Chemical modifications of a recombinant bovine stress-inducible 70 kDa heat-shock protein (Hsp70) mimics Hsp70 isoforms from tissues

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A cDNA clone for the stress-inducible 70 kDa heat-shock protein (Hsp70) has been isolated from a bovine skeletal-muscle cDNA library. This mRNA encodes a protein with a calculated molecular mass of 70250 Da. The cDNA has one continuous open reading frame capable of encoding a 641-amino-acid protein. Expression of this cDNA in a bacterial expression system produced a protein with a mobility identical with that of the inducible Hsp70 protein from bovine skeletal muscle as determined by SDS/PAGE. Two-dimensional gel electrophoresis demonstrated this protein to have focusing properties identical with that of a minor isoform from bovine skeletal muscle. Upon carbamylation of this bacterially expressed protein, a train of charged proteins with charge differences of −1 were produced. These carbamylated proteins were shown to have similar focusing mobilities to the Hsp70 isoforms isolated from bovine skeletal muscle. These results demonstrate the identification of a skeletal-muscle inducible Hsp70 gene and suggest that the presence of multiple Hsp70 isoforms may be the product of post-translational modifications to the Hsp70 proteins.

INTRODUCTION

It is well established that, in response to environmental stresses, cells synthesize a group of proteins called 'heat-shock proteins' [reviewed by Gething and Sambrook (1992), Welch (1992) and Hendrick and Hartl (1993)]. This response, initially known as the 'heat-shock response', is characterized by a rapid increased expression of this select group of proteins. It is thought that the function of these induced proteins is to help the cell survive the stress by preventing unproductive protein–protein interactions or the production of aggregates that occur during stress, but the mechanism by which this occurs is unclear (Gething and Sambrook, 1992; Hartl and Martin, 1992).

Previous studies in this laboratory using a quantitative e.l.i.s.a. assay and Western blots have demonstrated elevated levels of the inducible bovine skeletal-muscle 70 kDa heat-shock proteins (Hsp70s) under unstressed conditions and shown that these proteins are further elevated by heat stress (Guerriero et al., 1989; Gutierrez and Guerriero, 1991). Using two-dimensional electrophoresis, these proteins have been shown to exist as a group of at least four isoforms with similar molecular masses. The cause of these isoelectric differences, or their relationship to the proposed functions of Hsp70, remains unknown. There are three possible ways in which these distinct isoforms might be formed. First, it is possible that the cell expresses only one Hsp70 gene and that each isoform represents a form of Hsp70 that has been uniquely post-translationally modified to cause the charge shifts. Secondly, it is possible that each isoform is the product of distinct Hsp70 genes expressed independently of each other. And thirdly, it is possible that a combination of both mechanisms occurs in bovine skeletal muscle.

Multiple genes for the inducible Hsp70 proteins have been identified in yeast (Ingolia et al., 1982), humans (Wu et al., 1985), cattle (Grosz et al., 1992), mouse (Lowe and Moran, 1984; Hunt et al., 1993) and the fruitfly *Drosophila* (Craig et al., 1979). However, studies have not been reported characterizing their expression in the cell or their relationship to the Hsp70 isoforms. It is also possible that some of these genes are part of 'silent' sequences called 'pseudogenes' (Moore et al., 1990).

Post-translational modifications, including methylation (Wang et al., 1992), phosphorylation (Leustek et al., 1992) and ADP-ribosylation (Leno and Ledford, 1990), have been reported in Hsp70 proteins. However, the relationship of these modifications to specific isoforms or to their function remains unknown. It is only for immunoglobulin-heavy-chain-binding protein (BiP) that an insight into the relationship between modifications and function is evident. It is proposed that the modifications of BiP [an endoplasmic-reticulum (ER) counterpart of Hsp70] alter its abilities to oligomerize with other proteins (Blond-Elguindi et al., 1993).

Current evidence suggests that both mutiple genes and post-translational modification are possible, and that neither of these mechanisms may be exclusive. The present paper describes the identification and characterization of an inducible Hsp70 cDNA from bovine skeletal muscle. Chemical modifications of the purified bacterially expressed protein created a pattern of isoforms similar to the pattern observed in bovine skeletal muscle. These results suggest that the distinct isoform patterns observed in bovine skeletal muscle may be the result of post-translational modifications of Hsp70 proteins.

MATERIALS AND METHODS

Reagents

Nitrocellulose (0.22 Nm and 0.45 Nm) was obtained from Schliecher and Schuell (Keene, NH, U.S.A.). Bicinchoninic acid (BCA) protein-analysis reagent was purchased form Pierce Immunochemicals (Rockford, IL, U.S.A.). Random-priming oligo-labelling kits, nested deletions kits and cDNA synthesis

Abbreviations used: Hsp70, stress-inducible 70 kDa heat-shock protein; Hsc70, constitutive 70 kDa heat-shock protein; BiP, immunoglobulin-heavy-chain-binding protein; RACE, rapid amplification of cDNA ends; BCA, bicinchoninic acid; ER, endoplasmic reticulum.

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The nucleotide sequence reported in this paper has been submitted to the GenBank Nucleotide Sequence Database under the accession number U09861.

kits were obtained from Pharmacia–P-L Biochemicals (Milwaukee, WI, U.S.A.). Sequencing-quality double-stranded plasmid DNA was obtained using a QIAGEN miniprep kit (Chatsworth, CA, U.S.A.). Poly(A) RNA isolation kits (Fast-Track RNA isolation kit) were obtained from Invitrogen (San Diego, CA, U.S.A.), and Lambda ZAP II vector kits and Gigaplus *in-vitro* Lambda Packaging kits were obtained from Stratagene (La Jolla, CA, U.S.A.). Restriction and modifying enzymes were obtained from Promega (Madison, WI, U.S.A.). ^{32}P-radiolabelled nucleotides (dCTP) and ^{35}S-labelled dATP were obtained from New England Nuclear (Boston, MA, U.S.A.). DE-52 resin was obtained from Whatman (Maidstone, Kent, U.K.). Unless otherwise specified, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Purification of Hsp70 and constitutive 70 kDa heat-shock protein (Hsc70)

Bovine (*Bos taurus*) skeletal muscle and recombinant proteins were purified by the procedure of Welch and Feramisco (1985) and as modified by Guerriero et al. (1989). Briefly, bacterial pellets were resuspended in the same volume as the induction media in a buffer consisting of 20 mM Tris/acetate, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, and 5 mM 2-mercapthoethanol (column buffer). Bovine skeletal muscle was trimmed of connective tissue and fat, cut into 1–2 cm squares, suspended in 10 vol. (w/v) of column buffer and homogenized three times for 30 s each in a Waring blender at the highest setting. Both the bacterial and skeletal-muscle suspensions were homogenized in a Polytron homogenizer at a medium setting for 1 min and centrifuged at 10000 ***g*** for 20 min at 4 °C. Hsp70 proteins were purified from the supernatants using ion-exchange chromatography (DE-52), hydrophobic chromatography (phenyl-Sepharose), and ATP-affinity chromatography (Guerriero et al., 1989).

Protein quantification, one- and two-dimensional electrophoresis and Western-blot analysis

Proteins were quantified using the BCA method with BSA as the standard (Smith et al., 1985). One-dimensional SDS/PAGE was performed as described by Laemmli (1970). Proteins for two-dimensional analysis were electrofocused using pH 3–10 (0.4%) and pH 5–7 (1.6%) ampholytes prior to analysis by SDS/PAGE (O'Farrel, 1975). The fractionated proteins were revealed by staining with Coomassie Brilliant Blue.

Library construction and screening

A random-primed lambda ZAPII library was constructed from bovine skeletal-muscle mRNA using the time-saver cDNA synthesis kit (Pharmacia–Biotech) and the lambda ZAPII vector (Stratagene). Library screening was performed by infecting *Escherichia coli* XL-1 Blue cells with recombinant phage to yield approx. 10000 plaques/plate. Filters were hybridized to ^{32}P-labelled cDNA fragments (Feinberg and Vogelstein, 1984) of the human inducible Hsp70 cDNA (pH 2.3) or Hsp70 cDNA fragments obtained from screening the library. Positive clones were isolated and excised as cDNA containing pBluescript SK(−) phagemids from lambda ZAP phage by using helper phage R408 (Stratagene).

Nested deletions, and sequencing

Sequencing of DNA fragments on both strands was accomplished by the generation of deletion mutants as described by Henikoff (1987) using a Nested Deletions kit from Pharmacia–Biotech. Double-stranded DNA was denatured and sequenced using dideoxynucleotide sequencing with the Sequenase kit (version 2.0) from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.).

Sequence analysis

The deduced nucleotide and amino acid sequences were analysed with the Genetics Computer Group software. Nucleic acid comparisons with other Hsp70 genes were performed with the program BLASTN to the GenBank and EMBL databases. Nucleic acid similarities were identified by FastA comparisons with specific sequences, and amino acid similarities were identified by TFastA comparisons by the method of Pearson and Lipman (1988).

3′ Rapid amplification of cDNA ends (RACE)

RACE (Frohman, 1993) was used to generate the 3′ end of a cDNA clone obtained from the bovine skeletal-muscle cDNA library. A primer containing a poly(T) tail was first used to make cDNA from poly(A) RNA (5′-ATCGATAAGCTTGGATCC-TTTTTTTTTTTTTTTT-3′).The adaptor primer (5′-ATCG-ATAAGCTTGGATCC-3′) and the gene-specific primer for Hsp70 (5′-TGCTAAGAACGCGCTGG-3′) were then used to amplify the 3′ end by PCR. Amplification reactions (1 μl) were subcloned into the TA vector (Invitrogen, San Diego, CA, U.S.A.) for sequence analysis and subcloning.

Site-directed mutagenesis

Site-directed mutagenesis was performed by the PCR as described by Reikofski and Tao (1992) using the mutagenic primer 5′-AAGCTTCTGCAGGAATTCCAGGGCACCG*CCATGG*CG-AAAAAC-3′ to introduce a *Nco*I restriction site and a downstream primer over the *Bst*XI restriction site (5′-GTGAAG-GCCACGTAGCTG-3′). The amplified product was subcloned into the TA cloning vector for sequencing and subcloning. *Hin*dIII- and *Bst*XI-digested PCR products were subcloned into pBS(−) containing the 1200-bp Hsp70 *Pst*I fragment. The mutagenized 1200-bp *Pst*I fragment was then re-cloned back into the full-length Hsp70 clone, and the entire coding sequence for Hsp70 (*Nco*I to *Bam*HI) was cloned into the pET-3d (pET.Hsp70) vector for bacterial expression.

Expression of recombinant Hsp70 and protein carbamylation

Expression of the recombinant Hsp70 gene was performed using the pET system as described by Studier et al. (1990). Hsp70 protein was purified as described above. To determine progressive charge shifts of −1 on Hsp70 molecules, recombinant bovine Hsp70 was carbamylated as described by Dunbar (1987). Briefly, recombinant bovine skeletal-muscle Hsp70 was adjusted to 0.3 mg/ml in 0.1 M borate buffer, pH 8.5. This protein solution was made to 9 M urea and heated at 100 °C. Timed aliquots of 50 μl at 0, 1, 2, 3, 4, and 5 min were removed and electrofocused as described above.

RESULTS

Cloning of the bovine Hsp70 cDNA

A probe was derived from a human Hsp70 cDNA (Hunt and Morimoto, 1985) that consisted of the *Pst*I to *Hin*dIII fragment of the clone pH 2.3. This encompasses approx. 450 bases of the 3′ coding region and 450 bases of the 3′ non-coding domain. This

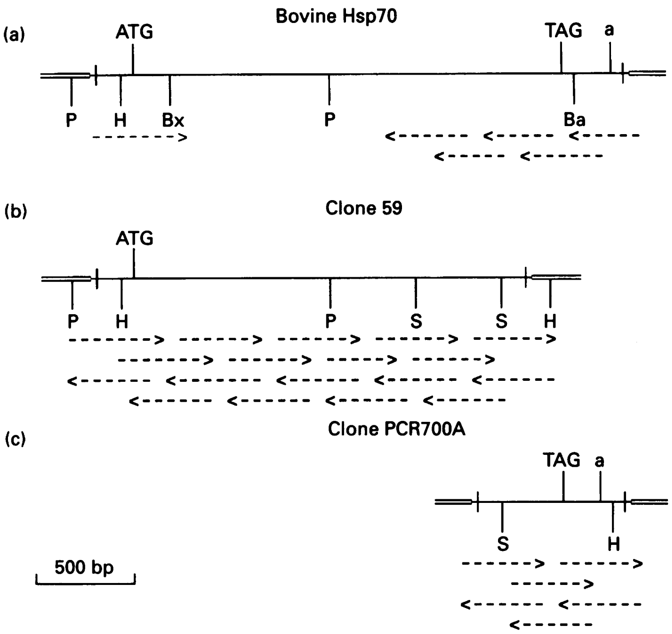

Figure 1 Physical map for the cloning and sequencing strategies of the bovine Hsp70 clone

The bar diagram (**a**) indicates the final open reading frame encoding the 641-amino-acid protein. Bar diagram (**b**) shows the cDNA clone, 59, derived from the lambda ZAP library and the restriction sites used for subcloning. Bar diagram (**c**) denotes the 3′ RACE derived clone, PCR700A, with the termination sequences and subcloning restriction sites used for assembly of the complete Hsp70 clone. ATG and TAG show the sites for initiation and termination of translation, and 'a' denotes the site of the polyadenylation signal. Double lines denote vector sequences. Broken arrows under each line diagram show the sequencing strategy for each clone, and capital letters denote sites of specific endonucleases: P, *Pst*I; Bx, *Bst*XI; Ba, *Bam*HI; S, *Sun*I; H, *Hin*dIII.

region was chosen as a probe because of convenient restriction sites, and it contained the greatest nucleotide divergence from the constitutive Hsc70. Initial screening of 2×10^5 plaques yielded five clones. Partial sequencing of each of these clones from both ends identified them as Hsp70 clones spanning the 5′ end of the bovine Hsp70 molecule. These clones were compared with the porcine Hsp70 sequence (Peelman et al., 1992). The largest contiguous clone extended approx. 150 bp 5′ of the initiation codon (ATG), but continued only 1300 bp into the coding domain. Further screening of the library with this clone yielded 17 additional clones, but none contained the 3′ coding region of the bovine Hsp70. Further screening of the library with a probe composed of 400 bp from the 3′ end of the partial clone resulted in 27 additional Hsp70 clones. None encoded the 3′ termination region of the bovine Hsp70. In summary, approx. 1.1×10^6 recombinant phage were screened and a total of 49 Hsp70 clones were isolated and partially sequenced. These clones contained regions that went from 300 bp of the 5′ untranslated region to within 70 bp of the termination codon. Clone 59, which contained the longest coding region of 1800 bp, was used as a base clone to subclone a product generated by 3′ RACE. Furthermore, this clone was completely sequenced in both strands by creating nested deletion mutants (Figure 1).

To isolate the missing portion of the Hsp70 from clone 59, a Hsp70-specific primer was designed from information obtained by partial sequencing, and used in a 3′ RACE cloning procedure (Frohman, 1993). The 700-bp 3′ RACE product was subcloned into the TA cloning vector for further subcloning and sequencing.

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                                                                      24
   1 AAAACTTGCGGCTTAGTCCGTGAGAACAGCTTCCGCAGACCCGCTATCTCCAAGGACCGC 60
  61 CCCGAGGGCACCAGAGCTTCACGATGTTGATCCTGTGGGCCGTTTTCAGGTTTGAAGCTT 120
 121 ATCTCGGAGCCGAAAAGGCAGGGCACCGGCATGGCGAAAAACATGGCTATCGGCATCGAC 180
                                   M  A  K  N  M  A  I  G  I  D
 181 CTGGGCACCACCTACTCCTGCGTAGGGGTGTTCCAGCACGGCAAGGTGGAGATCATCGCC 240
     L  G  T  T  Y  S  C  V  G  V  F  Q  H  G  K  V  E  I  I  A
 241 AACGACCAGGGCAACCGCACCACCCCCAGCTACGTGGCCTTCACCGATACCGAGCGGCTC 300
     N  D  Q  G  N  R  T  T  P  S  Y  V  A  F  T  D  T  E  R  L
 301 ATCGGCGATGCGGCCAAGAACCAGGTGGCGCTGAACCCGCAGAACACGGTGTTCGACGCG 360
     I  G  D  A  A  K  N  Q  V  A  L  N  P  Q  N  T  V  F  D  A
 361 AAGCGGCTGATCGGCCGCAAGTTCGGAGACCCGGTGGTGCAGTCGGACATGAAGGAGTGG 420
     K  R  L  I  G  R  K  F  G  D  P  V  V  Q  S  D  M  K  E  W
 421 CCTTTCCGCGTCATCAACGACGGAGACAAGCCTAAGGTGCAGGTGAGCTACAAAGGGGAG 480
     P  F  R  V  I  N  D  G  D  K  P  K  V  Q  V  S  Y  K  G  E
 481 ACCAAGGCGTTCTACCCGGAGGAGATCTCGTCGATGGTGCTGACCAAGATGAAGGAGATC 540
     T  K  A  F  Y  P  E  E  I  S  S  M  V  L  T  K  M  K  E  I
 541 GCCGAGGCGTACCTGGGCCACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTC 600
     A  E  A  Y  L  G  H  P  V  T  N  A  V  I  T  V  P  A  Y  F
 601 AACGACTCGCAGCGGCAGGCCACCAAGGACGCGGGGGTGATCGCGGGGCTGAACGTGCTG 660
     N  D  S  Q  R  Q  A  T  K  D  A  G  V  I  A  G  L  N  V  L
 661 AGGATCATCAACGAGCCCACGGCCGCCGCCATCGCCTACGGCCTGGACAGGACGGGCAAG 720
     R  I  I  N  E  P  T  A  A  A  I  A  Y  G  L  D  R  T  G  K
 721 GGGGAGCGCAACGTGCTCATCTTTGATCTGGGAGGGGGCACGTTCGACGTGTCCATCCTG 780
     G  E  R  N  V  L  I  F  D  L  G  G  G  T  F  D  V  S  I  L
 781 ACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCCGGGGACACGCACCTGGGCGGG 840
     T  I  D  D  G  I  F  E  V  K  A  T  A  G  D  T  H  L  G  G
 841 GAGGACTTCGACAACAGGCTGGTGAACCACTTCGTGGAGGAGTTCAAGAGGAAGCACAAG 900
     E  D  F  D  N  R  L  V  N  H  F  V  E  E  F  K  R  K  H  K
 901 AAGGACATCAGCCAGAACAAGCGGGCCGTGAGGCGGCTGCGCACCGCATGCGAGCGGGCC 960
     K  D  I  S  Q  N  K  R  A  V  R  R  L  R  T  A  C  E  R  A
 961 AAGAGAACCTTGTCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTCGAGGGC 1020
     K  R  T  L  S  S  S  T  Q  A  S  L  E  I  D  S  L  F  E  G
1021 ATCGACTTCTACACGTCCATCACCAGGGCGCGGTTCGAGGAGCTGTGCTCCGACCTGTTC 1080
     I  D  F  Y  T  S  I  T  R  A  R  F  E  E  L  C  S  D  L  F
1081 CGGAGCACCCTAGAGCCCGTGGAGAAGGCGCTACGCGACGCCAAGCTGGACAAGGCGCAG 1140
     R  S  T  L  E  P  V  E  K  A  L  R  D  A  K  L  D  K  A  Q
1141 ATCCACGACCTGGTCCTGGTGGGGGGCTCCACCCGCATCCCCAAGGTGCAGAAGCTGCTG 1200
     I  H  D  L  V  L  V  G  G  S  T  R  I  P  K  V  Q  K  L  L
1201 CAGGACTTCTTCAACGGGCGCGACCTCAACAAGAGCATCAACCCCGACGAGGCGGTGGCG 1260
     Q  D  F  F  N  G  R  D  L  N  K  S  I  N  P  D  E  A  V  A
                                                                      25
1261 TACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGACAAGTCGGAGAACGTGCAGGAC 1320
     Y  G  A  A  V  Q  A  A  I  L  M  G  D  K  S  E  N  V  Q  D
1321 CTGCTGTTGCTGGACGTGGCTCCCCTGTCGCTGGGACTGGAGACGGCCGGAGGCGTGATG 1380
     L  L  L  L  D  V  A  P  L  S  L  G  L  E  T  A  G  G  V  M
1381 ACCGCCCTGATCAAGCGCAACTCCACCATCCCCACGAAGCAGACGCAGATCTTCACCACC 1440
     T  A  L  I  K  R  N  S  T  I  P  T  K  Q  T  Q  I  F  T  T
1441 TACTCGGACAACCAGCCGGGCGTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACG 1500
     Y  S  D  N  Q  P  G  V  L  I  Q  V  Y  E  G  E  R  A  M  T
1501 CGGGACAACAACCTGCTGGGGCGCTTCGAGCTGAGCGGCATCCCGCCGGCCCCGCGGGGG 1560
     R  D  N  N  L  L  G  R  F  E  L  S  G  I  P  P  A  P  R  G
1561 GTGCCCCAGATCGAGGTGACCTTCGACATCGACGCCAATGGCATCCTGAACGTCACGGCC 1620
     V  P  Q  I  E  V  T  F  D  I  D  A  N  G  I  L  N  V  T  A
1621 ACGGACAAGAGCACGGGCAAGGCCAACAAGATCACCATCACCAACGACAAGGGCCGGCTG 1680
     T  D  K  S  T  G  K  A  N  K  I  T  I  T  N  D  K  G  R  L
1681 AGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCGGAAAAGTACAAGGCGGAGGACGAG 1740
     S  K  E  E  I  E  R  M  V  Q  E  A  E  K  Y  K  A  E  D  E
1741 GTGCAGCGCGAGAGGGTGTCTGCTAAGAACGCGCTGGAGTCGTACGCCTTCAACATGAAG 1800
     V  Q  R  E  R  V  S  A  K  N  A  L  E  S  Y  A  F  N  M  K
1801 AGCGCCGTGGAGGATGAGGGGCTGAAGGGCAAGATCAGCGAGGCGGACAAGAAGAAGGTG 1860
     S  A  V  E  D  E  G  L  K  G  K  I  S  E  A  D  K  K  K  V
1861 CTGGACAAGTGCCAGGAGGTGATTTCCTGGCTGGACGCCAACACCTTGGCGGAGAAGGAC 1920
     L  D  K  C  Q  E  V  I  S  W  L  D  A  N  T  L  A  E  K  D
                 <-----------| Clone 59
1921 GAGTTTGAGCACAAGAGGAAGGAGCTGGAGCAGGTGTGTAACCCCATCATCAGCAGACTG 1980
     E  F  E  H  K  R  K  E  L  E  Q  V  C  N  P  I  I  S  R  L
1981 TACCAGGGGGCGGGCGGCCCCGGGGCTGGCGGCTTTGGGGCTCAGGGCCCTAAAGGGGGC 2040
     Y  Q  G  A  G  G  P  G  A  G  G  F  G  A  Q  G  P  K  G  G
2041 TCTGGGTCTGGCCCCACCATTGAGGAGGTGGATTAGGAATCCTTCCCTGGATTGCTCATG 2100
     S  G  S  G  P  T  I  E  E  V  D
2101 TTTGTTATGGAGACTGTTGGGATCCAAGGCTTTGCATTGCCTTATATATCTGCCTTTCAT 2160

2161 CAGCCATCAGCTATGCAAGCTGTTTGAGATGTTGAACTGTCCCTTTTATGAAATTAGGAA 2220
                                   *
2221 CTCTTTTTTCCAGAGTCTTAAGTATAGAGCTGAATGTATAGTGCCATCTTTTGTCAGTTT 2280

2281 CTTTTTGTAGTATTCATGCCAAACTCAAGCTATTTTTCACCCGTTTCTGTTTACTTCCAA 2340
       *-----
2341 GTAAATAAACTCAAATAATTCGAAAAAAAAAAAAAAAAAAA 2381
```

Figure 2 Nucleotide sequence and predicted amino acid sequence of the bovine Hsp70

The top line shows the numbered nucleotide sequence and the bottom line shows the predicted amino acid sequence. Underlined amino acids represent the highly conserved Hsp70 motifs, and the underlined nucleotides between positions 1761 to 2381 represent the region corresponding to the 3′ RACE product cloned. Nucleotide sequences overlined and bearing an asterisk (*) represent polyadenylation signal. Nucleotide 2250 (*) represents the only nucleotide observed to be different in one of the three clones isolated by 3′ RACE.

Table 1 Amino acid similarities between bovine Hsp70 cDNA and other Hsp70 and Hsc70 sequences

Comparisons of amino acid sequences were performed with the FastA program using the algorithm of Pearson and Lipman (1988) of the Genetics Computer Group ('GCG'). Sequences were identified with the program BLASTN and recovered with the GenBank and EMBL data bases with the program FETCH.

Protein	Amino-acid-sequence similarly to bovine Hsp70
Bovine Hsp70-II	99
Human Hsp70	98
Pig Hsp70	97
Mouse Hsp70	95
Drosophila Hsp70	81
Bovine Hsp70-I	79
Mouse Hsc70	85
Bovine Hsc70	84
Rat Hsc70	84
Yeast Hsc70	74
Rat BiP (ER Hsp70)	64
Bacterial Hsp70 (DnaK)	48

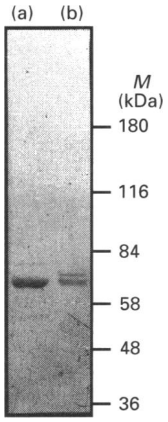

Figure 3 Comparison of recombinant purified Hsp70 with bovine skeletal-muscle Hsp70

Recombinant Hsp70 (**a**) and Hsp70 purified from bovine skeletal muscle (**b**) were fractionated in 7.5%-(w/v)-polyacrylamide gels containing SDS and stained with Coomassie Brilliant Blue. Recombinant Hsp70 shows a slight contaminant band of approx. 75 kDa, while Hsp70 proteins purified from skeletal muscle show the inducible Hsp70 (72 kDa) and the constitutive Hsc70 (73 kDa). Values on the right margin are the molecular masses (*M*) of marker proteins.

Three 3′ RACE product clones were completely sequenced on both strands to reveal that two of the three clones analysed were 100% identical. The third clone differed by one base. The two clones encoded a C at position 2250 of the final clone assembled and the third clone contained a T at this position. It is believed that this is a misincorporation by the polymerase during the amplification reaction, and the location of this base change is approx. 180 bp 3′ from the translation termination codon (Figure 2). One of the two identical clones was subcloned into the clone 59.

Sequence analysis of clone 59 revealed an open reading frame for a protein highly similar to the porcine Hsp70. Sequence comparisons between the primer site (bp 1765) and the termination of clone 59 (1950) (the region of overlap) revealed this region to be identical. Alignment of the two sequences using the program SEQED from the GCG program revealed that the initial reading frame continued and that the protein encoded was highly similar to other Hsp70s cloned (Table 1).

The sequence of the assembled bovine Hsp70 cDNA is shown with the predicted amino acid sequence (Figure 2). The nucleotide sequence contains the initiation codon at nucleotide 151 and the termination codon at nucleotide 2076. This sequence encodes for a 641-amino-acid protein with a polyadenylation signal at nucleotide 2344. Amino acid sequences shown to be highly conserved in the Hsp70 family of proteins are located at amino acids 9–16 and 131–139 and are underlined. Computer-simulated translation of the same reading frame as ATG 151 upstream from this ATG demonstrated an in-frame stop signal, suggesting that there is no alternate initiation methionine prior to it. The site of clone 59 termination is shown at nucleotide 1945.

Comparison of the predicted amino acid sequence of the assembled Hsp70 gene with sequences of members of the Hsp70 family of proteins revealed that the product is more similar to the inducible Hsp70 proteins than to the Hsc70 (Table 1). The data from Table 1 also shows the presence of a bovine Hsp70 sequence that is 99% similar to the sequence presented here. Amino acid comparisons using the program FASTA demonstrated that these two proteins only differ in two amino acids. The bovine skeletal-muscle Hsp70 encodes for methionine at amino acid 4 and glutamic acid at amino acid 89, while the bovine Hsp70-II encodes threonine and histidine amino acids at these respective positions. Nucleotide comparisons of the coding sequences showed these to be almost identical, but, in contrast, the 5′ and 3′ non-coding regions showed very little nucleotide identity. These results suggest that these are the products of two distinct genes.

Bacterial expression of bovine Hsp70

Expression of the bovine Hsp70 was achieved by subcloning the *Nco*I–*Bam*HI fragment (149–2110) into the pET.8C vector to create the pET.Hsp70 and introducing it into BL21 pLysS bacterial cells. Recombinant Hsp70 was purified from induced bacterial cells and, starting with 1 litre of induced bacteria, 4.0 mg of purified protein was obtained. The purity of the recombinant protein was verified by SDS/PAGE (Figure 3). Results of SDS/PAGE demonstrate the recombinant protein to be highly pure, with a small amount of a contaminating protein at approx. 75 kDa (Figure 3a). When the recombinant Hsp70 was compared with the bovine skeletal-muscle Hsc70 and Hsp70 (Figure 3b), it was observed that it migrated with the inducible Hsp70. These results demonstrated that the cDNA cloned encoded a protein with a mobility on SDS/PAGE identical with that of the bovine inducible Hsp70.

Two-dimensional gel electrophoresis of recombinant Hsp70

An additional experiment was performed to identify which isoform was encoded by the recombinant Hsp70. Two-dimensional gel electrophoresis of bovine skeletal-muscle Hsp70 revealed the previously described isoform pattern (Figure 4a). When recombinant Hsp70 was similarly fractionated, a major isoform was observed with a measured pI of 6.1 (Figure 4b). The precise identity of the recombinant Hsp70 was observed when purified bovine Hsp70 and recombinant Hsp70 were electrofocused simultaneously (Figure 4c). These results clearly demonstrated that the recombinant Hsp70 co-migrated with the in-

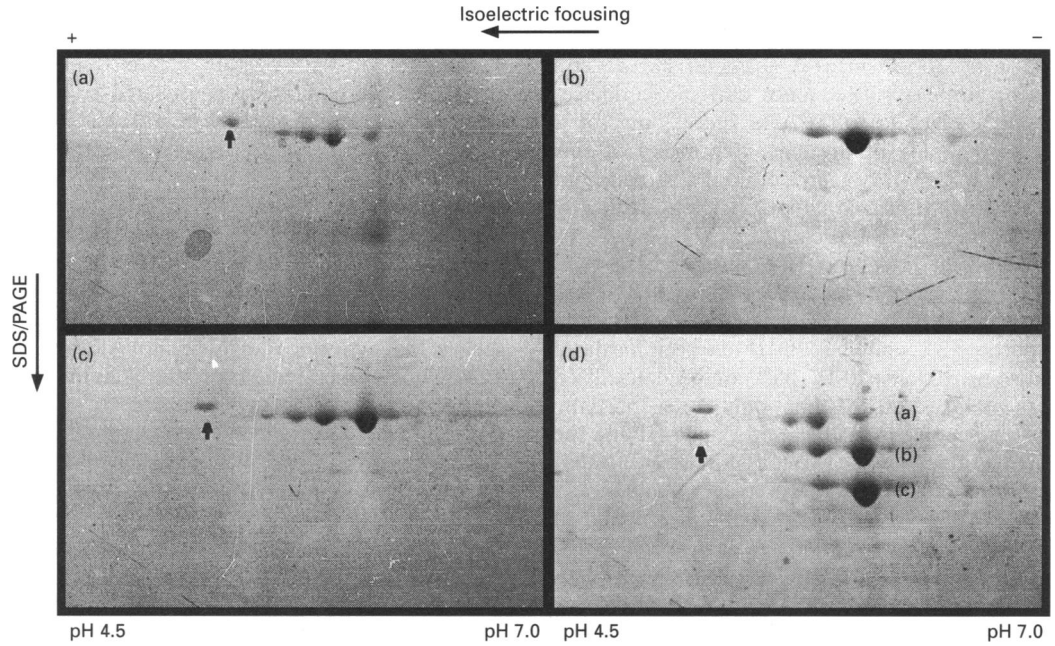

Figure 4 Comparison of recombinant Hsp70 and Hsp70 purified from bovine skeletal muscle

Bovine skeletal-muscle Hsp70 (**a**), recombinant Hsp70 (**b**), and bovine skeletal-muscle Hsp70 spiked with recombinant Hsp70 (**c**) proteins were subjected to two-dimensional gel electrophoresis and stained with Coomassie Brilliant Blue. Gels (**a**), (**c**) and (**b**) were overlapped and photographed (**d**) to demonstrate the migrational relationship of the isoforms. The location of the constitutive Hsc70 is marked by an upward-pointing arrow.

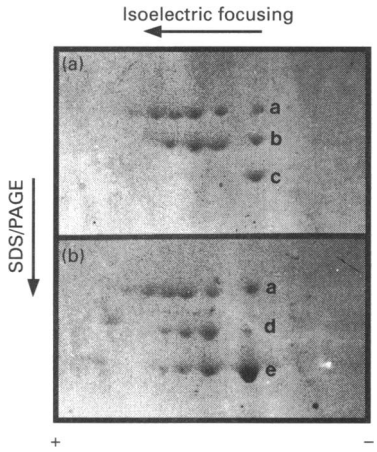

Figure 5 Comparison of carbamylated recombinant Hsp70 and Hsp70 from bovine skeletal muscle

(**a**) Shows the generation of −1 charge-shift forms of recombinant Hsp70 protein after 0 (band c), 2 (band b) and 4 (band a) min of carbamylation. (**b**) Shows the relationship of 4 min-carbamylated recombinant Hsp70 (band a) to bovine skeletal-muscle Hsp70 isoforms (band d) and bovine skeletal-muscle Hsp70 in the presence of recombinant Hsp70 (band e). Gels were overlayed and isoforms were aligned for photography.

ducible bovine skeletal-muscle Hsp70 of pI 6.1, a minor isoform of the bovine Hsp70. Figure 4(d) was created by overlaying gels for Figures 4(a), 4(b) and 4(c), and it clearly shows the relationship of the recombinant Hsp70 to the bovine skeletal-muscle Hsp70 isoforms. The presence of the constitutive Hsc70 was observed (upward-pointing arrows).

Recombinant Hsp70 carbamylation

A time course was performed with recombinant Hsp70 exposed to 9 M urea and boiling followed by two-dimensional electrophoresis to observe the charge shifts, and the protein isoform patterns were aligned by overlaying gels. Recombinant Hsp70 carbamylation created an isoform train with approximate charge shifts of −1. At zero time (Figure 5a, band a), the pattern and location (pI 6.1) previously observed for the recombinant Hsp70 was observed. After 2 min (Figure 5a, band b), three chemically modified protein forms appear. By 4 min, at least five chemically modified forms appear (Figure 5a, band a). By 5 min of carbamylation, the protein isoforms have migrated to the acidic end of the focused gel (results not shown). Isoform alignment of 4 min-carbamylated recombinant Hsp70 (Figure 5b, band a) with the bovine skeletal-muscle Hsp70 isoforms (Figure 5b, band d) and the combination of bovine skeletal-muscle Hsp70 plus recombinant Hsp70 (Figure 5b, band e) by gel overlaying, demonstrated that the −1 charge shifts generated by the carbamylation of recombinant Hsp70 co-migrated with the isoform pattern observed in bovine skeletal-muscle Hsp70 (Figure 5b). These results suggest that the Hsp70 isoform pattern observed in tissues may be the result of post-translational modifications of these proteins.

DISCUSSION

It was previously reported by our laboratory that a heat-inducible protein with a mobility and an antibody cross-reactivity identical with that of Hsp70 existed in bovine skeletal muscle at elevated levels (Guerriero et al., 1989; Gutierrez and Guerriero, 1991). Partial sequencing of the 49 clones identified showed these to be identical in the regions sequenced (results not shown), suggesting

the possibility that, in the bovine skeletal muscle, one major form of Hsp70 mRNA may be expressed. In the present paper the complete cDNA for an Hsp70 mRNA from bovine skeletal muscle is described. Amino-acid-sequence and nucleotide-sequence analysis clearly place this cDNA as one coding for a member of the Hsp70 family of proteins. Expression of this cDNA in a bacterial expression system produced a protein that migrates in one- and two-dimensional gel systems identically with bovine Hsp70 from skeletal muscle.

In the bovine species at least four Hsp70 genes have been identified from a genomic sperm library (Grosz et al., 1992). Two genes, Hsp70-I and Hsp70-II, have been shown to be tandemly localized in chromosome 23, while Hsp70-III has been localized to bovine chromosome 10. Hsp70-IV has not been localized (Gallagher et al., 1993). Hsp70-I has only been partially sequenced. Amino acid comparisons between Hsp70-III and the bovine cDNA obtained from bovine skeletal muscle reveals that the two proteins are only 82% similar (Table 1). Amino acid comparison with Hsp70-II reveals a protein that is 99% identical at the amino acid level, but nucleotide comparisons between the 5′ and 3′ untranslated domains for Hsp70-II and the skeletal-muscle cDNA reveals that these are encoded by different genes. These results suggest that, if the gene for Hsp70-II is functional, very similar Hsp70 proteins may be encoded by different genes in the bovine species.

The mere presence of genomic sequences identifying a particular gene does not signify that this sequence is transcriptionally functional. In studies attempting to identify mouse Hsp84, it was found that five out of six distinct Hsp84 genes, identified from a genomic library, were not functional, and they suggested that these pseudogenes arose by some erratic replication process (Moore et al., 1990).

Several researchers have reported the presence of multiple isoforms for the constitutive Hsc70 in mouse L-cells (Lowe and Moran, 1984), mouse spermatogenic cells (Allen et al., 1988) and the inducible Hsp70 in HeLa cells (Welch and Feramisco, 1985), in rat bladder and adrenal tissue (Currie and White, 1983) and in bovine skeletal muscle (Gutierrez and Guerriero 1991), yet the nature of these post-translational modifications is unclear. Carbamylation of recombinant Hsp70 created a set of proteins with progressive charge shifts of −1 which migrate in a pattern very similar to that shown by Hsp70 isolated from bovine skeletal muscle. These results indirectly show that a series of post-translational modifications of the Hsp70 proteins cause the Hsp70 isoforms.

There are several types of modifications that may cause progressive charge shifts of Hsp70 proteins in the cell. Carboxylation of glutamic acid residues, hydroxylation of lysine residues, phosphorylation, methylation and ADP-ribosylation are some of the potential post-translational modifications. Carboxylation or hydroxylation of glutamic acid or lysine residues are unlikely post-translational modifications, since the enzymes responsible are specifically located in the lumen of microsomes (Kivirikko and Myllyla, 1980; Suttie, 1980) or extracellularly. Phosphorylation has been speculated to occur to cytosolic mammalian Hsp70s, since it has been shown that DnaK, *Drosophila* and mitochondrial Hsp70s are phosphorylated *in vivo* (Leustek et al., 1992). This created an impetus to look for phosphorylated cytosolic mammalian Hsp70s; however, it is proposed that Hsp70s are not normally phosphorylated *in vivo* in vertebrate cells (Lindquist and Craig, 1988). Phosphorylation of Hsp70s would create an isoform train very similar to the charge-shift pattern observed during recombinant Hsp70 carbamylation.

Wang et al. (1992) demonstrated in Balb/c 3T3 cells that Hsc70, Hsp70, and BiP were methylated on lysine and arginine residues; however, it is not believed that methylation of lysine and arginine residues causes the isoform train observed. Multiple methylation of lysine residues creates lysine residues with quaternary amines, and these methylated residues would have an alkaline effect on the pI of the Hsp70 proteins. The modifications of Hsp70 molecules are acidic in their measured pI.

ADP-ribosylation of BiP molecules has been clearly demonstrated to be reversible and functionally correlated to the binding of maturing proteins of the ER (Leno and Ledford, 1990). ADP-ribosylation occurs in arginine residues, and this modification creates a more acidic isoform as analysed by two-dimensional electrophoresis. However, there are two pieces of evidence to suggest that cytosolic Hsp70s are not ADP-ribosylated. First, the ADP-ribosylase is located specifically in the ER, and it produces only one isoform of BiP with a charge of −2 against at least four isoforms observed in bovine skeletal muscle with charge shifts of −1. Secondly, the ADP-ribosylation of BiP appears to be a very specific event, since resolution of cellular proteins by two-dimensional electrophoresis has shown that BiP is the only protein modified (Leno and Ledford, 1990).

The mechanism by which a single Hsp70 protein can produce multiple isoforms is unclear and subject to potential speculation. It is suspected that if indeed the accumulation of a single type of modification in Hsp70 proteins is responsible for the isoform pattern observed in bovine skeletal muscle, then it is possible that this train may represent a maturation train where the most basic Hsp70 proteins being less modified are 'younger', while those that are more acidic are 'older' and perhaps being targeted for degradation. Alternatively it is possible that each cumulative modification group may represent a pool of Hsp70 molecules with distinct biochemical properties (i.e. rate of ATP hydrolysis, rate of substrate turnover or substrate specificity). For either of these ideas, the critical question arises as to the exact form of Hsp70 isoform(s) that interact(s) with its protein target and the sequence of events that accompanies the ATP-dependent release of the target protein. Specifically, do more modified isoforms perform their potential activities more or less efficiently or more selectively? The identification of these specific modifications must be recognized, and the functionality of each of these modifications may be the aim of Hsp70 structure–function studies.

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