

Hibernation-Associated Gene Regulation of Plasma Proteins with a Collagen-Like Domain in Mammalian Hibernators

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In mammals, hibernation is expressed by only a limited number of species, and the molecular mechanisms underlying hibernation are not well understood. Recently, we have found plasma proteins which disappear from blood specifically during hibernation in a mammalian hibernator, the chipmunk. Here, we report the cDNA cloning of these chipmunk hibernation-related proteins, HP-20, -25, and -27, and analyses of their expression. All three proteins contain a collagen-like domain near the N terminus and are highly homologous to each other. Their mRNAs were detected only in liver in nonhibernating chipmunks, and in hibernating chipmunks, the amounts were reduced to less than 1/10 of those in nonhibernating chipmunks, indicating that HP-20, -25, and -27 mRNA expression is regulated similarly in association with hibernation. Southern blot analyses of the squirrel family with each of chipmunk HP-20, -25, and -27 cDNA revealed that a nonhibernating species (tree squirrel) as well as another hibernating species (ground squirrel) retained the corresponding genes. However, their transcripts were detected only with the hibernating species, and in hibernating ground squirrels, their levels were greatly reduced compared with those in nonhibernating animals, as were the cases with the chipmunk. These observations are the first line of evidence for occurrence of hibernation-associated gene regulation. The results would indicate the commitment of HP-20, -25, and -27 to hibernation and support the idea that genetic controls are involved in mammalian hibernation.

Most mammals maintain a high, steady body temperature throughout their adult lives, and only certain small mammals, primarily in the orders Rodentia, Insectivora, and Chiroptera, can undergo hibernation. During hibernation, the body temperatures of mammalian hibernators drop to below 10 or even 5°C and metabolic rate is reduced to only a few percent of the euthermic level. Heart rate and breathing rate also fall. However, unlike the lower vertebrate and invertebrate hibernators, mammalian hibernators maintain the thermoregulatory control with a lowered set point during hibernation and retain the capacity to arouse from hypothermia by physiological means.

Mammalian hibernation accompanies various physiological changes (21). Several histological and morphological studies reveal that polyglandular involution takes place prior to the onset of hibernation and that endocrine functions are generally depressed during the first half of the hibernation season (20). Also, the lymphoid organs undergo an involution in autumn, and the immune system is depressed during hibernation (17). Recently, through comparison of plasma proteins between hibernating and nonhibernating Asian chipmunks (*Tamias asiaticus*) by using gel permeation high-performance liquid chromatography, we have found that the 140-kDa fraction is markedly reduced in the hibernating animals (9). Four kinds of proteins (20, 25, 27, and 55 kDa [referred to as HP-20, -25, -27, and -55, respectively]) in this 140-kDa fraction of the nonhibernating chipmunks are practically absent in the corresponding fractions of the hibernating ones, which results in the reduction of this fraction in the hibernating chipmunks, and HP-27 is scarcely detectable in

the total plasma proteins with anti-HP-27 antibody in the hibernating state (9). The 140-kDa fraction starts to decrease before the onset of hibernation, stays in the reduced level during hibernation, and starts to increase just before the cessation of hibernation.

Although the physiological changes associated with hibernation are assumed to be initiated by endogenous factors and under genetic controls (15), we have no evidence for hibernation-associated gene regulation. In this study, to reveal whether HP-20, -25, and -27 gene expression is regulated in association with hibernation, we isolated these cDNA clones and compared the levels of these transcripts between nonhibernating and hibernating chipmunks. Moreover, to understand the relationship between HP-20, -25, and -27 gene expression and hibernation, we analyzed the gene expression by employing a nonhibernating species as well as another hibernating species of the squirrel family.

MATERIALS AND METHODS

Cloning procedure. Total RNA was prepared from a nonhibernating chipmunk liver by the guanidium isothiocyanate method (12), and poly(A)⁺ RNA was purified by using oligo(dT)-cellulose. Size fractionation of poly(A)⁺ RNA was carried out as follows: sucrose gradients were made by three freeze-thaw cycles of a solution containing 15% sucrose, 1 mM EDTA, and 20 mM sodium acetate (pH 5.2) in a 13PA tube (Hitachi), and 40 µg of poly(A)⁺ RNA was fractionated by centrifugation through the gradients at 27,500 rpm for 21 h at 4°C (RPS40T rotor, Hitachi). Oligo(dT)-primed cDNA was synthesized by using poly(A)⁺ RNA or its fraction and cloned into the *Eco*RI site of lambda gt10 or ZAPII (Stratagene). Nondegenerate oligonucleotides (59-, 32-, and 62-mer

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for HP-20, -25, and -27, respectively) were synthesized on the basis of the partial amino acid sequences (9): HP-20, 5'-GTTTTTAATACGGTTGAGCCTGGTAATTATCATTTTCTTTTTGATGTTGAGCTGTATCA-3' (VFNTVEPGNYHF SFDVELYH, residues 85 to 104); HP-25, 5'-TATAATCAGGAGGTCATTTAATATGGCTAC-3' (YNQEGHFNMAT, residues 86 to 96); and HP-27, 5'-CAGACTGATCTGAGCGGGGTACGGTTCAGGCTGTTTTTCTGGTTTCTTGATTCATGAGAA-3' (QTDLERGTVQAVFSGFLIHEN, residues 165 to 185). These oligonucleotides were labeled with T4 polynucleotide kinase and [γ - 32 P]ATP and used to screen the cDNA library prepared from total poly(A)⁺ RNA by the method of Staunton et al. (18). Screening of the cDNA libraries prepared from fractionated poly(A)⁺ RNA was carried out by using 32 P-random-prime-labeled HP-20 or -25 cDNA fragments as follows: hybridization was at 42°C in 50% formamide, 5× SSC (standard saline citrate) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate buffer (pH 7.0), 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg of denatured salmon sperm DNA per ml, and 32 P-labeled cDNA fragment, and the final wash was at 65°C in 0.1× SSC-0.1% SDS. Positive clones were purified, and cDNA inserts were isolated by *Eco*RI digestion and subcloned into the *Eco*RI site of pBlue-script KS(+) in the case of lambda gt10 clones or excised *in vivo* to form pBluescript plasmids in the case of lambda ZAP clones.

DNA sequencing. cDNA sequences were determined with single-stranded DNA by using at least two cDNA clones carrying cDNA inserts in the opposite orientation. Single-stranded DNA was prepared as previously described (2). DNA was sequenced by the dideoxy chain termination method (16) by using synthetic primers that hybridize with cloned internal sequences.

Northern (RNA) blot analysis. Total RNA was prepared from tissues of nonhibernating and hibernating adult animals by the guanidium isothiocyanate method, and poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography. Next, 5 to 10 μ g of total RNA or 0.5 μ g of poly(A)⁺ RNA was fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel, transferred to a nitrocellulose filter, and fixed by Stratalinker (Stratagene). The filters were hybridized in 50% formamide, 5× SSC, 50 mM sodium phosphate buffer (pH 7.0), 1× Denhardt's solution, 0.1% SDS, and 0.1 mg of denatured salmon sperm DNA per ml with a 32 P-random-prime-labeled chipmunk cDNA fragment at 42°C for 16 h. The filters were washed with 0.1× SSC-0.1% SDS at 65°C for chipmunk RNA or with 0.5× SSC-0.1% SDS at 60°C for the squirrel family RNA.

Southern blot analysis. High-molecular-weight chromosomal DNA was prepared from liver. A total of 10 μ g of DNA was digested with *Eco*RI, subjected to a 0.7% agarose gel electrophoresis, and transferred to a nitrocellulose filter. The filter was hybridized with 32 P-random-prime-labeled chipmunk cDNA fragment under the same condition as for Northern blot analysis. The filter was washed at 60°C with 0.5× SSC-0.1% SDS.

RESULTS

Isolation of HP-20, -25, and -27 cDNA clones. Taking account that HP-20, -25, and -27 disappear from blood during hibernation (9), to isolate chipmunk HP-20, -25, and -27 cDNA clones, a cDNA library was constructed by using liver poly(A)⁺ RNA prepared from a nonhibernating chipmunk. Nondegenerate oligonucleotide probes (59-, 32-, and

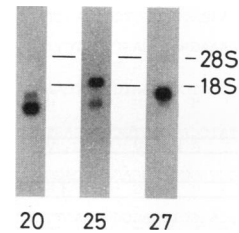


FIG. 1. Northern blot analysis of HP-20, -25, and -27 mRNA. A total of 0.5 μ g of liver poly(A)⁺ RNA from a nonhibernating chipmunk was resolved on a 1.0% agarose-formaldehyde gel and probed with the corresponding chipmunk cDNA. The positions of 18S and 28S rRNA are indicated on the right.

62-mer for HP-20, -25, and -27, respectively) were synthesized on the basis of the partial amino acid sequences (9). Labeled probes were used to screen the cDNA library by the method of Staunton et al. (18), and several positive hybridizing recombinant phages were obtained. Northern blot analysis of liver poly(A)⁺ RNA of a nonhibernating chipmunk with each of the HP-20, -25, and -27 cDNA fragments revealed a single transcript of approximately 1.5 kb as HP-27 mRNA and the presence of two distinct size classes of mRNA, with approximate sizes of 0.8 and 1.3 kb for HP-20 mRNA and 0.9 and 2.0 kb for HP-25 mRNA (Fig. 1). To isolate and characterize HP-20 and -25 cDNA clones corresponding to each size class of mRNA, liver poly(A)⁺ RNA of a nonhibernating chipmunk was fractionated by sucrose gradient centrifugation, and after hybridization of fractionated RNA with HP-20 and -25 cDNA, cDNA libraries were constructed by using poly(A)⁺ RNA fractions containing only one class of each mRNA. From the corresponding cDNA libraries, HP-20 cDNA clones carrying a 0.7- or 1.3-kb insert and HP-25 cDNA clones carrying a 0.9- or 2.0-kb insert were obtained.

HP-20, -25, and -27 are highly homologous proteins with a collagen-like domain. Nucleotide sequences of HP-20, -25, and -27 cDNA with their deduced amino acid sequences are shown in Fig. 2 to 4. DNA sequence analyses of HP-20 cDNA clones with 0.7- and 1.3-kb cDNA inserts and HP-25 cDNA clones with 0.9- and 2.0-kb cDNA inserts revealed that the shorter transcripts of both HP-20 and -25 mRNA were shortened at the 3' end and that the two size class transcripts of each mRNA contained the same open reading frame. The 1.3-kb HP-20 cDNA clones possessed a consensus polyadenylation signal, AATAAA (nucleotides 1238 to 1243), 22 bp upstream from the poly(A) tract, and the shorter 0.7-kb HP-20 clones had an AATAAA sequence (nucleotides 717 to 722) 18 bp upstream from the poly(A) tract. The 0.9- and 2.0-kb HP-25 cDNA clones possessed a polyadenylation signal, AATAAA (nucleotides 887 to 892 and 1983 to 1988, respectively), 15 and 17 bp, respectively, upstream from the poly(A) tract. Thus, the two different size transcripts of both HP-20 and -25 mRNA are generated by alternate utilization of two polyadenylation signals within the 3' noncoding region.

HP-20, -25, and -27 were composed of 173, 187, and 185 amino acids, respectively. These three proteins were highly homologous and share 61 identical amino acids (Fig. 5). There was 44, 48, and 54% amino acid sequence homology between HP-20 and -27, HP-20 and -25, and HP-25 and -27, respectively. All of them contained uninterrupted collagen-like Gly-X-Y repeats (13 repeats in HP-20 and -27 and 14 repeats in HP-25) preceded by a short noncollagenous seg-

CAAGCCTCAGGCTCCTGAGTCAAGCAACACAGGAACC 37
 TTTTGCCTGGGAGAACCCACCAAAGAACTCAGCCTTTCTACCTGAGAATGACCGAC 97
 M T D -21
 GTGTGGAGACTGGCCATATTTGTTCTCATGGTAATGTCTCTCAACGATCAGGTGAGTGC 157
 V W R L A I F V L M V N V L N D Q V S C -1
 TCTGGACCTCCAGGACCTGTAGGATATCCAGGAGTGGCCGAGTGGCCGGGCTCGTGGC 217
 S L G P P I G P V I G Y P I G V P I G V P I G P R I G 20
 CCCCAGGTCAACAGGCGCTGCGGGTAGCCAGGAGACCAGGACCAAAAGGCCAGT 277
 P P I G Q P I G A A G R P I G D P I G P K I G P S 40
 GTAAAATGCCCTGCAGAGAGAGGTGACCTTACCGTGAAGTTCAGCGGAAGGCTCCCT 337
 V K C P C R E R S A F T V K F S G R L P 60
 CCACCTTCGGAGCCTGTGGTCTTACAGAGGTCCTGTACAATCCAGAGGACTTGAAG 397
 P P S E P V F V F E N A S G A M I M P L 80
 GAGACACAGGAGTCTTAACCTGCGTGGAGCCTGAAATTTACCTTTGAGTGT 457
 E S T G V F N C V E P G N Y H F S F D V 100
 GAGCTTTACCCTGCAAGGTGAAGTTGGGTTGATGAAGAACCACATCCAAGTCCGAA 517
 E L Y H C K V K I G L M K N H I Q V M E 120
 AAGCATCAGCTCTCAAAAATGAATATGAANAATGCTTCTGGTCCATGATTATGCCGCTG 577
 K H Q L S K N E Y E N A S G A M I M P L 140
 AGACAGGGGACAGGTTGGCTAGAACGAGATGTTGAAACAGAGGAACCAGATCAAGCA 637
 R Q G D K V W L E A D V E T E E P D Q A 160
 AAAGTGTATCTATTTCTCAGGTTTCTGATTTCTTCTGAACTCTCAACCTTGTGTG 697
 K V V I Y F S G F L I S S * 173
 AGTCTTTCACCTGAGAAGGAATAAACCTAGTTTCCAGATCTTGAAGTTTGTGCTTATT 757
 CTTTCAGTATCTGAGAAAAACAATGTATAAAAATAGGCATCTTACCCCTCGTTAAAGCT 817
 GGGCCTTCTACTGTCAAATCAGAAAATTTTCAAGCAACACTATCTCAACAATCAAGC 877
 CTGAAGGTGAAAAATGCAACCGTTGTGTAACAATAAGTCTTTCCCAAACTGTCTCA 937
 ATTTTTGAAAAATGACCCCTCCCTAGCAGTACAGCTGACCCAAACTTATTCTTGTTC 997
 CTTATTTGTCTAGCTTGAACAAACCGGGTGGCGGATCACCCCTCCCTAGCAGTACGGCT 1057
 GACCCAGAATCAAAAGGAATGCTTTGACGTGACTTTATATGTTAACAATCAAAAGAG 1117
 CGCTATGAGCCCCCTTAACCTGATGATTTTCAATATAAAAACCTGCTAAATCTGGG 1177
 CTCGGGGCTCTTCCGCTACCCGGTACCGAGTGGCGGAGGACCAAGATAGAGCTTGCT 1237
AATAAATGACTCTTGTATTATACATTGAAAAAATAAAAAA

FIG. 2. The nucleotide and deduced amino acid sequences of 1.3-kb chipmunk HP-20 cDNA (pCM20-7). The N-terminal amino acid of the mature HP-20 is numbered as 1. The collagen-like Gly-X-Y repeats are boxed. The potential N-linked glycosylation site is indicated by a wavy line. The amino acid sequence used for the design of the oligonucleotide probe is underlined. The putative polyadenylation signals (AATAAA) for 0.7-kb (nucleotides 717 to 722) and 1.3-kb (nucleotides 1238 to 1243) mRNA are underlined. The poly(A) sequence of 0.7-kb mRNA starts from nucleotide 741. The nucleotide sequences were also determined with 0.7-kb HP-20 cDNA clones, pCM20-1 and -2, and another 1.3-kb cDNA clone, pCM20-11, and their comparison revealed base substitutions: T, C, and C at nucleotides 107, 121, and 538, respectively, for pCM20-1; C at nucleotide 538 for pCM20-2; T, T, C, T, and T at nucleotides 45, 421, 538, 808, and 1075, respectively, for pCM20-11.

ment (1 to 14 residues). There was one potential N-glycosylation site in each of HP-20, -25, and -27 at similar positions (Fig. 5).

Tissue-specific expression of HP-20, -25, and -27 mRNA and hibernation-associated changes in mRNA levels. To examine tissue-specific expression of HP-20, -25, and -27 mRNA, total RNA was isolated from several tissues of a nonhibernating chipmunk and Northern hybridization was carried out (Fig. 6). HP-20, -25, and -27 mRNAs were detected in liver. No detectable amounts of these transcripts were seen in brain, heart, kidney, or lung. In hibernating chipmunks, HP-20, -25, and -27 mRNA were also found only in liver (data not shown). However, the amounts of HP-20, -25, and -27 mRNA in hibernating chipmunks were greatly reduced and were less than 1/10 of those in nonhibernating chipmunks, while albumin mRNA did not show any definite quantitative difference between nonhibernating and hibernating chipmunks (Fig. 7). In addition, when RNA dot analysis was performed by probing with three cDNA clones isolated arbitrarily from a nonhibernating chipmunk cDNA

ATTTTCAGCAATCATCC 17
 TTCAGCAATCAAGCTTCAAATCAAACCAAGTGAGAGAAGAACTAAAGAGGAGAGAG 77
 GCCCATTTTCAGTGGCTTCTCCACGCTGTCTGAATGCTGCACAAAGAGGAGGCGCT 137
 M P A Q R G G A -21
 CTCTCCATGGAGCTGTGGCTTCTGGATTTTGGCTGTCCATCAGTCCAGGCTGGCT 197
 L S M G A A G F W I L V L S I T S A L A -1
 GATTTCCAAATCAAGAAATCTGAACCTGTGGACCTCCAGGTCCTCCAGGACCTCCA 257
 D S N N N Q G N S E P C G P P I G P I G P P 20
 GGCATCCAGGCTTTCCAGGCGCTCCTGGAGCACTTGGTCCACAGGACCCAGGTTG 317
 G I P I G P P G A P G A L G P P G P P G V 40
 CCAGGAATACAGGACCAAGGTCCTCCTGGAGCCTTGAAGTGTCTATCGCGACC 377
 P G I P I G P Q I G P P G D V E K C S S R P 60
 AAATCGCTTGTGTGAGCTGAGTGAACGCCCCAGAGCCCTCCAGCCATGTC 437
 K S A F A V K L S E R P P E P F Q P I V 80
 TTCAGGAGGCTCTGTAACATCAGGAGGCTTCAACATGCCACAGGAGAGTTCAGC 497
 F K E A L Y N Q E G H F N M A T G E F S 100
 TGTGCTCCCTGGTGTACAATTTGGCTTTGACATTCGACTGTTTCAGAGTTCGTG 557
 C V L P G V Y N F G F D I R L F Q S S V 120
 AAGTACAGGCTCAGGGATGATCCAGGTCAGAGAAAGAACCCAGCAATGAC 617
 K I R L M R D G I Q V R E K E A Q A N D 140
 AGTACAACATGCAATGGGCTGTGATTTGGCCCTGGGAGGGGGGCAAGTCTGG 677
 S Y K H A M G S V I M A L G K G D K V W 160
 CTGGAATCCAAGTGAAGGAAACCGAATCTGAAAAAGGAATCACTCAGATTTGTCTTT 737
 L E S K L K G T E S E K G I T H I V F F 180
 GGGTATTTGGTGTACGGCAAGTAAATTTGGCTTGGAGCCCTCCATCCCTTGTGTAATCTG 797
 G Y L Y Y G K * 187
 AATTAGTGAGCAAAATTTCTATTTTCCATGCTGCTTATGACGGAATGAGGGAAAGTCT 857
 ATAAAAACTTAAGAGTCTCAGTTGAAAGAAATAAATGTAACATGTATCCAAGAAATGTG 917
 TAAATGATAAGACAGCTTGAANAATACATGGTCCCACTGATGGGTAGGGGTCT 977
 GCAAGAATCAGAGAAAGGATGAGGGCCAGATGAATGGATTTTGGGAGAAAGTTC 1037
 CCACTCAATCTTTGGGAAGTAAAGCCCTGTCTGTCCAGGCTTCTGCGCCAGGCT 1097
 TTACTAAGAAATGACCAAGTGAATTTCCCTGTAGAGGTTGGGAAGTCTTGAACCT 1157
 CCAACGAAATGTGAACACTTAACCTCAGTTCCTTTCTGGGAGGATATTTGATGGATG 1217
 GATGGGAAGAGGCTGCAATATGATTAGAGGAGGATGTACCATTGCTTACTGCCAAT 1277
 GACTGAATTTGTCACCCACGCCCTGCTCAAATTCCTGTGCAGAGCCCAAGCCCTAA 1337
 CATAACGGTATCTGGATGCAGGCTCTCGGGAAGATATTAAGTTAAATGAGGTCATAAGA 1397
 ATGAGGCCCTGTGTAGTCAAGTTCATCATTTGATCAATAACCAAAAAGAACAAAC 1457
 TCAGAGGAGGAATGATTTATTTGGGCTCATGGTTTTCAGAGGATTTAGTCCATGGTGTAGCT 1517
 GACTCTAAGGCAGACCCATCATGGCAAAAGCGTATAGCAGAGAAAGCTGTGACTTCCAG 1577
 GCAGCTGGGAAGCAGAGAGAAAGAAAGGACTGGGAAGAAGATAAACCTTGAAGGGG 1637
 AACCCACCCCTGGGACTCGATTATCAACTAGACCCCACTTCCCTGATGCCATTCAGC 1697
 TATCGTGATGGCTGATGAAGTCAACATTTCTCACACCCAGTCAATGTCTAAAAGCCCCA 1757
 GCTCTGAACCTTGTGTGTTCAAGGCCACACTTCCACACGTTAGCTTTTGGGGACATT 1817
 CCACATCAAACTGCAACTGGCCCTACATGACAGTGGCCTTATGAAAGAAGGCATGGG 1877
 CGGACACAACAAGGAAGGCCCTCATCAAGCCAGGAGGAAAGACTTCCACATGAACCGA 1937
 ATCGGACAGCAGTGAATCTGGACTTCCAGCCTCCAAAATTAATAAATCTGTGTTG 1997
 TTTCAACCAAAAAAAAAAAAAA

FIG. 3. The nucleotide and deduced amino acid sequences of 2.0-kb chipmunk HP-25 cDNA (pCM25-3). The N-terminal amino acid of the mature HP-25 is numbered as 1. The amino acid sequence used for design of the oligonucleotide probe is underlined. The poly(A) sequence of 0.9-kb mRNA starts from nucleotide 908, and the putative polyadenylation signals for 0.9-kb (nucleotides 887 to 892) and 2.0-kb (nucleotides 1983 to 1988) mRNA are underlined. The nucleotide sequences were also determined with 0.9-kb HP-25 cDNA clones pCM25S-1 and S-2 and 2.0-kb cDNA clones pCM25-1 and -2, and their comparison revealed a base substitution: C for pCM25S-1 and S-2 at nucleotide 191.

library, there was almost no difference in their transcript levels between the nonhibernating and hibernating chipmunks (data not shown).

Analysis of HP-20, -25, and -27 gene expression among hibernating and nonhibernating species of the squirrel family. HP-20, -25, and -27 mRNA levels showed hibernation-associated changes in the chipmunk. To inquire further into the relationship between these gene expressions and hibernation, we investigated in other squirrel species the presence

GATGGTCAAACCTGGAATAACAAGAACAACATCTC 34
 AAAATCTCAATCTTCCTTTTGAAGCTTAGAATGTATGAAGCAGGAAAACAGCTTCCTCTC 94
 M Y E A G K R A S F -21
 ATGGGAGGGGAGGCATCTGGATTTTGGCCCTGTCTGTACTAATGCACGTGGTGTCTT 154
 M G G A G I W I L A L S V L M H V V C S -1
 GAAACTCAAGAAATCCTGAATCCTGTAAATGTTCCAGGACCTCAAGGACCTCCAGGCATG 214
 E T Q G N P E S C N V P G P Q G P P G M 20
 CGGGTCCCGGAGTACACCTGGAAAACAGGCCACCAGGATGGAATGGTTTTCCAGGA 274
 R G P P G T P G K F G P P G W N G F P G 40
 CTACCAGGGCCACCAGGGCCACCAGGCATGACTGTGAAGCTGCCACAGCAAAGGAACATCG 334
 L P G P P G P P G M T V N C H S K G T S 60
 GCCTTTGACGTAAGGCAATGAGCTGCCCCAGCTCCCTCCAGCCCGTATCTTCAAG 394
 A F A V K A N E L P P A P S Q P V I F K 80
 GAAGCCCTGCATGACGCTCAGGGACACTTTGATCTGGCCACTGGTGTGTACCTGCCCA 454
 E A L H D A Q G H F D L A T G V F T C P 100
 GTCCAGGACTTACCAGTTTGGATTTTCACATTTGAAGCTGTCCAGGGCTGTGAAGTGT 514
 V P G L Y Q F G F H I E A V Q R A V K V 120
 AGCCTCATGAGAAATGGCACCAGTCAATGGAGAGAGAGAGAGAGAGAGAGAGAGAGAG 574
 S L M R N G T Q V M E R E A E A Q D G Y 140
 GAGCACATTTGAGAACTGCCATCTTACAGCTGGGCATGGAAGACAGGGTCTGGCTGGAG 634
 E H I S G T A I L Q L G M E D R V W L E 160
 AACAGCTGAGCCAAACAGATCTCGAAGGGGACGTTTACAGGCTGTGTTCTCGGGGTTT 694
 N K L S Q T D L E R G T V Q A V F S G F 180
 CTGATTCATGAAATGAGAGTACTGGTCTGTATCATTCTTACCACCCCTCCCTACGAG 754
 L I H E N * 185
 CGTCCAATTTGAGAGAGAGAAATATTGCTCCTTCTTATAAGGATTCAATTTCTTTCAAAA 814
 TCATAACAGAAAAAGGTTTAAAAAATCTCAGCACATAAAAACCTGATACTATTTCGGGA 874
 GATAAAGTGGTTTGTATCAGTACACATTTGTATGCGTATATTGGATTATAATATTCTATTTC 934
 ATAAATAAGTACAAATCACAAGTCAAAATCAGTAATAATAATCAGTCAAAATCACAAG 994
 TAAATAATCTAAAAAGAATATTCTCTTTATGGTAGTACTTTAAAATGATCATTGGGGT 1054
 ATGAATAAATACATGATGAAATAAGGTGATTTTAAAGCAGATCCCTGAACTAGGAAGT 1114
 GAATGTGCATTAGATTGTCAACTAATTCATATTATTTTAAAGTATATTGGATTATTTTACC 1174
 TACGTAGGAAAAATTTATGTAATATGAAGCATGGAGAATCTCAATTTATGGTGCATCAAA 1234
 AAAAGAATCTTGGGGCTGGGATTTGGCTCAGTGGTAGAGCGCTCGCCTAGCACATGGGC 1294
 GACCCGGTTCGATCTCAGCACCAGTCAAAATAAATGCAATGAATAAAACGCATTGT 1354
 GTACAACATAAGCAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA

FIG. 4. The nucleotide and deduced amino acid sequences of chipmunk HP-27 cDNA (pCM27-3). The N-terminal amino acid of the mature HP-27 is numbered as 1. The amino acid sequence used for design of the oligonucleotide probe is underlined. Comparison of the nucleotide sequence with that of another HP-27 cDNA clone, pCM27-4, revealed base substitutions: G, G, G, A, C, and A at nucleotides 27, 51, 69, 143, 186, and 216, respectively, in pCM27-4. The latter four base substitutions caused amino acid changes: C, M, A, and Q at residues -30, -4, 11, and 21 in pCM27-4.

of the genes corresponding to chipmunk HP-20, -25, and -27 genes and their expression. The 13-lined ground squirrel (*Citellus tridecemlineatus*) is another hibernating species, while the Formosan tree squirrel (*Callosciurus caniceps*) is a closely related species that does not hibernate. Southern blot analyses of chipmunk, ground squirrel, and tree squirrel chromosomal DNA probed with the chipmunk cDNAs revealed specific hybridization signals with all three species (Fig. 8). Expression of HP-20, -25, and -27 mRNA in liver was further analyzed. These transcripts were detected in ground squirrels, and in the hibernating state, their levels were greatly reduced, as were the cases with the chipmunk (Fig. 9). On the other hand, in tree squirrels, the transcripts could be detected neither in liver (Fig. 9) nor in any of the other tissues examined: brain, heart, kidney, lung, muscle, or spleen (data not shown).

DISCUSSION

Mammals in hibernation are in special physiological states, and the physiological changes associated with hibernation are considered to be under genetic controls (15). However, there is no evidence for hibernation-associated

HP-25	DSNNQGNSEF	CGPFPFGPP	GIPGFPAGP	ALGPPGPGV	PGIPGQGGP	50
HP-27	ETQGNPES	CNVPGQGGP	GMRGPPGPG	KPGPPGNGF	PGLPGPPGP	48
HP-20		SGPPGPV	GYPGVGVP	PRGPPQPGA	AGRPGDGGK	37
	GDVEKCSSRP	KSFAVKLSE	RPPEFPOIV	FKEALYNQEG	HFNMATGEFS	100
	GMTVNCHSKG	TSFAVKANE	LPPAPQPMV	FKEALHDAQF	HPDLATGVPT	98
	GPSVKPCRE	RSAPTVKFSG	RLPPPSEPVV	FTEVLYNTQR	DLKESGVFN	87
	CVLPGVYVNF	PDIRLQFSSV	KIRLMRDGIQ	VREKQAQND	SYKHAMGSKI	150
	CPVPLGYQFG	FHIEAVQRAV	KVSLMRNGTQ	VMEREAEAQD	GYEHSIGTAI	148
	CVEPGNYHFS	PDVELYHCKV	KIGLMRNHIQ	VMEKHQLSKN	EYENASGAMI	137
	MALGKGDQVW	LESKLGKTES	EKGITHIVFF	GYLLYGK		187
	LQLGMEDRVV	LENKLSQTDL	ERGTVAQVFS	GFLIHEN		185
	MPLRQGDQVW	LEADVETEPE	DQAKVVIYFS	GFLISS		173

FIG. 5. Comparison of the amino acid sequences (single-letter code) of chipmunk HP-20, -25, and -27. The potential N-linked glycosylation sites are underlined. Amino acids identical between HP-25 and -27 or between HP-20 and -27 are indicated by double dots. Conservative amino acid substitutions between HP-25 and -27 or between HP-20 and -27 are indicated by single dots. Conservative amino acids are classified as follows: D, E, N, and Q; H, K, and R; A, G, P, S, and T; I, L, M, and V; and F, W, and Y.

gene regulation, and the molecular mechanisms used in control of hibernation still remain to be elucidated. The finding that the chipmunk plasma proteins HP-20, -25, and -27 disappear from blood prior to the onset of hibernation (9) suggests that the reduction of these proteins in the blood would be necessary in preparation for hibernation and that their gene expression would be coupled with hibernation. In this study, to investigate the relationship between the gene expression of chipmunk HP-20, -25, and -27 and hibernation, we isolated these cDNA clones and compared the transcript levels between hibernating and nonhibernating chipmunks. These transcripts were detected only in liver in both nonhibernating and hibernating animals. In hibernating animals, however, the amounts of the transcripts were greatly reduced in comparison with those in nonhibernating animals, while albumin mRNA did not show any specific quantitative difference between the two states (Fig. 7). These observations indicate that the gene expression of chipmunk HP-20, -25, and -27 is regulated in association with hibernation, which would possibly result in the hibernation-associated quantitative changes of HP-20, -25, and -27 in the blood.

Furthermore, the comparative study employing another hibernating species, the ground squirrel, and a closely related nonhibernating species, the tree squirrel, supports the idea of the involvement of HP-20, -25, and -27 in hibernation. Genomic Southern analysis with the chipmunk cDNA probes showed that the tree squirrel as well as the ground squirrel retained the genes corresponding to chipmunk HP-20, -25, and -27 genes (Fig. 8). The corresponding transcripts could be detected in the ground squirrel, but not in the tree

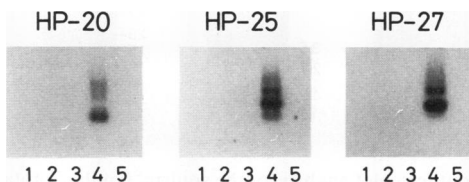


FIG. 6. Tissue-specific expression of HP-20, -25, and -27 mRNA in a nonhibernating chipmunk. Each lane contained 10 µg of total RNA and was probed with chipmunk HP-20, -25, or -27 cDNA fragment. Lanes: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung.

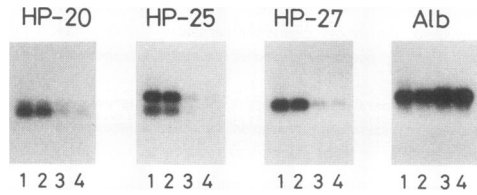


FIG. 7. Comparison of HP-20, -25, and -27 mRNA levels between nonhibernating and hibernating chipmunks. A total of 0.5 μ g of liver poly(A)⁺ RNA from nonhibernating (lanes 1 and 2) and hibernating (lanes 3 and 4) animals was fractionated on a 1.0% agarose-formaldehyde gel and probed with a chipmunk HP-20, -25, or -27 or albumin (Alb) cDNA fragment.

squirrel (Fig. 9). The finding that HP-20, -25, and -27 gene expression is restricted to the hibernating species, the chipmunk and the ground squirrel, suggests their certain roles in hibernation. To understand the extinction of these transcriptions in the tree squirrel, comparative studies of the promoter regions of HP-20, -25, and -27 genes between the chipmunk and the tree squirrel would be required.

HP-20, -25, and -27 contain collagen-like Gly-X-Y repeats immediately downstream of a short N-terminal noncollagenous sequence. Among proteins with a collagen-like domain such as complement protein C1q (13), mannose-binding protein (4), pulmonary surfactant apoprotein SP-A (1), macrophage scavenger receptor (8), conglutinin (11), and the asymmetric forms of acetylcholinesterase (10), the overall organizations of HP-20, -25, and -27 into the N-terminal collagen-like domain preceded by a short segment and the C-terminal noncollagenous domain are very similar to those of complement protein C1q, conglutinin, mannose-binding protein, and pulmonary surfactant apoprotein. Although the collagen-like domains of HP-20, -25, and -27 (13 or 14 Gly-X-Y repeats) are a little shorter compared with the corresponding portions of C1q (25 to 27 repeats), conglutinin (55 repeats), mannose-binding proteins (18 to 20 repeats), and pulmonary surfactant apoprotein (24 repeats), HP-25 and -27 have a cysteine residue within the short noncollagenous segment preceding the collagen-like domain, and HP-20, -25, and -27 are considered to form complexes through the collagen-like domains and by interchain disulfide bonds as these proteins (9, 19). In addition, in the cases of HP-20, -25, and -27, the presence of a cysteine residue immediately downstream of the collagen-like domain may stabilize the triple helix structure formed by the collagenous

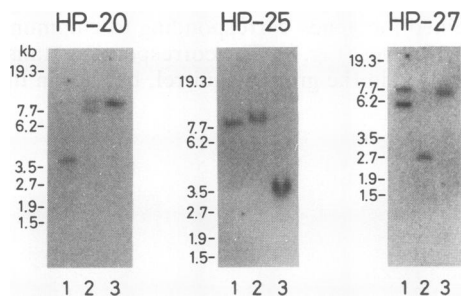


FIG. 8. Southern blot analysis of the squirrel family. A total of 10 μ g of liver chromosomal DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel, and probed with a chipmunk HP-20, -25, or -27 cDNA fragment. Lanes: 1, chipmunk; 2, ground squirrel; 3, tree squirrel.

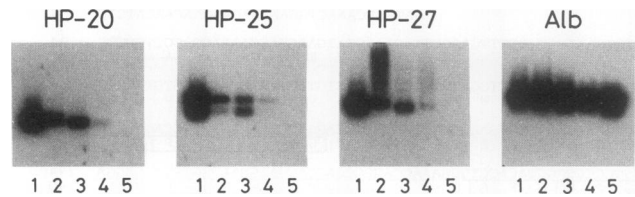


FIG. 9. Northern blot analysis of the squirrel family. A total of 0.5 μ g of liver poly(A)⁺ RNA was fractionated on a 1.0% agarose-formaldehyde gel and probed with a chipmunk HP-20, -25, or -27 or albumin (Alb) cDNA fragment. Lanes: 1, nonhibernating chipmunk; 2, hibernating chipmunk; 3, nonhibernating ground squirrel; 4, hibernating ground squirrel; 5, tree squirrel. The chipmunks used in this figure are different from those used for Fig. 7.

domains. Conglutinin, mannose-binding protein, and pulmonary surfactant apoprotein belong to the C-type lectin family and retain a carbohydrate recognition domain within the C-terminal noncollagenous portion (3, 19). The C-terminal domain of C1q is also involved in binding with the Fc regions of immunoglobulin G and immunoglobulin M (14). To understand the functions of HP-20, -25, and -27, whether their C-terminal noncollagenous portions are involved in specific ligand binding, and if so, what the ligand is are to be determined.

HP-20, -25, and -27 form a 140-kDa complex with HP-55 in the plasma (9). The partial amino acid sequence of HP-55 reveals its similarity to α_1 -antitrypsin, a member of the serpin (serine protease inhibitor) superfamily (9). Taken together that HSP47, a heat shock protein identified in chicken embryo fibroblasts, is a serpin superfamily member and binds to collagen (7), HP-55 may bind to the triple helical region formed by the collagenous domains of HP-20, -25, and -27. There is a possibility that a role of HP-20, -25, and -27 may be to form a complex with HP-55 and, consequently, regulate the functions of HP-55. The serpin superfamily includes hormone-binding proteins such as thyroxine-binding globulin (5) and corticosteroid-binding globulin (6). HP-55 may also function as a hormone-binding protein, and not as a protease inhibitor, to transport a certain hormone or regulate its half-life. To understand the roles of the chipmunk hibernation-related proteins in hibernation, the entire structure of HP-55 and its function are also to be elucidated.

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