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Constitutively Expressed Rat mRNA Encoding a 70-Kilodalton Heat-Shock-Like Protein

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A nearly full-length cDNA clone isolated from the rat pheochromocytoma cell line, PC12, revealed extensive nucleotide sequence similarity between the rat cDNA and the *Drosophila melanogaster* hsp70 gene. The rat recombinant clone encodes a 71,000-dalton protein that is 70% identical with the dipteran hsp70 protein. Remarkably, a truncated segment of this cDNA clone was originally isolated by immunoreactivity with antisera raised to catecholamine-synthesizing enzymes, suggesting that this heat shock protein and these catecholamine enzymes shared antigenic determinants. The rat hsp70-related mRNA is responsible for the production of a constitutive hsp70 protein, because it is present in abundant amounts in various tissues at normal growth temperatures and is only minimally induced by hyperthermia. The rat hsp70-related sequence is part of a multigene family that extends across species to mice and humans.

In response to a sudden alteration in temperature, both procaryotic and eucaryotic cells increase the transcription of a small subset of relatively inactive genes, the products of which have been termed heat shock proteins (hsp) (31). First noted in *Drosophila* species (1), a heat shock response has been observed in yeasts (25, 27), *Dictyostelium discoideum* (21), tobacco and soybeans (4, 18), hamsters (16), chickens (16), mice (22), rats (10), and humans (17, 35). Many agents other than temperature elicit the heat shock response in a number of species (2, 11, 21, 31). Therefore, the alteration in gene expression appears to be universal and is thought to be a general response to metabolic disturbance. In most organisms, the major heat shock protein produced has an apparent subunit molecular weight of 70,000 to 75,000 and is coded for by the hsp70 gene (31). In *Drosophila* and yeast cells, a multigene family related to the hsp70 gene has been identified (12, 14). A constitutively expressed set of hsp70-like proteins are also produced in unstressed cells in all species examined. The eucaryotic hsp70 genes examined to date are those that function after heat shock or stress. hsp70-related sequences, referred to as cognate genes, have been isolated in *Drosophila* and yeast cells, but their relationship to the constitutive hsp70-like proteins has not been confirmed.

Recently, we reported the isolation of a rat cDNA clone, pDBH-1, whose mRNA is enriched in rat pheochromocytoma (PC12) cells derived from a rat pheochromocytoma. On the basis of the subunit molecular weight, immunoreactivity, and peptide mapping by partial proteolysis of the translated protein, we concluded that this clone coded for dopamine-β-hydroxylase (DBH) (28, 29). In this paper, we report that this initial assignment was incorrect and that the nucleotide sequences of this rat cDNA clone and a full-length counterpart clone, pRC62, identify it as an hsp70-related sequence. Transcripts related to pRC62 do not respond significantly to heat shock and accumulate abundantly in all tissues examined. Furthermore, these constitutively expressed sequences are encoded by a multigene family in several mammalian species. We thus identify this cDNA as encoding a constitutively expressed, minimally regulated hsp70-like protein. Recent data (29a) suggest a nuclear role for hsp70 in the reassembly of damaged preribosomal ribonuclear proteins. If this is the case, then the constitutively expressed mRNA cloned in pRC62 may play a role in the normal physiological assembly of ribosomal or other ribonuclear proteins.

MATERIALS AND METHODS

Cell culture and labeling. PC12 cells and human cervical carcinoma (HeLa) cells were grown in 100-mm plastic dishes (Corning Glass Works) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cells, maintained in 10% CO_2, were grown to near confluence (3×10^7) and heat shocked by the addition of fresh medium prewarmed to 42°C, and then they were incubated at this temperature. After an appropriate time, the medium was aspirated, and the cells were washed twice with prewarmed DMEM without methionine. Cells were further incubated at 37 or 42°C for 30 min in the same medium plus 10 μCi of [^{35}S]methionine (Amersham Corp.; specific activity, 660 Ci/mmol). After being labeled, cells were washed once with cold phosphate-buffered saline and then solubilized by the addition of $NaDodSO_4$-electrophoresis buffer (20). RNA preparations were made from matched cultures of PC12 and HeLa cells that had been treated in an identical manner (33).

Hybridization and selection of mRNA. Plasmid DNA (10 μg) was immobilized on a nitrocellulose filter (1 by 3 cm) and hybridized with 40 μg poly(A)$^+$ RNA for 2 h at 50°C in 65% formamide–20 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid (PIPES); pH 6.4)–0.2 $NaDodSO_4$–0.4 M NaCl with 0.1 mg of yeast tRNA (Boehringer Manheim Biochemicals). The filters were washed extensively with 0.01 M Tris (pH 7.5)–0.15 M NaCl–0.5% $NaDodSO_4$ at 65°C. Two final washes were done in the same buffer without $NaDodSO_4$. Hybridized RNA was eluted thermally and ethanol precipitated. Hybrid-selected RNA and cytoplasmic poly(A)$^+$ RNA were translated in vitro by using a rabbit reticulocyte lysate system (New England Nuclear Corp.) in the presence of [^{35}S]methionine. Labeled products were analyzed by one-

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dimensional electrophoresis on 10% $NaDodSO_4$-acrylamide gels both before and after antibody immunoprecipitation. Immunoprecipitation and the tyrosine hydroxylase antibody were as described previously (28). The monoclonal and polyclonal antibodies to purified soluble DBH were generously provided by Ruth Hogue-Angeletti (University of Pennsylvania). After electrophoresis, the gels were fixed and fluorographed with En^{3}Hance (New England Nuclear). The gels were exposed to Kodak XAR-5 film at −70°C.

Sequence determination. Nucleotide sequencing was performed by the chemical method of Maxam and Gilbert (24) and by the dideoxy method of Sanger et al. (30). In the former method, restriction fragments were labeled at their 5′ termini by using T_4 polynucleotide kinase (New England Nuclear). Dideoxy sequencing was performed with single-stranded templates prepared from an M13mp8 phage vector propagated in *Escherichia coli* JM101 (26). Fragments from both strands were sequenced at least twice in separate experiments. Sequence data management was performed by using the GEL program of IntelliGenetics.

Southern blot analysis. Genomic DNA prepared from either rat thymus, mouse thymus, or HeLa cells (29) was digested with *Eco*RI restriction endonuclease. The DNA digests were electrophoresed on 0.8% agarose gels at 40 V overnight, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled nick-translated coding region probe by the method of Southern (32). After prehybridization for 6 h at 65°C in 0.5 M NaCl–0.06 M sodium citrate (pH 7.0)–0.05% polyvinylpyrrolidone–0.05% Ficoll–0.05% bovine serum albumin–50 mM NaP_i (pH 7.0) the blots were hybridized for 18 h with 10^6 cpm/ml of nick-translated DNA probe in the above buffer plus 10% dextran sulfate. Filters were washed extensively in 0.06 M NaCl–0.006 M sodium citrate (pH 7.0)–0.1% sodium dodecyl sulfate at 65°C.

RNA isolation and analysis. Various Sprague-Dawley rat tissues were dissected on ice and immediately frozen at −70°C. RNA was prepared by the method of Chirgwin et al. (6). Poly(A)$^+$ RNA was fractionated by electrophoresis on a 1% agarose–formaldehyde gel (23). The RNA was transferred to a nitrocellulose filter (23) and probed with the nick-translated coding region probe. Hybridization buffer was prepared as described above, with the addition of 50% formamide, 0.1% sodium dodecyl sulfate, and 50 μg of denatured calf thymus DNA per ml. Hybridizations and filter washing (0.075 M NaCl, 0.0075 M sodium citrate, 0.1% sodium dodecyl sulfate) were done at 42°C. After autoradiography, blots were scanned with an Optronics P-1000 digitizer driven by a PDP-10 computer generously put at our disposal by Jerry Latter of The Linus Pauling Institute for Science and Medicine, Palo Alto, Calif.

RESULTS

Hybridization selection and immunoprecipitation. Previously, Joh et al. (T. H. Joh, E. E. Baetge, B. B. Kaplan, M. E. Ross, M. J. Brodsky, V. R. Albert, D. H. Park, D. J. Reis, Abstr., Soc. Neurosci. Symp. **9**:206, 1981) reported that tyrosine hydroxylase (TH) antisera would immunoprecipitate DBH. Recently, this group demonstrated (15) that bovine cDNA clones encoding DBH and a third catecholamine biosynthetic enzyme, phenylethanolamine *N*-methyltransferase, contain regions of nucleotide sequence homology. These findings suggest that all three of these enzymes of the catecholamine pathway contain cross-reacting antigenic determinants. Polyclonal antibodies against purified TH immunoprecipitated the *in vitro* translation product synthesized by using as template the mRNA hybrid selected by clone pRC62 (Fig. 1). We have renamed this clone from its original designation as pDBH-3 (29). Additionally, both a polyclonal antibody and a monoclonal antibody raised against purified, soluble DBH immunoprecipitated the hybrid-selected translation product (Fig. 1). On the basis of the immunoreactivity with these antisera—and the identity of the cleavage fragments generated by partial proteolysis—of the peptide produced by hybrid-selected translation, we originally suggested that pRC62 encodes part of the 72-kilodalton subunit of DBH (28, 29). Based on the nucleotide sequence data presented below, this is clearly not the case.

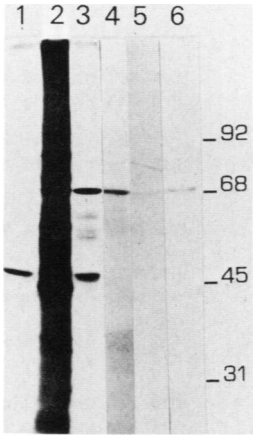

FIG. 1. Autoradiograms of [^{35}S]methionine-labeled in vitro translation products synthesized with mRNA complementary to the rat hsp70-related DNA. Hybridization selection of RNA and in vitro translation were performed as described in Materials and Methods. Lanes: 1, translation products when no mRNA was added to the system; 2, translation products of total PC12 cell RNA; 3, total translation products synthesized with PC12 cell RNA selected by pRC62; 4, immunoprecipitation with TH antisera of the translation product synthesized with RNA selected by pRC62; 5, immunoprecipitation by polyclonal anti-DBH antisera of the translation product synthesized with RNA selected by pRC62; 6, immunoprecipitation with monoclonal antibody raised against purified soluble DBH of the translation product synthesized with RNA selected by pRC62. Molecular weight markers (×10^3) are to the right.

Sequence analysis of pRC62. The nucleotide sequence of pRC62 is shown in Fig. 2. This cDNA clone carries a 2,072-base insert plus a poly(A) tail. A 51-base 5′ untranslated region, which is probably truncated, precedes the initiation codon. The deduced amino acids from the single, long open reading frame in the mRNA are printed below the nucleotide sequence. Comparison of the nucleotide sequences derived from this nearly full-length cDNA with those present in the National Institutes of Health Genbank library indicated extensive nucleotide sequence homology between pRC62 and the published *Drosophila* heat shock protein gene, hsp70 (13).

The amino terminus of this protein has three additional residues when compared with the *Drosophila melanogaster* hsp70 amino terminus assignments but is coincident with the amino terminus predicted for a trout hsp70-like protein (Fig. 3) (19). On the basis of this amino-terminal assignment, the molecular weight of the encoded rat polypeptide is 71,500. This correlates well with our estimates, from $NaDodSO_4$-acrylamide gels, that the hybrid-selected translation product (Fig. 1) has a molecular weight of 72,000 (28). The nucleotide residues encoding the aspartic acid at position 1987–1989 are

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....GTCTCTGTGTGGTCTCGTCATCAGCACAGCTGGGCCTACACGCAAGCAACC ATG TCT AAG GGA    63
                                                        Met Ser Lys Gly

CCT GCG GTT GGC ATT GAT CTT GGC ACC ACC TAC TCC TGT GTG GGT GTC TTC CAG   117
Pro Ala Val Gly Ile Asp Leu Gly Thr Thr Tyr Ser Cys Val Gly Val Phe Gln

CAT GGA AAG GTG GAA ATA ATT GCC AAT GAC CAG GGT AAC CGC ACC ACG CCG AGC   171
His Gly Lys Val Glu Ile Ile Ala Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser

TAT GTT GCT TTC ACC GAC ACA GAA CGA TTA ATT GGG GAT GCG GCC AAG AAT CAG   225
Tyr Val Ala Phe Thr Asp Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln

GTT GCA ATG AAC CCC ACC AAC ACA GTT TTT GAT GCC AAA CGT CTG ATC GGA CGT   279
Val Ala Met Asn Pro Thr Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg

AGG TTC GAT GAT GCT GTT GTT CAG TCT GAC ATG AAG CAC TGG CCC TTC ATG GTG   333
Arg Phe Asp Asp Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe Met Val

GTG AAC GAT GCA GGC AGG CCC AAG GTC CAA GTC GAA TAC AAA GGG GAG ACA AAA   387
Val Asn Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys Gly Glu Thr Lys

AGT TTC TAT CCT GAG GAA GTG TCT TCA ATG GTT CTG ACA AAA ATG AAG GAA ATT   441
Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu Thr Lys Met Lys Glu Ile

GCA GAA GCT TAC CTT GGA AAG ACT GTT ACC AAT GCC GTG GTC ACC GTG CCA GCT   495
Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr Asn Ala Val Val Thr Val Pro Ala

TAC TTC AAT GAC TCT CAG CGA CAG GCA ACA AAA GAT GCT GGA ACT ATT GCT GGC   549
Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly

CTC AAC GTA CTT CGA ATT ATC AAT GAG CCA ACT GCT GCT GCT ATT GCC TAT GGC   603
Leu Asn Val Leu Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly

TTA GAT AAG AAG GTC GGG GCT GAA AGG AAT GTG CTC ATT TTT GAC TTG GGA GGT   657
Leu Asp Lys Lys Val Gly Ala Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly

GGC ACT TTT GAT GTG TCA ATC CTC ACT ATC GAG GAT GGA ATT TTT GAA GTC AAA   711
Gly Thr Phe Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys

TCA ACA GCT GGA GAC ACC CAC TTG GGC GGA GAA GAC TTT GAC AAC CGA ATG GTC   765
Ser Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Met Val

AAC CAT TTC ATT GCT GAG TTT AAG CGA AAG CAC AAG AAG GAC ATC AGT GAG AAC   819
Asn His Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp Ile Ser Glu Asn

AAG AGA GCT GTC AGG CGT CTC CGC ACT GCC TGT GAG CGG GCC AAG CGC ACC CTC   873
Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu Arg Ala Lys Arg Thr Leu

TCC TCC AGC ACC CAA GCC AGT ATT GAG ATT GAT TCT CTC TAT GAG GGA ATT GAC   927
Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile Asp Ser Leu Tyr Glu Gly Ile Asp

TTC TAC ACC TCC ATT ACC CGT GCT CGA TTT GAG GAG TTG AAT GCT GAC CTG TTC   981
Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe Glu Glu Leu Asn Ala Asp Leu Phe

CGT GGC ACA CTG GAC CCT GTA GAG AAG GCC CTT CGA GAT GCC AAA CTA GAC AAG  1035
Arg Gly Thr Leu Asp Pro Val Glu Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys

TCA CAG ATC CAT GAT ATT GTC CTG GTG GGT GGT TCT ACC AGA ATC CCC AAG ATC  1089
Ser Gln Ile His Asp Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Ile
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followed by a single in-frame translational stop codon, TAA. Accordingly, the 3′ noncoding region of the mRNA is composed of an untranslated region of 81 nucleotides incuding a polyadenylation signal AATAAA at base 2050 (9).

At the nucleotide level, the translated regions of pRC62 and *D. melanogaster* hsp70 are 71% similar from the start codon to base 1680 in the pRC62 sequence, but they are less well conserved beyond that point (50%; data not shown). This is reflected in the poor amino acid sequence conservation (53%) between these species in the carboxy-terminal 156-amino-acid segment (Fig. 3). Deletion mapping experiments (29a) suggest that functional domains of hsp70 are present in the more conserved amino-terminal segment of the proteins. The amino-terminal 492 residues shown in Fig. 3 are 81% conserved between the rat hsp70-like protein and hsp70 itself. The amino acid sequence derived from the partial sequence of a cDNA clone of *Xenopus levis* hsp70-like mRNA (5) and two partially sequenced trout hsp70-like mRNAs (19) are included in the comparisons shown in Fig. 3.

Heat shock inducibility of pRC62-related transcripts. The likelihood that pRC62 encodes a mammalian hsp70-like protein led us to test whether transcripts related to the rat cDNA were induced by hyperthermia. We subjected nearly confluent cultures of rat pheochromocytoma-derived PC12 and human fibroblast-derived HeLa cells to heat shock at 42°C for 1 or 8 h (35), followed by radiolabeling of proteins with [^{35}S]methionine. Both heat-shocked cells and control cultures were divided into two groups, one for the preparation of labeled proteins and the second for total cytoplasmic

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CAG AAA CTT CTG CAA GAC TTC TTC AAT GGA AAA GAG CTG AAT AAG AGC ATT AAC  1143
Gln Lys Leu Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn

CCC GAT GAA GCT GTT GCC TAT GGT GCA GCT GTC CAG GCA GCC ATT CTA TCT GGA  1197
Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu Ser Gly

GAC AAG TCT GAG AAT GTT CAG GAT TTG CTG CTC TTG GAT GTC ACT CCT CTT TCC  1251
Asp Lys Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val Thr Pro Leu Ser

CTT GGG ATT GAA ACT GCT GGT GGA GTC ATG ACT GTC CTC ATC AAG CGC AAT ACC  1305
Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val Leu Ile Lys Arg Asn Thr

ACC ATT CCC ACC AAG CAG ACC CAG ACT TTC ACC ACC TAC TCT GAC AAC CAG CCA  1359
Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe Thr Thr Tyr Ser Asp Asn Gln Pro

GGT GTA CTC ATC CAG GTG TAT GAA GGT GAA AGG GCC ATG ACC AAG GAC AAC AAC  1413
Gly Val Leu Ile Gln Val Tyr Glu Gly Glu Arg Ala Met Thr Lys Asp Asn Asn

CTG CTT GGG AAG TTT GAG CTC ACA GGC ATA CCT CCA GCA CCC CGT GGG GTT CCT  1467
Leu Leu Gly Lys Phe Glu Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro

CAG ATT GAG GTT ACT TTT GAC ATT GAT GCC AAT GGC ATC CTC AAT GTT TCT GCT  1521
Gln Ile Glu Val Thr Phe Asp Ile Asp Ala Asn Gly Ile Leu Asn Val Ser Ala

GTA GAT AAG AGC ACA GGA AAG GAG AAC AAG ATC ACC ATC ACC AAT GAC AAG GGC  1575
Val Asp Lys Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly

CGC TTG AGT AAG GAG GAT ATT GAG CGC ATG GTC CAA GAA GCT GAG AAG TAC AAA  1629
Arg Leu Ser Lys Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys Tyr Lys

GCT GAG GAT GAG AAG CAG AGA GAT AAG GTT TCC TCT AAG AAC TCG CTG GAG TCT  1683
Ala Glu Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn Ser Leu Glu Ser

TAT GCT TTC AAC ATG AAA GCA ACT GTT GAG GAT GAG AAA CTT CAA GGC AAG ATC  1737
Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu Lys Leu Gln Gly Lys Ile

AAT GAT GAA GAC AAA CAG AAG ATT CTT GAC AAG TGC AAC GAA ATC ATC AGC TGG  1791
Asn Asp Glu Asp Lys Gln Lys Ile Leu Asp Lys Cys Asn Glu Ile Ile Ser Trp

CTG GAT AAG AAC CAG ACT GCG GAG AAG GAA GAA TTT GAG CAT CAG CAG AAA GAA  1845
Leu Asp Lys Asn Gln Thr Ala Glu Lys Glu Glu Phe Glu His Gln Gln Lys Glu

CTG GAG AAG GTC TGC AAC CCT ATC ATC ACC AAG CTG TAC CAG AGT GCT GGT GGC  1899
Leu Glu Lys Val Cys Asn Pro Ile Ile Thr Lys Leu Tyr Gln Ser Ala Gly Gly

ATG CCT GGA GGA ATG CCT GGT GGC TTC CCT GGT GGA GGA GCT CCT CCA TCT GGT  1953
Met Pro Gly Gly Met Pro Gly Gly Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly

GGT GCT TCT TCA GGC CCC ACC ATT GAA GAG GTC GAT TAA GTCAAAGTAGAGGGTATAG  2011
Gly Ala Ser Ser Gly Pro Thr Ile Glu Glu Val Asp

CATTGTTCCACAGGGACCCAAAACAAGTAACATGGAATAATAAAACTATTTAAATTGGCACC(poly[A])  2073
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FIG. 2. Nucleotide sequence of the insert from pRC62. Nucleotides 1 to 2091 contain an open reading frame that is translated into the amino acid sequence shown. The polyadenylation signal, AATAAA, is underlined.

RNA preparation. Several proteins appeared to be increased in the heat-treated PC12 cell lysate when compared with the labeled proteins of the nontreated control (Fig. 4A). Increases in proteins of 90,000 and 70,000 daltons (Fig. 4A) were similar to those seen by Welch et al. (35) with a similar regimen of treatment for rat fibroblasts and HeLa cells. We conclude that the proteins of PC12 cells show a typical mammalian heat shock response.

Equal amounts of RNA prepared from the remaining cells were dot blotted onto nitrocellulose and probed with radiolabeled pRC62 DNA. mRNA complementary to pRC62 was, at most, increased twofold by hyperthermia (Fig. 4). Northern blot analysis (data not shown) on RNA prepared from cells heat shocked for 1 h showed that radiolabeled pRC62 DNA hybridizes to a single 2.7-kilobase RNA (see Fig. 6) in both HeLa cells and PC12 cells, and no hybridizing mRNAs of different size were detected after heat shock treatment. The possibility remains that the 2.7-kilobase RNA is made up of more than one mRNA species, but this seems unlikely. The data support our conclusion that there is only a minimal change in the steady-state levels of a single transcript in response to heat shock.

hsp70 multigene family in the rat, mouse, and human genome. To assess the number of pRC62 hsp-related sequences present in the rat genome, a nick-translated probe derived from the coding region of pRC62 was hybridized to nitrocellulose blots of electrophoretically separated, *Eco*RI-digested, rat, mouse, and human DNAs. The resulting autoradiograms are shown in Fig. 5. It is apparent that the rat hsp70-related sequences make up a multigene family of between 20 and 25 DNA segments. The intensities of the most heavily hybridizing genomic fragments represent single copies per haploid genome as estimated by comparisons with the intensity generated from hybridization to known amounts of pRC62 DNA (data not shown). The less intense autoradiographic bands probably represent sequences with various degrees of base-pairing complementarity with the probe. Clearly, some homologous sequences are observed

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MSKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPTNTVFDA  Rat
M---..I.............Y.........................S.....EP.........R.......  Dm70a
.....................................................S.................C.......  THS70.14

KRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPKVQVEYKGETKSFYPEEVSSMVLTKMKEI-AEAYLGKTV  Rat
......KY..PKIAE.......K..S.G.K..IG......S.R.A...I...........TA......ESI  Dm70a
..........G...........E.I..ST...L...............I.....V.....-.........B  THS70.14
         .P...C.L.....Q..S.E.K...K......S...F...I                         Xen
                                                                .......QK.  THS70.7

TNAVVTVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKKVGAERNVLIFDLGGGTFDVSI  Rat
.D..I...................H.................L......NLKG.....................  Dm70a
N.........................S.................RT.............................  THS70.14
S...I...................V.................S....M..GMSR.....................  THS70.7

LTIEDGI-FEVKSTAGDTHLGGEDFDNRMVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTLSSST  Rat
...DE.SL...R................L.T.LAD.....Y...LRS.P..L......AE..........  Dm70a
....                                                                       THS70.14
.......-....A................L.S..VE...........Q....L.................S  THS70.7

QASIEIDSLYEGIDFYTSITRARFEELNADLFRGTLDPVEKALRDAKLDKSQIHDIVLVGGSTRIPKIQK  Rat
E.T....A.F..Q....KVS.......C.....N..Q......N...M..G................V.S  Dm70a
.........F.................MCS.......E......G...M..A....V...........V..  THS70.7

LLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIKRNT  Rat
......H..N..L.......................Q.GKI..V..V..A..............K.....C  Dm70a
........R............G..L.I.....................A...........              THS70.7

TIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNV  Rat
R..C...K..S..A......S................A..T.D.S.................L........  Dm70a

SAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSKNSLESYAFNMKATVEDEKL  Rat
..KEM....AKN...K.......QAE.D...N.....AD....R.QR.T.R.A...HVL.V.QA..QAPA  Dm70a

QGKINDEDKQKILDKCNEIISWLDKNQTAEKEEFEHQQKELEKVCNPIITKLYQSAGGMPGGMPGGFPGG  Rat
-..LDEA..NSD.....DT.R...S.T.......D.KLE..TRH.S..M..MH.QGA.AGA.G..ANC.Q  Dm70a

GAPPSGGASSGPTIEEVD                                                      Rat
Q.GGF..Y-..R.V....                                                      Dm70a
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FIG. 3. Comparison of pRC62 and hsp70-like proteins. The single-character amino acid code is presented for the entire rat sequence as determined from the nucleotide sequence (Fig. 2). The other proteins include the entire *D. melanogaster* protein (DM70a) (12), two related trout sequences (THS70.7 and THS70.14) (19), and a short segment of an *X. levis* protein (Xen) (5). Only the amino acids that differ from the rat protein are presented. Identical residues are indicated by a period. Deletions are indicated by a dash. The trout and *X. levis* proteins are incomplete.

across species, because both mouse and human genomes also contain multiple hybridizing fragments.

Tissue distribution. To examine the tissue distribution of heat-shock-related mRNA, total RNAs from various tissues were prepared, and equal amounts were electrophoresed on a denaturing agarose gel. Nitrocellulose blots were probed with the same nick-translated coding region probe used for the genomic blots. The resulting autoradiograms were scanned by computer, and the relative amounts of RNA were determined. Whereas 2.7-kilobase mRNAs complementary to pRC62 were present in every tissue tested, they accumulated to high levels in two related pheochromocytoma cell lines (Fig. 6). The steady-state levels of mRNA present in each tissue type relative to that in PC12 or PCG2 cells as determined by scanning of several exposures of the blot shown in Fig. 6 were: heart, 29%; liver, 9%; kidney, 26%; and adrenal medulla, 33%. In an identical experiment, various isolated brain mRNAs were probed and quantitated as described above. All brain areas examined, including the cerebellum, hypothalamus, olfactory bulb, cortex, brain stem, hippocampus, and caudate striatum, appeared to accumulate pRC62 mRNA on the order of 40 to 50% of the steady-state level observed in PC12 cells. Thus, all brain regions tested had higher levels of hsp70-like mRNA than did nonneuronal tissue. These blots were also hybridized with a probe designed to detect a major class of abundant mRNAs—a probe derived from the coding region of a human β-actin cDNA (8)—and of similar length and specific radioactivity as the pRC62-derived probe. By using this β-actin probe, we detected bands of intensity similar to those observed in brain RNAs when the pRC62 probe was used (data not shown). Because nonmuscle actin mRNAs are highly abundant, we conclude that the hsp70-like RNAs are also highly abundant in essentially all tissues examined and more abundant than actin in the proliferating tissue culture cells.

DISCUSSION

A heat shock response has been observed in species ranging from *Escherichia coli* to humans. Because this response can be elicited by many different agents, including certain drugs, amino acid analogs, and metals (31), this phenomenon probably represents a general response to metabolic disturbance or stress. It is therefore interesting that antibodies raised against purified catecholamine-synthesizing enzymes, regulators of the stress response of an animal, would immunoprecipitate at least one member of the hsp70 multigene family. Data presented in Fig. 1 show that antibodies prepared against either purified TH or DBH immunoprecipitate the heat-shock-like protein translated from stringently selected mRNA hybridized to filters carrying the heat-shock-like cDNA clone, pRC62. Not only do these polyclonal antisera immunoprecipitate this protein, but a monoclonal antibody directed against the C-terminal portion of soluble DBH (a gift of R. Hogue-Angeletti) also does. These results, together with evidence reported earlier (28), erroneously led us to identify the product encoded for by pRC62 as the 72,000-molecular-weight subunit of DBH.

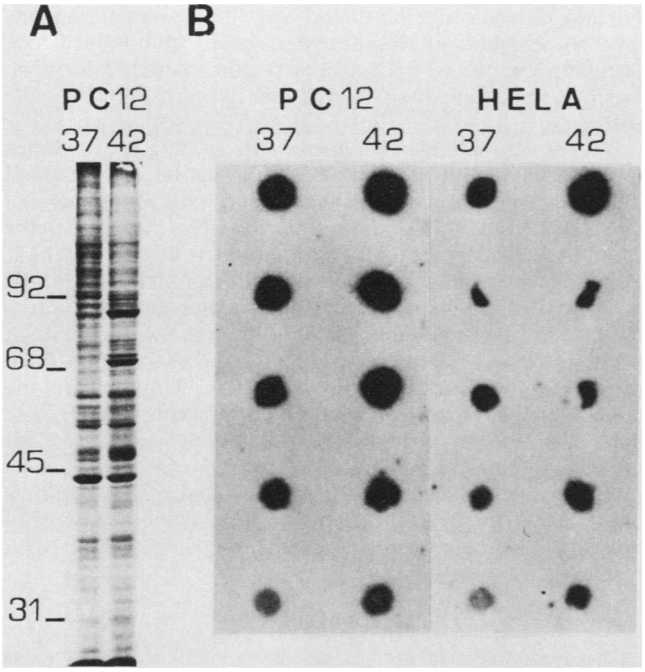

FIG. 4. Effect of hyperthermia on rat pRC62y-related mRNA. (A) [^{35}S]methionine-labeled proteins from normal (37°C) and heat-shocked (42°C) PC12 cells. Cells were heat shocked as described in the text and solubilized in electrophoresis sample buffer, and the proteins were separated on a 10% $NaDodSO_4$-acrylamide gel. Molecular weight markers ($\times 10^3$) appear to the left. (B) Analysis by dot hybridization of pRC62-related cytoplasmic mRNA. Various amounts of cytoplasmic RNA from normal or stressed cells were spotted onto nitrocellulose and hybridized to nick-translated pRC62 as described in Materials and Methods. The total amount of RNA spotted on the filter represented a doubling serial dilution starting with 2 μg.

DNA sequencing of pRC62 and subsequent comparison with published data in the National Institutes of Health Genbank data base revealed the sequence homology between the rat cDNA and genes coding for hsp70 proteins.

It is unclear at present whether the shared antigenic determinants described above extend to the nucleotide level, i.e., whether there is a region of conserved sequences between rat catecholamine enzymes and heat-shock-related sequences. pRC62 bears no significant homology with the recently reported sequence of the rat tyrosine-hydroxylase-encoding cDNA (7). Additionally, the rat hsp70-like protein and the TH protein have no major segments of protein identity; four tetrapeptides are identical, but their potential for generating cross-reacting antibodies is uncertain. Furthermore, the high levels of pRC62 mRNA present in both neuronal and nonneuronal tissues argue against this clone coding for a catecholamine-synthesizing enzyme. It may be that the pRC62 protein and DBH share only antigenic determinants.

A comparison of the predicted amino acid sequences of the rat hsp70-like clone and *Drosophila* hsp70 showed a remarkable 70% sequence conservation between these diverse species. *E. coli* DNA K gene, which was recently sequenced (3) shows 57% identity to *Drosophila* hsp70 at the nucleotide level and 48% identity at the amino acid level. This identity increased to over 90% in one 46-amino-acid segment. In this same region, residues 139 to 185, the rat cDNA maintained 85% homology with DNA K protein (data not shown) and 94% homology in the same region of *Drosophila* hsp70. This region of high amino acid sequence conservation suggests the presence of a conserved functional domain in the protein. For the limited sequence available for *X. levis* hsp70-like genes (5), the similarity with the rat sequences is 76% for both nucleotides and amino acids. It is interesting to note that Bienz found that *Drosophila* hsp70 genes did not cross-hybridize with *X. levis* DNA (5). However, this dipteran gene does cross-hybridize with mouse DNA and RNA prepared from mouse L cells (34).

Related antigenicity has been shown between hsp70-like proteins from yeasts, *Drosophila* species, chickens, mice, and humans (17). That this antigenic conservation can be extended to the nucleotide level is demonstrated by the ability of the rat cDNA clone to cross-hybridize with both mouse and human genomes (Fig. 5). Additionally, nitrocellulose blots of HeLa RNA probed with the rat coding region probe indicated that the hybridizing messages were the same size as those hybridizing in the rat PC12 cells.

A multigene family related to hsp70 has been identified in *Drosophila* species (2). Two members of this family are induced by hyperthermia (12, 14), whereas three additional genes are constitutively transcribed at normal temperatures and not induced by heat treatment (2). These non-heat-inducible genes have been called hsp cognate genes (12). A similar multigene family of about 10 genes has been de-

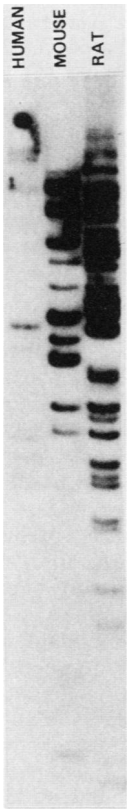

FIG. 5. Determination of the number of *Eco*RI fragments in rat, mouse, and human genomes homologous to the rat cDNA probe. Southern blots of *Eco*RI-digested DNA from three species were probed with a radiolabeled fragment of pRC62 cDNA as described in Materials and Methods. The filters were exposed to X-ray film.

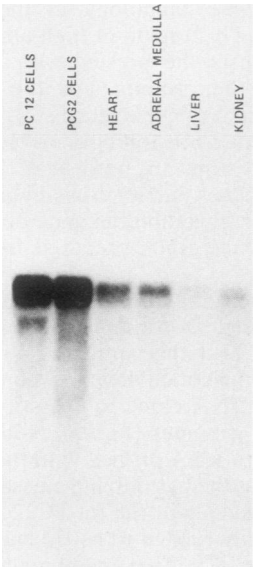

FIG. 6. Abundance of rat pRC62 mRNA in PC clonal cell lines and different rat tissues. Northern blots of cellular RNA hybridized with a radiolabeled probe derived from the coding region of pRC62. Each lane contained 20 μg of total RNA isolated from the indicated tissues. Hybridization and washing conditions were as described in Materials and Methods. The major band of hybridization represents an RNA 2.7-kilobases long based on the mobilities of marker RNAs visualized before the gel was blotted.

scribed in the yeast *Saccharomyces cerevisiae* (14). This yeast multigene family also contains both heat-inducible members and constitutively expressed genes. The results shown in Fig. 4 suggest that the rat gene corresponding to the hsp70-like clone described here is only marginally heat inducible. Furthermore, because the probe used to enumerate genomic *Eco*RI fragments (Fig. 5) contained the coding region of pRC62, it is unlikely that these fragments encode mRNAs that are significantly induced by heat, because their transcripts also would have been detected by the probe. Which of these DNA segments represent genes or pseudogenes or both remains to be seen. G. Mues, T. Z. Munn, and J. D. Raese (J. Biol. Chem., in press) have recently described a human hsp70-like pseudogene homologous with pRC62.

Traditionally, heat shock proteins have been identified by the heat-induced shift in the amounts of a few proteins from nearly undetectable to greatly overproduced. The mRNAs associated with these proteins also shift from nondetectable, or very low, to very abundant amounts (2). The tissue distribution of rat transcripts seen in Fig. 6 indicated that at normal growth temperatures the rat hsp70-related sequences are transcribed at high levels in all tissues examined. These levels are equal to or greater than the accumulation of nonmuscle actin mRNA. Hightower, White, and Currie (10, 36) have described an abundant 73-kilodalton protein (P73) present in most rat cells. They showed by peptide mapping that this protein is closely related to another protein, SP71, that is not normally synthesized by rat cells but which can be induced to high levels by hyperthermia. Possibly, the rat hsp70-like mRNA described here is related to P73. Similarly, the lack of heat inducibility and high (but variable) message levels observed in all tissues tested suggest that the rat gene encoding the pRC62 cDNA is analogous to the *Drosophila* hsp70 cognate gene (hsp70c) (12) and the yeast YG101 gene (14).

Because both these *D. melanogaster* and yeast clones were derived from genomic DNA, it has been speculated that they represent pseudogenes and not functional sequences (2, 11, 34). Conversely, because the hsp70-like clone described in this paper was derived from a poly(A)$^+$ cDNA, its gene almost certainly encodes a functional protein product. Pelham (29a) has elegantly demonstrated a potential role for heat-inducible hsp70 in the restoration of rRNA metabolism and nucleolar structure after heat shock. Such responses to environmental stress are likely to provide temporary respite until normal cellular proteins recover. The demonstration here that a constitutively expressed rat hsp70-like gene is functionally expressed suggests that its product functions routinely. If the model of Pelham is correct, then the pRC62 rat gene product may be involved in normal, non-stress-related nucleolar physiology and rRNA metabolism.

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