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The hsc70 gene which is slightly induced by heat is the main virus inducible member of the hsp70 gene family

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Abstract We have found that SV40 infection of CV1 cells induces the synthesis of a 72 kDa protein that upon molecular cloning was shown to be the product of the hsc70 gene. The above gene product was found to be mainly virus inducible, in contrast to the hsp70 gene product which was mainly heat inducible. The two genes were found to be cell cycle regulated in a distinctively different manner.

Key words: hsc70; hsp70; SV40; Cell cycle

1. Introduction

The heat shock or stress proteins are synthesized by almost all cells after exposure to elevated temperatures or to a variety of other physical or chemical stimuli [1,2]. A large number of genes from a wide range of organisms encoding members of the hsp70 gene family have been isolated and their expression was extensively studied. Induction of stress genes was observed following infection of cells by a variety of DNA tumor viruses such as adenovirus, SV40, polyoma and cytomegalovirus. The early viral oncogenes, which are implicated in the process of cellular transformation, were shown to be responsible for heat shock protein induction [3–6]. However, there is still conflicting evidence concerning the specificity of the above induction. For example, it was reported that SV40 infection of CV1 cells does not result in induction of any of the hsp70 family genes, in contrast to Ad5 and HSV-1 infection of HeLa cells which specifically induces the expression of the hsp70 gene [7]. On the other hand, using the same SV40/CV1 cell system, Khandjian and Turler [4] observed that hsp70 as well as a 72 kDa protein are both SV40- and heat inducible. Moreover, their findings demonstrated that the 72 kDa protein was the main virus inducible protein. Finally the hsp70 gene expression was found to be cell cycle regulated and this finding led to the suggestion that the oncogene induction of the same gene is related to cell proliferation [8,9].

Studying the effects of heat shock and SV40 infection in monkey cells, we observed that their impact on the hsp70 gene family expression is quite distinct. We report here that the main virus inducible member of the family is the hsc70 gene, which is only slightly induced by heat. We also demonstrate that the induction of the hsc70 gene occurs early in infection and its expression is cell cycle regulated.

2. Materials and methods

2.1. Cells, viruses, thermal treatment and cell synchronization

Monkey kidney CV1 cells, COS cells and HeLa cells were grown as monolayers in DMEM medium supplemented with 10% newborn calf serum at 37°C. Infections were carried out with wild-type SV40 or adenovirus type 5 at a multiplicity of 50 pfu/cell in DMEM without serum. After 90 min of virus absorption, the cells were fed with complete medium and allowed to grow. Thermal treatment was performed by placing the cells in a water bath set at 43°C for 90 min. For synchronization, cells were grown to confluency and subsequently incubated in DMEM without serum. After 40 h the cells were split, re-plated and stimulated to grow by addition of fresh complete medium. DNA synthesis was monitored by incorporation of [^{3}H]thymidine at 3 h time intervals following stimulation.

2.2. RNA and protein analysis

Total cellular RNA was prepared from cultured cells by guanidinium thiocyanate extraction as described [10]. For Northern analysis 20 μg of RNA was fractionated on 1.2% formaldehyde agarose gels, transferred to nitrocellulose filters and hybridized to nick-translated DNA probes as described [11]. Two-dimensional gel electrophoresis of proteins from whole cell extracts was carried out as previously described [12].

2.3. Cloning and sequencing

A λzap cDNA library derived from COS cells (Stratagene, La Jolla, CA) was screened with a 2.3 kb *Bam*HI–*Hin*dIII fragment covering the entire coding region of the human hsp70 gene [13]. The probe was nick translated and used to screen 5×10^5 plaques by standard methods [14]. Positive clones in duplicate filters were selected, amplified and verified by re-screening. In vivo excision of the inserts was performed as described [15]. For further analysis, restriction fragments of the appropriate size were subcloned into pBluescript and sequenced with the dideoxy chain termination method using the Sequenase version 2.0 system (USB Corp.). Sequences were determined by complete sequencing of both cDNA strands. The hsc70 and hsp70 sequences were deposited to the EMBL data bank and have been assigned accession numbers x73685 and x70684, respectively.

3. Results and discussion

In our effort to determine the specificity of the viral and heat induction of the hsp70 genes we utilized as a model system monkey kidney CV1 cells and COS-1 cells which are transformed CV1 cells expressing the SV40 T antigen [16]. Analysis of the hsp70 gene family products by two-dimensional gel electrophoresis revealed that viral infection of CV1 cells significantly increased the synthesis of hsp70, but at the same time it led to a dramatic increase in the synthesis of a 72 kDa protein (Fig. 1, panels M and 2). Both hsp70 and the 72 kDa protein were also found to be stimulated after heat shock, but in a reverse manner. In other words, the 72 kDa protein was slightly induced after heat treatment of CV1 cells but the induction of the hsp70 following the same treatment was profound (Fig. 1,

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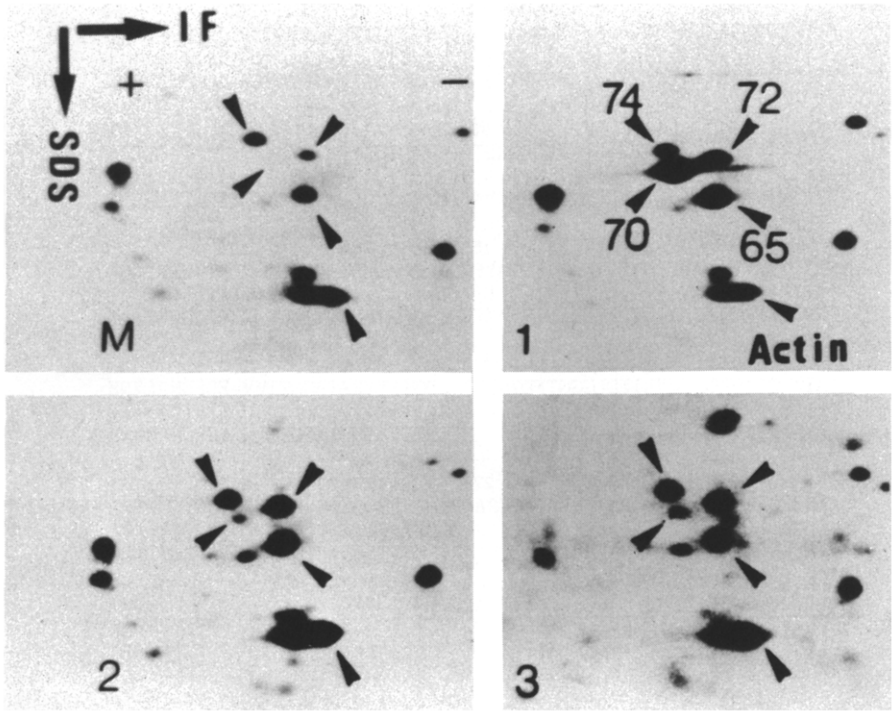

Fig. 1. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins from CV1 cells (panel M), CV1 cells heat shocked for 90 min (panel 1), CV1 cells infected with SV40 for 16 h (panel 2), and COS cells (panel 3). Viral infection and thermal treatment of cells were carried out as described in section 2. Arrows depict the major heat- and/or virus-induced proteins and numbers indicate their approximate molecular mass. The position of actin is also indicated.

panels M and 1). Moreover elevated levels of both hsp70 and the 72 kDa protein were observed in COS cells (Fig. 1, panel 3) at a ratio consistent with our finding that the 72 kDa protein and not the hsp70 is the main virus inducible protein. It was also evident from the above experiment that the induction of the 72 kDa protein was the result of the T antigen function since this is the only viral product expressed in COS cells. Therefore we concluded that SV40 infection of CV1 cells induces mainly the synthesis of a 72 kDa protein which, based on its response to heat treatment, we assumed to be a member of the hsp70 gene family.

In order to identify the gene which encodes the virus inducible 72 kDa protein, we screened a COS cell λzap cDNA library with the human hsp70 gene as a probe. The screening of 5×10^5 plaques yielded twenty positive signals and their corresponding phages were isolated. Following in vivo excision the resulting phagemids were used as probes for Northern blot analysis of cellular COS and CV1 RNAs. This analysis revealed that three clones showed a pattern of RNA hybridization identical to that of the hsp70 gene. Hybridization by a representative clone is shown in Fig. 2 (clone 12). Clone 12, with an insert of 2.5 kb

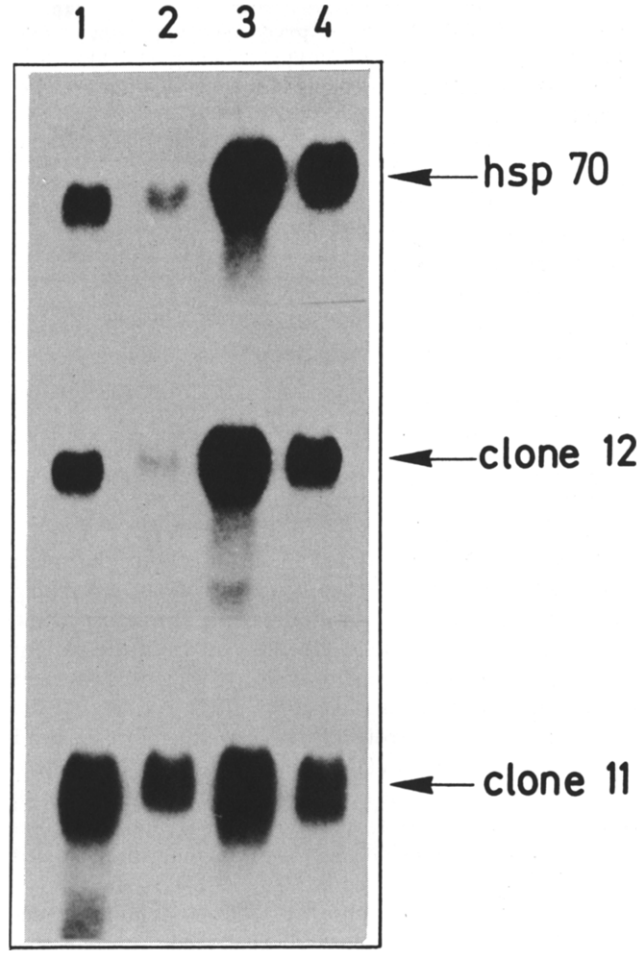

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Fig. 2. Equal amounts of total RNA (20 μg) prepared from COS cells (lane 1), CV1 cells (lane 2), heat shocked COS cells (lane 3), and heat shocked CV1 cells (lane 4), were separated in a 1.2% agarose-formaldehyde gel, blotted to nitrocellulose filters and hybridized with the human hsp70 gene and two of the isolated cDNA clones, namely clone 12 and clone 11. The probes were prepared by nick translating the gel-purified inserts of the indicated plasmids.

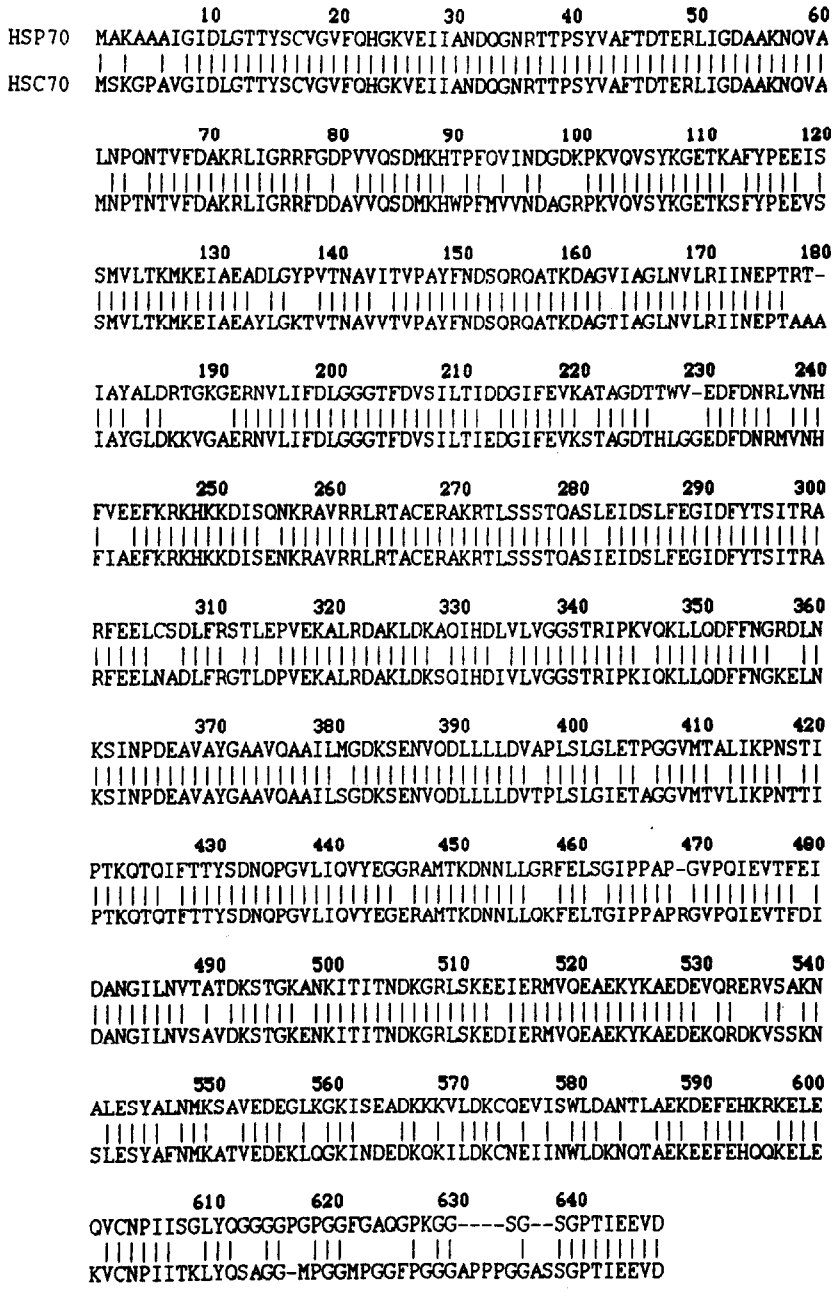

Fig. 3. Amino acid sequence alignment of monkey hsc70 and hsp70. Identical residues are shown by vertical lines.

in size, was fully sequenced and found to contain the entire coding region of the monkey hsp70 gene plus 5′ and 3′ flanking sequences. The RNA hybridization pattern of another of the isolated clones (Fig. 2, clone 11), different from the pattern obtained with the hsp70 gene, was clearly in good agreement with the expected accumulation of the mRNA coding for the virus inducible 72 kDa protein (Fig. 1). We therefore assumed that clone 11 contains a cDNA belonging to the hsp70 gene family that encodes a virus inducible protein other than hsp70. Both strands of this clone were fully sequenced and a subsequent search in the Genebank revealed that the obtained sequence corresponded to the hsc70 gene [17]. A striking homology between our monkey hsc70 cDNA and its human counterpart (98% at the nucleotide level) was noted, with only two out of the 646 amino acids differing, at positions 320 and 633. We therefore concluded that the virus inducible 72 kDa protein is the product of the hsc70 gene. Comparison of the predicted amino acid sequence of the cloned monkey hsc70 with that of the also isolated and characterized hsp70 cDNA showed 84% homology, with the most divergent parts located at the C-terminal regions (Fig. 3).

Using the isolated and characterized monkey hsc70 cDNA, we directly measured the hsc70 mRNA levels during the course of viral infection. For that purpose CV1 cells were infected with SV40 and RNA samples were collected at various times post-infection and analyzed by Northern blotting. As shown in Fig. 4 the abundance of the hsc70 mRNA increased dramatically as a result of SV40 infection. As expected, a similar pattern of

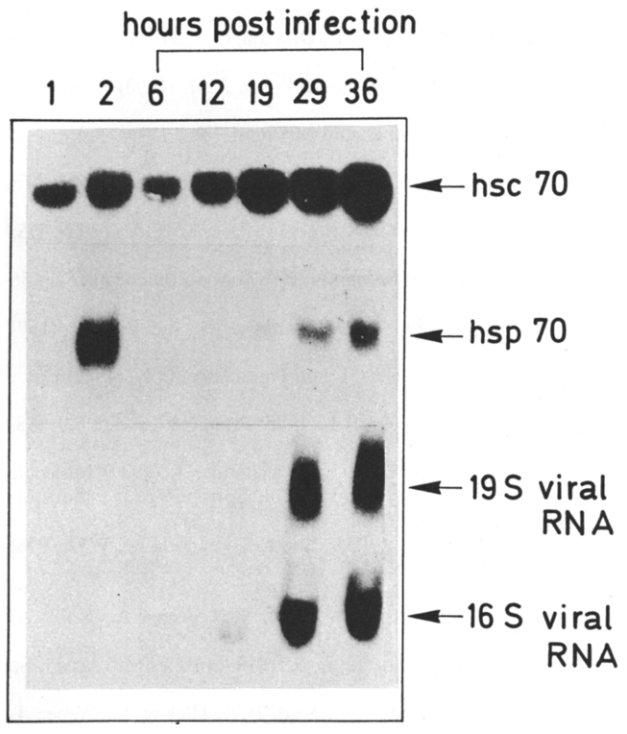

Fig. 4. CV1 cells were infected with SV40 as described in section 2 and total cellular RNA was prepared at the indicated times post-infection. Total RNA was also prepared from mock infected CV1 cells (lane 1) and from heat treated CV1 cells (lane 2). Equal amounts of the above RNAs (20 μg) were analyzed by Northern blotting utilizing the monkey hsc70 and hsp70 cDNAs as probes. The same samples were hybridized to a *Pst*I–*Bam*HI fragment derived from the late region of the SV40 virus to monitor the expression of the late viral genes and consequently the progression of viral infection.

induction was observed when the mRNA levels of the hsp70 gene were monitored under the same conditions. Here again the early viral genes seem to be responsible for the hsp70 mRNA induction, but the appearance of this activation is somewhat delayed (Fig. 4). Our results indicate that although both genes are inducible by the early viral gene products, there seems to be a timely order of activation, with the hsc70 gene being the first to be stimulated.

Expression of the hsp70 gene in HeLa cells was shown to be cell cycle regulated and this finding led to suggestions implicating hsp70 in the process of virus stimulated cell proliferation [18,19]. Since our findings demonstrated that hsc70 is also virus inducible, we investigated its growth-related properties. For this purpose CV1 cells were synchronized by serum starvation and the mRNA levels of hsc70 and hsp70 were monitored at various times following re-entry into the cell cycle. Cyclin B expression was monitored as an indicator of G_2/M. As shown in Fig. 5 the levels of hsc70 mRNA were barely detectable in G1 cells, peaked at 15 h post-release corresponding to S phase, and then declined following cell entry into the G_2 phase. As expected, the hsp70 gene was also found to be cell cycle regulated, but the pattern of its mRNA accumulation was clearly different from that of the hsc70. Maximal levels of hsp70 mRNA were observed during the late S/G_2 phase, in contrast to hsc70 mRNA which reached maximal levels during the S phase (Fig. 5). From the above results, it became evident that serum stimulation of CV1 cells, a phenomenon comparable to viral infection with respect to activation of DNA synthesis, results in induction of the hsc70 gene expression prior to hsp70 gene stimulation. The fact that maximal levels of hsc70 mRNA accumulate during the early/middle S phase of the cell cycle, well before the appearance of hsp70, suggests that, as in the case of viral infection, the hsc70 gene is the first member of the hsp70 gene family to respond to cell proliferation stimuli.

Identical results were obtained in virus-infected HeLa cells. Induction of the hsc70 gene expression was observed in HeLa cells infected with adenovirus 5, and hsc70 was found to be cell cycle regulated in a manner identical to CV1 cells (data not shown).

Therefore the results presented in this study establish the viral induction of yet another member of the hsp70 gene family,

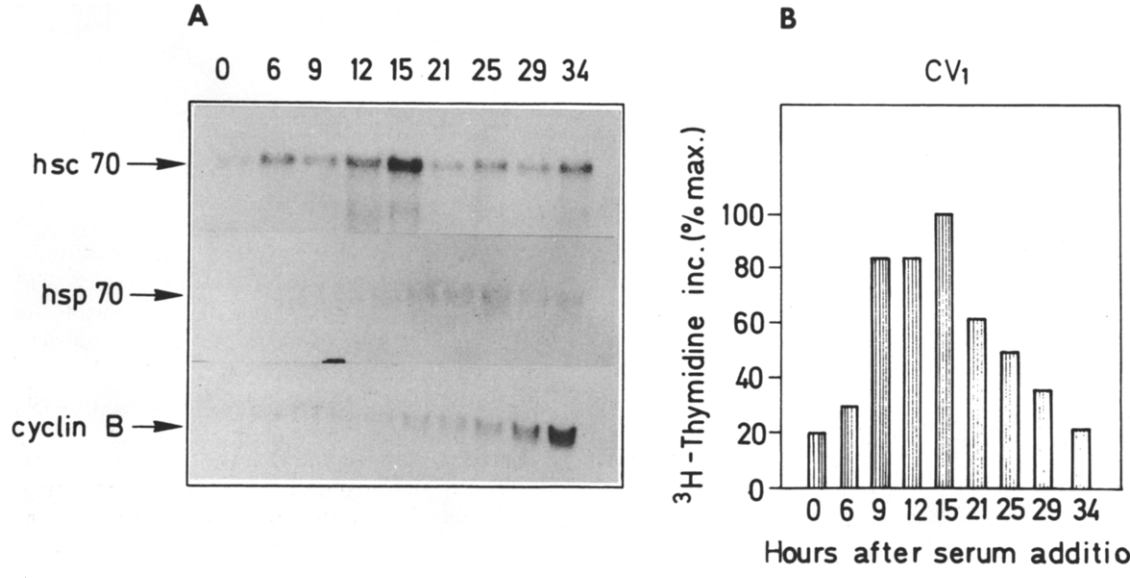

Fig. 5. (A) CV1 cells were synchronized by serum starvation and then released by feeding with fresh media containing 10% serum. Total RNA was isolated at the indicated times after release and analyzed by Northern blotting using hsc70, hsp70 and cyclin B specific cDNA probes. (B) DNA synthesis was measured by [^{3}H]thymidine incorporation at the indicated times after serum addition.

namely the hsc70 gene. A clear distinction, however, should be made between the viral and the heat shock activation mechanisms responsible for the induction of the hsp70 family genes. At least three members of the hsp70 gene family have been identified as heat inducible but not virus inducible, and from all the family members studied so far, only the virus inducible members are cell cycle regulated [19]. Although there are genes which are both virus and heat inducible, e.g. hsp70, stimulation of hsp gene expression seems to be a rather specific event. Indeed, our findings revealed the regulation of another member of the hsp70 gene family by virus, and this gene is only slightly heat inducible. The sequential expression of members of the hsp70 family during the cell cycle indicates a role for these proteins during cellular proliferation, a role that is exploited by oncogenic viruses.

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