*Proc. Natl. Acad. Sci. USA*
Vol. 92, pp. 7172–7176, August 1995
Biochemistry

# Positive regulation of general transcription factor SIII by a tailed ubiquitin homolog

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*Communicated by Roger D. Kornberg, Stanford University School of Medicine, Stanford, CA, April 25, 1995*

**ABSTRACT** **General transcription factor SIII, a heterotrimer composed of 110-kDa (p110), 18-kDa (p18), and 15-kDa (p15) subunits, increases the catalytic rate of transcribing RNA polymerase II by suppressing transient pausing by polymerase at multiple sites on DNA templates. Here we report molecular cloning and biochemical characterization of the SIII p18 subunit, which is found to be a member of the ubiquitin homology (UbH) gene family and functions as a positive regulatory subunit of SIII. p18 is a 118-amino acid protein composed of an 84-residue N-terminal UbH domain fused to a 34-residue C-terminal tail. Mechanistic studies indicate that p18 activates SIII transcriptional activity above a basal level inherent in the SIII p110 and p15 subunits. Taken together, these findings establish a role for p18 in regulating the activity of the RNA polymerase II elongation complex, and they bring to light a function for a UbH domain protein in transcriptional regulation.**

Eukaryotic messenger RNA synthesis is a complex biochemical process controlled in part by the concerted action of a set of general transcription factors that regulate the activity of RNA polymerase II at both the initiation (1) and elongation (2, 3) stages of transcription. At least six general initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) have been identified in eukaryotic cells and found to promote selective binding of RNA polymerase II to promoters and to support a basal level of transcription (1). In addition, three general elongation factors (SII, TFIIF, and SIII) have been identified in eukaryotic cells and defined biochemically. The elongation factors fall into two functional classes.

The sole member of the first class is SII. SII is an ≈38-kDa protein originally identified and purified to homogeneity by Natori and coworkers (4). SII binds to RNA polymerase II and promotes readthrough by polymerase through a variety of transcriptional impediments, including attenuation sites found in such genes as the human histone H3.3 (5, 6), adenovirus 2 major late (AdML) (7–9), and adenosine deaminase genes (10), as well as DNA-bound proteins (11) and drugs (12). SII is believed to promote readthrough by an unusual mechanism involving the reiterative shortening and reextending of nascent transcripts. Interaction of SII with the RNA polymerase II elongation complex activates a latent ribonuclease activity that shortens nascent transcripts from their 3′ ends (13, 14). Shortened transcripts remain in the polymerase active site and can be reextended.

The second class of elongation factors includes TFIIF and SIII. TFIIF is unique among the general transcription factors because of its ability to control the activity of RNA polymerase II at both the initiation and elongation stages of transcription. TFIIF from higher eukaryotes is a heterodimer of ≈30-kDa (RAP30) and ≈70-kDa (RAP74) subunits (15–18) and was initially purified to homogeneity from rat liver (15) and *Drosophila melanogaster* (16), although its RAP30 and RAP74 subunits were originally identified by Greenblatt and coworkers among a small group of proteins capable of binding to immobilized RNA polymerase II (19–21). TFIIF from *Saccharomyces cerevisiae* is a heterotrimer of ≈105-, 54-, and 30-kDa subunits; the ≈105- and 54-kDa subunits are homologs of RAP74 and RAP30, respectively (22). A role for TFIIF in transcription elongation was demonstrated by Greenleaf and coworkers (16). SIII is a heterotrimer of ≈110-, 18-, and 15-kDa subunits and was purified to homogeneity from rat liver (23). Mechanistic studies indicate that both SIII and TFIIF potently increase the overall catalytic rate of transcribing RNA polymerase II by a mechanism involving suppression of transient pausing by polymerase at multiple sites on DNA templates (16, 24).

In this report, we describe the isolation, structure, and expression of a full-length cDNA encoding the functional SIII p18 subunit,** which is shown to be a ubiquitin homolog that belongs to a growing ubiquitin homology (UbH) gene family. In addition, we demonstrate that p18 functions as a dissociable regulatory subunit that is capable of activating overall SIII transcriptional activity through interactions with the SIII p110 and p15 subunits.

## MATERIALS AND METHODS

**Isolation of a cDNA Encoding the SIII p18 Subunit.** SIII was purified to near homogeneity from rat liver nuclear extracts (23). Approximately 300 pmol of the SIII p18 subunit was isolated by reverse-phase HPLC (23). After reduction, *S*-carboxyamidomethylation, and digestion with trypsin, the resultant mixture was further fractionated by microbore HPLC. Peptides to be sequenced were identified by differential UV absorbance and matrix-assisted laser desorption mass spectrometry (Lasermat; Finnigan-MAT, San Jose, CA) and submitted to automated Edman microsequencing (25). The N-terminal sequences of two tryptic peptides (I and II) were obtained and were as follows, with uncertain residues in lowercase: I, LYKDDQLLDDGKTLGECGFTSQTARPQap; and II, ADDTFEALRIEPFSSPPELPDVMKPQDSGgsANe. A partial p18 cDNA was isolated from a rat liver λgt11 cDNA library (Clontech) by PCR, using as primers the sense and antisense degenerate oligonucleotides 5′-TNTAYAARGAY-GAYCARYT-3′ and 5′-TGNGGYTTCATNACRTCNGG-3′, which encode residues 1–7 of tryptic peptide I and residues 20–26 of tryptic peptide II, respectively (R is A or G; Y is C

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Abbreviations: AdML, adenovirus 2 major late; UbH, ubiquitin homology; ORF, open reading frame.

†K.P.G. and T.A. contributed equally to this work.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. L42855).

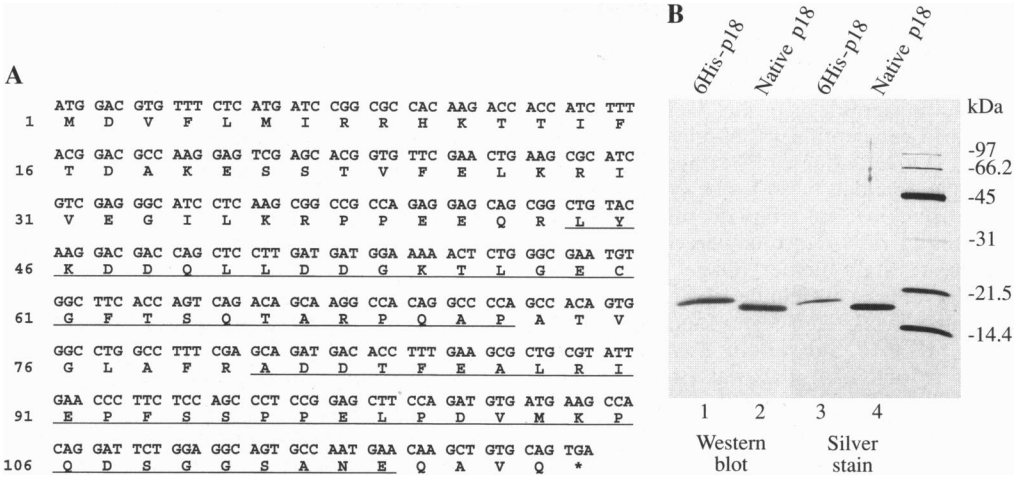


FIG. 1. Sequence and expression of the SIII p18 subunit. A full-length cDNA encoding rat p18 was isolated as described in the text. (*A*) Nucleotide sequence of the SIII p18 cDNA and predicted amino acid sequence of p18. Amino acid sequences matching those determined for tryptic peptides are underlined. (*B*) SDS/PAGE and Western blot analysis of bacterially expressed and native p18. Bacterially expressed p18 with an N-terminal histidine tag (lanes 1 and 3) prepared as described in the text and native rat p18 purified from rat liver as described (23) (lanes 2 and 4) were subjected to SDS/8% PAGE. Lanes 1 and 2 were transferred to nitrocellulose (Millipore) and analyzed by Western blotting as described (35), using rabbit antiserum raised against recombinant histidine-tagged p18. To prepare anti-p18 antiserum, rabbits were immunized with recombinant p18 that had been expressed and purified by nickel nitrilotriacetate affinity chromatography and further purified by preparative SDS/PAGE. Lanes 3 and 4 were visualized by silver staining. 6His-p18, histidine-tagged p18.

or T; N is A, C, G, or T). PCR was performed for 30 cycles of 1 min at 94°C, 1 min at 46°C, 2 min at 72°C with 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of *Taq* DNA polymerase, and 0.02 $A_{260}$ unit of each primer. PCR products encoding SIII p18 polypeptide sequences were identified by Southern blotting (26), using as probe the 5′-$^{32}$P-labeled degenerate oligonucleotide 5′-GCNGAYGAYACNTTYGARGC-3′, which encodes residues 1–7 of tryptic peptide II; isolated by preparative

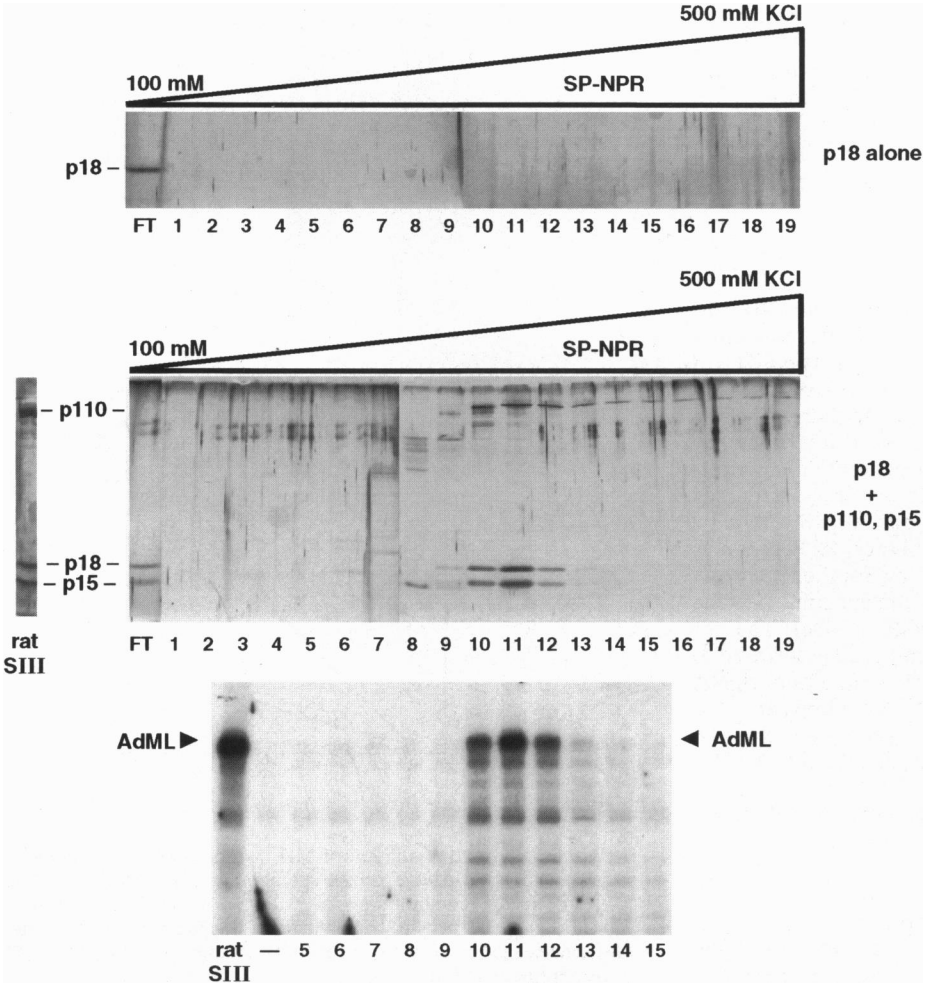


FIG. 2. Interaction of bacterially expressed p18 with the SIII p110 and p15 subunits. Renaturation of bacterially expressed p18 in the presence and absence of the SIII p110 and p15 subunits was carried out essentially as described (23) with 15 μg of histidine-tagged p18, 15 μg of histidine-tagged p15 (29), and 100 μg of histidine-tagged p110 (T.A., unpublished results). After dialysis, renatured protein was applied to a TSK SP-NPR column (35 mm × 4.6 mm, Hewlett–Packard) equilibrated in a buffer containing 40 mM Hepes–KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, and 100 mM KCl. The column was eluted with a 9-ml gradient of 100 mM KCl to 800 mM KCl in the same buffer. Aliquots of each column fraction were analyzed by SDS/8% PAGE, and protein was visualized by silver staining. Aliquots of each column fraction were also assayed for SIII transcriptional activity. (*Top*) SDS/PAGE of column fractions from TSK SP-NPR HPLC analysis of recombinant p18 renatured alone. (*Middle*) SDS/PAGE of column fractions from TSK SP-NPR HPLC analysis of recombinant p18 renatured together with the SIII p110 and p15 subunits. (*Bottom*) Assay of SIII transcriptional activity in column fractions from TSK SP-NPR HPLC carried out in *Middle*. FT, flow-through.

PAGE; and subcloned by blunt-end ligation into pBluescript KS (−) (Stratagene). Bacteria harboring the recombinant plasmid (pKG2) carrying the partial SIII p18 cDNA were identified by colony hybridization (26) using the same 5′-$^{32}$P-labeled degenerate oligonucleotide as probe. A cDNA encoding the complete SIII p18 polypeptide was obtained by screening a rat brain λZAP II cDNA library (Stratagene) with an internally labeled, single-stranded DNA probe synthesized by asymmetric PCR (27) using pKG2 as template. pBluescript SK (−) phagemids containing cDNA inserts were rescued with VCS-M13 interference-resistant helper phage (Stratagene) and sequenced by the dideoxynucleotide chain-termination method using a Sequenase kit (United States Biochemical).

**Expression of the SIII p18 Subunit in *Escherichia coli*.** Bacterially expressed recombinant p18 was prepared with an N-terminal six-histidine tag by using the bacteriophage M13mpET expression system (28). The expression vector m13mpET-6Hp18, which encodes p18 with an 11 amino acid N-terminal extension of sequence MHHHHHHNVD, was constructed by insertion of the entire open reading frame (ORF) encoded by the SIII p18 cDNA into the previously described vector M13mpET-6H (28). Histidine-tagged p18 was isolated from inclusion bodies and purified by nickel nitrilotriacetate affinity chromatography as described (29).

**Assay of SIII Transcriptional Activity.** Preinitiation complexes were assembled at the AdML promoter at 28°C by a 30-min preincubation of 60-μl reaction mixtures containing 20 mM Hepes–NaOH (pH 7.9), 20 mM Tris·HCl (pH 7.9), 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, bovine serum albumin at 0.5 mg/ml, 2% (wt/vol) polyvinyl alcohol, 7% (vol/vol) glycerol, 6 units of RNasin, 100 ng of the *Eco*RI–*Nde* I fragment from pDN-AdML (30), 10 ng of recombinant TFIIB (31), 10 ng of recombinant TFIIF (32), 7 ng of recombinant TFIIE (32), 40 ng of TFIIH (rat δ, fraction VI) (33), 50 ng of yeast TATA-binding protein (AcA 44 fraction) (33), and 0.01 unit of RNA polymerase II (34). Transcription was initiated by addition of 7 mM $MgCl_2$, 50 μM ATP, 1 μM UTP, 10 μM CTP, 50 μM GTP, and 10 μCi (1 μCi = 37 kBq) of [α-$^{32}$P]CTP and was allowed to proceed at 28°C for 30 min. Runoff transcripts were analyzed by electrophoresis through 6% polyacrylamide/7 M urea gels.

## RESULTS

**Isolation of a Full-Length cDNA Encoding the SIII p18 Subunit.** General transcription factor SIII was purified to near homogeneity from rat liver nuclear extracts (23). The SIII p18 subunit was purified free of the p110 and p15 subunits by reverse-phase HPLC and digested with trypsin. The N-terminal sequences of two tryptic peptides were determined by sequential Edman degradation. Using degenerate oligonucleotide primers selected from regions of low codon degeneracy within the two peptides, we obtained a partial p18 cDNA by PCR amplification of a rat liver cDNA library. PCR products encoding p18 sequences were identified by Southern blotting using as probe a degenerate oligonucleotide encoding peptide sequence predicted to be within the PCR product. The appropriate PCR product was subcloned and used as a probe to isolate the full-length p18 cDNA from a rat brain cDNA library. As shown in Fig. 1*A*, the p18 cDNA contains an ORF that includes the sequences of both tryptic peptides and encodes a 118-amino acid protein with a calculated molecular mass of 13,170 Da.

**p18 Functions as a Positive Regulatory Subunit of SIII.** We have confirmed by several criteria that the isolated cDNA encodes the *bona fide* SIII p18 subunit, and we demonstrate that p18 functions as a positive regulator of SIII activity. First, as shown in Fig. 1*B*, the cDNA directs expression in *E. coli* of an ≈18-kDa protein (lanes 1 and 3). Second, polyclonal antisera raised against the recombinant 18-kDa protein recognize both recombinant and native rat p18 in Western blots (Fig. 1*B*, lanes 1 and 2). Third, like native rat p18, recombinant p18 assembles with the p110 and p15 subunits to reconstitute heterotrimeric SIII (Fig. 2). In this experiment, recombinant p18 was renatured in the presence or absence of p110 and p15 and subjected to ion-exchange HPLC on TSK SP-NPR (Hewlett–Packard). Analysis of TSK SP-NPR column fractions by SDS/PAGE revealed that, when renatured alone, the majority of recombinant p18 did not bind to the TSK SP-NPR column at 100 mM KCl (Fig. 2 *Top*) and was recovered in the flow-through (FT) fraction. In contrast, when renatured with p110 and p15, a substantial fraction of recombinant p18 bound to the TSK SP-NPR column and co-eluted at ≈250 mM KCl with p110 and p15 (Fig. 2 *Middle*); as shown in Fig. 2 *Bottom*, TSK SP-NPR column fractions containing p18, p110, and p15 had substantial SIII transcriptional activity. Fourth, recombinant p18 is transcriptionally active (Fig. 3). Various combinations of recombinant p18 and the SIII p110 and p15 subunits were renatured together and tested for their abilities to increase the rate at which RNA polymerase II elongates RNA chains initiated at the AdML promoter. As shown in Fig. 3, recombinant p18 strongly stimulates SIII transcriptional activity above the level observed with the p110 and p15 subunits alone, indicating that p18 functions as a positive regulator of SIII transcriptional activity. Like native p18 (23), recombinant p18 had no detectable transcriptional activity in the absence of p110 and p15 (data not shown).

**p18 Is a Ubiquitin Homolog.** Although p18 contains no structural motifs characteristic of transcription factors, such as zinc finger, leucine zipper, or helix–turn–helix (HTH) do-

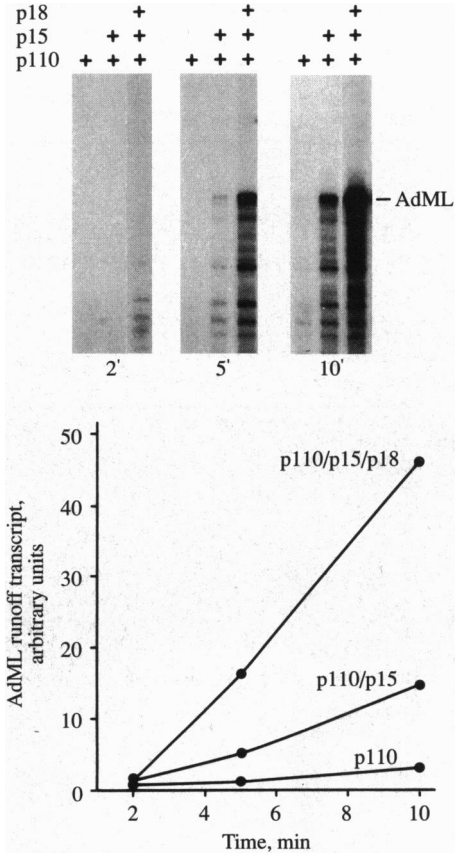


FIG. 3. Positive regulation of SIII transcriptional activity by bacterially expressed p18. To test recombinant p18 for transcriptional activity, various combinations of ≈100 ng of bacterially expressed histidine-tagged p18, ≈100 ng of histidine-tagged p15, and ≈500 ng of histidine-tagged p110 were renatured together and assayed for SIII transcriptional activity.

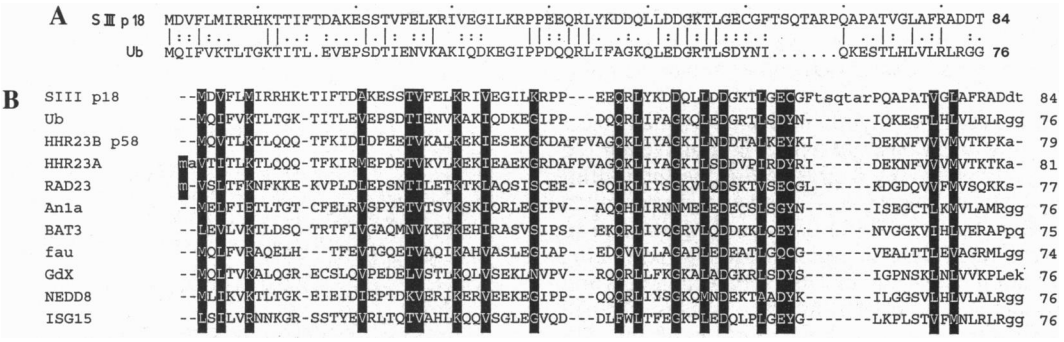


FIG. 4. p18 is a member of the UbH gene family. (*A*) Alignment of p18 and vertebrate ubiquitin. Sequences were aligned by using the BESTFIT program of the Genetics Computer Group package (36). |, Identical residues; : and ·, residues with alignment scores (36) ≥0.5 and ≥0.1, respectively. (*B*) Alignment of members of the UbH gene family. The MACAW program (37) was used to determine the best alignment of ubiquitin (Ub) with the UbH domains of p18, HHR23B p58 and HHR23A (38), RAD23 (39), An1a (40), BAT3 (41), fau (42), GdX (43), NEDD8 (44), and ISG15 (45). Residues that fall outside of homology blocks identified by the MACAW program are shown in lowercase letters.

mains, a search of the Swiss-Prot data base revealed that the first 84 amino acids of the p18 N terminus bear striking sequence similarity to ubiquitin (Fig. 4*A*). As determined by the BESTFIT program of the Genetics Computer Group (GCG, Madison, WI) package, the two proteins are ≈28% identical and ≈58% similar and have an alignment score of ≈7.5 SD. The SIII p18 subunit is therefore composed of an 84-amino acid N-terminal UbH domain fused to a 34-amino acid C-terminal tail. In possessing an N-terminal UbH domain, p18 joins a growing family of proteins known to contain N-terminal UbH domains (Fig. 4*B*); other members of the UbH gene family include the proline-rich *BAT3* gene (41) found in the major histocompatibility complex, the interferon-inducible *ISG15* gene (45), and yeast *RAD23* (39) and its human homolog *HHR23B* (38). Although the p18 UbH domain bears a striking resemblance to ubiquitin, p18 cannot be replaced by ubiquitin in regulation of SIII transcriptional activity (data not shown), indicating that p18 sequences differing from those of ubiquitin are important for p18 function.

## DISCUSSION

Molecular cloning of a full-length cDNA encoding the functional 18-kDa subunit of RNA polymerase II general elongation factor SIII has revealed that p18 is a new member of the ubiquitin gene family. The ubiquitin family comprises a diverse collection of proteins that fall into three classes. The first class is composed solely of ubiquitin, which is found covalently attached through its C-terminal glycine residue to a variety of cytoplasmic, cell surface, and nuclear proteins, where it either tags them for degradation in the ubiquitin-dependent proteolytic pathway or plays other less well defined roles in DNA repair, cell cycle regulation, stress responses, and lymphocyte homing (46, 47). The second class is composed of a small set of ribosomal proteins, which contain N-terminal ubiquitin that is proteolytically removed after incorporation of these proteins into ribosomes (48, 49). The third class is composed of proteins that contain N-terminal UbH domains fused to variable-length C-terminal tails. By virtue of its N-terminal UbH domain, p18 belongs to this family, whose members are listed in Fig. 4*B* and

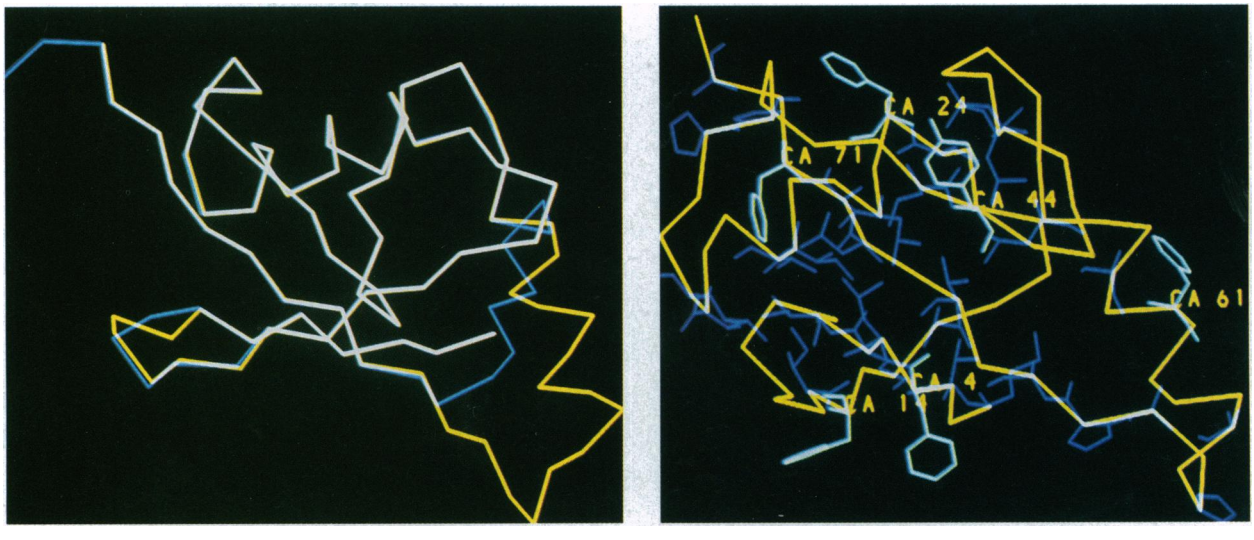


FIG. 5. Structural conservation of the p18 UbH domain and ubiquitin. The computer drawings show a model for the tertiary structure of the p18 UbH domain. The model was constructed by conservative molecular modeling using a ubiquitin homology template and geometric regularization with the LORE program (Version 2.2) (50). The 7-amino acid insertion in p18 between residues 62 and 68 is modeled as a surface loop. The loop is one possible structural motif derived from a nonredundant dictionary of 183 crystal structures of resolution 2.5 Å or better from the Brookhaven Protein Data Bank. The loop extends the ubiquitin α-helix at position 56 and forms a compact helix–arch–β-sheet structure. (*Left*) Tracing of the predicted α-carbon backbone of p18. Blue, ubiquitin backbone; yellow, predicted p18 backbone; white, conserved backbone. (*Right*) Predicted hydrophobic p18 residues. Residues in the computer drawing are numbered according to the ubiquitin sequence. A notable feature of the p18 model is the striking conservation of the ubiquitin hydrophobic core. In addition, in keeping with ubiquitin, which has three hydrophobic residues on its surface, the p18 model conserves the hydrophobic character of these residues and adds two additional surface hydrophobic groups, thus forming an unusual hydrophobic ring around the circumference of the model. Dark blue, predicted p18 hydrophobic core residues; yellow, predicted α-carbon backbone of the p18 UbH domain; light blue, aromatic residues.

include the products of the proline-rich *BAT3* gene (41) found in the major histocompatibility complex, the interferon-inducible *ISG15* gene (45), and yeast *RAD23* (39) and its human homolog *HHR23B* (38).

Given ubiquitin's compact, tightly hydrogen-bonded structure (38) and its previously described roles as a molecular tag for targeting proteins to be degraded in the ubiquitin-dependent proteolytic pathway (46, 47) and as a chaperone in ribosome biogenesis (49), the UbH domain appears ideal to function as a protein–protein interaction module regulating the specificity of interactions among UbH family members and the cellular proteins with which they interact. Because the p18 UbH domain constitutes such a large portion of the p18 protein, it is likely that it will ultimately be proven to play some role in controlling the interaction of p18 with the SIII p110 and p15 subunits or with RNA polymerase II itself.

Whether the UbH domains encoded by UbH gene family members actually adopt tertiary structures similar to that of ubiquitin is presently unknown. It is noteworthy that molecular modeling studies indicate that the UbH domain of HHR23B p58 could adopt a ubiquitin-like conformation (38). Likewise, the p18 UbH domain can be modeled as ubiquitin with striking conservation of the ubiquitin hydrophobic core (Fig. 5 and data not shown). In the case of the p18 UbH domain, the 7-amino acid insertion (FTSQTAR) between residues 62 and 68 is readily modeled as a surface loop, since amino acids 62 and 70, which flank the potential loop, would be at the surface of a ubiquitin-like structure (51).

Compared with the quantity of information available on the structures and functions of protein motifs such as SH2, SH3, and immunoglobulin domains, information on the role(s) of UbH domains in biological processes is scant. Given that UbH domains are appearing in a growing number of cellular proteins, it is clear that the UbH domain is an important structural motif that will demand a more thorough investigation. Because it possesses a specific and readily assayable biochemical activity, the SIII p18 subunit should provide an ideal model for such studies.

**Note Added in Proof.** Human SIII p18 (GenBank accession no. L42856) is nearly identical to rat SIII p18, differing in only 3 of 118 amino acids.

We thank G. Zurawski, S. Zurawski, D. Ligget, and T. Mather for helpful discussions, R. Robinson of the Harvard Microchemistry Facility for peptide isolation, K. Jackson of the Molecular Biology Resource Center at the Oklahoma Center for Molecular Medicine for oligonucleotide synthesis, and R. Irish for artwork. This work was supported by Grant GM41628 from the National Institutes of Health and by funds from the H.A. and Mary K. Chapman Charitable Trust to the Oklahoma Medical Research Foundation.

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