Volume 14 Number 14 1986 Nucleic Acids Research

Induction of B2 RNA polymerase III transcription by heat shock: enrichment for heat shock induced sequences in rodent cells by hybridization subtraction

Albert J.Fornace Jr and James B.Mitchell

Radiobiology Section, Radiation Oncology Branch, N.I.H., Bethesda, MD, USA

Received 9 March 1986; Revised and Accepted 19 June 1986

ABSTRACT

When hybridization subtraction was used to enrich for sequences induced by heat shock in Chinese hamster cells, B2 sequences were found to be one of the major sequences enriched. With cloned B2 probes, we found that the level of the short, 0.1 to 0.6 kb, polyadenylated RNA polymerase III transcripts of this repetitive genetic element increased approximately 10 to 20 fold after heat shock. Transcription of B2 RNA by RNA polymerase III was rapidly induced after heat shock based on time course studies and nuclear runoff experiments. The induction of B2 RNA was not a nonspecific response to lethality or cellular injury because maximum B2 RNA induction was observed with even nontoxic heating while no induction occurred with other agents such as UV or X-radiation. Since B2 RNA increased after heat shock in several different Chinese hamster and mouse cell lines, induction of B2 RNA by heat shock is probably common in rodent cells. B2 RNA may also be the most abundant transcript induced by heat shock because the level of B2 RNA was substantially higher than several other abundant transcripts induced by heat shock including a rodent HSP70. Our finding of the induction of high levels of RNA polymerase III B2 transcripts in different rodent cells raise the possibility of a role in the heat shock response.

INTRODUCTION

The B2 sequence is one of the major short repetitive genetic elements in rodent cells; approximately 10^5 copies of this sequence are scattered throughout the rodent genome (1). The B2 sequence is specifically transcribed by RNA polymerases III and contains RNA polymerase III promoter regions; B2 sequences are also found in the introns and 3' nontranslated regions of some RNA polymerase II transcripts (1-5). B2 RNA is present in hnRNA and cytoplasmic RNA (1-5). Recently, there has been much interest in B2 RNA because the level of certain RNA polymerase III (RNA pol III) transcripts has been found to vary in different tissues and to be increased in malignant cells (2, 4). B2 RNA levels are modulated by changes in the physiologic state of the cell since these transcripts are expressed in undifferentiated mouse embryonal carcinoma cells but not in their differentiated derivatives (6). In addition, the level of these RNA pol III B2 transcripts has been found to be increased in mouse cells after SV40 transformation (7). These RNA pol III B2 transcripts are polyadenylated at their 3' ends in a manner very similiar to mRNA (8); B2 RNA localizes to the cytoplasm and varies from 0.1 to 0.6 kb in length (1-3). Both strands of the B2 element can be found in higher molecular weight RNA, however only one

strand is transcribed in the short RNA pol III transcripts (4). In this paper, we report that these short RNA pol III B2 transcripts are rapidly induced by heat shock (HS) in rodent cells.

Besides the induction of HS proteins, HS has other profound effects on both transcription and translation in eukaryotic cells (9), but induction of RNA pol III transcription from single copy or repetitive genes has not been described previously. HS has been shown to induce transcription from two repetitive elements in lower eukaryotes, the DIRS-1 of Dictyostelium (10) and copia of Drosophilia (11). In vertebrate cells, HS induction of transcription of repetitive genes has not been observed previously. We have found that HS rapidly induced increased levels of short pol III B2 transcripts in several different rodent cell lines. This B2 RNA was found to be a major transcript induced by HS; on a molar basis, B2 RNA was probably the most abundant polyadenylated (polyA) transcript induced by HS.

Hybridization subtraction was used to enrich for B2 and other sequences induced by HS. Our approach differed from others (12) in that low ratios of control RNA : HS cDNA was used. In the past, high ratios of RNA : cDNA have been used in hybridization substraction in order to drive the reaction as much as possible to completion. In such experiments, only cDNA complementary to sequences absent or present at much lower levels in the control RNA will remain single stranded and be enriched by hybridization subtraction (12). Theoretically, low ratios of RNA : cDNA can be used if the hybridization is driven towards completion by a high concentration of RNA. The advantage of low ratios of RNA : cDNA is that sequences only several fold more abundant in the cDNA may be enriched for by hybridization subtraction. In the case of the reponse(s) of higher eukaryotic cells to a cellular injury such as HS, induced transcripts can be present in untreated cells at substantial levels (9) such that low ratios of RNA : cDNA will be required to enrich for these sequences by hybridization subtraction. With a ratio of control RNA : cDNA of 1:1, we have demonstrated greater than 20 fold enrichment by hybridization subtraction for sequences induced 3 to 10 fold by HS.

MATERIALS AND METHODS

Cell Culture

Chinese hamster V79 cells which were derived originally from lung fibroblasts, mouse NIH 3T3 cells, and Chinese hamster ovary (CHO) cells were grown in F12 medium with 10% fetal calf serum, penicillin, and streptomycin (13). The CHO-K1 and CHO-16B cell lines were provided by T. Stamato. Exponentially growing cultures were used for all experiments. Cell survival was assessed by colony forming ability as described previously (13). For heat shock (HS) experiments, cells were heated in 175 cm^2 flasks containing 35 ml of medium in a 45.5^{o} water bath; approximately 5 min was required for these flasks to equilibrate to this temperature. The standard heating time was 17 min unless otherwise noted; this heating time reduced cell survival to approximately 10 to 20% (Fig. 6). In some experiments, 5 μg/ml of actinomycin D (Calbiochem) was added to the cells 10 min before heating; after HS, the medium in these flasks was replaced with fresh medium containing actinomycin D for the indicated times.

RNA Preparation and Blotting

4 M guanidine thiocyanate/15 mM Na_2EDTA/50 mM TRIS/0.5% sarkosyl/10 mM ßmercaptoethanol, pH 7.5, was added directly to tissue culture flasks, and whole cell RNA was then isolated by standard techniques (14). The yield of RNA per 10^9 cells was similiar in all samples. All RNA samples were treated with RNase-free DNase (Worthington). RNA, 1 µg per lane unless otherwise specified, was size separated on formaldehyde agarose gels (15, 16); size markers included human liver ribosomal RNA and end labelled single strand DNA. RNA was transferred to nylon filters, Gene Screen Plus (New England Nuclear), according to the manufacturer's directions by capillary blot for 20 h (14). RNA (Fig. 7, lanes 1-4 only) was transferred to nitrocellulose as previously described (14). For dot blots, RNA or linearized DNA was bound to nylon filters in 50 mM sodium phosphate, pH 6.8, or nitrocellulose in 1.5 M ammonium acetate. In Figures 4 and 8C, the exact concentration of DNA in each sample was determined by fluoresence with Hoechst 33258 (Aldrich) in a fluorimeter.

Hybridization Subtraction

cDNA was synthesized with polyA RNA (oligo dT cellulose selected) and AMV reverse transcriptase (Life Sciences) (see Fig. 7 lanes 5-8 and Fig. 8A) or MMLV reverse transcriptase (BRL) (see Fig. 7 lanes 1-4 and Fig. 8B). The AMV enzyme was used at 600 u ml^{-1}in 0.5 mM dCTP/50 µCi ml^{-1}[^{32}P]dCTP/1 mM dATP/1 mM dGTP/1 mM dTTP/7 mM $MgCl_2$/300 u ml^{-1} RNasin (Promega)/2 mM dithiothreitol/ 60 µg ml^{-1} actinomycin D/15 mM KC1/50 mM TRIS (pH8.3)/35 µg ml^{-1} oligo $dT_{(12-18)}$ (PL Biochemicals)/40 µg ml^{-1}polyA RNA for 1.5 h at 42°. The MMLV enzyme was used for 1 h at 37° in the same solution at 6000 u ml^{-1}, except for the following changes: 60 mM KCL, 50 mM TRIS (pH 7.5), 7 µg ml^{-1} oligo $dT_{(12-18)}$, plus 200 µg ml^{-1} random primers, an oligodeoxynucleotide mixture $pd(N)_6$ (PL catalogue no. 27-2166).

For hybridization subtraction, cDNA which had been synthesized from 30 to 60 µg of polyA RNA was hybridized with 30 to 90 µg of control polyA RNA to an uncorrected ROT of 10^3 (12). Single strand cDNA was isolated by hydroxylapatite chromatography at 62° (12). Hybridization subtraction removed 85 to 93% of the starting cDNA. The remaining single strand cDNA was hybridized with HS polyA RNA to an uncorrected ROT of 200 to remove small and other non-hybridizing species. This second hybridization resulted in a 1.5 to 4 fold enrichment. In two experiments, the single strand cDNA from the second hybridization was used as template for probe as in Fig. 7, and no hybridization was observed (data not shown). The hybrid duplex cDNA from the second hybridization subtraction, the enriched HS specific cDNA, was treated with base to remove the RNA. In some experiments (Fig. 8), this enriched HS specific cDNA was used as template in the synthesis of radioactive probes with random primers (17). For northern analysis, 20 ng of this enriched HS specific cDNA was made into double strand DNA with the Klenow fragment of DNA polymerase I, random primers, 10^{-10} moles of dCTP, and excess of the other nucleotides (17). This reaction was monitored with a small amount of [^{32}P] dCTP, 1 µCi; over 70% of the cDNA was made double stranded in all samples. High specific

activity radioactive probes were generated using a portion of this double strand cDNA as template as described previously (17); briefly, the double strand cDNA was denatured by heating to 100° and then incubated at 25° with deoxynucleotides, the Klenow fragment of DNA polymerase I, and random primers. With this protocol both strands of the double strand cDNA were labelled.

Plasmids

Several plasmid clones were used which will be described in more detail elsewhere. The plasmid clone pHPa2 was provided by T. Fanning and was isolated from the cosmid clone pHTKA after rescue from transfected mouse cells, (L Yun-Fai and Y Wai Kan, 1984, Proc. Natl. Acad. Sci. USA 81: 414). The plasmid clone pHPa2 was found to contain mouse DNA; a 144 bp AluI fragment was sequenced and found to be a B2 sequence with 139 bp identical in sequence to the published B2 consensus sequence (T. Fanning, personal communication). pM1.8, a mouse HSP70 (70 kdalton heat shock protein) genomic clone, was provided by R. Morimoto. pMl.8 is from an intronless gene, starts approximately 280 bp 5' from the translation start site, and is 1.7 kb in length; it contains over 1.6 kb of sequence found in the gene's mRNA by sequence analysis (R. Morimoto, personal communication). Both V79 cells and mouse cells showed a very similar 2.6 kb HS inducible mRNA on northern analysis with pM1.8 (data not shown).

V79 cDNA plasmid clones were isolated from a library derived from polyA RNA extracted from V79 cells treated with HS followed by a 4 h incubation at 37°. This cDNA library was constructed in E. coli HB101 by synthesizing double strand cDNA as previously described but without DNA ligase (18), standard GC tailing, and cloning fragments > 0.5 kb into the Pst I site of PBR322 (14). p14 was a randomly chosen plasmid which hybridized with a discrete 2 kb band on northern analysis; p35 hybridized with a 2.5 kb RNA species; the level of p14 and p35 as measured by quantitative RNA dot blots was unchanged by HS (data not shown). Multiple cDNA clones containing B2 elements were isolated from this library by screening (14) with labelled enriched HS specific cDNA obtained by hybridization subtraction; several including pHS18 were sequenced and found to contain typical B2 elements. pH8 and pH37 were obtained by screening this library with labelled cDNA probes derived from control and HS V79 cells (19); these plasmids preferentially hybridized with HS cDNA (data not shown). pH37 was the most abundant isolate other than B2 cDNA clones. pH8 hybridized at high stringency with human HSP27 cDNA provided by D. L. Weber; pH8 and human HSP27 probes hybridized with the same V79 cell RNA on northern analysis (data not shown).

Radioactive Probes

DNA was labelled with [^{32}P] dCTP, 3000 Ci/mmol (Amersham), random primers, and the Klenow fragment of DNA polymerase as described previously (17). Radioactive cDNA probes were synthesized with reverse transcriptase and random primers (14), except for Fig. 8C where oligo dT $_{(12-18)}$ (PL Biochemicals) was used as a primer. For RNA probes, the 252 bp Pst I fragment of pHS18, which contains a B2 sequence, was cloned into pGEM-1 (Promega); high activity strand specific RNA probes were synthesized with SP6 or T7 RNA polymerase according

to the manufacturer's (Promega) instructions. PolyT probes were synthesized with AMV reverse transcriptase as described above but with polyadenylic acid (Sigma) as template, only dTTP as precursor, and without actinomycin D such that the average length was 200-300 bases.

Hybridization

Gel blots or dot blots were hybridized with 10^6 dpm ml^{-1}, unless otherwise noted. RNA nitrocellulose blots were hybridized at 42° for 40 h in 50% formamide with 10% dextran sulfate (14). The DNA dot blots on nitrocellulose were for 18 h at 65° (14). The nitrocellulose blots were washed in buffered salt solutions containing decreasing concentrations of Na^+ at 65°; the final rinse was in 0.02 M Na^+ for 0.5 h (14). Nylon filters were hybridized in the same solution as Ref. (20) for 18 h at 65°; the filters were rinsed for 1 h at 65° in the high salt wash, 0.21 M Na^+, and a total of 40 min at 65° in the low salt wash, 0.075 M Na^+ (20).

In order to estimate the polyA content of various RNA samples, RNA dot blots were hybridized with labelled polyT, 25 to 200 ng ml^{-1}, for 2 to 4 h at 44° in the hybridization solution of Ref. (20); they were rinsed with high salt wash (19), 0.21 M Na^+, for 50 min at 25°, 30 min at 44° and 0.5 min rinse at 25° in 0.075 m Na^+. A representative experiment is shown in Fig. 3A. Hybridization was measured by scintillation counting and the polyA content normalized to that of the control (untreated) sample. In most experiments, the polyA content varied by less than 20% when equal amounts of RNA, as measured by absorbance at 260 nm, were used. To confirm that the polyA content could be accurately estimated with this polyT probe, the polyA content was estimated in several RNA samples (such as in Fig. 1C) with 2 non-HS inducible probes, p14 or a ß-actin probe; the relative hybridization with these probes was proportional to that seen with the polyT probe (all data not shown).

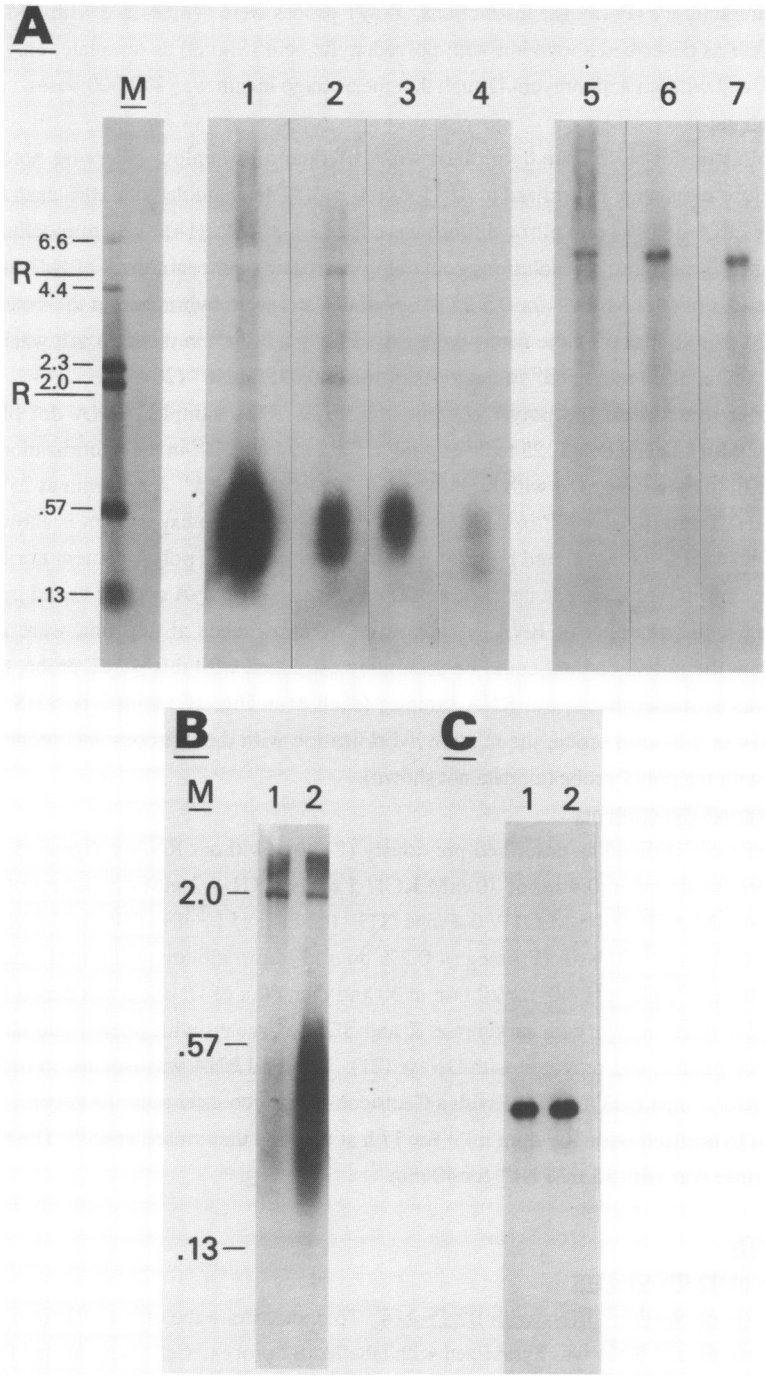
Nuclear Runoff Experiments

Nuclei were isolated as described previously (21) but without RNAse digestion. 5 x 10^7 nuclei were incubated in 0.4 ml of 70 mM KCl/ 5 mM $MgCl_2$/ 2 mM dithiothreitol/ 0.1 mM EDTA/ 0.4 mM ATP/ 0.4 mM CTP/ 0.4 mM GTP/ 0.2 mCi of [^{32}P] UTP (0.33 µM, 3000 Ci $mmol^{-1}$)/ 600 u ml^{-1} RNasin (Promega)/ 0.5% bovine serum albumen/ 0.5 mM $MnCl_2$/10% glycerol/ 10 mM TRIS, pH 8.0, for 20 min at 30 and then 60 u of DNase I was added for 5 min. The samples were treated with proteinase K and SDS, extracted with phenol and chloroform, ethanol precipitated, and retreated with DNase (21). Labelled RNA was isolated in 0.25% SDS/ 10 mM TRIS/ 1 mM EDTA, pH 7.1 with a Centricon-30 microconcentrator (Amicon). DNA dot blots were hybridized with 10^6 dpm ml^{-1} for 17 h at 65° and then rinsed at 68°. The final most stringent rinse was with 75 mM Na^+ for 40 min.

RESULTS

Induction of B2 RNA by HS

Analysis of Northern Blots with B2 Probes. To demonstrate the effect of HS on B2 RNA, Chinese hamster cell RNA was hybridized with labelled B2 probes. Cells were heated for 17 min.


A
M
1
2
3
4
5
6
7
6.6
R
4.4
2.3
2.0
R
.57
.13
B
M
1
2
2.0
.57
.13
C
1
2

at 45.5° which reduced survivial to 10 - 20% of unheated cells, and incubated at 37° for 2 - 4 h to allow sufficient time for the accumulation of HS induced transcripts (9) prior to RNA extraction. In Figure 1, strand specific probes of a Chinese hamster B2 sequence were hybridized with V79 cell RNA. As seen in this figure, short B2 RNA was approximately 10 times more abundant in polyA (polyadenylated) RNA from HS treated cells compared to control (untreated) cells. In this figure, only one strand specific probe (lanes 1-4) hybridized with the 0.1 - 0.6 kb HS induced B2 RNA. This probe (minus strand) was complementary to the published (1) B2 sequence. In contrast, no hybridization was seen to this short RNA with the plus strand probe (lanes 5-7) which was the same orientation as the B2 RNA. The RNA pol III B2 transcripts described by others were of the same size and showed the same pattern with strand specific probes as Fig. 1 (4). B2 probes also hybridized with higher molecular weight RNA with prominent hybridization to ribosomal size RNA; however, in all experiments, HS did not noticeably affect the level of these higher molecular weight B2 transcripts. The same results were obtained with other B2 probes including a mouse B2 sequence (data not shown).

Several other observations can be made from northern analysis of RNA from HS treated cells. The first is that the HS induced B2 RNA was probably polyadenylated since it was 30-50 fold more abundant in polyA RNA compared to whole cell RNA (Fig. 1A and 3A). The HS induced B2 transcripts were not a discrete length but varied from 0.1 - 0.6 kb; in Fig. 1B, the size range of these HS induced B2 transcripts are clearly shown. As described in Methods, equal amounts of polyA RNA were used in control and HS samples based on relative hybridization with a polyT probe; this was confirmed in Fig. 1C with the use of a β-actin probe. Hybridization to actin RNA was very similiar in HS and control samples; these bands were cut out and measured by scintillation counting and found to vary by less than 25%.

In Fig. 2A, the same effect of HS on B2 RNA was observed in 2 Chinese hamster ovary (CHO) cell lines. The intensity of hybridization in lane 1 was equivalent to that of V79 cell RNA treated in the same manner. Since B2 RNA was also induced by HS in CHO cells and mouse cells (see Fig. 3), this effect of HS on B2 RNA is probably common in rodent cells.

B2 RNA Is Rapidly Induced By HS. Timecourse experiments were performed to determine how quickly the B2 RNA level rose after HS. As seen in Fig. 3B, the level of B2 RNA increased

Figure 1. Northern blot analysis with strand specific B2 probes. In A, V79 cell RNA was size separated in a 1.2% agarose formaldehyde gel, transferred to a filter, and hybridized with labelled B2 RNA synthesized from a pGEM-1 vector with SP6 (minus strand RNA, lanes 1-4) or T7 RNA polymerase (plus strand RNA, lanes 5-7). Samples are:

1, 5 polyA RNA, HS and 2 h at 37°
2, 6 polyA RNA, control
3 same as lane 1 except 10% the amount of RNA (100 ng)
4, 7 whole cell RNA, HS and 2h at 37°

In B, control (lane 1) and HS (lane 2) polyA RNA were analyzed on a 3% agarose gel (Nusieve, FMC) and hybridized with labelled minus strand B2 RNA.
In C, control (lane 1) and HS (lane 2) polyA RNA were hybridized with a labelled β–actin probe (28); these lanes were a portion of the same gel used in panel A.
Size markers, M, were single strand DNA and ribosomal RNA (R).

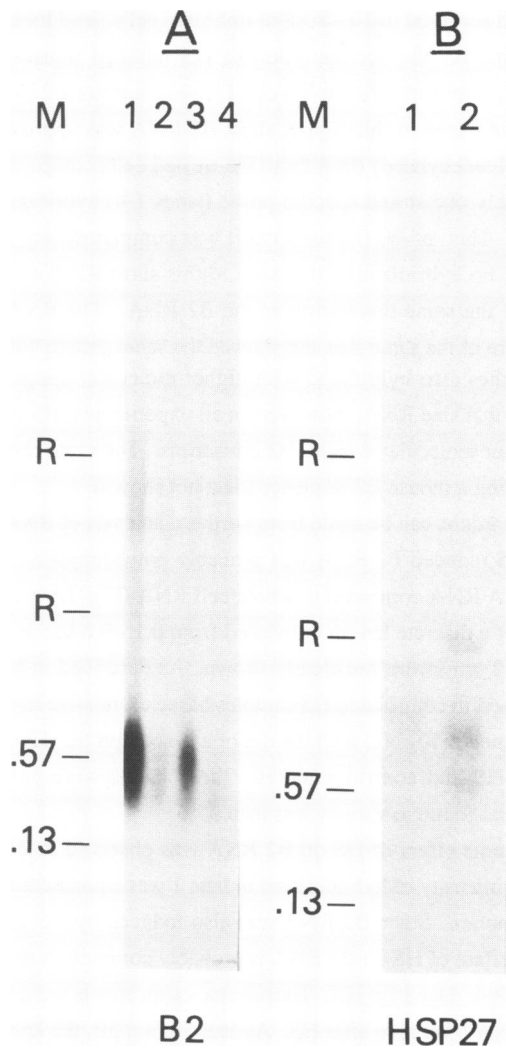


Figure 2. Northern blot analysis with B2 or HSP27 probes. In A, whole cell RNA from CHO K1 cells (lanes 1-2), and CHO 16B cells (lane 3-4) was hybridized with labelled B2 DNA (the 252 bp fragment of pHS18); RNA in lanes 1 and 3 was isolated from HS treated cells and lanes 2 and 4 from control cells. In B, control (lane1) and HS (lane 2) polyA RNA was hybridized with HSP27 (pH8) probe. Size markers were as in Fig. 1; R refers to the 5 and 1.7 kb RNA markers.

appreciably within 15 min after HS. In typical experiments with HS, B2 RNA content was elevated 6-9 fold over control by 4 h after HS and remained at this level for 24 h (Fornace et al, manuscript in preparation). The RNA dot blots in Fig. 3 and subsequent figures underestimated the HS induction of this short B2 RNA by approximately a factor of 2 since the control samples

also contained substantial levels of unrelated longer B2 containing transcripts which were not affected by HS. As shown in Fig. 1A, the level of short B2 transcripts in cells 2 h after HS was approximately 10 fold greater than control cells. In order to accurately measure the increase in the short HS induced B2 transcripts, the 0.1 - 0.6 kb portion of several B2 northern blots (such as in Fig. 1) were cut out and measured by scintillation counting; B2 hybridization in HS samples was 10-20 fold greater than control samples.

The Increase in B2 RNA Is Due to Increased Synthesis. The increase in B2 RNA seen after HS required RNA synthesis since no increase in B2 RNA occurred in the presence of actinomycin D (Fig. 3B). The B2 RNA level actually decreased in the presence of actinomycin D with a T1/2 of approximately 1 h. B2 transcription was increased for a considerable time after HS since the actinomycin D experiments indicate a rapid turnover of B2 RNA.

Nuclear runoff experiments were performed in Fig. 4 to confirm that the increase in B2 RNA after HS was due to increased synthesis. Hybridization to B2 DNA was more than 10 fold greater with labelled runoff RNA from nuclei of HS treated cells than nuclei of untreated cells. Hybridization to pH37 DNA was also markedly greater with RNA from nuclei of HS treated cells. Addition of the RNA polymerase II inhibitor α-amanitin had no effect on B2 RNA synthesis, but did markedly inhibit the synthesis of RNA which hybridized with pH37. These results indicate that both B2 and pH37 locus RNA synthesis was increased in HS treated cells. The α-amanitin experiments indicate that the HS induced B2 RNA was transcribed by RNA polymerase III while RNA from the pH37 locus was transcribed by RNA polymerase II.

B2 RNA Is Induced By HS But Not By Other Types of Cellular Injury Such as UV or X-Irradiation. Experiments were performed to determine if the induction of B2 RNA was specific for HS compared to other unrelated types of cellular injury. Equal amounts of polyA RNA from V79 cells treated with equitoxic doses of HS, X-rays, or UV-radiation were hybridized with B2 probe in Fig. 5. As seen in this figure, treatment of the cells with X-rays or UV had no effect on the B2 RNA level, while HS increased the B2 RNA level 5-7 fold. These results indicate that the induction of B2 RNA by HS was not a nonspecific response to cellular injury.

B2 RNA Induction By HS Is Not Related To Cell Lethality. Cells were heated for varying times in order to determine the relationship of B2 RNA induction by HS to the cellular toxicity of HS. In Fig. 6, both cell survival and the B2 RNA level were determined after HS. B2 RNA was induced by even nontoxic cell heating; maximum induction was seen with a HS treatment that produced no cell lethality. Ethanol induces increased levels of HS proteins in most cells including rodent cells (9). Ethanol treatment also induced a significant increase in the level of B2 RNA with no appreciable cell lethality. These results indicate that the induction of B2 RNA by HS or ethanol was not dependent on cell lethality.

Enrichment for HS Induced Sequences by Hybridization Subtraction

The induction of B2 RNA by HS was originally observed during hybridization subtraction experiments. These experiments can be used to illustrate that B2 RNA is a major transcript

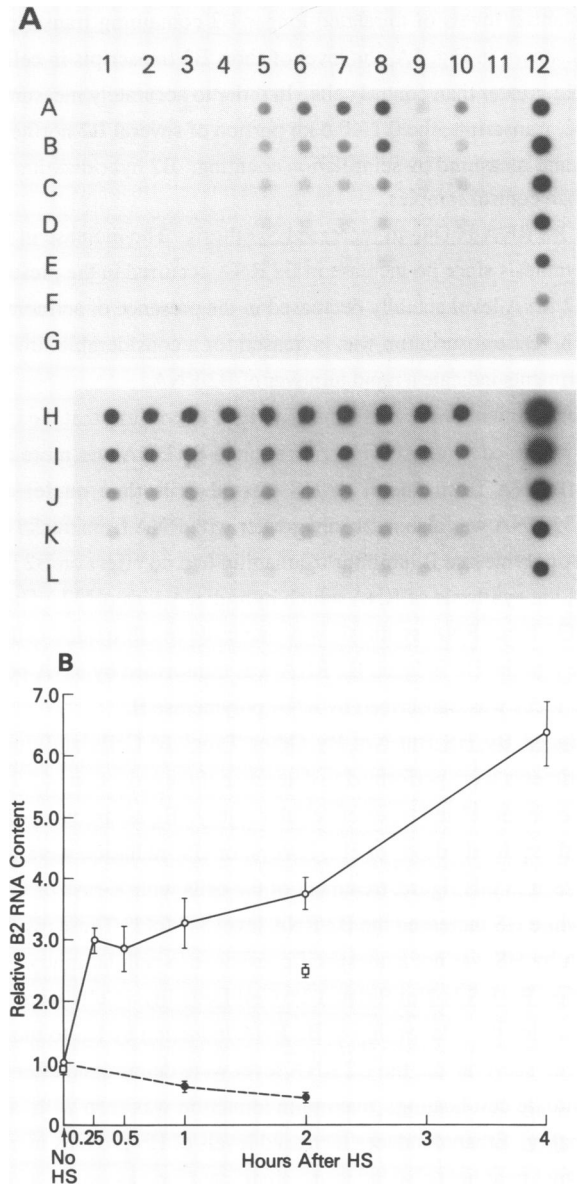


Figure 3. Timecourse for B2 RNA induction. Cells were heated, incubated for varying times at 37° after HS, and then analyzed by RNA dot blot hybridization for B2 RNA content (panel A, rows A-G) or polyA content (panel A, rows H-L). Samples are:

1, 2 whole cell V79 RNA, control
3 whole cell V79 RNA, HS and 1 h at 37° with actinomycin D
4 whole cell V79 RNA, HS and 2 h at 37° with actinomycin D
5 whole cell V79 RNA, HS and 0.25 h at 37°

6 whole cell V79 RNA, HS and 0.5 h at 37°
7 whole cell V79 RNA, HS and 1 h at 37°
8 whole cell V79 RNA, HS and 2 h at 37°
9 whole cell mouse 3T3 RNA, control
10 whole cell mouse 3T3 RNA, HS and 2 h at 37°
11 yeast tRNA
12 polyA V79 RNA, HS and 2 h at 37°

500 ng of RNA was used in row A (except 250 ng in sample 12) with successive 2 fold dilutions in rows B-G; 250 ng was used in row H (except 125 ng in sample 12) with 2 fold dilutions in rows I-L. The Chinese hamster B2 probe was the same as in Fig. 2. PolyA content was measured by hybridization with a polyT probe (see Methods). In panel B, dot blots were measured by scintillation counting and the relative B2 content was normalized to that of RNA from control V79 cells after adjusting for differences in polyA content. Open circles indicate V79 samples ± SD; closed circles indicate V79 cells also treated with actinomycin D. Open square indicates mouse NIH 3T3 cells.

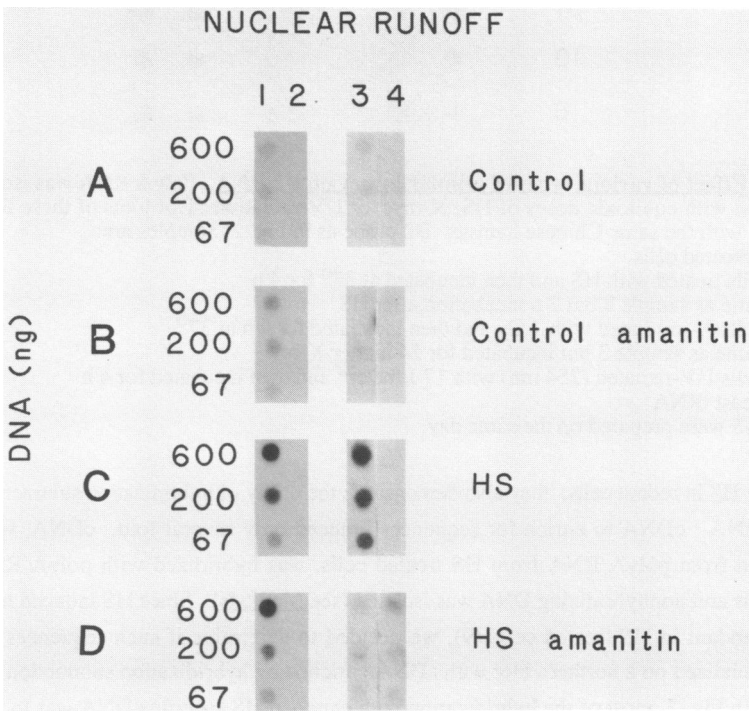


Figure 4. Nuclear runoff. Equal amounts of pHS18 which contains a B2 sequence (lane 1), pH37 (lane 3), or PBR322 (lanes 2, 4) DNA were hybridized with radioactive RNA labelled by nuclear runoff. In A and B, labelled RNA was isolated from nuclei of untreated V79 cells (Control); in C and D nuclei from cells treated with HS and 1 h at 37° were used. α-amanitin, 1 μg ml^{-1}, was included in the labeling reaction of B and D. The autoradiography exposure time was approximately 10 times longer for lanes 3 and 4 than lanes 1 and 2.

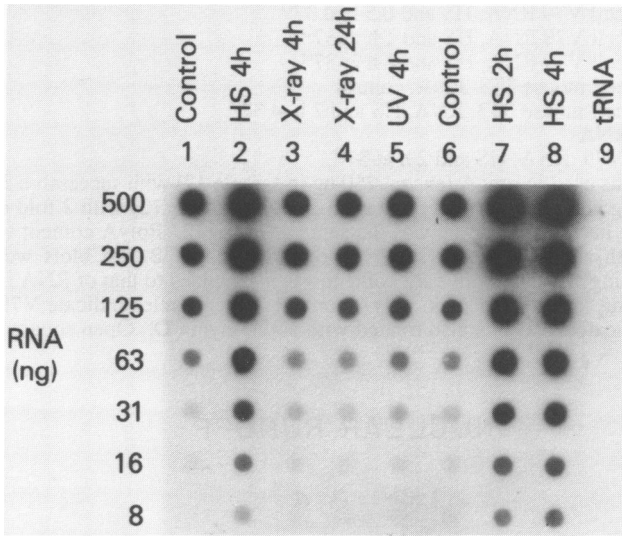


Figure 5. Effect of various types of cellular injury on B2 RNA. PolyA RNA was isolated from cells treated with equitoxic doses of HS, X-rays, or UV-radiation. Dot blots of these RNAs were hybridized with the same Chinese hamster B2 probe as in Fig. 2. Samples are:

1, 6 untreated cells
2, 8 cells treated with HS and then incubated at 37° for 4 h
7 same as sample 8 but 2 h incubation after HS
3 cells X-irradiated with 7 Gy and then incubated for 4 h at 37°
4 same as sample 3 but incubated for 24 h after X-ray
5 cells UV-radiated (254 nm) with 17 J/meter^2 and then incubated for 4 h
9 yeast tRNA

Samples 1-5 were prepared on the same day.

induced by HS in rodent cells; they also demonstrate the utility of hybridization subtraction at low ratios of RNA : cDNA to enrich for sequences induced only several fold. cDNA, which was synthesized from polyA RNA from HS treated cells, was hybridized with polyA RNA from control cells and nonhybridizing DNA was isolated (see Methods). Since HS induced transcripts can be abundant in HS treated cells (9), we decided to determine if such sequences could be directly visualized on a northern blot with cDNA enriched by hybridization subtraction as probe. As shown in Fig. 7, most of the hybridization with enriched HS specific cDNA was to 0.1 to 0.6 kb transcripts which were more abundant in HS (lanes 4 and 8) than control RNA (lanes 3 and 7). With probe synthesized in the same manner from unfractionated cDNA, very little hybridization can be seen in lanes 1 and 2 at the same exposure time where the small transcripts were clearly visible with the enriched HS specific cDNA probe in lane 4. In lanes 5 to 8, a similiar experiment was performed but the autoradiographs were exposed for much longer. With the unfractionated HS cDNA probe, a broad smear was observed with most of the hybridization to RNA of 1 to 5 kb. With the enriched HS specific cDNA probe obtained by hybridization

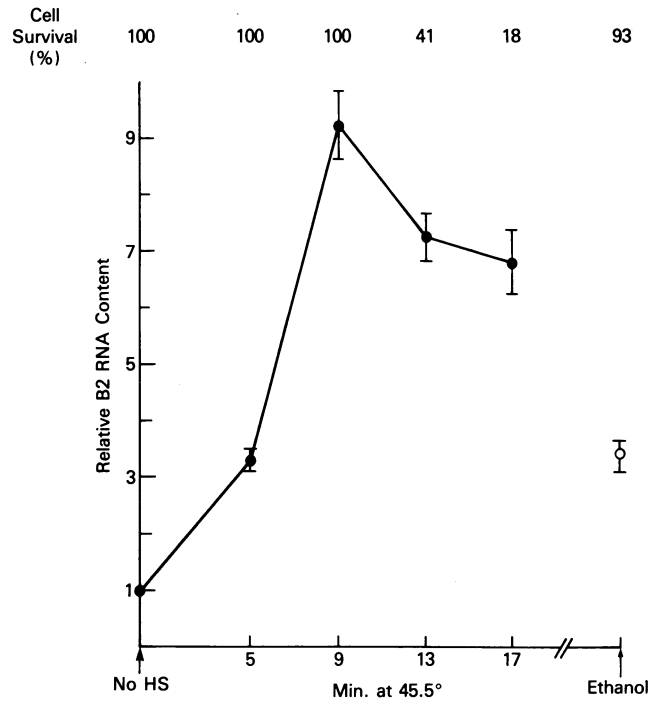


Figure 6. Induction of B2 RNA by various HS treatments or ethanol exposure. V79 cells were incubated at 45.5° for the indicated time and then incubated at 37° for 4 h. For ethanol, V79 cells were incubated in 6% ethanol in tissue culture medium for 1 h, rinsed, and then incubated in medium without ethanol for 4 h at 37°. RNA dot blots were performed as described in Methods and quantitated by scintillation counting. Cell survival, as measured by relative colony survival, is shown for each treatment at the top of this figure.

subtraction, bands could also be distinguished at 2 and 5 kb; this pattern is typical for that seen with a B2 sequence probe (see Fig. 1 and 2, and ref. 3, 4, 7). In 12 different experiments, the ratio of control polyA RNA to HS polyA RNA used to make cDNA was varied from 1:1 to 3.3:1, and the same result was obtained as shown in Fig. 7. To rule out an anomolous reason for B2-like enrichment by hybridization subtraction, experiments similiar to those in Fig. 7 were carried out with cDNA synthesized from polyA RNA extracted from cells treated with a dose of X-rays or UV which reduced cell survival to the same level as HS, and no B2-like hybridization was detected (data not shown).

Estimation of Enrichment for HS Induced Sequences by Hybridization Subtraction. The enrichment for B2 sequences by hybridization subtraction was estimated in Fig. 8 by DNA dot blot analysis. In panel A, there was 21 times more hybridization with the enriched HS specific cDNA probe than with the unfractionated HS cDNA probe to Chinese hamster or mouse B2 DNA.

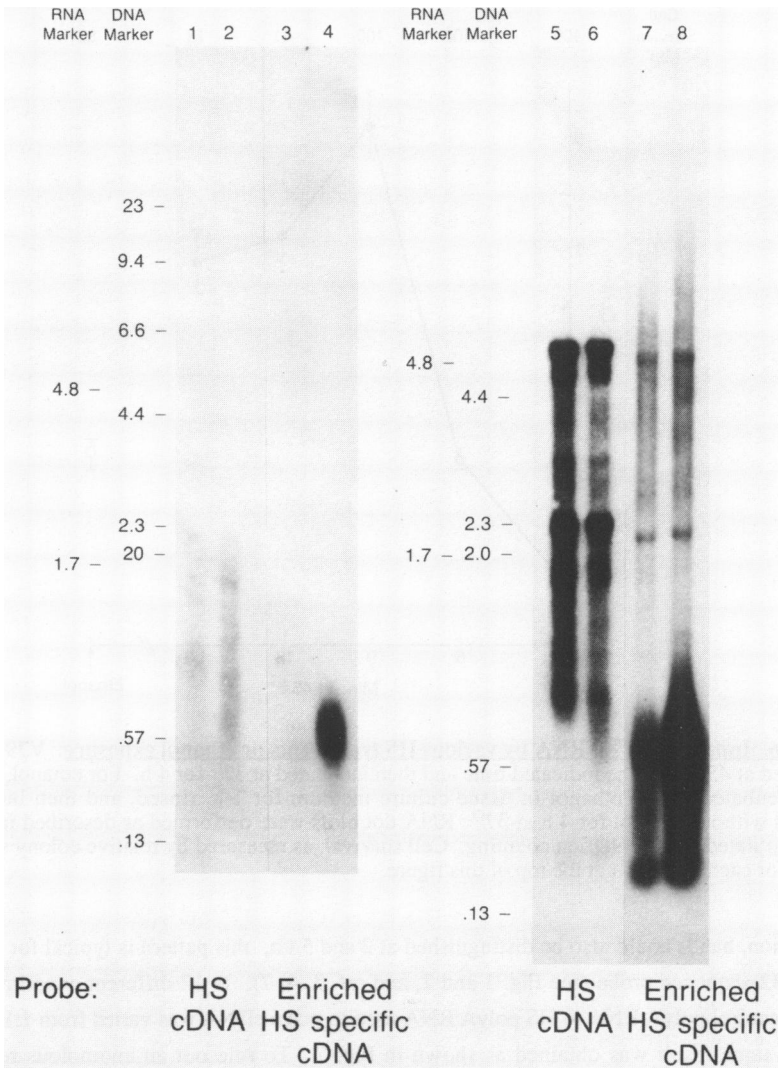


Figure 7. Northern blot analysis with cDNA enriched by hybridization subtraction. PolyA RNA from control (lanes 1, 3, 5, 7) or HS treated (lanes 2, 4, 6, 8) cells was hybridized with cDNA probes. As described in Methods, radioactive probes complementary to RNA were synthesized from cDNA enriched by hybridization subtraction (lanes 3, 4, 7, 8) or unfractionated cDNA (lanes 1, 2, 5, 6). Hybridization subtraction was with a 1:1 ratio of control RNA to HS cDNA in the experiment shown in lanes 3 and 4, while a 3:1 ratio was used in lanes 7 and 8. X-ray film exposure was approximately 10-20 times longer in lanes 5-8 compared to lanes 1-4.

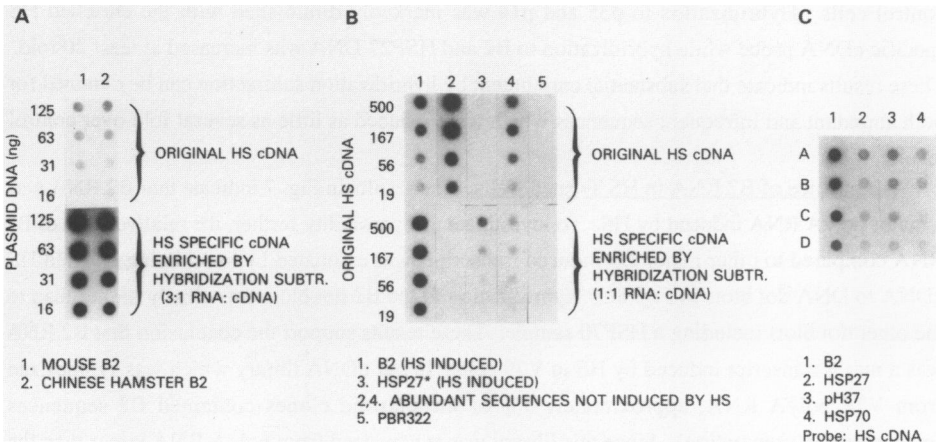


Figure 8. Estimation of enrichment by hybridization subtraction for various cDNA clones. In A and B, plasmid DNA was hybridized with labelled cDNA derived from HS treated cells (see Methods); in the upper portion of these figures unfractionated cDNA was used, while in the lower portion of these figures cDNA enriched by hybridization subtraction was used. Plasmids used were:

A, lane 1, pHPa2 (mouse B2)
lane 2, pHS18 (Chinese hamster B2)
B, lane 1, pHS18
lane 2, p35
lane 3, pH8 (HSP27); * the autoradiography exposure time was increased 2 fold for this lane
lane 4, p14
lane 5, PBR322

In C, radioactive cDNA was synthesized (14) with polyA RNA from V79 cells treated with HS and 2 h at 37°, and hybridized with equimolar amounts of plasmid DNA. The DNA concentration was determined using a fluorometric assay, and 0.05 pmoles (165 ng for pHS18) was dot blotted in row A with sequential 2 fold dilutions in rows B to D. Plasmids used were pHS18 (B2) (lane 1), pH8 (HSP27) (lane 2), pH37 (lane 3), and pM1.8 (HSP70) (lane 4).

Additional DNA dot blot studies in Fig. 8B demonstrate that hybridization subtraction at low ratios of RNA : cDNA can be used to enrich for induced sequences. Enriched HS specific cDNA was obtained by hybridization subtraction at a ratio of 1:1 control RNA : HS cDNA and experiments similiar to Fig. 8A were performed. Again, substantial enrichment was seen for B2 sequences. Enrichment for HSP27 sequences by hybridization subtraction was also determined in this experiment. HSP27 was chosen since it was much less abundant than B2 transcripts at this time (2 h) after HS. The HS RNA used in this hybridization subtraction contained only 3.3 times more HSP27 RNA than the control RNA. This was determined by RNA dot blot analysis (data not shown) and by northern analysis; the bands in Fig. 2B were cut out and measured by scintillation counting. As seen in Fig. 8B, HSP27 sequences were clearly enriched by hybridization subtraction. As additional controls, two cDNA clones were chosen which represent RNA species not elevated by HS; p35 RNA is present at high levels and p14 at moderate levels in both HS and

control cells. Hybridization to p35 and p14 was markedly diminished with the enriched HS specific cDNA probe while hybridization to B2 and HSP27 DNA was increased at least 20 fold. These results indicate that substantial enrichment by hybridization subtraction can be obtained for both abundant and infrequent sequences which were induced as little as several fold over control levels.

Abundance of B2 RNA in HS Treated Cells. The results in Fig. 7 indicate that B2 RNA was a major polyA RNA induced by HS. To investigate this possibility further, the relative level of B2 RNA compared to other major HS induced transcripts was estimated by hybridizing labelled HS cDNA to DNA dot blots in Fig. 8C. Hybridization to the B2 dot blots was clearly higher than to the other dot blots including a HSP70 sample. These results support the conclusion that B2 RNA was a major transcript induced by HS in V79 cells. In our cDNA library which was synthesized from V79 polyA RNA, approximately 1% of the plasmid clones contained B2 sequences (manuscript in preparation). Since this library was synthesized from polyA RNA larger than the HS induced B2 transcripts, we estimated that over 10% of the polyA transcripts in HS treated cells contained B2 sequences.

DISCUSSION

Induction of B2 RNA Polymerase III Transcription By Heat Shock

In this report, B2 RNA polymerase III transcripts were shown to be rapidly induced by HS to high levels in several rodent cell lines. This HS induced B2 RNA shared many of the characteristics of certain B2 transcripts described previously by others (1-5, 7) - they were 0.1 - 0.6 kb in length and polyadenylated, they were synthesized by RNA pol III, and only one strand of the B2 sequence was transcribed. The increase in B2 RNA after HS was clearly due to increased synthesis based on experiments with actinomycin D and nuclear runoff. The levels of RNA pol III transcripts have usually been found to be relatively constant in most cells. When changes in RNA pol III transcription have been observed, thay have been over longer timescales such as after malignant transformation or in different developmental stages. On the other hand, HS B2 RNA pol III transcription rapidly increased within 15 min. The signal for increased B2 transcription was specific for HS or ethanol, which induces HS protein synthesis, and was not related to cell lethality. If this signal for HS induced B2 transcription is a trans factor(s) which induces B2 transcription from multiple genomic loci, than such a factor may be relatively abundant. B2 induction by HS should prove to be a valuable approach in the study of RNA polymerase III transcription. Our studies demonstrate that RNA polymerase III transcription can respond rapidly to a specific cellular treatment, HS, by synthesis of high levels of B2 RNA.

HS Induction of Repetitive Genetic Elements. Two other eukaryotic repetitive sequences have been shown to be HS inducible. In lower eukaryotes, HS induction of transcription of the slime mold DIRS-1 repetitive element (10) and of the Drosophilia copia repetitive element (11) has been found. Both these repetitive genes are well characterized long transposable elements which are

very dissimiliar from the short B2 sequence. In addition, both the DIRS-1 and copia HS induced RNA are longer pol II transcripts unlike the rodent B2.

Possible Implications Of B2 Induction By HS

B2 transcription could be regulated by a cellular factor which is induced by HS or malignant transformation. For example, both the level of B2 RNA (4, 7) and HS proteins (22) have been found to be elevated with malignant transformation. HSP70 was increased in cells expressing E1A, suggesting some cellular E1A-like activity may play a role in the regulation of HSP70 (23). E1A also increased RNA polymerase III transcription (24). If such a cellular E1A-like activity is induced by HS, it may also affect B2 RNA pol III transcription.

B2 as a HS Identifier Sequence? The B2 sequence has several similiarities to the neuronal ID sequence. The neuronal ID sequence is a short repetitive genetic element found in rodent cells (25). Like the B2 sequence, it is abundant in nuclear and cytoplasmic RNA of cells from different organs (26), and is found in both RNA polymerase II and III transcripts (25, 26, 27). The ID sequence also shares sequence homology with the B2 sequence (8). Originally, the ID sequence was proposed to be present in a substantial portion of brain transcripts but not in RNA from other organs (25); this hypothesis is controversial based on the recent finding that the abundance of the ID sequence is similiar in nuclear RNA from different organs including brain (26). However, the short polyadenylated RNA polymerase III ID transcript, BC1, has recently been found to be transcribed specifically in brain cells in three different rodent species (27). This could be analogous to the B2 sequence and HS treated cells - in the case of B2, this sequence is found in many RNA pol II and III transcripts normally, but with HS the short B2 polyA RNA transcribed by RNA pol III increase dramatically.

Hybridization Subtraction At Low Ratios of RNA : cDNA.

We have shown that substantial enrichment can be obtained by hybridization subtraction at low ratios of control RNA : HS cDNA for both abundant and infrequent cDNA's whose transcripts are induced by HS. In higher eukaryotic cells containing polyA RNA of high complexity, hybridization subtraction has usually been used with high ratios of control RNA to cDNA (12). With our approach, less enrichment was probably obtained than with the conventional approach (12); for example, the non-HS induced sequences were still present to some extent in our enriched HS specific cDNA (Fig. 8B). However, the advantage of our approach is that sequences induced only several fold can be enriched. With a 1:1 ratio of RNA to HS cDNA, at least a 20 fold enrichment was observed for B2 and also HSP27 which represents a transcript induced only 3.3 times. This enrichment would be expected if uninduced sequences, particularily abundant sequences, were substantially reduced by hybridization subtraction - for example, p14 was reduced approximately 30 fold and p35 was reduced even more in Fig. 8B in the enriched HS specific cDNA. However at a 3:1 ratio of RNA:cDNA, enrichment was seen for B2 (Fig. 8A) while HSP27 was not enriched (data not shown).

Hybridization subtraction has several advantages over the conventional approach using

differential hybridization with labelled cDNA probes synthesized from control and induced RNA (14, 19). The major limitation with differential hybridization screening of plasmid or phage libraries is the requirement that induced sequences must be relatively abundant to give a positive signal with such a complex cDNA probe. The practical lower limit in vertebrate cells for the abundance of a particular cDNA is 0.1% of the total cDNA which represents more than 200 polyA transcripts per cell (14). With our hybridization subtraction approach, this sensitivity can be increased more than 20 fold so that cDNA clones representing transcripts which are present at less than 10 per cell can be detected. A second advantage is the increase in positive signal obtained after hybridization subtraction; e.g., with the HSP27 clone the difference in hybridization with HS versus control cDNA probes would only be several fold while the difference with the enriched versus unenriched HS cDNA probes in Fig. 8B was over 20 fold. Hybridization subtraction at low ratios of RNA : cDNA should prove to be useful in the isolation of other induced sequences; we have successfully used this approach in the molecular cloning of infrequent (<0.1% total polyA RNA) sequences induced less than 10 fold by certain types of cellular injury (manuscript in preparation).

Acknowledgement The authors would like to thank Drs. T. Fanning, R. Morimoto, and L. Weber for providing plasmid clones. The authors would also like to thank Drs. D. Levens and G. Crabtree for reviewing this manuscript.

References

1. Krayev, A.S., Markusheva, T.V., Ryskov, A.P., Skryabin, K.G., Bayev, A.A., Georgiev, G.P. (1982) Nucleic Acids Res. 10, 7461-7474.
2. Kramerov, D.A., Lekakh, I.V., Samarina, O.P., Ryskov, A.P. (1982) Nucleic Acids Res. 10, 7477-7491.
3. Kramerov, D.A., Tillib, S.V., Lekakh, I.V., Ryskov, A.P., Georgiev, G.P. (1985) Biochem. Biophys. Acta 824, 85-98.
4. Ryskov, A.P., Ivanov, P.L., Kramerov, D.A., Georgiev, G.P. (1983) Nucleic Acid Res. 11, 6541-6557.
5. Ryskov, A.P., Ivanov, P.L., Tokarskaya, O.N., Kramerov, D.A., Grigoryan, M.S., Georgiev, G.P. (1985) FEBS Letters 182, 73-76.
6. Bennett, K.L., Hill, R.E., Pietras, D.F., Woodworth-Gutal, M. (1984) Molec. Cell. Biol. 4, 1561-1571.
7. Singh, K., Carey, M., Saragosti, S., Botchan, M. (1985) Nature 314, 553-556.
8. Kramerov, D.A., Tillib, S.V., Ryskov, A.P., Georgiev, G.P. (1985) Nucleic Acids Res. 13, 6423-6437.
9. Schlesinger, M.J., Ashburner, M., Tissieres, A. (1982) HEAT SHOCK from bacteria to man (Cold Spring Harbor Laboratory)
10. Zuker, C., Cappello, J., Lodish, H.F., George, P., Chung, S. (1984) Proc. Natl. Acad. Sci. USA 81, 2660-4.
11. Strand, D.J. and McDonald, J.F. (1985) Nucleic Acids Res. 13, 4401-9.
12. Sargent, T.D. and Dawid, I.B. (1983) Science 222, 135-9.
13. Russo, A., Mitchell, J.B., McPherson, S. (1984) Br. J. Cancer 49, 753-8.
14. Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning a Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor).
15. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5.
16. Fornace, A. J. Jr., Cummings, d.E., Comeau, C.M., Kant, J.A., Crabtree, G.R. (1984) J. Biol. Chem. 259, 12826-12830.

17. Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
18. Gubler, U. and Hoffman, B.D. (1983) Gene 25, 263-9.
19. Crabtree, G.R. and Kant, J.A. (1981) J. Biol. Chem. 256, 9718-9723.
20. Church, G. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991-5.
21. Dony, C., Kessel, M., Gruss, P. (1985) Nature 317, 636-639.
22. Imperiale, M.J., Kao, H.T., Feldman, L.T., Nevins, J.R., Strickland, S. (1984) Molec. Cell. Biol. 4, 867-874.
23. Nevins, J.R. (1982) Cell 29, 913-9.
24. Hoeffler, W.K. and Roeder, R.G. (1985) Cell 41, 955-963.
25. Sutcliffe, J.G., Milner, R.J., Gottesfeld, J.M., Reynolds, W. (1984) Science 225, 1308-1320.
26. Owens, G.P., Chaudhari, N., Hahn, W.E. (1985) Science 229, 1263-5.
27. Sapienza, C. and St-Jacques, B. (1986) Nature 319, 418-420.
28. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J., Kirschner, M.W. (1980) Cell 20, 95-105.