The EMBO Journal vol.3 no.13 pp.3087–3093, 1984

Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of *Drosophila* hsp70

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Communicated by M.Bretscher

We have developed a technique which allows specific detection of proteins expressed from cloned genes. The method involves fusion of an oligonucleotide coding for part of the neuropeptide substance P to the 3′ end of the gene; the protein can then be detected with a monoclonal antibody that recognises this peptide. We have used this method to determine the properties of deletion mutants of the major *Drosophila* heat shock protein, hsp70, expressed in monkey COS cells. The results suggest that this protein has two distinct domains. Both are capable of accumulating in the nucleus of unstressed cells, but only the more highly conserved N-terminal domain is able to bind to nucleoli following a heat shock. This implies that nucleolar binding and nuclear migration are distinct properties of the protein, and suggests that the former may be of functional importance. In addition, we observed a novel effect of heat shock on cellular metabolism: protein fragments that are normally rapidly degraded are stabilized. The effect persists for several hours after the heat shock, but does not require expression of heat shock proteins. Together with previously published data, these results suggest an intimate relationship between protein degradation and the heat shock response.

Key words: COS cells/heat shock/hsp70/monoclonal antibodies/substance P

Introduction

The major heat shock protein, hsp70, is synthesised by cells of a wide variety of organisms in response to stress (for reviews, see Ashburner and Bonner, 1979; Schlesinger *et al.*, 1982a, 1982b). Its sequence has been highly conserved throughout evolution and it is assumed to have an important protective function. However, although several genes for hsp70 have been isolated and sequenced, the precise function of the protein has proved difficult to deduce.

The protein has been reported to be associated with a variety of cellular components (for references, see accompanying paper). Some of this variety may reflect the presence of multiple hsp70-like proteins, but in the following paper it is shown that the protein from a single cloned *Drosophila* hsp70 gene, when expressed in mammalian cells, can be found both in the cytoplasm and nucleus, and after heat shock associates transiently with the nucleolus.

These multiple interactions might be due to a single property of the protein, such as an ability to bind to RNA, or could reflect multiple independent functional domains. To investigate this, we have constructed mutants of the hsp70 gene, expressed them in COS cells, and looked at the intracellular distribution of the altered proteins. Such a strategy requires a sensitive and specific method for detecting the products of altered genes, which does not depend on the presence of any one region of the protein. We have achieved this by fusing to the gene an oligonucleotide that codes for part of the neuropeptide substance P. The protein can then be detected with a monoclonal antibody that recognises this peptide. Using this method, we have obtained evidence for two distinct domains in hsp70; both have the ability to accumulate in the nucleus of COS cells, but only the N-terminal one associates with nucleoli after heat shock. In addition, we made the unexpected observation that heat shock leads to the stabilization of protein fragments that are otherwise rapidly degraded in COS cells.

Results

Tagging the hsp70 gene

To be able to detect proteins expressed from mutant genes, we decided to fuse the coding sequence for a known antigenic site to the hsp70 gene. We chose to use the C-terminal portion of substance P, which is not normally expressed in COS or other tissue-culture cells, and for which a highly specific and tight binding monoclonal antibody was available. Substance P has the sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2, but competitive binding assays have shown that the antibody (NCl/34) recognises only the C-terminal pentapeptide (Cuello *et al.*, 1979). The 14-nucleotide long oligonucleotides with a 10-base complementary sequence were synthesized, annealed, blunt-ended with DNA polymerase and cloned into the *Bam*HI site of M13 mp8. Phage plaques were screened by sequencing to identify monomer and dimer inserts (Figure 1a). The sequences were chosen so that the dimer codes for a suitable hydrophilic ‘spacer’ before the substance P sequence. The cloned synthetic sequences were then inserted at the *Sal*I site which covers the last two codons of the *Drosophila* hsp70 gene, in the expression plasmid pAH5 (see Materials and methods). After suitable adjustment of the reading frame, we obtained plasmids coding for the entire hsp70 protein plus either nine or 14 extra amino acids (pAPH.1, pAHP.2, Figure 1b). These plasmids contain an SV40 origin of replication and the adenovirus major late promoter (Figure 1c) and are transcribed efficiently and constitutively in COS cells.

Preliminary experiments established that the altered plasmids produced the same level of transcripts (as detected by S1 mapping) as the parental plasmid. Indirect immunofluorescence using monoclonal antibodies against *Drosophila* hsp70 showed normal levels of protein with an intracellular distribution indistinguishable from that of the normal protein both at low temperature and after heat shock (see the following paper). Thus the short extensions of the C terminus do not noticeably affect the behaviour or stability of hsp70.

Natural substance P has an amide group at its C terminus, and it turned out that the antibody NC1/34 was completely unable to bind either to non-amidated tagged protein or to other constructions which had additional amino acids after

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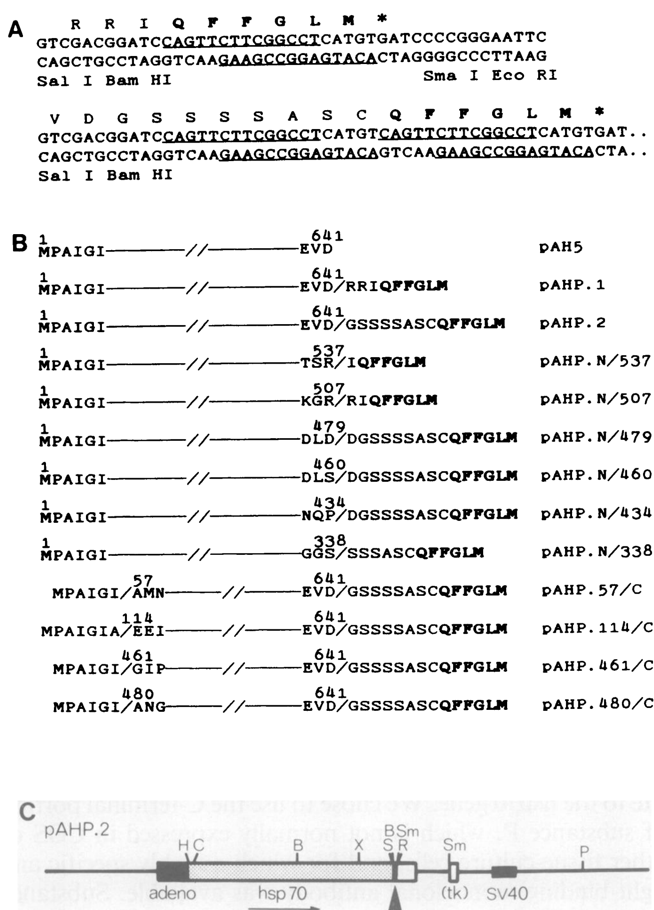

Fig. 1. Plasmid structures. **(A)** Sequences of the synthetic oligonucleotides inserted into M13 mp8; the synthesised sequences are underlined, and the coded amino acid sequences are also indicated (one-letter code). **(B)** Structure of the proteins encoded by the various plasmids. Numbers refer to residues of wild-type hsp70; the sequences around the junctions are shown. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val. **(C)** Structure of a typical plasmid. The adenovirus major late promoter and SV40 origin of replication are shown in black. The hsp70 gene sequences are shown as a box, with the coding region shaded. The arrowhead indicates the inserted synthetic sequence (see **A**). Because of the way the plasmid was constructed, a small fragment of the herpesvirus thymidine kinase gene is also present. Selected restriction sites are indicated: B, *Bam*HI; C, *Cla*I; H, *Hind*III; P, *Pvu*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

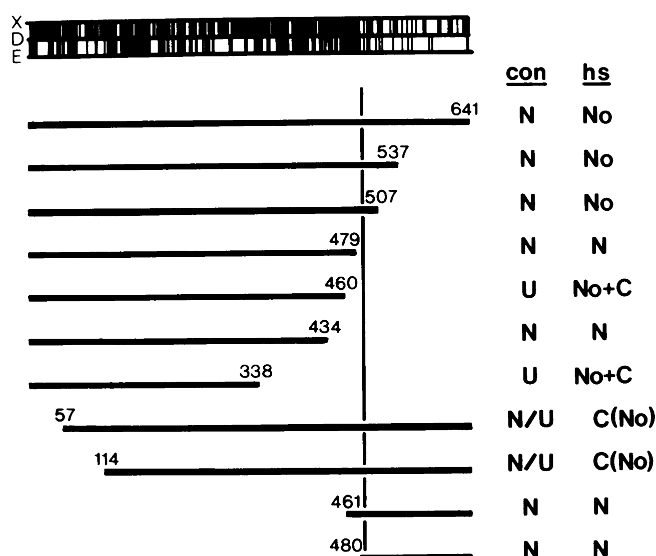

Fig. 2. Deletion mutants and their behaviour. The upper part of the figure shows a diagrammatic comparison between the amino acid sequences of hsp70 from *Xenopus* (X), *Drosophila* (D) and *E. coli* (E); black regions indicate homology. The C-terminal region is much less homologous than the N-terminal part. The bars beneath indicate the regions of the protein retained in each deletion mutant, and the intracellular distribution of these proteins before and after heat shock, as determined by immunofluorescence, is also indicated. N, nuclear; No, nucleolar; C, cytoplasmic; U, unstable. The stability of the N-terminal deletions to positions 57 and 114 in cells not subjected to heat shock varied between experiments. After heat shock they were usually cytoplasmic, but in one experiment nucleolar staining was visible after a mild heat shock.

the antigenic site (data not shown). In order to detect tagged proteins by immunofluorescence or on protein blots we had to chemically convert all the protein carboxyl groups to amides. This proved quite easy to achieve simply by incubating the blots or fixed cells in a solution containing a water-soluble carbodiimide and NH_4Cl (see Materials and methods). With the inclusion of this step, tagged hsp70 with either the 9- or 14-amino acid extension was readily detectable (see Figures 3 – 6).

Deletion mutants of hsp70

Little is known of the three-dimensional structure of hsp70, but comparison of published sequences of the protein from *Drosophila* (Karch *et al.*, 1981; Ingolia *et al.*, 1980), *Xenopus* (Bienz, 1984) and *Escherichia coli* (Bardwell and Craig, 1984) shows that the N-terminal 500 amino acids have been highly conserved, whereas the C-terminal 140 amino acids have drifted considerably during evolution (Figure 2), suggesting that these two regions may form independent structural domains. With this in mind, we constructed a number of deletion mutants, using convenient restriction sites within the gene, in which part or all of each of these domains had been removed. The predicted structures of the mutant proteins are indicated in Figures 1b and 2. To confirm that the plasmids do code for proteins of the expected sizes, and that these remain intact, we transfected them into COS cells, ran cellular extracts on an SDS-polyacrylamide gel, transferred the proteins to nitrocellulose and stained the tagged proteins using the monoclonal antibody against substance P. Figure 3 shows that proteins of the appropriate sizes were present and that in most cases only very low levels of degradation products were detectable (the small fragment from pAPH.2 was not found reproducibly and probably results from degradation after lysis of the cells). Several of the mutant proteins were present at low levels because they are unstable (see below); however, mutants that retain just one of the two putative domains (e.g., N/507, 480/C) are quite stable, which supports the idea that these two regions can fold independently.

Stabilization of proteins after heat shock

We noticed during immunofluorescent staining experiments that proteins such as N/338 that were completely undetectable before heat shock became readily detectable 2 h after a heat shock. This was confirmed by analysis of the proteins on blots (Figure 4): heat shock induces a large increase in the level of intact mutant proteins. To see whether this was due to an increase in the corresponding mRNAs we repeated the experiment but added 10 $\mu g/ml$ actinomycin D prior to the heat shock; the results were identical. Controls showed that actinomycin did block expression of an inducible hsp70 gene under these conditions.

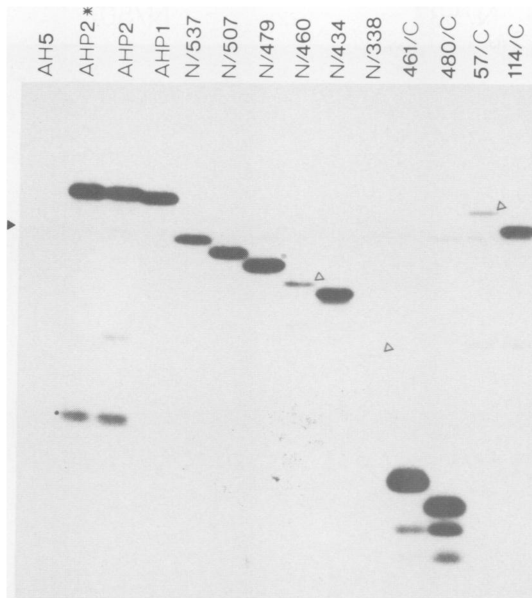

Fig. 3. Detection of mutant proteins on blots. COS cells were transfected with the indicated plasmids, whole cell extracts separated on an SDS gel, blotted to nitrocellulose, and the tagged proteins detected as described in Materials and methods. The largest protein in each track corresponds to undegraded mutant protein; some of the fainter bands are indicated by open triangles. The solid triangle indicates a background band detectable even in untransfected cells. The band indicated by a dot is a degradation product not found reproducibly. APH2* refers to cells that had been heat shocked for 45 min at 43.5°C 2 h before harvesting. Note that pAH5 codes for untagged hsp70, which is not detected by this procedure.

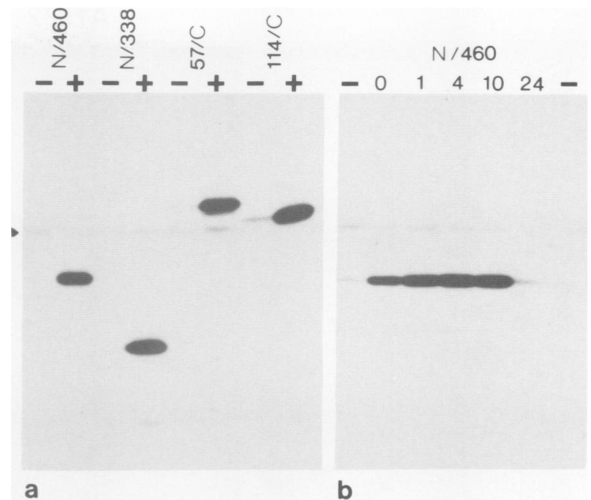

Fig. 4. Stabilization of mutant proteins by heat shock. Proteins were detected by protein blotting as in Figure 3. **(a)** Cells were transfected with the indicated plasmids and harvested without heat shock (−) or 2.5 h after a 45-min heat shock at 43.5°C. **(b)** Cells transfected with pAPH.N/460 were exposed to 43.5°C for 30 min and harvested immediately (0), or after a further 1, 4, 10 or 24 h at 37°C. Cells not treated by heat shock (−) were harvested at the 24-h time point.

In a number of experiments we found that the conditions required for this effect (temperature, length of heat shock) correlated very well with those required to induce heat shock gene expression. However, the failure of actinomycin D to block protein accumulation indicates that it does not depend on the synthesis of endogenous heat shock proteins. It seems that heat shock directly inhibits protein degradation, allowing newly synthesized 'unstable' proteins to accumulate. The level of mutant proteins reaches a new steady-state ~2 h after a 30-min heat shock at 43.5°C; this persists for at least 10 h, but within 24 h it returns to its pre-stressed level (Figure 4). These results are consistent with a partial but irreversible inactivation of some component of the protein degradation system by heat shock.

It is apparent from Figures 3 and 4 that 'unstable' mutant proteins can accumulate to levels similar to that of intact hsp70 within 2.5 h of a heat shock. This implies that the pool of 'stable' *Drosophila* hsp70 must actually turn over with a lifetime of only a few hours, which contrasts with the much longer lifetime that has been reported for mammalian hsp70. Indeed, intact *Drosophila* hsp70 is also somewhat stabilized by heat shock of COS cells. This instability may reflect the fact that *Drosophila* proteins are adapted to lower temperatures than mammalian ones, but clearly it limits the yield of the heterologous protein in COS cells.

Nuclear migration

As described in the following paper, *Drosophila* hsp70 accumulates in the nucleus of COS cells at 37°C, but is excluded from the nucleoli. Figures 5 and 6 show representative cells transfected with the various mutants and stained with the anti-substance P antibody. The staining pattern of pAPH.2 at 37°C is indistinguishable from that of untagged hsp70 (see accompanying paper). Deletion of part or all of the C-terminal domain (pAHP.N/537, .N/507, .N/479) did not affect this distribution (Figure 5). Surprisingly, deletion of the N-terminal domain also did not prevent nuclear accumulation (pAHP.461/C, .480/C) (Figure 6). Thus there must be two separate regions of the protein that both have an affinity for the nucleus. In fact, all the mutants that are stable enough to be detected at 37°C show nuclear accumulation (summarised in Figure 2). The fact that no one region appears essential for accumulation suggests that relatively non-specific interactions may be involved in this phenomenon.

We can rule out some trivial explanations; the nuclear localisation is not dependent on the tagging amino acids, for example, because versions of pAPH.N/537, .N/507 and .461/C that lack these amino acids are detectable with anti-hsp70 monoclonals and give the same pattern of staining (data not shown). The distribution of the mutant proteins also remains unchanged from 36 to 72 h after transfection of the COS cells. Since this is much longer than the lifetime of the proteins, the observed distribution represents a true steady-state. This is important because, as pointed out by Dingwall and Allan (1984), when proteins are transiently introduced into cells by injection they may appear to be concentrated in the nucleus because they are more rapidly degraded in the cytoplasm; in the steady-state, however, any difference in concentration between nucleus and cytoplasm must reflect selective binding or transport. Nuclear binding need not be particularly strong, just stronger than any cytoplasmic interaction. In fact the mutant proteins are rapidly released from nuclei during Nonidet P-40 (NP-40) lysis of unfixed cells at physiological ionic strength (unpublished observations).

Nucleolar binding after heat shock

As described in the accompanying paper, heat shock has a dramatic effect on the intracellular distribution of hsp70: the protein rapidly accumulates in nucleoli and remains there for several hours. Figures 5 and 6 show the distribution of the various mutant proteins 2 h after a heat shock (summarised

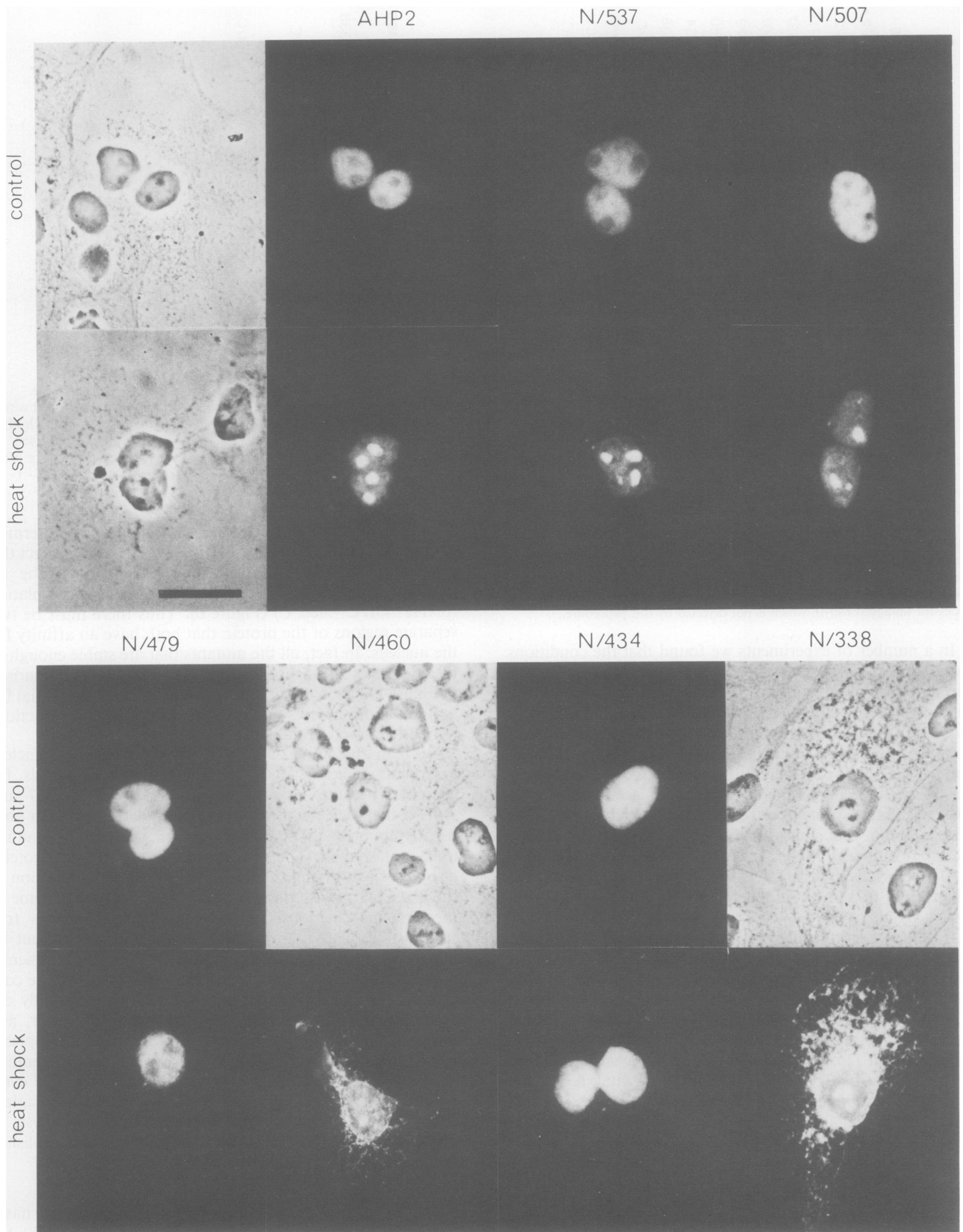

Fig. 5. Immunofluorescent staining of cells containing C-terminal deletions of hsp70. Cells were fixed without heat shock, or 2 h after heat shock for 30 min at 43.5°C. Photographs are exposed to show details of nuclear staining, and in most cases the cytoplasm is not visible. No stained cells were detected with N/460 or N/338 without heat shock; instead phase contrast images of the corresponding heat-shocked cells are shown (note nucleolar staining). Phase images of the pAHP.2-containing cells (which contain intact hsp70) are also shown. Bar = 25 μm.

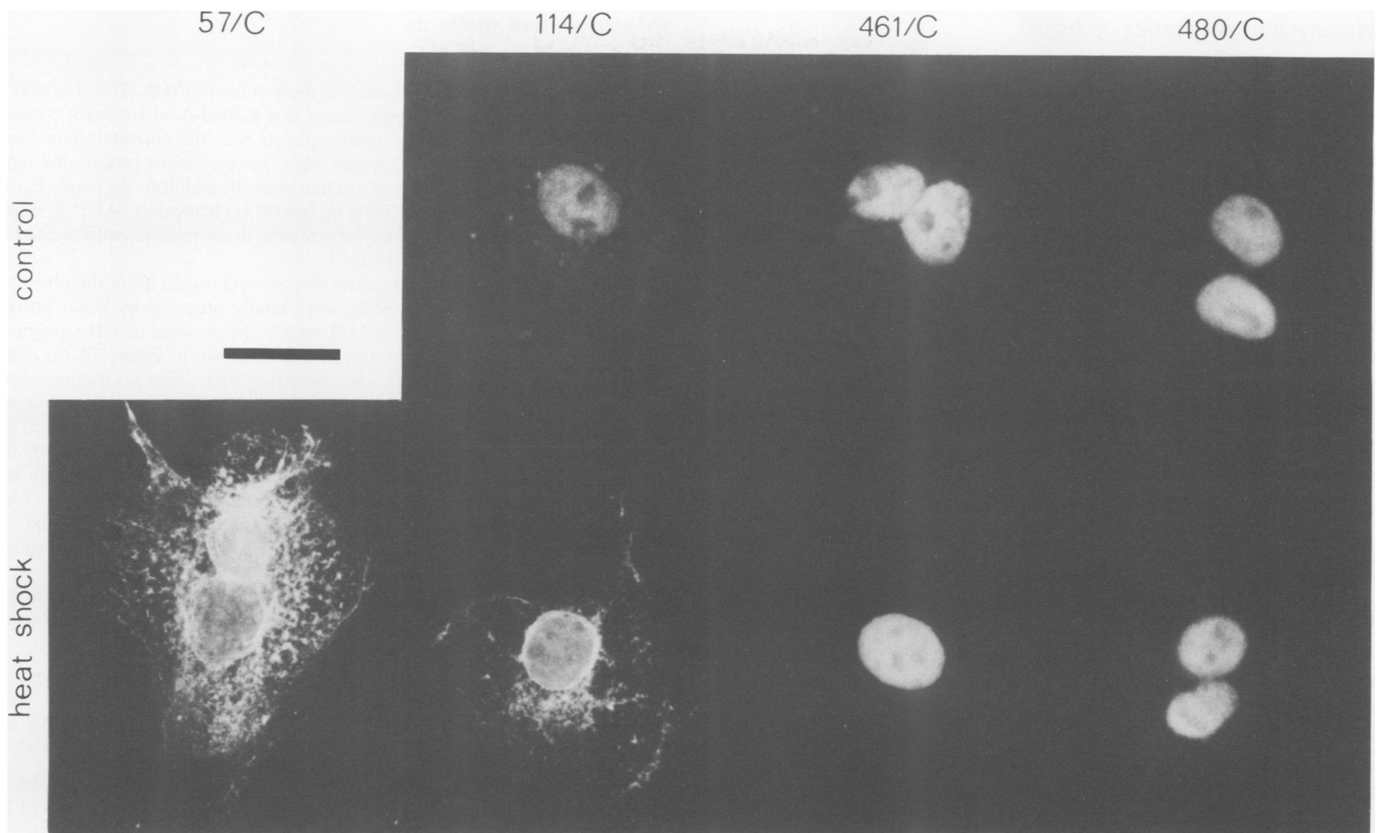

Fig. 6. Immunofluorescent staining of cells containing N-terminal deletions of hsp70. Conditions were the same as for Figure 5. Cells not treated by heat shock containing 57/C did not stain brightly enough to be photographed. Bar = 25 μm.

in Figure 2). As expected, the intact hsp70 is substantially concentrated in nucleoli at this time. Deletions from the C terminus that remove the C-terminal domain (N/537, N/507) do not prevent the movement of the protein into nucleoli (Figure 5). They do have some effect, however: whereas normal *Drosophila* hsp70 (with or without a tag) is found in nucleoli immediately after a 45-min heat shock, the N/537 protein takes several hours to move in, and after mild heat shock both N/537 and N/507 show a less marked concentration and/or a lower proportion of cells with clear nucleolar staining. Nevertheless, the N-terminal domain is clearly sufficient for nucleolar binding.

In contrast, mutant proteins that retain only the C-terminal domain (461/C, 480/C, Figure 6) are always excluded from the nucleolus, before and after heat shock, and apparently have no affinity for it at all. This indicates that nuclear migration and nucleolar binding can be quite distinct phenommena, and are not just different manifestations of a single property of the protein.

The deletion mutants that lack part of the N-terminal domain show a variety of responses to heat shock. Two of them, N/479 and N/434, are stable but do not accumulate in nucleoli after heat shock. The other four are unstable to varying degrees. After heat shock they are found mostly in the cytoplasm, apparently precipitated or bound to some structure. However, a portion of the protein in cells transfected with pAHP.N/460 or pAHP.N/338 is found in nucleoli after heat shock (Figure 5). In one experiment we also observed nucleolar staining with 57/C and 114/C after a mild heat shock, although the levels of protein were very low. We interpret these results as follows: the N-terminal region of hsp70 apparently folds as a single domain, and thus deletions tend to destabilize it; however, those molecules that do manage to fold in a manner similar to the wild-type protein retain the ability to bind to nucleoli. The key sequences for this binding must lie in the first 338 amino acids, and probably between residues 114 and 338.

Discussion

Tagging expressed proteins

We have described a sensitive method for detecting the proteins encoded by genes mutated *in vitro*, by tagging them with a short peptide to which an antibody is already available. Such a method may be useful in other cases where it is desirable to distinguish the expressed protein from similar or identical endogenous products. However, some care is required with this approach; in preliminary experiments we found that C-terminal fusions of various fragments of human interferon or of the frameshift-generated sequence LKPNRKLFSSWLKFLKVILFIWL caused hsp70 to stick completely in the cytoplasm, apparently in insoluble form.

We were unfortunate in having to modify chemically our mutant proteins in order to detect them with the anti-substance P monoclonal antibody, but this minor inconvenience was largely outweighed by the benign properties of the fused peptides and the excellent performance of the antibody. Moreover, recent experiments indicate that the tagged proteins can be detected without modification by the use of polyclonal antisera against substance P. This should extend the usefulness of the method by allowing immunoprecipitation of native proteins.

Structure and properties of hsp70

We have examined two properties of *Drosophila* hsp70: its accumulation in the nucleus of unstressed COS cells and its ability to move transiently into the nucleolus after heat shock. The results indicate that the nucleolar localisation is a property of the evolutionary conserved N-terminal portion of the protein, particularly sequences between residues 114 and 338. The C-terminal domain seems relatively unimportant, at least for localisation of the protein, which is consistent with the considerable divergence of its sequence between species.

Nuclear accumulation in unstressed cells, on the other hand, is a property shared by both N-terminal and C-terminal domains. The results can be explained most easily by free diffusion of the protein fragments into the nucleus, with the final distribution being determined by a weak affinity of the proteins for some nuclear component. Of course, this does not mean that there are no specific extranucleolar functions for hsp70 – specific interactions would merely be masked by the non-specific ones.

The more stringent requirements for nucleolar migration suggest that this process may be of physiological significance, and in the following paper it is shown that hsp70 can actually catalyse the recovery of nucleoli from heat shock. An obvious question concerns the nucleolar component with which hsp70 interacts. rRNA and ribosomal proteins are likely candidates, and our ability to detect mutant proteins on blots will allow us to study their chromatographic and other properties, and see whether there is a correlation between their (presumed) abilities to bind these components and to enter nucleoli.

Heat shock stabilizes proteins

The finding that unstable mutant proteins are stabilized following a heat shock was unexpected. Stabilization does not require *de novo* synthesis of heat shock proteins, although it could involve activation of a pre-existing protein. Alternatively, some component of the protein degradation system may be directly sensitive to elevated temperature. For example, Finley *et al.* (1984) have recently reported that the enzyme that adds ubiquitin to abnormal proteins (an early step in the degradative pathway) is partially inactivated by exposure to 45°C *in vitro*, and there is evidence that ubiquitination of histones is reduced in heat-shocked *Drosophila* cells (Glover, 1982).

It might be coincidental that inhibition of protein degradation and induction of the heat shock response occur at the same temperature, but both systems help to protect cells and it is possible that they are regulated in a complementary fashion. If a function of the heat shock proteins is to repair denatured proteins and protein-containing structures (e.g., following paper), it would clearly be advantageous for the cell to cease degrading the altered proteins while repair proceeds. Conversely, when degradation is impossible, repair becomes necessary. This is consistent with the observation that a mutant cell line with a temperature-sensitive ubiquitinating enzyme expresses hsp70 at the non-permissive temperature even though this is below that normally required for a heat shock response (Ciechanover *et al.*, 1984). We have recently devised a way to select *Drosophila* mutants that respond to heat shock at a lower temperature than normal (Bonner *et al.*, 1984) and it will be interesting to see whether these also have an altered protein degradation system.

Materials and methods

Plasmid constructions

The plasmids used in this work were all derived from pAH5. This is similar to pAH3 (see accompanying paper), except that a *Pvu*I-*Nru*I fragment containing vector sequences only has been replaced with the corresponding *Pvu*I-*Pvu*II fragment from pUC12, which has a more efficient procaryotic replication origin and lacks certain restriction sites. In addition, the single *Eco*RI site in pAH3 was removed by filling in. [pAH5 is identical to pAHP.2, shown in Figure 1C, except that it lacks the synthetic insert together with the associated *Bam*HI, *Sma*I and *Eco*RI sites.]

The two synthetic oligonucleotides (Figure 1A), made using the phosphotriester method (Gait *et al.*, 1982), were kindly provided by Brian Sproat. They were ligated to *Bam*HI-cut M13 mp8 in the presence of DNA polymerase (Klenow fragment) to form the structures shown in Figure 1A (as determined by dideoxy sequencing). The *Hin*dIII-*Eco*RI (filled in) fragment containing the mp8 cloning cassette with the dimer insert was then inserted between the *Hin*dIII and *Acc*I (filled in) sites of AH5 (*Acc*I cuts at the *Sal*I site at the end of the hsp70 coding sequence; the *Eco*RI site was reformed by this ligation). The hsp70 gene was then re-inserted as a *Hin*dIII-*Sal*I fragment into the *Hin*dIII and *Sal*I sites derived from the mp8 cloning cassette to form pAPH.2 (Figure 1C). pAPH.1 was formed similarly, but as an additional step the *Sal*I site was cut, filled in and re-ligated to restore the appropriate reading frame.

pAPH.2 was grown in the *dam*$^-$ *E. coli* strain EQ106 to allow cutting at the *Cla*I site, which overlaps a *dam* methylation site. Deletions of the hsp70 gene were then constructed using convenient restriction sites as follows.

pAPH.57/C, .114/C, .461/C and .480/C. Fragments extending 5′ from the *Xba*I site in the hsp70 gene to *Bal*I (aa57), *Ava*I (aa114; site filled in), *Msp*I (aa461) or *Aha*II (aa480; partial digest) were inserted between the *Cla*I and *Xba*I sites of pAPH.2. The *Cla*I site was filled in for .57/C and .114/C.

pAPH.N/338. pAPH.2 was cut with *Bam*HI and re-ligated to delete the hsp70 coding region between the site at aa338 and the site at the start of the synthetic oligonucleotide dimer.

pAPH.N/434, .N/460, .N/479 and .N/507. The *Hin*dIII-*Acc*I (cuts at *Sal*I site) fragment containing the vector portion of pAPH.2 and the *Hin*dIII-*Bam*HI fragment containing the 5′ end of the hsp70 gene were ligated together with various fragments extending 3′ from the *Bam*HI site in the middle of the gene (see Figure 1C). These extended to *Msp*I (aa434), *Aha*II (aa479), *Msp*I (aa460; partial digest) or *Aha*II (aa507; partial digest). For pAHP.N/507 a *Hin*dIII-*Bam*HI (partial digest) fragment spanning the entire, deleted, hsp70 gene was then transferred to the corresponding position in pAPH.1, thus replacing the out-of-frame synthetic oligonucleotide dimer at the end of the gene with an in-frame monomer.

pAPH.N/537. The hsp70 gene was cut out of pAPH.1 with *Hin*dIII and *Bam*HI, and replaced with the *Hin*dIII-*Xba*I fragment (the *Bam*HI and *Xba*I sites being filled in), thus deleting from the *Xba*I site at aa537 to the *Bam*HI site at the start of the synthetic oligonucleotide.

Detection of tagged protein in cells

COS cells were transfected, transferred to slides, heat-shocked, fixed and stained with the anti-substance P monoclonal antibody NC1/34 (Cuello *et al.*, 1979) and fluorescent goat anti-(rat IgG) exactly as described in the accompanying paper. However, after the cells had been fixed and rinsed in phosphate-buffered saline (PBS), it was necessary to modify them chemically as discussed in the text. A 0.5 M solution of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide was prepared in ice-cold 1 M NH_4Cl and immediately applied to the wells of the PTFE-coated slides. After 20 min at 0°C, a large excess of 1 M NH_4OH was added, and incubation continued for 20 min at room temperature. The slides were then rinsed thoroughly in PBS and stained as normal. This procedure should convert all protein carboxyl groups to amides (cf. Sheehan and Hess, 1955).

Protein blots

Samples corresponding to the total protein from 1/30th of a 25 cm² flask of COS cells were separated on a 15% polyacrylamide-SDS gel and transferred electrophoretically to nitrocellulose (Towbin *et al.*, 1979). The filter was rinsed in PBS, and the immobilized proteins chemically modified as described above. In some experiments the carbodiimide treatment was performed at room temperature, and the NH_4OH step omitted; this gave a somewhat higher sensitivity of detection, but also resulted in some non-specific binding of the second antibody to proteins on the blot. In either case, good blocking of the modified filters proved essential: they were incubated for 30 min in 2% (w/v) dried skimmed milk (Cadbury's Marvel) in PBS, rinsed in PBS, incubated

with monoclonal antibody NC1/34 in 20% foetal calf serum/0.5% Tween-20/PBS for 1 – 2 h, rinsed again, and incubated for 1 h with 10^6 c.p.m./ml ^{125}I-labelled monoclonal mouse anti-rat κ chain in 20% serum/0.5% Tween-20/PBS. After washing well in PBS the filters were dried and exposed to X-ray film for 3 – 16 h at –70°C with an intensifying screen.

Acknowledgements

We thank César Milstein for the monoclonal antibody against substance P, Brian Sproat for the synthetic oligonucleotides, Mike Lewis for invaluable assistance and numerous colleagues for useful advice and comments.

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Received on 7 August 1984; revised on 24 September 1984