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HPV16 E7 oncoprotein induces expression of a 110 kDa heat shock protein

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Abstract Heat shock protein genes are induced by various kinds of stress. Besides stress, the heat shock family gene hsp70 has been shown to be induced by growth-stimulating agents such as the DNA virus oncoproteins and serum. Here, we report cloning of a novel cDNA that encodes a 100 kDa heat shock protein-related polypeptide as a human papillomavirus oncoprotein E7-inducible gene. E7 induces expression of this heat shock protein at the level of RNA synthesis. Moreover, the induction of this heat shock protein-mRNA was dependent on the conserved region 2 of the E7 protein, which is essential for binding to the proteins of the retinoblastoma family.

Key words: Human papillomavirus; E7; Heat shock protein

1. Introduction

The heat shock proteins (HSPs) are cellular stress response proteins that are induced in response to elevated temperature or different kinds of stress [1]. These proteins in mammalian cells have been divided into families based on their structural similarities and apparent molecular weight [2]. Several functions have been ascribed to the HSPs. These proteins have been shown to possess a molecular chaperone function, which includes participation in protein folding, assembly and translocation across the membrane. For example, hsc70 has been shown to interact with nascent proteins, and is believed to play a role in proper folding of the nascent proteins [3]. Recently, it has been shown that the yeast high molecular weight heat shock protein hsp104 resolubilizes heat-inactivated insoluble aggregates of proteins [4]. The heat shock proteins are also involved in protein degradation. Ubiquitin itself and two of enzymes, UBC4 and UBC5, responsible for conjugation of ubiquitin to target proteins are also heat shock proteins [5].

Several proteins of the hsp70 family were found to be expressed in a cell cycle dependent manner; and their accumulation reaches the highest level in the S-phase of the cell cycle [6]. The oncogenes of adenovirus, SV40, polyoma and cytomegalovirus that induce entry into S-phase were also shown to induce expression of hsp70 [7]. Thus, the HSPs may also be involved in cell-growth, and their induction may be a part of the cell cycle progression.

The immortalizing oncogene E7 of the human papillomavirus possesses transcriptional transactivation function. We and others have shown that E7 stimulates expression of cellular genes [8,9]. To identify the cellular genes that might be involved in E7-induced cell cycle progression we have made stable cell lines that inducibly express either the wild type or a mutant E7 protein. The cDNAs from wild type E7-expressing cells were used to make a cDNA library. Also, we made a subtracted cDNA probe by subtracting the cDNAs made from the mutant E7-cells from the cDNAs obtained from the wild type E7-cells. The subtracted probe was used to screen the cDNA library to isolate the E7-induced genes. Here, we report isolation of a cDNA clone (hsp-E7I) that encodes a 859 amino acid polypeptide. The predicted amino acid sequence exhibits 94% sequence identity with a recently cloned heat shock protein from hamster hsp110 [10]. Antiserum raised against recombinant hsp-E7I specifically recognizes a 110 kDa polypeptide. We also show that the levels of hsp-E7I mRNA and protein are increased by heat shock. The induction of hsp-E7I depends upon the conserved region 2 of the E7 protein, which is critical for E7's binding to the retinoblastoma protein.

2. Materials and methods

2.1 Stable cell lines

The stable cell line, derived from the NIH3T3 and expressing HPV16 E7 under Zn-inducible promoter, have been described before [9]. The cells with mutant E7 were generated following a similar procedure as it was described for the wild type E7 [9]. The mutant E7 harbors a deletion of the amino acids DLYC in the conserved region 2 of the E7 polypeptide. This mutant does not bind cellular tumor suppressor pRB [11].

2.2. Cell labeling and immunoprecipitation

The cells were grown to confluence in DMEM supplemented with 10% fetal bovine serum. Then, they were starved in media containing 0.4% FBS for 36 h. Following starvation, cells were washed with the cysteine/methionine-free media and incubated for 3 h with 3 ml/10 cm dish of this media containing 300 μCi of [^{35}S]methionine (ICN) and 4 μM zinc chloride. After the incubation, plates were washed 3 times with PBS, cells were harvested. Harvested cells from two 10 cm plates were lysed with 600 μl of the NP40-lysis buffer (150 mM NaCl, 50 mM TrisHCl pH 8.0, 1% NP40) for 20 min on ice. The cell debris was removed by centrifugation for 5 min at 12 000 × g. The lysates were immunoprecipitated by using a monoclonal antibody against E7 (150 ng of antibody per 2 mg of total protein, Triton Diagnostics, Cat. No. 100201). The immunoprecipitates were subjected to 15% SDS-polyacrylamide gel electrophoresis.

2.3 cDNAs and library

NIH3T3-E7 cells were grown to confluence using DMEM and 10% fetal bovine serum. Cells were serum-starved in media containing 0.4% fetal bovine serum for 36 h. E7 expression was induced by adding 4 μM zinc chloride to the media for 3 h. Total RNA was isolated [12]. Poly(A)$^+$ RNA was purified using Poly(A) Quick mRNA Isolation Kit from Stratagene (cat # 200348). The cDNA library was made using ZAP-cDNA Synthesis Kit from Stratagene (cat # 200400).

2.4 Subtracted probe

The subtraction procedure was based on the representational difference analysis of cDNA [13]. 1 μg of the cDNA from wild type or mutant E7-expressing cells was digested with *Sau*3A restriction enzyme. The oligo t: 5'-GACCTGGCTCTAGAATCCACGACA was annealed to the oligo t': 5'-GATCTGTCGTGGATTCTAGAGCCAGG. The oligo

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d: 5′-GACTCGACGTTGTAACACGGCAGT was annealed to the oligo d′: 5′-GATCACTGCCGTGTTACAACGTCGAG. 0.5 μg of the annealed oligos t/t′ were ligated with the 1 μg of the *Sau*3A digested cDNA (tester) from the wild-type E7-expressing cells. 0.5 μg of the annealed oligos d/d′ were ligated with the 1 μg of the *Sau*3A digested cDNA (driver) from the mutant E7-expressing cells. Unligated oligos were separated from the ligation products by using a Sephacryl 200 spin column as described in the Protocols for the ZAP-cDNA Synthesis Kit from Stratagene. 5% of the ligation products were subjected to the PCR with the oligo t as primer for the tester and with the oligo d as primer for the driver. The amplification was carried out in 25 cycles and the annealing temperature was 55°C. The PCR products (amplicons) were separated from the free oligos by using Sephacryl 200 spin columns. For subtraction, 5 μg of the driver amplicon were co-precipitated with 1 μg of the tester amplicon; the precipitate was dissolved in 4 μl buffer containing 30 mM EPPS (Sigma) pH 8.0 and 3 mM EDTA and denatured for 5 min in boiling waterbath. 1 μl of 5 M NaCl was added and the DNA was hybridized at 66°C for 18 h. 30 μl water, 4 μl of 10 × mung bean nuclease buffer (0.5 M NaCl, 10 mM ZnCl, 50% glycerol) and 1 μl (20 U) of the mung bean nuclease were then added to the hybridization mixture and the incubation was continued for 30 min at 30°C The reaction was stopped by the addition of 160 μl of 0 1 M Tris-HCl pH 8.8. 8 μl of the solution was used as a template for PCR with the t-primer. The PCR products (tester after the first round of subtraction) were purified by Sephacryl S 200 column. Nine rounds of subtraction were performed using 5 μg of the driver and 1 μg of tester DNA for each round.

2 5 Screening

Hybridization and screening was done following a procedure provided by Stratagene. The subtracted cDNA was used as probe. The probe was labelled with ^{32}P in the following manner. An aliquot of the subtracted cDNA was subjected to PCR amplification using the t primer in the presence of 200 μCi of ^{32}P-labelled dATP in a total reaction volume of 20 μl The PCR was carried out under the same conditions as before, except that the concentration of the deoxynucleotides was reduced by 100 fold and dATP was omitted.

2 6 Nuclear run-on

3 μg of the each plasmid containing a cDNA clone was applied onto nitrocellulose membrane and probed with ^{32}P labelled RNA obtained by labelling the nuclei of cells expressing either wild type or mutant E7. To obtain labelled nuclear RNA, the cells were first starved with media containing 0.4% FBS for 24 h and induced with 4 μM zinc chloride for 3 h The nuclei were isolated, and run-on labelling was performed as described before [14]

2.7 Sequencing

Both strands of the 3387 bp insert were sequenced using Sequenase Version 2.0 DNA Sequencing kit (Amersham).

2 8 Northern hybridization

Total cellular RNA was isolated following the protocol described by Chomczynski et al. [12] Northern blot assay was performed following a previously described procedure [15]. To make an antisense probe for the hsp-E7I a DNA-fragment corresponding to first 521 nucleotide of the hsp-E7I cDNA was cloned into pGEM4 at the *Pst*I site. The pGEM clone was linearized with *Hin*dIII and a 600 nt ^{32}P-labelled cRNA, which contained hsp-E7I sequences from +521 to +1 along with 80 nucleotides of the vector sequence, was synthesized by using sp6-polymerase

2 9. Heat-shock and Western blots for the hsp-E7I encoded protein

NIH3T3 cells were heat-shocked at 42°C for different periods of time. After heat shock, the cells were incubated at 37°C for 18 h to allow for heat shock proteins to accumulate. The cells from a 60 mm flask were harvested and lysed in 300 μl NP40 lysis buffer (150 mM NaCl, 50 mM TrisHCl pH 8, 1% NP40) for 20 min on ice. Cell lysates (50 μg of protein) were separated in 7.5% SDS-PAAG followed by blotting to a nitrocellulose membrane. The blot was probed with either an hsp-E7I antiserum (1:3000 dilution) raised against GST-fusion protein containing C-terminal 135 amino acids of hsp-E7I or with the peptide antibodies raised against hamster hsp110. The immunoblots were processed according to Amersham ECL protocol.

3. Results

3.1. Stable cell lines expressing the wild-type and a mutant HPV16 E7

HPV16 E7 was shown to possess transcription regulatory properties. It could activate transcription from promoters with E2F-sites [11]. Moreover, E7 has been shown to stimulate transcription of cellular genes such as c-*fos* [9], and c-*myb* [8]. To identify the other cellular genes that are induced by E7, we made a stable cell line that expresses E7 upon addition of zinc chloride in the media ([9], and see Fig. 1). We have also constructed a cell line that expresses a mutant form of E7 upon zinc-induction (Fig. 1). The mutant lacked four amino acids in the conserved region 2 of the E7 polypeptide and was not able to bind the retinoblastoma protein. This mutant was, also, shown to be defective in transactivation of heterologous promoter through the E2F-site [11], as well as in the activation of the c-*fos* gene transcription [9]. As shown in Fig. 1A, the stable transfectants expressed E7 mRNA upon induction by zinc chloride (4 μM) in culture media. The extracts of the zinc-induced cells were analyzed for the E7-protein. The cells were labelled

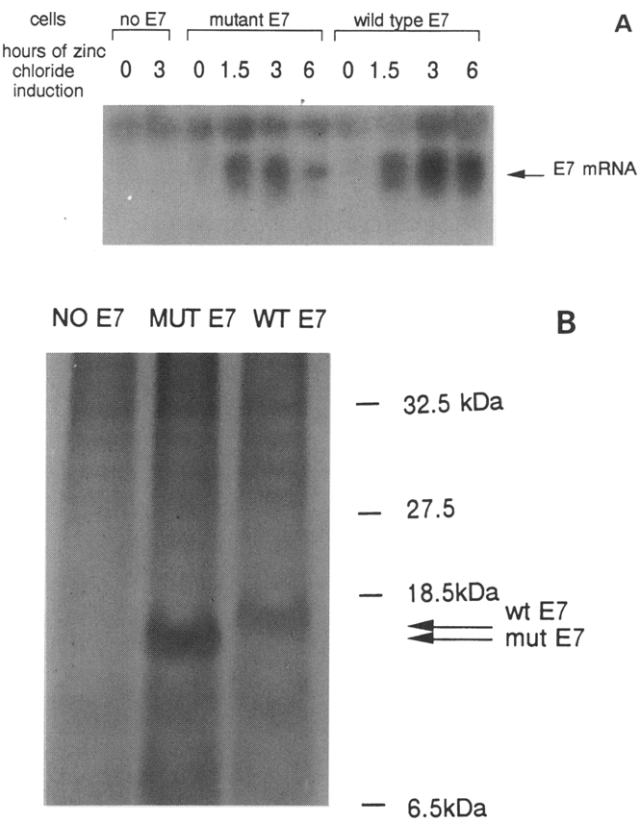

Fig. 1. Zn-induced expression of the wild type and mutant E7 in stably transfected NIH3T3 cells. (A) Northern blot for E7-RNA. NIH3T3 cells expressing no E7 or a mutant E7, or the wild type E7 were induced with zinc chloride as described in section 2. Cells were harvested at the indicated time-points after the addition of zinc chloride in the media. Total cellular RNA (2.5 μg) was analysed by Northern blot assay using a ^{32}P-labelled antisense E7-specific RNA-probe. (B) Immunoprecipitation of ^{35}S-labelled E7. Cells were labelled with [^{35}S]methionine as described in section 2. After a 3 h induction with zinc chloride, cells were harvested. Cell-lysates were subjected to immunoprecipitation by using a monoclonal antibody against E7 as described in section 2.

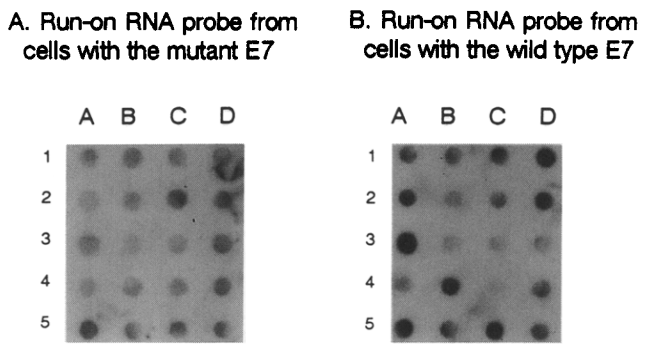

Fig. 2. Identification of cDNA clones differentially induced by the wild type E7. Plasmids with cDNAs were excised and purified. 5 μg of the plasmids was applied onto a nitrocellulose membrane using a dot-blot apparatus. The nitrocellulose blot was then hybridized with the ^{32}P-labelled nuclear run-on RNA from cells with the mutant, or the wild type E7. A representative blot showing differential induction of the cDNA in clones 2A, 3A, 3D and 4B are shown.

with [^{32}S]methionine, and the lysates of these cells were immunoprecipitated using a monoclonal antibody against E7 as described in section 2. The E7 protein was detected in the extracts of these cells (Fig. 1B).

3.2. *cDNA cloning of an HSP-related gene induced by the HPV16 E7 gene*

In order to clone genes induced by the wild type E7 in quiescent cells, we have made a cDNA-library using poly(A)$^+$ RNA from serum-starved cells, where expression of the wild type E7 was induced by adding zinc chloride. To select cDNA clones of genes, which are induced by E7, we generated a subtracted probe as described in section 2. We used c-*fos* as a positive control to monitor the subtraction, because the c-*fos* expression in NIH3T3-cells was induced by the wild type E7, but not by the mutant (data not shown, and see ref. [9]).

We screened 200,000 plaques and 93 positive plaques were isolated. Since we suspected that the subtraction would not be

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        10         20         30         40         50         60         70
MSVVGLELGSQSCYIAVARAGGIETIANEFSDRCTPSVISFGSKNRTIGVAAKNQQITHANNTVSSFKRF  HSP-E7I
......DV.....................................P.........................  hsp110

        80         90        100        110        120        130        140
HGRAFNDPFIQKEKENLSYDLVPMKNGGVGIKVMYMDEEHFFSVEQITAMLLTKLKETAENNLKKPVTDC  HSP-E7I
.....S.........S.........................L.............................  hsp110

       150        160        170        180        190        200        210
VISVPSFFTDAERRSVLDRAQIVGLNCLRLMNDMTAVALNYGAYKQDLPNAEEKPRVVVFVDMGHSSFQV  HSP-E7I
.................A.......................I........D...QGSGVCGH.P.....  hsp110

       220        230        240        250        260        270        280
SACAFNKGKLKVLGTAFDPFLGGKNFDEKLVEHFCAEFKTKYKLDAKSKIRALLRLHQECEKLKKLMSSN  HSP-E7I
............................................................................  hsp110

       290        300        310        320        330        340        350
STDLPLNIECFMNDKDVSGKMNRSQFEELCAELLQKIEVPLHSLMAQTQLKAEDVSAIEIVGGATRIPAV  HSP-E7I
.................A.........................E..H..T.....................  hsp110

       360        370        380        390        400        410        420
KERIAKFFGKDVSTTLNADEAVRRGCALQCAILSPAFKVREFSVTDAVPFPISLVWNHDSEETEGVHEVF  HSP-E7I
......................A.....................................................  hsp110

       430        440        450        460        470        480        490
SRNHAAPFSKVLTFLRRGPFELEAFYSDPQDVPYPEAKIGRFVVQNVSAQKDGEKSRVKVKVRVNTHGIF  HSP-E7I
.............................G.........................K.....................  hsp110

       500        510        520        530        540        550        560
TISIASMVEKVPTEEEDGSSLEADMECFQNQRPTESSDVDKNIQQDNSEAGTQPQVQTDGQQTSQSPPSP  HSP-E7I
...T..........D....V.......-P..K.A........S.............................  hsp110

       570        580        590        600        610        620        630
ELTSEESKTPDADKAANKKVDQPPEAKKPKIKVVNVELPVEANLVWQLGRDLLNMWIETEGKMIMQDKLA  HSP-E7I
..P...N.I......NE.....................................Y.............E  hsp110

       640        650        660        670        680        690        700
KERNDAKNAVEECVYEFRDKLCGPYEKFICEQEHEKFLRLLTETEDWLYEEGEDQAKQAYIDKLEELMKM  HSP-E7I
.............................Q..................................................  hsp110

       710        720        730        740        750        760        770
GTPVKVRFQEAEERPKVLEELGQRLQHYAKIAADFRGKDEKYNHNDESEMKKVEKSVMEVMEWMNNVMNA  HSP-E7I
.N.................................S.......I...........N.....................  hsp110

       780        790        800        810        820        830        840
QAKRSLDQDPVVRTHEIRAKVKELNNVCEPVVTQPKPKIESPKLERTPNGPNIDKKEDLEGKNNLGAERP  HSP-E7I
.............................N...................L.........D.F...A.  hsp110

       850
HQNGECHPNEKGSVNMDLD                                                       HSP-E7I
...................                                                       hsp110
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Fig. 3. hsp-E7I is homologous to hsp 110. The predicted amino acid sequence (1 letter code) of the E7-inducible clone and sequence comparison with the hamster hsp110 are shown. A dot (.) indicates identity with hanster hsp110, a dash (–) indicates amino acid missing in hamster hsp10 (this occurs only once at position 519), and the differences are indicated by 1 letter code below the sequence of hsp-E7I.

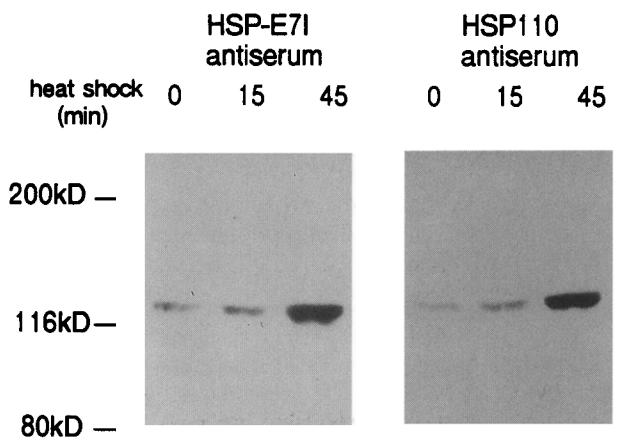

Fig. 4. Antuserum against hsp-E7I recognizes a 110 kDa heat-shock protein. NIH3T3 cells were heat-shocked at 42°C for the indicated time periods. Cell-lysates (50 μg) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis followed by blotting to a nitrocellulose membrane. The nitrocellulose blot was probed with an antiserum specific for the C-terminus of hsp-E7I or with a peptide-antibody raised against hamster hsp110 (1:3000 final dilution). The immunoblots were developed with ECL reagents as described in section 2

complete a second screening was employed to identify the clones that were actually induced by the wild-type E7. The 93 clones were subjected to a slot-blot analysis in which the cDNA clones were probed with in vivo labelled RNA from the nuclei of the E7-induced cells. The clones which hybridized with RNA from the wild type E7-expressing cells, but not with the mutant E7-expressing cells, were considered as 'true positives'. Fig. 2 shows a representative analysis of 20 cDNA clones. In this analysis 4 clones (2A, 3A, 3D, and 4B) were found to be differentially induced in the wild type E7-containing cells. 19 'true positive' clones were identified (not shown). 18 clones contained inserts corresponding to the same cDNA. It was confirmed by cross-hybridization and restriction analysis (data not shown). We have sequenced both strands of the largest insert (clone 4B1. The 3387 nucleotide insert contained open reading frame encoding a putative polypeptide of 859 amino acids (Fig. 3). The nucleotide sequence has been submitted to GenBank (accesion number L40406). The predicted amino acid sequence of the E7-induced clone (hsp-E7I) has strong homology with a recently cloned heat-shock family member protein hsp110 [10], as well as hsp70RY [23]. The hamster hsp110 that possesses about 94% amino acid sequence identity with the predicted amino acid sequence of our clone (see Fig. 3). An antiserum raised against the C-terminal 135 amino acids of hsp-E7I specifically interacted with a 110 kDa polypeptide (Fig. 4). Moreover, a peptide-antibody raised against the hamster hsp110 recognized a polypeptide that exhibited similar mobility as the hsp-E7I encoded polypeptide during an SDS-polyacrylamide gel electrophoresis. Thus, it is quite likely that hsp-E7I represents the mouse version of hamster hsp110.

3.3. Expression of the HSP-E7I-mRNA is induced by E7 as well as elevated temperature

To further confirm the induction of hsp-E7I by the HPV16 E7 protein and by elevated temperature, northern blot experiments were performed. For E7-induction, total RNA from E7-induced cells was subjected to Northern blot analysis. Expression of HPV16 E7 was induced by adding 4 μM $ZnCl_2$ into the culture media. This concentration of $ZnCl_2$ had no effect on the expression of hsp-E7I (see Fig. 5). For heat shock induction, NIH3T3 cells were incubated at 42°C for 15 min or 45 min; and total cellular RNA was used in Northern blot experiments. A ^{32}P-labelled antisense-RNA corresponding to hsp-E7I was used to probe the blots. As can be seen in Fig. 5A and 5B, a 3500 nt RNA band was specifically detected by the probe in the RNA from wild type E7-induced cells. Cells expressing the mutant E7 did not exhibit a significant induction of this RNA. Moreover, the level of the same RNA band was increased in cells incubated at 42°C relative to that in the uninduced cells. On the basis of this result and the structural relatedness with hsp110, we conclude that hsp-E7I is a heat shock protein.

4. Discussion

The DNA virus oncoproteins have been shown to induce expression of the heat shock protein hsp70. For example, adenovirus E1A was shown to increase expression of the hsp70 gene by stimulating the promoter activity [22]. The mechanism of E1A-activation has been studied by analyzing the hsp70 promoter [24–27]. It has been shown that both the 'TATA' and the 'CAAT' elements in the hsp70 promoter are targets of E1A-activation [25–27]. Consistently, it has been also shown that E1A associates with the 'TATA'-binding factor TBP [25,26] and 'CAAT'-binding factor CTF [27]. The interaction between E1A and TBP or CTF largely involves the conserved region 3 of the E1A protein. The papillomavirus E7 protein possesses sequence homology with the adenovirus E1A, however, no significant homology is detected in the conserved region 3 [11]. In addition, there is no evidence for an interaction between E7 and TBP or CTF. Here, we provide evidence that E7 stimulates expression of a distinct heat shock protein gene that is structurally very similar to hsp70.

Existence of a 110 kDa heat shock protein (hsp110) in mammalian cells was reported by Subjeck et al. [16,17] and Welch et al. [18,19]. Recently, Lee-Yoon et al. [10] isolated the cDNA clone of hamster hsp110. The predicted amino acid sequence of hsp-E7I exhibits 94% sequence identity with that of the hamster hsp110. Moreover, a peptide antibody raised against the hamster hsp110 reacts with the same size polypeptide (as judged by mobility in SDS-gel) as that recognized by an hsp-E7I-antiserum. Thus, hsp-E7I is related to hsp110. Two other high molecular weight heat shock proteins (HMM-HSP) hsp105A and hsp105B have been characterized in mammalian cells [20]. Recently, Hatayama et al. sequenced a peptide obtained after lysyl endopeptidase digestion of hsp105A [21]. This partial sequence (23 amino acids) can be found between residues 332 and 355 in the predicted amino acid sequence of hsp-E7I (Fig. 4). Thus, hsp-E7I is also related to hsp105A.

Very little is known about the cellular function of hsp110. Thus, the significance of its induction by the papillomavirus oncoprotein E7 remains speculative. It is possible that these heat shock proteins play important role in the immortalization induced by E7. In this study, we were able to analyze one mutant of E7 that lacked four amino acids DLYC in the conserved region 2. This mutant is defective in transformation assays and is unable to bind the retinoblastoma tumor suppres-

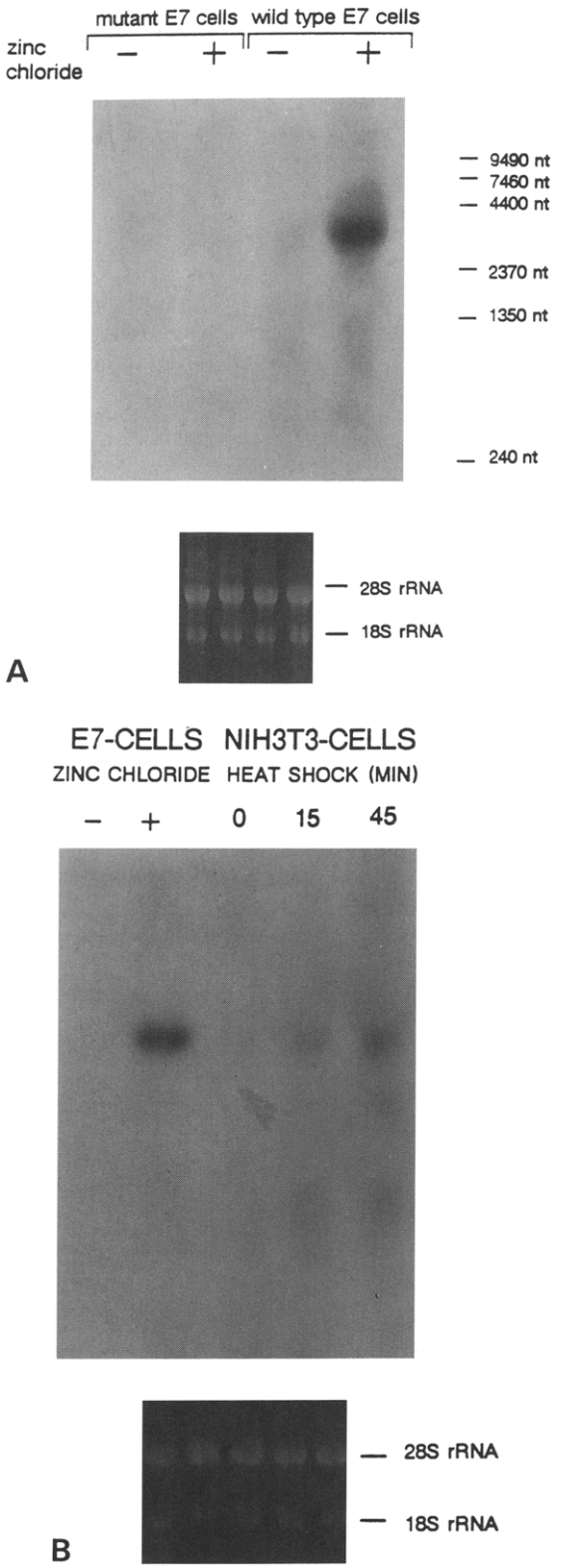

Fig. 5. Induction of the hsp-E7I mRNA expression by E7 and heat-shock. (A) Cells expressing the wild type or a mutant E7 were incubated with (+) or without (–) zinc chloride in the media for 3 h. Cells were harvested and total cellular RNA (5 μg) was analyzed in Northern blot assay using ^{32}P-labelled antisense hsp-E7I RNA-probe. (B) NIH3T3 cells were heat-shocked by incubating at 42°C for the indicated time-period. Total cellular RNA was subjected to Northern blot analysis. The Northern blot was probed with the antisense hsp-E7I RNA probe.

sor protein [11]. We observed that this mutant was much reduced in its ability to induce expression of hsp110. Expression of the wild type E7 causes quiescent cells to enter S-phase (data not shown). Entry into S-phase depends upon new protein synthesis (see ref. [28] for a review). It is possible that the hsp110 family proteins carry out the role of a chaperone for these newly synthesized proteins. Clearly, further studies on the function of this hsp110 related protein will be required to determine the significance of induction by the oncogene E7.

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