Molecular Cloning of cDNA Encoding a Human Heat-shock Protein Whose Expression is Induced by Adenovirus Type 12 E1A in HeLa Cells

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We have identified by differential plaque hybridization, human cDNA clones encoding a member of a heat-shock protein family (hsp 90 α) in the cDNA library of Adenovirus Type 12 E1A transfected HeLa cells. The complete nucleotide sequence of one of the clones (pHB76-114A) was identified. The sequence of 2912 base pairs had a single reading frame with a coding potential for an 84,672-Da protein. The amino acid sequence was highly homologous, but not identical, to that of the human hsp 90 α gene isolated from human peripheral blood lymphocytes [M. Yamazaki, K. Akaogi, T. Miwa, T. Imai, E. Soeda and K. Yokoyama, *Nucleic Acids Res.*, 17, 7108 (1989)]. This cDNA hybridized with RNA species which increased 5- to 20-fold upon heat shock and more than 5-fold in the differentiation stage of human Tera 2 cells.

Heat-shock proteins are expressed in response to stresses such as heat shock, cold shock, viral infection, ionizing radiation, and cytotoxic agents (metal ions, amino acid analogues, and chemical carcinogens). The major heat shock proteins can be grouped into three size-classes: 20–30, 68–73, and 80–90 kDa, the latter commonly referred to as hsp 90. Members within each class are evolutionarily related. Hsp 90 is an abundant cytoplasmic protein in unstressed cells¹⁾ and is post-translationally modified by phosphorylation.^{2,3)} Hsp 90 and another cellular protein of 50 kDa have been found to be complexed with pp60^{src} and other retroviral tyrosine kinases.^{4,5)} Hsp 90 has also been shown to be a component of the non-DNA binding form (8–9 S) of the steroid receptor complex,^{6,7)} and the level of hsp 90 itself is subject to estrogen regulation.⁸⁾ A number of other proteins have also been found to be associated with hsp 90, including

casein kinase II,⁹⁾ actin,¹⁰⁾ and the aryl hydrocarbon receptor.¹¹⁾ Thus, hsp 90 appears to be involved in the transport, stabilization, and regulation of several important intracellular proteins.

Several groups have reported that human hsp 90 consists of two forms which differ slightly in apparent relative molecular mass.^{12–14)} These two forms of hsp 90 are referred to as hsp 90 α and hsp 90 β and the sizes of their corresponding mRNAs are 2.5 kb and 2.7 kb, respectively.¹³⁾ Both mRNAs are coordinately induced by heat-shock in HeLa cells.¹²⁾ Of the two, only hsp 90 α is strongly induced by Adenovirus E1A. While this indicates that the gene expression of hsp 90 α differs from that of hsp 90 β , as does that of members of the hsp 70 gene family, the biological significance of this differential expression is not known.¹³⁾ In this paper, we report isolation and characterization of hsp 90 α cDNA clones from

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Adenovirus Type 12 E1A (Ad12-E1A)¹⁵⁾ transfected HeLa cells using a differential hybridization technique.¹⁶⁾

Materials and Methods

Materials and cells. All restriction enzymes, T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, T4 polynucleotide kinase, and dideoxy- and deoxy-nucleotides were either from Takara Co., Kyoto or from Toyobo Co., Tokyo. Oligodeoxynucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. HeLa cells and Tera 2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY.), 2 mM L-glutamine and antibiotics. Tera 2 cells were differentiated with 10^{-7} M retinoic acid.

Construction of a cDNA library. Total RNA was prepared from Adenovirus Type 12 E1A (Ad12-E1A)¹⁷⁾ transfected HeLa cells using guanidine isothiocyanate extraction and CsCl centrifugation.¹⁸⁾ Poly (A)⁺ RNA was selected through an oligo(dT)-cellulose column. The cDNA was synthesized using oligo(dT)₁₂₋₁₈ (Pharmacia, Piscataway, NJ) as a primer and cloned into a λ gt 10 vector to construct the cDNA library.^{19,20)} The insert of HB76 was digested with *Eco*RI and subcloned into the *Eco*RI site of Bluescript SK(-) (Stratagene, La Jolla, CA) before transformation to XL-1 blue cell. The 145-bp *Sau*3AI fragment (corresponding to nucleotides (nt) 369 to 514 of pHB76-114A) from an HB76 clone and the oligodeoxynucleotide 19 mer probe A (nt 252 to 270), 5'd(TAATTACTGGGATCTGTC)3', and probe B (nt 115 to 133), 5'd(CTGCCTGAAAGGCGAACGT)3' were prepared to obtain a full-length cDNA clone (pHB 76-114A). The cDNA probes used for differential hybridization and the 145-bp *Sau*3AI fragment were radiolabeled by the random-primed labeling method.²¹⁾ The oligodeoxynucleotide 19 mer was labeled by T4 polynucleotide kinase.²²⁾

Differential plaque hybridization. The λ gt 10 cDNA library was plated at about 1,000 to 1,500 plaques per 137-mm dish. The recombinant phage plaques were transferred to duplicate sets of nylon filters (NEN Research Products Boston, MA). After denaturation of the phage DNAs, the filters were incubated in 50% formamide 1% (w/v) SDS, 1 M NaCl, and 10% (w/v) dextran sulfate at 42°C for 16 hr. The filters were hybridized in buffer containing 100 μ g/ml sonicated salmon sperm DNA and 10⁶ cpm/ml of [α -³²P]labelled total cDNAs prepared from either Ad12-E1A transfected or non-transfected HeLa cells. The clones that preferentially hybridized to the cDNA probe from Ad12-E1A HeLa cells were selected and replated.¹⁵⁾

Nucleotide sequencing. Sequencing was done by the dideoxy chain termination method of Sanger *et al.*,²³⁾ using both stranded fragments truncated by partial digestions with exonuclease III²⁴⁾ and subcloned into Bluescript SK(-). The nucleotide sequences were analyzed on an NEC computer system using DNASIS software (Hitachi Co., Yokohama). Homology was measured with the computer program of Lagrimini *et al.*²⁵⁾ Hydrophilicity indices were calculated with the modified program PEPSYS (Fujiya Co., Tokyo) of Hopp & Woods.²⁶⁾

RNA preparation and analysis. Poly(A)⁺ RNAs extracted from Ad12-E1A transfected or non-transfected HeLa cells²⁷⁾ and from Tera 2 cells²⁸⁾ or differentiated Tera 2 cells were separated by a 1.1% agarose gel and transferred to a nitrocellulose membrane filter by the method of Thomas.²⁹⁾ For heat shock induction, cells were incubated at 42°C for 2 hr and allowed to recover for 1-2 hr at 37°C. For induction of differentiation, Tera 2 cells were treated with retinoic acid (10^{-7} M). Northern hybridization was done for 20 hr at 68°C with a *Sau*3AI probe, or at 5°C below the minimum melting temperature, with the oligodeoxynucleotide probe A, in 5 $\times$ SSPE (10 mM NaH₂PO₄ (pH 7.4), 0.18 M NaCl, 1 mM EDTA (pH 7.4)), 0.5% (w/v) SDS, 5 $\times$ Denhardt's solution, and 100 μ g/ml yeast tRNA as described.²⁹⁾ The filters were washed three times with 2 $\times$ SSC (0.15 M NaCl, 0.015 M sodium citrate), 1% (w/v) SDS, and twice with 1 $\times$ SSC, 1% (w/v) SDS at 68°C or room temperature and were autoradiographed on Fuji RX-film with intensifying screens for 24 hr at -80°C.

Results and Discussion

Heat shock protein genes from Ad12-E1A induced HeLa cells cDNA library

From about 5×10^4 λ gt 10 recombinant phages in the cDNA library of Ad12-E1A transfected HeLa cells, four phage clones were selected by differential plaque hybridization using two cDNA probes prepared from both poly(A)⁺ RNAs from Ad12-E1A transfected and non-transfected HeLa cells. One of the four clones that were induced by Ad12-E1A, designated as HB42, had an identical sequence to that of the human hsp 70 gene.³²⁾ This result is consistent with the report of Simon *et al.*¹³⁾ who found enhanced expression of the hsp 70 gene in Ad-E1A introduced cells. The second induced clone, HB76, had 76% homology at the nucleotide sequence level to that of the human hsp 90 gene.¹⁴⁾ To further analyze the clone HB76, northern blot hybridization of the total RNAs from Ad12-E1A transfected and

from the ATG codon at 61 nt to the termination codon at 2,257 nt with coding potential for an 84,672-Da protein. The entire 90-kDa α heat-shock protein was encoded by this full-length cDNA. In analogy to the amino terminal sequence of the homologous heat shock proteins from other rodent cells,^{2,3,12-14} the ATG at 61 nt is tentatively assigned as the initiation codon of pHB76-114A. We observed typical polyadenylation signals, AA-CAAA 639 nt downstream and AAGAAA 510 nt downstream from this termination codon and the poly A tail was seen 140 nt downstream from the AAGAAA site. The amino acid composition of this deduced protein corresponded well with those of the 90-kDa heat-shock or stress protein purified from HeLa

A

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1 MPEETQTGDQPMEEEEVETFAFGAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYETLTDP SKLDSGKELHINLIPNKQDRTLTI VDTGIGMTK 100
|||||
1 MPEEVHH.....GEEEVETFAFGAEIAQLMSLIINTFYSNKEIFLRELISNASDALDKIRYESLTDP SKLDSGKELKIDIIPNPQERTLT LVDTGIGMTK 95

101 ADLINNLGTIAKSGTKAFMEALQAGADISMIGGFGVGFYSAYLVAEKVTVITKHNDDEGYAWESSAGGSFTVR TDTGEPMGRGTKVILHLKEDQTEYLEE 200
|||||
96 ADLINNLGTIAKSGTKAFMEALQAGADISMIGGFGVGFYSAYLVAEKVVVIRKHNDDEGYAWESSAGGSFTVR ADHGEPIGMGTKVILHLKEDQTEYLEE 195

201 RRIKEIVKKHSQFIGYPITL FVEKERDKEVSDDEAEKEDEKEEKEKESEDKPEIEDVGSDEEEKKDGD KKKKKKKIKEYIDQEELNKT KPIWTRN 300
|||||
196 RRVKEVVKKHSQFIGYPITL YLEKEREKEISDDEAEKEKG...EKEEEDKDDEEKP KIEDVGSDEEDSGDKKKKKTKKIKEYIDQEELNKT KPIWTRN 292

301 PDDITNEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPDFL FENRKKKKNNIKLYVRRVFIMDNCEELIPEYLNFI RGVVDSEDLPLNISR 400
|||||
293 PDDITQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFIPRRAPDFL FENRKKKKNNIKLYVRRVFIMQSCDELIPEYLNFI RGVVDSEDLPLNISR 392

401 EMLQGSKILKVIRKNLVKKCLELFT ELAEDKENYKKFYEGFSKNIKLGIHEDSQNRKKLSELLAYYTSASGD EMVSLKDYCTRMKENQKHIYYITGETD 500
|||||
393 EMLQGSKILKVIRKNIVKKCLELFS ELAEDKENYKKFYEAFSKNLKLGIHEDSTNRRLSELLAYHTSQSGDEMTSL SEYVSAMKETQKSIYYITGESKE 492

501 QVANSASFVERLAKHGLEVIYMI EPIDEYCVQQLKEFEGKTLVSVTKEGLELPEDEEEKKKGEEKTKFENLCKIMKD ILEKKVEKVVVSNALVTSPCCIV 600
|||||
493 QVANSASFVERVRKRGFEVYVMTEPIDEYCVQQLKEFDGKSLVSVTKEGLELPEDEEEKKKMEESKAKFENLCKLMKE ILDKKVEKVTISNRLVSSPCCIV 592

601 TSTYGWTANMERIMKAQALRDNSTMGYMAAKKHLEINPDHSIIETLRQKAEADKN DKSVDLVILLYETALLSSGFSLED PQTHANRIYRMIKLGLGIDE 700
|||||
593 TSTYGWTANMERIMKAQALRDNSTMGYMAAKKHLEINPDHPIVETLRQKAEADKN DKAVDLVLLFETALLSSGFSLED PQTHSNRIYRMIKLGLGIDE 692

701 DDPTADDTSAAVTEEMPPL EGDDDTSRMEEVD 732
|||
693 DEVAEEPNAAVPDEIPPLEGDE DASRMEEVD 724

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B

Fig. 2. Comparison of the Deduced Amino Acid Sequence and the Hydropathy Profiles of the hsp 90 Family.

(A): The amino acid sequences are aligned to give the maximum homologies. Gaps introduced to obtain the maximum homologies are indicated by horizontal dots. Vertical bar represents identical amino acids. Upper lane, pHB76-114A; lower lane, hsp 90 β .¹⁴⁾

(B): Hydropathy is evaluated according to Hopp & Woods²⁶⁾ (hydrophilic, positive value; hydrophobic, negative value). Sequences of the *Drosophila* and yeast proteins are described.^{31,32)}

cells by Welch and Feramisco.³⁾ A comparison of the deduced amino acid sequence reported here with the sequence reported for a human hsp 90 β ¹⁴⁾ is shown in Fig. 2A. Extensive homology exists throughout the entire sequence with an overall homology of 86%. A major difference between the two human proteins is the presence of an additional block of 5 amino acids (QTQDQ), located near the amino terminus of pHB76–114. The amino acid sequence predicted from pHB76–114A was also 79% homologous to *Drosophila* hsp 90³⁰⁾ and 61% to yeast hsp 90.³¹⁾ Several long stretches of amino acid sequences were conserved among four species of the above-mentioned eukaryotic hsp 90s (amino acid numbers; 107–123, 125–141, 325–338, 354–369, 608–620, and 667–678). Furthermore, profiles in hydropathy of these four related hsp 90s from different species were virtually identical (Fig. 2B).

Induction of homologous message at elevated temperature and in differentiated Tera 2 cells

The 145-bp *Sau*3AI fragment and probe B derived from pHB76–114A were used for northern hybridization (Fig. 3). Poly(A)⁺RNA, hybridized with the probes, was approximately 2.9 kb in length. Densitometry of the autoradiograms showed that the elevation of hsp 90 transcript reported here ranged from 5–20 fold following heat treatment (Fig. 3A). The transcript of pHB76–114A was enhanced about 3–5 fold in Ad12-E1A transfected HeLa cells compared to that of non-transfected HeLa cells (Fig. 3B). The heat induction of the human normal lung fibroblast WI 38 elevated the expression of the pHB76–114A gene (data not shown). Thus, we conclude that the pHB76–114A encoded protein is a member of the human heat-shock protein family. It has been reported that decreased expression of hsp 70 is an early event in mouse erythroleukemic cell differentiation.³³⁾ In an attempt to study the gene expression of pHB76–114A, we measured mRNA levels in the differentiation stage of human Tera 2 cells.²⁸⁾ After induction of differentiation with retinoic acid, the enhance-

Fig. 3. Northern Blot Analysis.

(A): Poly(A)⁺RNAs were resolved on a denaturing 1.1% agarose gel, blotted onto a nitrocellulose filter, and probed with a random-primed [³²P]labeled *Sau* 3AI insert DNA (145-bp) of pHB76–114A or rat β -actin cDNA (*Hind*III–*Eco*RI)³⁷⁾. Lanes 1 and 3 contain 40 μ g RNA of the unbound fraction from an oligo dT column. Lanes 2 and 4 contain 5 μ g of poly(A)⁺RNA. HeLa cells were incubated at 37°C (lane 1, 2) or were heat shocked 2 hr at 42°C and allowed to recover at 37°C for an additional hour (lane 3, 4).

(B): Poly(A)⁺RNAs (10 μ g) from Ad12-E1A transfected and non-transfected HeLa cells were hybridized with oligodeoxynucleotide 19 mer (probe B). Lane 1, HeLa cell RNA; Lane 2, Ad12-E1A transfected HeLa cell RNA.

(C): poly(A)⁺RNAs (10 μ g) from human Tera 2 cells treated with retinoic acid were hybridized with oligonucleotide 19 mer (probe B). (1), hsp 90 α mRNA; (2), β -actin mRNA.

ment of hsp 90 α transcript was evident at 6 h. The mRNA increased by approximately 5 fold through hours 18 to 24 and then declined to near the original level (Fig. 3C). Conversely we found that the level of hsp 70 was decreased greatly at 6 hr (data not shown).

Hsp 90 α gene family

We have identified a cDNA clone homol-

ogous to, but slightly different from the hsp 90 α gene from human peripheral blood lymphocytes.³⁴⁾ Simon *et al.*¹³⁾ have previously isolated two different partial cDNA clones (α and β) from HeLa cells that encode 89-kDa heat-shock proteins. They have shown that protein encoded by hsp 90 α mRNA was induced by Adenovirus infection, especially with E1A, in a similar way to the 70-kDa heat-shock protein gene. The transcripts of both hsp 70 and hsp 90 α accumulated several fold in Ad12-E1A introduced HeLa cells. Recently Lees-Miller *et al.*³⁵⁾ have identified the N-terminal sequences of hsp 90 α and β proteins. The N-terminal 38 residues of hsp 90 α protein completely matched with the deduced protein sequence of pHB76-114A. Thus, it is highly possible that pHB76-114A is a second hsp 90-related gene, hsp 90 α . Recently Hickey *et al.*³⁶⁾ have reported the nucleotide sequence of a human 90 α genomic clone. The coding region of the genome sequence completely matches that of pHB76-114A. We have also reported the cDNA sequence of human hsp 90 α mRNA as expressed in human peripheral blood lymphocytes.³⁴⁾ Surprisingly we found differences in nucleotide sequence at positions 121, 122, 129, 543, and 558 between two hsp 90 α cDNAs isolated from either HeLa cells or peripheral blood lymphocytes. These differences caused 9 amino acid substitutions. It is not known whether the hsp 90 α gene family shows heterogeneity between HeLa cells and human peripheral blood lymphocytes. However, from our work it is clear that the cDNA sequence of pHB76-114A is different from that of human peripheral blood lymphocytes.³⁴⁾ The homologous transcripts of pHB76-114A were induced by heat shock, introduction of the E1A gene, and during the differentiation of human Tera 2 cells (Fig. 3).

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