
Localization of the hsp83 transcript within a 3292 nucleotide sequence from the 63B heat shock locus of *D. melanogaster*

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ABSTRACT

We have determined the complete nucleotide sequence of a 3292 bp cloned segment derived from the 63B heat shock cytogenetic locus of *D. melanogaster*. Within this segment we have positioned the start of transcription and RNA splice sites of the unique gene that encodes the 83,000 d heat shock polypeptide (hsp83 gene) by S1 mapping and synthesis of cDNA from restriction fragment primed mRNA. The sequence begins at a point 879 bp upstream from the transcription start and includes the 149 bp nontranslated first exon, the 1139 bp intron and extends 1125 bp into the protein coding region. These data identify a single open translation reading frame for the first 375 amino acids of the 83,000 d polypeptide, beginning with the first ATG codon located at the 3' intron-exon junction. We discuss and demonstrate the use of *E. coli* exonuclease III generated single-strand DNA probes as an alternative to strand separation for S1 mapping of mRNA. We also use homology search criteria based upon known protein-DNA binding sites to compare our hsp83 sequence with other sequenced *Drosophila* heat shock genes. These comparisons indicate that a large region of approximately 80 bp centered around the transcription initiation point of the hsp83 gene shares only a 31% homology with the corresponding region of the hsp70 gene, whereas the hsp22, 23, 26, and 27 genes share a 54% homology with hsp70 in this region. The lower homology of the hsp83 gene is consistent with the deviant nature of this heat shock gene.

INTRODUCTION

Exposure of *Drosophila* cells to temperatures several degrees above the normal growth temperature of 23°C induces a rapid and dramatic change in gene expression. Most genes active at 23°C are repressed while a specific set of heat shock genes is activated both at the transcriptional and translational levels (1). One of these induced genes, which encodes the 83,000 d heat shock polypeptide (hsp83), is a single copy gene located at the 63B cytogenetic locus of *D. melanogaster* (2). Several lines of evidence suggest that the expression of the hsp83 gene differs significantly from the other major heat shock genes of *D. melanogaster*. First, in several continuous cell culture lines the 83,000 d polypeptide (3-5) and RNA (6) are abundant at normal growth temperatures. Second, maximum induction of hsp83 protein synthesis occurs at 33°C, a temperature below optimal for synthesis of other abundant heat shock

proteins (4). Third, in cultured cells grown at 25°C this message is found primarily in poly(A)⁻ fractions, yet after induction, is found distributed between poly(A)⁺ and poly(A)⁻ fractions (3,6). Fourth, larval salivary glands incubated at 25°C produce hsp83 mRNAs that can be isolated and translated in vitro (5), yet that are inefficiently translated in these cells prior to induction (7). Fifth, the 63B region is induced to puff in isolated salivary gland nuclei in response to uninduced cellular extracts that do not cause puffing at other heat shock loci (8). Sixth, hsp83 is a common protein in early embryos kept at normal growth temperatures, and is also the only heat shock protein detectable in heat-induced unfertilized eggs (9). Furthermore, of the seven major heat shock genes only the mRNAs encoding hsp83, hsp27, and hsp26 are present during normal development in non-heat shocked adult ovaries and in preblastoderm embryos. These appear to be maternally derived RNAs synthesized in ovarian nurse cells and transported into oocytes (10). Seventh, hsp83 mRNA is found in uninduced immature ovaries and early egg chambers, whereas other heat shock mRNAs are not detected (10). Finally, the hsp83 gene is the only heat shock gene known to contain an intervening sequence (2). Together, these observations indicate that expression of the hsp83 gene involves regulatory events at the levels of transcription, processing, and translation that are not shared by other heat shock genes.

The basis for the differential expression of the hsp83 gene must ultimately reside in differences in regulatory nucleotide sequences. Therefore, our initial goal was to determine the nucleotide sequence of an interval sufficient to cover potential regulatory regions. Such regions should include the start of transcription and 5' flanking sequences that encompass known sites of DNase I hypersensitivity in chromatin (11) and extend through the start of translation into the protein coding sequence. Identification of these starts requires precise identification of the initiation and splice sites of the hsp83 transcript. Although 578 bp of interrupted DNA sequence from the 5' end of the hsp83 gene and at the splice junctions have been determined (12), the reported sequence omits regions containing the upstream DNaseI hypersensitive sites (11), the hsp83 intron, and fails to establish a translation reading frame for the 83,000 d protein. We present a more extensive, uninterrupted sequence and demonstrate a 375 codon open reading frame. Furthermore, using a rapid exonuclease III/nuclease S1 mRNA mapping procedure, we present high resolution data confirming the previously assigned initiation and 3' splice nucleotides of the hsp83 mRNA. The 5' splice junction is positioned by determining the exact length of cDNA

synthesized from restriction fragment primed heat shock mRNA. We compare sequences flanking the hsp83 gene and the four small heat shock genes at the 67B locus (13) to hsp70 gene flanking sequences (14). The five genes share an extended region of homology, but the homology in the case of the hsp83 gene is less than that shared by the other four genes.

MATERIALS AND METHODS

(a) Enzymes and Substrates

Restriction endonucleases were from Bethesda Research Laboratories or New England Biolabs, E. coli exonuclease III and T_4 polynucleotide kinase were from New England Biolabs, nuclease S1 was from Sigma, and AMV reverse transcriptase was a gift from Dr. J. Beard, Life Sciences, Inc. Calf intestinal alkaline phosphatase (Grade I) was obtained from Boehringer Mannheim and purified free of contaminating nucleases as described (15). [α - 32 P]deoxynucleoside triphosphates and [γ - 32 P]adenosine triphosphate were from Amersham Corp.

(b) Purification of in vitro labeled DNA fragments

Plasmids were propagated in E. coli K12 strains HB101 [F^- , hsdS20 (r_B^- , m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xy-15, mt1-1, supE44, λ^-], (16) or GM33 [F^- , dam-3, sup85], (17), and prepared by equilibrium gradient centrifugation as described (18). DNA preparations were routinely extracted with phenol, RNase A treated, and precipitated with polyethylene glycol (19). 5'-protruding restriction fragment ends were labeled with T_4 polynucleotide kinase or reverse transcriptase as described (20,21). Flush restriction fragment ends were labeled with high efficiency by either polynucleotide kinase or reverse transcriptase after limited treatment with E. coli exonuclease III to generate short (about 5 nucleotide) 5'-protruding ends, as described previously (20). All end-labeled fragments were separated on preparative polyacrylamide gels (20).

(c) DNA sequencing

DNA was sequenced by the Maxam and Gilbert method (21). The sequence for a total of 1859 base pairs, including 1125 base pairs within the protein coding region was determined from both DNA strands. Within this interval we observed 13 positions where band compression (21) required sequence from the opposite strand. From this error frequency of 0.007 we estimate that our sequence for 1433 bases derived from one strand is better than 99% correct.

(d) Isolation of Drosophila RNA

Heat shock poly(A)⁺ RNA was isolated from polysome pellets of D.

melanogaster K_{co} line tissue culture cells, essentially as described (22). Alternatively, poly(A)⁺ RNA was obtained from D. melanogaster adults after incubating approximately 2000 flies at 37° for 60 minutes, crushing with mortar and pestle in 15 ml ice-cold buffer (10 mM Tris-HCl, pH 8, 20 mM EDTA, 1% SDS, 0.1% diethylpyrocarbonate) and immediately extracting three times with equal volumes of equilibrated phenol and diethyl ether. The RNA was ethanol precipitated and treated with RNase-free DNase I (23) and a poly(A)⁺ fraction was collected (22).

(e) Exonuclease III/nuclease S1 mRNA mapping

Restriction fragments labeled at one 5' terminus were purified from polyacrylamide gels and digested to completion with an excess of E. coli exonuclease III using the conditions described by Guo and Wu (24). Briefly, purified fragments were treated in 100 µl of exo III buffer (66 mM Tris-HCl, pH 8, 77 mM NaCl, 5 mM MgCl₂, 10 mM dithiothreitol) with 1-2 µl of exonuclease III (56 units/µl) at 23°C for 50 minutes. The reaction was terminated by two extractions with an equal volume of equilibrated phenol, followed by ether extraction and ethanol precipitation. Generally about 2 µg of Drosophila heat shock poly(A)⁺ RNA was added with 23 µg of yeast carrier tRNA to an aliquot of phenol-extracted probe and co-precipitated. Probe aliquots without Drosophila RNA were precipitated with 25 µg of yeast tRNA only. These pellets were then resuspended in 10 µl of S1 hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA in 20% or 80% formamide), heat-denatured, hybridized, and then treated with nuclease S1 as described (25). The short RNA-DNA hybrid used to position the start of transcription was generated by a hybridization under nonstringent conditions in buffer containing 20% formamide at 30°C for 15 hours. The larger RNA-DNA hybrid used to position the 3' splice junction was generated in 80% formamide at 30°C for 13 hours. Nuclease S1 digestions were in 300 µl at 200 units/ml at 15°C for 60 minutes. Control samples hybridized without Drosophila RNA were incubated in S1 buffer with or without nuclease S1. S1 resistant hybrids were precipitated with isopropanol and loaded onto sequencing gels as described (25).

(f) Restriction fragment primed reverse transcripts

Primer extension was performed by hybridization of primer to RNA at 75°C in S1 hybridization buffer without formamide, and cDNA was synthesized with reverse transcriptase (20). Non-hybridized RNA was digested with RNase A at 250 µg/ml, and after phenol extraction the cDNA was recovered by ethanol precipitation.

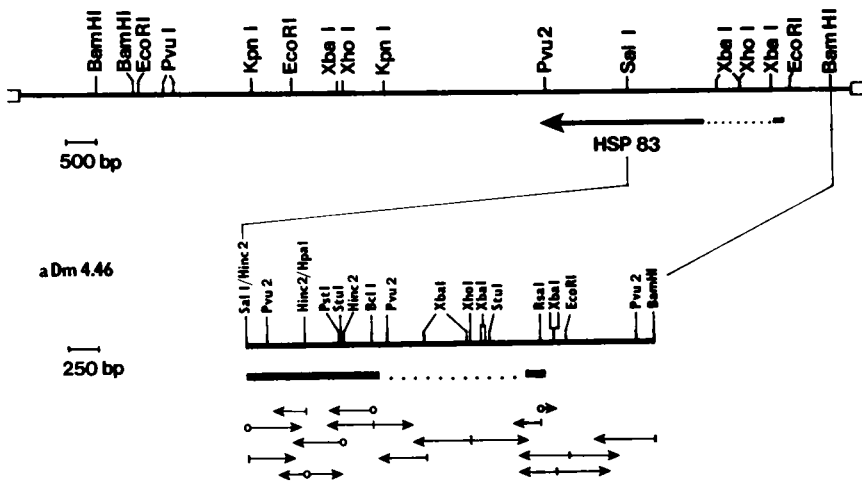


Figure 1. Restriction map of cloned segments from 63B and sequencing strategy.

A low resolution restriction map of the genomic clone λ Dm4L (6) carrying 13.3 Kb of *D. melanogaster* DNA (solid bar) inserted into λ sep6 (open bars) is shown. The solid arrow beneath designates the extent and 5'-3' orientation of the hsp83 transcribed sequences. Intron sequences are indicated by dots. A BamHI-SalI subclone, aDm4.46, is shown in expanded scale with key restriction sites. Each arrow in the sequencing strategy represents the direction and total distance read from a labeled end. Arrows beginning with circles represent 5' kinase end-labeling, and arrows beginning with bars represent 3' labeled ends.

RESULTS

(a) A 3292 bp sequence from the 5' end of the hsp83 gene

Figure 1 illustrates at low resolution the position of the 3292 bp BamHI-SalI insert of subclone aDm4.46 relative to the hsp83 transcript and the original *D. melanogaster*/ λ Sep6 hybrid phage from which it was isolated. In Figure 2 we present the complete nucleotide sequence derived from this subcloned segment, with key restriction sites, the TATA sequence (26), splice junctions (27), and predicted hsp83 amino acid sequence indicated. Holmgren *et al.* (12) have reported 578 bp of DNA sequence at the 5' end of a hsp83 gene isolated from a different genomic library, and apparent splice sites of the hsp83 transcript. Figure 2 indicates the regions sequenced by Holmgren *et al.* (12) and positions where our sequence differs. The nucleotide sequence and splice sites reported previously did not establish an open translation reading frame for the 83,000 d protein, and the first AUG codon within the spliced transcript (12) was followed by translational stop codons. In contrast, our

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5' BamHI	-870	-860	-850	-840	-830	481	491	501	511	521
	GGATCCTAAC	GGGAACCTGA	AGAAGTCGAT	ATTGGGGTTG	CGGCTAGAAC	CATGTGGTTT	CTAGAATACA	AGATTAATTT	TTGTTTAATT	AATGCAATGG
	-820	-810	-800	-790	-780	531	541	551	561	571
	CCACCCGACA	ATCACGAAAA	CAACACTTAG	TGCCGCCCAT	TTGTTTAAAT	TCTTTTAGGG	CTAAATCGAA	TTATGCCGCT	CTTCTTAGGG	GGTGACAAATG
	-770	-760	-750	-740	-730	581	591	601	611	621
	ATAAGCAAAC	AACTTTTATG	TTATTATGTC	TGGAAGGCTT	AGCGTCAAGT	CGCAAATCAC	ATTTGCCGCT	CGAGAATGCT	CTAGAGGTTT	CTATGCTTTA
	-720	-710	-700	-690	-680	631	641	651	661	671
	GGTGATATCG	ATGGGAGGCA	TGATAACAG	AATTGACCGA	AACCAAAATGA	GCATGAATTA	AACAGCGTGC	CAAATAGGGC	TTTTTAATTA	TAAATTAAGT
	-670	-660	-650	-640	-630	681	691	701	711	721
	TCGATATAAC	ACTTCTTAAT	TAATGAGAGA	TTTTTACTT	GACTGGGCTT	TCCTTCATTT	ACAATTACAC	GTGCTTTCTT	TGCATTCCCA	TTTTAAATG
	-620	-610	-600	-590	-580	731	741	751	761	771
	GTAGGAGGTT	TTGCACAGAA	GCAATTAATT	TCCGGAATGT	GAATGTCTG	CACATGCCGC	ATACGCACAC	ATGGACGGCC	ATGACTAATA	CTTTCAAGTA
	-570	-560	-550	-540	-530	781	791	801	811	821
	CTTTTACGCT	AATTACAACA	AAAACTTTCC	AATTTTGTCT	CTCCAAACCC	AAAATGTGGC	GTCAGTAAGC	AAATTAATGT	AAATCGGTTT	TATAACCOCTA
	-520	-510	-500	-490	-480	831	841	851	861	871
	ACTCAAGTGA	TTTCAAATTT	TACCGTCCGC	TTAAAATGGA	ACTAGTTTAT	TTTGCCTCAT	TTTAATTTGT	TGGTTCGCTA	CTAGCAACTT	GCTAGCGGAA
	-470	-460	-450	-440	-430	881	891	901	911	921
	TCCAGAGGAA	CCAGCTTGCA	CCACCAAGTC	TCTGAAACTC	TGGAATATTC	TTAGTTTTCCT	TTTGGCTTCT	AGATGCTTCC	ACAAACTTCC	TTGGTGAAGT
	-420	-410	-400	-390	-380	931	941	951	961	971
	GATAGTCTGG	TGGGAAAAAG	TATTCATAAA	TATAAAATAA	AATTAACAGG	ACGAATTTTC	AATGCAATTC	TCACTCACAC	AGACACGAGT	TTGCACACAG
	-370	-360	-350	-340	-330	981	991	1001	1011	1021
	TCATAAGCTG	ATTTCCTTAT	TATTTACTGT	TAAAACAAGT	AAAATAATAT	CAGGGGTAGA	AAAATTATCA	ACCCGCCCAA	TTTGCAATAT	TATATTTAAA
	-320	-310	-300	-290	-280	1031	1041	1051	1061	1071
	TGGGAACAAT	TAAATTTTCC	ATTTTCTCTA	TTACAGATA	AGCGTAGTGG	TATTTAAATTT	TAAAACATAA	TTTTGCAACT	TAAAATCAAT	TCTGTTGCGCT
	-270	-260	-250	-240	-230	1081	1091	1101	1111	1121
	CGGTTTTGAT	ATCCAATTGT	AATGTTTTAA	GCAACCCAGT	GGGTTTGTCT	AATTGAAATTT	AAAATTCAT	TTTACGGGTT	GCAAAGTGAA	TGCTATAAAT
	-220	-210	-200	-190	-180	1131	1141	1151	1161	1171
	CAATCGTTCC	GACCACTTAG	ACGAATTTCC	ACCAAACCTA	GTTTTCAGTTA	TTGACCACCA	CTGTAAGTTC	ATATGGCCAT	GTTAAATGAG	GCATGTGCAA
	-170 EcoRI	-160	-150	-140	-130	1181	1191	1201	1211	1221
	TAATTTTCTG	ATTCGGCCGC	ACAGGTTGGC	CACCTTTGCA	CCGTAATGCA	AAGAGAAGA	AAGAAAAGA	ATAAACCCG	AGCAGCTGCT	GAATAATGCA
	-120	-110	-100	-90	-80	1231	1241	1251	1261	1271
	ACACTCAACT	ACCGTAGTCT	TCACAGGAAG	TTGCATCCCT	GCATCCAGAA	CTGCTTTTCC	TTAGTGTGGA	ACCCACAGAC	TATAACTAAT	CCTAATGATT
	-70	-60	-50	-40	-30	1281	1291	1301	1311	1321
	GCCTCTAGAA	GTTTCTAGAG	ACTTCCAGCT	CGGTTGGGG	TTTTTCTATA	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	-20	-10	1	11	21	31	41	51	61	71
	TTGACAGAGC	CGCGGCGTTT	GCGGGTTGCA	GTTCTGAAAA	AAATTTGCTA	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	81	91	101	111	121	131	141	151	161	171
	AATTCATAC	AAAGCAAAGT	GAAAATATCT	GTATTTTTAC	CTTTATTCTG	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	131	141	151	161	171	181	191	201	211	221
	TGAATAGAAC	GA AAAACATA	CATACAAGCT	GAGTAATGCA	AATTACAAGA	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	181	191	201	211	221	231	241	251	261	271
	AAAGACTGAA	TAGTTTATCA	CTGCACTATG	CCCAATTCGC	AAATTTGCGT	GGTCTCTGTG	CATCTCGAAT	GTTCTTGACC	CAAACTGTGAG	ATATTGATTT
	231	241	251	261	271	281	291	301	311	321
	TTAAATTTCTA	GGAGCCAAAGT	TTAAGAATTT	TTTTTATTTT	AAATTAGAGGT	GGTCTCTGTG	CATCTCGAAT	GTTCTTGACC	CAAACTGTGAG	ATATTGATTT
	331	341	351	361	371	381	391	401	411	421
	GGCAACCGTC	AAATTAACCT	AAAATGCCGG	TTTCTTTTAT	TTTTTGTGGC	TTGGAAGCAT	CTCCAGAGGT	TTCTATGCTT	TAGCATCGAA	ATTAACATC
	431	441	451	461	471	481	491	501	511	521
	GTGCCAAATA	GGCCTTTTAA	TTATATATTA	CTGTCCCTTA	TTTACAATTA	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	531	541	551	561	571	581	591	601	611	621
	TCTTTTAGGG	CTAAATCGAA	TTATGCCGCT	CTTCTTAGGG	GGTGACAAATG	CGCAAATCAC	ATTTGCCGCT	CGAGAATGCT	CTAGAGGTTT	CTATGCTTTA
	631	641	651	661	671	681	691	701	711	721
	GCATGAATTA	AACAGCGTGC	CAAATAGGGC	TTTTTAATTA	TAAATTAAGT	TCCTTCATTT	ACAATTACAC	GTGCTTTCTT	TGCATTCCCA	TTTTAAATG
	731	741	751	761	771	781	791	801	811	821
	CACATGCCGC	ATACGCACAC	ATGGACGGCC	ATGACTAATA	CTTTCAAGTA	AAAATGTGGC	GTCAGTAAGC	AAATTAATGT	AAATCGGTTT	TATAACCOCTA
	831	841	851	861	871	881	891	901	911	921
	TTTGCCTCAT	TTTAATTTGT	TGGTTCGCTA	CTAGCAACTT	GCTAGCGGAA	TTAGTTTTCCT	TTTGGCTTCT	AGATGCTTCC	ACAAACTTCC	TTGGTGAAGT
	931	941	951	961	971	981	991	1001	1011	1021
	ACGAATTTTC	AATGCAATTC	TCACTCACAC	AGACACGAGT	TTGCACACAG	CAGGGGTAGA	AAAATTATCA	ACCCGCCCAA	TTTGCAATAT	TATATTTAAA
	1031	1041	1051	1061	1071	1081	1091	1101	1111	1121
	TATTTAAATTT	TAAAACATAA	TTTTGCAACT	TAAAATCAAT	TCTGTTGCGCT	AATTGAAATTT	AAAATTCAT	TTTACGGGTT	GCAAAGTGAA	TGCTATAAAT
	1131	1141	1151	1161	1171	1181	1191	1201	1211	1221
	TTGACCACCA	CTGTAAGTTC	ATATGGCCAT	GTTAAATGAG	GCATGTGCAA	AAGAGAAGA	AAGAAAAGA	ATAAACCCG	AGCAGCTGCT	GAATAATGCA
	1231	1241	1251	1261	1271	1281	1291	1301	1311	1321
	CTGCTTTTCC	TTAGTGTGGA	ACCCACAGAC	TATAACTAAT	CCTAATGATT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1331	1341	1351	1361	1371	1381	1391	1401	1411	1421
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1431	1441	1451	1461	1471	1481	1491	1501	1511	1521
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1531	1541	1551	1561	1571	1581	1591	1601	1611	1621
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1631	1641	1651	1661	1671	1681	1691	1701	1711	1721
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1731	1741	1751	1761	1771	1781	1791	1801	1811	1821
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1831	1841	1851	1861	1871	1881	1891	1901	1911	1921
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT
	1931	1941	1951	1961	1971	1981	1991	2001	2011	2021
	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT	TTGTAATTCG	ATTCGCTG	TTGCTGCT	TTGCTGCT	TTGCTGCT

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1663          1678          1693          2068          2083          2098
CAG TTC GGT GTG GGT TTC TAC TCC GCC TAC CTG GTC GCC GAC AAG GAC AAG GAT GCC AAG AAG AAG ACC ATC AAG GAG AAG TAC ACT
Gln Phe Gly Val Gly Phe Tyr Ser Ala Tyr Leu Val Ala Asp Lys Asp Lys Asp Ala Lys Lys Lys Lys Thr Ile Lys Glu Lys Tyr Thr

1706          1723          1738          2113          2128          2143
GTG ACT GTC ACC TCC AAG AAC AAC CAT GAC GAG CAG TAC GTG TGG GAG GAT GAG GAG CTG AAC AAG ACC AAG CCC ATC TGG ACC CGC AAT
Val Thr Val Thr Ser Lys Asn Asn Asp Glu Glu Tyr Val Trp Glu Asp Glu Glu Leu Asn Lys Thr Lys Pro Ile Trp Thr Arg Asn

1753          1768          1783          2158          2173          2188
GAG TCC TCT GCC GGA GCC TCT TTC ACA GTC CGT GCC GAC AAC TCT CCC GAT GAT ATC TCC CAG GAG GAG TAC GCC GAG TTC TAC AAA TCC
Glu Ser Ser Ala Gly Gly Ser Phe Thr Val Arg Ala Asp Asn Ser Pro Asp Asp Ile Ser Gln Glu Tyr Gly Glu Phe Tyr Lys Ser

1798          1813          1828          2205          2218          2233
GAG CCC CTG GCC CGT GCC ACC AAG ATC GTG CTG TAC ATC AAG GAG CTG ACC AAC GAC TGG GAG GAT CAT CTG GCC GTG AAG CAC TTC TCC
Glu Pro Leu Gly Arg Gly Thr Lys Ile Val Leu Tyr Ile Lys Glu Leu Thr Asn Asp Trp Glu Asp His Leu Ala Val Lys His Phe Ser

1843          1858          1873          2248          2263          2278
GAC CAG ACC GAC TAT CTG GAG GAG AAG ATC AAG CAG ATT GTT GTG GAG GGT CAG CTG GAG TTC CGT GCT CTG TTC ATT CCC CCG
Asp Gln Thr Asp Tyr Leu Glu Glu Ser Lys Ile Lys Glu Ile Val Val Glu Gly Gln Leu Glu Phe Arg Ala Leu Leu Phe Ile Pro Arg

1888          1903          1918          2293          2308          2323
AAC AAG CAC TCC CAG TTC ATT GCC TAC CCC ATC AAG CTG CTC GTA CGC ACC CCC TTC GAT CTC TTT GAG AAC CAG AAG AAG CCG AAC AAC
Asn Lys His Ser Gln Phe Ile Gly Tyr Pro Ile Lys Leu Leu Val Arg Thr Pro Phe Asp Leu Phe Glu Asn Gln Lys Lys Arg Asn Asn

1933          1948          1963          2358          2355          2368
GAG AAG CAG CCG GAG AAG GAG GTC AGC GAC GAT GAG GCT GAT GAT ATC AAG CTG TAC GTC TTT ATC ATG GAC AAC TTC GAG
Glu Lys Glu Arg Glu Lys Glu Val Ser Asp Asp Glu Ala Asp Asp Ile Lys Leu Tyr Val Arg Arg Val Phe Ile MET Asp Asn Cys Glu

1978          1993          2008          2383          2398          2413
CAG AAG AAG GAA GGT GAT GAG AAG AAG GAG ATG GAG ACT GAT GAG GAC CTC ATT CCA GAG TAC TTG AAC TTC ATG AAG GGT GTG CTC GAC
Glu Lys Lys Glu Gly Asp Glu Lys Lys Glu MET Glu Thr Asp Glu Asp Leu Ile Pro Glu Tyr Leu Asn Phe MET Lys Gly Val Val Asp

2023          2038          2053
CCC AAA ATC GAG GAT GTT GCC GAG GAT GAG GAT GCC GAC AAG AAG
Pro Lys Ile Glu Asp Val Gly Glu Asp Glu Asp Ala Asp Lys Lys

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Figure 2. Nucleotide sequence of the 3292 bp aDm4.46 insert.

The nucleotide sequence is shown oriented 5' to 3' from the BamHI site. Nucleotide +1 designates the start of hsp83 transcription. Several restriction sites from Figure 1 and the text are included. Solid boxes highlight the TATA sequence, the 5' splice junction, and the 3' splice junction. The unique open translation reading frame begins immediately adjacent to the 3' splice junction, and the predicted amino acids are shown. Positions where our sequence overlaps and agrees with that of Holmgren *et al.* (1981) are indicated by dots. At positions where the sequences differ, we show their nucleotides below ours. Arrows indicate bases reported by Holmgren *et al.* (1981) not found in our sequence.

DNA sequence contains two in-phase translational start codons, at positions +1289 and +1340 near the 3' splice junction, that could serve as the initiating AUG and specify the same open reading frame. In order to assign the start of translation to one of these, a precise determination of the 3' splice junction was required. Below we describe transcript mapping experiments that provide high resolution confirmation of the previously predicted initiation and splice sites of the hsp83 transcript, and which taken together with our DNA sequence, establish an open translation reading frame beginning immediately adjacent to the splice site.

(b) Determination of the 3' splice junction

To generate 5' terminally-labeled, single-strand probes for nuclease S1 transcript mapping, we have used the strategy shown in Figure 3(a). This

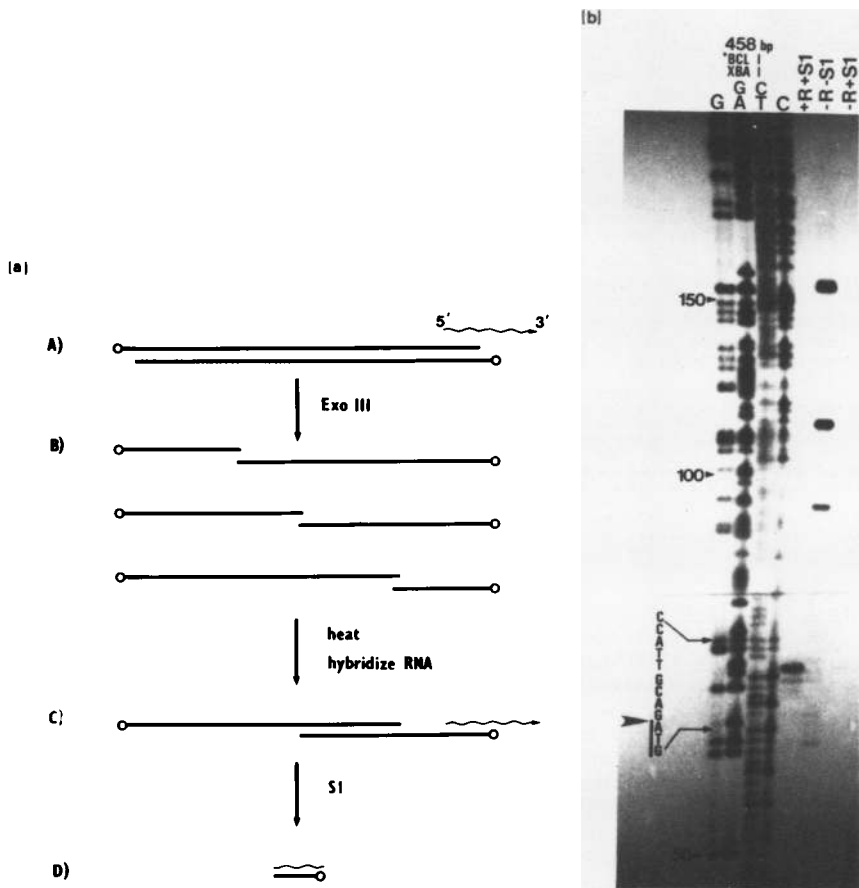


Figure 3.

(a) Nuclease S1 transcript mapping using exonuclease III generated single-strand probes.

A. A restriction fragment (solid lines) with 5' protruding ends (blunt end fragments also may be used) is shown with the transcript to be mapped (wavy lines). Open circles represent 5' phosphates. Both 5' termini may be labeled or just the one needed for mapping.

B. Digestion with *E. coli* exonuclease III yields molecules that have been more or less synchronously digested from both 3' ends.

C. The mixture of single-strand DNAs is heat denatured and hybridized to RNA. Competition from opposite DNA strands is eliminated. Any DNA reannealing does not interfere with hybridization to RNA, and results in unlabeled DNA fragments after S1 treatment.

D. Digestion with nuclease S1 leaves the RNA-DNA hybrid as the only labeled fragment.

(b) Nuclease S1 mapping of the 3' splice junction.

A 458 bp BclI-XbaI restriction fragment from adM4.46, kinase end-labeled at the BclI site, was treated with exonuclease III and hybridized to heat shock, poly(A)⁺ RNA. This same fragment was sequenced to provide exact size standards. Numbers designate distance

from the terminal labeled nucleotide. Hybridizations were with (+R) or without (-R) Drosophila RNA, and then treated with (+S1) or without (-S1) nuclease S1. A short stretch of sequence deduced for the opposite strand is aligned with the sequencing ladder at the splice point. The actual splice site, in agreement with consensus sequences (27), is indicated by an arrow. The first ATG codon is overlined. Samples were run on an 80 cm, 6% sequencing gel.

approach exploits the duplex-specific and processive digestion by E. coli exonuclease III of 3' hydroxy termini (24). Under controlled digestion conditions at 23°C, exonuclease III synchronously removes approximately 10 nucleotides per minute from each 3' hydroxy terminus and exhibits little sequence dependence (24). This exonuclease III digest provides a means of generating in high yield a single-stranded probe that is more rapid than strand separation.

As shown in Figure 3(b), exonuclease III digestion of the BclI-XbaI fragment (+890 to +1348 in Figure 2), which is labeled at the BclI site, produces an array of single-stranded fragments (-RNA -S1 lane) that yield no S1-resistant duplexes after hybridization in the absence of Drosophila RNA (-RNA +S1 lane). Addition of heat shock poly(A)⁺ Drosophila RNA to the hybridization mixture results in two S1-resistant fragments (+RNA +S1 lane). Since S1 generated, 5' end-labeled fragments migrate 1.5 nucleotides slower on high resolution sequencing gels than comparable Maxam-Gilbert cleavage fragments (28), these bands represent S1-resistant fragments that are 67 and 65 nucleotides long. This places the 3' splice junction at positions +1285 to +1287 in Figure 2. These S1 termini occur within a sequence (5'-TCCATTGCAGAT-3') that perfectly matches the consensus Drosophila intron-exon boundary sequence (5'-TCP_N^APy_NPy_N^GPy_N^CAG_C^{Pu}-3') (27) and the more general eucaryotic consensus (29). Holmgren et al. (12), using different conditions of nuclease S1 digestion, also localized the 3' splice junction to this consensus sequence. Since nuclease S1 may leave short overhangs (30), we position the 3' splice site at position +1288 in accordance with the established consensus splice sequence. This location agrees well with previous electron microscopic measurements of hsp83 R-loops (6). The adjacent ATG codon at +1289 is the first initiation codon of a long open reading frame, as we shall demonstrate.

(c) Determination of the hsp83 mRNA start

Figure 4 shows that the complete exonuclease III digestion of an end-labeled RsaI-Sau3AI fragment (-116 to +24) generates a series of single-strand probes (-RNA -S1 lane) that do not give rise to any S1-resistant

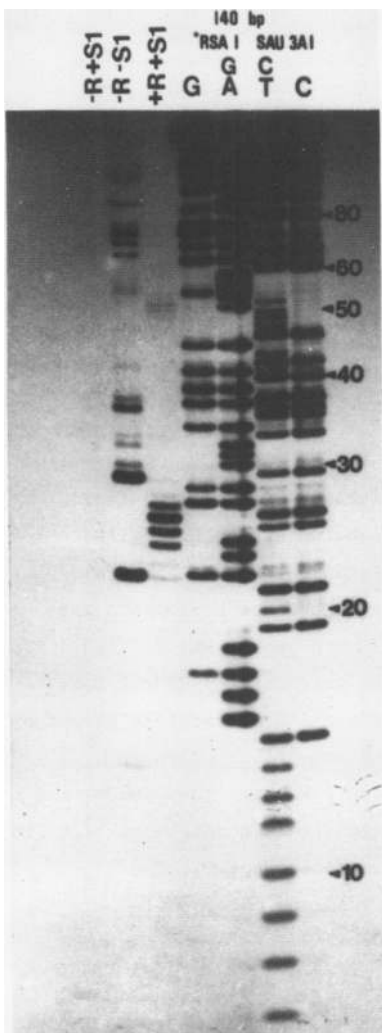


Figure 4. Nuclease S1 mapping of the *hsp83* mRNA start. A 140 bp *RsaI*-*Sau3AI* restriction fragment from *adm4.46*, kinase end-labeled at the *RsaI* site, was digested with exonuclease III and hybridized to heat shock, poly(A)⁺ RNA. This same fragment was sequenced to provide exact size standards. Numbers along the right margin indicate distance from the labeled nucleotide. Symbols for each lane are as in Figure 3(b). Samples were run on a 40 cm, 15% sequencing gel.

duplexes when hybridized without *Drosophila* RNA (-RNA +S1 lane). Under the same hybridization conditions with *Drosophila* heat shock poly(A)⁺ RNA, specific S1-resistant fragments are detected (+RNA +S1 lane). Again, 5' end-labeled S1 fragments migrate 1.5 nucleotides slower than their chemical cleavage counterparts (28). Therefore, the protected fragments vary from 20 nucleotides to 25 nucleotides in length. The 20 nucleotide S1-resistant fragment corresponds to an adenosine residue located within a sequence (5'-CGACTCT-3') that partly matches the eucaryotic consensus mRNA capping site

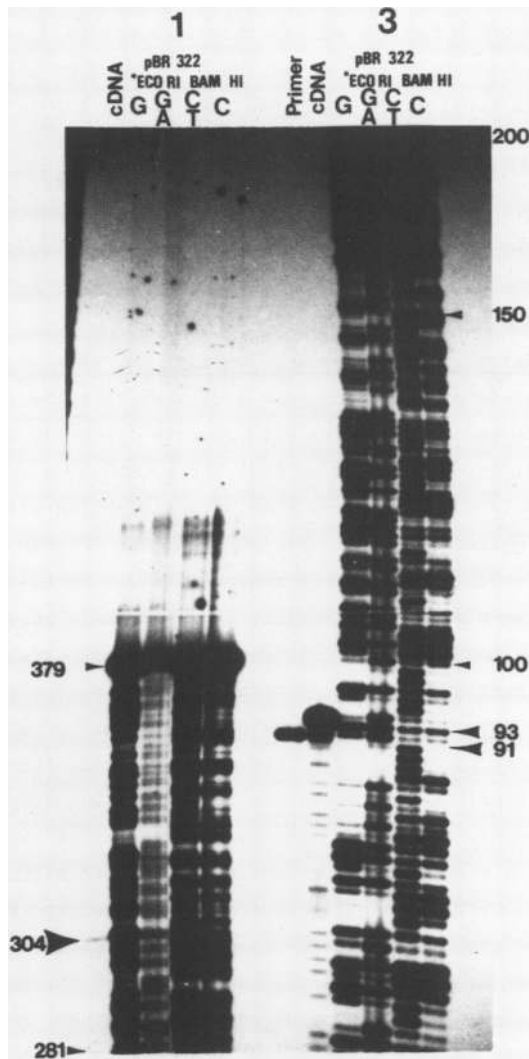


Figure 5. Determination of the 5' splice junction.

The staggered first and third loadings on an 80 cm, 6% sequencing gel are shown. The lane labeled "Primer" contains the 90 nucleotide 3' end-labeled strand of the BclI-HinI fragment from aDm4.46. The cDNA species primed from heat shock *Drosophila* RNA (cDNA lanes) is seen in the first loading. This same sample in the third loading shows the original primer strand, as well as a species two nucleotides larger generated by reannealing of the primer restriction fragment and reverse transcriptase repair of the BclI end. The sequencing standard is the 375 bp EcoRI-BamHI fragment from pBR322, 3' end-labeled at the the EcoRI site with [α - 32 P]dATP. Numbers indicate distance from the terminal labeled nucleotide at the EcoRI site.

(5'-PyCATTCPu-3') determined previously (31). Because many eucaryotic mRNAs initiate at an adenosine residue within this sequence (32), and because comparably situated adenosines occur at the 5' termini of other heat shock genes in *Drosophila* (12,13), we designate this nucleotide as +1 in Figure 2. Holmgren *et al.* (12) also detect an S1-resistant terminus corresponding to position -4 in our sequence; the additional fragments seen in Figure 4 probably represent short 3' DNA overhangs not removed by S1 nuclease (30). The hsp83 mRNA start at nucleotide +1 is also located an appropriate distance, 26 nucleotides, from a sequence (5'-TATAAAA-3') that matches the consensus eucaryotic TATA sequence (26).

(d) Determination of the 5' splice junction and size of the small exon

Previous analysis of the hsp83 gene indicated that the single intron is preceded by a short exon (2,12). We used reverse transcriptase to generate cDNA from restriction fragment primed heat shock mRNA as a means to determine the size of the small exon. Plasmid aDm4.46 was digested with BclI and 3' end-labeled with reverse transcriptase, dGTP and [α -³²P]dATP, and then cleaved with HinfI. The resulting BclI-HinfI fragment (+1347 to +1436) was gel purified and used as a primer to initiate cDNA synthesis after hybridization to hsp83 mRNA. The length of the RNA homologous primer strand from the HinfI terminus to the partially filled BclI terminus is 90 nucleotides. Reverse transcripts primed from BclI will extend an additional 61 nucleotides to the 3' splice junction at position +1288, thereby accounting for 151 nucleotides of the final length of the cDNA product. By subtracting this length from the total size of the cDNA, the size of the small exon may be determined. As shown in Figure 5, a single cDNA species is evident and migrates on an 80 cm long sequencing gel with a 304 nucleotide chemical cleavage fragment derived from an end-labeled pBR322 restriction fragment. We used the 375 bp EcoRI-BamHI fragment of plasmid pBR322, end-labeled at the EcoRI site with [α -³²P]dATP, as a convenient size standard since no single restriction fragment from plasmid aDm4.46 has the same sequence as the cDNA species. However, we have also compared the mobility of this cDNA on 80 cm gels relative to a sequenced, 3' end-labeled HpaI-SalI fragment from aDm4.46, and find an apparent mobility of 302 nucleotides (not shown). This difference may be explained by the slower mobility on high resolution sequencing gels of single strands with higher T content (33). Thus, the large pBR322 chemical cleavage fragments, with 19% T content, move faster relative to the *Drosophila* chemical cleavage fragments containing 29% T. Since the cDNA sequence has a 32% T content, we use 302 nucleotides as the apparent total length of the

cDNA. In addition, because chemical cleavage products of 3' end-labeled DNA migrate 1.0 nucleotide faster than reverse transcriptase generated fragments (28), the actual length of the cDNA species is 301 nucleotides, and thus the small hsp83 exon is 150 nucleotides long (301-151 nucleotides). From position +1 in our sequence, this places the 5' splice junction at position +150 in Figure 2. This site occurs within a sequence (5'-AAGGTGAGTA-3') that perfectly matches both the eucaryotic (5'- $\overset{C}{A}G/GT\overset{A}{C}AGT-3'$) exon-intron consensus boundary sequence (29), and a consensus sequence for *Drosophila* (5'- $\overset{G}{Pu}/GTPu\overset{A}{C}GTPu-3'$) exon-intron junctions (27). From this match to consensus splice sequences we conclude that the small exon of the hsp83 gene is 149 nucleotides in length. This value is in accord with previous determinations that estimated this exon to be 0.16 Kb long, based upon DNA-primed reverse transcripts, and closer to 0.15 Kb, based upon consensus sequence matches in this region of the hsp83 gene (12).

(e) Identification and evaluation of a translation reading frame for hsp83

Examination of sequences contained within the spliced hsp83 transcript, derived from the S1 nuclease and cDNA results, indicates that the first ATG codon downstream from the mRNA initiation nucleotide occurs at position +1289, immediately after the 3' intron-exon junction. This ATG codon specifies an open translation reading frame that extends through the remaining 1122 nucleotides in the DNA sequence, and is capable of encoding the first 375 amino acids of the 83,000 d polypeptide. The sequence at the first ATG codon (5'-CAAG/ATGC-3') matches in part the eucaryotic translation start consensus (34). Moreover, this ATG specifies the only open reading frame in our sequence. Although a second ATG codon in this same reading frame occurs at position +1340 in our DNA sequence, we adopt the first ATG codon in the 5'-noncoding region as the start of translation. In Figure 2 the first 375 amino acids predicted for the amino half of the hsp83 protein are shown. These amino acids account for about 41,250 d of the total 83,000 d determined by SDS-polyacrylamide gel electrophoresis (2). From our DNA sequence we note that the amino terminal half of hsp83, particularly between amino acids 210 and 275, should be rich in acidic and basic (hydrophilic) residues. Indeed, of the 64 amino acids specified in this region, 48 are glutamic acid, aspartic acid, lysine or arginine residues arranged as strings of hydrophilic groups. Such a composition of charged amino acids is consistent with the known cytoplasmic location of hsp83 (35). Finally, we have compared our hsp83 amino acid sequence to the current protein sequence database, as described by Barker

and Dayhoff (36). No significant homology was found. However, comparison to the yeast hsp90 gene nucleotide sequence determined by F. Farrelly and D. Finkelstein (personal communication) reveals four long (38-103 amino acid) stretches that are 60-90% conserved. The first stretch begins at codon 6 in the Drosophila hsp83 open reading frame.

(f) Comparison of the hsp83 nucleotide sequence to other Drosophila genes---a new twist in the method of searching for common sequences among different genes

We have used a package of computer programs (37) designed to identify homologies between DNA sequences and display the results in dot-matrix form. When a homology of the specified length and percent match is identified, a dot is printed on the graph whose (x,y) coordinates correspond to the position of the first nucleotide of the homology within each sequence.

The criteria used to search for homologies were derived from known procaryotic protein-DNA binding sites. We have examined two instances in E. coli where DNA binding sites for the same regulatory protein occur at several genes or operons that display similar regulation. Five different sites for lexA repressor binding have an average 64% match over twenty nucleotides (38). Five cAMP-CRP binding sites exhibit an average match of 50% over 24 nucleotides (39). In our comparisons of Drosophila flanking sequences, using the criteria of a minimum 60% match between 20 nucleotide blocks yields plots in which known homologies such as TATA sequences are detected, while random matches are not excessive.

DNA sequence data that include 5' flanking regions are available for the hsp70 (14,40), hsp68 (12), and hsp22, hsp23, hsp26, and hsp27 (13,41) genes of D. melanogaster. Because the hsp70 and hsp68 genes are very similar and probably reflect a relatively recent duplication event (2), we have omitted the hsp68 gene in the figures, though it shows striking homology to hsp70 in the region from -42 to +30 (12). Of the five copies of hsp70 (42), we use the sequence of the distal copy at the 87A7 locus (14) as the standard. A deletion ending upstream of this copy demonstrates that sequences necessary for heat induced expression in vivo do not extend beyond 479 nucleotides upstream from the start of transcription (43).

We have superimposed dot-matrix plots in order to illustrate homology between each of the heat shock genes and hsp70. We find three striking features in this matrix. First, the most conspicuous is an extended diagonal line representing a nested set of homologies at nearly identical positions surrounding the mRNA starts shown on an expanded scale in Figure 6. Second, a

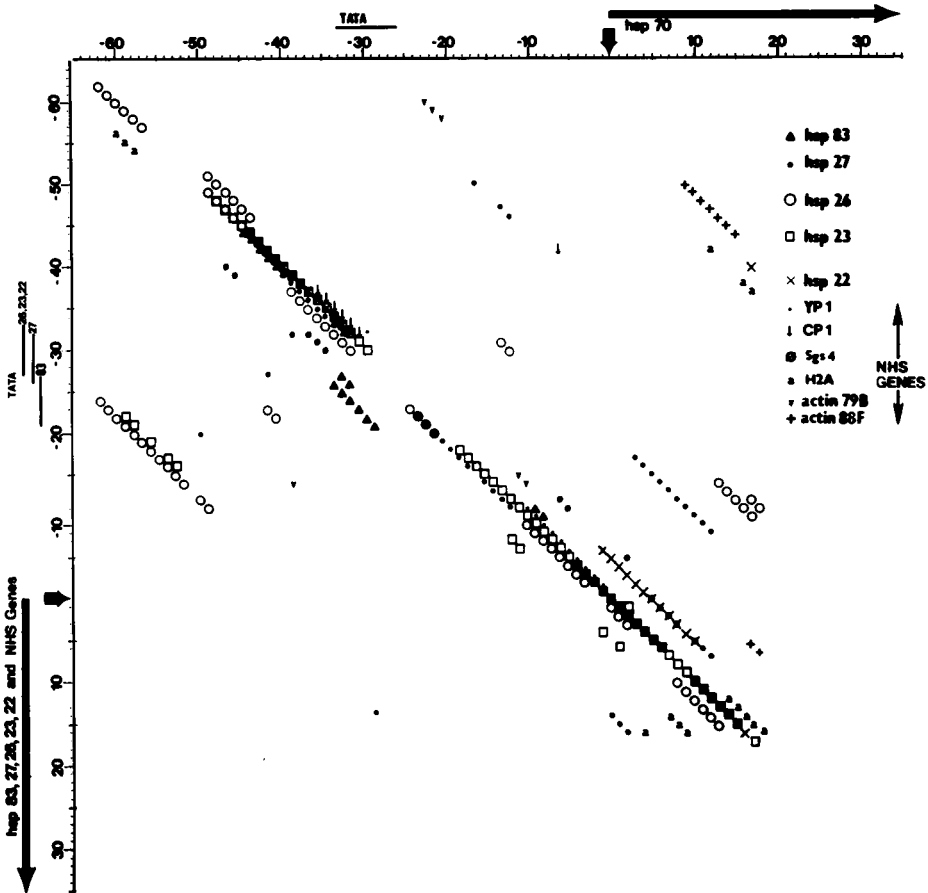


Figure 6. Expanded GRAPHOM outputs for five heat shock gene sequences compared to hsp70 sequences.

All five genes have been aligned at their transcription starts (+1). Criteria used to generate these plots were 20 nucleotide blocks with at least 60% match. Only 5' flanking and noncoding sequences have been compared, and the hsp83 intron has been deleted. Arrows indicate the transcribed sequences. We show similar comparisons to hsp70, using the same criteria, of sequences flanking other *Drosophila* genes that are not heat shock inducible (NHS genes), including yolk protein (52), cuticle protein (27), salivary glue protein (53), histone (54), and two actin (55) genes.

block of clustered homologies centered around coordinates (-150, -100, not included in Figure 6) is almost entirely due to repeated sequences common to hsp70 and hsp26 flanking regions. Third, a larger block of scattered

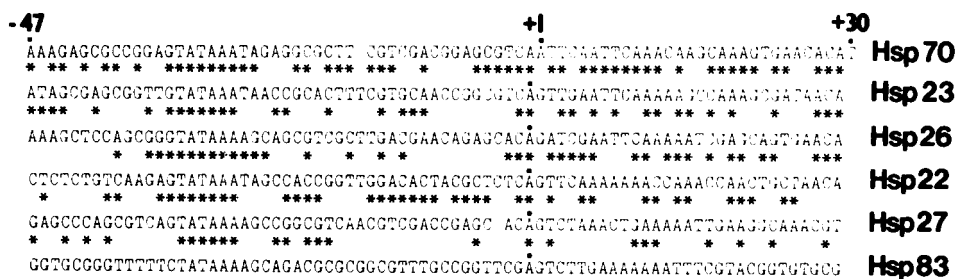


Figure 7. Alignment of heat shock gene homologous flanking sequences. The standard hsp70 sequence (14) from the distal copy at 87A7 is shown at top. All other sequences have been aligned with this sequence at the TATA homology. Numbering is from the start of transcription at +1. We have inserted a single base loopout in the hsp70 and hsp27 sequences, indicated by gaps, to permit a nonoverlapping display of the homologies from Figure 6. The matches to hsp70 are indicated by asterisks above each sequence. For hsp83, the six overlapping matches shown in Figure 6 do not align the TATA consensus sequences and are omitted here. Small heat shock gene sequences are from Ingolia and Craig (13).

homologies occurs between the transcribed, 5' noncoding sequences of all five genes and hsp70 (centered at +150 on hsp70). Interestingly, the hsp83 leader RNA sequence contributes fewer homologies than other heat shock genes.

The homologies arising from a particular gene are identified by symbols in Figure 6. The small heat shock gene sequences shares significant homology with the hsp70 gene. By comparison, the hsp83 and hsp70 genes share homology only at the TATA sequence. We also note similarities to other Drosophila genes that encode abundant non-heat shock RNAs, though the matches never equal those between the small heat shock genes and the hsp70 gene.

The nucleotide sequence of the hsp70 gene and the corresponding matches in the other heat shock genes are shown in Figure 7. The matches identified by the matrix analysis are indicated by asterisks above each sequence. Introducing a single nucleotide loopout in the hsp70 and hsp27 sequences allows us to present these matches in linear, nonoverlapping form. The two loopouts serve only to better align regions of homology already noted in the dot-matrix and do not alter the overall pattern of homologies. Examination of the aligned sequences shows that the percent match with hsp70 in this 77 bp interval is 47% for hsp26, 49% for hsp22, 52% for hsp27, and 68% for hsp23.

The small heat shock genes and the hsp70 gene in D. melanogaster have conserved flanking sequences that lie roughly between nucleotides -47 and +30 in each gene. Furthermore, the average match to hsp70 sequences within these

regions is 54%. These larger regions of lower average homology to hsp70 gene flanking sequences have not been noted in previous sequence comparisons (12,13) that focused on short (3-12 nucleotide), highly conserved sequence blocks. Surprisingly, in similar comparisons we find that the small heat shock genes, which are related by a gene duplication event, show more homology to the hsp70 gene in this region than to each other. In contrast to the small heat shock genes, sequences flanking the hsp83 gene do not exhibit extensive homology to hsp70 sequences. The match to hsp70 sequences within this 77 nucleotide region is only 31%. The TATA sequence forms the only homology longer than three continuous nucleotides. We conclude that sequences flanking the hsp83 gene are homologous only to a subset of those hsp70 sequences shared by the small heat shock genes.

DISCUSSION

We have sequenced a 3292 bp region that spans the 5' DNase I hypersensitive sites, the transcription start, the entire first exon, the intron, and translation start of the hsp83 gene. The transcription start and splice sites were mapped at high resolution and the results confirm previously made predictions (2,12). We assign the translation start to the first nucleotide of exon 2, as predicted from the open reading frame that begins at this ATG and extends at least 1125 bp. With these functional landmarks defined we have compared the hsp83 sequence to other heat shock gene sequences. Like the hsp70 and small heat shock genes, the 5' noncoding portion of mature hsp83 mRNA is long, 149 nucleotides. Unlike the other heat shock genes, this region is interrupted by an intron, possesses a lower adenosine content which is typical of some nonheat shock transcripts (13), and has the least homology with hsp70 of any major heat shock gene.

In an attempt to identify potential regulatory sequences in regions flanking several heat shock genes, we have conducted homology searches using criteria based upon defined procaryotic DNA-protein interactions. We find in all cases except hsp83 that sequences surrounding the transcription starts exhibit homology. How well does this flanking sequence homology agree with deletion analysis of the hsp70 gene? Genetic evidence from 87A deficiencies limits the maximum amount of 5' flanking sequence required for regulated expression in vivo to 479 nucleotides (43). In vitro generated deletions assayed in heterologous systems suggest that only about 70 nucleotides of flanking sequence are required for heat induction (44-47). The 5' extent of the -47 to +30 homologous regions lies within the limits defined by these

deletions. However, except in the comparison between the hsp26 and hsp70 genes, we do not detect homologies in the -65 to -48 sequences of these genes. This region is of interest because nucleotides -66 to -47 in hsp70 have been demonstrated to be essential for heat inducible expression in monkey COS cells (46). Furthermore, a consensus sequence derived from this region and comparable regions of other heat shock genes has been synthesized and appears to promote heat induced transcripts when inserted upstream of a HSV thymidine kinase gene and assayed in COS cells and in Xenopus oocytes (48). Under our search conditions this short 14 nucleotide sequence, which is not well conserved (33-67% match) between the hsp70 standard sequence and other heat shock genes, could not have been detected.

The fact that flanking sequences implicated in the regulation of the heat shock response were not detected by our sequence comparisons or previous comparisons (12,13) may be explained in several ways. First, the various heterologous assay systems used, and the presence of hsp70 genes on plasmid vehicles, may alter the normal functioning of regulatory sequences active in Drosophila chromosomes. Second, these regulatory sequences may not be protein binding sites, but may act instead to induce a particular DNA structure (e.g., Z DNA) and/or chromatin configuration that regulates the response (47). Finally, it is possible that established procaryotic protein-DNA regulatory interactions are not adequate models for these interactions in eucaryotes. Of particular interest in this regard are the sequences shown to be necessary for induction during amino acid starvation of the his 3 and his 4 genes in yeast (49), which are only 8 nucleotides in length.

Eucaryotic promoters appear to consist of two sequence elements. One element is defined by the TATA sequence and directs RNA polymerase II to the correct initiation point in a variety of genes in vivo (50). The second element, usually situated a variable distance upstream from the TATA sequence, appears to modulate the efficiency with which RNA polymerase II initiates transcription in vivo (50). The -66 to -47 region and the more conserved -47 to +30 region of several Drosophila heat shock genes appear to constitute such promoter elements. From the deletion analysis of hsp70 and assay of activity in heterologous systems, it appears that the upstream element functions to enhance promotion by RNA polymerase, since deletions in this region result in low levels of expression during heat shock, instead of constitutive expression (46). In addition, analysis of the in vitro transcriptional activity of mutant globin gene templates has suggested that sequences at the capping-initiation site (-10 to +7) increase the efficiency of transcription

by RNA polymerase II (51). From these results we suggest that the -47 to +30 homologous regions in several Drosophila heat shock genes may function in the proper initiation and perhaps also the efficiency of transcription of these genes during induction. In addition, the sequence disparity of the hps83 gene in these regions relative to the other Drosophila heat shock genes may ultimately be reflected in the differential regulation of this gene at normal and elevated temperatures.

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