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The Third Subunit of Protein Phosphatase 2A (PP2A), a 55-Kilodalton Protein Which Is Apparently Substituted for by T Antigens in Complexes with the 36- and 63-Kilodalton PP2A Subunits, Bears Little Resemblance to T Antigens

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The small and middle T (tumor) antigens of polyomavirus have been shown previously to associate with the 36-kDa catalytic subunit and the 63-kDa regulatory subunit of protein phosphatase type 2A, apparently substituting for a normal third 55-kDa regulatory subunit (D. C. Pallas, L. K. Shahrik, B. L. Martin, S. Jaspers, T. B. Miller, D. L. Brautigan, and T. M. Roberts, Cell 60:167–176, 1990). To facilitate a comparison of the normal regulatory subunit and T antigens, we isolated a 2.14-kb cDNA clone encoding this 55-kDa subunit from a rat liver library. Using a probe from the coding region of this gene, we detected a major 2.4-kb mRNA transcript in liver and muscle RNAs. The 55-kDa protein phosphatase 2A subunit purified from rat skeletal muscle generates multiple species when analyzed on two-dimensional gels. Transcription and translation of the clone in vitro produced a full-length protein that comigrated precisely on two-dimensional gels with three of these species, indicating that the 55-kDa protein is apparently modified similarly in vivo and in reticulocyte lysates. Additional species in the purified preparation were not found in the translate, suggesting that there are probably two or more isoforms of this protein in rat muscle. Somewhat surprisingly, there was no clear homology with T-antigen amino acid sequences.

Some oncogenic viruses, such as Rous sarcoma virus, transform cells by virtue of the fact that their genomes encode an altered version of a cellular protein or proteins involved in control of normal cell growth and division. Other tumor viruses, such as the papovaviruses polyomavirus and simian virus 40 (SV40), encode proteins that specialize in associating with and modulating the activity of such normal cell growth control proteins. Papovavirus tumor (T) antigens, for example, interact with and affect the normal function of a number of cellular proteins, of which $pp60^{c\text{-}src}$ (4), phosphatidylinositol-3 kinase (44), and p53 and the retinoblastoma gene product (12) are probably the best known examples. Whether the T antigens are themselves altered versions of other cellular growth control proteins remains to be seen; if such cellular homologs of T antigens exist, they certainly have been difficult to detect. One possibility that appears more likely is that T antigens represent a patchwork quilt of domains, each of which is biochemically active on cellular control proteins. For example, the amino acid sequence around tyrosine 315 in middle T (MT) antigen of polyomavirus that is necessary for association of phosphatidylinositol-3 kinase activity is related to the kinase insert region of the platelet-derived growth factor receptor, which also binds the phosphatidylinositol-3 kinase (7). According to this hypothesis, other parts of MT might be related to other as yet unidentified proteins.

More recently, two cellular proteins of 36 and 63 kDa that associate with polyomavirus small and middle T antigens and with SV40 small t antigen (13, 25, 27, 31, 34–36, 41, 46) were demonstrated to be, respectively, the catalytic and regulatory subunits of protein phosphatase 2A (PP2A) (29). This result was subsequently confirmed by others (43).

The biochemically purified 36-kDa/63-kDa PP2A heterodimer from cells not expressing MT is often found associated with an additional subunit (for a review, see reference 3). Several different species of additional regulatory subunits, with molecular masses of 54, 55, and 72 kDa, have been identified; the 55-kDa species has been the most studied. None of these third subunits is found associated with T antigen/PP2A complexes from T-antigen expressing cells (29). Because of this and other similarities between the association of T antigens and the 55-kDa subunit with the PP2A heterodimer (see discussion in reference 29), it seemed likely that T antigens and the 55-kDa subunit might bind at the same site. Our working hypothesis is that interaction of the 36-kDa/63-kDa PP2A heterodimer with small t or MT in vivo might substitute for binding of a normal regulatory subunit of PP2A.

Consistent with this hypothesis, Yang and colleagues (45) recently reported reassociation experiments demonstrating among other things that SV40 small t antigen cannot bind simultaneously to the same PP2A 36-kDa/63-kDa heterodimer as the 55-kDa subunit in vitro. Although in those experiments SV40 small t was unable to displace the 55-kDa subunit from the PP2A 36-kDa/63-kDa/55-kDa heterotrimer (45), there is evidence that in vivo all the PP2A 36-kDa protein in the cell is associated with SV40 small t upon SV40 viral infection (30). One possible explanation consistent with these results is that PP2A exists for some or most of the cell cycle as the 36-kDa/63-kDa heterodimer form and that only

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upon proper stimulus does some portion of it associate with the 55-kDa subunit, modulating its activity. Homogenization of tissue for purification of PP2A may artificially trigger extensive association that does not reflect the status in vivo prior to this disruption. According to this model, small and middle T antigens might commandeer the heterodimer in a cell-cycle independent manner to help stimulate progression through the cell cycle. PP2A has been implicated recently in control of cell cycle both in yeast cells (19) and in frog oocyte systems (8, 11, 22), apparently negatively regulating entry into mitosis.

If small and middle T antigens bind to the same site on the PP2A heterodimer as the 55-kDa regulatory subunit, it would not be unreasonable to expect that this might be reflected by some homology in their primary sequences. To facilitate a comparison of these two proteins, we undertook to isolate cDNA clones encoding the PP2A 55-kDa regulatory subunit.

MATERIALS AND METHODS

Purification of 55-kDa subunit of PP2A. The heterotrimeric form of PP2A ($2A_1$) was isolated from rat skeletal muscle as described previously (17). Briefly, 1 kg of rat skeletal muscle was homogenized in a buffer containing 20 mM Tris (pH 7.6), 10 mM EDTA, 2 mM EGTA, 0.1% 2-mercaptoethanol, 2% glycerol, 1 mM phenylmethylsulfonyl fluoride and benzamidine, and 1 μg each of leupeptin, pepstatin, and chymostatin per ml. The cytosol was prepared by centrifugation at 140,000 × *g*, and the fraction of the cytosol precipitating between 30 and 60% saturation with ammonium sulfate was dialyzed and run on a DE52 anion-exchange column. The phosphatase was eluted with a 0 to 0.5 M NaCl gradient, and following dialysis, the phosphatase preparation was run on an aminohexyl-agarose column. Following elution from this column with a 0.2 to 1 M NaCl gradient, the phosphatase was dialyzed and concentrated under vacuum. The concentrated preparation was subjected to size exclusion chromatography using a semipreparative Bio-Rad TSK-250 high-pressure liquid chromatography (HPLC) column (21.5 by 600 mm). The fractions eluting at a molecular size corresponding to approximately 210 kDa and containing the peak phosphorylase phosphatase activity were pooled and dialyzed. The preparation was subjected to a final cleanup by using a Waters analytical DEAE-5PW anion-exchange HPLC column equilibrated in 20 mM triethanolamine (pH 7.0)–1 mM EGTA–1 mM dithiothreitol–5% glycerol. The purified phosphatase was eluted with a 0 to 0.4 M NaCl gradient, and the fractions were analyzed for phosphorylase phosphatase activity and for purity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The subunits of the purified heterotrimeric form of PP2A were separated by electrophoresis on a 10% SDS-polyacrylamide gel, and the proteins were electrophoretically transferred to nitrocellulose and visualized by staining with 1% Ponceau S (1).

Protein sequence analysis. Except for HPLC, internal amino acid sequence analysis was done by the method of Abersold et al. (1). Resultant tryptic, chymotryptic, or staphylococcal V-8 protease digestion mixtures were run essentially as described previously (38) on a Hewlett-Packard 1090 HPLC equipped with a model 1040 diode array detector and a Vydac C18 column (2.1-mm inside diameter by 150 mm long). Peptides were eluted essentially by using the gradient described elsewhere (38). Peptides were applied to an Applied Biosystems model 477A pulsed liquid protein sequencer equipped with an online model 120A HPLC using the manufacturer's fast cycle program.

Isolation and sequencing of cDNA clones. Multiple oligonucleotide probes of 20 or 23 nucleotides each containing a minimum of 17 contiguous nucleotides either completely degenerate or with an occasional inosine substitution were designed from tryptic peptide sequences. This guaranteed that at least one subspecies of the degenerate probe was a perfect 17-nucleotide match to the authentic gene, or at most contained one inosine pairing. On one or both sides of the 17-nucleotide stretch, an extra 3 nucleotides were added that usually contained one guess based on codon frequencies. These probes were end labeled with polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (Dupont, NEN Research Products) and used to screen a rat liver Zap II cDNA library (Stratagene 936507) plated on XL1-blue cells (Stratagene) according to the manufacturer's protocol with the following modifications. Aliquots (2 ml) of XL1-blue cells in 10 mM $MgSO_4$ (optical density at 600 nm = 0.5) were infected with 120,000 PFU of bacteriophage, mixed with 23 ml of top agar (0.7% agarose), and then plated on bioassay dishes (24.5 by 24.5 cm) (Nunc). After approximately 8 h at 37°C, the plates were chilled and four sequential plaque lifts were performed with nitrocellulose filters (Stratagene). The lifts were performed at 4°C for 4, 7, 10, and 13 min, respectively. Phage were lysed and filters were baked according to the manufacturer's protocol. Plaque prehybridizations and hybridizations were performed at 37°C in 3.33× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% SDS–20% formamide–200 μg of salmon sperm DNA per ml–20 mM NaH_2PO_4 (pH 7.5). Filters were washed twice for 30 min at room temperature in 3 M tetramethylammonium chloride–2 mM EDTA–50 mM Tris-HCl (pH 8.0) (TMAC buffer) (6), once for 30 min at 40°C in TMAC buffer plus 0.2% SDS, and finally once for 30 min at 50°C in TMAC buffer plus 0.2% SDS. Positive plaques were picked and plaque purified, and pBluescript SK(−) phagemids were excised according to Stratagene's in vivo excision protocol. The result of in vivo excision from lambda Zap II is a Bluescript plasmid with T3 and T7 promoters on either end of the gene. Large plasmid DNA preparations were done by standard protocols, and inserts were sequenced by the dideoxy chain termination method (32), first with primers corresponding to the T3 and T7 promoters and then with conventional methods to sequence longer inserts. The first authentic cDNA clone isolated for the 55-kDa PP2A subunit contained 940 bp of the 3′ end of the coding region and 472 bp of 3′ untranslated sequence (UTS). A restriction fragment containing 380 bp of the 5′ end of the first authentic cDNA clone isolated for the 55-kDa PP2A subunit (a partial clone; see Results) was labeled by random priming with the Multiprime DNA Labelling System (Amersham) and used to rescreen the library. Prehybridization and hybridization were done the same as for oligonucleotide probes except the temperature was 50°C. Filters were washed with 0.2× SSC at room temperature twice for 10 min and then twice for 45 min and finally at 60°C for 10 min in 0.2× SSC containing 0.1% SDS.

Northern (RNA) blot analysis. Total RNAs from rat heart and aorta were a gift from Kevin Claffey, and poly(A)$^-$ and poly(A)$^+$ rat RNAs were a gift from Greg Robinson. Samples of 15 μg of each total or poly(A)$^-$ RNA and an approximately 3-μg sample of poly(A)$^+$ RNA were analyzed on a 1% agarose–3.7% formaldehyde gel as described by Maniatis et al. (23). RNA transfer to nylon membrane and Northern blot hybridization were performed by the methods

of Virca et al. (40) with three exceptions: transfer was by vacuum blotting, 60 μg of denatured salmon sperm DNA per ml was included in the prehybridization and hybridization solutions, and washes were performed with 0.5× SSC. An *Alw*NI-to-*Pst*I restriction fragment beginning 7 nucleotides into the coding region and ending 16 nucleotides after the stop codon was isolated, labeled by random priming with Multiprime DNA Labelling System (Amersham), and used as a probe. Prehybridization, hybridization, and final wash were all performed at 65°C.

2D gel electrophoresis and fluorography. SDS-polyacrylamide gel electrophoresis (10% acrylamide) was performed by the method of Laemmli (21). Two-dimensional (2D) gel analysis was performed as described previously (26). Gradients of pH 4.8 to 7.4 were obtained by mixing 400 μl of pH 3.5 to 10 Ampholine (LKB) with 150 μl of pH 6 to 8 Ampholine (LKB) per 10 ml of gel solution. Gels of methionine-labeled proteins were preincubated with En^{3}Hance before exposure. All exposures were on XAR-5 film (Kodak) at −70°C.

Immunoblotting. Immunoblotting was done by standard procedures (39). Briefly, proteins in 2D gels were electrophoretically transferred to nitrocellulose membranes at 0.5 A for 3.5 h in an electroblotting apparatus (Hoefer). The membranes were blocked with 3% bovine serum albumin in phosphate-buffered saline, washed, and probed for 3 h at room temperature with affinity-purified sheep antibody directed against the 36-, 55-, and 63-kDa subunits of rat skeletal muscle PP2A (17). They were again washed and then probed with alkaline phosphatase-conjugated second antibody (Jackson ImmunoResearch Laboratories). The blots were incubated with color development substrates. All antibody solutions incubated with the blots contained 0.5% bovine serum albumin to reduce nonspecific binding.

Nucleotide sequence accession numbers. The nucleic acid sequences of clones 7-4 and 7-1 have been submitted to GenBank/EMBL and were assigned accession numbers M83297 and M83298, respectively.

RESULTS

Purification and microsequencing of the 55-kDa PP2A subunit. To obtain partial amino acid sequence of the 55-kDa PP2A subunit for use in designing oligonucleotide probes for cDNA cloning of this protein, the PP2A 36-kDa/55-kDa/63-kDa heterotrimer was purified from rat muscle as described in Materials and Methods, and the subunits were resolved on SDS-polyacrylamide gels. The proteins were then electrophoretically transferred to nitrocellulose; the 55-kDa subunit was trypsinized, and the resulting peptides were resolved and sequenced as described in Materials and Methods. A total of 141 amino acids of high-confidence sequence were obtained, corresponding to 12 separate peptides. Two of the peptides contained eight identical contiguous amino acids but differed at several other positions; although they could theoretically correspond to a repeated sequence within the 55-kDa subunit, they more likely are derived from two isoforms of the protein (see the legend to Fig. 2 and Discussion).

cDNA cloning of the 55-kDa PP2A subunit. The rat liver cDNA library utilized in this study to obtain clones for the 55-kDa PP2A subunit was chosen for three reasons. First, our protein sequence of the 55-kDa PP2A subunit was obtained from rat protein, and although we expected the protein to be conserved, using a rat library avoided the potential problem of mismatches during hybridization because of nonconserved residues. Second, liver is known to be a good source of PP2A (16). Third, this particular library was shown by polymerase chain reaction to contain the full-length gene for the PP2A catalytic subunit and to have a reasonable number (1/50,000 plaques) of cDNAs for the 63-kDa PP2A regulatory subunit (data not shown).

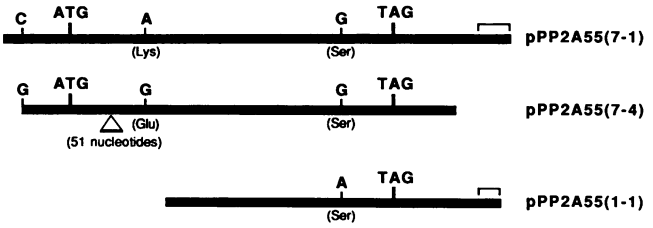

FIG. 1. Schematic representation of three independent cDNA clones isolated for the 55-kDa PP2A subunit. The cDNA clone inserts of three plasmids [pPP2A55(7-1), pPP2A55(7-4), pPP2A55(1-1)], each derived from an independently isolated rat liver clone, are shown aligned colinearly to indicate their similarities and differences. ATG and TAG, respectively, indicate the start and stop codons of each clone. The individual nucleotides indicated above each clone indicate the nucleotide found in each when there was a difference with at least one other clone. The three such nucleotides indicated for pPP2A55(7-1) are at positions −197, 313, and 1146, respectively, where position 1 is the first nucleotide of the start codon (see Fig. 2). The amino acid encoded by the codon at that position is indicated underneath in parentheses. The triangle denotes the position of insertion of 51 additional nucleotides present in clone 7-4 but not in clone 7-1. The brackets indicate divergent 3′ UTS found in two of the clones.

The library was screened with several different oligonucleotides designed from tryptic peptides as described in Materials and Methods. Of the probes to be used, the first to successfully identify an authentic cDNA clone for the 55-kDa PP2A subunit was oligonucleotide GTCTGGGA(TC)(TC)TIAA(TC)ATGGA(AG)AA, designed from the first eight amino acids of the tryptic peptide VWDLNMENR PVETYQVHEYLR. The first clone obtained contained 940 bp of the 3′ end of the coding region and 472 bp of 3′ UTS. To obtain a clone containing the remainder of the 5′ coding region, we labeled a restriction fragment containing 380 bp of the 5′ end of this partial clone and used it to rescreen the library. Two additional clones encoding the full-length 55-kDa PP2A subunit were isolated in this way and subsequently sequenced.

DNA sequence and predicted amino acid sequence of the 55-kDa PP2A subunit cDNA clones. Figure 1 shows a schematic comparison of the three different cDNA clones obtained for the PP2A 55-kDa subunit. Excluding linkers, clone 7-1 is 2,142 nucleotides long, clone 7-4 is 1,877 nucleotides long, and clone 1-1 is 1,412 nucleotides long. All three appear to represent the same gene, as there are only minor differences between them.

Within the coding region, three differences are found. First, relative to clone 7-1, clone 7-4 has an additional 51 nucleotides in frame near the 5′ end which encode an additional 17 amino acids. This is likely due to differential splicing. One of the tryptic peptide sequences from the purified 55-kDa protein spans this position (Fig. 1) and corresponds to amino acid sequence predicted by clone 7-1. This does not rule out the existence of a protein corresponding to clone 7-4; the appropriate peptide might not have been among those sequenced. Second, clone 7-4 has a deoxyguanosine instead of a deoxyadenosine at position 313 of the

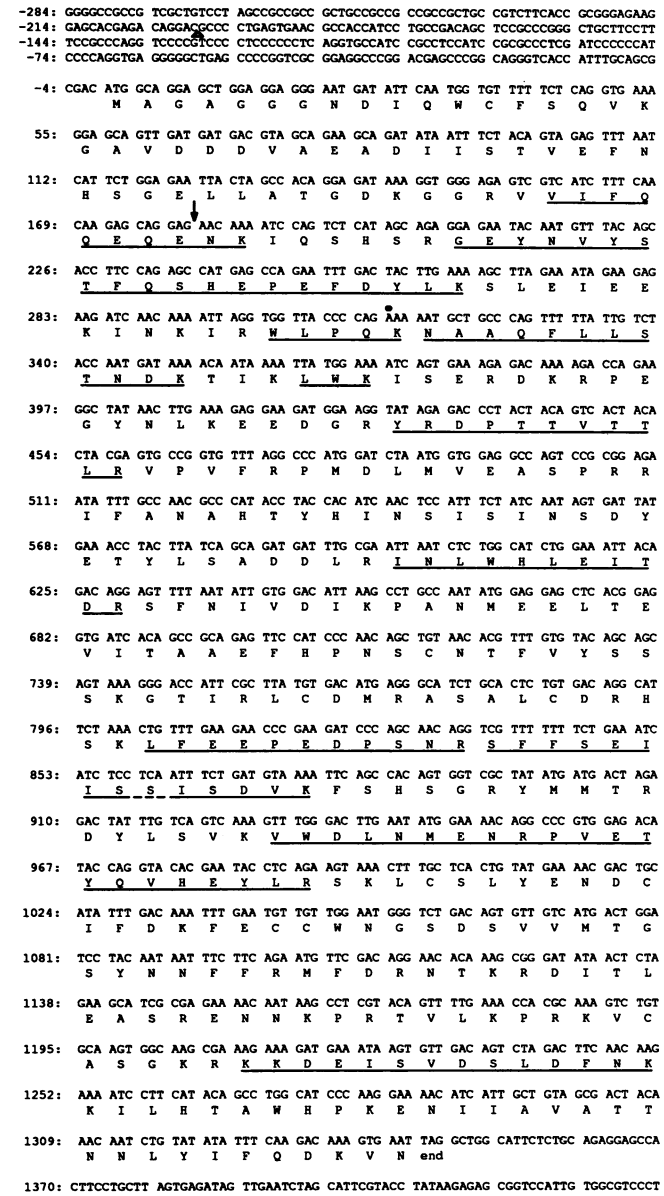

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the 55-kDa PP2A clone pPP2A55(7-1). The cDNA insert nucleotide sequence of PP2A 55-kDa subunit clone pPP2A55(7-1) excluding *Eco*RI linkers is shown along with the predicted 447-amino-acid polypeptide sequence. Peptide sequences obtained from microsequencing tryptic peptides from the purified protein (see Materials and Methods) are underlined. Serine 287, underlined with a dashed line to indicate that it lies within a peptide that was sequenced, was not able to be detected during microsequencing. Two peptides of similar sequence were microsequenced, LFEEPEDPSNR and FFEEPEDPSS, only the first of which is found in the predicted amino acid sequence of the 55-kDa PP2A subunit clone. The second peptide eluted at a different position in the HPLC gradient and most likely indicates the presence of a second isoform of the 55-kDa subunit in the purified preparation (see Discussion). Open triangles indicate the start and stop positions of clone 7-4. The arrow indicates the location of the 51 additional nucleotides found in clone 7-4 but not in clone 7-1. The 51 additional nucleotides are AGT TTTAAGTGCATGCAGCATTGAGAGAGGCCTCTAACTTGTCA ATGCAG; the 17 amino acids they enclode are SFKVHAAL REASNLSMQ. The small filled circle denotes nucleotide 313, which is a G in clone 7-4, encoding a glutamate at that position instead of the lysine found both in clone 7-1 and in one of the tryptic peptides (see Results). Clone 1-1 begins at nucleotide 405 and diverges from the clone 7-1 3′ UTS beginning at nucleotide 1729.

coding sequence. The most likely explanation for this the tryptic peptides sequenced from the purified 55-kDa protein spans this position and has a lysine at the position in question, again consistent with clone 7-1. The third difference in the coding region is that clone 1-1 has a deoxyadenosine at a position in which both clones 7-1 and 7-4 have a deoxyguanosine (position 1146 of clone 7-1). This difference would not result in an amino acid change in the predicted protein and may represent a reverse transcriptase error during synthesis of the clone 1-1 cDNA.

Figure 2 shows the DNA sequence of the longest clone obtained, clone 7-1, and the predicted 447-amino-acid sequence of the 55-kDa protein. The 284 bp of putative 5′ UTS contain no additional upstream ATG codons in any frame. The predicted molecular weight of the protein is 51,685, close to the value estimated in SDS-polyacrylamide gels. Eleven of 12 tryptic peptide sequences obtained from the purified protein are represented precisely in the predicted protein sequence. Peptide 12, FFEEPEDPSS, which differs only at two positions from one of the other peptides, probably corresponds to another isoform of the protein.

Transcription and translation of the 55-kDa clone 7-1. To verify that the 55-kDa clone 7-1 encodes the full-length protein, this clone was transcribed and translated in vitro. [^{35}S]-methionine was included in the translation reaction, and the radiolabeled translation product was analyzed on an SDS-polyacrylamide gel (Fig. 3A). A product of 55 kDa was synthesized only when RNA transcribed from clone 7-1 was added to the translation (compare lanes 1 and 2). The migration of the translated protein was then compared with that of the authentic 55-kDa protein by mixing the translate with purified trimeric PP2A prior to electrophoresis. The gel was stained to visualize the PP2A subunits (Fig. 3B), and an autoradiogram was prepared to determine the position of the translated protein (Fig. 3C). The relevant proteins comigrate indicating that the clone is indeed full length.

Based on precedent from the 36- and 63-kDa PP2A subunits, it seemed probable that there would be multiple isotypes of the 55-kDa protein. In addition, 2D Western immunoblots with an antibody to this protein suggested the existence of multiple isoforms of similar size (unpublished data). When the preparation of purified PP2A used in this study to isolate the 55-kDa protein for microsequencing was analyzed on 2D gels, more than six species of nearly identical size were seen (Fig. 4). This pattern could represent the products of multiple genes or various modified species of one or more genes. The translation product of the 55-kDa protein clone 7-1 obtained in vitro was analyzed on 2D gels to see whether it comigrated with one of the purified 55-kDa forms. The resulting pattern is shown in Fig. 4a. Interestingly, multiple species of approximately identical size were generated in the translation reaction. When the radiolabeled translate was premixed with the purified, unlabeled PP2A preparation prior to analysis on 2D gels, the pattern shown in Fig. 4b was seen. As can be seen by comparing panels b and c of Fig. 4, the 55-kDa translate species comigrate perfectly with the more acidic set of 2D variants bracketed in panel c.

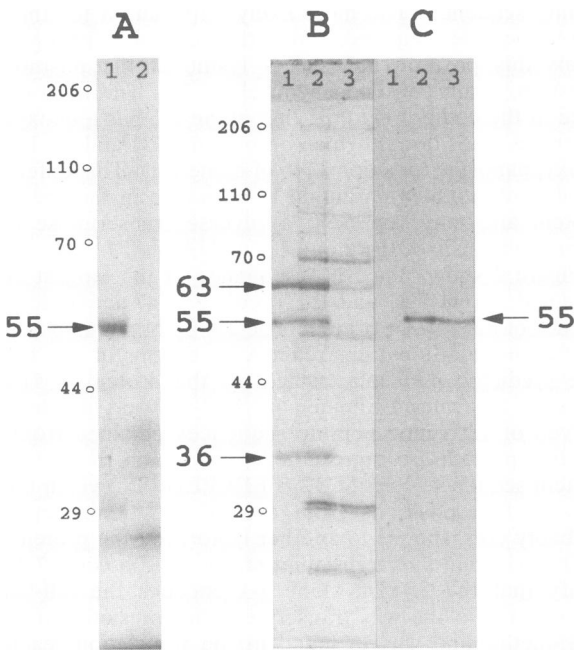

FIG. 3. PP2A 55-kDa clone 7-1 encodes the full-length protein. Clone 7-1 was transcribed in vitro by using the Promega Riboprobe system and T7 polymerase and then translated in vitro in the presence of [^{35}S]methionine by using the Promega rabbit reticulocyte lysates according to the manufacturer's protocols. A translation in the absence of added RNA was performed in parallel as a control. The radiolabeled translation products were analyzed on an SDS-polyacrylamide gel. (A) Lanes: 1, 5-μl translate with clone 7-1 RNA added; 2, 5-μl translate with no RNA added. (B and C) Migration of the translated protein was compared with that of the authentic 55-kDa protein by mixing the translate with purified trimeric PP2A prior to electrophoresis. The gel was stained to visualize the PP2A subunits (B) and an autoradiograph was prepared to determine the position of the translated protein (C). Lanes 1, 15 μg of purified trimeric PP2A; lanes 2, 15 μg of purified trimeric PP2A and 5 μl of ^{35}S-labeled clone 7-1 translate; lanes 3, 5 μl of ^{35}S-labeled clone 7-1 translate. Positions and sizes in thousands of molecular weight markers (small numbers) and PP2A subunits (large numbers) are indicated. Autoradiographs were aligned with the aid of dots on the gels made with ink containing [^{35}S]methionine.

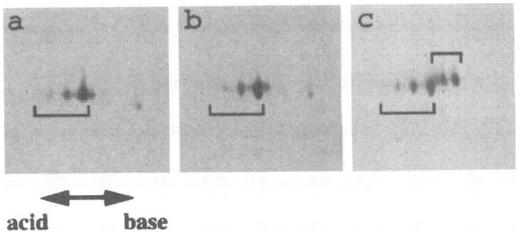

FIG. 4. PP2A 55-kDa clone 7-1 gene product is posttranslationally modified. The ^{35}S-labeled in vitro translation product of the PP2A 55-kDa protein clone 7-1 was analyzed on 2D gels by itself (a) or after being premixed with the preparation of purified PP2A used in this study to isolate the 55-kDa protein for microsequencing (see Materials and Methods) (b). The proteins in the gels were electrophoretically transferred to nitrocellulose, and the purified PP2A 55-kDa species (c) were visualized by immunoblotting with affinity-purified sheep antibody directed against heterotrimeric PP2A as described in Materials and Methods (17). Autoradiograms were prepared to visualize the radiolabeled proteins from the translate (a and b). Only the portion of the blot and autoradiograms where the major 55-kDa species migrate is shown. Separation horizontally was by isoelectric point; acid and basic ends of the gel are indicated. The second-dimension gel was a 10% SDS-polyacrylamide gel. Brackets indicate two sets of variants, the leftmost of which comigrates precisely with the radiolabeled, translated 55-kDa products. Autoradiographs were aligned with the aid of dots on the blot made with ink containing [^{35}S]methionine.

The 7-4 clone also transcribes and translates efficiently in vitro and should produce a protein with 17 additional amino acids. Its translated product does not comigrate with any of the species seen, nor does the product of a modified version of it which, like clone 7-1 (Fig. 1), has a lysine instead of a glutamate at amino acid 105 (data not shown).

Analysis of primary sequence of the 55-kDa PP2A subunit. Computer analysis of the 55-kDa PP2A subunit indicates that it does not correspond to any known protein from GenBank, nor is it highly related to any known protein. When the primary sequence of the 55-kDa clone is compared with that of small t antigens of polyomavirus and SV40, no convincingly significant similarities can be found. Since residues conserved between the small t antigens of papovaviruses known to associate with PP2A are more likely to be important for binding PP2A, comparisons were also done with a consensus pattern for small t antigens known to associate with PP2A. However, once again the results were equivocal. Because the 63-kDa regulatory subunit of PP2A is known to possess an internal repeat structure (14, 42), the 55-kDa sequence was examined to see whether it had an analogous structure, but no repeat structure could be detected. A number of consensus phosphorylation sites for casein kinase II and protein kinase C are found in the sequence. One of the potential sites for casein kinase II phosphorylation corresponds to a serine at position 287 which lies within a peptide that was among those sequenced from the purified protein. During the sequencing of this peptide, no amino acid was able to be detected at position 287. In contrast, all other amino acids of this peptide including three other upstream serines and one other downstream serine gave good yields at their respective cycles (data not shown). This result suggests that phosphorylation of this serine may occur in vivo.

5′ and 3′ UTS of the 55-kDa PP2A subunit. The three clones isolated for the 55-kDa subunit have two differences in their UTS. The first, a difference of 1 nucleotide in the 5′ UTS (position −197 in clone 7-1; Fig. 1), probably resulted from a reverse transcriptase error. The second difference is that the 3′ UTS of clones 7-1 and 1-1 diverge completely at a point past the end of clone 7-4 (position 1729 of clone 7-1; Fig. 2), perhaps indicating a point of alternate splicing or a cloning artifact.

The first 105 nucleotides of the rat 55-kDa regulatory subunit clone 5′ UTS (−1 to −105; Fig. 2) are 84% identical to the 105 nucleotides of 5′ UTS obtained for the human clone by Mayer and colleagues (24) during this study. The 3′ UTS for the rat and human 55-kDa subunit clones are 89% identical over the first 397 nucleotides. Rat 55-kDa clone 1-1 3′ UTS (Fig. 2) continues to be homologous to the human clone after it diverges from clone 7-1, indicating that this sequence is genuine and not a cloning artifact; it is 90% identical over a stretch of 479 nucleotides. This value is very close to that for the coding regions of the rat and human 55-kDa subunit clones—93% identity at the nucleotide level.

Northern blot analysis. When a probe specific for the coding region of the 55-kDa clone 7-1 was used to detect transcripts from rat liver, heart, and aorta (Fig. 5), a major

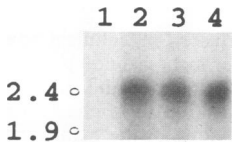

FIG. 5. Northern blot analysis of liver and muscle RNAs. RNAs from rat liver and muscle tissues were separated on formaldehyde gels as described in Materials and Methods. A probe specific for the coding region of the 55 kDa clone 7-1 was used as described in the text to probe the blot under stringent conditions. Lanes: 1, poly(A)$^+$-depleted rat liver RNA; 2, poly(A)$^+$-selected rat liver RNA; 3, rat heart total RNA; 4, rat aorta total RNA. Positions of migration and sizes in kilodaltons of RNA molecular size markers (Bethesda Research Laboratories) are indicated.

transcript of 2.4 kb was detected in all three tissues. Clones 7-1 and 7-4, therefore, are close in size to the full-length transcript. As expected, the transcript was specifically detected in poly(A)$^+$-selected rat liver RNAs (lane 2) but not in poly(A)$^+$-depleted RNAs (lane 1).

DISCUSSION

The T antigens of papovaviruses appear to specialize in binding to and altering the function of key cellular proteins involved in control of cell growth and division. In any given case, the effect of a T antigen on a particular cellular protein is likely to mimic an event which is normally cell cycle regulated. In this study, we describe a rat cDNA clone encoding the full-length 55-kDa PP2A subunit that is substituted for by the small and middle T antigens of all papovaviruses examined to date. Perhaps surprisingly, no clear homology was found between the primary sequence of this protein and that of the T antigens. Notably, the 55-kDa subunit exists in vivo in multiple forms, at least some of which are due to chemical modifications. No such chemically modified forms have been detected for the catalytic and 63-kDa regulatory subunits, making the 55-kDa subunit the most promising candidate at this point for regulating PP2A-dependent cellular processes such as cell cycle control.

The 2D analysis of the purified 55-kDa protein demonstrates that it is quite heterogeneous. The various species shown in Fig. 4c could be accounted for by two separate gene products each modified in the same way, yielding the two sets of variants indicated by the brackets. Alternatively, the two sets of variants could represent the same protein and differ from one another by yet another modification. Other more complicated possibilities also exist. We favor the first interpretation for two reasons. First, 2 of the 12 tryptic peptides sequenced differed at several positions from one another. One sequence variant was found in the predicted protein sequence (Fig. 2), while the other was not, indicating that it probably derives from another isoform of the 55-kDa protein in our purified preparation. Second, the results of translation in vitro in reticulocyte lysates, in which our single clone generated the more acidic set of variants seen in purified preparations of the 55-kDa protein (Fig. 2), suggest that this set derives from our clone, and that the other set of variants represents another gene product. It is striking that even the relative ratios of the species are similar in the translate and the purified preparation. This pattern could not have been generated by multiple translation starts at alternate, in-frame ATGs because, while the 2D species do not vary significantly in size, the next such potential start ATG is 160 amino acids after the first methionine. One variant in each set could represent the unmodified primary translation product, while another, slightly more acidic variant might have been generated by acetylation of the amino terminus. The additional variants would have to be explained by another modification(s). In particular, the pattern is consistent with the 55-kDa protein having multiple phosphorylation sites. Initial attempts to simplify the pattern by incubation with potato acid phosphatase have yielded negative results (data not shown). However, the putative phosphates might not be accessible to this enzyme. The fact that we obtained a blank during peptide microsequencing at serine 287, a casein kinase II consensus site, while getting good yields at all other positions including four other serines in the same peptide is consistent with this serine being phosphorylated in the native protein. Although more study is necessary to determine what the modifications may be, the translation results indicate that the 55-kDa protein is post-translationally modified. It will be interesting to see whether these modifications affect either the association of the 55-kDa subunit with the 36-kDa/63-kDa PP2a heterodimer or the activity of the resulting heterotrimer or both.

There may be other species of the 55-kDa protein as well, not seen in our preparation shown in Fig. 3. The species shown are the major ones found in a particular set of fractions of purified heterodimer. Western blotting with an antibody raised to the PP2A holoenzyme suggests the existence of additional forms (data not shown). We are presently raising antibody to bacterially produced 55-kDa protein to be able to identify more clearly variants of the 55-kDa protein immunologically. The 7-1 clone we isolated appears to account for the most predominant set of variants in our purified preparations. The 7-4 clone, on the other hand, which contains a 51-nucleotide insert, does not appear to be responsible for any of the major species in our preparations, raising the question of whether it represents a species translated in vivo.

The lack of clear homology between the 55-kDa protein and T antigens was somewhat surprising, given that their binding appears to be mutually exclusive. There are some limited similarities, but in the absence of mutational analysis, these similarities will remain of questionable significance. We previously proposed that interaction of the PP2A heterodimer with small t or MT in vivo might substitute for the binding of a normal regulatory subunit of PP2A (29). Several species of additional PP2A regulatory subunits had been identified (for a review, see reference 3), and although the PP2A 36-kDa/63-kDa heterodimer was often found associated with a third subunit when purified, none of these species was present in our T-antigen immunoprecipitates from NIH 3T3 cells expressing small t or MT (29). In addition, there was evidence that both T antigens and the 55-kDa subunit might bind the 63-kDa subunit (15, 18). More recently (45), it has been shown that purified SV40 small t antigen will bind to the 36-kDa/63-kDa PP2A heterodimer in vitro, but not to the 36-kDa/63-kDa/55-kDa heterotrimeric form, further supporting the idea that T antigens and the 55-kDa subunit bind to the same region of the PP2A heterodimer. It is possible that T antigens and the 55-kDa subunit bind distinct sites on the PP2A heterodimer that are close enough to prevent both proteins from associating with the same heterodimer. Alternatively, they may bind the same site but the primary structure information necessary for binding may be limited and not easily identified. A known case in which limited homology turned out to be quite significant is the motif, originally identified in E1A and in

SV40 large T antigen, which is necessary for association with the retinoblastoma gene product (Rb) (5, 9, 10, 37).

Small t antigen contains all the sequence information necessary for association with the phosphatase. Monoclonal antibodies directed against determinants in the first 79 amino acids of polyomavirus T antigens invariably have been unable to immunoprecipitate the T antigen/PP2A complex (for a discussion, see reference 28), suggesting that this region is necessary for association. Given that the first 79 amino-terminal residues of all three polyomavirus T antigens are identical and that large T does not associate with PP2A, a portion of the sequence common between small t and MT antigens but not in common with large T may also be necessary for association with PP2A. This is supported by the fact that polyomavirus *hrt* mutations such as NG59, which abolish T-antigen ability to associate with PP2A, are localized in this region (33).

After completion of this study, Mayer and colleagues (24) published a characterization of several cDNAs for the 55-kDa subunit from humans and rabbits. Two, designated as the human and rabbit beta forms, appeared to be neuron specific. One, designated as the human alpha form, was found in all cell lines tested. The rat cDNA clone 7-1 described in this present work encodes a protein corresponding to the alpha form. There is only one conservative change in the primary sequence: valine 310 in clone 7-1 is an isoleucine in the human clone. This extreme conservation between species at the amino acid level is similar to that found for the PP2A catalytic subunit. This is consistent with our finding that anti-55-kDa PP2A subunit antibodies raised against rat 55-kDa protein recognize human, monkey, and mouse versions in immunoblotting experiments (25a). A clone corresponding to the more basic set of variants seen in Fig. 4c apparently has yet to be isolated. Based on its migration on 2D gels, one would expect a protein very related to clone 7-1, with a slightly more basic net charge.

The 5′ and 3′ UTS of the PP2A 55-kDa subunit are quite conserved between species (see Results). The 5′ and 3′ UTS of the other PP2A subunits are also very conserved between species. The first 108 nucleotides of 5′ UTS of the rat (20) and human (2) catalytic alpha-subunit clones are 78% identical. The first 451 nucleotides of 3′ UTS from these same clones are 89% identical. The 3′ UTS of porcine and human 63-kDa regulatory beta-subunit clones have been reported to be 84% conserved (14). The conservation of the 55-kDa subunit 3′ UTS between rats and humans (~90%) is very similar to the conservation of coding region sequence (~93%). The reason for the conservation is not known. No long open reading frame is present in the 3′ UTS; in addition, small deletions (1 to 4 nucleotides) are found periodically, suggesting that these sequences are not part of a conserved open reading frame even if spliced alternatively. These conserved sequences could be involved in mRNA stability or regulation at transcription or posttranscriptional levels, although it is not clear why such extensive regions of sequence would be necessary for these purposes. It is worth noting that similar conservation of UTS is not found between clones for the different phosphatase subunits, or between alpha and beta clones for the same subunit, suggesting that each isoform of each PP2A subunit is individually regulated. However, the elucidation of the specific functions of both the very conserved protein primary sequences and the conserved untranslated regions of mRNAs awaits more dissection of the important roles PP2A plays in cell growth and development.

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