Essential role of the first intron in the transcription of hsp90β gene

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Abstract The human HSP90 gene family contains introns. There are two typical heat shock elements (HSE) in the first intron of human hsp90β gene. As detected by chloramphenicol acetyl transferase (CAT) reporter activity assays, the HSE-containing intron is essential in maintaining high constitutive expression and is critical for heat shock inducibility of the human hsp90β gene. Cellular heat shock factor 1 (HSF 1) shows much higher binding affinity toward the intronic HSEs in comparison to an atypical HSE in the 5′ flanking sequence. Novel initiation sites found in the first intron probably also contribute to constitutive and heat-inducible expression of the hsp90β gene in Jurkat cells.

Key words: hsp90β gene; Heat shock element (HSE); The first intron; Transcription initiation site; Chloramphenicol acetyl transferase (CAT)

1. Introduction

Eukaryotic heat shock gene (hsp) transcription is usually *cis*-regulated through a specific heat shock element (HSE) located in the 5′ flanking sequences of the hsp genes. A typical mammalian HSE consists of at least three sets of the conserved nGAAn five nucleotide motif in a head-to-head or tail-to-tail alignment [1]. The HSE is the binding site for a heat shock responsive transcription factor, HSF. Complementary DNAs encoding human HSE binding proteins, heat shock factor 1 and 2 (HSF1 and HSF2), have been cloned [2,3]. HSF activation is regulated by oligomerization, DNA binding or phosphorylation [4]. Additionally, a third component designated as constitutive HSE binding activity [5] was later shown to be the Ku autoantigen [6] which is involved in hsp70 gene regulation.

In contrast to most of the eukaryotic genes, heat shock genes are usually composed only of exons. However, human hsp90 gene contains introns [7]. The two copies of human hsp90 gene, hsp90α and hsp90β, have been sequenced entirely [8,9], and share much less homology compared with their protein products, particularly in the 5′ and 3′ non-coding region, the introns and the regulatory 5′ flanking sequences [8]. The protein products encoded by the hsp90 genes play the role of specific molecular chaperones in the regulation of cellular signal transduction, as shown in the signaling of steroid hormone and vitamin receptors [10,11], and further suggested through the findings of their participating in complex formation with a number of protein tyrosine kinases and serine/threonine kinases [12–14].

While the chaperone functions of HSP90 have been intensively studied, the regulation of hsp90 gene expression remains unexplored [15–17]. Hsp90 genes are usually constitutively expressed in most mammalian cell types and can be further induced by heat shock. We have found that hsp90 gene expressed in human T lymphocytes can not only be induced at an elevated temperature (40–42°C heat shock) but also expressed in mitogen activated T cells [18–20]. Evidently, the two stimulants induce different functions in T cells. Preliminary findings from our laboratory indicated that sequences upstream of the human hsp90β gene, while conferring efficient constitutive expression in human Jurkat cells, were insufficient in directing heat shock-inducible expression [21]. In this paper, we present evidence of the essential role of the first intron and the synergistic role of the intron and the upstream sequence in the regulation of human hsp90β gene expression.

2. Materials and methods

2.1. Preparation of whole cell extracts (WCE)

The WCE was prepared according to Mosser's method [22] with slight modifications. 2×10^7 Jurkat cells cultured under normal condition (37°C), heat shocked at 43°C for 30 min, or activated by PHA (1% v/v, GIBCO-BRL) for 18 h were suspended in 50 μl of PBS respectively, and stored at −70°C overnight. Three hundred μl of lysis buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) were added into the frozen pellets with gentle mixing, then centrifuged at $12\,000\times g$ for 40 min at 4°C. The resulting supernatants were stored at −70°C until use. The concentration of protein in the supernatants was determined by the method of Bradford [23].

2.2. Polymerase chain reaction (PCR)

In each PCR experiment, 0.5–0.6 μg of template genomic DNA, 12.5 pmole of each primer, each one of the dATP, dGTP, dCTP and TTP at 2.5 mM, 0.6 U of Taq DNA polymerase, 2.5 μl of the 10×PCR reaction buffer (100 mM KCl, 100 mM Tris-HCl pH 8.5, 15 mM $MgCl_2$, 0.1% Triton X-100) were added and didistilled water was added to make up to 25 μl. Each PCR cycle applied in the first experiment was 94°C for 1 min, 57°C for 1 min, 72°C for 4 min; in the second experiment was 94°C for 1 min, 45°C for 1 min and 72°C for 4 min. After 30 cycles, the reaction mixture was further incubated at 72°C for 10 more minutes.

2.3. Cloning of the regulatory region of the hsp90β gene

By using a pair of synthesized oligonucleotide primers (the 5′ primer with a *Sac*I site, P1: 5′-GCGAGCTCCGGCTGCCCTGCAC-3′; the 3′ primer with an *Eco*RI site, P2: 5′-GCGAATTCGCAAC GTAGGCTTGCTTTCCGA-3′) and the template DNA extracted from a λGEM-11 genomic library from human peripheral blood leukocyte, the −1102/+67-bp DNA fragment containing the 5′ flanking sequence of hsp90β gene was obtained by PCR. The two ends of the amplified PCR product were first filled-in to yield a blunt-ended fragment. Following digestion at a *Eco*RI site that was designed specifically in the

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3′ primer, the fragment was inserted into pGEM-4Z vector (Promega) at the *Eco*RI and *Hin*cII sites. The recombinant plasmid was then digested at *Sac*I site and *Eco*RI site, to release a fragment spanning −1102 through +67 of the hsp90β gene. The recovered fragment was then inserted into the respect cloning sites of a pBluescript-KS vector. The construct was designated as pHSP90β-1. To orient the *Pst*I and *Eco*RI fragment (−1039/+67) of pHSP90β-1, it was recovered and then inserted into a second pBS-SK vector.

The first exon and intron spanning from −2 to +1531 bp of hsp90β was also amplified from the above mentioned genomic DNA template with another pair of oligonucleotide primers (the 5′ primer with an internal *Xho*I site, P3: 5′-GTAGCTCTCTCGAGTCACT-3′; the 3′ primer with a *Vsp*I site, P4: 5′-AAAATAAAAATCTCATTAAT-3′) and then inserted into a pCRII vector (Invitrogen) designated as pHSP90β-2. The plasmid was partial digested with *Xho*I to recover a fragment started from +8 of the first exon of hsp90β gene and ended at a *Xho*I site in the multi-cloning site of the pCRII vector. The 1.5-kb recovered fragment covering +8 ∼ +1531 bp of the hsp90β gene and an additional 38-bp fragment from the pCRII vector in its 3′ was inserted into the *Xho*I site at +7 of the first exon of pHSP90β-1 in the oriented intermediate plasmid, we thus obtained a 2.5-kb fragment of hsp90β gene in the pBS-SK vector designated as pHSP90βWT.

2.4. Construction of the chloramphenicol acetyl transferase (CAT) reporter plasmids

The −1039/+1531 fragment in the plasmid pHSP90βWT was recovered through digestion with *Pst*I and partially digested with *Xho*I, and then ligated to the upstream of a CAT plasmid (pBLCAT3) to form the reporter plasmid hsp90βwt/CAT according to the techniques described by Sambrook et al. [24]. Depending on the construct, mutants with either 5′ or 3′ truncations, or with internal deletions were individually constructed through restriction cleavage either at the *Bam*HI site of the upstream sequences, the *Xho*I site of the first exon or at the *Bgl*II sites in the first intron of hsp90β gene, and ligation of the mutant fragments to the upstream of the CAT reporter gene.

2.5. Cell culture and transfection

Jurkat cells were cultured in RPMI 1640 medium (GIBCO-BRL) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2. The procedure of transfection was performed by using Lipofectamine (GIBCO-BRL) following the manufacturer's instruction with slight modifications. The mixture of 2 μg of total DNA, which consisted of reporter plasmid and pSV-β-galactosidase plasmid (Promega) used as an internal control at the ratio of 3:1, and 10 μl of lipofectamine in 200 μl reduced serum medium of Opti-MEM 1 (GIBCO-BRL) were added to 800 μl of cell suspension at a density of 2–5×10^6 cell/ml in 35 mm plastic dishes. After incubation at 37°C for 5 h, 9 ml of RPMI 1640 medium with 10% FCS was added and cells were incubated for an additional 48 h, then followed by treatment of heat shocked at 42°C for 40 min or 1% PHA (GIBCO-BRL) activation at 37°C for 18 h. For each reporter gene construct, three independent transfection experiments were performed.

2.6. CAT and β-galactosidase activity assays

Transfected cell extracts were prepared by three cycles of freeze-thawing in 100 μl of 0.25 M Tris-HCl (pH 7.8). Extracts were clarified by centrifugation at 12000×*g* for 10 min at 4°C. For CAT assay [24], to each 20 μl aliquot of cell extract, 30 μl of 0.1 M Tris-HCl (pH 7.8) were first added and incubated at 65°C for 15 min, and then, 200 μl of CAT reaction buffer [0.125 M Tris-HCl (pH 7.8), 1.25 mM chloramphenicol, 0.1 mCi ^{3}H-acetyl CoA (10 mCi/mmol, Amersham)] was added, mixed and incubated at 37°C for 4–8 h. CAT activities were detected by transferring samples into individual counting vials containing 5 ml of scintillation liquid for ^{3}H counting in a Beckman Scintillation Counter LS-6500. For β-galactosidase assay, 5 μl of cell lysates were mixed with 20 μl of LumiGal reaction buffer (Clontech Laboratories) and kept at room temperature for 60 min followed by adding 30 μl of LumiGal accelerator, the fluorescence was then measured in the Monolight 2010 Luminometer. CAT activity was individually normalized by comparing the activities with those of β-galactosidase from the same cell extract.

2.7. Electrophoretic mobility shift assay (EMSA)

DNA fragments were labelled by α-^{32}P-dCTP (3000 Ci/mmol) filling-in reaction and used as probes at 5×10^4 cpm in each experiment in the presence of 10^4-fold excess of sonicated salmon sperm DNA, with or without 10 μg of individual WCE. For competition analysis, unlabeled DNA fragment were added in excess as indicated. Binding reaction was carried out in DNA binding buffer [10 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM EDTA and 50% glycerol] at 22°C for 30 min as described by Ausubel et al. [25] with slight modification [26]. DNA-protein complexes were analysed on 4% polyacrylamide gels (acrylamide/bisacrylamide, 19:1) run in Tris-borate/EDTA buffer, pH 8.3. Gels were then dried and autoradiographed. The same amount of WCE used in an individual EMSA was dotted onto nitrocellulose filter (Schleicher & Schuell), stained with 0.1% Amidoblack 10B and was shown at the bottom of each lane in Fig. 2. In the supershift assay, whole cell extracts were reacted at 4°C for 30 min with the anti-HSF1 serum diluted at different ratio in PBS before adding to the mobility shift assay system.

2.8. Primer extension

Primer extension was performed with the method introduced by Sambrook et al. [24] with slight modifications. Synthesized 30-mer primer (P5 shown in Fig. 3c) was labeled at the 5′ end with [γ-^{32}P]ATP (5000 Ci/mmol) by T4 polynucleotide kinase. 20 μg of total RNA extracted from Jurkat cells with or without heat shock or after 1% PHA (GIBCO-BRL) stimulation was hybridized with 10^5 cpm of a ^{32}P-labeled P5 in 30 μl hybridization solution [80% formamide, 40 mM PIPES, 0.4 M NaCl, 1 mM EDTA] at 22°C overnight after denaturing at 85°C for 10 min. Following the addition of 90 μl of water, 12 μl of 3 M NaAc and 360 μl of ethanol into the mixture, the hybrids were precipitated at −70°C for 30 min, centrifuged at 12000×*g* for 20 min at 4°C and washed with 75% ethanol sequentially. The hybrids were dissolved in 25 μl of reverse transcription buffer [50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM of each dNTP, 10 mM DTT, 25 U of RNasin]. Reverse transcription was then carried out at 42°C with the addition of 40 U of AMV reverse transcriptase (GIBCO-BRL) for 120 min. The reaction was then stopped by adding 1 μl of 0.5 M EDTA and 1 μl of 1 mg/ml RNase A and incubating at 37°C for 30 min. The primer extension products were electrophoresed on 6% polyacrylamide gel containing 7 M urea in Tris buffer pH 8.3, followed by drying and autoradiography.

2.9. RNase protection assay

^{32}P-labeled RNA probe was transcribed in vitro from a linearized template DNA by T3 RNA polymerase at 30°C for 60 min in a reaction system following Sambrook et al. [24] with slight modifications. After digestion with RNase-free DNase at 37°C for 15 min, the probe RNA was subjected to phenol/chloroform extraction and ethanol precipitation.

Cellular RNAs from differently treated cells were prepared by GITC/phenol method [24] and precipitated in ethanol. The pellet of 150 μg cellular RNA was first dissolved in 30 μl of hybridization buffer [40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 400 mM NaCl, 80% formamide] containing 5×10^7 cpm of probe RNA, incubated at 85°C for 10 min, and then quickly transferred to 52°C for hybridization overnight. RNase digestion was performed by the addition of 300 μl RNase digestion buffer [300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 7.5), 40 μg/ml RNase A, 500 U/ml RNase T_1] to the hybridization mixture and incubated at 30°C for 30 min. The digestion was then stopped by the addition of 20 μl of 10% SDS and 10 μl of a 10 mg/ml solution of Proteinase K and incubated at 37°C for 15 min. The protected dsRNA was then extracted with phenol/chloroform, precipitated in ethanol, and then analyzed on an 8% sequencing gel.

3. Results

To investigate the regulation of the human hsp90β gene, we constructed three classes of promoter fusions to the chloramphenicol transacetylase (CAT) gene (Fig. 1). These constructs carry different portions of a wild-type 2571-bp region (−1039/+1531) encompassing the 5′ upstream sequence, the first exon, and the major part of the first intron which ended at +1532. The 5′ upstream region of hsp90β gene contains a cAMP

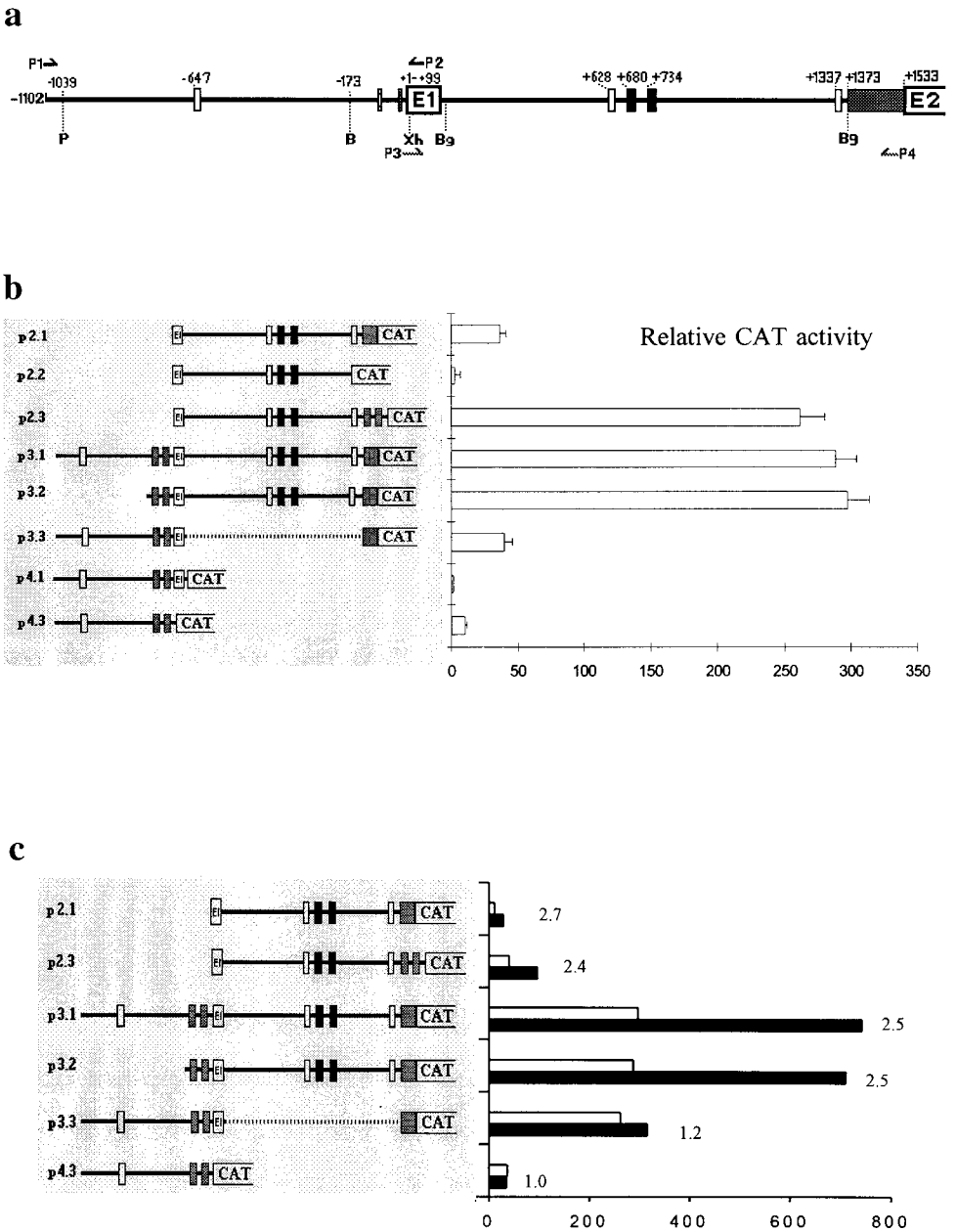

Fig. 1. Schematic diagram of the regulatory regions of the human hsp90β gene and levels of constitutive expression and heat inducibility from different hsp90β-CAT fusion constructs. (a) Diagram corresponds to sequences (−1102 bp) from within the 5′ upstream sequences of the human hsp90β gene through to the second exon (E2). Arrows indicated the locations of primers for PCR. The endonuclease restriction sites used in the cloning of the reporter plasmids are indicated [P: *Pst*I (−1039); B: *Bam*HI (−173); Bg: *Bgl*II (+109, +1373); Xh: *Xho*I (+7)]. The three open boxes, one in the 5′ flanking region and two in the first intron, indicate atypical HSEs and the two filled boxes in the intron indicate typical HSEs. The two narrow boxes between −173 and +1 represent a CAAT sequence at −87 and a TATA box at −27. The shaded box at the 3′ end of the first intron corresponds to the AT-rich region spanning +1373 to +1531. (b) The relative levels of CAT activities are defined by the ratio of the CAT activity of the hsp90β fusion constructs relative to the activity of a co-transfected β-galactosidase control. Two shaded boxes at the 5′ of exon 1 (E1) indicate the UPE/CP fragment (−173/+7); another shaded box at the 5′ end of the CAT gene corresponds to the AT-rich region (+1373/+1531). The three open boxes indicate atypical HSEs and the two filled boxes in the intron indicate typical HSEs. The dotted line corresponds to the fragment (+109/+1372) deleted in p3.3. Standard deviation of each experiment is shown at the top of each bar. (c) The filled and open bars in the right panel indicate the constitutive (37°C) and heat inducible (43°C for 30 min) levels of CAT activities driven by individual deletion constructs. The numbers on the right side of the figure show folds of heat induction. Other label markers are the same as in Fig. 1b.

response element (CRE) at −126, the upstream promoter element (UPE) carrying a CAAT sequence at −87/−84 and a SP1 site at −51, the core promoter region (CP) which includes a TATA box at −27, and the initiation site at +1. The first intron contains two typical HSEs and a 3′ atypical HSE, followed by an AT-rich region at the extreme 3′ end of the intron (Fig. 1a).

The first series of CAT fusion constructs contain only the first exon and the major part of the first intron of the human hsp90β gene (Fig. 1b). p2.1 carries sequences between

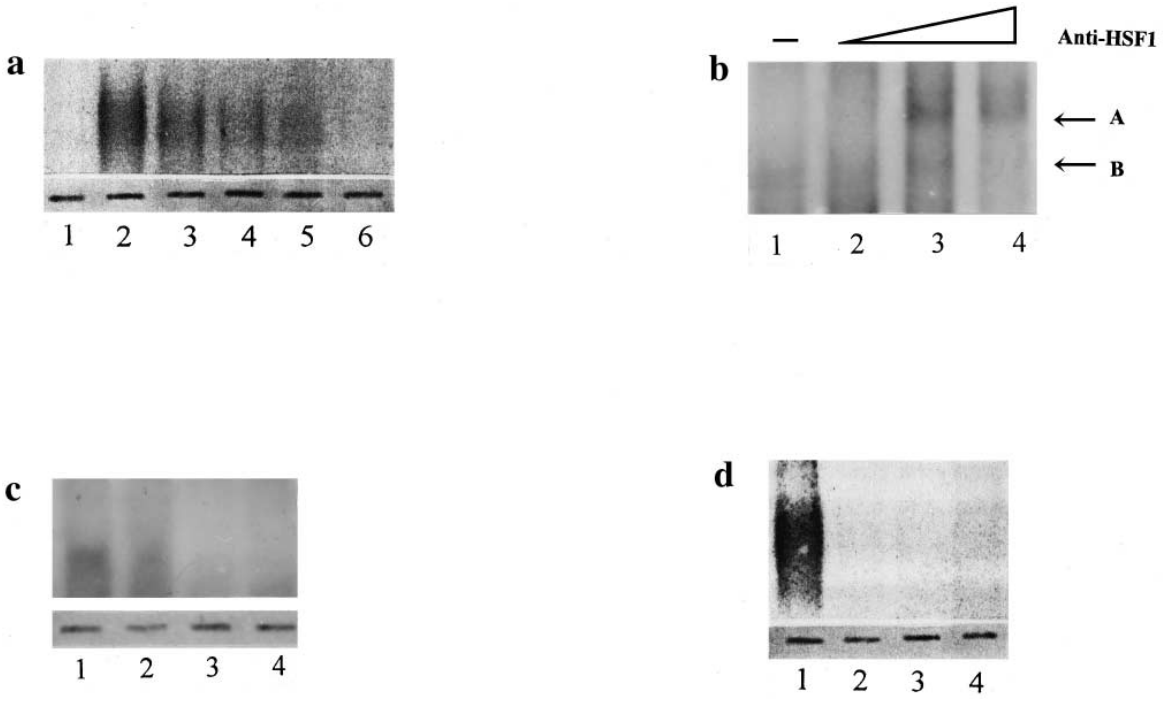

Fig. 2. Electrophoretic mobility shift assays of HSF binding to different HSEs in hsp90β gene. (a) An intronic DNA fragment of +634/+827 carrying the two typical iHSEs was used as the probe in EMSA. 4% PAGE profile of the DNA-protein complex formed with heat shocked cell extracts (lanes 2–5). The binding was competed by the addition of an 8-, 16-, and 32-fold excess of a specific competitor (unlabeled fragment of +634/+827) (lanes 3–5). Extracts of control cells (lane 1) and PHA-activated cells (lane 6) did not show binding. (b) Specificity of DNA binding protein in the complex with ^{32}P-labeled probe of +633/+827. EMSA was performed in the absence of serum (lane 1), 1:50, 1:10 and 1:2 dilutions of the anti-HSF1 sera individually incubated with the whole cell extract before starting the EMSA (lanes 2–4, respectively). (A) The supershift band with the addition of anti-HSF1 serum. (B) The protein/DNA complex without specific antiserum. (c) EMSAs carried out with the same probe as in (a) (lane 1); over 100-fold molar excess of each of the followings were added as competitors: a fragment from the 5′ flanking region with atypical HSE (−771/−567) of the hsp90β (lane 2); a synthesized oligonucleotide with 4–6 typical HSE motifs in a tail-to-tail alignment (lane 3); and the unlabeled fragment of +633/+827 (lane 4). (d) EMSAs carried out using a labeled 19-mer oligonucleotide corresponding to +735/+753 of the first intron. The binding activity from the heat shocked cells (lane 1) was competed by a longer fragment of the intron +634/+827 at 8-, 16-, and 32-fold molar excess (lanes 2–4). Equal amounts of whole cell extracts were added to each of the lanes of the EMSA and the slots shown at the bottom of the lane.

−2/+1531, encompassing the intronic HSEs (iHSEs) and the AT-rich region. p2.2 is a mutant of p2.1 with the 3′ atypical iHSE and AT-rich region deleted (−2/+1195). In p2.3, the AT-rich fragment (+1373/+1531) is replaced by the proximal 5′ sequence between −173/+7. The second series of constructs are derived from the full length construct p3.1 (−1039/+1531) which carries 5′ flanking sequences, the first exon, and the first intron. p3.2 (−173/+1531) has a 5′ truncation to −173. p3.3 is a mutant of p3.1, which carries a truncation of the most part (+110/+1372) of the first intron. The third series of constructs carry only the 5′ flanking sequences, p4.3 (−1039/+7), and the 5′ flanking sequences together with the first exon, p4.1 (−1039/+109).

3.1. Constitutive expression of the hsp90β gene

Using luciferase as a reporter, we previously found that the upstream sequence of hsp90β, from −1039 to +7, is very efficient in conferring a high level of constitutive expression in human Jurkat cells but it is ineffective in heat shock induction of the gene [21]. By determining the CAT activities of the wild type and mutant constructs described above, we found that the wild type, full length construct (p3.1) is 8–10-fold more efficient than the 5′ flanking sequence alone (p4.3) in conferring constitutive expression (Fig. 1b). A deletion of 5′ upstream sequences (p3.2) only slightly affected the level (97% of p3.1) of reporter activity; this construct p3.2 (−173/+1531) carries the upstream promoter element, the core promoter, the first exon and the first intron. A further deletion of the upstream sequence to −2 (p2.1) removing the UPE and CP reduced dramatically the expression of CAT (3.6% of p3.1). Together, these results show that the UPE, CP, and sequences within the first exon or the first intron are critical for high constitutive expression.

To further delineate the sequences in the first exon and first intron required for high constitutive expression, we examined additional constructs. In the 3′ end of the first intron, starting from a *Bgl*II site at +1373 downstream to the last nucleotide of the first intron, there is an AT-rich (65.8% A or T) region. If only the last 90 bp of the 3′ region of the first intron are taken into account, the AT percentage is even higher (~70%). However, in our experiments, we designate the 158-bp segment between +1373 to +1531 as the AT-rich region. Two constructs, with truncations in either the 5′ flanking sequence (p3.2) or deleted for most parts of the first intron (p3.3), show similar constitutive activities in CAT assay (96.9% and 88.0% of p3.1, respectively). These results suggest that the AT-rich region of the first intron together with the upstream promoter (the UPE and CP sequences) is sufficient for efficient constitutive expression of the hsp90β gene. When the AT-rich fragment is replaced with the UPE/CP sequences in construct

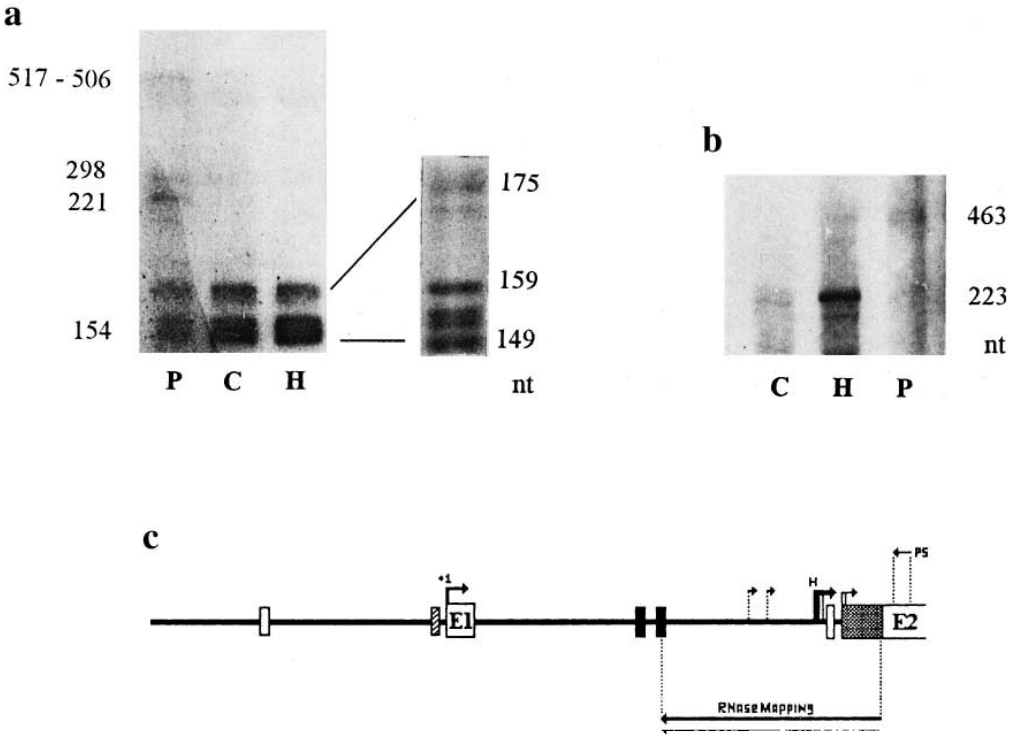

Fig. 3. Transcription initiation sites in the first intron of hsp90β gene. (a) Electrophoretic profile of primer extension products initiated from P5 within the second exon and run on a 1.5% agarose gel (left panel). The right panel indicates the lengths (nt) of the extension products separated on a 6% denaturing polyacrylamide gel. mRNA was extracted from control cells (C), from heat shocked cells (H), and from PHA-activated cells (P). (b) Electrophoretic profile of RNase protection products run on an 8% denaturing polyacrylamide gel. C, H and P correspond to the same samples as in (a). nt: the approximate lengths of the protected products. (c) Schematic map of the initiation sites within the first intron of the hsp90β gene determined by both the primer extension assays and the RNase protection assay. Arrows with solid lines indicate the initiation site at +1 as well as the defined initiation sites in the first intron. Dotted lines indicated some unidentified sites within the intron. The H designates the heat-inducible start site.

p2.3, the CAT activity was reduced to only 13.4% of p3.1 activity.

Transcription initiated from UPE/CP (−173/+1) is equally efficient under the control of either the untruncated 5′ flanking region (p4.3) or the first exon and intron lacking the AT-rich sequence (p2.3), suggesting that neither of these regions significantly contribute to promoter activity. The observation that some promoter activity is observed in the complete absence of the UPE/CP sequences (p2.1) suggests the presence of additional initiation sites, and the fact that the expression of CAT gene is completely abolished when both of the UPE/CP and AT-rich fragments are deleted (p2.2) indicate that the AT-rich region in the 3′ of the first intron is required for the residual activity. One additional conclusion from our promoter activity assays is that the first exon may contain certain sequences that significantly down-regulate the transcription activity of the UPE/CP since the construct carrying the first exon (p4.1) shows significantly lower activity than a construct lacking the first exon (p4.3).

3.2. Heat-inducible expression of the hsp90β gene

We examined the 5′ flanking sequences for the conserved 5-nucleotide motif of the heat shock element but only found one HSE-like sequence located at −648/−634 (**GGAAACTG-CTGGAAA**). Since one nucleotide differs from the typical mammalian HSE motif we have designated this sequence an atypical 5′ HSE (indicated with an open box in the related figures). After further analysis, we found two typical HSEs in the first intron designated iHSE-1 at +680/+695 (**GTTCTG-GAAGATTCA**) and iHSE-2 at +733/+747 (**GTTCTG-GAAGCTTCT**). Both of these typical intron HSE sequences contain three sets of the nGAAn consensus motif and are designated by filled boxes in the figures. In the first intron, there are two more HSEs at +628/+642 (**CTTCCA-GATCTTTCT**) and +1337/+1346 (**TGAATTTTCA**) designated iHSE-3 and iHSE-4 respectively, which contain either one mutation in the three motifs or only two of the typical motifs and are therefore atypical, and were shown in Fig. 1 as two open boxes, one upstream and the other downstream of the typical two iHSEs. In comparison to the 14-mer CnnGAAnnTTCnnG consensus sequence identified in *Drosophila* HSE [1,27] the sequence spanning +1335/+1348 shows only one variation. We examined the functions of these typical and atypical iHSEs using the constructs described above.

In preliminary experiments, we found that the 5′ flanking sequence of hsp90β gene does not respond to an elevated temperature of 42°C, regardless of the presence of the 5′HSE [21]. In this paper, we confirmed that the 5′ flanking region (p4.3) does not confer heat inducibility to CAT expression (Fig. 1c). However, the wild type full-length construct (p3.1) not only showed high constitutive expression, but was also induced 2.5-fold by heat shock. In addition, a construct in which the 5′ upstream sequences were deleted (p3.2) showed similar heat inducibility. This suggests that the atypical 5′ HSE is not essential and does not by itself regulate heat inducibility. In contrast, when sequences including the typical intronic HSEs are removed (p3.3), heat inducibility is almost abolished. These results suggest that iHSEs of the first intron

play a unique role in heat inducible expression of hsp90β gene. In addition, our observation that both p2.1 (carrying the AT-rich sequence but lacking the UPE/CP sequences) and p2.3 (lacking the AT-rich sequence but carrying the UPE/CP sequences) are heat inducible despite low promoter activity suggests that the intronic sequences can confer heat inducibility to either type of promoter element.

3.3. HSF binding to HSEs of the hsp90β gene

To compare the function of the upstream atypical HSE with the functions of the typical HSEs in the intron, we prepared cell lysates from heat shocked, PHA activated and untreated Jurkat cells. We then carried out electrophoretic mobility shift assays (EMSA) with different promoter and intronic fragments. We found very strong binding activity toward the intronic HSEs (+634/+827) in the lysates of heat shocked cells; while the lysates from control and PHA-activated cells did not show any binding activity (Fig. 2a). The binding complex was found to consist mostly of heat shock factor-1 (HSF1), as determined by antibody supershifting (Fig. 2b). This binding of HSF1 to the iHSE fragment was specific, as it could be competed by higher level of unlabeled iHSE fragment (Fig. 2c, lane 4). However, binding could not be competed by the 5′ HSE-containing fragment of either −771/−567 shown in lane 2 of Fig. 2c, or by another fragment of −651/−174 (data not shown), indicating that the upstream HSE is a substantially weaker site for HSF-1. As shown in lane 3 of Fig. 2c, the binding complex of intronic HSEs is readily competed by a synthetic oligonucleotide with typical tail-to-tail HSE motifs [1] indicating iHSEs in the 193-bp probe are the binding motif of the HSF1 in the cell extracts. In addition, when a synthetic 19-bp oligonucleotide (+735/+753) carrying the sequence of one iHSE was used as a labeled binding site in EMSA, a clear complex was formed that was fully competed with an 8-fold excess of the +635/+827 intron fragment (Fig. 2d). This observation indicates that nucleotides immediately flanking the iHSEs have a modest contribution to high affinity binding, although it is also likely that this may be due to an end-effect of the short DNA fragment. In summary, our results show strong binding of HSF1 to the iHSEs of the first intron of the hsp90β gene. We have also found that low concentrations of recombinant HSF1 bound tightly to the intronic HSE fragment while HSF2 shows much lower affinity to the fragment (data not shown).

3.4. Novel initiation sites within the first intron

Our findings that a construct (p2.1) containing only the first exon and first intron but lacking the upstream promoter element and core promoter (UPE/CP) still showed some constitutive expression (Fig. 1b), as well as a 2.7-fold heat inducibility (Fig. 1c), suggested that initiation sites may be present in the first intron of the hsp90β gene. To explore this possibility, we synthesized a 30 nucleotide oligonucleotide complementary to the 5′ end of the second exon (+1579/+1608, see Fig. 3c) and carried out primer extension assays (Fig. 3a). An extension fragment of 175 nucleotides and several bands between 149 and 159 nucleotides were observed on a sequencing gel. The 175 nucleotide band could correspond to initiation at either the G residue at +1434 in the intron, or at the known +1 initiation site of the spliced hsp90β transcript identified by Rebbe et al. [9]. The smaller bands could correspond to transcripts initiating from the 3′ end of the first intron or to start sites within the first exon of the spliced transcript.

To further test for the existence of novel initiation sites within the first intron, we performed a RNase protection assay by using a RNA probe transcribed from the 3′ end of the first intron toward a *Hin*dIII site located in one of the two typical iHSEs (+1531/+740). Results from this assay confirmed that some constitutive transcripts from the hsp90β gene initiate from sites within the first intron (lane C, Fig. 3b). Importantly, an initiation site located downstream of the two typical iHSEs was particularly evident in the heat-induced RNA sample (lane H, Fig. 3b).

4. Discussion

The major points of this paper can be summarized as follows: (1) the first intron participates in the high level constitutive expression of the human hsp90β gene in Jurkat cells; (2) the first intron is critical for heat inducibility of the gene; (3) HSF1 tightly binds to two typical HSE elements in the first intron; (4) initiation sites are present within the first intron and some of these are strongly inducible by heat shock. We have also shown that an AT-rich fragment located in the 3′ region of the first intron is sufficient for efficient constitutive expression of hsp90β gene in Jurkat cells; however, it does not confer heat inducibility in the absence of other intronic sequences.

The primer extension and RNase protection experiments show that there are multiple transcription start sites located within the 3′ of the first intron, we thus examined whether there is any TATA box or initiators in the region. We found that there is no TATA box which fits the TATA consensus sequence in the first intron, whereas there are at least 8 consensus sequences (PyPyATN/APyPy) of an eukaryotic initiator [28] located in the 3′ of the intron between +1007 and +1530 of the hsp90β gene. Among them, there are 5 motifs located at +1097/+1103, +1117/+1123, +1160/+1166, +1234/+1240, +1268/+1274 which carry the most commonly seen sequences of a C at the −1 position and an A at +1 (initiation) site, and are very likely to be related to the multiple transcription initiation sites within the intron of the gene.

It has been known that the start site of the translation of hsp90β mRNA is the ATG (+1533/+1535) at the beginning of the second exon. The transcripts initiated from the multiple sites within the intron may result in alternative 5′ ends of the hsp90β mRNA. In order to understand the importance of these distinct 5′ ends of the transcripts in translational control, we have scanned for all the possible ATGs in the sequences transcribed from the first intron. We found that each one of the five ATGs found in this region is always followed by an in-frame stop codon and suggested that the ATGs available upstream of the known start codon do not have any impact on the N-terminal coding sequences of HSP90β. However, the production of some small peptides from the 5′ non-coding region of a mRNA may decrease the translational efficiency of the long open reading frame of the mRNA. Whether this is also applicable to hsp90β gene and its importance in translation control are remained to be explored.

Comparing the relative CAT activities driven by the upstream sequences (p4.3) and that of the full length construct including the first intron (p3.1), we have found that the p3.1 is 7.8-fold more efficient than p4.3 for the constitutive expres-

sion (Fig. 1b), while the efficiency difference further expands to 21.2-fold upon heat shock (Fig. 1c). Using the 3′ fragment of the intron and the first exon as individual probes to hybridize to the total cellular RNA extracted either from the untreated or the heat shocked Jurkat cells, we found that the RNA from untreated cells is 4.2 times more efficient to hybridize to the intron fragment than to the first exon of the gene; in the heat shock cells, the hybridization efficiency of RNA to the intron probe is approximately 20.2-fold higher than that of the first exon (Wang, X. and Shen, Y.F., to be published). In other words, in the constitutive expression of hsp90β gene, 80.9% of the transcripts are initiated from the intron promoters; while upon heat shock, almost all of the inducible transcription are driven by the intron promoters (95.3% of the total). These results indicates that the multiple transcription initiation sites within the first intron play an absolutely dominant role in heat shock inducible expression of the human hsp90β gene in Jurkat cells.

Based on our findings, we conclude that the typical iHSEs in the first intron of the human hsp90β gene play critical role as heat shock-dependent enhancers of transcription and that initiation from within the first intron is important for the high constitutive expression as well as highly efficient heat inducibility of the hsp90β gene.

The constitutive expression of hsp90β gene is extremely efficient in Jurkat cells, generally 8- to 20-fold higher than the expression of the gene driven by a CMV promoter or a SV40 early promoter (data not shown). The high expression of the gene is at least in part due to multiple promoters some lying within the first intron. These observations for the hsp90β gene are interesting in relation to a proposal by Westwood et al. [29] suggesting that during heat shock, the binding of HSF to the HSEs downstream of the initiation sites of some developmentally regulated *Drosophila* genes may repress gene transcription. In our case, the HSEs located in the intron clearly do not play an inhibitory role; but they apparently stimulate transcription from initiation sites located still further downstream in the intron.

Sequence comparisons of the upstream sequences of mouse hsp84 and human hsp90β genes show that the promoters share 85% homology suggesting that the regulation of mouse and human should be similar. However, Dale et al. [30] reported that a 0.6-kb upstream sequence of the mouse hsp84 gene was sufficient for the constitutive and heat inducible expression of the mouse gene. This observation is somewhat at variance with our previous results [21] and with the data in Fig. 1b and c of this paper, although the mouse hsp84 study did not evaluate possible contributions of the first intron of the mouse gene. Further studies will be required to determine whether role of intron sequences on hsp90 gene regulation is conserved amongst mammals. Studies are now in progress to examine aspects of the mechanism of transcriptional regulation from the first intron of the hsp90β gene.

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