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Ubiquitin mRNA is a major stress-induced transcript in mammalian cells

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ABSTRACT

Ubiquitin mRNA was found to be an abundant transcript which was induced by heat shock (HS), and certain other stresses in mammalian cells. In Chinese hamster cells, the 2 major ubiquitin transcripts of 2.6 kb and 1.7 kb were induced coordinately, while a minor ubiquitin transcript of 0.8 kb was not induced; the response was similar in human cells with induction of the 2.5 kb Ub C and 1.0 kb Ub B transcripts. A representative ubiquitin cDNA clone, isolated from a cDNA library derived from HS-treated Chinese hamster cells, coded for a typical tandem repeat polyubiquitin transcript. Only a portion of the 5' nontranslated sequence of this clone had homology with the previously published corresponding region in human Ub B mRNA. Oligonucleotide probes complementary to the portion of the 5' nontranslated sequence with homology to the human sequence and also portions with no homology hybridized only to the 1.7 kb transcript. There was coordinate induction of ubiquitin, HSP27, and HSP70 mRNA by HS as determined by both increased RNA and increased transcription. Ubiquitin mRNA was induced by certain DNA damaging agents, in particular the alkylating agent methylmethane sulfonate, or incubation in isoleucine-deficient medium under conditions where the other HSP mRNA were not.

INTRODUCTION

Ubiquitin, as it name implies, has been found in many eukaryotic cells and probably has multiple functions including an essential role in the cellular response to stress. This small, highly conserved protein is abundant in many cells; e.g., human fibroblasts contain approximately 10^8 molecules per cell (1). Ubiquitin is found free in cells and also conjugated to many proteins including histones, where it may play some role(s) in chromatin organization and regulation (2). Its role as a factor in ATP-dependent proteolytic degradation has been well documented (reviewed in 2), and recently ubiquitin has been reported to have intrinsic proteolytic activity (3). There is much evidence that conjugation of ubiquitin to proteins, particularly damaged or denatured proteins, can target them for degradation (discussed in 2). Many cellular stresses can damage or denature proteins. After heat shock (HS), there is a transient acceleration of proteolysis and changes in the levels of ubiquitin conjugates (2, 4). In yeast, the ubiquitin gene *UB14* is necessary for resistance to stresses such as HS and starvation, and is also induced by these stresses (5). Ubiquitin was induced by HS in chicken cells (6), and a chicken ubiquitin gene was found to contain a HS promoter (7). In chicken cells (6), 2 ubiquitin transcripts of 1.2 and 1.7 kb increased in abundance after HS. Most eukaryotic cells contain 2 major ubiquitin transcripts of

approximately 1 and 2 kb which code for tandem repeats of the 76-aa coding unit, and a third lower molecular weight transcript coding for a single-copy-ubiquitin fusion protein(s). For example in human cells (8, 9, 10), there are 3 ubiquitin transcripts of 2.5, 1.0, and 0.6 kb which have been termed Ub C, Ub B, and Ub A respectively; Ub B and Ub C are transcribed from typical polyubiquitin genes and Ub A from a ubiquitin fusion gene(s).

In addition to stresses such as HS which can lead to protein damage, ubiquitin was also induced in yeast cells during meiosis and by exposure to DNA-damaging agents (11), and in mammalian cells by viral infection (12). A role for ubiquitin in the response to genotoxic stress was raised by the finding that the mutation in the yeast DNA repair mutant *RAD6* involved an ubiquitin-conjugating enzyme (13). In the current work, we have found that ubiquitin was induced, as measured by increased transcription and increased ubiquitin mRNA, in mammalian cells by a variety of stresses including HS and exposure to certain alkylating agents.

MATERIALS AND METHODS

Cells and Cell Treatment

Chinese hamster cell lines, V79 lung fibroblasts and CHO-K1 ovarian cells, and human cells, skin fibroblast cell strain AG1522 (Correll Institute for Medical Research) and HeLa cell line, were grown as previously described (14, 15). All experiments were performed with exponentially growing cells at a density 1.5 to 2.5 x 10^7 per 175 cm^2 flask or 15 cm dish for Chinese hamster cells, unless otherwise noted. To study nongrowing G_0 phase cells, CHO cells were grown to ~5 x 10^7 cells per 15 cm dish and incubated for 2 days without medium change in medium containing15 mM HEPES buffer. G_0 phase cells were also studied by incubating CHO cells in medium lacking isoleucine; dialyzed serum was used in this experiment. For studies with nongrowing human fibroblasts, cells were grown to confluence, and maintained for an additional wk without replating.

For heat shock experiments, cells were heated in 175 cm^2 flasks containing 35 ml of medium in a water bath at the specified temperature (15). For treatment with chemical agents, a stock solution was added directly to the tissue culture medium for the indicated time, and a portion of the original medium without the chemical agent was then added or the cells were harvested. Cells were UV-irradiated and x-irradiated as previously described (14).

cDNA Clones

pH37, pH8, pM3, and pA2 were isolated from a cDNA library which has been described previously (15). Briefly, this library was constructed with polyA RNA from V79 cells which nad been heated to 45.5° for 17 min followed by a 4 hr incubation at 37°. pH37, pH8, and pM3 were isolated by differential screening (16) with labelled cDNA derived from untreated and heat shock-treated cells (15). pH8 hybridized at high stringency with a human HSP27 cDNA clone, pHS208 (17); pH8 and pHS208 hybridized with the same V79 cell RNA on northern analysis (data not shown). pM3 hybridized at high stringency with a human HSP70 cDNA clone, pHS709 (17),

and also with a mouse HSP70 genomic clone, pM1.8 (provided by R. Morimoto); pM3, pHS709, and pM1.8 hybridized with the same V79 cell RNA on northern analysis (data not shown). A further description of pH8, pM3, and other Chinese hamster HSP cDNA clones will be presented elsewhere. pA2, which contained a 1.1 kb insert, was isolated by screening the library with a chicken ß-actin cDNA probe (18); pA2 hybridized, at high stringency, with the same size Chinese hamster RNA as the chicken ß-actin probe (data not shown).

A human ubiquitin cDNA clone, pBRL26, was kindly provided by R.T. Baker and consisted of a portion of the coding region, 2 complete and part of a third ubiquitin coding sequence, and also the complete 3' nontranslated sequence (10).

DNA Sequence Analysis

pH37 double-stranded plasmid DNA was sequenced using both the method of Hattori (19) and also the Sequenase (USB) method according to the manufacturer's recommendations. Both strands were sequenced using oligomer primers to the flanking plasmid regions (New England Biolabs) and also with custom synthesized oligomer primers (Midland Certified Reagents) to unique sequences in the cDNA.

RNA Isolation and Analysis

Cells were lysed *in situ* with guanidine thiocyanate for RNA isolation, and polyA or whole cell RNA was bound to nylon filters for RNA dot blots or was size-separated in denaturing gels prior to transfer, as previously described (15). For hybridization, labelled (20) cDNA, which had been excised from its plasmid vector, was used at 3×10^6 dpm ml^{-1}. Oligonucleotide probes were synthesized with a Biosearch 8700 DNA synthesizer (New Brunswick Scientific) and end-labelled with T4 polynucleotide kinase (New England Biolabs) (16). The hybridization conditions were the same as in (15) except for the oligonucleotide probes where the hybridization temperature was 50° for the 24-mer probe and 62° for the 38-mer probe; blots hybridized with oligonucleotide probes were washed extensively at 25° and for 10 min at the hybridization temperature in 240 mM Na^+. The polyA content of all RNA samples was estimated using a labelled polythymidylic acid probe (15); this correction was small and usually varied from the RNA content measured by OD_{260} by less than 25%. Hybridization was quantified by densitometry measurements of autoradiographs. Relative hybridization was determined by normalizing to the result with RNA from untreated cells isolated in the same experiment on the same day. Hybridization was nearly linear when the signal varied by less than 10 fold, but deviated from pseudo-linear (21) when the signal was much greater. For this reason a computer program ("RNA Analysis") was written in TurboPascal for the Macintosh computer which generated a standard curve for dilutions of RNA from untreated cells. Values for experimental samples were then compared to this standard curve: the amount of RNA from control cells was divided by the amount of RNA from treated cells, which gave the same densitometry reading; this result was defined as induction. Each value in *Results* represents the mean of 4 dot blot determinations at different dilutions (15); the SD usually varied by less than 20% of the mean.

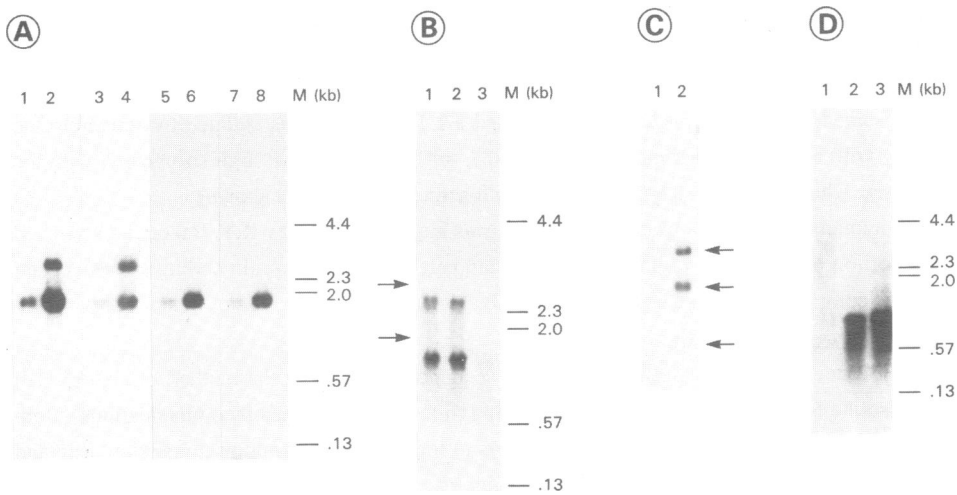

Fig. 1. RNA (northern) blot analysis of heat shock-treated and MMS-treated cells. Equal amounts of RNA from untreated or treated cells were size-separated in 1% formaldehyde agarose gels, blotted, and hybridized with the indicated probes. In A, polyA RNA from untreated (lanes 1,3, 5, and 7) or heated (lanes 2, 4, 6, and 8) V79 cells were hybridized with the Chinese hamster cDNA probe pH37, (lanes 1 and 2), the human cDNA probe (2) pRBL26 (lanes 3 and 4), an oligonucleotide probe complementary to positions 67 to 90 of pH37 (lanes 5 and 6), and an oligonucleotide probe complementary to positions 29 to 66 of pH37 (lanes 7 and 8). In B, RNA from heated (lanes 1 and 2) or untreated (lane 3) HeLa cells was hybridized with pBRL26; the arrows on the left indicate the positions of the major ubiquitin transcripts in Chinese hamster cells. The heat shock treatment for all samples of A and B except lane 1 of panel B consisted of heating cells to 45.5° for 9 min, with a subsequent 4 hr incubation at 37°; in lane 1 of panel B, cells were heated to 42° for 1 hr and then incubated at 37° for 1 hr. In C, polyA RNA from untreated CHO cells (lane1) or the same cells treated with 200 μM MMS (methylmethane sulfonate) for 4 hr was hybridized with pRBL26; arrows indicate the position of the 3 ubiquitin transcripts. In D, the same RNA as in Fig. 3A from untreated cells (lane 1) and cells heated to 45.5° for 5 min (lane 2) or 9 min (lane3) was hybridized with pH8. Size markers, designated M, consisted of labelled single-stranded DNA (15).

Nuclear Runoffs

Nuclei were isolated as described previously (15) but with RNAse A treatment of isolated nuclei (22). 2 x 10^7 nuclei were incubated in 0.1ml of 70 mM KCl / 5 mM $MgCl_2$ / 2 mM dithiothreitol / 0.1 mM EDTA / 0.4 mM ATP, CTP, and GTP / 0.24 mCi of [^{32}P] UTP (800 Ci mmol^{-1}) / 200 units 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°. Labelled RNA was isolated as described previously (15). DNA dot blots were hybridized with 3.5 x 10^6 dpm ml^{-1} for 48 hr at 66°. Blots were washed extensively including 15 min in 0.3 M Na^+ with 1 μg ml^{-1} RNAse A; the final most stringent wash was with 40 mM sodium phosphate (pH6.8)/1% SDS/1 mM EDTA at 66°.

RESULTS

Ubiquitin mRNA is a Major HS-induced Transcript in Chinese Hamster Cells

The ubiquitin cDNA clone, pH37, was isolated from a V79 cell library constructed from HS-treated cells. pH37 was one of 57 ubiquitin cDNA clones which were isolated by differential screening of 4500 clones; these cDNA clones hybridized preferentially with labelled cDNA derived from HS-treated cells compared to that from untreated cells. pH37 was identified by DNA sequence analysis (see below); the other 56 cDNA clones hybridized with pH37 at high stringency and all, which were tested, hybridized with the same size RNA on northern blots. As seen in Fig. 1A, pH37 hybridized with 2 major transcripts, of approximately 1.7 and 2.6 kb, which were induced (increased in abundance) in HS-treated cells. With longer autoradiograph exposure time, a third low molecular weight transcript of approximately 0.8 kb was detected which was not induced by HS or any other agent (data not shown). The same result was obtained also with a human ubiquitin cDNA probe in Fig. 1, except that the human probe hybridized equally with the 1.7 and 2.6 kb RNA while pH37 hybridized more strongly with the 1.7 kb transcript. Other Chinese hamster ubiquitin probes showed similar variability in hybridization; e.g., some hybridized equally with 1.7 and 2.6 kb transcripts while others hybridized more strongly with one of the transcripts (data not shown). In all cases, the relative increase in the 1.7 and 2.6 kb RNA compared to that of untreated cells was equivalent; e.g., in Fig. 1A, both size transcripts increased 5- to 10-fold after HS with either probe.

Sequence analysis demonstrated that pH37 coded for a typical polyubiquitin transcript. This cDNA clone contained a portion of the 5' nontranslated sequence and the complete sequence for two plus partial sequence for a third tandem ubiquitin. The predicted amino acid sequence for these ubiquitin repeats was identical and corresponded to the published sequences for human (2), bovine (23), chicken (6), and insect (24) ubiquitin. The nucleotide sequences of the 3 ubiquitin repeats were very similar with only 5 differences which are indicated by asterisks in Fig. 2. The last 18 nt of the 5' nontranslated sequence were identical to that in the same position of the human Ub B mRNA (10) with only 1 substitution, but the more 5' sequence had no convincing homology. When an oligonucleotide probe complementary to the region with homology to the human Ub B mRNA was hybridized to V79 RNA in Fig. 1A (lanes 5 and 6), hybridization was only seen to the 1.7 kb transcript even with very long exposures or after hybridization at 42° (data not shown). The same result (lanes 7 and 8) was obtained with an oligonucleotide probe to 5' region which lacked homology to the human mRNA. We conclude from these results that pH37 codes for the 1.7 kb polyubiquitin transcript. Since a portion of the 5' nontranslated region of pH37 had homology with the human Ub B mRNA, this indicates that the 1.7 kb ubiquitin transcript may be the Chinese hamster equivalent of this human 1.0 kb transcript. As shown in Fig. 1B, both the Ub B and Ub C mRNA were clearly induced by HS in human cells, but differed from the 2 Chinese hamster HS-inducible ubiquitin transcripts in that they were somewhat

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TTGTCCACTTCGGAGCTCCTTGCGTCTAGGTGTTTCTACGCGCTCGGCGGTTTGTGGTCTCATCACATCCGTTAACAGGTCAAA
cttgttgggtgagcttgtttgtgtcccgtgggtggacgtggttggtgattggcaggatcctggtatccgctaacaggtcaaa  Ub B

  87
ATG CAA ATC TTC GTG AAG ACC CTG ACC GGC AAG ACC ATC ACC CTA GAG GTG GAG CCC AGT GAC ACC ATC GAA AAT GTC
atg cag atc ttc gtg aaa acc ctt acc ggc aag acc atc acc ctt gag gtg gag ccc agt gac acc atc gaa aat gtc  Ub B

AAG GCC AAG ATC CAG GAT AAA GAG GGC ATC CCC CCC GAC CAG CAG CGA CTC ATC TTT GCC GGC AAG CAG CTG GAA GAT
aag gcc aag atc cag gat aag gaa ggc att ccc ccc gac cag cag agg ctc atc ttt gca ggc aag cag ctg gaa gat  Ub B

GGC CGC ACT CTT TCT GAT TAC AAC ATC CAG AAA GAG TCC ACC CTG CAC CTG GTC CTC CGC CTC AGG GGT GGC
ggc cgt act ctt tct gac tac aac atc cag aaa gag tcg acc ctg cac ctg gtc ctg cgt ctg aga ggt ggt  Ub B

 315
ATG CAG ATT TTC GTG AAG ACC CTG ACC GGC AAG ACC ATC ACC CTA GAG GTG GAA CCC AGT GAC ACC ATC GAG AAT GTC
Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile Glu Asn Val

AAG GCC AAG ATC CAG GAT AAG GAG GGC ATC CCC CCC GAC CAG CAG CGA CTC ATC TTT GCC GGC AAG CAG CTG GAA GAT
Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp

GGC CGC ACT CTT TCT GAT TAC AAC ATC CAG AAA GAG TCC ACC CTG CAC CTG GTC CTC CGC CTC AGG GGT GGC
Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly

 545
ATG CAG ATC TTC GTG AAG ACC CTG ACT GGC AAG ACC ATC ACC CTA GAG GTG GAG CCC AGT GAC ACC ATC GAG AAT GTG

AAG GCC AAG ATC CAG GAT AAA GAG GGC ATC CCC CCC GAC CAG CAG CGA CTC ATC TTT GCC GGC AAG CAG CTG GAA GAT

GGC CGC ACT CTT TCT GAT TAC AAC ATC CAG AAA GAG TCC ACC CTG CAC CTG GTC CT
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shorter. The human Ub B transcript differed also from the Chinese hamster 1.7 kb mRNA in that the 38-mer oligonucleotide probe used in Fig. 1A (lanes 7 and 8) did not hybridize to this or any other human RNA even when the hybridization temperature was reduced 20° (data not shown).

Dose Response and Kinetics of Induction after HS

Fig. 3 shows the dose response (panel A) and timecourse (panels B-D) for the induction of ubiquitin mRNA (measured by relative abundance in HS-treated samples compared to untreated samples prepared on the same day). Since the 0.8 kb RNA was only a minor transcript and the 1.7 and 2.6 kb transcripts were induced to the same extent (see above), induction was measured by RNA dot blots. Results equivalent to that shown with pH37 were obtained when other ubiquitin probes were used (data not shown). Results with cDNA probes for two other major HS proteins, HSP27 and HSP70, have been been included for comparison only and will be presented in more detail elsewhere (manuscript in preparation). Values for HSP27 and HSP70 mRNA induction are shown on the right axes of this figure. In all these experiments, the patterns of induction were similar for ubiquitin, HSP27, and HSP70. In the dose response experiment both ubiquitin and the other HS protein transcripts were maximally induced 4 hr after HS with the same HS dose of 9 min at 45.5°. In different experiments done on different days with Chinese hamster cells (including results of Fig. 3 and Table 1), this HS dose (4 hr after 9 min at 45.5°) always strongly induced HSP mRNA; e.g., induction of HSP27 mRNA was 22-, 30-, 33-, 49-, and 55-fold and induction of HSP70 was 11-, 26-, 44-, 51-, and 72-fold in different experiments. The greater variation in induction of HSP70 mRNA resulted probably because pM3 hybridized to 2 different transcripts, one of which was HS-inducible while the other only appeared after HS (manuscript in preparation); in addition, the level of HSP70 mRNA in unheated cells varied with growth rate and cell cycle while ubiquitin and HSP27 did not (Table 1). At higher HS doses in Fig. 3A, induction was less for all. With mild heating of only 5 min, neither ubiquitin nor HSP70 mRNA increased much, while HSP27 differed in that induction was nearly maximal. In Fig. 1D, the northern blot analysis for the 5 min and 9 min samples was similar for HSP27: the 1 kb transcript was markedly increased in both HS samples while it was barely visible in the unheated sample. A second difference was that the magnitude of the relative induction for HSP27 and HSP70 mRNA was greater than ubiquitin mRNA which was much more abundant in untreated cells (data not shown).

The kinetics of induction of ubiquitin mRNA was studied in timecourse experiments with different heating protocols which were done on different days. In panel B, induction was studied

Fig. 2. Chinese hamster ubiquitin sequence (EMBL accession no. X08013). The complete sequence of pH37 cDNA is shown. The 5' nontranslated region and the first ubiquitin repeat are compared to the human Ub B sequence (lower case letters) (10). The predicted amino acid sequence of the 3 ubiquitin repeats was identical and only one is shown. Differences in the DNA sequence between the ubiquitin repeats are indicated by asterisks and differences between pH37 and the human sequence by underlining. G and C tails added during the cloning have been omitted.

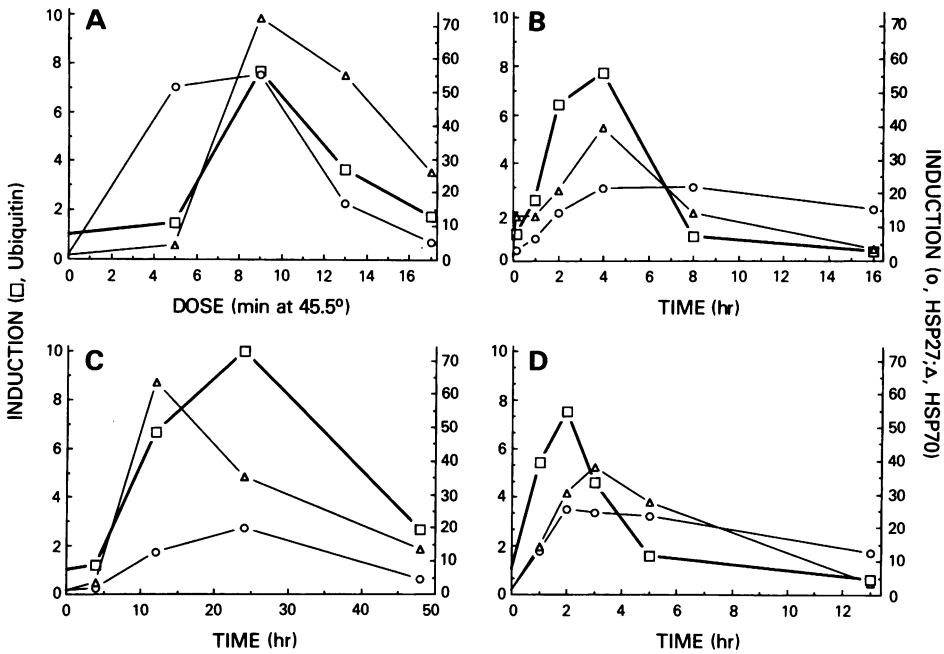

Fig. 3. Dose response and timecourse for heat shock induction in V79 cells. Induction, as measured by relative RNA abundance normalized to unheated samples prepared on the same day, was determined by dot blot hybridization using ubiquitin (pH37), HSP27 (pH8), and HSP70 (pM3) cDNA probes. Values for induction of ubiquitin mRNA are shown on the left axes and those for HSP27 and HSP70 on the right. Equal amounts of whole cell RNA (see *MATERIALS AND METHODS*) were used. In A, CHO cells were heated to 45.5° for the indicated times and then incubated at 37° for 4 hr. In B, cells were heated to 45.5° for 9 min and then incubated at 37° for the indicated times; the earliest timepoint represents cells which were harvested immediately after heat shock. In C, cells were heated to 45.5° for 17 min and then incubated at 37° for the indicated times. In D, cells were heated to 42° for 1 hr then incubated at 37°; the time axis represents the time from the start of heating. The 0 hr values in panels B, C, and D represent untreated samples.

after heating to 45.5° for 9 min which produced maximum induction in the dose response experiment of panel A. Both ubiquitin and the other HS protein transcripts were rapidly induced with a clear increase by 1 hr after HS and a maximum by 2 to 4 hr. When transcription was blocked with actinomycin D, no induction of ubiquitin, HSP27, or HSP70 mRNA was seen (data not shown). With the more severe HS treatment in panel C, induction was delayed for all 3 transcripts such that maximum levels were not attained until 10 or more hours after heating. In contrast to 9 min at 45.5° which produced no cell killing, 17 min at 45.5° produced substantial cell killing and reduced cell survival to 18% (15). In panel D, cells were heated to 42° and both ubiquitin and the other transcripts increased appreciably during the hour at this temperature; maximum induction for all 3 transcripts occurred within 2 hr of returning the cells to 37°. For all

TABLE 1. Induction of ubiquitin mRNA by different cell treatments[a]

Cell type	cell treatment[b]	cDNA probe			
		ubiquitin	HSP70	HSP27	ß-actin
V79	45.5°, 9 min[c]	7.5	72.	55.	0.7
	Ethanol (6%)[c]	3.2	25.	23.	1.1
	MMS (100 µg/ml)[d]	3.9	2.1	2.1	1.5
	H_2O_2 (400 µM)	1.6	0.5	1.2	1.2
CHO	45.5°, 9 min	20.	26.	33.	0.7
	MMS (200 µg/ml)	8.7	3.0	10.0	1.1
	MMS (100 µg/ml)	4.9	2.5	1.8	1.3
	DDP (45 µg/ml)	2.4	1.1	1.5	0.8
	HN2 (40 µM)	2.0	1.0	1.5	1.0
	H_2O_2 (400 µM)	1.7	0.3	0.8	0.9
	Adriamycin (2 µg/ml)	1.3	0.7	1.3	1.1
	TPA (20 ng/ml)	1.2	0.9	0.9	1.3
	AAAF (20 µM)	1.2	0.9	2.1	0.8
	254 nm UV radiation (14 Jm^{-2})	1.2	0.4	2.8	1.0
	MNNG (30 µM)	1.1	0.5	1.2	0.9
	X-rays (40 Gy)	0.9	0.9	1.8	1.1
	G_0 phase, isoleucine deficient[e]	3.2	0.7	1.0	0.5
	G_0 phase, depleted medium[f]	1.2	0.2	0.8	0.4
	G_0 phase, 0.5% serum[g]	1.1			0.6
Human fibroblast	MMS (100 µg/ml)	22.			0.9
	G_0 phase, contact-inhibited[h]	1.3			0.4
	G_0 phase, depleted medium[i]	1.0			0.3

[a] Cells were harvested 4 hr after heat shock, irradiation, or the addition of chemical agents; relative abundance was determined by RNA dot-blot hybridization and was normalized to untreated cells isolated at the same time.
[b] The treatment time was 1 hr for H_2O_2, ethanol, and adriamycin; and 4 hr for methylmethane sulfonate (MMS), cis-Pt(II) diamminedichloride (DDP), nitrogen mustard (HN2), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), *N*-acetoxy-2-acetylaminofluorene (AAAF), and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG).
[c] The HS sample is the same as in Fig. 3A and the ethanol sample was part of the same experiment.
[d] PolyA RNA was used for this sample and all samples below this in the table. The MMS and H_2O_2 samples were prepared on the same day. In the same experiment, induction by HS was 9.6, 11, 30, and 1.1 for ubiquitin, HSP70, HSP27, and ß-actin respectively.
[e] Cells were incubated in isoleucine deficient medium for 1 day.
[f] Cells were grown to high density and then incubated for 2 days without medium change.
[g] Cells were incubated in medium containing reduced serum, 0.5%, for 24 hr.
[h] Cell growth was prevented by contact inhibition; fresh medium was added 1 day before the cells were harvested.
[i] Contact-inhibited cells were incubated for 7 days without medium change.

3 timecourse experiments, the kinetics of ubiquitin mRNA induction was quite similar to that of HSP27 and HSP70 which indicates that these 3 transcripts were coordinately induced.

Induction of Ubiquitin by Non-HS Stresses

The monofunctional alkylating agent methylmethane sulfonate (MMS) can damage a wide variety of cellular macromolecules and was found to induce the same ubiquitin transcripts, 1.7 kb and 2.6 kb, as HS (Fig. 1C). In Table 1, treatment with this agent induced ubiquitin mRNA in 2 Chinese hamster cell lines and also in an untransformed human fibroblast cell strain. As seen in

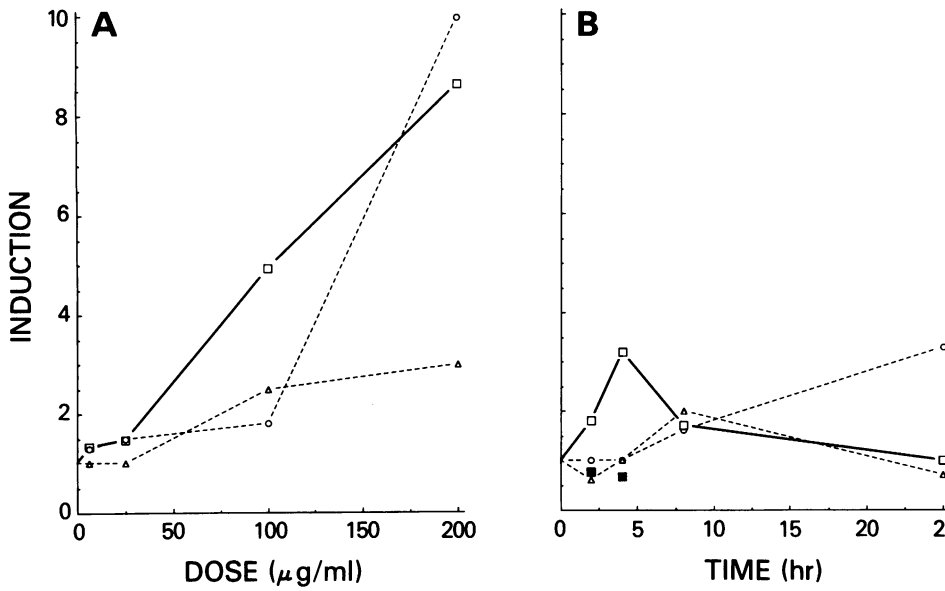

Fig. 4. Dose response (panel A) and timecourse (panel B) for MMS induction in CHO cells. Induction of ubiquitin (□, ■), HSP27 (○), and HSP70 (△) mRNA was measured as in Fig. 3 with polyA RNA. In A, cells were exposed to the indicated dose of MMS for 4 hr. In B, 100 μg/ml MMS was added at the start of the experiment and removed after 2 hr for the earliest timepoint and 4 h for the other timepoints. Samples designated with a solid symbol (■) were also treated with actinomycin D, 5 μg/ml, which was added 10 min before MMS. The scale for the y-axis is the same in panels A and B.

Fig. 4A, induction of ubiquitin mRNA was approximately proportional to MMS dose after a 4 hr exposure. The maximum induction of ubiquitin mRNA by MMS or HS was similar, while the increase in HSP27 and HSP70 mRNA was less for MMS than HS. The kinetics of ubiquitin induction by MMS (Fig. 4B) was similar to that seen after HS in Fig. 3B and 3D; in both cases, induction was rapid with a maximum by 4 hr. No induction of ubiquitin mRNA was seen in the presence of actinomycin D. In this timecourse experiment which was done on a different day than the dose response, there was not coordinate induction of ubiquitin, HSP27, and HSP70 mRNA. In contrast to ubiquitin, HSP27 and HSP70 induction by MMS was slower; e.g., the maximum increase in HSP27 mRNA occurred more than 20 hr after MMS treatment. Ubiquitin mRNA was also induced by the bifunctional alkylating agent HN2 and by another crosslinking agent DDP (Table 1); induction was less than 2 fold with lower doses of these agents (data not shown). As seen in Table 1, HN2 and DDP induced HSP27 mRNA only 1.5-fold and had no effect on the level of HSP70 mRNA at the same dose where ubiquitin mRNA was induced 2- and 2.4-fold. These results indicate that even though ubiquitin and the other HS protein transcripts were coordinately induced by HS or the HS-mimetic agent ethanol, they were not coordinately induced by other types of stress.

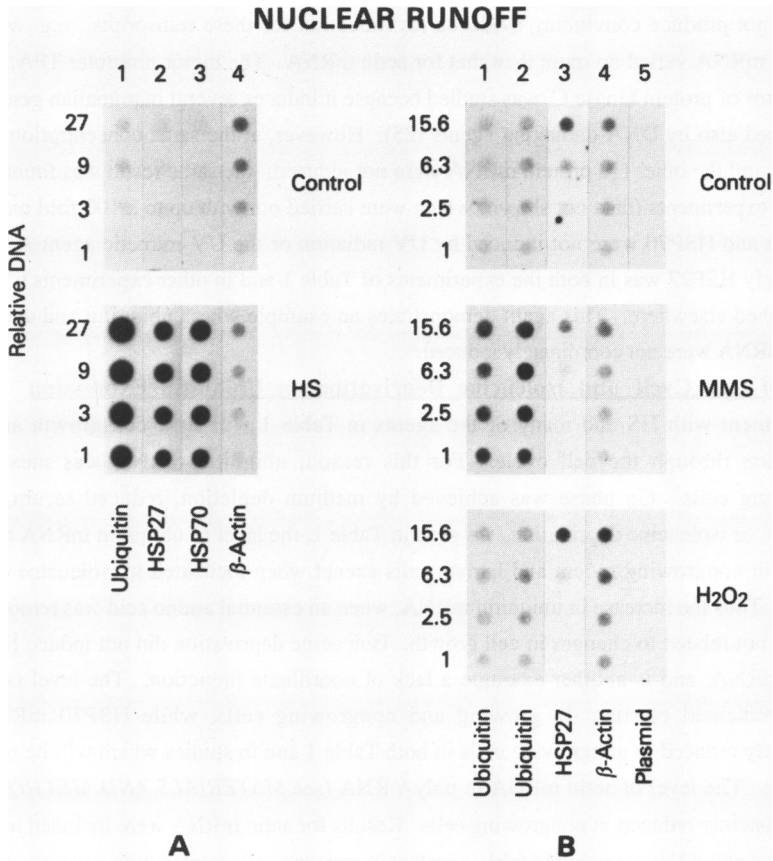

Fig. 5. Nuclear runoff transcription assay. Replicate DNA dot blots were hybridized with labelled RNA synthesized from isolated nuclei. In A, nuclei from untreated CHO cells (control) or cells heated to 45.5° for 9 min and incubated at 37° for 1 hr (HS) were used. Replicate dot blots of plasmid DNA were used with decreasing 3-fold dilutions in each row. In lane 1, 7 μg of pBRL26 plasmid DNA was dotted on the top row; lane 2, 1.8 μg of pH8; lane 3, 1.8 μg of pM3; and lane 4, 0.4 μg of pA2. In B, nuclei from untreated cells, cells treated with 100μg/ml MMS for 2 hr (MMS), or cells treated with 400 μM H_2O_2 for 1 hr and incubated in fresh medium for 0.5 hr (H_2O_2) were used. In lane 1, 7 μg of pBRL26 plasmid DNA was used in the top row; lane 2, 1.4 μg of pH37; lane 3, 1.8 μg of pH8; lane 4, 0.4 μg of pA2; and lane 5, 6 μg of pBR322 (plasmid vector alone). As in A, replicate dot blots were used but with decreasing 2.5-fold dilutions in each row. The exposure time for lane 3 was 5 times longer than the other lanes.

Evidence for ubiquitin induction was not found after treatment with several other agents in Table 1. After exposure to H_2O_2 at a dose which strongly induced multiple damage-inducible transcripts in the same cells (Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) DNA-damage-inducible transcripts in mammalian cells. J. Cell. Biochem. 12A, 312), ubiquitin mRNA increased less than 2 fold, the level of HSP27 mRNA was essentially unchanged, and HSP70 mRNA actually decreased. Treatment with adriamycin, the alkylating agent MNNG, or X

rays did not produce convincing evidence for induction for these transcripts; e.g., values for ubiquitin mRNA varied no more than that for actin mRNA. The tumor promoter TPA, which is an activator of protein kinase C, was studied because it induces several mammalian genes which are induced also by DNA-damaging agents (25). However, at the same concentration of TPA, ubiquitin and the other HS protein mRNA were not induced; the same result was found in dose response experiments (data not shown) which were carried out with up to a 100-fold more TPA. Ubiquitin and HSP70 were not induced by UV radiation or the UV-mimetic agent AAAF, but surprisingly HSP27 was in both the experiments of Table 1 and in other experiments which will be published elsewhere. This again demonstrates an example where ubiquitin and another HS protein mRNA were not coordinately induced.

Effect of Cell Cycle and Isoleucine Deprivation on Ubiquitin Expression

Treatment with HS and many of the agents in Table 1 will slow cell growth and delay progression through the cell cycle. For this reason, ubiquitin mRNA was measured in nongrowing cells. G_0 phase was achieved by medium depletion, reduced serum, contact inhibition, or isoleucine deprivation. As seen in Table 1, the level of ubiquitin mRNA remained constant in nongrowing rodent and human cells except when incubated in isoleucine deficient medium. Thus the increase in ubiquitin mRNA, when an essential amino acid was removed, was probably not related to changes in cell growth. Isoleucine deprivation did not induce HSP27 or HSP70 mRNA, and is another example a lack of coordinate induction. The level of HSP27 mRNA remained constant in growing and nongrowing cells, while HSP70 mRNA was consistently reduced in nongrowing cells in both Table 1 and in studies which will be published elsewhere. The level of actin mRNA in polyA RNA (see *MATERIALS AND METHODS*) was also consistently reduced in nongrowing cells. Results for actin mRNA were included in Table 1 as a control and its level remained fairly constant in growing cells treated with a variety of agents, but actin mRNA is a poor control when comparing growing and nongrowing cells.

Nuclear Runoff Studies

Transcription in nuclei isolated from untreated (control) cells or cells treated with HS, MMS, or H_2O_2 was measured in Fig. 5. After HS, transcription of ubiquitin, HSP27, and HSP70 mRNA increased markedly; in the same sample actin transcription decreased slightly compared to control. As seen in panel A, hybridization was maximal with several dilutions of ubiquitin, HSP27, or HSP70 plasmid DNA which indicates that the filter bound DNA was clearly in excess. Under these conditions where the filter bound DNA is in excess, hybridization of labelled RNA should be proportional to the initial concentration of RNA (21), and thus the ratio of the relative abundance of induced RNA compared to control should be proportional to the hybridization signal in the HS (induced) compared to the control runoff experiments (where the same amount of DNA was dotted on the filter). By densitometry measurements after various autoradiograph exposure times, hybridization was consistently more than 40-fold greater for ubiquitin, HSP27, and HSP70 dot blots in the HS compared to dot blots containing the same amount of DNA in the control

samples; this indicates that transcription of these genes was increased more than 40-fold. In nuclei of cells treated with 100 μg ml^{-1} MMS for 2 hr, ubiquitin transcription was also markedly elevated, while HSP27 transcription was not increased (panel B). The same result as HSP27 was obtained in other experiments with multiple HS protein cDNA including HSP70 (data not shown). In panel B, actin transcription also remained fairly constant. It should be noted that the yield of RNA in the runoff assays of panel B were similar and varied by <10%. In the H_2O_2 sample, neither ubiquitin nor HSP27 were induced; under the same conditions transcription was clearly increased for several damage-inducible transcripts (Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) DNA-damage-inducible transcripts in mammalian cells. J. Cell. Biochem. 12A, 312) which will be described elsewhere.

DISCUSSION

Our studies demonstrate that the HS protein cDNA clone pH37 codes for the 1.7 kb ubiquitin transcript and that ubiquitin mRNA is a major stress-induced RNA in mammalian cells. pH37 coded for a typical tandem repeat polyubiquitin transcript which has been found in both yeast (7) and higher eukaryotes (6, 10). The predicted protein sequence of ubiquitin in the tandem repeats was identical and was also identical to the ubiquitin sequence in other vertebrates (6, 10, 23) and insects (24). The DNA sequence of the ubiquitin repeats in pH37 was also highly conserved with less than 2 substitutions per repeat; in contrast, the nucleotide sequence of the first repeat in Fig. 2 had 18 or 30 base changes compared to a human Ub B or chicken (position 13-240 of clone 7 [6]) ubiquitin sequence respectively. Thus, the nucleotide sequence of ubiquitin tandem repeats must be conserved by some gene conversion-type events between the repeats. The pattern of ubiquitin mRNA in northern blots was very similar to that of other species including yeast (7), chicken (6) and human (8, 9, 10) (Fig. 1B). For example, in yeast 2 larger mw, 1.5 and 2.6 kb, transcripts were transcribed from polyubiquitin genes, while a third low mw transcript of 0.7 kb was transcribed from a ubiquitin fusion gene(s) which was not induced by HS. Based on northern blot analysis, both the Chinese hamster genes coding for the 1.7 and 2.6 kb ubiquitin transcripts were coordinately induced. In human cells, the Ub B and Ub C transcripts were induced by HS. The effect of HS was similar in Chinese hamster and human cells – i.e., the 2 major ubiquitin bands were clearly induced in both species in Fig. 1. As described earlier, the last 18 nt of the 5' nontranslated portion of the pH37 sequence were homologous to the same region in human Ub B mRNA (Fig. 2). The implication of these results is that the 1.7 kb Chinese hamster mRNA is the equivalent of the human Ub B mRNA. However if this is the case, then the most 5' portion of pH37 has clearly diverged from the corresponding region in Ub B mRNA. In the human Ub B gene, there is a 0.8 kb intron in the 5' nontranslated region 6 nt from its 3' end (10). The homology of the 5' nontranslated region of pH37 (position 67-84 in Fig. 2) with Ub B spans the site where the intron is spliced out of the Ub B mRNA but stops abruptly 12 nt 5' from this site. In addition, the most 5' portion of pH37 (which was clearly in the 1.7 kb mRNA of

both untreated and HS-treated cells [Fig. 1A]) had no convincing homology with this Ub B intron. Thus, the mechanism for this divergence between the 1.7 kb Chinese hamster ubiquitin and the human Ub B 5' sequences can not be explained on the basis of any obvious change in splice sites.

Ubiquitin transcription, as measured by nuclear runoffs, increased even more (relative to untreated control) after HS or MMS than the relative level of ubiquitin mRNA increased. This could be due to decreased stability of ubiquitin mRNA in stressed cells; however the $T_{1/2}$ for ubiquitin mRNA in the presence of actinomycin D was approximately 4 hr in MMS-treated cells (Fig. 4B) and in untreated cells (data not shown).

An examination of the characteristics of the agents in Table 1, which induced ubiquitin, reveal both similarities and differences. HS has a variety of effects on cells including perturbations in protein folding and other forms of protein denaturation (2, 26). Much evidence indicates that the induction of HS proteins has a protective effect against HS toxicity (discussed in 2, 26); in Chinese hamster cells the HS dose in Fig. 3A, which maximally induced HS protein mRNA, also was the most effective dose in inducing thermotolerance (data not shown). Many have proposed that HS proteins can have a protective effect against HS by stabilizing cellular macromolecules such as proteins and, for ubiquitin in particular, by facilitating the removal of damaged proteins (2, 26). In the case of MMS, many of its toxic effects are probably due to DNA damage (27), but it also alkylates proteins. In Chinese hamster cells (27), MMS damaged DNA and protein to similar extents: 110 $\mu g\ ml^{-1}$ MMS produced approximately 1 alkylation per 10^6 daltons in both DNA and protein. DDP and HN2 at the concentrations used in Table 1 produced less adducts in DNA and protein than MMS, but many of their adducts will be crosslinks which are far more toxic than monoadducts in DNA and may perturb protein structure more than simple methylation (28). MNNG is also a methylating agent but differs from MMS in that it alkylates nucleophilic sites by a different substitution reaction and produces a different spectrum of lesions in DNA and probably also protein (28). In addition, the dose of MNNG in Table 1 produced approximately the same frequency of alkylations in DNA of Chinese hamster cells as MMS, but 5- to 10-fold less protein alkylation (28). The same dose of MNNG was as effective as MMS in inducing mRNA of the DNA repair enzyme DNA polymerase ß in CHO cells (Fornace *et al*, manuscript submitted for publication) and induced other DNA-damage inducible transcripts (Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) DNA-damage-inducible transcripts in mammalian cells. J. Cell. Biochem. 12A, 312).

Our results can be used to speculate on the signal for ubiquitin induction. There are two general possibilities: the first is that all the inducing agents activated a common signal for induction, while the second is that there may be multiple signals and that the Ub B and Ub C mammalian polyubiquitin genes may be coordinately regulated by several different promoters (which both genes contain). All the agents in Table 1, which induced ubiquitin, can either damage proteins or, in the case of isoleucine deprivation, perturb protein synthesis such that abnormal and

truncated proteins may accumulate in the cell. Thus, the signal for ubiquitin induction could be increased levels of damaged or abnormal proteins.

The second possibility, that there are multiple signals for ubiquitin induction, is more likely because ubiquitin was coordinately induced with the other HS proteins by HS, but was not by MMS or some other agents. For example, ubiquitin mRNA was clearly induced by a 4 hr exposure to 100 $\mu g\ ml^{-1}$ MMS in the 2 experiments of Fig. 4A and 4B, while induction was <2-fold for HSP27 mRNA. The nuclear runoff experiment demonstrates that ubiquitin transcription was markedly increased soon after HS or MMS treatment, while HSP27 was only increased after HS. The major HS protein genes in many organisms have been shown to contain a common regulatory sequence which is induced by a HS-activated trans factor (26). The mammalian polyubiquitin genes probably also contain this HS promoter since a chicken polyubiquitin gene does (7). If this HS promoter was induced by MMS, then coordinate induction of ubiquitin, HSP27, and HSP70 by MMS would be expected. Our results in mammalian cells were similar to that seen in simpler organisms. In *E. coli*, the *htpR* HS regulon contains at least 15 genes which are coordinately induced by HS or the HS-mimetic agent ethanol. These bacterial HS genes are not coordinately induced by other stresses; e.g., 3 of 15 were induced by H_2O_2 and only 1 of 15 by isoleucine deprivation (29). Induction of the *recA* SOS DNA repair regulon has been shown to lead to induction of some but not all *htpR* genes (29, 30). Similar results have been found in yeast; e.g., some but not other DNA-damage-inducible genes were induced by HS (31). The yeast polyubiquitin genes were induced by both HS (5) and DNA damaging agents (11). In mammalian cells there are many examples of genes which contain multiple regulatory elements; e.g., the regulatory regions in metallothionein genes for metal, glucocorticoid, and the inflammatory response inductions map to different 5' regions (32). Thus, it is likely that the mammalian polyubiquitin genes contain more than 1 promoter which can be induced by different types of stress, and that ubiquitin plays a role in the cellular response to such stresses.

At the doses of MMS and other DNA-damaging agents which induced ubiquitin, the target size for damage was larger than most cell proteins which raises the possibility that damage to a larger macromolecule may signal induction. Possible larger targets could be nucleoproteins such as ribosomes or chromatin. In yeast, ubiquitin plays a role in the cellular response to DNA damage because the *RAD6* protein is an E2 ubiquitin-conjugating enzyme and because RAD6 mutants are hypersensitive to DNA-damaging agents (13). It is reasonable to speculate a similar role for an ubiquitin-conjugating enzyme in mammalian cells.

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