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Synthesis and Processing of Small B2 Transcripts in Mouse Embryonal Carcinoma Cells

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B2 genes are short repeated sequences which are transcribed by RNA polymerase III. Abundant transcripts accumulate in embryonic and transformed cells, but transcripts are rare or absent from normal differentiated cell types. During retinoic acid-induced differentiation of P19 embryonal carcinoma cells, an early transient increase in B2 RNA levels is followed by a rapid drop in expression. The marked changes in B2 RNA levels are most likely due to transcriptional modulation since B2 RNA stabilities are unaffected by differentiation. At least four short-lived B2 RNAs with apparent lengths of 150, 180, 240, and 500 nucleotides were characterized. The two larger RNAs are polyadenylated and are more stable in cells. A cDNA of a B2 gene was isolated which was over 99% identical to the consensus sequence. This B2 cDNA can be transcribed in human cells and yields at least two distinct transcripts. We propose a model for B2 RNA metabolism which describes transcription, posttranscriptional modification and processing, and nucleocytoplasmic transport.

Mammalian genomes contain numerous single-copy genes, a small number of pseudogenes, and families of highly repeated DNA sequences. The latter include long and short interspersed elements (33). Short interspersed elements are generally less than 500 base pairs (bp) long, are present at a high copy number (>10^4 per genome), and are dispersed throughout the genome (33, 42). B2 is a rodent short interspersed element family comprising approximately 10^5 related sequences, which is thought to have spread throughout rodent genomes by retrotransposition (5, 27, 42).

The consensus B2 element is 180 bp long and contains intragenic sequences homologous to the RNA polymerase III (pol III) promoter (27, 33). In addition, a pol III termination signal marks the 3′ end of the 180-bp consensus sequence. Transcription of these sequences by pol III requires transcription factors TFIIIB and -C, like tRNA (7). B2 also contains a poly(A) addition signal near its 3′ terminus, and its transcripts are reportedly polyadenylated (4, 26). Short transcripts hybridizing to B2 generally form a heterogeneous smear at 200 to 600 nucleotides (nt) in Northern (RNA) blots (5). It has been suggested that B2 RNAs play a role in regulating gene expression, mRNA transport, or RNA processing (12, 26). To date, however, no function has been assigned to B2 transcripts.

B2 elements are expressed at very high levels in early embryos (4, 41), transformed cells (8, 36), and the germ line (36) but are virtually silent in differentiated cell types (33, 36). Expression of B2 elements can be induced in somatic cells under conditions of stress, including serum starvation and stimulation (14) and heat shock (16). These transcriptional changes in B2 expression are not shared by other pol III genes, such as tRNA or 5S rRNA (8).

We investigated the nature of B2 transcripts in mouse embryonal carcinoma (EC) cells and regulation of these transcripts during retinoic acid (RA)-induced differentiation of these cells.

MATERIALS AND METHODS

Cell culture. P19S18 (P19) EC cells were cultured as described previously (34). Cells were grown on tissue culture-grade plastic dishes in α-minimal essential medium (α-MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 7.5% calf serum and 2.5% fetal calf serum (Bockneck Industries, Rexdale, Ontario, Canada). Cells were incubated at 37°C in a 5% CO_2 atmosphere. A 10^{-3} M stock solution of all-*trans* RA (Eastman Kodak, Co., Rochester, N.Y.) was made in ethanol. P19 cells were differentiated in monolayer by adding RA diluted to 10^{-6} M in fresh α-MEM. P19 cells were also induced to differentiate in 10^{-6} M RA or in 0.5% (vol/vol) dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) after aggregation in bacterial grade plastic dishes (34).

Human 293 cells (19) were cultured in Joklik modified α-MEM (GIBCO) supplemented with 10% calf serum and were subcultured after incubating in EDTA (GIBCO) for 20 min at 37°C to release them from the dish surface.

Cell fractionation. P19 cells were separated into nuclear and cytoplasmic fractions by the following procedure. Cells were trypsinized to remove them from tissue culture dishes and were washed extensively with α-MEM and phosphate-buffered saline. Approximately 5×10^7 cells were suspended in 1 ml of hypotonic buffer (13) and set on ice for 15 min. Cells were slowly taken up in and ejected from a 1-ml syringe fitted with a 26-gauge needle. After four repetitions, Triton X-100 was added to the final concentration of 0.1% (vol/vol) and the mixture was gently swirled on ice for 2 to 3 min. Alternatively, Triton X-100 was added to 0.5% (vol/vol) and cells were sheared twice more. Similar results were obtained by both methods. Nuclei were pelleted by centrifugation at 1,000 × *g* for 5 min. No cytoplasmic tags were visible on nuclei when viewed by phase-contrast microscopy. RNA was extracted from either fraction as described below.

Cloning of B2 cDNA. A cDNA library was constructed from neuron-enriched cultures of P19 cells treated with RA. Total RNA was isolated from these cultures by the method described below. Poly(A)$^+$ RNA was isolated (3) by two passages of adsorption to and elution from oligo(dT)-cellulose (type 3; Collaborative Research, Inc., Lexington, Mass.). This fraction was used as a template for oligo(dT)-primed first-strand cDNA synthesis (15) with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) Second-strand cDNA was synthesized by

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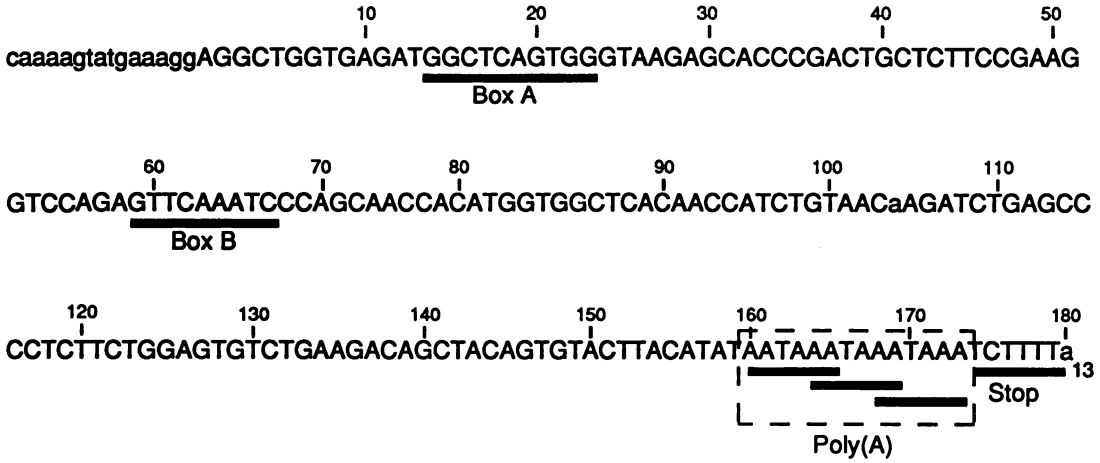

FIG. 1. Nucleotide sequence of the cDNA containing the B2 sequence. The pol III promoter A and B boxes and transcription termination signal are underlined. Three nested poly(A) addition signals (AATAAA) are present near the 3′ end and are boxed. Numbering of the bases corresponds to a consensus sequence of B2 (33), with nt 104 being the only divergent base (A instead of G). Fifteen nucleotides which are not part of the consensus are found at the 5′ end of our cDNA (lower case), and an oligo(A) tail follows the B2 sequence.

using the *Escherichia coli* Klenow fragment (Dupont, NEN Research Products, Boston, Mass.) The double-stranded DNA was extended with avian myeloblastosis virus reverse transcriptase and was subsequently treated with nuclease S1 (Boehringer Mannheim Canada Ltd., Dorval, Quebec). The ends were filled with the Klenow fragment and ligated to *Bam*HI linkers (New England BioLabs, Inc., Beverly, Mass.) (30). These molecules were cloned into the *Bam*HI site of pBR322, and the plasmids were used to transform *E. coli* DH1.

Transfections. Plasmid DNAs were purified from bacteria by alkaline extraction, followed by binding to and elution from glass powder (32). 293 cells were transfected by calcium phosphate precipitation (10). Approximately 1.5×10^6 cells in normal α-MEM were plated in 10-cm dishes the day before transfection. Plasmid DNA (20 μg) was dissolved in 50 mM BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (pH 6.96) (Sigma)–280 mM NaCl–1.5 mM Na_2HPO_4. After 6 h and the following day, the medium was replaced and the cells were grown for approximately 48 h before RNA isolation.

RNA half-life. To determine RNA stabilities, cells were treated with actinomycin C_1 (AMC) and transcript levels were quantified after various time intervals. Cells were plated the day before an experiment, and media were replenished just before the addition of inhibitor. AMC (Boehringer Mannheim), made up as a 5-mg/ml stock solution in 95% ethanol, was added to cells at a final concentration of 20 μg/ml. At timed intervals, cultures were quickly washed with phosphate-buffered saline and were frozen at −20°C for RNA extraction and Northern blot analysis. The levels of B2 transcripts were quantified by scanning X-ray films with an LKB Ultrascan XL enhanced laser densitometer. The areas under absorbance peaks were integrated to yield RNA levels. Various exposure times of the Northern blots were used to ensure that band intensity was proportional to RNA amount. Results were normalized by reprobing films with a probe to β-actin mRNA (35), a long-lived species. In some experiments, the inhibitor cordycepin (3′-deoxyadenosine; Boehringer Mannheim) was added to cultures at a final concentration of 10 μg/ml.

RNA isolation and electrophoresis. RNA was isolated from cells or subcellular fractions by LiCl-urea extraction (2). All solutions were treated with 0.1% diethyl pyrocarbonate overnight and autoclaved, and all steps were performed at 4°C. Isolated RNA was suspended in diethyl pyrocarbonate-treated distilled deionized water, and the concentration was determined by measuring A_{260}. RNA was separated in dena-

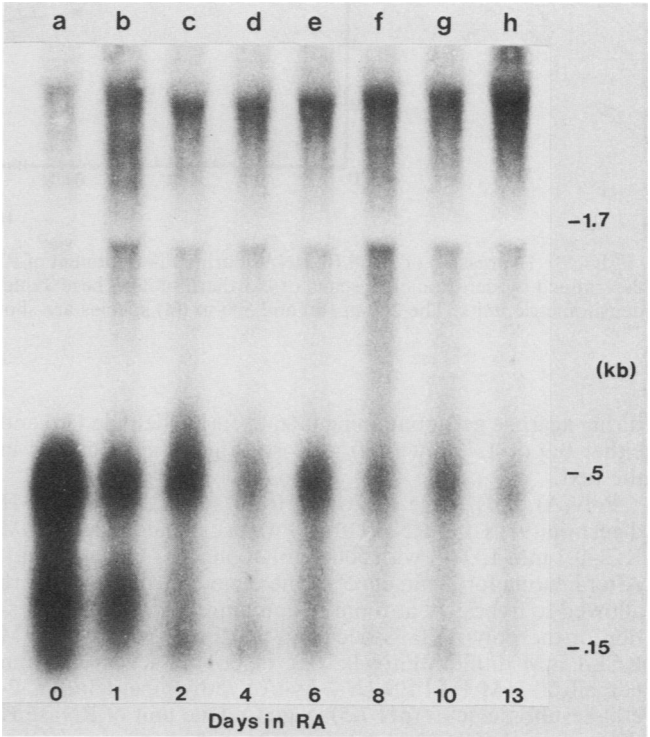

FIG. 2. Disappearance of B2 RNA during differentiation of P19 cells. Aggregated P19 cells were treated with RA for the indicated number of days before RNA was isolated and probed with the B2 cDNA. The most abundant species appeared near 200 and 500 nt. RNA sizes were interpolated from the positions of ethidium bromide-stained tRNA and rRNA in the gel. kb, Kilobases.

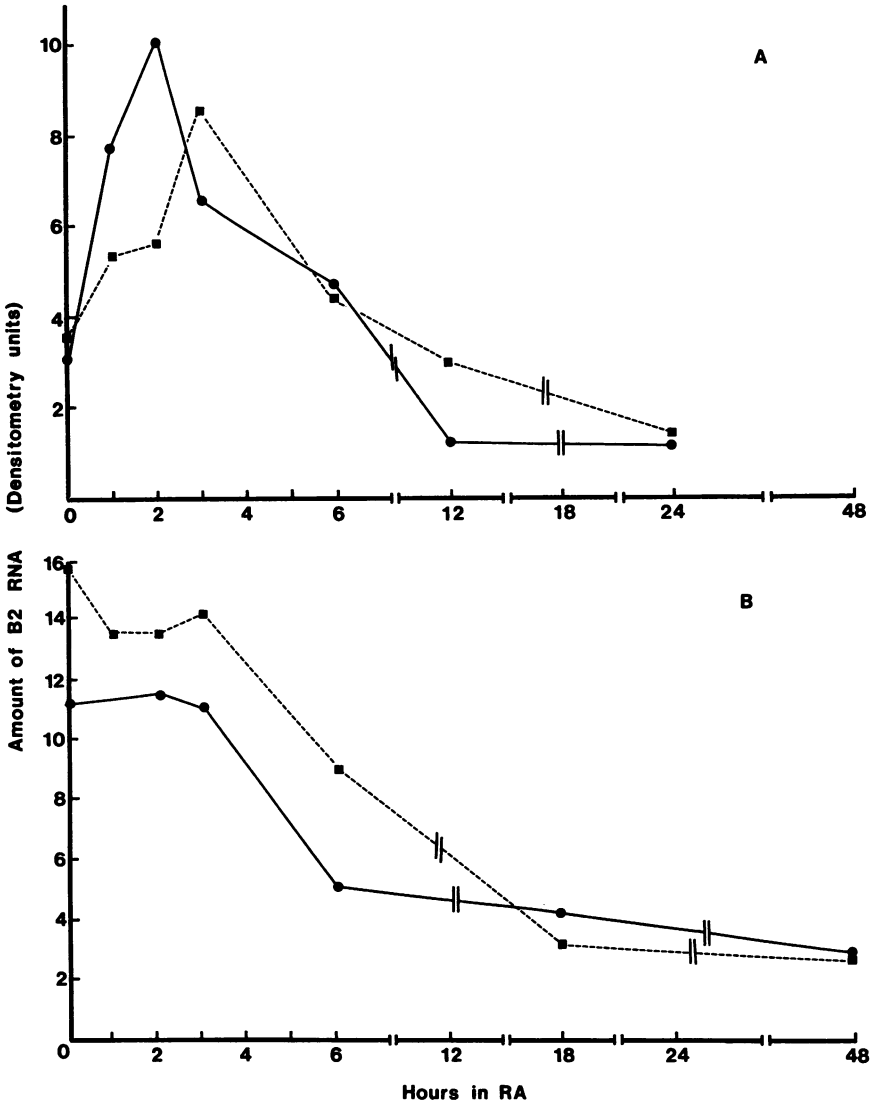

FIG. 3. Expression of small B2 RNAs during RA treatment of P19 (A) and RAC65 (B) cells. The quantities of small B2 RNA species were determined by densitometric scans of Northern blots. These values, corrected for the amount of β-actin mRNA, are indicated in arbitrary densitometric units. The 200-nt (●) and 500-nt (■) species are shown separately.

turing agarose gels containing 0.66 M formaldehyde (30) and either 0.9 or 1.7% (wt/vol) agarose (Sigma) as indicated in the text.

Poly(A) tails were removed from RNA by RNase H digestion (9). Total RNA (10 μg) was suspended in 100 mM KCl–0.1 mM EDTA with 500 pmol of oligo(dT)$_{12-18}$ (Sigma). After heating for 2 min at 65°C, the oligo(dT) and RNA were allowed to hybridize at room temperature for 30 min. Digestion of the poly(A) tails occurred in 10 mM $MgCl_2$–80 mM KCl–1 mM dithiothreitol–0.5 mg of bovine serum albumin per ml–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5) (Sigma). One unit of RNase H (Pharmacia LKB Biotechnology, Montreal, Quebec, Canada) was added to each reaction, and digestion proceeded for up to 30 min at 37°C. Parallel control reactions lacked oligo (dT) or RNase H. Digested RNA was extracted once each with phenol-chloroform and chloroform and used for Northern blot analysis.

To fractionate B2 transcripts, poly(A)$^+$ RNA was isolated from P19 total RNA by using an oligo(dT)-cellulose column.

Northern blot analysis. RNA separated in formaldehyde gels was transferred overnight to Hybond-N filter paper (Amersham Canada Ltd., Oakville, Ontario) and cross-linked to the filter by UV light (254 nm) for 1 to 2 min.

Filters were prehybridized for at least 2 h at 57°C in 50% (vol/vol) deionized formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2.5× Denhardt solution–0.1% sodium dodecyl sulfate–1% denatured salmon DNA–50 μg of yeast tRNA (Sigma) per ml. [α-^{32}P]CTP-labeled riboprobes were transcribed from pGEM3B2 and other derivatives with SP6 or T7 polymerase according to the instructions of the manufacturer (Promega Corp., Bio/Can Scientific Ltd., Mississauga, Ontario, Canada). Filters were incubated overnight at 57°C in (pre)hybridization solution with added probe and were washed at high stringency (three times; 20 min; 0.1% sodium dodecyl sulfate–0.1× SSC,

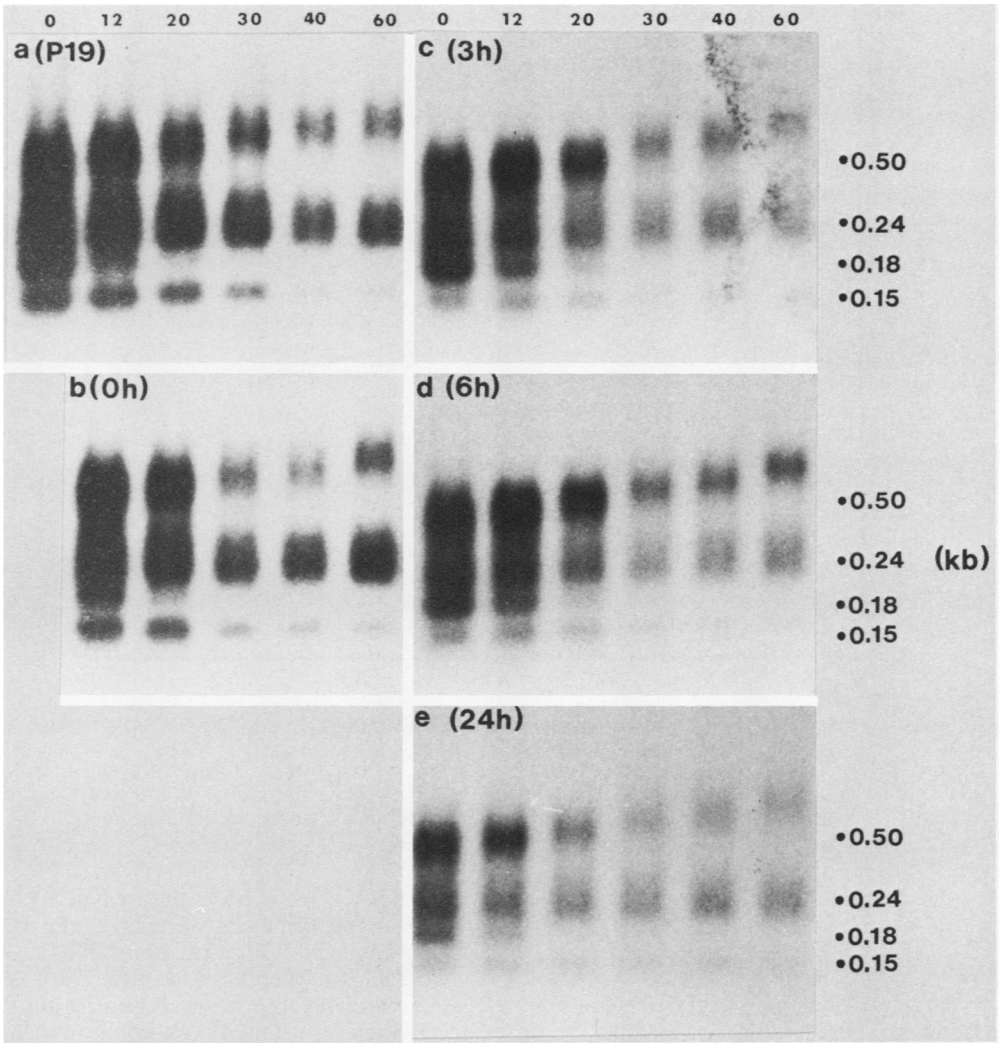

FIG. 4. Determination of B2 transcript stabilities during RA-induced differentiation. RNA for Northern analysis was isolated from cultures at 0, 12, 20, 30, 40, and 60 min after the addition of AMC. Cultures were untreated (a) or treated with RA for 0 h (b), 3 h (c), 6 h (d), or 24 h (e) before the addition of AMC. kb, Kilobases.

65°C). Hybridization was visualized by exposing Kodak XAR-5 film to filters at −70°C in cassettes containing two intensifying screens.

RESULTS

A cDNA clone carrying the B2 sequence was identified from a library made from RA-treated P19 cells based on its differential hybridization to RNA from undifferentiated and differentiated P19 cells. The sequence of the 207-bp insert of this cDNA clone is shown in Fig. 1. The insert was found to be nearly identical to a consensus sequence of the repeated B2 (short interspersed) element, differing by only 1 of 180 nucleotides (33). No substantial open reading frames were found, and stop codons were detected in each reading frame. Our cDNA clone contained 15 nt at the 5′ end which were not part of the consensus, as well as a short (13-nt) oligo(A) tail at the 3′ end. As with other B2 sequences, this clone carried a pol III intragenic split promoter, three nested polyadenylation signals near the 3′ end, and a pol III transcription termination signal.

Expression of B2 sequences in EC cells. Northern blot analyses were done to determine the expression pattern of B2 sequences during the differentiation of P19 cells. Two heterogeneous bands of approximately 200 and 500 nt were present at very high levels in undifferentiated EC cells (Fig. 2); however, induction of differentiation of these cells with RA caused a marked decrease in B2 expression over several days. Induction of differentiation with dimethyl sulfoxide also resulted in a rapid decline in the level of B2 expression (data not shown). B2 transcripts were not detected in adult brain, heart, or liver cells (data not shown).

Shortly after the addition of RA to P19 cells, there was a transient increase in the levels of small B2 RNAs followed by a rapid drop in expression (Fig. 3). The amount of B2 RNA as much as doubled in the first 2 h of RA treatment. The size and duration of this transient increase were somewhat variable but generally peaked between 2 and 4 h after RA addition. Actin mRNA levels did not change after RA treatment in these experiments (data not shown).

RAC65 cells are a clonal derivative of P19 which do not differentiate in the presence of RA (24). Unexpectedly, we observed a drop in B2 RNA levels after treatment of RAC65

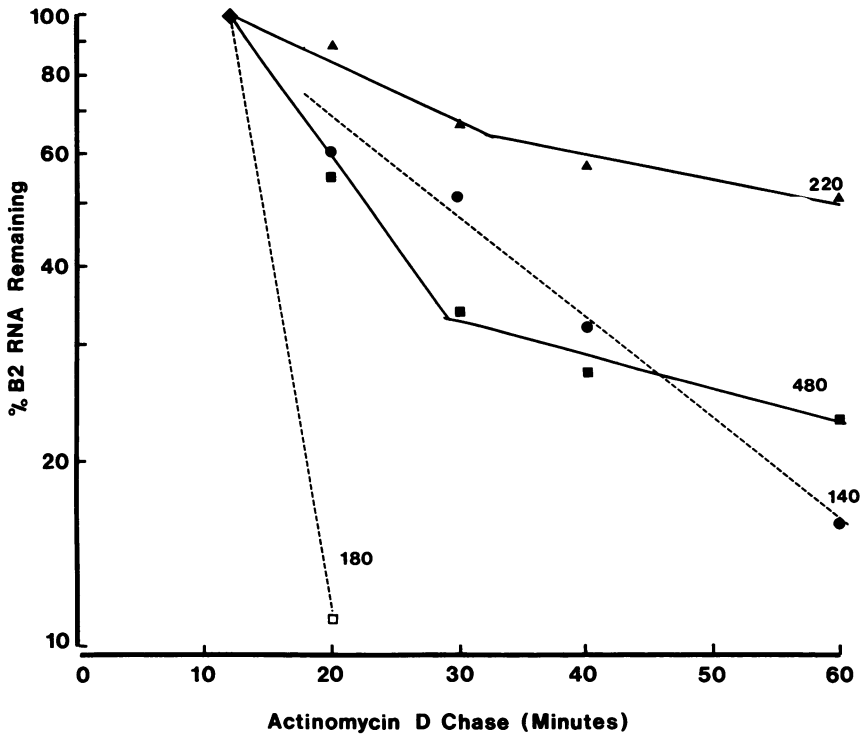

FIG. 5. Calculation of half-lives of B2 RNA species. Northern blots such as those shown in Fig. 4 were scanned, and the amount of each B2 RNA species was quantified by comparison with the β-actin mRNA. The RNA species were 150, 180, 240, and 500 nt. Decay curves were fitted by eye. The results of a representative experiment are shown. Other experiments gave comparable results.

cells with RA. However, unlike P19 cells, the early increase in B2 expression was not observed in RA-treated RAC65 cells (Fig. 3B).

B2 RNA stability. Since the levels of B2 RNAs changed after induction of differentiation, we sought to determine whether this was due to altered transcript stability or to changes in transcription rates. The stabilities of B2 RNAs were measured in P19 cells after various intervals in RA. This was done by treating cells with AMC and measuring the rates of decrease in B2 transcript levels.

For these experiments, RNA was electrophoresed in high-percentage (1.7%) agarose gels. Under these conditions, we observed four, rather than two, RNA species hybridized to B2 probes. These species were approximately 140 to 150, 180, 220 to 240, and 480 to 500 nt long (Fig. 4). The same four B2 transcripts were seen after treatment with RA. The rate of migration of the largest transcript decreased with prolonged AMC exposure. This change in mobility was eliminated when cordycepin (3'-deoxyadenosine) was added together with AMC (data not shown), suggesting that the increase in size of this transcript was due to posttranscriptional addition of extended poly(A) tails to this RNA species. When the half-lives of each of the B2 RNA species were calculated, they were found to be different (Fig. 5 and Table 1). The expected size of the full-length primary transcript from B2 sequences is 180 nt. This transcript was extremely unstable, having a half-life of only 3 to 4 min. The 240- and 500-nt species were more stable, with half-lives approaching 60 min. The decay of these two species was often biphasic, suggesting the presence of both unstable and stable components. The 150-nt species was of intermediate stability. Half-lives were determined upon the addition of RA (0 h), at 3 h during the peak in B2 expression, at 6 h during the drop in B2 RNA levels, and at 24 h when B2 RNA levels were reduced. The stabilities of each of the B2 RNAs did not change appreciably after treatment of P19 cells with RA (Fig. 5 and Table 1). Therefore, the drop in B2 RNA levels during induction of differentiation did not result from preferential degradation or instability of these transcripts. Rather, these data suggest that the changes in levels of B2 RNAs in P19 cells after RA treatment were due to alterations in transcriptional regulation.

Nuclear and cytoplasmic forms of B2 RNA. P19 cells were fractionated into nuclear and cytoplasmic compartments to determine the subcellular location of each B2 transcript.

TABLE 1. Half-lives of B2 transcripts in P19 cells

RA treatment (h)	Stability (min) of B2 RNA species			
	140–150 nt	180 nt	220–240 nt[a]	480–500 nt[a]
None	19	3.5	(1) 55 (2) 78	(1) 22 (2) 50
0	19	3.3	(1) 40–70[b] (2) 63	(1) 28 (2) 34
3	22	4	(1) 70 (2) 52	(1) 18 (2) 53
6	21	3	(1) 66 (2) 72	(1) 44 (2) 42
24	19	2.5	(1) 30, 90[c] (2) 73	(1) 11, 50[c] (2) 18, 40[c]

[a] Data from two experiments in which these species were resolved are shown.

[b] Point scatter did not allow accurate determination. Values are high and low calculations.

[c] In some samples, biphasic curves were observed, suggesting the presence of both unstable and stable species.

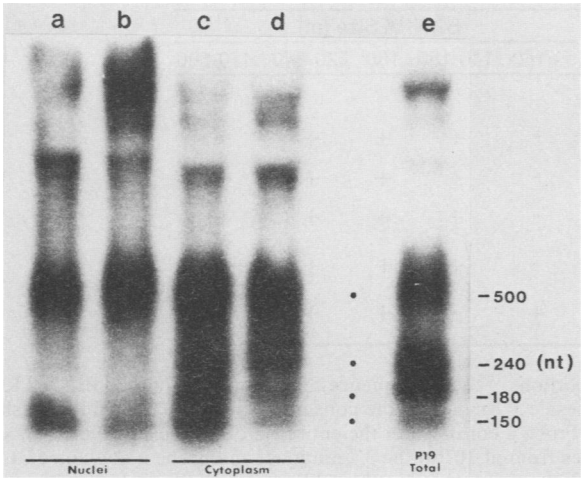

FIG. 6. Location of B2 RNAs in nuclear and cytoplasmic cellular compartments. P19 cells were fractionated into nuclear and cytoplasmic fractions, and RNA was isolated from each. The results of two separate fractionation experiments are shown. Lanes a and b, Nuclei; lanes c and d, cytoplasm; lane e, total RNA.

Nuclei contained 150- and 500-nt RNAs, with the appearance of the 180-nt transcript in some experiments (Fig. 6). The 240-nt transcript was not detected in the nuclear fraction in any experiment. Cytoplasmic RNA contained all four species of B2 RNA, with the two larger species predominating. Large RNAs containing B2 in the sense orientation were found primarily in EC cell nuclei.

Polyadenylation of transcripts. To determine whether any of the B2 transcripts were polyadenylated, we fractionated P19 cell RNA on an oligo(dT)-cellulose column to separate poly(A)$^+$ and poly(A)$^-$ components. The four transcripts were cleanly partitioned into two classes; the 240- and 500-nt RNAs bound to the column, whereas the 150- and 180-nt

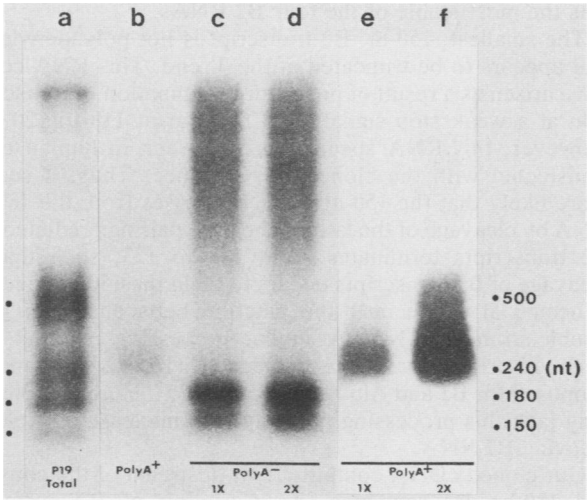

FIG. 7. Separation of poly(A)$^+$ and nonadenylated B2 RNAs. Total P19 cell RNA was chromatographed on oligo(dT)-cellulose columns. Lane a, Total RNA; lane b, poly(A)$^+$ RNA; lanes c and d, nonadenylated RNA corresponding to 10 and 20 μg of loaded RNA, respectively; lanes e and f, poly(A)$^+$ RNA from 10 and 20 μg of loaded RNA with film exposure time doubled compared with lanes a to d.

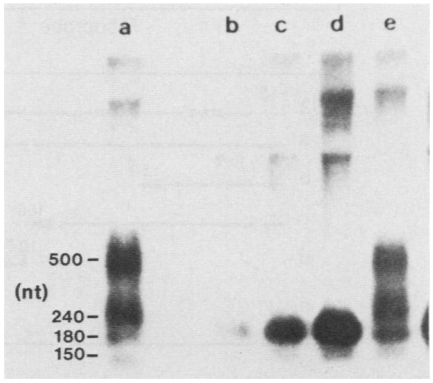

FIG. 8. Removal of poly(A) tracts from B2 RNAs. P19 cell RNA (10 μg) was hybridized to oligo(dT)$_{12-15}$ and digested with RNase H to selectively degrade poly(A) tracts. Lane a, Undigested total RNA; lanes b to d, RNA digested for 5, 15, and 30 min, respectively; lane e, control reaction lacking RNase H, incubated for 30 min. Slightly less RNA was loaded in lane b.

RNAs did not (Fig. 7). Some degradation of the poly(A)$^+$ RNA likely occurred since longer exposures of the filter were required to show the 240- and 500-nt transcripts. However, even extended exposures did not show the 240- and 500-nt transcripts in the poly(A)$^-$ fraction.

To confirm that the larger B2 transcripts contained poly(A) tracts, RNA was hybridized to oligo(dT)$_{12-18}$ and digested with RNase H to selectively remove those RNA sequences which hybridized to the oligo(dT). Within 5 min of RNase H digestion, the 240- and 500-nt B2 RNAs were lost and apparently chased into the 180-nt band (Fig. 8). Digestion of the RNA for longer times resulted in the appearance of increasing amounts of the smallest (150-nt) B2 RNA, with a reduction in the amount of 180-nt RNA. This may have resulted from digestion of A-rich sequences near the 3′ end of the 180-nt RNA.

B2 RNA structure. Northern blots of P19 cell RNA were probed with various portions of the B2 cDNA to determine which regions of the B2 sequence were present in each band. Probes to the entire cDNA or to the 5′ end of the B2 sequence hybridized to all four bands (Fig. 9). However probe e, complementary to the 3′-terminal 20% of the B2 sequence, did not detect the 140- to 150-nt transcript. The A+T-rich nature of this probe required the use of very low stringency hybridization and washing conditions, resulting in extensive cross-hybridization to the 18S rRNA. Nevertheless, probe e did hybridize to a novel short (<140-nt) RNA. These data suggest that the four major RNAs hybridizing to B2 probes share sequences at their 5′ ends and that the 140- to 150-nt species is a processed or prematurely terminated transcript.

Transcription of B2 cDNA in human cells. Although most B2 motifs in DNA have a very similar size and sequence, it was possible that the four B2 transcripts present in P19 cells were the products of four different genes. We wanted to determine whether our cloned B2 cDNA could be transcribed and whether the transcripts were unique or heterogeneous in size. Since human cells contain no B2 sequences, plasmids carrying our B2 cDNA were transfected into human 293 cells. RNA from transfected cells contained at least two species which hybridized to B2 at approximately 200 and 400 to 500 nt (Fig. 10). Untransfected cells lacked these RNAs.

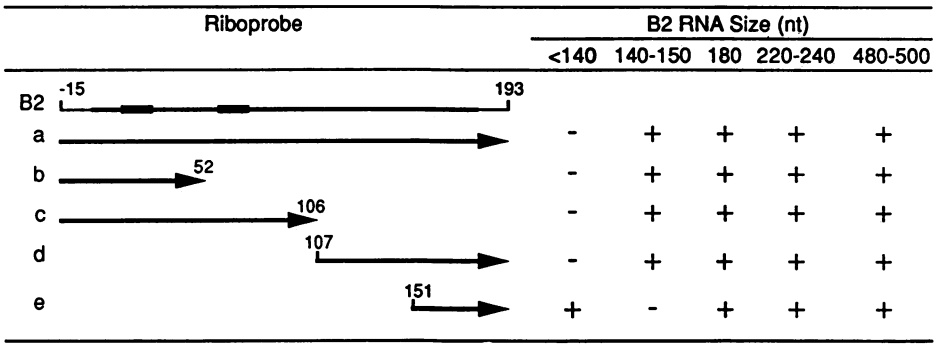

FIG. 9. Hybridization of B2 RNA species to probes recognizing different portions of the B2 sequence. Northern blots of total P19 cell RNA were hybridized with the probes indicated in the diagram. The heavy line beside B2 indicates the consensus element. The thin lines show flanking sequences in our cDNA, and the boxes represent promoter motifs. Probe a consisted of the entire B2 cDNA, probe b contained nt −15 to +52, probe c contained nt −15 to +106, probe d contained sequences from nt 107 to the 3′ terminus, and probe e contained nt 151 to the 3′ terminus. Symbols indicated presence (+) or absence (−) of hybridization signals in Northern blots. Relative signal strength varied between some probes.

Secondary structure models for B2 RNA. It has been proposed that mouse B2 sequences represent pseudogenes of tRNA (28, 38). However, our models for the secondary structure of B2 RNAs are very different from those of tRNA. At least three stable structures were predicted (Fig. 11). Free energies ranged from −39 to −40 kcal/mol for these foldings. The 5′ portion of B2 forms a large hairpin, with G-3 always pairing with C-72. This structure places the promoter A and B boxes near each other in the double-stranded RNA (Fig. 11, iii). Three possible stable foldings for the central portion of B2 RNA were predicted, each with unique hairpin or stem-loop structures. The extreme 3′ terminus is A+T rich and does not form stable structures.

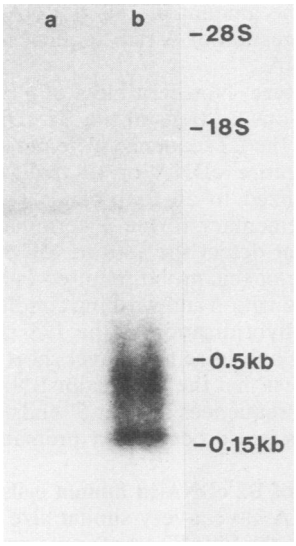

FIG. 10. Transient expression of B2 cDNA in human cells. The isolated cDNA was transfected into human 293 cells and RNA was extracted from cultures after 48 h and separated in agarose gels. No human RNAs hybridized to the B2 riboprobe, as shown by the untransfected control (lane a). In RNA from transfected cells, (lane b), two small species hybridizing to B2 are visible near 200 and 400 to 500 nt. The positions of ribosomal bands were determined from ethidium bromide-stained gels, whereas the positions of the small B2 RNAs (0.5 and 0.15 kilobases [kb]) were observed in P19 nuclear RNA separated in the same gel (not shown).

DISCUSSION

Although B2 sequences in the genome are almost uniformly 180 bp long, there are at least four small transcripts derived from these elements in EC cells and in early mouse embryos (4). We believe that the 180-nt B2 RNA species is the primary pol III transcript because of its size and its extreme instability. The apparent cytoplasmic location of the 180-nt transcript was likely artifactual, resulting from leakage of the nascent transcript out of nuclei (17). The B2 sequence has several polyadenylation signals near its 3′ end, and our evidence indicates that the relatively stable 500- and 240-nt B2 transcripts are polyadenylated. Poly(A) tails could protect these RNAs from endonucleases, perhaps through association with poly(A)-binding protein (6). In addition, association of the 500-nt B2 RNA with poly(A)-binding protein may facilitate its transport from the nucleus to the cytoplasm and subsequent shortening of the poly(A) tail (37). The 240-nt transcript was present only in the cytoplasm and was the most stable of the four B2 RNAs.

The smallest, 150-nt, B2 transcript is not polyadenylated and appears to be truncated at the 3′ end. This RNA could have arisen as a result of premature termination of transcription at a weak stop signal (TCTTCT) at nt 118 to 120 (4). However, this RNA species was not seen in human cells transfected with the cloned B2 sequence. Thus, it seems more likely that the 150-nt transcript arises from the 180-nt RNA by cleavage of the latter. The base pairing predicted for B2 transcripts terminates at nt 148 to 153, such that if cleavage of B2 transcripts occurs to yield the 150-nt species, it occurs at a site near the junction between single- and double-stranded RNA. A similar processing reaction has been described for tRNA molecules (18, 22, 44) and is proposed for B1 and Alu transcripts (1, 31). Human 293 cells may lack this processing pathway or a nuclease capable of cleaving B2 RNA.

Our cloned cDNA contains 15 nt upstream of the consensus 180-bp B2 sequence. Transcription of B2 normally initiates at the first nucleotide of the consensus sequence, 12 to 13 nt upstream of the promoter A box (11). The sequence GGAGGCTGG extending from nt −2 to 7 in our cloned cDNA resembles a promoter A box (RRYNNARYGG [11]) and may function as a promoter element, resulting in transcription initiation at nt −15 for this particular B2 element. A

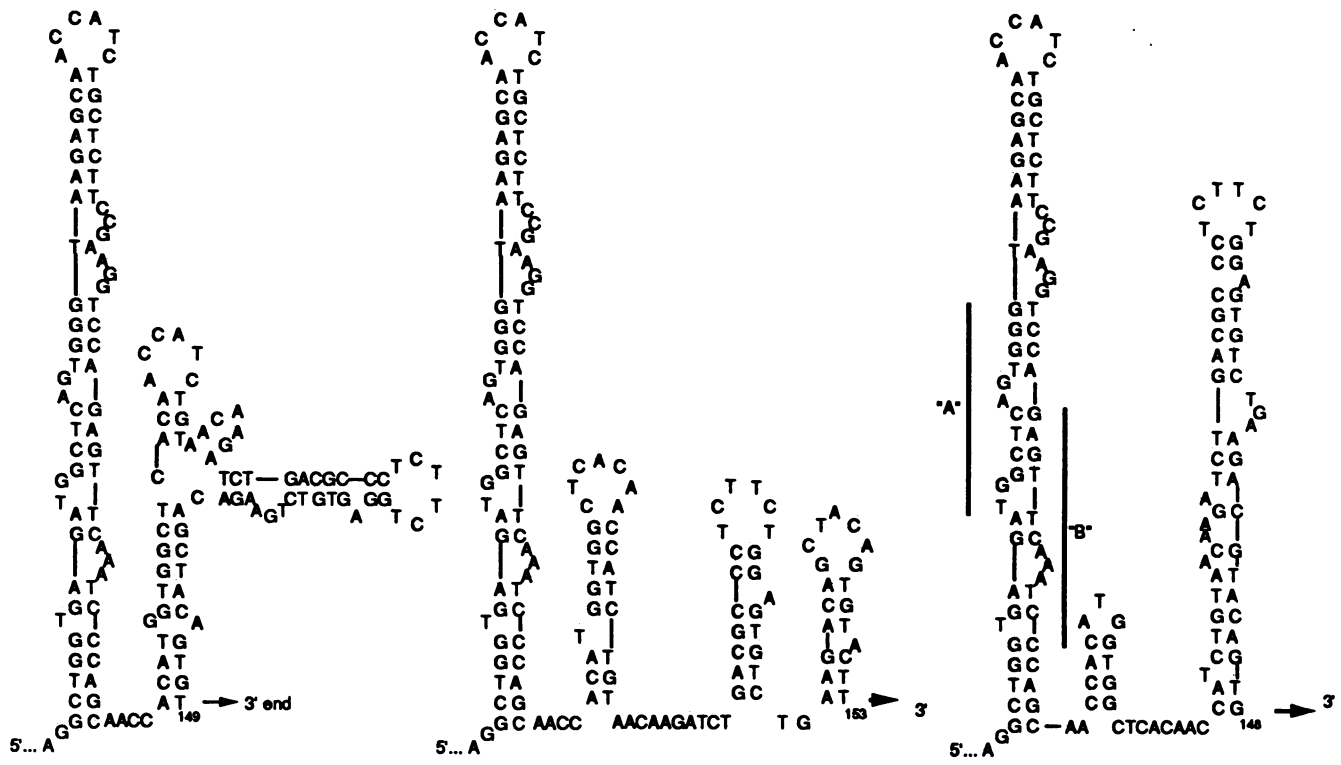

FIG. 11. Predicted secondary structures for B2 RNA. Stable folding patterns for RNA from our B2 cDNA were calculated based on the algorithms of Zuker and colleagues (23, 45). Three possible foldings of the canonical strand are represented, with the relative stabilities indicated below each. The first hairpin-loop structure is present in all models. The sequences extend from the first nucleotide of the consensus to the last nucleotide involved in forming stable secondary structure. The numbering of nucleotides is that of Fig. 1, and positions of the promoter boxes are shown in structure iii.

similar 5′ sequence and extended terminus have been observed in another B2 element (21).

After the addition of RA to P19 cells, the levels of small B2 RNA transiently increased before decreasing to very low levels in the differentiated cells. The stabilities of each B2 transcript remained unaltered in RA-treated cells, suggesting that the modulation of levels of B2 RNA was due primarily to fluctuations in transcription initiation. Recent evidence with differentiating F9 cells (43) indicates that availability of transcription factors can indeed account for the fluctuations in B2 expression. It is interesting that the abundance of B2 RNAs decreases in P19 and RAC65 cells at almost the same rate. Since RAC65 cells do not differentiate in response to RA, this observation indicates that the reduction in B2 RNA is not causally involved in the induction of cellular differentiation.

The biological function, if any, of B2 transcripts remains unclear. Small pol III transcripts can play roles in the control of gene expression. For example, VAI RNA is a 160-nt pol III transcript encoded by adenovirus which binds and inactivates a double-stranded RNA-induced kinase and results in stimulation of translation (25). VAI RNA also associates with ribosomes to stabilize mRNAs and increase translation (40). Mouse 4.5S RNA is a small pol III transcript similar to B2 which binds to poly(A)$^+$ RNAs and may regulate mRNA transport or translation (29). B2 RNAs are found in ribonuclear protein particles of various sizes and bound to cytoplasmic mRNAs (26, 39). Like VAI RNA, B2 RNAs might function in translational control and/or mRNA transport (12, 26; T. S. Bladon and M. W. McBurney, submitted for publication); however, no such role has yet been demonstrated.

B2 elements are thought to have spread throughout rodent genomes by retrotransposition (42), the same mechanism responsible for the creation of most pseudogenes. B2 sequences are very much more abundant than other pseudogenes because (i) B2 sequences are expressed at high levels in cells of the germ line (4, 36, 41) and (ii) each retroposed B2 element carries with it an internal promoter which should allow each new element to become a potential source of further retrotransposons (20). We found that our cDNA derived from a B2 transcript was indeed able to be transcribed, confirming that the internal pol III promoter did remain functional after reverse transcription of a B2 transcript back into DNA.

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