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The tumor-selective over-expression of the human *Hsp* 70 gene is attributed to the aberrant controls at both initiation and elongation levels of transcription

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ABSTRACT

The tumor selective over-expression of the human Hsp70 gene has been well documented in human tumors, linked to the poor prognosis, being refractory to chemo- and radio-therapies as well as the advanced stage of tumorous lesions in particular. However, both the nature and details of aberrations in the control of the Hsp70 expression in tumor remain enigmatic. By comparing various upstream segments of the Hsp70 gene for each's ability to drive the luciferase reporter genes in the context of the tumor cell lines varying in their p53 status and an immortal normal liver cell line, we demonstrated in a great detail the defects in the control mechanisms at the both initiation and elongation levels of transcription being instrumental to the tumor selective profile of its expression. Our data should not only offer new insights into our understanding of the tumor specific over-expression of the human Hsp70 gene, but also paved the way for the rational utilization of the tumor selective mechanism with the Hsp70 at the central stage for targeting the therapeutic gene expression to human tumors.

Key words: *Hsp 70, Tumor, transcription elongation, 5' UTR, Over-expression.*

INTRODUCTION

Under the adversary conditions including heat shock (hyperthermia), the abundance of a group of proteins (heat shock proteins, Hsp) rapidly goes up in cell. Chiefly via direction interaction, heat shock proteins assist diverse protein substrates with vital biological functions in their folding, translocation, and refolding of intermediates. The majority of member of heat-shock proteins would mend or degrade the stress-induced misfolded proteins, thus restoring protein homeostasis and promoting cell survival. According to the molecular weight initially and the sequence homology late on, the heat-shock protein family consists of six groups, i.e., Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 ad small Hsps. It becomes evident that the heat shock proteins display tremendous diversity in all their attributes. Some members are constitutively rather than inducibly expressed. Furthermore, distinctions in function, structure, regulation of expression, intracellular compartmentation and interaction partners have their representation in all subgroups. Finally, it has been paradoxically demonstrated that expression of the heat-shock protein genes is also influenced by various physiological events that are not typically associated with cell stress, including cell cycle, cell proliferation, and differentiation[1], and reference within].

In addition to its unique function in protecting the cells from stress-related damages, the heat shock proteins including Hsp70, have caught a great attention in cancer field by its aberrant expression in all the human tumors in general and physically in-

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teracted with the cellular proteins of vital biological importance including the tumor suppressors, p53. The over-expressed Hsp70 at levels of both the steady state mRNA and protein has been detected in almost all classes of tumors examined[2, 3] and is closely associated with the poor prognosis, resistance to the chemo- and radio- therapies, as well as the advanced stage (metastastic) of human cancers[4-7] For instance, the Hsp70 RNA was found five-fold higher in the metastastic derivative than in the otherwise sibling in an expression profiling type study with an elegant mouse model of the metastatic melanoma[8]. The suggested etiological role of the Hsp 70 in tumor has been substantiated by the observation of induced apoptosis and tumor regression in animal tumor models being directly linked to the artificially forced repression of its expression[9]. However, the alternative explanation remains difficult to be eliminated for tumor selective over-expressed Hsps, including Hsp70, as simply the consequence with no causative significance in the tumor genesis of the hostile environments where the tumor lesions reside and proliferate.

The Hsp70 gene expression is subjected to the multiple controls. Beside the transcription initiation, the mechanisms at the levels of transcription elongation[10], RNA turn-over[11, 12] and translation[13] are also actively involved. The molecular dissection of the Hsp genes of various organisms in the content of heat-shock response resulted in identification of the key heat-shock responsive element (HSE, Heat-shock element) and its cognate transcription factor (HSF)[14]. It comprises multiple adjacent inverted arrays of the binding site (5'-nGAAn-3'), interaction of which with its cognate transcription factors (HSFs) after polymerization and nucleus-entering lead to both elevation of the transcription initiation and abolishing pausing of the engaged RNA polymerase II in the stressed cells (+25 to +45 at downstream of the transcription initiation site)[15-18]. However, little details are available of the underlying mechanisms for the transcription initiation and elongation of the Hsp70 gene in both normal and disease conditions, including tumors.

The tumor suppressor protein, p53 is mutated in over 50% human tumors of various origins, indicating its paramount role in preventing the malignant transformation. It acts as a dual functional transcription regulator, either positively or negatively controlling upon the transcription of a spectrum of genes, many of which have the proven roles in the cell cycle progressing and apoptosis of cells[19, 20] The Hsp70 gene is one such gene, transcription of which is repressed by wild type p53(wtp53)[21]. Not only some mutant p53 proteins (mtp53) fail to repress, some but also gain the capacity of trans-activating the Hsp70 promoter activity, which seemed to be mediated by the proximal HSE element via the unknown mechanisms[22], and reference within]. Therefore, a plausible working model has been proposed that the tumor selective over-expression of the hsp70 gene is at least partially attributed to the prevalent loss of the wtp53's transcription repressing function and gaining of the mtp53 trans-activating function in human tumors. This property has been utilized in a novel tumor targeting strategy of the therapeutic gene expression to the p53 defective tumor[42].

In consideration of the importance of the regulation over the elongation process of transcription mediated largely by the region between the transcription and translation initiation sites[10, 23, 24], it is desirable to find out whether the elongation control mechanism is altered in human tumors and contributes the tumor selective over-expression of the human Hsp 70 gene. In this work, we have employed a spectrum of the molecular genetic approaches with a panel of cell lines including three tumor cell lines differing in the p53 status and an immobilized "normal" cell line to address these questions at a considerable detail. Our data demonstrated that the tumor selective aberration of the elongation control mechanism contribute significantly to the elevated expression of the Hsp70 gene in human tumors and completed the some characterization of the cis-elements involved in this process.

MATERIALS AND METHODS

Reporter constructs and mutagenesis

The desired upstream fragments of the human Hsp70 gene (Accession: M11717, M15432) were PCR cloning from the genomic DNA of the normal human embryonic fibroblast cells with the primers listed below, into the luciferase based reporter constructs pGL3 basic (Promega, USA)at Bgl II site. The constructs were named as cd (-273 to +215), cb (-273 to +26), ab (-116 to +26) and ad (-116 to +215), respectively (Fig 1).

Oligonucleotide a: 5' -ATA GAT CTG CGA AAC CCC TGG AAT ATT C -3'C
Oligonucleotide b: 5' -ATA GAT CTA GCA GCT CCT CAG GCT AGC -3'
Oligonucleotide c: 5' -ATA GAT CTC GCC ATG GAG ACC AAC AC -3'
Oligonucleotide d: 5' -ATA GAT CTA AGC TTG CGG TTC CCT GCT CTC TG -3'

N.B., the underlying sequence is the recognition site of Bam HI, or Hind III.

Various fragments of the Hsp70 promoter created by PCR

Primer	cd(491bp)	ab(142bp)	cb(299bp)	ad(334bp)
oligo a		√		√
oligo b		√	√	
oligo c		√	√	
oligo d	√			√

Eleven linker scanner mutants of the bd (+26 to +215) region of the human Hsp70 gene in cd were made essentially according to the previously described protocol[25, 26] , except for which the 18mer linker (5' TCT AGA GAA TTC GGT ACC 3': the recognition sequences of Xba I, Eco RI, and Kpn I, sequentially) was used to replace the 18 nucleotide sequence beginning from +26 sequentially to result in eleven cd based constructs (M1-M11) with the primer pairs below:

m1L: 5'-TCT AGA GAA TTC GGT ACC CAG CAG CTC CTC AGG -3'
m1R: 5'-GGT ACC GAA TTC TCT AGA TTC GAG AGT GAC TCC -3'
m2L: 5'-TCT AGA GAA TTC GGT ACC AAA GGT AGT GGA CTG -3'
m2R: 5'-GGT ACC GAA TTC TCT AGA TGT CCC AAG GCT TCC -3'
m3L: 5'-TCT AGA GAA TTC GGT ACC ACG GGA GTC ACT CTC -3'
m3R: 5'-GGT ACC GAA TTC TCT AGA AGC GAA CCT GTG CGG -3'
m4L: 5'-TCT AGA GAA TTC GGT ACC CTG GGA AGC CTT GGG -3'
m4R: 5'-GGT ACC GAA TTC TCT AGA CAG GCA CCG GCG CGT -3'
m5L: 5'-TCT AGA GAA TTC GGT ACC CAG CCG CAC AGG TTC -3'
m5R: 5'-GGT ACC GAA TTC TCT AGA GTT TCC GGC GTC CGG -3'
m6L: 5'-TCT AGA GAA TTC GGT ACC TCG ACG CGC CGG TGC -3'
m6R: 5'-GGT ACC GAA TTC TCT AGA GAC CGA GCT CTT CTC -3'
m7L: 5'-TCT AGA GAA TTC GGT ACC CTT CCG GAC GCC GGA -3'
m7R: 5'-GGT ACC GAA TTC TCT AGA GAT CCA GTG TTC CGT -3'
m8L: 5'-TCT AGA GAA TTC GGT ACC CGC GAG AAG AGC TCG -3'
m8R: 5'-GGT ACC GAA TTC TCT AGA CAG CCC CCA ATC TCA -3'
m9L: 5'-TCT AGA GAA TTC GGT ACC GAA ACG GAA CAC TGG -3'
m9R: 5'-GGT ACC GAA TTC TCT AGA CCG AGC CGA CAG AGA -3'
m10L: 5 '-TCT AGA GAA TTC GGT ACC CTC TGA GAT TGG GGG -3'
m10R: 5 '-GGT ACC GAA TTC TCT AGA GGG AAC CGC AAG CTT -3'
m11L: 5 '-TCT AGA GAA TTC GGT ACC TCG GCT CGG CTC TGA -3'
m11R: 5 '-GGT ACC GAA TTC TCT AGA AAG CTT GGC ATT CCG -3'

All the constructs were verified by the detail diagnostic restriction digestion.

The HA tagged p53 constructs were made by inserting the wt p53 and mutant p53 (Arg175his) cDNA into the CMV promoter based expression vector with three HA tag at the 5' end (unpublished result).

Cell lines, Cell Culture and transient-transfection assay

U20S (ATCC No. HTB-96, an osteosarcoma cell line, p53 status: wild type), 5637 (ATCC No: HTB-9, a bladder carcinoma cell line, p53 status: nil), C33A (a cervical cell line, p53 status: arg273cys) and Lo-2 (a human embryonic liver derivative cell line, p53 status: wild-type)[27], were cultured in DMEM medium (Gibco, USA) with 10% calf serum (Four Season Co. ltd. China) at 37 °C with 5% CO_2 atmosphere.

Transfection of the reporter constructs with $CaCl_2$/phosphate method was carried out on the cells at the log-phase of proliferation (no more than 70-80% confluence in the 24-well plate) as previously described[25, 26]. To each well, 0.5 μg of cd, ab, cb, and ad, respectively was co-transfected with 0.05 μg CMVRL (Promega, USA, where the renilla luciferase gene is driven by CMV promoter). The whole experiment was performed in duplicate and both luciferase activities of the cell extracts were measured with the dual reporter kit (Promega, USA), approximately 16-24 h post-transfection. The relative activity of the firefly luciferase over the renilla luciferase (representing the CMV promoter potency as the internal control for the transfection efficacy) was calculated. The mean and standard deviation (SD) of the each experiment was calculated according to the following formulas: SD (SQRT) = $[(\text{ratio1-mean})^2 + (\text{ratio2-mean})^2]/(2-1)$ and plotted. To quantify the p53's effect on the activity of each of the upstream segments,

the relevant value of which over the mock transfected (as 1, arbitrarily) was calculated and plotted.

To determine the relative abundance of the steady status luciferase mRNA, or Hsp70 RNAs, a semi-quantitative PCR was undertaken[25, 26]. The primer pair for the human b-actin (GI: 1474796, the sense: 5' AAGTACTCCGTGTGGATCGG 3', the antisense: 5' TCAAGTTGGGGACAAAAG 3' the product size : 614bp), the human Hsp 70 (M11717, sense: 5' CCCACCATTGAGGAGGTAGA 3' and antisense: 5' GGAATAAAAGGGCATCACTTG 3'; the product size is 331 bp) and the firefly luciferase (U47295, sense: 5' TGGGACGAAGACGAACAC and anti-sense: 5' TGACTGGCGACGTAATCC 3' 236 bp). The total RNA was made with Trizol solution according to manufacturer's instruction (Gibco, USA), and cDNA was made by the Supertranscript plus reverse transcriptase with the oligo-dT as primers (Gibco. USA). The PCR with either Hsp70 primers or luciferase primers for 15 cycles followed by another 15 cycle PCR reactions in presence of the b-actin primers (the parameter of each cycle is 94ºC 20", 60 ºC for 20" and 72 ºC for 30"). The resulted PCR products were visualized under UV illumination after an electrophoretic separation on a 1.2 % agarose and densitometrically scanned.

The protein extracts were made from various cells with the cell lysis buffer (20 mM Tris. HCl, 1 mM EDTA, 50 mM KCl, 0.5% NP-40) plus proteinase inhibitor cocktail (Roch, USA) and quantified with BCA protein assay kit (Pierce, USA). The proteins were separated in a 7.5 % denaturing polyacrylamide gel electrophoresis and blotted onto the immobiline membrane (Millipore, USA), as previously described[28]. The blot was subjected to anti-human Hsp70 antibody (HSP 70 (K-20) sc-1060, Santa Crutz, USA) and anti-PCNA (PCNA (PC10) sc-56, Santa Crutz, USA) detected by ECL (Pierce, USA), sequentially. The relative densities of the scanned bands were analyzed with SmartView Bioimage Analysis Software.

RESULTS

Tumor-cell-selective over-expression of the Hsp 70 gene

Tumor selective elevation of the Hsp70 gene expression has been well documented in both established cell lines and tissues of a variety of human tumors, which has tentatively been implicated to both function loss of the wtp53's trans-repressing ability and gaining trans-activation of the mutated p53 over the Hsp 70 promoter, likely mediated by the interaction with the proximal HSE cis-element[21, 22] . Therefore, we recruited four cell lines including three tumorous with different p53 status and an immortal "normal" cell lines. They are, L0-2 cells [27], an immortal "normal" cell line of the embryonic liver organ, U20S, an osteosarcoma cell line with wild-type p53 status, C33A, an ovarian cancer cell line with a mutant p53(Arg273his), and 5637, a cell line with no p53(p53nil). The level of the Hsp70 protein in each of these four cell lines were compared by Western analysis with anti-Hsp70 antibody. To control the loading variation, the same blot was subsequently probed with the antibody verse the proliferation cell nuclear antigen (PCNA), the expression of which varies little among the different cell lines. With such adjustment, the Hsp70 protein level was found as high in U20S, C33A and 5637, respectively as 5.6 fold, 7.7 fold and 9.7 fold of the level of L02 cells (Fig 2).

We further asked whether the heat shock response remains intact in tumor cells by quantifying both mRNA (a semi-quantitative PCR) and protein (Western blotting) of the Hsp70 gene in cells prior and post to the heat shock treatment (a 30 min at 42 oC followed by a 24 h 37 oC incubation). Despite of the detectable elevation of the hsp70 expression induced by the heat-shock in all three tumor cell lines (RNA/protein: U20S:5/2.6; C33A:12.8/3.1; 5637:18/5.3), the extents were significant less drastic than that in L0-2 cells (RNA/protein: 80/14.8) (Fig 3). It was also noticed that at the mRNA level (via the semi-quantitative PCR), the heat-shock induced elevation was more dramatic than that at the protein level (via the Western analyses), raising an interesting question: the translational regulation may indeed act for the fine control of the Hsp 70 expression both in physiological and pathological condition as recently suggested[13, 29]. However, considering the technical limitation of the semi-quantitative PCR method used in this analysis, it is not possible to rule out completely the possibility of the observed difference between mRNA and protein analysis simply being an experimental bias.

Beside, it was noticed that the wtp53 type tumor cells (U20S) expressed Hsp70 protein less than the p53 nil (5637) and mtp53 cells (C33A) (Fig 2) and responded less-efficiently to the heat-shock treatment (Fig 3). Such observation conformed well to the notion that the p53/HSE mediated event is instrumental to tumor specificity of the Hsp70 over-expression well, but much ratification remains to be done.

The impaired elongation control contributes significantly to the tumor specific upregulation of the Hsp 70 expression

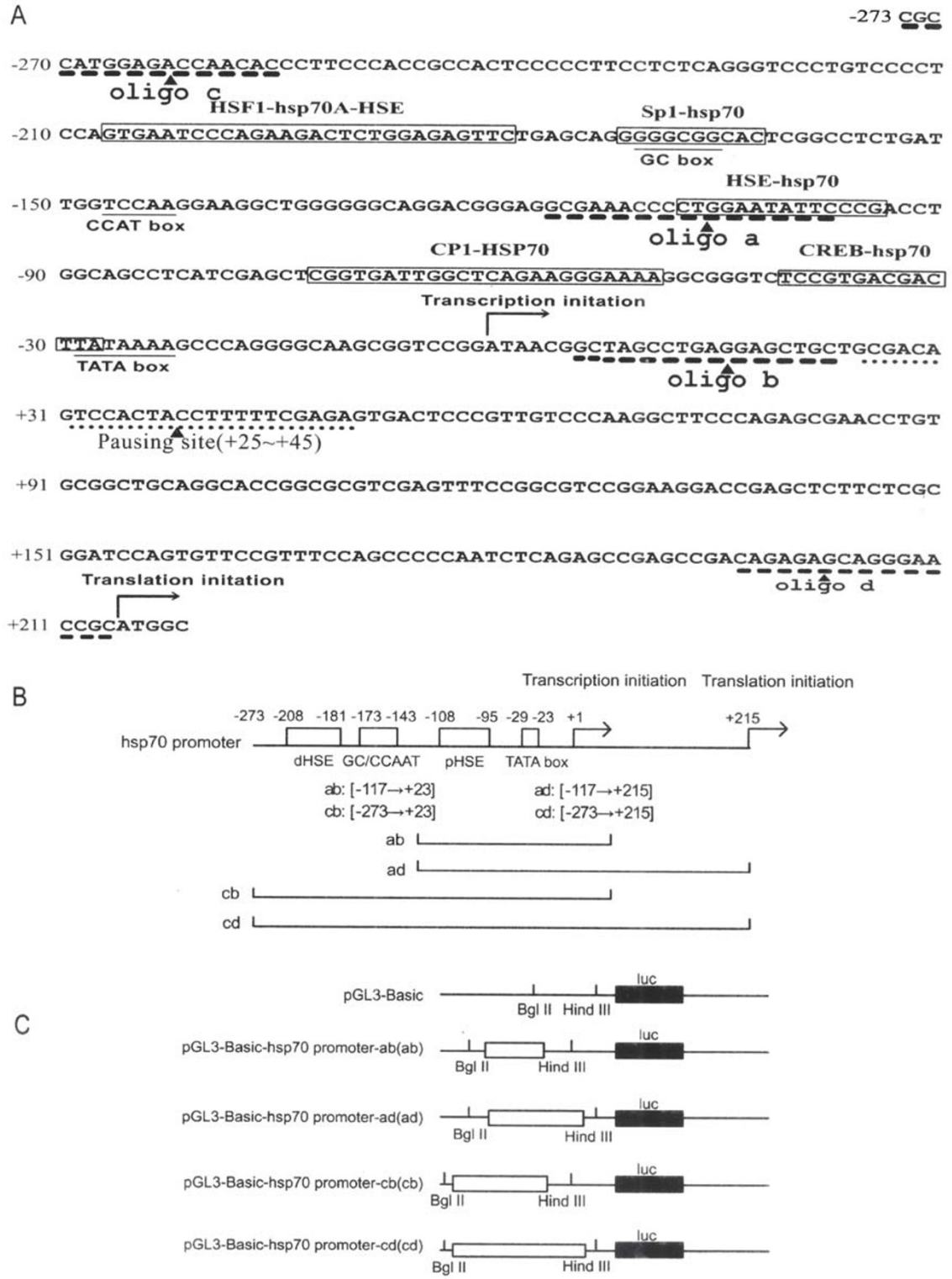
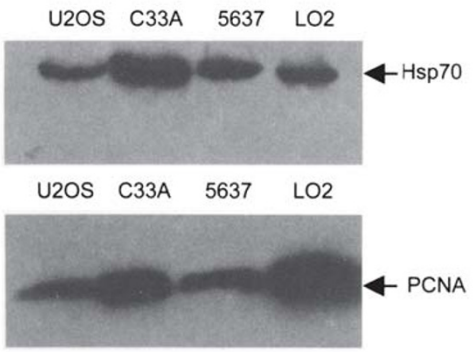


Fig 1. The upstream region of the Hsp70 gene and report constructs
A, The primary sequence of the upstream region (-273 to +219, +1 refers to the first nucleotide of mRNA and indicated by an arrow). HSE (heat-shock response element, -208 to -181 and -108 to -95), respectively.
B and **C**, The schematic illustration of the upstream region of the human Hsp70 gene and the segments under study in this report are marked along with the relevant reporter constructs.

The basal level of transcription of the full length Hsp70 gene is extremely low, a small part of which can be attributed to the initiation event, but the premature termination (paused region) of initiated transcription at the 20 bp region downstream of the transcription initiation (+25 to +45) would account for the rest[14, 30, 1]. Therefore, the region between the transcription initiation (+1) and the translation initiation sites (+215) should embrace the cis-elements for the elongation control.

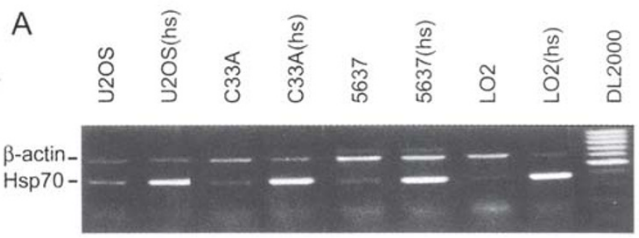
There are two classical HSE elements, proximal (-95 to -108) and distal (-181 to -208), within the upstream region of the Hsp70 gene, both contribute to the heat-shock inducible elevation of the transcription as well as release of the pausing of the engaged transcriptional machineries via the interaction with the nucleus localized, polymerized HSF[15, 32]. In this study, various upstream regions of the translation initiation site were cloned in front of the firefly luciferase gene and four reporter constructs were made: 1, cd(-273 to +215), cb(-273 to +26), ad(-116 to +215), and ab(-116 to + 26), respectively (Fig 1).



	U2OS	C33A	5637	LO2
Hsp70(a)	1	4.3	2.1	1.3
PCNA(b)	1	3.1	1.2	7
a/b	1	1.39	1.75	0.18
tumor/normal	5.6	7.7	9.7	1

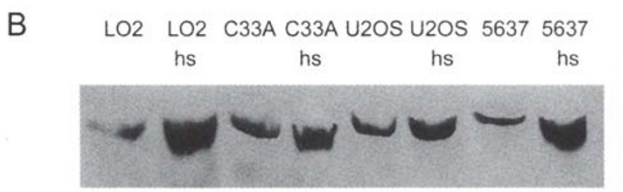
Fig 2. The basal level of the Hsp70 protein in various cell lines
The basal level of the Hsp70 protein in the cell lines was assessed by Western blotting analysis with the anti-Hsp70 protein antibody by taking the pcna level as reference, both were visualized with the relevant peroxidase linked secondary antibodies by the ECL based assay.
The bands were quantified by densitometry and calculated after standardization with the pcna level.

The relative potency of each of these four upstream segments in each of the four cell lines were compared (Fig 4). By taking the ab promoter activity in 5637 cells as 1, the strength of other upstream segments was calculated accordingly. Both p53 nil and mutant cells supported the more drastic reporter activities by cd than the wtp53 type cells, being in a good agreement with (within an acceptable margin of error) the expression profile of the endogenous Hsp70 genes (Fig 2 and 3).



Result of RT-PCR (non-hs+hs quantification of the RT-PCR results (A)

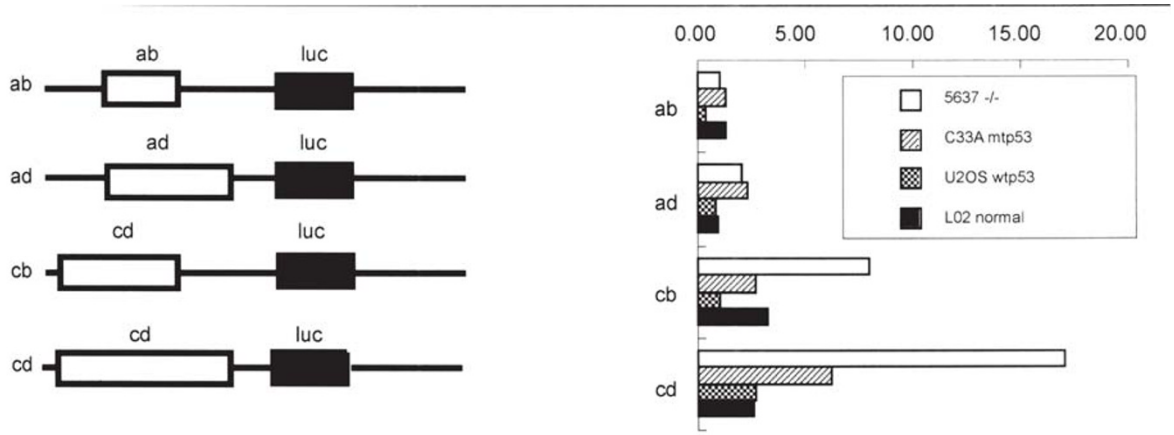
	U2OS	U2OS (hs)	C33A	C33A (hs)	5637	5637 (hs)	LO2	LO2 (hs)
Hsp70(a)	1.2	6.6	0.8	7.5	0.6	8	0.4	8
β-actin(b)	1	1.1	1.5	1.1	3	2.2	2	0.5
a/b	1.2	6	0.53	6.8	0.2	3.6	0.2	16
hs/non-hs		5		12.8		18		80



Result of western blot (non-hs+hs) Quantificationof the western blot results (B)

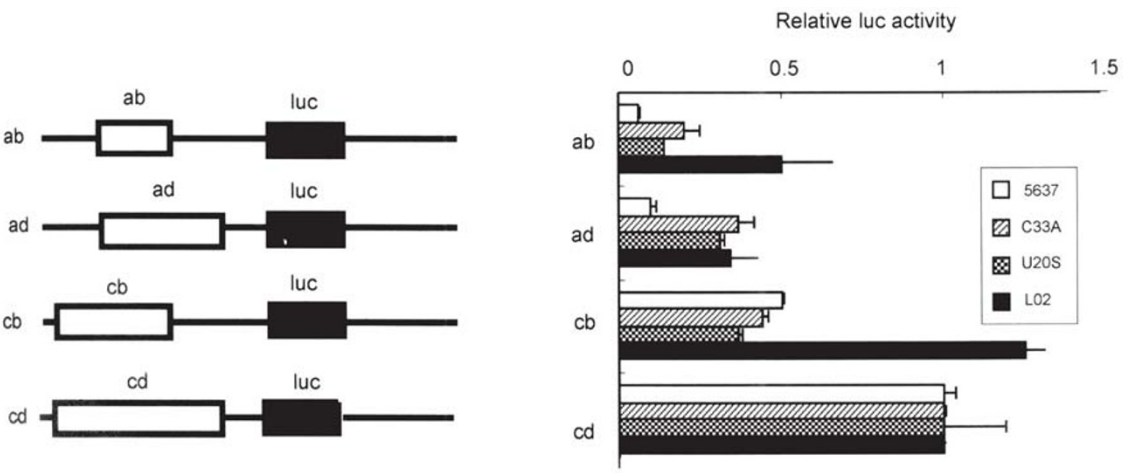
	LO2	LO2 hs	C33A	C33A hs	U2O2	U2O2 hs	5637	5637 hs
Hsp70(a)	1	14.8	2.1	6.5	1.8	4.7	1.9	10
hs/non-hs		14.8		3.1		2.6		5.3

Fig 3. The steady state mRNA and protein level of the Hsp70 gene at either non-stressing and stressing conditions. **A,** the expression profile of the Hsp 70 gene prior to and post to heat-shock response by the semi-quantitative PCR where the β-actin level was used as the reference for the loading. **B,** the expression profile of the Hsp 70 gene prior to and post to heat-shock treatment by the Western analysis with anti-Hsp70 antibody. The bands were quantified by densitometry, calculated and presented in the tables below.



Cell lines	p53 status	ab	ad	cb	cd
5637	-/-	1	2	8	17
C33A	mtp53	1.25	2.32	2.69	6.19
U2OS	wtp53	0.40	0.80	1.00	2.70
LO2	normal	1.33	0.91	3.27	2.60

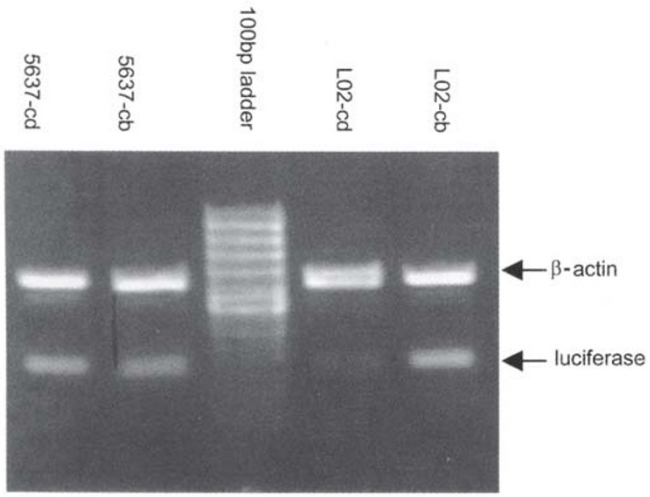
Fig 4. The relative activity of the upstream regions of the Hsp70 gene in various cell lines
The relevant capacity of the different upstream segments (cd, cb, ad and ab) driving the luciferase expression in each of the four cells lines used in this study. CMV RL was included as the internal control, and the capacity of each fragments directly reflected by the firefly luciferase activity was standardized with the RL activity in the same experiments. Both the mean and SD was calculated from the duplicate experiments and plotted by taking the figure of ab arbitrarily as 1.



Cell lines	ab	ad	cb	cd
5637	0.06	0.1	0.5	1
C33A	0.2	0.37	0.44	1
U2OS	0.14	0.31	0.37	1
LO2	0.5	0.34	1.25	1

Fig 5. Relative strength of various upstream segments of the Hsp70 gene.
The relevant capacity of different upstream segments (cd, cb, ad and ab) driving the luciferase expression in each of the four cells lines used in this study in the context of different upstream region of the Hsp 70 gene. In this plot, the ratio between each over cd (arbitrarily taken as 1) was calculated and plotted.

On the contrary, the results with the shorter segments (ab, cb, and ad) resulted in rather diverse profiles, implying that the missed regions in the shorter segments have the important cis-elements for the in vivo expression profile of the Hsp70 gene. By quantifying the potency between the cd and cb, and the ad and ab in pair, we found that the activities of cd and ad were significantly higher than ab and ab in all the three tumor cell lines, irrespective of the p53 status while the reverse was true in L0-2 cells (Fig 5). For instance, in 5637 cells, the relative luciferase activities of cd (1) was approximately two folds of cb (0.5) whereas ad (0.1) was approximately two fold of that of ab (0.06). But in L0-2 cells, cd (1) is 0.25 fold lower than that of cb (1.25), and ad (0. 34) is 0.5 fold less than that of ab (0.5). This implies that the elongation control was lost in all these tumor cell line, while relatively intact in the immortal "normal" cells. A recent study suggested the active participation of the translational events operating at the 5' UTR region of the Hsp70 mRNA, to control the Hsp70 expression[13, 33]. It is therefore, desirable to assess the relative contribution from the elongation related and the translation associated events to the observed tumor selective activities of cd verse cb. To this end, a semi-quantitative PCR was done to quantify the luciferase mRNAs in the transiently transfected 5637 cells or L0-2 cells with cd and cb constructs, respectively. As shown in Fig 6, the relative level of luciferase (presented as the its ration over that of the b-actin, the third lane of the table) was higher in the cd transfected than the cb transfected 5637 cells (0.218 verse 0.18), whereas was lower in the cd transfected than in the cb transfected L0-2 cells (0.06 verse 0.25, the third lane of the table). This observation confirmed the previous conclusion that the elongations control that operates normally in L0-2 cells and fails in 5637 cells. The aberrant elongation control likely contribute predominantly, if not fully, to the over-expression of the Hsp 70 gene in tumor cells.



	5637-cd	5637-cb	L02-cd	L02-cb
Luciferase(a)	0.24	0.18	0.07	0.38
β-actin(b)	1.1	1	1.1	1.5
a/b	0.218	0.18	0.06	0.25

Fig 6. The luciferase RNA level in the recipient cells transfected by cd or cb
The luciferase RNA level in the either 5627 or L0-2 cells transfected with cd and cb respectively. The separate transfection in parallel with that presented in Fig 5 were used for the semi-quantitative PCR analysis. The luciferase RNA level was standardized with the β-acin level and represented in the attached table, from where the cb transfected 5637 was taken as 1 arbitrarily.

In comparison with cd and cb, both ad and ab lack the 156 bp upstream sequence (represented by fragment ca) that contains a number of the cis-elements, including the distal HSE (heat-shock responsive element) (Fig 1A). Both cd and cb conferred a higher level of luciferase activity than ca deleted counterparts in all the cell lines tested with little variation, suggesting that although ca region (-273 to -117) may contribute significantly to the potency, it has relative minor role (Fig 5), if any, to play for the tumor selectivity of the over-expression of the Hsp70 gene. Instead, the bd fragment (+26 to +216) embraces the key cis-elements for the elongation related over-expression of the Hsp70 expression in the human tumor cells.

The impaired tumor-specific heat-shock responses

It is also interesting to determine whether the defective heat shock response in tumor cell lines (Fig 3) can be reflected by the transient transfection assay with various upstream segments of Hsp70 gene. The heat shock treatment was firstward optimized for both temperature and duration of the post-heat-shock incubation in the context of the cd transfected 5637 cells (Fig 7). It was concluded that the treat-

ment consisting of a 30 min 42 °C incubation followed by one 24 h incubation at 37 °C is the best. As shown in Fig 8, heat-shock treatment resulted in more drastic elevation of the cd, cb, ad, and ab activities in L0-2 cells (109, 83.3, 5, and 2) than in p53 nil 5637 cells (33, 8, 3.1 and 2), C33A cells (10. 6, 3.8, 2, and 1.2) and U20s cells (22, 5.3, 2.4 and 1. 8), respectively. This indicates that the heat-shock response was at least partially damaged in all three tumor cell lines. However, it is intriguing to notice little difference between cd (109) and cb (83.3) in L0-2 cells, an indication that relief of the elongation pausing for the heat-shock induced elevation of the Hsp70 expression contributed to no more than 1/5th (25.7 fold) elevation of the total. It was only slightly higher than in 5637 (33-8=25), in C33a (10. 6-3.8=6.8), and in U20S (22-5.3=16.7). The distal HSE within ca segment (cd and cb) seems critical to the more efficient heat-shock induced elevation of transcription initiation and elongation in association with the proximal HSE element (within the ab).

The p53 status of the recipient cells and its contribution to the over-expression of the Hsp70 gene in tumor cells

In addition to the Hsp70 over-expression, functional loss of the tumor suppressor gene is another prominent hallmark of the human tumors[19, 20]. Coincidentally, the majority of mtp53 variants not only lose the repressive capacity, but also may gain the ability to trans-activate the Hsp70 promoter, probably mediated by the proximal HSE element [21, 22]. Therefore, it is of importance to decide whether the elongation control mechanisms overseeing the Hsp70 gene expression are also under the p53's influence.

The protein level of the endogenous Hsp 70 gene in tumor cells was found in a descending order from 5637 (p53 nil, 9.7), C33A (p53 mutant, 7.7), U20S (wtp53, 5.6) to the immobilized normal cells L0-2 (wtp53, 1), showing the tentative association between loss of wtp53 function in tumor cells and the elevation of the Hsp70 expression (Fig 2). Incidentally, we found that cd's activity in the transfected cells was significantly higher in the p53 nil (5637, 17) and mtp53 cells (C33A, 6.19) than that in the wtp53 cells (U20S, 2.7) and L0-2 cells (2.6), respectively (Fig 8). A similar profile was obtained from the experiment with the ad. However, the corresponding profiles of the ab and cb were quite different (Fig 8). Considering the published evidence confining the cis-element responding to the wtp53

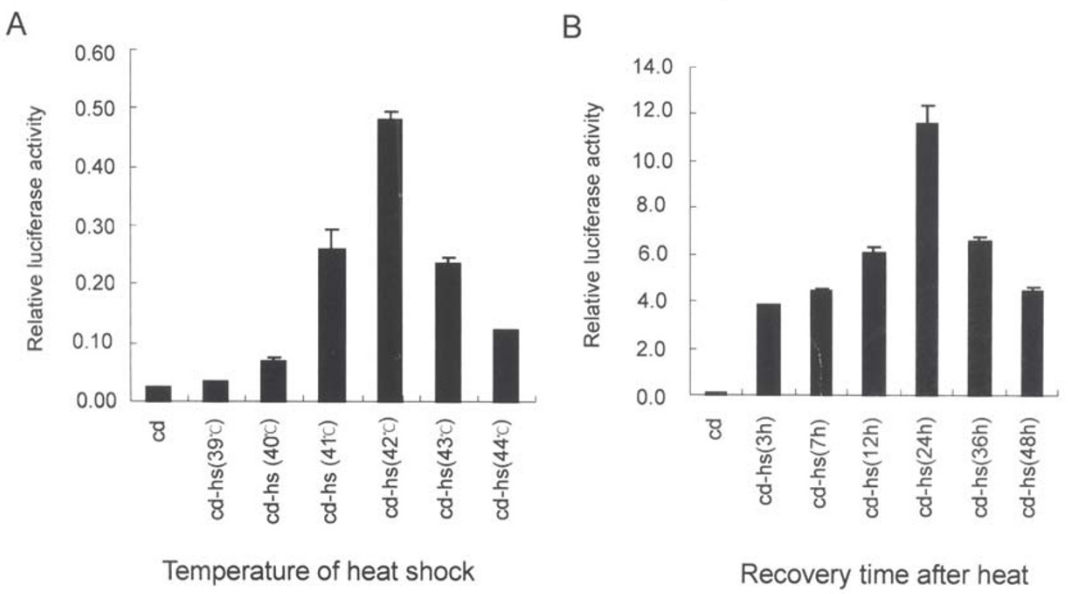
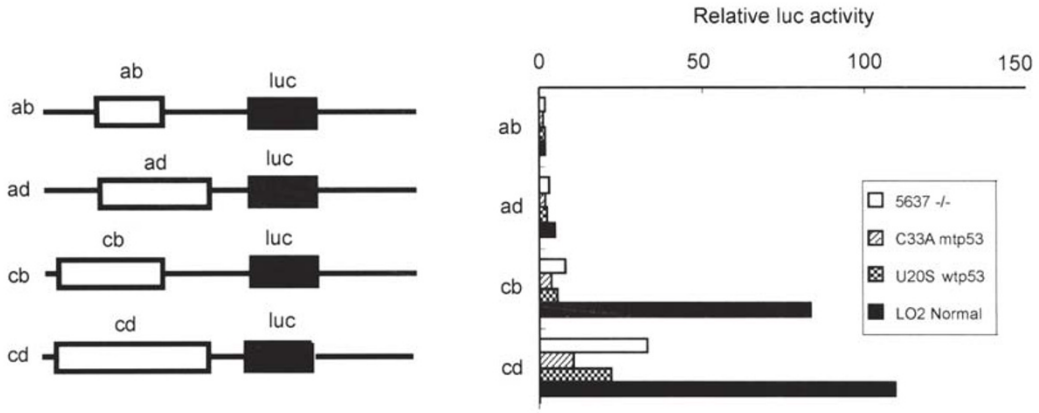
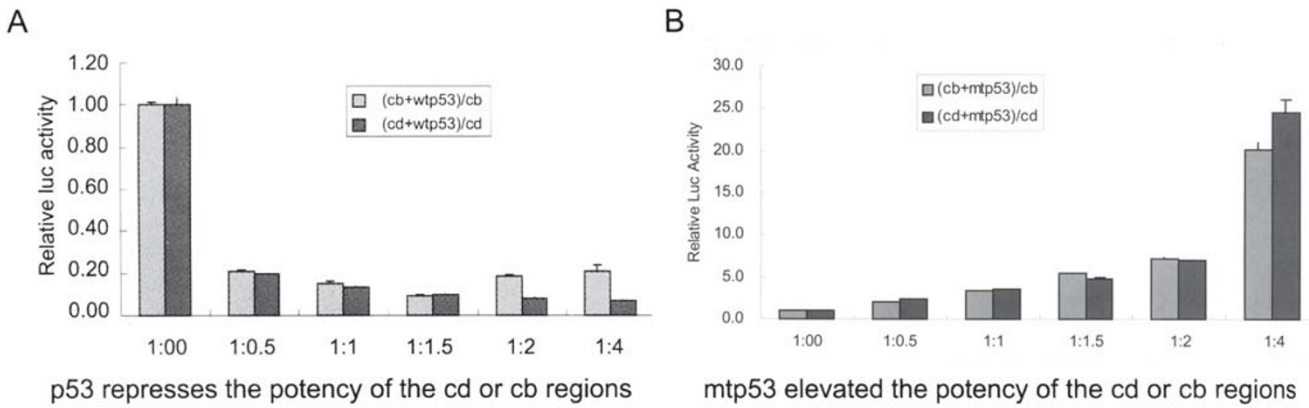


Fig 7. Optimization of the heat shock conditions
The cd transfected 5637 cells were treated with various temperature (A) for 30' and incubated for various length of time after the treated cells by 42 °C for 30' had been returned back to 37 °C. The relative reporter activities were analyzed and plotted.



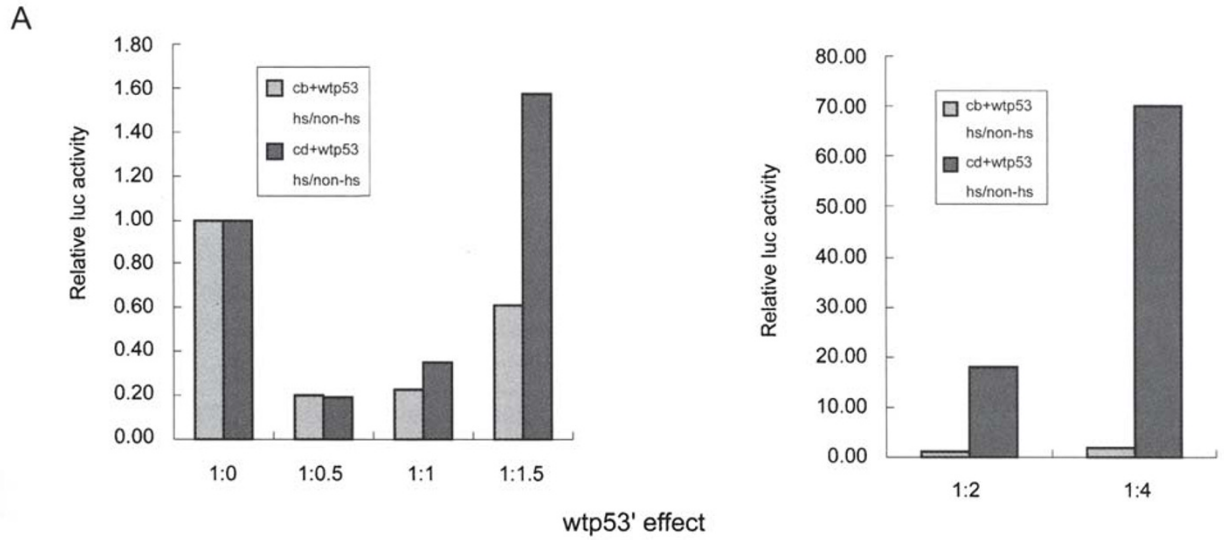
Cell lines	p53 status	ab	ad	cb	cd
5637	-/-	2	3.1	8	33
C33A	mtp53	1.2	2	3.8	10.6
U2OS	wtp53	1.8	2.4	5.3	22
LO2	normal	2	5	83.3	109

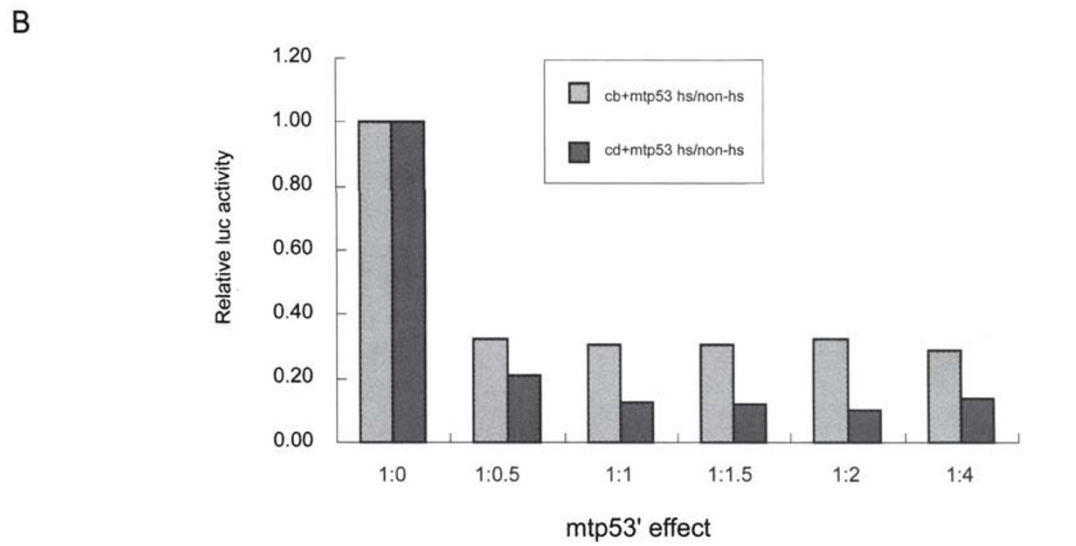
Fig 8. The strength of various upstream segments of the Hsp70 gene over the reporter gene activity at the non-stressing and stressing conditions
Please take the legends of Fig 5-7 for the relevant information.



ratio	(cb+wp53) /cb	(cd+wp53) /cd	(cb+wp53) /cb	(cd+wp53) /cd
1:1	1	1	1	1
1:0.5	0.21	0.2	2.0	2.4
1:1	0.15	0.13	3.3	3.5
1:1.5	0.09	0.1	5.4	4.8
1:2	0.19	0.08	7.2	6.9
1:4	0.21	0.07	20.2	24.6

Fig 9. The p53's effect on the activity of various unpstream segments (cd or cb) of the Hsp70 gene.
Various ratio of the reporter/ p53 constructs were co-transfected into 5637 cells in duplicate and results were presented in the plot (A for wtp53, and B for mtp53) where x-axis was the input ratio of the reporter/p53 constructs, presented in the attached table.





ratio	cb+wtp53 hs/non-hs	cd+wtp53 hs/non-hs	cb+mtp53 hs/non-hs	cd+mtp53 hs/non-hs
1:00	1	1	1	1
01:00.5	0.2	0.19	0.33	0.21
1:01	0.23	0.35	0.31	0.13
01:01.5	0.62	1.58	0.31	0.12
1:02	1.17	18.15	0.33	0.1
1:04	2.05	70.23	0.29	0.14

Fig 10. The effect of the p53 plus heat-shock treatment over thecd or cb's activities. Please take the legends of Fig 5-7 for the relevant information.

repression being the proximal HSE within the ab region, an attractive interpretation is that p53 may also affect the elongation control of the Hsp 70 gene expression via the unknown mechanisms.

To address this issue without any interference of the endogenous p53 status, we co-transfected the p53 nil cell line, 5637 with the wtp53 and mutp53 (arg175his) in conjunction with each of the four

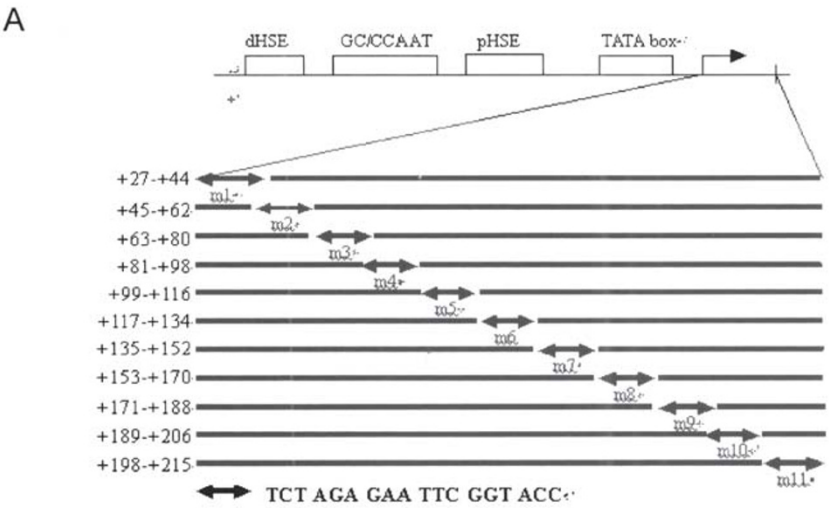
Hsp70 promoter reporter constructs. As shown in Fig 9A, the activities of cd and cb were diminished along with the increased input of the wtp53 with a slightly different kinetics. In the former, the repression by the wtp53 increased gradually and reached its maximum when the most wtp53 was added into, while, for cb, it reached its maximum with the moderate level of wtp53 and reduced in association with more wtp53 present. It is quite likely that the bd region embracing the cis-elements for the elongation control may be instrumental to such differences. On the contrary, the mutp53 was able to trans-activate the Hsp 70 promoter in a dosage dependent manner. When four fold mutp53 was added, the trans-activation of both cd and cb reached its top (Fig 9B) by a similar slope. It appears that the mtp53's effect may chiefly operate at the region shared by both cd and cb.

We further established the responding profile to the heat-shock treatment of cd and cb in presence of various amount of the wtp53 input. As shown in Fig 10A, with the lower input (? 1:1.5 of the reporter contructus/wtp53) of the wtp53, the heat-shock inducible response of both cd and cb was completely suppressed. However, when more wtp53 provided, the heat shock treatment relieved the wtp53's repression. However, in this areas, wtp53 (1:4) plus heat-shock led to 3.5 fold increase of cd activity while essentially no increase of cb activity. Probably as a consequence of the mtp53's trans-activation of the Hsp70 promoters, the heat-shock treatment did not result in any significant increase in cd and ab activity till the mutp53 input reached the plateau (Fig 10B).

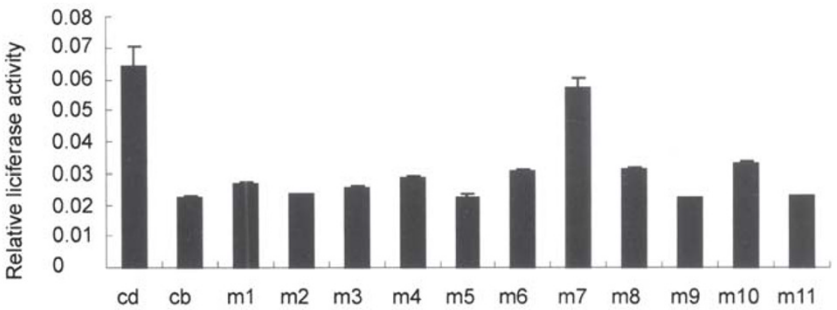
Changes in temperature can lead to the conformational alteration of p53 proteins[34]. And Hsp70 forms a complex with p53 protein, mtp53 in particular in vivo with a number of functional consequences[35]. Therefore, there should not be any simple interpretation for the observed effect of the treatment with p53 and heat shock on the cd and cb (Fig 10). However, the detected difference (even trivial in quantity), in aforementioned response between cd and cb might be suggestive of the participation of the p53 protein in the control of the transcription elongation mediated by the cis-element within the bd region, at least in the case of wtp53. In this connection, it has been reported the 5' UTR of the Hsp70 mRNA present a negative constraint for the translation and wtp53 can repress the relevant translation process[13, 29]. Hence, what we have observed might be at least, partially attributed to the translation regulation provided by wtp53, which was lacked by mtp53 in this context, respectively.

The sequence integrity of the bd region and the regulation at the levels of the initiation and elongation of transcription of the Hsp70 genes expression.

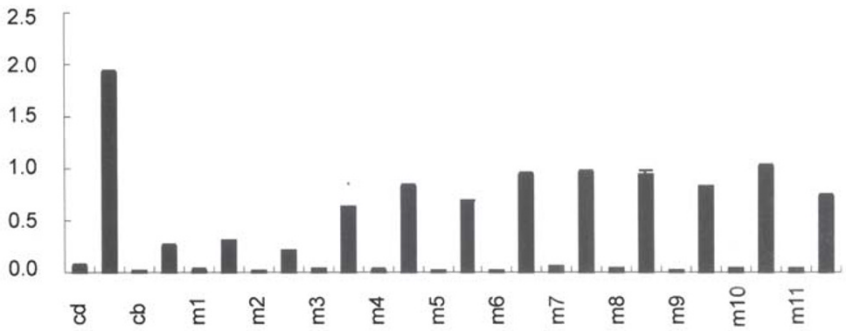
Despite of significant progresses being made as to the protein factors modulating the transcription initiation process (http://transfac.gbf.de/TRANSFAC/)[36], the conventional bioinformatics approach of identifying the cis-elements for sequence-specific DNA-protein interaction that may involve in the control of the transcription elongation has yielded little, hardly existence of any cis-elements recognized by the known transcription factors within the bd region (not shown, the output of this region from the transfactor data base, http://ifti.org/cgi-bin/ifi/Tfsitescan.p1). Hence, we carried out and functionally tested in 5637 cells, a set of eleven cd mutants, within bd region of which the 18 nucleotides were sequentially and individually replaced with the following sequence: TCTAGAGAATTCGGTACC (Fig 11A). We found, except for the mutant 7, where the sequence +135 to +152 was replaced, all the other ten mutants gave rise to a similar level of reporter activity as the cb, where the bd fragment (+27 to +215) where the key cis- elements for the elongation control reside was missed (Fig 11B). Therefore, every 18 nucleotide segments except for the +135 to +152 in the context of cd region, plays an equally important role in the elongation and/or translational control of the Hsp70 gene expression. We further tested in the heat-shock induced response of the wild type cd with the mutants (Fig 11C) and found that m1 (+27 to +44), and m2 (+45 to +62) displayed a similar extent (11.4 and 8.8) of heat-shock response as the cb (11). Considering the paused site is located between + 25 to +45, the defective heat-shock response may be directly linked to the lack of pausing site in m1. However, it remains mysterious how the sequence mutated in m2 (+45 to +62) affect the luciferase



The schematic illustration of the linker scanner mutants



The basal level of the activities of various linker scanner mutants of cd



The heat-shock response of the linker-scanner mutants of cd

Cell line	cd	cb	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11
5637	30	11	11.4	8.8	25	29.3	30.7	33.5	14	28.7	32.1	30.7	27.8

Fig 11. The linker-scanner mutants of the db region (+26 - +215) in cd and their capacity of driving the reporter gene in 5637 cells.

The effects of the mutation within bd region over the cd capacity of driving the luciferase activity in 5637 cells. The linker scanner mutants where the bd regions was sequentially replaced at 18 nucleotide in turn was transfected into 5637 cells for assessment of the basal and heat-shock induced level of expression.

expression in prior and post to the heat-shock. It was also noticed that the m7 (+135 to +152) had a reduced extent(14) of heat-shock response, the mechanism underlying remains to be unveiled.

DISCUSSION

The tumor-specific over-expression of the Hsp70 gene is attributed to the aberrant control at the levels of initiation and elongation of transcription

Over-expression of the human Hsp70 genes in human tumors has been well established[1, 14]. In this report, our data concerning the endogenous Hsp70 protein level and the promoter activities of the longest promoter fragment (cd, -273 to +215) in the tumor cell lines with various p53 statuses in comparison with the immortal "normal" liver cell line indeed concur with this general notion. The tight association of the over-expressed Hsp70 gene with the poor prognosis, resistance to chemo- or radiotherapy, as well as the advanced stage of human tumors[2, 5-7] have honored it with the "hallmark" or "biomark" of cancer and elicits the great interests in the underlying molecular mechanisms, our comprehension of which remains very limited. Indeed, our assessment (Fig 2 and 3) of the Hsp70 expression in tumor cell lines and an immortal "normal" liver cells did lend the same support. In view of the paramount importance of the elongation control over the Hsp70 expression at both non-stressed and stressed conditions, we have confined our efforts at the detail dissection of the 215 bp region spanning from transcription initiation site (+1) to translation initiation site (+216), the RNA copy of which exists in the mRNA molecule of this gene. This regions contains the cis-elements for the pause site, located from +26 to +45, and is enriched in GC (63%), abundant in potential prone to the secondary structures. We employed the transient transfection/reporter assays, to compare the capability of the various segments a, cd, having the longest segment covering -273 to +215, cb, having the segment from -273 to +26, ad, having the segment from -116 to +215, and ab, having the segment from -116 to +26, to produce the luciferase activities in various cell lines. In the immobilized " normal" liver cell line, L0-2 cells, the bd (+26 to +215) containing segments, cd and ad were less potent than the bd nil segments, cb and ab, whereas the opposite was true in all the three tumor cell lines, irrespective of the p53 status (Fig 4). This highly suggested that the elongation control remained functional in L0-2 cells, but failed in all the tumor cell lines tested. In view of the published data suggesting the translation control mediated by 5'UTR of the human Hsp70 gene is also involved[13, 29], the same region associated with the transcription elongation control, the distinction has to be made as to the relative contribution by the defects at the elongation level verse the translation level. We semi-quantified the steady state mRNA level of the luciferase gene in the transient transfected 5637 in comparison with L0-2 cell lines with cd and cb constructs, respectively by RT-PCR (Fig 5). It was clearly showed that the difference in luciferase activity in the context of cd verse cb, between 5637 verse L0-2 cells was well reflected by the level of the corresponding mRNA (Fig 5). Therefore, the aberrant elongation control mechanism is the predominant determinant, if not the only, for the observed tumor selective over-expression of the Hsp 70 gene.

The cellular p53 status and the Hsp70's over-expression in human tumors

The bond between Hsp70 and p53 has been well established despite of lacking the molecular details [35]. p53 is a well-known tumor suppressor, negatively regulating the cell proliferation via either imposing arrests of the damaged cells at a given phase in cell cycle or promoting apoptosis of cells with lethal lesions[19, 20]. It acts as a versatile transcription factors either negatively or positively regulates the transcription of a cohort of genes, products of which have been implicated growth control, angiogenesis, redox regulation, metastasis, nitric oxide production and protein degradation in normal cell. Being at such a paramount position, abundance, conformation, intracellular localization as well as function of p53 are under the continuous and close monitoring in cell. The functional loss attributed to the genetic changes in itself or the key players at either upstream or/and downstream of the regulatory network where the p53 presides has been widely recognized in tumors of all the tissue origins and regarded as "the most infamous hallmark" of tumors. Several heat shock proteins including Hsp70

and Hsp90 can bind to and affect the p53 stability, tetramerilzation, as well as intracellular localization via the intimate association[1, 20, 35]. Failure to be bound by the heat-shock protein complexes makes the wtp53 vulnerable to the unbiquitinization mediated protein degradation. Changes in temperature can influence the conformation and tetramerization of p53[34] as well as the expression of the Hsp70 protein. Therefore, how to explain our data concerning the effects of co-transfected p53 gene plus heat-shock treatment on the cd and cb fragments in the reporter assay (Fig 8) remains an enigma.

On the other hand, the hsp70 gene is one of the p53's target genes, transcription of which was subjected to the wtp53's repression in normal cells[21, 22]. Besides the loss of the wtp53's repressive function, a number of mtp53 gained new function, including the trans-activating capacity[20], which has been attributed to the over-expressed Hsp70 gene in tumor cells. In the report, we have deliberately included the tumor cell lines with different p53 status, namely, U20S (wtp53), C33A (mtp53 arg273his) and 5637 (p53 nil) along with an immortal "normal" liver cell lines (L0-2). Indeed, there was an inverse correlation between the p53 status (p53 nil/mtp53 versus wt p53) and the expression level of the endogenous Hsp70 (Fig 2 and 3), as well as the relative potency of the cd over cb fragments to drive the luciferase activity in transient cells (Fig 5). Although, the forced over-expression of the mtp53 (arg175his) elevated both cd and cb fragments activities drastically, whereas the wtp53 repressed both equally impressively with the noticeable difference in detail. There was no more endogenous Hsp70 expression and more elevation of the cd and cb potency in C33A (mtp53 273) than in 5637 cells (p53 nil). This may be attributed to the lacking in mtp53 (arg273his, the endogenous mutant p53 in C33A cell) of trans-activation ability of the mtp53 (arg175his). In this context, it was noticed that the wtp53's repression of cd and cb activities differs at high range of the p53 input, the saturation level for cb is 1:1.5 (reporter:wtp53) and > 1:4 for cd (Fig 9A). This may indicate that the bd region may also being involved in the wtp53 repression. On the contrary, there was no distinction between cd and cb's response to the mtp53'stransactivation (Fig 9B), implying the different mechanism likely involved.

At the normal circumstances, elevation of the Hsp70 gene expression occurs only in the stressed cells. One aspect of great interests concerns the integrity of the heat-shock response of the tumor cells. As shown in Fig 3, the heat-shock response in L0-2 cells (80/13.8, protein/ RNA levels) was more dramatic than that in tumor cells (U20S:2.6/5, C33A:3. 1/12.8; and 5637:5.3/18). Since the basal level expression of the Hsp70 was higher in tumor cells than in L02 cells, the reduced heat shock response in all three tumor cell lines may be partly due to the safety-guiding mechanism that limits the maximum level of the expression of the Hsp70 in any given cell. In the co-transfected experiment (Fig 10), at the low input range (the ratio of reporter/wtp53 < 1.1.5), the heat shock response was completely suppressed for both cd and cb (from 6.5/26 to 4 and 4.1 for cb and cd, respectively in this experiment, reporter/p53: 1: 0 to 1.1.5), and fully recovered when the high wtp53 input was involved (reaching 13.3 and 1826 for cb and cd, respectively). This may highlight the dosage effect of the wtp53 on the transcription initiation and elongation of the Hsp 70 gene in this artificial system and the involvement of bd region in shaping the profile in the cells with the high level of wtp53 via the unknown mechanisms. As wtp53 is able to repress the translation of its own mRNA and others sharing the GC rich 5' UTR [36], the observed profile shown in Fig 10, may be, at least, partially contributed by p53's role in translation control of the Hsp70 mRNA. This notion was substantiated by the observation with mtp53, (Fig 10B) where the mtp53 devoid of the translation repressing function led much less suppression of the heat-shock response in this system. Obviously, much needs to be done before the sensible answers can be offered.

The 5' UTR and elongation control of the Hsp70 gene expression

It has been amply established that the transcription elongation is tightly associated with the transcription initiation of gene expression, which has 5' UTR elements and comprises of the heat shock genes and a number of the early responsive genes such as c-myc, c-myb and c-fos[10, 15, 23, 24, 31, 37-40]. Both processes are under the similar control as a whole, but distinct at some molecular details includ-

ing players and the operating roles. Searching for the important cis-elements within the 5' UTR region of the genes subjected to the elongation control with the approaches to the transacting factors for the promoter activity has yield little (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) (not shown)[10, 24]. This concurs with the notion that the relevant protein-nucleic acid interactions is not exclusively DNA based, rather than the RNA or RNA/DNA based, the later is more likely modulated at the level of the secondary structures than the primary sequences. Incidentally, the region between the transcription initiation and translation initiation sites is GC rich (GC content: 63%). Although the paused site has been mapped at the +25 to +46[16, 18], how pausing of the engaged RNA polymerase II may be achieved is largely unknown. In this report, we assessed the activities of the cd and cb along with eleven linker-scanner mutants, in each mutants where 18 nucleotide within bd region were sequentially replaced (Fig 10). Except for mt 7 (where +135 to +152 was replaced), all the mutants displayed a similar potency in driving the luciferase expression in 5637cell as cb where all bd region (+26 to +215) was deleted. The tentative conclusion is drawn that all the region except for +135 to +152 form an integral part of the cis aspect of the control over the elongation of Hsp 70 gene expression in cells. The only sensible explanation for these data is that the wild type secondary structure is sensitive to any changes that resulted from the changes in sequences represented by each of the rest ten mutants. However, the heat shock response was reduced by approximately to the level of ab (deleted in bd region) in M1 (+27 to +44), M2 (+45 to +62) and M7 (+135 to +152). The underlying mechanisms for the defect of each of these three mutants can be different. With the paused site destroyed in M1, the affected extent of the heat-shock response might simply reflect the absence of the relief of the pausing suppression.par In the novel targeting of therapeutic gene to the p53 defective human tumors at the level of transcription[42], the ab fragment of the hsp70 promoter was utilized, simply because it may contains a cis-element (s) for the wtp53's repression and the mtp53's mediated transactivation. In this report, we demonstrated the apparent advantages of cd over ab fragments in driving the tumor specific expression of the reporter gene in tumor cells disregarding to each's p53 status, than the immortal "normal" liver cell line (Fig 4), and exploitation of cd's potential as the promoter to drive the therapeutic gene expression to the p53 defective tumor cells in the dual control system[42], is currently underway.

Although this report provides further insights into our understanding the tumor selective over-expression of the Hsp 70 genes and possible explanation of the p53 status of tumor cells being etiologically relevant, more questions have been raised and more future investigations have been warranted

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