNA topoisomerase I activity copurifies with PARP (Ferro et al., 1983), supporting the data in the current work indicating that the two proteins associate *in vivo*. Similar results were obtained with DNA polymerase α and PARP, both of which were also found to copurify in a 400 kDa and a larger 700 kDa complex containing both activities (Simbulan et al., 1993). The effects of PARP *per se* and/or poly(ADP-ribosyl)ation on the other components of the complex, such as on DNA helicases, DNA polymerase *δ*, and accessory proteins PCNA and RF-C, however, will be elucidated in ensuing studies.

It has been suggested that the interaction of PARP with the replicative apparatus indicates that it may function as a molecular nick sensor, controlling the progression of the replication fork when DNA strand breaks are present, such as during DNA damage, ensuring that lesions are not replicated before repair. Consistently, the binding of PARP to DNA termini suppressed SV40 DNA replication *in vitro* by inhibiting the elongation of Okazaki fragments by DNA polymerase α (Eki & Hurwitz, 1991). When unmodified PARP locates and binds to DNA ends generated in the initial stages of DNA repair/replication, its subsequent automodification results in its dissociation from the DNA ends, allowing access to DNA repair or DNA replication enzymes (Satoh & Lindahl, 1992). Using bacterially expressed

deletion mutants of PARP, we further showed that deletion mutants with an intact DNA-binding domain inhibit DNA repair, whereas mutants with deletions in this domain do not inhibit the *in vitro* assay (Smulson et al., 1994).

In the current work, contrary to our initial expectations, we observed that about 15 proteins of the approximately 30 polypeptides of the MRC (Malkas et al., 1990) were poly(ADP-ribosyl)ated when probed with highly specific anti-(ADP-ribose) polymer antibodies (Figures 4−6). Some of these modified proteins (i.e., DNA topoisomerase I, DNA pol α, DNA ligase, and PCNA) were initially identified on the basis of their mobilities in identical membranes in our earlier studies (Wu et al., 1994) and previous reports of their modification by poly(ADP-ribosyl)ation *in vitro* and/or *in vivo* (Kasid et al., 1989; Ferro & Olivera, 1984; Darby et al., 1985; Eki & Hurwitz, 1991; Yoshihara et al., 1985). Results of immunoprecipitation with the anti-polymer antibody and antibodies to several of the replication proteins further confirm that DNA pol α, topoisomerase I, and PCNA are poly(ADP-ribosyl)ated protein components of the MRC (Figure 6). The antigenic component on these proteins was characterized as authentic poly(ADP-ribose) by its ability to be cleaved by snake venom phosphodiesterase (Figure 7). It therefore appears that modification by poly(ADP-ribosyl)-ation and possibly modulation of the catalytic activities of a subfraction of the component proteins may additionally be involved and play an important role in the functions of the replication complex.

About 50−70% of the total poly(ADP-ribose) residues existing *in vivo* are tightly associated with the nuclear matrix (Cardenas-Corona et al., 1989) which has also been implicated as the site of DNA replication foci (Tubo & Berezney, 1987). This putative membrane-associated property of the DNA replicative process may account for the relatively high levels of poly(ADP-ribosyl)ated replicative enzymes which we observed in the purified complex; however, this must be quantitated in later studies. The poly(ADP-ribosyl)ation of component proteins of the MRC is especially surprising since we have established that the half-life of polymer on acceptor proteins *in vivo* is transient (approximately 1−2 min; Wielckens et al., 1983). In general, it is quite difficult to detect poly(ADP-ribosyl)ated proteins, derived from *in vivo* experiments. Thus, the high levels of poly(ADP-ribosyl)-ation found associated with purified proteins in the complex will be explored in greater detail in future experiments.

Large complexes (100−150S) of replication-essential proteins have been described which are composed of groups of smaller protein complexes (20S) organized into a replication center around a nuclear matrix filament (Tubo & Berezney, 1987). Although the smaller protein complexes (20S) are approximately the same size as the MRCs studied in the current work, it remains to be established whether they represent the same complexes. Large multienzyme complexes for DNA replication have been shown to contain DNA-precursor synthesizing enzymes juxtaposed with a smaller "replication apparatus" comprising DNA polymerases, other enzymes, and auxiliary proteins (Reddy & Pardee, 1980). Additionally, topoisomerase II, recognized as an essential nuclear matrix-associated protein (Cockerill & Garrard, 1989), is tightly associated with the core proteins in the human (Applegren et al., 1995) and mouse MRC (Wu et al., 1994); thus, it has been suggested that one of its roles in the MRC is to promote association of the MRC with the nuclear matrix. Since topoisomerase II activity is inhibited by poly(ADP-ribosyl)ation (Darby et al., 1985), PARP may also play a role in the association or dissociation of the MRC with this nuclear matrix substructure.

Even if unmodified PARP itself is not generally bound to the nuclear matrix, the majority of the poly(ADP-ribosyl)-ated proteins, including automodified PARP, appear to be associated with it (Brauer et al., 1989). The association of a number of prominent proteins to the nuclear matrix appears to be mediated in part by poly(ADP-ribosyl)ation (Cardenas-Corona, 1989). It is of interest that apparent molecular masses of these proteins, (i.e., 170, 116, 100, 70, 67, 55, 36, and 20 kDa) approximate the sizes of the ADP-ribosylated proteins found in the replication complex (Figures 4−6).

Although various replicative enzymes have been shown to be acceptors for poly(ADP-ribose) *in vitro*, future studies will be directed at elucidating which of the replicative enzymes and protein factors in the MRC serve as physiological poly(ADP-ribose) acceptor proteins *in vivo* and ultimately how the modification affects their functions within the complex. The functional integrity of the MRC has been reported to be impaired when one of its essential components is inactivated (Reddy & Pardee, 1980). Studies will be directed at the effects of incubation of the complex in the presence or absence of NAD on its ability to support *in vitro* viral DNA replication. Additionally, it will be informative to assess the effects of cleavage of the polymer from the ADP-ribosylated proteins in the complex by phosphodiesterase or poly(ADP-ribose) glycohydrolase on both the enzymatic activities of the component proteins and the overall replicative competence of the purified MRCs. Studies are now underway to determine the effects of PARP antisense RNA expression (Ding et al., 1993; Smulson et al., 1995; Rosenthal et al., 1995) on the individual enzyme activities of the component proteins in the MRC, i.e., DNA polymerase α activity. The MRC from 3T3-L1 PARP antisense cells will be purified in ensuing studies to determine the effect of antisense PARP RNA expresssion on the *in vitro* replicative competence of these MRCs from PARP-depleted cells.

MRC component enzymes exist in complex form throughout the cell cycle; however, only the complex isolated during the S phase is capable of supporting *in vitro* DNA replication (Na et al., 1995). It is possible that accessory factors (for example, PARP) or posttranslational modification [for example, phosphorylation or poly(ADP-ribosyl)ation] of MRC component proteins may be responsible for the conversion of the MRC from a latent to an active form. The induction of differentiation-associated DNA replication in 3T3-L1 cells provides a model experimental system with which to clarify further the overall significance of PARP and poly(ADP-ribosyl)ation in the MRC.

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