In vitro transcription of a human hsp 70 heat shock gene by extracts prepared from heat-shocked and non-heat-shocked human cells

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Received 31 July 1986; Revised and Accepted 23 October 1986

ABSTRACT
A cDNA clone homologous to HeLa cell mRNA encoding the 70 kDa human heat shock protein (hsp 70) was used to isolate a recombinant phage containing an entire human hsp 70 gene. Sequence analysis of the 5' and 3' flanking regions revealed the structural integrity of the regulatory elements. The functional integrity of this hsp 70 gene was substantiated by in vitro transcription studies with nuclear extracts. Specific run-off transcripts, synthesized by RNA polymerase II, were obtained with extracts prepared from heat-shocked cells. Extracts from non-heat-shocked cells were found inactive in hsp 70 gene transcription. However, 5' deletion mutants lacking the heat shock element (HSE) were transcribed by both heat shock and nonheat shock HeLa extracts.

INTRODUCTION
The heat shock response is widely used as a model system to study gene expression in bacteria, plants and animal cells (for a review see ref.1). These studies have demonstrated that the expression of heat shock genes is regulated at various levels. These include gene activation at the chromatin level (2-4), initiation of transcription (5,6), translational control (7,8) and stability of cytoplasmic messenger RNA (9,10). In addition to the ubiquitous occurrence of the heat shock response, in recent years mainly two observations have attracted considerable attention to the heat shock system. One is the heat shock gene expression during normal animal development (11-13) and the other consists in the activation of heat shock genes by transforming genes (14,15).

The response of human cells to elevated temperatures (43°-45°C) consists in the induction of three major polypeptides of 89, 70 and 27 kilo Daltons molecular mass (hsp 89, hsp70 and hsp 27),
respectively (16,17). In order to extend our knowledge on the mechanisms regulating the heat shock gene expression in mammalian cells, we have started the isolation of the corresponding human genes. Such genes provide an excellent basis for a detailed investigation of the compounds affecting the heat shock gene expression at the level of transcription and translation and may help to gain insight in the physiological role of the heat shock response.

In this report we describe a human gene encoding the major heat shock protein hsp 70. The structural analysis of the regulatory elements provides evidence for a functional gene. The gene is accurately transcribed in vitro by nuclear extracts prepared from heat-shocked cells. Nonheat-shocked extracts were found active in hsp 70 gene transcription only with 5' deletion mutants lacking upstream sequences and the heat shock element (HSE,18).

MATERIALS AND METHODS

Materials: Restriction enzymes, DNA polymerase I and Klenow fragment were from Boehringer, 32P-labeled dATP, dCTP and UTP from NEN and nylon membranes and filters from Pall. Cell culture media and sera were obtained from Gibco and electrophoresis supplies from Serva (Heidelberg).

Screening of a human genomic library: The human genomic library described in detail by Lawn et al.(19) was screened with nick-translated DNA of the cDNA clone P7, encoding 850 nucleotides of the human hsp 70 gene (Drabent and Benecke, in preparation), by plaque-lift hybridization (20). Out of 600,000 individual clones, twelve positive signals were obtained and the phages purified. Restriction analysis of DNA isolated from minilysates revealed that all twelve clones were identical, thus representing reisolates of the same phage which was designated as λ Hh7.

Sequence analysis: Sequence determinations of the 5' and 3' flanking regions of the hsp 70 coding sequence present in λ Hh7 was by the dideoxy-chain-termination method (21) after subcloning of restriction fragments into M13 vectors.

Hybridization conditions: Prehybridization of filters and membranes was for 8 hours at 65°C in 5 x SSC, 5 x Denhardt's reagent (22), 0.1% SDS and 250 μg/ml of E.coli nucleic acids.
Hybridization was in the same solution with labeled DNA added for 36 hours at 65°C. The membranes were washed twice in 2 x SSC -0.1% SDS at room temperature and 4 x with 1 x SSC-0.1% SDS at 65°C for 30 minutes, respectively. Isolation of DNA and Southern blotting were by standard techniques (23).

In vitro transcription: HeLa cells were grown in MEM supplemented with 5% newborn calf serum and 293 cells, an adenoviral transformed line of human embryonic kidney cells, in MEM supplemented with 10% fetal calf serum. Nuclear extracts (24) were prepared from either control cells or cells shifted to 43°C for 30 minutes. The assay for in vitro synthesis of run-off transcripts was essentially as described for the RNA polymerase II system (24), with the following modifications: 300 ng of gel-purified DNA fragments were used as template with 10 uCi of α-32P-UTP present per assay. The reaction was terminated after 60 minutes at 30°C by addition of 0.5% SDS. Phenol extracted RNA was analyzed in 5% polyacrylamide gels containing 8 M urea with 0.09 M borate, 0.09 M Tris and 0.01 M EDTA as running buffer. Exposure to Fuji RX films was for 16 hours using intensifier screens.

RESULTS

Isolation and characterization of genomic hsp 70 DNA: We have shown by northern blot hybridization and hybridization selection experiments of control and heat shock messenger RNA that our cDNA clone P7 is complementary to hsp 70 messenger RNA (Drabent and Benecke, in preparation). Nick-translated DNA of this cDNA clone was used as a probe to screen the human genomic library described above. Twelve phages showing homology with P7 DNA were isolated and purified. Restriction mapping revealed that all twelve clones contained the same human DNA insert. Figure 1A shows the restriction pattern of two of these phages, designated as λ Hh7, obtained with the Bam HI and Hind III enzymes. The inserted human DNA of λ Hh7 is represented by a set of subfragments with a characteristic quadruplet between two and three kilobases in length. Southern blot analysis (25) of these fragments showed that only the 2.3 kb fragment did hybridize to P7 cDNA (Fig.1B). The additional signal on top represents undigested phage DNA, reproducibly obtained with partially puri-
Figure 1. Restriction analysis and southern blot hybridization of genomic clones: The 12 clones identified in the library by cDNA hybridization were purified and DNA was isolated from minilysates. The Bam HI-Hind III fragments were separated in 0.8% agarose gels, transferred to membranes and hybridized to labeled cDNA of P7. A: Ethidium bromide staining of two clones; insert specific fragments are indicated by size. B: Southern blot hybridization of A with P7 cDNA. C: Restriction map of λ Hh7 and sequencing strategy.

fied DNA of minilysates. Analysis of λ Hh7 with a variety of restriction enzymes established the map shown in Figure 1C, with the bold arrow indicating the direction of transcription. Furthermore, this arrangement of restriction fragments lead to the conclusion that the hsp 70 gene present in λ Hh7 is very similar to that described previously (26), though our phage certainly spans a different section of human DNA. After subcloning, we have determined the sequence of the promotor region, the 5' leader and the 3' untranslated region as well as the beginning and the end of the open reading frame, following the sequencing strategy outlined in Figure 1C. These sequencing
Figure 2. Sequence analysis of hsp 70 DNA: The 5' (part A) and the 3' (part B) ends as well as flanking sequences of the hsp 70 gene were determined. 1 = heat shock element; 2 = CAAT box; 3 = TATA box; 4 = polyadenylation signal. The nucleotides below the sequence depict divergences to the sequence determined by Hunt and Morimoto (27).

results (Fig.2) indicate that our clone very likely represents a functional gene containing an intact 5' regulatory region including the heat shock element (18), which is identical to the heat shock consensus sequence in 12 out of 14 nucleotides, at position -105, an inverted CAAT-box at -80 and the TATA-box at position -31 relative to the cap site (+1) which has been determined by S1 nuclease mapping. The amino terminus of the protein coding sequence is found at position +214 with the first AUG. At the 3' end (Fig.2B) the last 26 triplets have been determined including the stop codon UAG. Downstream to the stop codon, the polyadenylation signal (AATAAA) preceeding an AT-rich
cluster of nine nucleotides, was found after 242 nucleotides of 3' flanking region. Together, these are the typical regulatory elements characteristic for a wide variety of eucaryotic structural genes. Furthermore, the sequence data presented here are in full agreement with those found in the other human hsp 70 gene (27), again indicating that both hsp 70 genes are closely related or even might be identical.

**In vitro transcription of the hsp 70 gene:** In order to further substantiate our assumption that the hsp 70 gene isolated from λ Hh7 represents a functional gene, we have set up in vitro transcription experiments with nuclear extracts. An isolated 835 nucleotides human hsp 70 gene fragment (-276 to +559) preceded by 176 base pairs of vector DNA was transcribed in vitro with nuclear extracts prepared from heat-shocked HeLa cells. As is shown in Figure 3A, these extracts were found to be highly active in RNA polymerase II directed in vitro transcription of the adenoviral Sma F fragment, which contains the major late promoter, resulting in an α-amanitin sensitive run-off transcript of 536 nucleotides (24). Figure 3B shows the in vitro transcription of the hsp 70 gene fragment described above, with the same extract, resulting in a set of major products, most of which are insensitive to 0.5 ug/ml of α-amanitin (compare lanes 1 and 2). However, the synthesis of one prominent RNA (arrow) is completely inhibited by these concentrations of the toxin, demonstrating that RNA polymerase II is involved. This RNA is slightly larger than the 536 nt

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**Figure 3. In-vitro transcription of adenoviral Sma F and hsp 70 gene fragments:** Isolated DNA fragments (300 ng) were used to direct in-vitro run-off transcription by nuclear extracts. Products were analyzed in 5% acrylamide gels in the presence of 8 M urea with labeled Alu I fragments of plasmid pY-H-48 (40) as marker (m). Part A: Transcription of the adenoviral Sma F fragment without (lane 1) or with (lane 2) 0.5 ug/ml α-amanitin by nuclear extracts prepared from heat-shocked HeLa cells. Part B: Transcription of an isolated 835 bp hsp 70 fragment (-276 to +559) by the same extract without (lane 1) or with (lane 2) inhibitor. Lane 3 - mixing experiment with the Sma F and the hsp 70 fragment (150 ng each) without inhibitor. Lanes 4-8 same as 1 but in the presence of 20, 50, 100, 150 and 200 ug/ml of α-amanitin. Part C: Transcription of a 847 bp hsp 70 fragment restricted with the Sst II enzyme at +227, without (lane 1) or with α-amanitin.
adenoviral run-off transcript observed in lane 3 and corresponds to the expected size of 559 nt for a specific hsp 70 run-off transcript. The most prominent RNA product of about 1000 nucleotides (arrowhead) very likely represented an end-to-end transcript of the DNA fragment. The synthesis of this and most other major RNA products is inhibited by high concentrations of α-amanitin only (lanes 4 to 8), indicating that RNA polymerase III is involved, since this enzyme has been shown to be completely inactivated only by α-amanitin concentrations of 150 ug/ml (28).

Comparable in vitro transcription experiments were performed with the 847 bp Nco I fragment (-272 to +575), however restricted with the Sst II enzyme. The resulting two fragments representing hsp 70 sequences from -272 to +227 and +228 to +575, respectively, were together transcribed in vitro with nuclear extracts as above. As seen in Figure 3C, again the synthesis of most of the products obtained is resistant to low concentrations of α-amanitin, with the two major bands at about 500 and 300 nucleotides (arrowheads) representing end-to-end transcripts of both fragments. However, one RNA polymerase II transcript is observed which closely migrates with the 226 nt marker band (lane 1). Though low in intensity, the length of this RNA again corresponds to the size of the expected run-off transcript of 227 nucleotides, demonstrating that initiation of transcription did occur at the same site as before.

In vitro transcription with heat shock and nonheat shock extracts: Since the overall in vitro transcription efficiency of this hsp 70 gene, which is generally believed to be under the control of a very strong cellular promotor, appeared comparatively low, we wanted to analyze the effect of different extracts. For these experiments, nuclear extracts were prepared from heat-shocked and nonheat-shocked HeLa and 293 cells. First, these nuclear extracts were again assayed with the adenoviral Sma F fragment. The results presented in Figure 4 demonstrate that run-off transcription from the major late promotor is much more effective with nuclear extracts prepared from heat-shocked HeLa cells (lane 5) than with those obtained from nonheat-shocked control cells (lane 3). The run-off products
observed with the control extract formed a weak and much more diffuse band as compared with the heat shock extract. On the other hand, under these conditions massive end-to-end transcription by RNA polymerase II did occur (top region of
lane 3). However, specific transcription by RNA polymerase II was greatly enhanced if the assay was supplemented with a small amount of a cytoplasmic S100 extract (lane 1). Concomittant with this strong increase in run-off transcription was a significant reduction in end-to-end transcription (arrowhead). Thus it appears that during cell fractionation a considerable amount of transcription factor(s) is lost to the cytoplasm. It is quite conceivable that in heat-shocked cells this leakage is strongly reduced, due to a massive aggregation of the collapsed cytoskeleton at the cell nucleus (29). In fact, addition of S100 to a nuclear extract from heat-shocked cells did not increase transcription of the adenoviral Sma F fragment (data not shown).

Lane 7 of Figure 4 shows the in vitro transcription pattern with a nuclear extract prepared from heat-shocked 293 cells. These extracts reproducibly revealed the highest activity in transcription of the Sma F fragment, which might be explained by the fact that 293 cells represent an adenoviral transformed human cell line.

Subsequently, the different extracts described above were analyzed with the same DNA fragment as in Figure 3B for hsp 70 run-off transcription. The results in Figure 5A show that the HeLa control system did not efficiently transcribe the hsp 70 gene. As is evident from lane 2 of Figure 5A, the nuclear extract supplemented with S100 revealed only a very faint and diffuse band at the position of the expected 559 nt run-off product. Although very active in adenoviral transcription, this system only showed low, if any, activity at all in hsp 70 transcription. A different result was obtained with extracts prepared from 293 cells. As expected, the extract prepared from heat-shocked cells was found most active, with the major product representing the specific run-off transcript (Fig. 5A, lane 4). In contrast to HeLa cells, however, the transcription pattern obtained with 293 control extracts supplemented with S100 clearly revealed the hsp 70 run-off transcript (lane 6), though the rate of synthesis was reduced if compared to the corresponding extract from heat-shocked cells. This result is in good agreement with the previous observation that 293 cells, even in the absence
Figure 5. In vitro transcription of the native hsp 70 gene (A) or the 5' deletion mutant (B) with heat-shocked and non-heat-shocked extracts: Part A: In vitro RNA synthesis with the same DNA as in Fig.3B was in the presence (lanes 1,3,5) or absence (lanes 2,4,6) of 0.5 ug/ml of a-amanitin. Extracts used were: Lanes 1+2 - HeLa control extract with S100; 3+4 heat-shocked extract from 293 cells; 5+6 - control extract from 293 cells. Part B: Transcription with the deletion mutant lacking the upstream sequences beyond -79 was in the presence (lanes 3+5) or absence of 0.5 ug/ml of a-amanitin (lanes 1,2,4,6). Nuclear extracts used were: Lane 1 - heat-shocked HeLa cells with S100; lane 2 - nonshocked HeLa cells with S100; lanes 3+4 - nonshocked HeLa cells without S100; lanes 5+6 - nonshocked HeLa cells with S100. The 596 nt run-off transcript (arrow) and the end-to-end transcript of the fragment (arrowhead) are indicated.

of elevated temperatures, constitutively express the major heat shock genes (14).

The extremely inefficient hsp 70 gene transcription observed with HeLa control extracts somehow appeared to be inconsistent
with the integrity of the properly positioned promotor elements such as the TATA and the CAAT-box. Therefore, we supposed that this seeming paradox might be explained by a negative regulatory effect of upstream sequences, possibly the heat shock element itself. In order to investigate this hypothesis, a 5' deletion mutant was analyzed for in vitro transcription by control extracts. This deletion mutant enclosed the Sst I - Bgl II fragment (-79 to +556) cloned in the Sst I and Bam HI sites of the pUC 18 vector. The isolated 680 bp Eco RI - Hind III fragment of this 5' deleted subclone should direct the synthesis of a 596 nt run-off transcript. As is shown in Figure 5B, lane 1, a major transcript of this size is obtained with a nuclear extract from heat-shocked HeLa cells (arrow). However, with this deletion introduced to the hsp 70 gene, the same run-off transcript is synthesized with comparable efficiency by a HeLa control extract (lane 2). It should be mentioned that both assays were performed with S100 supplementation. The effect of this supplementation is seen by a comparison of lanes 4 and 6 of Figure 5B. Addition of this cytoplasmic fraction (lane 6) caused an increase in run-off transcription accompanied by a decreased rate of synthesis of the end-to-end transcript (arrowhead). Both these transcripts are synthesized by RNA polymerase II, indicated by their sensitivity towards low concentrations of α-amanitin (Fig.5B, lanes 3 and 5). Together, these results reveal that upon removal of upstream flanking sequences, the human hsp 70 gene is efficiently transcribed by a cell-free extract derived from non-heat-shocked HeLa cells. In contrast, efficient transcription of the wild type gene depends on components present in heat-shocked cells only.

DISCUSSION

The heat shock induction of proteins and among these that of a 70,000 dalton polypeptide is highly conserved throughout evolution (1). Comparable to the situation observed in Drosophila (30), human DNA sequences coding for the major heat shock protein hsp 70 apparently belong to a gene family. In order to avoid the selection of hsp 70 related sequences such as cognate or pseudogenes distributed within the human genome
(31), the recombinant phage described here (\(\lambda\) Hh7) was isolated using a homologous cDNA as probe. Based on restriction mapping and sequence analysis, the hsp 70 gene present in \(\lambda\) Hh7 appears to be very similar or even identical to the human gene described previously (26,27). Within the 5' flanking region only three base substitutions were observed in comparison to that other sequence, none of which is located within essential regulatory elements such as the TATA and CAAT-box or in the heat shock element. In contrast, the 5' flanking sequence identified here is not shared by another member of the human hsp 70 gene family (32), although that promotor too clearly is functional and under heat shock control, but does not respond to adenoviral E1A activation. That gene contains a much shorter 5' leader and lacks both the typical TATA and CAAT boxes. Furthermore, two overlapping sequence elements at position -66, each of which corresponds to the heat shock consensus sequence in 7 out of 14 positions were observed.

In addition to the structural integrity of the regulatory elements, we have shown that the hsp 70 promotor described here directs the RNA polymerase II catalyzed synthesis of specific run-off transcripts. However, in contrast to results obtained with the Drosophila system (33), in vitro transcription of the native human hsp 70 gene is efficient only with extracts prepared from heat-shocked cells. Nonshocked extracts obtained from control cells revealed inefficient, if any, synthesis of specific run-off transcripts. Since under heat shock the rigidity of the cell nucleus is significantly enhanced, one might explain this effect by a differential loss of nuclear proteins including transcription factors to the cytoplasm. Our results show, however, that supplementation of the nuclear extract with a cytoplasmic S100 fraction, though strongly increasing the transcriptional activity with adenoviral DNA, did not lead to efficient in vitro transcription of the hsp 70 gene by nonshocked extracts. Therefore, the seeming similarity of increased transcription rates of the adenoviral gene and the hsp 70 gene in heat shock extracts as compared to control extracts, is due to two different effects. Whereas adenoviral transcription with control extracts is strongly increased by
readdition of factors lost to the cytoplasm, the failure to stimulate hsp 70 gene transcription in the same system shows that in this case a heat shock specific factor must be involved. These results confirm a previous suggestion that extracts of heat-shocked and non-heat-shocked HeLa cells possess different transcriptional specificities, although in those experiments the HeLa extracts were used to transcribe the heterologous Drosophila heat shock gene (34). One possible explanation for the different transcriptional activities of both extracts might be a positive regulation mediated by a heat shock specific transcription factor. Such a heat shock specific transcription factor (HSTF) has been isolated from Drosophila nuclear extracts and was analyzed in detail (33). HSTF was found in both types of extracts at approximately the same level, albeit significantly reduced in activity within nonshocked extracts. With these reduced amounts of the active form of HSTF present in nonshocked extracts, the question remains why RNA polymerase II together with other transcription factors, such as the TATA box binding factor B (33), does not use the hsp 70 core promotor to initiate transcription. One might speculate that "upstream" sequences which were found to be essential for efficient transcription of other eucaryotic genes (35-38), are blocked by the inactive form of HSTF which still may be able to bind to the heat shock element without promoting transcription. An approach to this hypothesis was made by in vitro transcription experiments performed after deletion of upstream sequences including the heat shock element. Removal of these sequences indeed now allowed the efficient transcription of this hsp 70 gene in the nonshocked system. This result was quite surprising, since removal of upstream sequences from "normal" cellular (35-37) and viral genes (38) strongly interfered with transcription initiation. The hsp 70 gene described here includes two of the putative upstream binding sequences GGGCGG for the SP1 transcription factor (39). Our deletion mutant still contained one of these two binding sites. This may explain why the nonshocked system still is able to use this truncated promotor for efficient hsp 70 transcription. A detailed investigation is currently performed in our laboratory to determine the molecular mechanism by which factors and/or
upstream sequences prevent the native hsp 70 gene from being transcribed efficiently by extracts prepared from non-heat-shocked cells.

ACKNOWLEDGEMENTS
We wish to thank Dr. T. Maniatis for providing the human genomic library and Dr. O. Pongs for providing research facilities. Thanks are also due to V. Lardong for skilful technical assistance and to K. Grabert for the photographs. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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