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Silk worm Bm1 SINE RNA increases following cellular insults

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ABSTRACT

The effect of cell stresses upon the expression of the Bm1 short interspersed element (SINE) family in cultured silk worm cells is examined. Primer extension analysis shows that Bm1 repeats are transcribed by RNA polymerase III (Pol III) into cytoplasmic RNAs. Five consecutive T residues, which would normally terminate Pol III transcription, occur within the Bm1 consensus and are included within cDNA sequences representing these transcripts. In analogy to mammalian SINEs, the level of the Bm1 transcripts increases in response to either heat shock, inhibiting protein synthesis by cycloheximide or viral infection. The lifetime of Bm1 RNA increases following cell insults so that post-transcriptional events partially account for stress induced increases in its abundance. In the case of heat shock, the increase in Bm1 RNA follows the transient increase in hsp70 mRNA indicating that this response is temporally regulated to occur later in heat shock recovery. These results support the proposal that SINE RNAs serve a role in the cell stress response that predates the divergence of insects and mammals implying that SINEs are essentially a class of cell stress genes.

INTRODUCTION

Repetitive short interspersed elements (SINEs) that are present in most eukaryotic genomes belong to either tRNA or SRP RNA superfamilies (1,2). The structure of the tRNA SINE superfamily consists of a 5' sequence derived from an ancestral tRNA and a 3' tRNA-unrelated region that includes elements required for retrotransposition (3). SINEs in almost all eukaryotes investigated belong to the tRNA superfamily; SRP RNA-related SINEs are evidently restricted to primates and rodents (1,2). Genes for tRNAs and for SRP RNA contain internal A box and B box promoter elements for RNA polymerase III (Pol III) endowing all SINEs with internal promoters and potential template activity.

As the most thoroughly studied example, the Alu family exemplifies known features of SINE transcription. Since Alus like other SINEs are frequently interspersed within genes, these elements are often included within Pol II directed transcripts. However, these Alu transcripts are largely removed during pre-mRNA processing (4,5). Here, we are concerned with Pol III directed SINE transcription. Initiating at a site that corresponds exactly to the 5' end of the Alu consensus sequence, Pol III transcribes through Alu elements until encountering the first run of four or more T residues which suffices to terminate Pol III directed transcription. Since external sequences that are 3' to the Alu insertion site supply this signal, transcription of each Alu is uniquely terminated (2). Accordingly, full-length (fl) Alu transcripts initiate at a common 5' site but are heterogeneous in length because of their termination at variable 3' positions. The half-life of flAlu RNA in the cytoplasm is ~30 min (6). Some flAlu RNA is processed into a stable 5' part of the transcript called small cytoplasmic (sc) Alu RNA (7).

The level of flAlu RNA is usually very low in cell lines and tissues (5,8). However various cellular insults, including viral infection, heat shock and exposure to cycloheximide raise the abundance of flAlu RNA by as much as 50-fold (8–11). The level of scAlu RNA is relatively insensitive to these same treatments (8). Rodent and rabbit SINE RNAs also increase in response to these insults (8,12–14). This survey implies that the increases in SINE RNA caused by these insults occur in all mammals, and suggests that Pol III transcribed SINE RNAs may serve a previously unrecognized role in the cell stress response. Pursuing this lead, we found that overexpressed flAlu RNA stimulates protein synthesis and apparently does so by inhibiting the activity of PKR (15). These observations lead to the proposal that Alus and, by implication, other mammalian SINEs regulate protein homeostasis under cell stress conditions.

The possibility that such a fundamental physiological role for SINEs is restricted to mammals seems unlikely implying that SINE RNAs in other species should also respond to cell stress (2). Silk worm contains a well-characterized SINE (3), providing a distant outgroup to mammals to test this prediction.

The 439 bp consensus sequence of the silk worm Bm1 SINE consists of a 5' tRNA-related region (positions 1–85) followed by a non-tRNA region (3). The 5' end of the Bm1 consensus sequence corresponds to the initiation site for Pol III directed transcription (3,16). However, five T residues are located immediately after the tRNA-related region at position 91 within the Bm1 consensus sequence. Although four Ts normally suffice to terminate Pol III transcription, Pol III is universally believed to transcribe the Bm1 RNA intermediates

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that are required for retrotransposition (3). Termination *in vitro* of Pol III directed transcription at this internal site is leaky allowing a single template to produce two transcripts: one terminating at position 91 and a longer 354 nt transcript (16). In the template transcribed in that study, a single base substitution at position 352 created a non-consensus terminator which accounts for the longer transcript (3,16). Here we refer to the 91 nt transcript as 'scBm1 RNA' because this shorter transcript like scAlu contains the A and B box promoter elements but not the 3' regions required for retrotransposition (3). In addition to the possibility of other non-consensus terminators (Discussion), the Bm1 consensus sequence contains several alternating AT-rich motifs (3) which might terminate Pol III transcription (17). As a consequence of their dispersion within other transcription units, much of the Bm1 RNA present *in vivo* is transcribed by Pol II (18,19). These considerations raise the questions of whether Pol III produces Bm1 RNA *in vivo* and whether such transcripts extend beyond the internal terminator at position 91.

MATERIALS AND METHODS

Cell culture

Adherent *Bombyx mori* cells (BmN-4) were grown at 26–28°C in 75 cm^2 flasks containing TC-100 (Sigma) insect medium at pH 6.0 supplemented with heat inactivated 7% fetal bovine serum (Gibco BRL). As reported in the text, cells were subjected to various heat shock temperatures in a water bath and allowed to recover at normal growth temperature for the times indicated. Cells were also either grown in the presence of 100 μg/ml cycloheximide (8) or infected with wild type baculovirus (*B.mori* nuclear polyhedrosis virus from Dr George Kamita) at a m.o.i. of 3 for the times indicated prior to RNA extraction.

To monitor the lifetime of Bm1 RNA transcripts under various conditions, actinomycin D was added, 27 μg/ml, for the indicated times prior to harvesting RNA (6). This concentration is higher than that used for mammalian cells (6) and was selected as the value at which any further increase in concentration had no effect upon the upon the kinetics with which the Bm 1 RNA decreased. The effects of α-amanitin on Bm1 RNA were similarly tested (18).

RNA extraction

Cells scraped from 60–80% confluent flasks were collected by centrifugation for 5 min at 4°C. Prior to either extraction or storing at –20°C, 10 μl of Vanadyl Ribonuclease Complex (Gibco BRL) were added to each flask equivalent of the cell pellet to prevent RNA degradation. No difference was observed in the level of Bm1 RNA from frozen and freshly harvested cell pellets. Cell pellets were resuspended in 500 μl RNA extraction buffer [50 mM Tris–Cl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) NP-40] and then incubated on ice for 10 min prior to centrifugation. The supernatant was extracted twice with 500 μl of a 50/50 mixture of phenol and chloroform, then with phenol and finally with chloroform. During these extractions, Phase Lock Light (5 Prime 3 Prime) was added prior to centrifugation. RNA was ethanol precipitated and the pellet was washed twice with 70% and once with 100% ethanol prior to vacuum drying. The pellet was dissolved in DEPC treated water and concentrations were measured using UV absorbance.

Primer extension analysis

RNA with a radiolabeled primer mixture was denatured at 75°C prior to incubation at the annealing temperature (Table 1). In nearly all experiments, a primer for 5S rRNA (5S 119) was included with the Bm1 RNA primer (Bm1 146) as an internal control for sample processing. Otherwise, primer extensions were performed as described and the products were assayed on 5% polyacrylamide (20:1 acrylamide to bis, 7 M urea) (20). Gels were photographed with either a Fuji BAS 1000 PhosphorImager screen or Fuji blue X-ray film with an

Table 1. Oligonucleotide primers and probes

Oligonucleotide Name	Sequence (5'–3')	Annealing temp (°C)	Position
Bm1 409	TCA CAG CCC ACC TGG TGTT	42	409–391
Bm1 318	CTC AGT ATA GTT ACA ACG GCT	42	318–298
Bm1 216	AAG AGG TCC TAC CAC CAG TA	42	216–197
Bm1 146	TCC TTC ACC GTG GAA GTC AA	56	146–127
Bm1 139	CCG TGG AAG TCA ATC GTG AA	56	139–120
Bm1 78	CCT GCG GGA TTC GAA CAC	56	78–61
Bm1-1	GAA GTC GTC GTG GCC TAA A	56	1–19
5S 119	AGC CAA CAT CAC GTA GTG TT	56	119–100
hsp70 114	TGT GAT ACG GTT GCC CTG GT	55	114–95
hsp70 116	GGT GTG ATA CGG TTG CCC TG	55	116–97
hsp70 122	TAC GAT GGT GTG ATA CGG TT	55	122–103
hsp70 126	GAC GTA CGA TGG TGT GAT AC	55	126–107
hsp70 127	CGA CGT ACG ATG GTG TGA TA	55	127–108
hsp70 383	TTC ATC TTG GTA AGC ACC AT	56	383–364
hsp70 79	GAT CAT CGC CAA CGA CCA	56	79–98

The base identity and numbering of Bm1 oligonucleotides refer to the silk worm Bm1 SINE consensus sequence (3). For hsp70 mRNA, oligonucleotides 114 through 127 refer to a partial cDNA for silk worm hsp70 (Materials and Methods). The numbering of these silk worm hsp70 sequences assigns the translational initiation site of *Drosophila* hsp70 mRNA (GenBank accession no. L01500) as being position 1. The oligonucleotide to 5S rRNA is for the silk worm transcript (GenBank accession no. K03316).

intensifier screen. BAS 1000 images were quantified with Molecular Dynamics Storm 860 hardware and Molecular Dynamics ImageQuant software.

Northern blot analysis

RNA (10 μg) was first dissolved in DEPC water (7.2 μl) and then 10× MESA (Sigma) buffer (2 μl) and formamide (10 μl) were added prior to heating for 10 min at 65°C. After this initial heating, 37% formaldehyde (0.8 μl) was added and the sample was reheated for 10 min at 65°C. Gels (1.5% agarose dissolved in running buffer, 1× MESA and 0.4 M formaldehyde) were pre-run for 10 min at 60 V in running buffer. RNA loading buffer (2 μl, Promega) was added to the sample prior to loading it on the gel. Markers were either visualized by ethidium bromide staining prior to loading or by methylene blue staining of the blotted filter (21).

RNA was transferred to nylon filters which were dried in a vacuum oven and then cross-linked by using a FisherBiotech FB-UVXL-1000 uv crosslinker (21–23). The filter was prehybridized at 42°C in 6× SSPE, 5× Denhardt's and 0.5% SDS with 5 μg/ml of salmon sperm DNA and then hybridized overnight with the ^{32}P-end-labeled oligonucleotide probe (300 ng). The filter was washed twice in washing buffer (5× SSPE, 0.5% SDS) at room temperature and once at 42°C for 5 min prior to imaging (see above).

Construction of Bm1 and Bm hsp70 cDNA clones

Primer extension products resulting from oligonucleotide Bm1 146 (Table 1) were synthesized as described above, excised from the polyacrylamide gel and used for PCR amplification with oligonucleotides Bm1 1 and Bm1 146 (Table 1). The resulting cDNAs were cloned in the TA vector (Invitrogen) for sequence analysis (GenBank accession nos AF117242–AF117251).

Conserved regions in human hsp70 (GenBank accession nos M11717 and M15432) and *Drosophila* hsp70 (GenBank accession no. L01500) were used to design primers (hsp70 383 and hsp70 79; Table 1) for both reverse transcription of silk worm RNA and PCR amplification of the resulting primer extension product. Sequence analysis of the cDNA clones identifies a silk worm hsp70 homolog (GenBank accession no. AF117252). Primer extension analysis using a series of primers (hsp70 114–127) derived from this sequence (Table 1) were used to map the 5' end of the corresponding mRNA and to verify its heat shock induction (text). Primer hsp70 116, which gives a 225 nt extension product, is used here to measure the abundance of hsp70 mRNA.

In vitro nuclear transcription

Cells were permeabilized, using 0.5% of NP-40 in place of lysolecithin, and nascent RNA was labeled in the presence of 20 μg/ml of α-amanitin (24). The labeled transcripts were hybridized to DNA clones (5 μg) complementing 5S rRNA and Bm1 RNA (25; GenBank accession no. AF117242) that had been immobilized on nitrocellulose filters by use of a slot blot apparatus. Prior to heat denaturing and loading, the DNA clones were linearized by *Eco*RI digestion. The hybridization conditions were identical to those used for northern analysis except that salmon sperm carrier DNA was omitted.

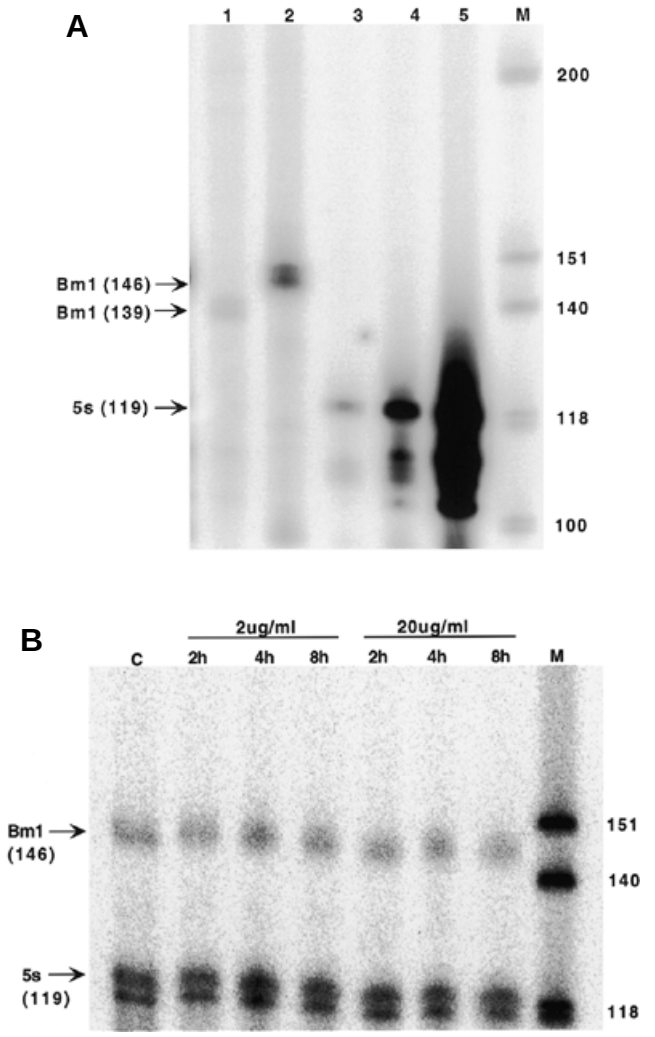

Figure 1. Pol III directs Bm1 RNA transcription. (**A**) Primer extension analysis of Bm1 RNA. Cytoplasmic RNA (10 μg in lanes 1, 2 and 5, 0.1 μg in lane 3 and 1 μg in lane 4) was analyzed using primers (Table 1) either to Bm1 RNA, Bm1 139 and Bm1 146 (in lanes 1 and 2, respectively), or to 5S rRNA, 5S 119 (in lanes 3–5). In this and subsequent experiments, the expected positions of the corresponding primer extension products are indicated and M refers to a marker lane. (**B**) Primer extension was performed using a mixture of primers Bm1 146 and 5S 119 on 10 μg of RNA from control cells (C) and cells that had been treated with treated with either 2 μg/ml or 20 μg of α-amanitin for the indicated times.

RESULTS

Pol III transcribes Bm1 RNA *in vivo*

Primer extension analysis is used to identify Pol III directed SINE transcripts according to the position of their 5' initiation site (20). Using primers starting at positions 146 and 139 within the Bm1 consensus sequence (Table 1), we observe the extension product lengths predicted for Pol III initiated Bm1 SINE transcripts (lanes 1 and 2; Fig. 1A).

As an internal control for RNA loading and the relative abundance of SINE RNA in subsequent experiments, we routinely use a mixture of primers for Bm1 RNA and 5S rRNA. In addition to a product having the expected length, several

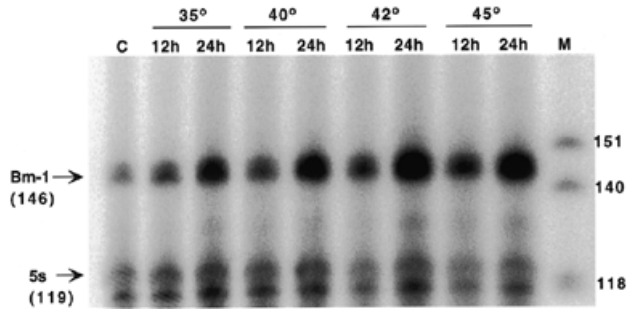

Figure 2. Heat shock increases Bm1 RNA. Following heat shock for 45 min at the indicated temperatures, cells recovered at the normal growth temperature for either 12 or 24 h prior to harvesting RNA. Cells grown without heat shock provide a control (C).

truncated products are observed for 5S RNA (lanes 3–5, Fig. 1A). The cause of this heterogeneity is unknown but the reproducible pattern of 5S rRNA product bands serves as the intended control for RNA loading. The abundance of the Bm1 RNA products approximates 1% of the abundance of the 5S rRNA products (Fig. 1A). Bm1 RNA is expressed at a high level relative to human Alu RNA (20,24).

Low concentrations of α-amanitin decrease the abundance of Pol II directed transcripts which contain Bm1 sequences (18,19). However, the intensity of the Bm1 RNA primer extension product is unchanged after exposing cells to α-amanitin for as long as 8 h (Fig. 1B). This resistance to α-amanitin also indicates that Bm1 RNA is transcribed by Pol III.

The 139 and 146 nt primer extension lengths (Fig. 1A) indicate that Pol III transcribes through the consensus termination signal at position 91 (Introduction). The integrity of this termination signal in the corresponding transcripts is confirmed by sequence analysis. Ten cDNA clones derived from the primer extension product closely match the Bm1 consensus sequence but each differs from the others by point and length mutations (GenBank accession nos AF117242–AF117251). Many member of this repeat family are transcribed. Nine of the ten cDNA sequences have five T residues at position 91; this terminator is leaky in *vivo*.

Bm1 RNA increases during heat shock recovery

The abundance of Bm1 RNA increases in cells that have been heated and allowed to recover at the normal growth temperature (Fig. 2). The magnitude of this increase (~5-fold) depends upon both the severity of the heat shock and the recovery time, but is comparable to the increases observed for flAlu RNA during heat shock recovery (8). As a control for the Bm1 RNA specificity of this effect, the steady state abundance of another Pol III directed transcript, 5S rRNA, is virtually unchanged following heat shock (Fig. 2 and data not shown).

To compare the heat shock induced increases of Bm1 RNA with a known heat shock gene, a silk worm hsp70 cDNA clone was used to design oligonucleotides for primer extension analysis (Materials and Methods). The abundance of hsp70 mRNA increases during heat shock at 37°C and then decreases rapidly during recovery at the normal growth temperature (Fig. 3). The kinetics and intensity of this response depend upon the severity of the initial heat shock (see below) but are generally similar to

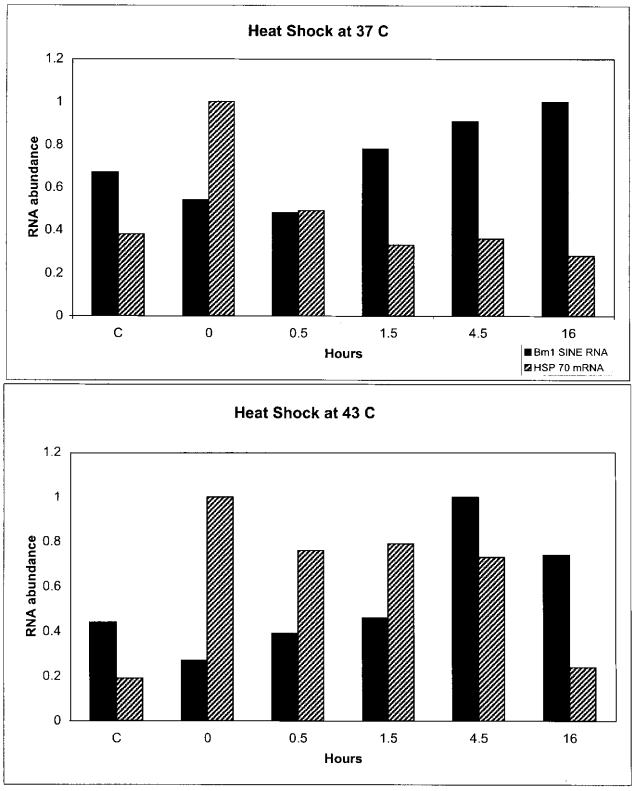

Figure 3. Heat shock kinetics of Bm1 RNA and hsp70 mRNA. Primer extension analysis was used to determine the relative abundance of Bm1 RNA as compared to 5S RNA (in this and Figs 4 and 5) and hsp70 mRNA in cells that had been heat shocked at either 37 or 43°C for 1 h and then allowed to recover for the times indicated. The abundance of these RNAs is compared to their expression in untreated control cells (C).

the response in *Drosophila* in which hsp70 mRNA increases during heat shock and then rapidly decreases after the onset of recovery (26,27). The heat shock response of Bm1 RNA shows slower kinetics: the abundance of Bm1 RNA increases after hsp70 mRNA has returned to its basal level (Fig. 3). Compared to hsp70 mRNA, the increase in Bm1 RNA is a later heat shock response. Following heat shock at a higher temperature, 42°C, the maximum level of hsp70 mRNA shifts to a later recovery time but still precedes the increase in Bm1 RNA (Fig. 3). Again, the increase in Bm1 RNA is a later heat shock response. At yet higher heat shock temperatures, the maximum level of hsp70 mRNA shifts to even later recovery times approaching that of the Bm1 RNA response (data not shown).

In contrast to hsp70 mRNA, which exhibits a transient increase following heat shock, the increase in Bm1 RNA apparently persists for longer times (Fig. 3). As discussed later, we attribute this continuing response to an increased stability of Bm1 RNA.

Other cellular insults increase Bm1 RNA

The effects of cycloheximide and viral infection upon Bm1 RNA were also investigated (Fig. 4). Bm1 RNA increases within 12 h after the addition of cycloheximide to cells, and after 72 h increases to 30 times its initial concentration (Fig. 4). As in

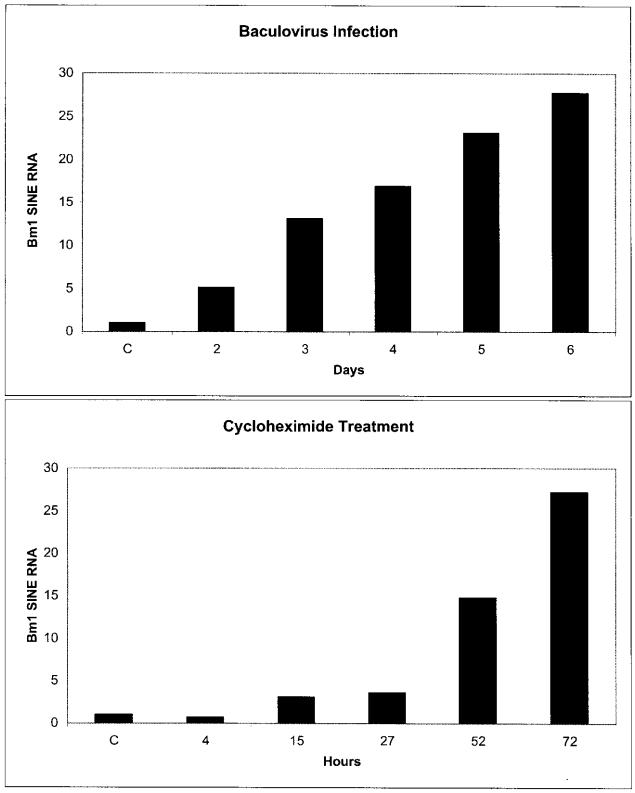

Figure 4. Other cell insults increase Bm1 RNA. Primer extension analysis was used to determine the abundance of Bm1 RNA in cells that had been exposed to cycloheximide or infected with baculovirus for the indicated times. The relative abundance is compared to the level in control cells (C).

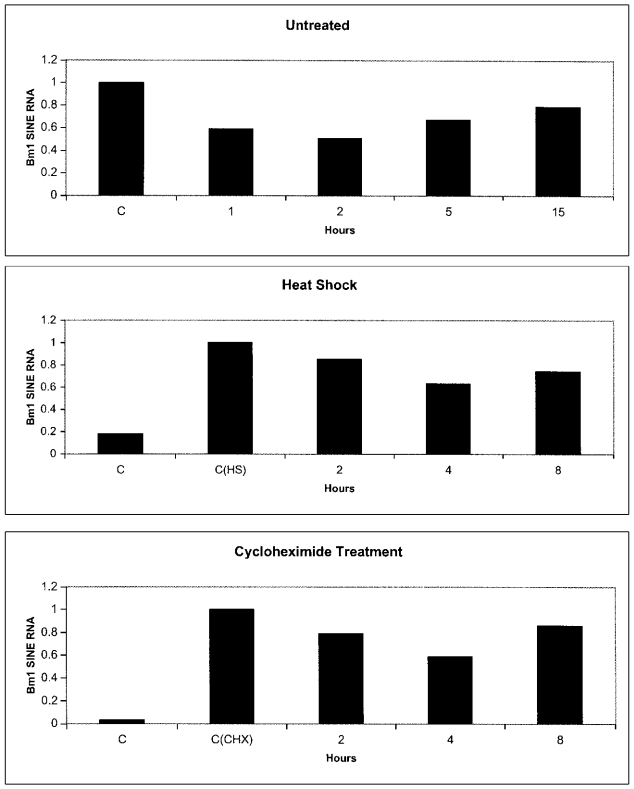

Figure 5. Bm1 RNA stability. Cells were grown in the presence of actinomycin D (27 μg/ml) for the times indicated and Bm1 RNA was assayed by primer extension. The abundance of Bm1 RNA is reported relative to its level in the appropriate control cells. The untreated cells were not stressed and this control (C) is taken at the time actinomycin was added. Cells were also heat shocked at 42°C for 45 min and allowed to recover for 3 days [C(HS)] prior to the addition of actinomycin D. The additional unstressed control (C) demonstrates the effect of heat shock on Bm1 RNA. Cells were also exposed to cycloheximide for 3 days [C(CHX)] prior to the addition of actinomycin D. The additional unstressed control (C) demonstrates the effect of cycloheximide on Bm1 RNA.

the case of heat shock, cycloheximide did not change the abundance of 5S RNA so that again this increase is specific for Bm1 RNA (data not shown). Very long term passage in the presence of cycloheximide (e.g. 1–2 weeks) with periodic changes in cell growth media results in as much as a 300-fold increase in Bm1 RNA (data not shown). Infecting silk worm cells with baculovirus also increases Bm1 RNA by as much as 30-fold (Fig. 4). In summary, cellular insults that increase the abundance of mammalian SINE RNAs, namely heat shock, inhibiting protein synthesis and viral infection, also increase Bm1 RNA.

Cell insults stabilizes Bm1 RNA

After inhibiting transcription with actinomycin D, the abundance of Bm1 RNA has been compared to that of 5S rRNA, a long-lived transcript. In control cells, the level of Bm1 RNA decreases by approximately one-half within 1 h of administering actinomycin D (Fig. 5). Bm1 RNA is relatively short-lived. However, following this initial decrease, the level of Bm1 RNA is stabilized and even begins to increase upon longer exposure times (Fig. 5). Although these kinetics are complicated, the first half-life of Bm1 RNA in cells recovering from heat shock is noticeably longer, ~4–8 h (Fig. 5). Consequently, the stabilization of this transcript (~4–8-fold) accounts for much of the increase (~5-fold) in its abundance during heat shock recovery. This stabilization of Bm1 RNA must also affect the kinetics of its increase. As previously discussed, the elevated level of Bm1 RNA following heat shock persists even after hsp70 mRNA has returned to its basal level.

Exposing cells to cycloheximide increases the half-life of Bm1 RNA to as long as 8 h (Fig. 5). As discussed above, long term exposure of cells to cycloheximide increases the abundance of Bm1 RNA by more than two orders of magnitude indicating that post-transcriptional events might account for a fraction of this increase. Presumably, transcriptional activation is responsible for the other fraction.

The transcriptional activity of Bm1 SINEs relative to that of 5S rRNA has been determined in the presence of α-amanitin (Table 2). The relative rates of Bm1 transcription *in vitro* from nuclei isolated from control and heat shock cells are virtually identical. In contrast, cycloheximide causes a 3–4-fold increase in the rate of Bm1 transcription. These data complement the results of the RNA half life experiments discussed above: the increased stability of Bm1 RNA entirely suffices to account for its accumulation following heat shock but does not account for the significantly greater increase in Bm1 RNA caused by exposing cell to cycloheximide. Viral infection also increases the transcription of Bm1 SINEs (Table 2).

Table 2. Hybridization analysis of nuclear run-off transcripts

	Bm1 SINE transcriptional activity			
	Control	Heat shock	Cycloheximide	Virus
EXPT. 1	'1'	1.1	2.8	n.d.
EXPT. 2	1.0	1.1	4.0	1.8

Cells recovered for 2 days following a 43°C heat shock for 1 h or cells were exposed to cycloheximide for 3 days or cells were infected for 3 days with baculovirus (Materials and Methods). Nuclei from these and control cells were transcribed *in vitro* using [γ-^{32}P]UTP and the RNA was hybridized to filters containing duplicate spots (5 μg) of both 5S rDNA and Bm1 cDNA (positions 1–146). The hybridization intensity of the Bm1 RNA signal relative to that of 5S RNA was determined by phosphorimager analysis and each data entry is based on average of the duplicate hybridization signals for both 5S rRNA and Bm1 RNA. The intensity of the Bm1 RNA signal divided by that of the 5S rRNA signal in the control cells of experiment 1 (observed value = 3.30) is arbitrarily taken as '1' and used as the basis for normalizing all other data entries.

Apparent length homogeneity of Bm1 RNA

Northern analysis has been used to identify Bm1 transcripts which might be responsible for the 146 nt primer extension product (Fig. 6A, B and C). Using oligonucleotide Bm1 146 as the hybridization probe, a prominent band having a length of ~200 nt is detected in control cells (Fig. 6A, B and C; lane 1). This band shows a marginal increase (1.7-fold) following heat shock (Fig. 6A, lane 2) and a convincing increase (>15-fold) following either cycloheximide treatment or viral infection (Fig. 6B and C, lane 2). Since this band is the major hybridization signal detected by the Bm1 146 probe and exhibits a significant increase following either cycloheximide treatment or viral infection, we conclude that the accumulation of the 200 nt transcript accounts for much of the increase observed for the 146 nt primer extension product in the experiments reported above. The apparent length homogeneity of this 200 nt band is surprising as primary Bm1 transcripts are expected to have 3' length heterogeneity (Introduction).

An oligonucleotide directed toward the tRNA-related region of the Bm1 consensus (oligonucleotide Bm1 78) should hybridize to this same 200 nt transcript was well other Bm1 RNAs that are too short to detect with oligonucleotide Bm1 146 (Table 1; Fig. 6). In agreement with the preceding results, the hybridization of this probe to the 200 nt transcript shows a slight increase following heat shock and large increases following either cycloheximide treatment or viral infection (Fig. 6A, B and C, lanes 3 and 4). However, a second low molecular weight (~100 nt) band is apparent in these same blots (Fig. 6, lanes 3 and 4). We presume this species is scBm1 RNA which has an expected length of 90 nt (Introduction). The abundance of this presumptive scBm1 RNA increases in cycloheximide treated cells but the effects of heat shock and viral infection on its abundance are less certain. Certainly, this shorter transcript cannot be responsible for the 146 nt primer extension products observed in the preceding experiments so that the effects of stress on the 100 nt transcript and its exact relationship to the 200 nt transcript both remain to be determined.

DISCUSSION

Emerging generalization of SINE structure and expression

As expected from their internal Pol III promoter structure, Bm1 repeats, like other SINEs, are transcribed by Pol III *in vivo*. An unusual feature of the Pol III transcription of Bm1 RNA is the presence of a terminator within the coding region (3). The 200 nt Bm1 transcript clearly results from transcription through this terminator whereas the presumptive scBm1 RNA observed by northern analysis could result from either termination or processing at this same site. The results for these two *in vivo* Bm1 transcripts are therefore consistent with previous results showing that termination at this site is leaky *in vitro* (16).

We expected primary Bm1 transcripts to be both longer than 439 nt and heterogeneous in length (Introduction); therefore, the apparent length homogeneity of 200 nt Bm1 RNA is surprising. Some length heterogeneity in an RNA may be undetectable by northern analysis and the exact 3' ends of these transcripts remain to be more precisely determined. With this qualification, either termination or processing must account for the unexpectedly short length and apparent homogeneity of 200 nt Bm1 RNA. There is no consensus terminator located near position 200 (3). However, in the one Bm1 template systematically examined, a single base substitution adjacent to three pre-existing T residues created a non-consensus Pol III termination signal *in vitro* (16). There are 10 different sites within the Bm1 consensus sequence at which a single T substitution would create a non-consensus terminator (3). One is located at position 190 and another at position 214 corresponding approximately to the length of the 200 nt Bm1 RNA. Increasing the possibilities, alternating TA dinucleotides can also terminate Pol III transcription (17) and two such motifs are present in the Bm1 consensus sequence. According to these considerations, a number of discrete length RNAs might be directly transcribed from the different members of the Bm1 repeat family.

Conservation of the SINE RNA response to cell stress

We previously observed that heat shock, cycloheximide treatment and viral infection increase the abundance of mammalian SINE RNAs (8). These observations coupled with results linking Alu RNA to the regulation of protein synthesis lead to the proposal that mammalian SINE transcripts have a defined role in the cell stress pathway (15). However, the *de novo* adaptation of mammalian SINEs to this central regulatory function is highly unlikely, leading to the further prediction that the response of SINEs to cell insults should be evolutionarily conserved (2). In agreement with this prediction, each of the three cell insults investigated here increases Bm1 RNA. Silkworm represents both a distant outgroup of mammals and an ectotherm supporting the notion that this response is deeply rooted in SINE evolution (2,3).

One criticism of this interpretation is that cellular insults might merely disrupt normal transcriptional regulation thereby causing an aberrant increase in SINE RNA. However during heat shock recovery, the increase in 200 nt Bm1 RNA occurs significantly after the transient increase in hsp70 mRNA. The transient increase in hsp70 mRNA is an early heat shock response in *Drosophila* (26,27) and also appears to be an early response in silk worm. These kinetics imply that the increase in

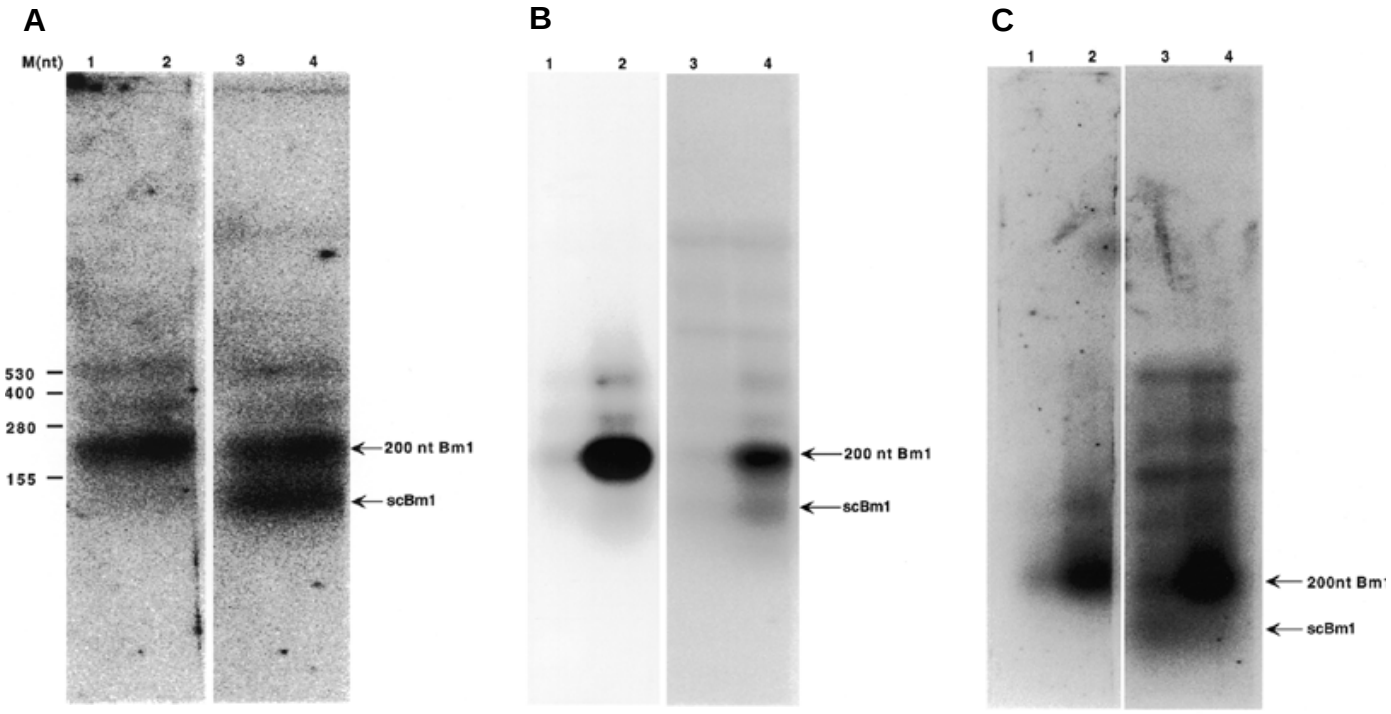

Figure 6. Northern analysis of Bm1 RNAs. RNA was extracted from either control cells (lanes 1 and 3 in all figures) or treated cells (lanes 2 and 4) that were either heat shocked (A), exposed to cycloheximide (B) or infected with virus (C) as described below. Blots were hybridized with either oligonucleotide Bm1 146 (lanes 1 and 2) or oligonucleotide Bm1 78 (lanes 3 and 4). Positions are indicated for a 200 nt transcript, which is detected with both probes (lanes 1–4), and for a lower molecular weight RNA, called scBm1 RNA, which is detected with oligonucleotide Bm1 78 (lanes 3 and 4). A ladder of higher molecular weight bands, which is more or less intense on the different blots, depends on details of the sample preparation (data not shown) and is partially attributable to formaldehyde cross-linking of RNA. Our analysis is therefore restricted to the lowest molecular weight bands which are detected by the two probes (text). (**A**) Following heat shock at 42°C for 45 min, cells were allowed to recover for 1 day (lanes 2 and 4). Comparing either lanes 1 and 2 or 3 and 4, there is a 1.7-fold increase in 200 nt Bm1 RNA following heat shock. Comparing lanes 3 and 4, the abundance of scBm1 RNA does not change following heat shock. (**B**) Cells were grown in the presence of cycloheximide for 3 days (lanes 2 and 4). Comparing either lanes 1 and 2 or lanes 3 and 4, 200 nt Bm1 increases by 15 in cycloheximide treated cells. Comparing lanes 3 and 4, scBm1 RNA increases by 5-fold following cycloheximide treatment. (**C**) Cells were grown for 5 days following baculovirus infection (lanes 2 and 4) Comparing either lanes 1 and 2 or lanes 3 and 4, 200 nt Bm1 RNA increases by nearly 20-fold following virus infection. Comparing lanes 3 and 4, viral infection has no effect on the level of scBm1 RNA.

Bm1 RNA occurs relatively later in cells that are already recovering from the initial insult. This is inconsistent with a mere loss of transcriptional control. In fact, the rate of transcription of Bm1 RNA is essentially unchanged following heat shock and the increase in its abundance seems to result primarily from changes in its post-transcriptional regulation. The stabilization of Bm1 RNA caused by heat shock and also by cycloheximide seem to be specific for Bm1 RNA as we do not observe any significant increase (or decrease) in the abundance of 5S rRNA even in cells that are cultured for many days following either heat shock or exposure to cycloheximide.

The stabilization of Bm1 RNA following heat shock potentially accounts for its relatively slow accumulation as compared to the more rapid heat shock response of hsp70 mRNA. Post-transcriptional regulation might effectively schedule the resulting increase in Bm1 RNA to occur during later heat shock recovery. In contrast, mammalian SINE RNAs increase more rapidly following heat shock and heat shock has no apparent effect on the stability of Alu RNA (6,8, unpublished). As yet another difference in the kinetic responses of silk worm and mammalian cells, the elevated level of Bm1 RNA persists indefinitely following heat shock recovery. We can attribute this persistent response to the continued stabilization of Bm1 RNA that heat shock induced. Presently, we have not determined the rate at which the stability of Bm1 RNA is restored to its preheat shock level. Since hyperthermia must present very different problems to mammals and silk worm, an ectotherm, the different kinetics of the heat shock responses of SINE RNAs in these organisms might be rationalized in several different ways.

The stabilization of Bm1 RNA is insufficient to account for the increases in its abundance following either viral infection or exposure to cycloheximide**.** Cycloheximide also increases the rate of transcriptional initiation of Bm1 repeats. Coupling this transcriptional activation with the stabilization of the resulting transcripts causes up to 300-fold increases in the level of Bm1 RNA making it an abundant RNA.

By the criterion of their induced expression, SINEs behave like cell stress genes. The conservation of this regulated response in evolutionarily distant groups lends support to the

proposal that SINE RNAs serve a function in the cell stress response. The effect of cell stress on gene expression genes has been extensively studied, raising the question of whether it is even reasonable to imagine that a whole class of 'heat shock genes' might have been overlooked. Previously, there had been no reason to suspect that SINEs might participate in the cell stress response so that this possibility has been largely uninvestigated. As now observed for silk worm cells and previously for mammalian cells (8,12) the abundance of SINE RNAs can dramatically increase in response to various stresses.

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