Properties of the nuclear P1 protein, a mammalian homologue of the yeast Mcm3 replication protein

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ABSTRACT

Polyclonal antibodies were raised against a multiprotein 'holoenzyme' form of calf thymus DNA polymerase α -primase and used to probe a human cDNA-protein expression library constructed in the λ gt11 vector. The probe identified a series of cDNA clones derived from a 3.2 kb mRNA which encodes a novel 105 kDa polypeptide, the P1 protein. In intact cells, the P1 protein was specifically associated with the nucleus, and in cell extracts, it was associated with complex forms of DNA polymerase α -primase. The synthesis of human P1-specific mRNA was stimulated upon addition of fresh serum to growth-arrested cells, and RNA blot analyses with the human P1-cDNA probe indicated that P1 is encoded by a strictly conserved mammalian gene. The amino acid sequence deduced from a 240-codon open reading frame resident in the largest human P1-cDNA (0.84 kb) displayed >96% identity with that deduced from the equivalent segment of a 795-codon open reading frame of a larger mouse P1-cDNA (2.8 kb). Throughout its length, the primary structure of mammalian P1 displayed strong homology with that of *Mcm3*, a 125 kDa yeast protein thought to be involved in the initiation of DNA replication (Gibson et al. 1990. *Mol. Cell. Biol.* 10: 5707–5720). The P1-*Mcm3* homology, the strong conservation of P1 among mammals, its nuclear localization, and its association with the replication-specific DNA polymerase α strongly suggest an important role of the P1 protein in the replication of mammalian DNA.

INTRODUCTION

DNA polymerase α -primase (Pol α) is an essential replication enzyme (1) which purifies as a multiprotein complex. The simplest form of Pol α is that prepared by purification on column matrices bearing an immobilized monoclonal antibody directed against the enzyme's catalytic polypeptide (2–4). After a rigorous

pre-elution salt wash to strip the immobilized antibody-enzyme complex of loosely associated proteins, elution typically yields an enzyme preparation consisting of four tightly associated polypeptide subunits—a 165–180 kDa DNA polymerase protein, a 70 kDa subunit of unknown function, and a 58 kDa and a 48 kDa doublet which together constitute the primase function of the enzyme (1).

Purification procedures which preserve weak protein-protein interactions typically yield 'holoenzyme' forms of Pol α containing considerably more polypeptide chains than the four resident in the immunoaffinity purified enzyme. For example, conventionally purified 'holoenzyme' forms of human and bovine Pol α contain as many as 6–12 additional, 'accessory' polypeptides (5–13).

We have sought to determine what, if any, function the accessory proteins found in conventionally purified Pol α preparations have in mammalian DNA replication. Our first step in this effort has been to identify, clone, and sequence cDNAs specifying these proteins and exploit this information to obtain clues to their structure and function.

To clone these protein-specific cDNAs, we have raised polyclonal antibodies against a conventionally purified Pol α 'holoenzyme' and used these antibodies to detect antigen-specific clones in the λ gt11 expression system (14). We have isolated a number of probe-positive clones. One group of cDNA clones possessed an open reading frame encoding a novel nuclear mammalian protein, the P1 protein. Sequence comparisons revealed that the P1 protein is related to a family of yeast proteins which play a fundamental role in regulation of DNA replication.

MATERIALS AND METHODS

Cell culture

Primary human embryonic lung fibroblast cells (Hel 299), HeLa cells and Balb/3T3 mouse cells (American Type Culture Collection) were grown under standard cell culture conditions with 5–10% fetal calf serum. Hel 299 cells were growth-arrested

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by serum starvation and restimulated to proliferate by addition of fresh serum as previously described (15). DNA replication was monitored by pulse-labeling with [^3H]-thymidine (15).

Cell fractionation

Prior to fractionation, cells were washed in phosphate-buffered saline (PBS) and swollen in hypotonic buffer A (10 mM Hepes, pH 7.8; 1.5 mM MgCl_2 ; 0.5 mM dithioerythritol; 10 mM NaCl; and aprotinin, pepstatin, leupeptin, each at 10 $\mu\text{g}/\text{ml}$). Cells were disrupted by Dounce homogenisation. Nuclei were separated from the homogenate by low-speed centrifugation, and the resulting supernatant was centrifuged for 1 hr at 100,000 $\times g$ to obtain the clarified cytosolic extract.

Preparation of Pol α

Polymerase α 'holoenzyme' was assayed and isolated from a cytosolic extract, prepared from fresh calf thymus tissue, by successive purification on DEAE-cellulose, phosphocellulose and hydroxyapatite essentially as described (13, 16). Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (17) revealed more than 15 polypeptides (see: Fig. 1).

Preparation of Pol α -antibodies

The holoenzyme form was used as an antigen to immunize rabbits (18). Immunoglobulines (IgG; rabbit anti-pol- α antibodies) were prepared from antisera by chromatography on protein A-Sepharose CL-4B as suggested by the manufacturer (Pharmacia) (18). The antibodies reacted with 4–5 polypeptides of the Pol α preparation.

Isolation and analysis of P1-specific cDNAs

A $\lambda\text{gt}11$ cDNA library prepared from human HeLa cell mRNA (18, 19) was probed with rabbit anti-Pol α antibodies generating 18 probe-positive clones. DNA-DNA hybridization indicated 17 of these to be P1-specific (19). The largest human P1-cDNA clone (0.84 kb; P1h) was used as a hybridization probe to isolate a 2.8 kb P1-specific mouse cDNA clone (P1m) from an Okayama-Berg (20) murine lymphoblast library (21). Both strands of the

P1h- and of the P1m-cDNAs were sequenced by the dideoxy chain termination technique (22).

RNA blotting

Cytoplasmic RNA was prepared by the method of Favarolo et al (23). Total RNA (75 μg) was denatured in 50% formamide, 6.5% formaldehyde and separated on 0.8% agarose gels containing 2.2 M formaldehyde. The RNA was transferred and uv-cross-linked to Hybond N membranes (Amersham-Buchler). The membrane-bound RNA was hybridized with [^{32}P]-labeled P1h-cDNA and processed for autoradiography by standard procedures (24). Filters to be used for rehybridization were stripped by soaking in 5 mM Tris-HCl, 2mM EDTA (pH8) for several hours at 65°C. Probes used for rehybridizations included cDNAs encoding β -actin (from H. Arnold, Hamburg), histone H4 (from D. Schümperli, Zürich) and rRNA (from M. Duguët, Paris). All cDNA probes were radioactively labeled to a specific radioactivity of at least 10^9 dpm/ μg DNA using a random-primed labeling kit from Amersham-Buchler.

Preparation of protein A-P1 fusion protein and P1-specific antibodies for use in immunoblotting

P1h-cDNA was fused in-frame with the protein-A-coding region of the pRIT2T *E. coli* expression plasmid (Pharmacia). The P1-protein A fusion protein was overexpressed and purified to homogeneity on IgG-agarose as suggested by the manufacturer (Pharmacia). The P1-protein A fusion protein was then used to produce IgG antibodies in rabbits and IgY antibodies in chicken as described by Gassmann et al. (25).

For Western blotting, cellular proteins were separated by polyacrylamide gel electrophoresis (17), transferred to PVDF membranes (Millipore) and probed with anti-P1 antibodies as described by Twobin et al. (26). The second antibody consisted of either goat-anti-rabbit or rabbit-anti-chicken antibodies linked to alkaline phosphatase (Sigma).

Monospecific antibodies against the large, 180 kD subunit of Pol α were prepared from the complex rabbit-anti-pol α antiserum (see above) by the method of Lewis et al. (27). For this purpose we used immunoaffinity-purified Pol α blotted onto Nylon membranes.

Fig. 1. Characterization of the antigen. Pol α was prepared from calf thymus essentially as described (13, 16). A sample of this preparation was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. A lane of this gel was stained by Coomassie blue (left lane). The same sample was used for Western blotting after polyacrylamide gel electrophoreses performed as above. In one experiment, rabbit antibodies against the P1-protein A fusion product (see: Methods) were used as a probe (central lane). In an additional experiment, we investigated the same Pol α preparation using as a probe monospecific antibodies against the Pol α large subunit (see: Methods) (right lane).

Fig. 2. Identification of the P1 protein in unfractionated protein extracts. The cytosolic extract (CE) from HeLa cells and the Pol α -holoenzyme from calf thymus (pol α) were prepared, subjected to polyacrylamide gel electrophoresis, and transferred to membranes for Western blotting as described in Methods. The chicken anti-P1 antibody was used in 1:500 and 1:1000 dilutions as indicated. Preimmune chicken antibodies (PIY) at the equivalent of a 1:500 dilution served as a control. The cytosolic extract was processed immediately after its preparation, whereas the Pol α preparation had been stored at -20°C for several months. Faint bands as visible in the 30–40 kDa range are unspecific (see: Fig.4).

Indirect immuno-fluorescence assays

Indirect immunofluorescence was performed according to a method adapted from that described by Bensch et al. (28). Cells were cultured on cover slips, washed with PBS and fixed with 2% formaldehyde. The cells were washed extensively with PBS, permeabilized with 1% *nonidet* 40 in PBS and incubated for 1 hr at 25°C with anti-P1 antibodies or control pre-immune antibodies. The cells were washed with PBS, developed with FITC-coupled anti-rabbit antibody (Fab fragment; Sigma) and processed for photography as described (28).

RESULTS

Immunodetection of the P1 protein

In Fig. 1, we show the results of a gel-electrophoretic analysis of a conventionally purified Pol α -‘holoenzyme’ preparation from calf thymus cells (see: Methods). One lane of the gel was stained

Fig. 3. Cosedimentation of P1 protein and Pol α . A protein extract from HeLa cells was sedimented through a 30–10% glycerol gradient (300 mM KCl; 20 mM Hepes buffer, pH 7.8), using the Beckman SW 55 rotor for 35 hs at 45,000 rpm and 2°C (15). Sedimentation direction is from the right to the left. (a) DNA polymerizing activity. The incorporation of [32 P]-labeled nucleotides into acid-precipitable DNA was determined as described (16). (b) Identification of the large subunit of Pol α by Western blotting (see Methods) using a monospecific antibody as a probe (see Methods and Fig. 1). (c) Identification of P1 protein by Western blotting as described in Methods and under Fig. 1 and 2.

with Coomassie blue, other lanes were immunoblotted and probed with antibodies against the P1-protein A fusion product or with monospecific antibodies against the 180 kD subunit of Pol α . Coomassie-staining revealed that our Pol α -preparation consisted of more than a dozen polypeptides. The P1-specific antibodies recognized two bands of polypeptides with apparent molecular weights of 105 kDa and of 85 kDa. The monospecific Pol α -antibodies reacted, as expected, with one band in the molecular weight range of 180 kDa and, in addition, with a series of smaller polypeptides, including a prominent 105-kDa species, a common proteolytic degradation product of the large Pol α -subunit. This result showed that our enzyme preparation was partially degraded, and suggested that the 85 kDa polypeptide, reacting with P1-antibody, could also be a degradation product.

To investigate this possibility, we prepared crude extracts of calf thymus as well as of human and mouse cells, and subjected them to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The respective electropherogram was blotted and probed with antibodies generated against the P1-protein A fusion product. In the crude extracts which were analyzed fresh—without freezing and thawing—we detected a 105 kDa P1-specific polypeptide. The results obtained with the calf thymus extract are shown in Fig. 2 (lanes marked CE), those obtained with extracts from human and mouse cells were very similar (see below). Blots of the same extracts subjected either to storage at -20°C or repeated freezing and thawing indicated an increasing amount of an 80–85 kDa immunoreactive band, which was apparently derived from degradation of the 105 kDa polypeptide. The 105 kDa/85 kDa doublet was particularly pronounced in the holoenzyme Pol α preparation, which was frozen and thawed several times during its preparation (Fig. 2, lanes labeled pol α ; see also Fig. 1).

In further experiments, we have determined (i) that Pol α holoenzyme preparations from human and mouse cells also contain the immunoreactive P1 protein, (ii) that a fraction of this

Fig. 4. Extraction of P1 protein from isolated nuclei. HeLa cells were ruptured in hypotonic buffer as described in Methods, and the cytoplasmic and nuclear fraction were separated by low-speed centrifugation. The cytosolic fraction was centrifuged at $100,000\times g$ to obtain a high speed supernatant (s) and a pellet (p) which was resuspended in its original volume. The nuclear pellet was also resuspended in its original volume, and identical samples were adjusted to 0.1, 0.4, 0.8, and 1.6 M NaCl as indicated, kept for 30 min on ice and centrifuged at $100,000\times g$ at 4°C . Samples, representing approximately 1/25th of a near confluent 14.5 cm dish of HeLa cells, were processed and blotted as described under Fig. 1. Left, reactions with chicken anti-P1 antiserum; right, reactions with chicken pre-immune serum at an equivalent dilution.

polypeptide cosediments with Pol α activity during glycerol gradient sedimentation (Fig. 3), and (iii) that the Pol α 'core' enzyme, isolated by immunoaffinity purification and consisting of the 4 basic subunits (2, 4), does *not* contain the P1 polypeptide (not shown).

Nuclear localization

We exploited the P1-specific antibodies to explore the subcellular distribution of P1, using human cell lines convenient for this purpose. We first investigated the distribution between nuclear and cytoplasmic fractions prepared from ruptured HeLa cells with a method favoring nuclear integrity (see: Methods). The results obtained by immunoblotting of the resultant cytosol and nuclear extracts are summarized in Fig. 4. Although a minor fraction of P1 was detectable in the cytosol, the majority remained in the nucleus, and concentrations of 0.5–1.0 M NaCl were required to extract it suggesting that the protein was bound to a nuclear structure and not free in the nucleoplasm. A similar set of experiments was performed with the human fibroblast Hel 299 cell line. The results were essentially identical to those of Fig. 4, and therefore are not shown.

We further investigated the subcellular distribution of P1, using indirect immunofluorescence analysis of glutaraldehyde-fixed permeabilized Hel 299 cells. The results, displayed in Fig. 5, also indicated a distinct nucleus-specific location of P1. The data of Fig. 5 also show that there was a clear variation in the intensity of nuclear staining in different cells. This was detected in several independent experiments of this type. It remains to be shown whether the variation in nuclear staining correlates with physiological parameters.

Fig. 5. Nuclear localization of P1 protein in human Hel 299 cells. Glutaraldehyde-fixed proliferating cells were incubated with P1-antibodies and counterstained with secondary goat anti-rabbit IgG coupled to FITC as described in Methods. Staining of the same population of cells using preimmune IgG yielded no significant immunofluorescence in the cell nuclei (not shown).

P1-specific mRNA: size, distribution, and fluctuation of levels in response to serum starvation and replenishment

Using the largest human cDNA clone as a probe we analyzed by high stringency RNA ('Northern') blotting, poly (A⁺) mRNA derived from calf thymus tissue and from exponentially growing mouse (Balb/3T3), and human (HeLa and Hel 299 fibroblast) cells. The results which are not shown indicated that each preparation contained a major probe-reactive RNA species with an electrophoretic mobility suggesting a length of 3.0–3.2 kb. The presence of this strong probe-reactive mRNA band in preparations from each species strongly suggested that P1 is specified by a highly conserved mammalian gene.

We investigated the relative amounts of P1-specific mRNA in a serum-deficient, growth-arrested cell population vs. that in a serum-stimulated population. Hel 299 cells were held in serum-deprived medium for 48 hrs by which time cell proliferation and uptake of [³H]-thymidine were essentially zero (15). Fresh serum was then added, and the entrance of the cell culture into S phase was monitored by determination of the rate of [³H]-thymidine incorporation into DNA (15). Total RNA was prepared from samples of cells taken at various times subsequent to serum addition, and the samples were blotted and probed with [³²P]-labeled P1-cDNA as described under Methods. The

Fig. 6. P1-specific mRNA levels in growth-arrested and serum-stimulated Hel 299 fibroblasts. Total RNA was extracted from serum-starved cells just prior to readdition of 10% fetal calf serum (0 hr) and at 6, 12, 18 and 24 hrs thereafter. Samples containing approximately 75 μ g total RNA were processed and Northern blotted as described in Methods, using the following [³²P]-labeled probes (a) human P1-specific cDNA; (b) β -actin and histone H4 cDNA as indicated; (c) human 18S rRNA.

results, summarized in Fig. 6, indicate a relatively low level of the 3.2 kb P1-specific mRNA in resting cells and a time-dependent increase of the level following serum addition, reaching a maximum at the time when the cells enter into S phase, at about 18 hrs after stimulation (15).

Determination of P1 primary structure and its comparison with that of the yeast *Mcm3* protein

The largest P1-specific cDNA available to us at this writing is a 2.8 kb mouse cDNA which we isolated from a size-fractionated Okayama-Berg library (21) using the 0.84 kb P1-specific human

cDNA as a probe. Although neither cDNA was sufficient in length to encode the entire 105 kDa P1 polypeptide, the determination of their respective sequences provided valuable information regarding P1 structure and its possible function.

The deduced amino acid sequences of the two DNAs were, as expected, very similar. The 841 bp human cDNA (EMBL Data Library accession number X62153) contained a 246-codon open reading frame specifying an amino acid sequence >95% identical with that deduced for the C-terminal end of the 794-codon open reading frame of the mouse cDNA (accession number X62154) (Fig. 7).

The mouse sequence displayed in Fig. 7 is sufficient to encode a polypeptide with a calculated molecular weight of 89.6 kDa, a value which is 85% that of the full sized protein as determined by electrophoretic means. The deep internal location of the first Met codon and the position of an AATAAA motif in a 425 bp untranslated region downstream of the last codon of the open reading frame indicate that the cDNA is truncated at its 5' end. Albeit incomplete, the amino acid sequence of the P1 polypeptide displays a very hydrophilic character generated by a content of charged amino acids (glu, asp, arg, lys and his) exceeding 32%. The amino acid sequence contains a large number of potential phosphorylation sites. Our assessment of the sequence did not reveal any sequence motifs, commonly found in DNA or nucleotide binding proteins.

A computer search using the BESTFIT program revealed that the P1 amino acid sequence is just over 40% identical with that of the yeast *Mcm3* protein (29), and in one 185 codon segment the identity approaches 77% (Fig. 7).

We may add though that the antibodies, specific for the mammalian P1 protein, do not react with a polypeptide in yeast nuclear extracts as investigated by Western blotting (not shown).

DISCUSSION

We have sought in this work to characterize the basic features of the Pol α -holoenzyme-associated protein P1 and to obtain leads that will facilitate a comprehensive definition of its cellular function. We have demonstrated that P1 is a strongly conserved, hydrophilic mammalian protein of ca. 105 kDa. We have found that P1 is preferentially bound in the cell nucleus, and that part of it may be associated with a complex holoenzyme form of Pol α .

We have also observed that the level of P1-specific mRNA increases in response to serum stimulation of serum-deprived quiescent cells, peaking with the onset of S phase. Whether the synthesis and/or degradation of P1-specific mRNA is cell-cycle specific awaits more rigorous analysis in naturally synchronized cells.

What are the likely functions of mammalian P1 ? At present, the strongest lead to its function lies in its extensive sequence similarity with the yeast *Mcm3* protein (29, 30). This homology strongly suggests that P1 may be the mammalian equivalent of a recently discovered family of nuclear proteins for which *Mcm3* may be the prototype. *Mcm3* was discovered as a member of a set of mutants defective in maintenance of yeast minichromosomes that contain a yeast replication origin (ARS, autonomously replicating sequence) and a centromere (29, 30). Interestingly, *mcm3* mutants affect the maintenance of minichromosomes with some, but not all, ARS sequences, suggesting that the function of the *Mcm3*-protein may depend on a specific interaction with specific ARS sequences (11).

Fig. 7. Comparisons of mouse P1 protein (p1. m) and human P1 protein (p1.h) with the yeast *Mcm3* protein (mcm3). The amino acid sequences, as given in the one-letter-amino acid code, were deduced from the nucleotide sequence of the mouse P1-cDNA and the human P1-cDNA clones (EMBL Data Library. Accession numbers: X62154 and X62153). Since the amino termini have not yet been determined, the amino acids encoded by the 5'-most codons were arbitrarily designated as residues nr. 1. The P1 sequences were aligned with the yeast *Mcm3* sequence (29, 30) using the BESTFIT program. Identities between the mouse and the yeast sequences are indicated by dots, identities between the two mammalian sequences and the yeast sequence are indicated by vertical lines.

Homozygous *mcm3* mutants are not viable, because they are apparently unable to replicate their genomes to completion (29). The latter phenotype and the ARS sequence dependence of *Mcm3* action on minichromosomes maintenance clearly suggest a role for *Mcm* proteins in replication initiation. The possibility of an initiation function is strengthened by the observation that the *Mcm3* protein also displays homology with *S. cerevisiae* proteins encoded by the *MCM2* and *CDC46* genes and *S. pombe* proteins encoded by genes *Cdc21* and *Nda4*. The phenotypes of the mutants in these genes suggest that all their respective protein products are involved in reactions relevant to initiation of DNA replication (29–32).

If P1 were involved in replication initiation, how might it act? The homology of P1 with a yeast protein which may interact with an ARS sequence and its association with complex forms of Pol α prompt us to speculate that it functions in cellular DNA replication in a capacity analogous to that which T antigen serves in the initiation of SV40 viral DNA replication (33–39).

T antigen functions in SV40 DNA replication initiation in at least two important ways. It binds the replication origin of replication where it first untwists and then unwinds the bound DNA as an early step for the establishment of replication forks (38). A subsequent step is the specific interaction of origin-bound T antigen with DNA polymerase α -primase (35, 36). This reaction probably guarantees the correct positioning of the polymerase for the synthesis of RNA primers which are then extended forming the start points for leading and lagging progeny DNA strands. Pol α does not only interact with origin-bound T antigen but with soluble T antigen as well. In fact, the first evidence for specific complex formation was obtained investigating unfractionated extracts from SV40-infected cells where a significant fraction of cellular Pol α was found to be in association with T antigen (37, 38).

The P1 protein-Pol α complex described in this communication may have a function in cellular DNA replication that could be similar to the role played by T antigen-Pol α complexes in SV40 DNA replication. Experiments exploiting purified P1 and P1-deficient forms of Pol α have been initiated to examine these possibilities.

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