

Cloning and expression of cDNA for human poly(ADP-ribose) polymerase

(DNA repair/COS cell transfection/cell cycle/DNA strand breaks)

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ABSTRACT cDNAs encoding poly(ADP-ribose) polymerase from a human hepatoma λ gt11 cDNA library were isolated by immunological screening. One insert of 1.3 kilobases (kb) consistently hybridized on RNA gel blots to an mRNA species of 3.6–3.7 kb, which is consistent with the size of RNA necessary to code for the polymerase protein (116 kDa). This insert was subsequently used in both *in vitro* hybrid selection and hybrid-arrested translation studies. An mRNA species from HeLa cells of 3.6–3.7 kb was selected that was translated into a 116-kDa protein, which was selectively immunoprecipitated with anti-poly(ADP-ribose) polymerase. To confirm that the 1.3-kb insert from λ gt11 encodes for poly(ADP-ribose) polymerase, the insert was used to screen a 3- to 4-kb subset of a transformed human fibroblast cDNA library in the Okayama–Berg vector. One of these vectors [pcD-p(ADPR)P; 3.6 kb] was tested in transient transfection experiments in COS cells. This cDNA insert contained the complete coding sequence for polymerase as indicated by the following criteria: (i) A 3-fold increase in *in vitro* activity was noted in extracts from transfected cells compared to mock or pSV2-CAT transfected cells. (ii) A 6-fold increase in polymerase activity in pcD-p(ADPR)P transfected cell extracts compared to controls was observed by “activity gel” analysis on gels of electrophoretically separated proteins at 116 kDa. (iii) A 10- to 15-fold increase in newly synthesized polymerase was detected by immunoprecipitation of labeled transfected cell extracts. Using pcD-p(ADPR)P as probe, it was observed that the level of poly(ADP-ribose) polymerase mRNA was elevated at 5 and 7 hr of S phase of the HeLa cell cycle, but was unaltered when artificial DNA strand breaks are introduced in HeLa cells by alkylating agents.

The chromatin-associated enzyme poly(ADP-ribose) polymerase is a 116-kDa protein that uses NAD as substrate to catalyze both the covalent transfer of ADP-ribose to a variety of nuclear protein acceptors and subsequently catalyzes the transfer of an additional 60–80 ADP-ribose units to the initial moiety. Nuclear proteins that become predominantly poly(ADP-ribosyl)ated include nucleosomal core histones, histone H1, HMG proteins, topoisomerase I and II, and simian virus 40 large tumor antigen (1). Extensive autopoly(ADP-ribosylation) of the polymerase also occurs (2). A clue to the biological function of poly(ADP-ribosylation) is provided by the observations that the *in vitro* catalytic activity of the enzyme is exclusively dependent upon the presence of strand breaks in DNA (3–6). It is thus possible that the enzyme system functions in response to transient and localized DNA strand breaks in cells that may arise through a variety of

processes including DNA repair, replication, recombination, and gene rearrangement. Accordingly, the long-term rationale for the molecular cloning of the gene encoding this enzyme has been to precisely define the function(s) of this nuclear protein modification system.**

MATERIALS AND METHODS

Antibody Screening of λ gt11 Library. The λ gt11 library used in the study was constructed with mRNA isolated from human hepatomas and was screened by a modification of the method of Young and Davis (8). The antibody used in the screening was a rabbit antiserum against a pure preparation of HeLa cell poly(ADP-ribose) polymerase (9). A similar antibody has been described (10).

Hybrid Selection and Hybrid-Arrested Translation. The cDNA inserts of recombinant λ gt11 clones were excised by *EcoRI* digestion as described by the supplier (Bethesda Research Laboratories) and subcloned into the plasmid pBR322. Plasmid preparations containing 0.1–0.5 μ g of cDNA insert were linearized by digestion with *EcoRI* endonuclease and spotted on 5-mm nitrocellulose filters (Schleicher & Schuell). Filter-bound DNA was alkali-denatured, neutralized, washed, and hybridized to mRNA as described (11). Hybridized RNA was eluted from the filters and translated in a mRNA-dependent rabbit reticulocyte lysate. Hybrid-arrested translation studies were performed essentially as described by Miller *et al.* (11).

RESULTS

Isolation and Characterization of a Partial Human Poly(ADP-ribose) Polymerase cDNA. A λ gt11 cDNA expression library constructed from human hepatoma mRNA was screened with an antibody against human poly(ADP-ribose) polymerase (9). The polyclonal antibody reacted selectively with polymerase on immunoblots of total HeLa nuclear proteins (see below). This antibody specifically promotes only poly(ADP-ribose) polymerase immunoprecipitation from [³⁵S]methionine-labeled cell extracts and neutralizes catalytic polymerase activity in an *in vitro* assay (data not shown). We screened 2×10^6 plaques from the hepatoma λ gt11 library. Several clones were identified as producing a hybrid protein with polymerase antigenic determinants through five successive rounds of antibody screening and plaque purification (7). DNA was isolated from these clones

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and digested with *EcoRI*. The cDNA inserts ranged in size from 2.1 to 0.14 kilobases (kb). It should be noted that clone 2-1 had an internal *EcoRI* site yielding two fragments A and B of 1.3 kb and 0.7 kb, respectively.

Three selected phage clones were used to lysogenize bacterial strain BNN103. A bacterial lysate was prepared from the cells by incubation of the temperature-sensitive repressor at 42°C and induction of fusion protein production by isopropyl β-D-thiogalactoside. The lysate was analyzed by NaDodSO₄/PAGE and immunoblotting, as shown in Fig. 1. In three of the selected clones (O-1, W-1, and 2-1; lanes 3–5, respectively), the fusion protein reacted selectively with the anti-human poly(ADP-ribose) polymerase. There was no reactivity with β-galactosidase *per se* as indicated in lane 2 by immunoblotting of λgt11 phage-infected cells lacking a DNA insert.

Inserts in λgt11 phage were removed by cleavage with *EcoRI* and recloned in *EcoRI*-cleaved phosphatase-treated pBR322. The cDNA fragment A (derived from λgt11 clone 2-1) was used as a probe to hybridize against the other four selected cDNA fragments. Southern blot analysis indicated that insert A did not hybridize to insert B (which is from the same large insert in λgt11) nor was there hybridization to any other inserts.

RNA Gel Blot Analysis. The molecular weight of poly(ADP-ribose) polymerase (116 kDa) predicts that the mRNA for this protein must be at least 3 kb. Accordingly, the cDNA inserts were evaluated for use as probes to screen RNAs prepared from various human cells by RNA gel blot analysis. The smaller cDNA clones mainly hybridized to mRNA species that migrated considerably faster than the 18S ribosome marker (i.e., <2.5 kb). On the other hand, as shown in Fig. 2 A, lanes 1 and 2, and B, lanes 1 and 2, the 1.3-kb insert-A cDNA hybridized only to an mRNA species derived from either HeLa cell total or poly(A)⁺-selected RNA, which was slightly faster in mobility than the 28S marker and, hence, 3–4 kb in size. Identical results were achieved with human fibroblast mRNA and human bladder carcinoma strain 1080 total RNA

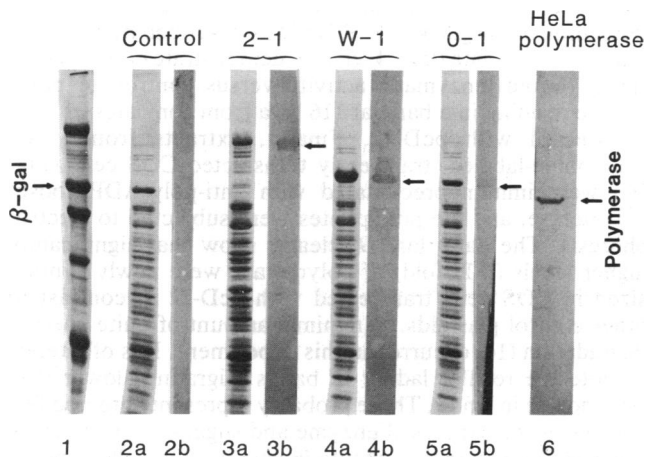


FIG. 1. Induction of the synthesis of antigenic fusion proteins in λgt11. The synthesis of the fusion proteins was induced essentially as described (8). Total proteins from *Escherichia coli* strain BNN103 and its λgt11 lysogens were isolated and analyzed by NaDodSO₄/PAGE. Duplicate lanes of the gel were either stained with Coomassie blue (lanes 1 and 2a–5a) or were transferred to a nitrocellulose membrane and subsequently treated with poly(ADP-ribose) polymerase antibody (lanes 2b–5b) and visualized using anti-rabbit peroxidase IgG. Lanes 3–5 represent polymerase positive λ strains 2-1, W-1, and O-1, respectively. Lane 1 contains molecular weight markers; lane 2 contains proteins from an induced λgt11 strain that contains no insert and only synthesizes β-galactosidase. Lane 6 is an immunoblot of a semi-purified preparation of HeLa cell poly(ADP-ribose) polymerase that comigrates on these gels at 116 kDa, with β-galactosidase (2, 9, 12).

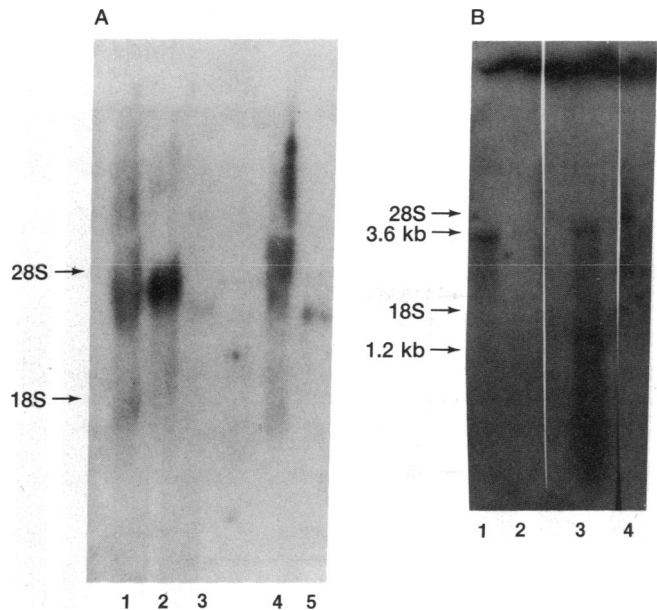


FIG. 2. RNA gel blot analysis of polymerase mRNA derived from various human RNA preparations and hybrid-selected RNA preparations. Labeled 1.3-kb cDNA fragment (insert A) from λ clone 2-1 was used as the hybridization probe. Total RNA (10–15 μg) or poly(A)⁺-selected RNA (<1 μg) was electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and processed for RNA gel blot analysis. (A) Lanes: 1 and 2, total RNA or poly(A)⁺-selected RNA from HeLa cells, respectively; 3, poly(A)⁺ RNA from human fibroblasts; 4 and 5, total RNA or poly(A)⁺ RNA from human cell line HT-1080, respectively. (B) Lanes: 1, total HeLa cell RNA; 2, poly(A)⁺-purified RNA; 3, hybrid-selected RNA; 4, poly(A)⁺ RNA not selected by the A fragment cDNA (as described in the legend to Fig. 3).

(Fig. 2A, lanes 3–5). Using RNA size markers, the HeLa cell mRNA species was calculated to be about 3.6 kb in size.

Hybrid Selection and Hybrid-Arrested Translation. Since amino acid sequence data is not as yet available for the human 116-kDa poly(ADP-ribose) polymerase, we verified that the cDNA insert A coded the polymerase cDNA sequences by a variety of techniques. First, insert A was bound to nitrocellulose and used to select and isolate the 3.6-kb mRNA species from total HeLa cell poly(A)⁺ RNA (Fig. 2B, lane 3). Some 1.3-kb insert DNA was also released from the filter but did not interfere with subsequent translation studies. Negligible residual 3.6-kb mRNA was noted in the mRNA not retained by the filter (lane 4). The hybrid-selected mRNA was translated in a reticulocyte lysate with [³⁵S]methionine. Total poly(A)⁺ RNA stimulated translation of many proteins in this assay (Fig. 3A, lane 1). NaDodSO₄/polyacrylamide gel analysis of the translated and immunoprecipitated products of hybrid-selected mRNA translation was performed. When immunoprecipitated with antipolymerase (lane 3), but not preimmune sera (lane 2), the largest product migrated identical to the 116-kDa poly(ADP-ribose) polymerase (also see Fig. 3B, lanes 3 and 4). A second band at ≈45 kDa is either nonenzymatic ³⁵S labeling of protein in the translation lysate or specific degradation peptides of polymerase (14), which we occasionally note during immunoprecipitation (i.e., Fig. 3B; see Fig. 5C, lane 9). The hybrid-selected translation product also comigrated with [³²P]ADP-ribose-labeled automodified polymerase.

To confirm the results obtained in the hybrid-selection experiment above, we attempted to arrest poly(ADP-ribose) polymerase mRNA translation by hybridization of mRNA at 48°C overnight with a full-length denatured cDNA derived from the 1.3-kb insert-A cDNA (DNA/RNA molar ratio was 0.0013).

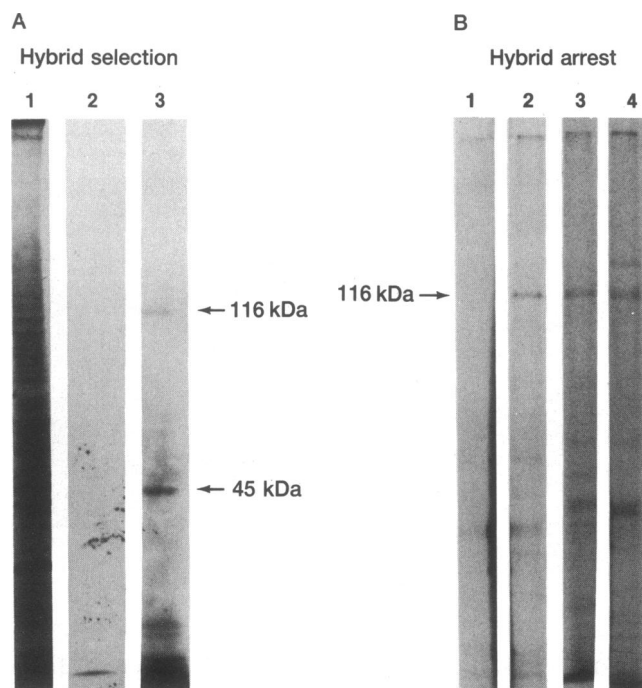


FIG. 3. Hybrid selection and hybrid-arrested translation. (A) Hybrid selection. The 1.3-kb cDNA fragment A was fixed to nitrocellulose, mock-prehybridized, hybridized to HeLa poly(A)⁺ mRNA, and washed to release unhybridized material. The selected mRNA was eluted and translated in a reticulocyte lysate with [³⁵S]methionine as described (13). The autoradiogram of an 8% NaDodSO₄/polyacrylamide gel containing ³⁵S-labeled translation products is shown. The reticulocyte lysate was programmed as follows: lane 1, nonhybrid-selected, total HeLa poly(A)⁺ RNA; lane 2, hybrid-selected RNA followed by immunoprecipitation with preimmune sera; lane 3, hybrid-selected RNA and immunoprecipitation with anti-poly(ADP-ribose) polymerase. (B) Hybrid arrest pcD-12 cDNA (0.1 μg) (see Fig. 4) was incubated with 10 μg of HeLa cell poly(A)⁺ RNA in buffer containing 65% formamide, 0.4 M NaCl, and 0.01 M Pipes (pH 6.4) at 48°C for 12 hr (lanes 1 and 2). As a control, HeLa poly(A)⁺ RNA was treated under the same conditions with the exception that cDNA was not added (lanes 3 and 4). After hybridization at 48°C (and melting at 100°C, when indicated) the samples were translated with [³⁵S]methionine, immunoprecipitated with anti-polymerase, and electrophoresed as described in A. Arrest: RNA was hybridized with pcD-12 cDNA (lane 1). Release: heat melt of lane 1 (lane 2). Arrest: RNA was hybridized in the absence of cDNA (lane 3). Release: heat melt of lane 3 (lane 4).

These data (Fig. 3B, lane 1) indicate that this DNA selectively inhibits the translation of polymerase mRNA. Examination of a nonimmunoprecipitated sample of lane 1 indicated a full complement of translated proteins (data not shown). Full recovery of polymerase translation was achieved upon heat melting of the hybrid sample (lane 2). Poly(A)⁺ RNA that had been mock-hybridized in the absence of added cDNA (lanes 3 and 4) directed the translation of polymerase in amounts equivalent to that of untreated mRNA.

Isolation of Full-Length Polymerase cDNA. Based upon the consistent detection of an mRNA species of ≈3.6 kb detected in a variety of RNA gel blot analyses (Fig. 2) an Okayama-Berg simian virus 40-transformed human fibroblast pcD-cDNA library that had been enriched for cDNAs 3–4 kb in size (15) was screened (5×10^6 colonies) with the labeled 1.3-kb insert-A cDNA. Approximately 100 positive clones were identified, and six were characterized. All of these clones hybridized with the 1.3-kb probe and contained inserts that were nearly consistent with the size of mRNA noted in Fig. 3. Clone pcD-19 was noted to possess 5 *Bam*HI sites, of which those at 1.1, 0.85, and 0.25 kb hybridized with the 1.3-kb probe (Fig. 4, lane 3). The remaining positive cDNAs were accord-

ingly analyzed with *Bam*HI restriction endonuclease (lanes 11–14). Four of the cDNA inserts gave essentially similar digestion and hybridization patterns; however, clone pcD-12 yielded a slightly larger *Bam*HI fragment at 2.0 kb that subsequently mapped toward the 5' region of the cDNA. Excluding vector sequences, the additive size of the *Bam*HI fragments in pcD-12 was 3.6 kb, which corresponded nearly exactly with the size of mRNAs noted earlier in Fig. 2.

pcD-12 Produces Enzymatically Active Polymerase in COS Cells. Based upon the above results a transient transfection of COS cells using DEAE-dextran was performed utilizing pcD-12 and pcD-19, which appeared to be shorter by ≈400 base pairs at the 5' region of the cDNA. Additional controls included duplicate plates of mock-transfected cells, and cells transfected with pSV2-CAT. After 48 hr, the cells were rinsed, scrapped, sonicated, and assayed for poly(ADP-ribose) polymerase activity utilizing [³²P]NAD in an *in vitro* assay that measures the initial velocity (16). The data were quite striking and indicated that indeed pcD-12 coded for nearly intact polymerase. Specific activity for pSV2-CAT transfected or mock-transfected cells was in the range of 1500 pmol of ADP-ribose per min per mg of protein, whereas cells transfected with pcD-12 yielded a value of over 4100, indicating a nearly 3-fold increase in specific activity. pcD-19, with a deletion at the 5' region, did not increase endogenous enzymatic activity. The labeled nuclear proteins from a similar assay were ethanol precipitated and separated by electrophoresis (Fig. 5A). The data indicated significantly increased automodification of the 116-kDa polymerase in the extract derived from pcD-12 transfected cells compared to controls.

To confirm conclusively that poly(ADP-ribose) polymerase was induced by pcD-12, two other experiments were performed. In Fig. 5B, unlabeled sonicates from the transfected cells were electrophoresed in NaDodSO₄/polyacrylamide gels that contained sonicated DNA as part of the gel matrix (since polymerase requires activated DNA for activity). Subsequently, the separated proteins in the gels were renatured and subjected to *in vitro* activity gel analysis by incubation of the gel with [³²P]NAD as described by Scovassi *et al.* (12). The data (lane 4) clearly demonstrate considerably higher (6-fold) enzymatic activity versus control for polymerase residing in a band at 116 kDa from sonicates of cells transfected with pcD-12. Finally, extracts from [³⁵S]-methionine-labeled, transiently transfected COS cells (Fig. 5C) were immunoprecipitated with anti-poly(ADP-ribose) polymerase, and the precipitates were subjected to electrophoresis. The data (lane 9) clearly show that significantly higher levels (>15-fold) of polymerase were newly synthesized in COS cells transfected with pcD-12 in contrast to other control plasmids. A minimal amount of quite specific degradation (14) occurred in this experiment. It is of interest to note the regular ladder of bands migrating slower than polymerase in lane 9. These probably represent automodified species of the expressed enzyme and suggest that enhanced poly(ADP-ribose)ylation occurs *in vivo* in COS cells expressing elevated levels of polymerase. At this stage, amino acid sequence information for peptides from the polymerase is not available for a direct DNA sequence comparison. From these data and the data presented earlier (7), we conclude that we have isolated a cDNA of sufficient size to code for human poly(ADP-ribose) polymerase [i.e., pcD-p(ADPR)P].

RNA Gel Analysis During the HeLa Cell Cycle. We screened for mRNA accumulation in HeLa cells during various stages of the synchronized cell cycle, since earlier data had suggested either higher catalytic activity and/or accumulation of poly(ADP-ribose)ylated proteins both in mid-S and at the end of S phase (18, 19). HeLa cells were synchronized by a double-thymidine block and were released into S phase. RNA samples were taken through the first 10.5 hr of the cell cycle

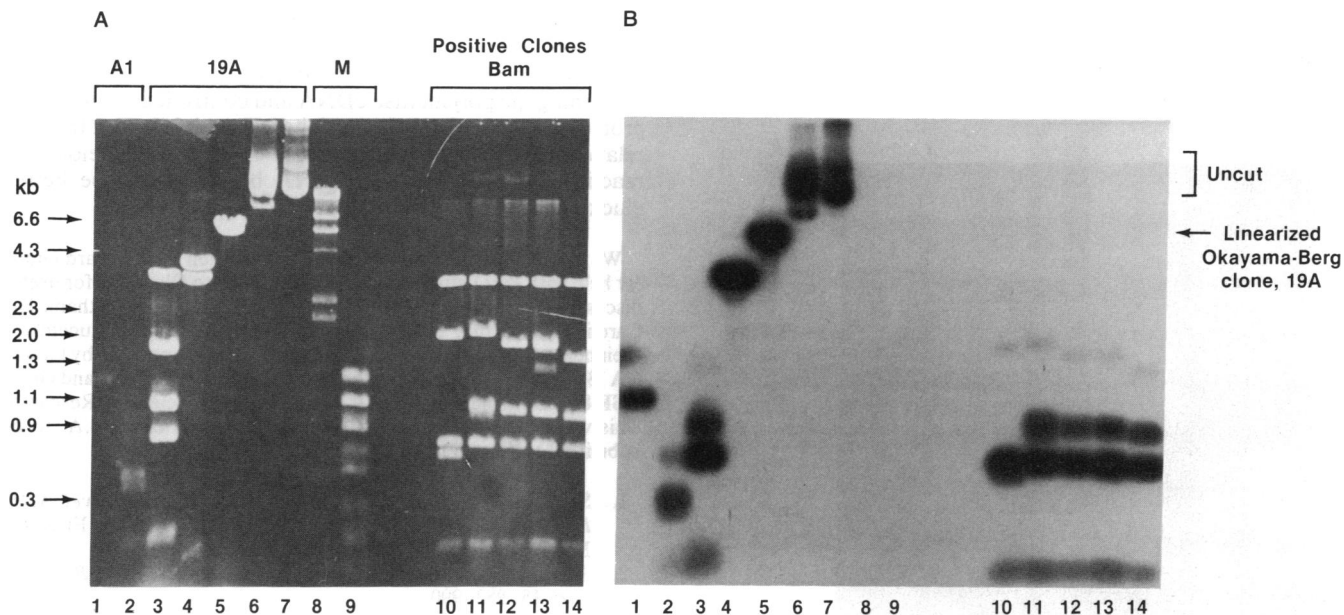


FIG. 4. Restriction enzyme analysis and Southern blot hybridization of cDNAs obtained from an Okayama-Berg 3- to 4-kb library. pcD-plasmid DNA (10–20 μ g) was isolated, digested with various restriction enzymes, electrophoresed on a 0.8% agarose gel (A shows the ethidium bromide stain), and, after Southern transfer, probed with 32 P-labeled A cDNA insert (B). Lanes: 1, cDNA insert A; 2, insert A digested with *Bam*HI; 3–7, pcD plasmid 19 digested with *Bam*HI, *Xho* I, *Sal* I, *Sac* I, and an undigested sample, respectively; 8, *Hind*III λ marker; 9, *Hae* III ϕ X174 marker; 10–14, pcD plasmids 10, 12, 13, 15, and 34 were digested with *Bam*HI, respectively.

and analyzed by RNA gel blot analysis with pcD-p(ADPRP) as probe. The data indicate (Fig. 6) that polymerase mRNA was consistently noted to be elevated (6-fold by scan) in periods between S phase and early G2 phase (at 5 hr and at 7 hr). In contrast, mRNA levels for histones H3 and H4 were elevated (10-fold) during 4–6 hr and negligible by 8 hr of these experiments (data not shown).

DISCUSSION

To our knowledge, this paper describes the first reported cloning and expression of a cDNA encoding poly(ADP-ribose) polymerase. The isolation of the large 3.6-kb insert in the Okayama-Berg expression vector and its expression into enzymatically active protein in COS cells provided the most

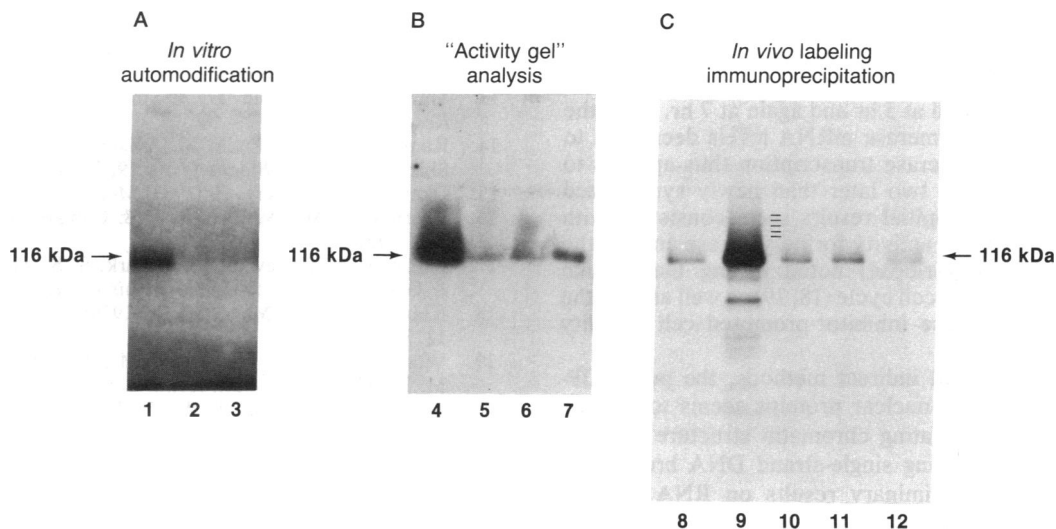


FIG. 5. Transfection of COS cells with pcD-12 produces enhanced expression of polymerase activity and immunoprecipitable polymerase protein. COS cells (10^6 cells) in duplicate flasks were treated in the presence or absence of plasmid DNA (25 μ g per 175-mm flask) for 4 hr at 37°C in the presence of DEAE-dextran as described (15). After 48 hr, the cells were rinsed, scraped from plates, sonicated, and assayed for poly(ADP-ribose) polymerase activity or immunoprecipitation (17). (A) Sonicated samples were assayed for activity for 10 sec with 2.8 μ Ci (1 Ci = 37 GBq) [32 P]NAD (2.1 mM), ethanol precipitated, electrophoresed on 7.5% NaDodSO₄/polyacrylamide gels, and exposed to x-ray film at –70°C. Lanes 1–3 show cells transfected with pcD-12, pSV2-CAT, or mock-transfected, respectively. (B) The sonicated samples were separated by electrophoresis in 7.5% NaDodSO₄/polyacrylamide (containing sonicated salmon sperm DNA at 100 μ g/ml). The gels were subsequently “renatured,” and the gel was assayed directly with [32 P]NAD for *in situ* poly(ADP-ribose) polymerase activity as described by Scovassi *et al.* (12). The washed gels were exposed to x-ray film at –70°C. Lanes 4–7 show cells transfected with pcD-12, pSV2-CAT, cells mock-transfected, or untreated, respectively. (C) The COS cells (2×10^7 cells per flask) were treated as above for 45 hr after which they were starved for methionine at 37°C in methionine-free media. [35 S]Methionine (250 μ Ci per flask) was added, and the cells were incubated at 37°C for 3 hr after which they were lysed; the proteins were immunoprecipitated with anti-polymerase, analyzed by NaDodSO₄/PAGE, and autoradiography. Lanes 8–12 show cells transfected with pBR322, pcD-12, pcD-sis, cells mock-transfected, or untreated cells, respectively.

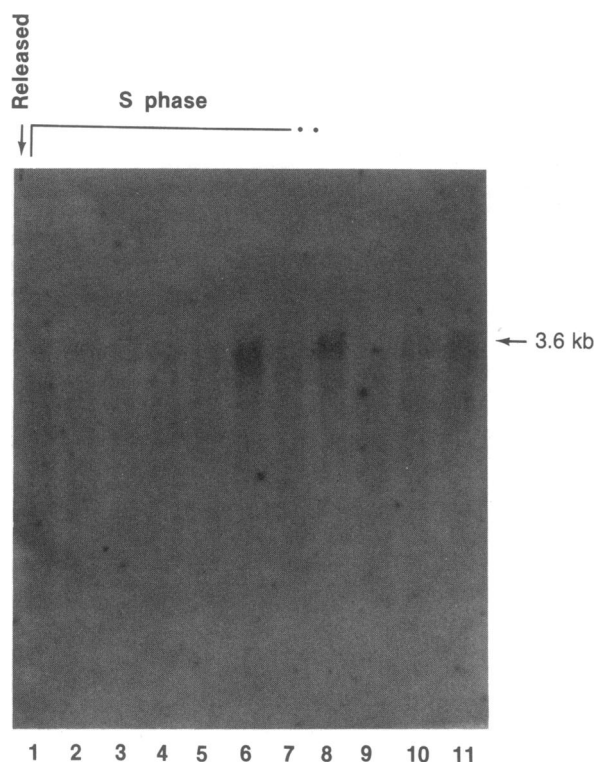


FIG. 6. Levels of polymerase mRNA during the cell cycle. HeLa cells were synchronized by a double thymidine block and released into S phase at 0 time (lane 1) as described in detail (20). Total cellular RNA samples were isolated hourly until 10.5 hr into the cell cycle, lanes 2–11, respectively. RNA (15 μ g) was loaded on each lane and hybridized to the pcD-p(ADPR)P insert.

impressive evidence for our isolation of the human poly(ADP-ribose) polymerase sequences. The transient expression data was verified by at least three criteria.

Using pcD-p(ADPR)P as probe, polymerase mRNA levels in synchronized HeLa cells were noted to be relatively steady and low during the first 4 hr of S phase; however, increased mRNA levels were noted at 5 hr and again at 7 hr. Near the end of S phase the polymerase mRNA levels decreased to basal levels. The polymerase transcription thus appears to occur about an hour or two later than newly synthesized histone mRNA. These initial results seem consistent with high levels of enzymatic activity for polymerase and for the synthesis of poly(ADP-ribose) noted during these same periods of S phase of the cell cycle (18, 19) as well as with the specificity of polymerase inhibitor-promoted cell lethality during S phase (21).

By various direct and indirect methods, the poly(ADP-ribose)ylation of various nuclear proteins seems to play an important role in modulating chromatin structure in those biological events involving single-strand DNA breaks (13, 22). Thus far, our preliminary results on RNA gel blot analysis using pcD-p(ADPR)P as probe with RNA isolated from cells exposed to various DNA damaging agents have not

indicated that these cellular conditions cause either immediate or long-term induction for poly(ADP-ribose) polymerase. However, in the future, once stable, transfected cell lines containing the polymerase cDNA and controlled by inducible promoters are available in our laboratory, it is hoped that the relationship between this enzyme system and pleiotropic reactions of cells involving DNA breakage can be better elucidated.

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