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Coordinated posttranscriptional control of gene expression by modular elements including *Alu*-like repetitive sequences

(cell transformation/growth control/B1 sequences)

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ABSTRACT **We previously reported that in rat fibroblasts, accumulation of a set of mRNAs ("pIL genes") was modulated as a function of cell growth and transformation, at a posttranscriptional stage, and by a mechanism that depends on a short nucleotide sequence containing an ID repetitive element. In mouse fibroblasts, hybridization with rat pIL probes identified mRNAs with the same pattern of expression, which did not contain ID sequences but contained a different regulatory element, encompassing a repetitive sequence of the B1 family. Expression in mouse cells of a reporter β-globin gene carrying this element inserted in its 3′ noncoding region was growth- and transformation-dependent. The nucleotide sequences of two murine and of three rat pIL cDNAs showed clear similarities in the region immediately adjacent to the ID and B1 repeats. Both the repeat and the flanking sequence were required to confer on β-globin constructs the pattern of expression characteristic of the pIL genes. The hypothesis is presented that repetitive sequences in the eukaryotic genome might be modular parts of complex regulatory elements ensuring the coordinated expression of various mRNA species.**

The rat ID repetitive sequences, described earlier in the small brain-specific BC1 transcripts (1), share a number of properties with the mouse B1 and B2 and the human *Alu* repetitive sequences (2, 3): size in the range of 100 nt, copy numbers in the range of 200,000 per genome, interspersion with single-copy genes, similarities of their primary and secondary structures with tRNAs, and RNA polymerase III promoter activity. We reported previously that in rat fibroblasts, ID sequences are present in a complex group of mRNAs ("pIL genes"), differentially expressed in arrested and growing cells and in normal and transformed cells (4). Their levels are minimal when growth has been arrested by serum starvation, they increase after addition of serum to reach maximal values during the G_2 period of the first cycle, and they are constitutively expressed in cells transformed by a variety of oncogenes. A regulatory role of the ID element was suggested by the observation that expression of a transfected reporter gene became similarly dependent on growth and transformation when it carried in its 3′ noncoding region a 130-nt fragment of one of the pIL cDNAs encompassing the repetitive element.

ID-related sequences may be found in the mouse genome but are found in numbers that are lower by orders of magnitude as compared with the rat and with a higher degree of sequence variability (5, 6). Because we assumed that the regulation of the pIL mRNAs during growth and transformation is physiologically significant, we decided to analyze the regulation of expression of the same genes in mouse cells. We now report that mouse pIL genes with the same characteristic pattern of expression can be identified on the basis of their homology with the nonrepetitive regions of the rat genes.‡ As in the rat, the regulated mouse transcripts include a repetitive element, but this time the repetitive element is of the B1 family. We further asked whether the presence of the repetitive block, either B1 or ID, was sufficient to confer growth-dependent expression. This question was raised by the large number of these repeats, present in various cell types in transcripts that are not growth- and transformation-dependent. It appears, in fact, that the *Alu*-like sequences (ID in rat and B1 in mouse) are necessary, but not sufficient, for regulation. Growth-dependent expression requires, in addition, a nonrepetitive sequence in the immediate vicinity of the repeats, in which blocks are conserved between the two species.

MATERIALS AND METHODS

Cell Lines. Cells were grown in Dulbecco's modified Eagle's medium/10% newborn calf serum. Serum starvation-stimulation experiments were done as described (4). Stable transformants were obtained from foci produced after transfection of plasmid pEJ6.6, which carries the c-Ha-*ras* oncogene (7).

cDNA Isolation and Characterization. A cDNA library made in λgt11 bacteriophage vector from polyadenylylated RNAs isolated from BALB/c 3T3 cells was screened for clones hybridizing with either the pIL8 or the pIL2 rat cDNA inserts (4). Positive clones were subcloned into a M13-derived phage vector by using standard procedures. Sequences were determined by using the dideoxynucleotide chain-termination technique (8). Searches in GenBank and European Molecular Biology Laboratory sequence libraries were conducted with the IFIND program (IntelliGenetics).

Construction of Plasmids. Sequences to be tested were inserted in the 3′ noncoding region of the rabbit β-globin gene of either pEP1 (9) or pFV1 (see below). In the pEP1 plasmid, the gene is expressed under control of the simian virus 40 enhancer–promoter region, whereas in pFV1 this region has been replaced by the *Sal* I–*Nru* I fragment of plasmid pΔ121 containing the H-2K promoter–enhancer region of the major histocompatibility K gene (10). Plasmid pFV0 was derived from pEP1, and plasmids pFV2, pFV3, pFV4, and pFV6 were derived from pFV1. Plasmids pFV0 and pFV2 (see Fig. 4) carry the *Rsa* I–*Eco*RI fragment of the mouse pIL8 cDNA containing both the B1 repetitive element and its flanking region; plasmid pFV3 carries the *Pst* I–*Eco*RI fragment containing only the B1 repetitive block, and plasmid pFV4 carries the *Rsa* I–*Pst* I fragment, which contains the region flanking the B1 sequence. The fragment cloned in plasmid pFV6 includes the flanking sequence and part of the B1

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M97159 and M97160).

repeated element (nt 230–350; see Fig. 2*A*). The insert was prepared by PCR amplification by using oligonucleotides with the two terminal sequences and a *Bgl* II restriction site (underlined) (OF1, 5′-GAAGATCTTCCGGTACAC-CCTAAACCC; OF2, 5′-GAAGATCTTCCTCCCGAGTGC-TGGG). Plasmid pFV5 contains the *Bgl* II–*Hin*dIII fragment of plasmid pBF1 [rat ID sequence (11)]. Before use, the structure of every construct was checked by restriction mapping and partial nucleotide sequencing.

Reverse Transcription and PCR Amplification. Cytoplasmic RNAs were prepared from exponentially growing BALB/c 3T3 cells and transcribed by using the rTth reverse transcriptase RNA PCR kit (Perkin–Elmer/Cetus) according to the supplier's methods. The amplification reaction was then done by using the following program for 30 cycles: denaturation for 1 min at 94°C, annealing for 1 min at either 48°C (for construction of pFV6) or 56°C (in the case of pIL2), and synthesis for 1 min at 72°C. Products were allowed to finish their elongation during 10 min at 72°C before cooling. For pIL2, PCR amplification products were first cloned in pUC19 vector, and three independent clones were sequenced.

Gene Transfer and G418 Selection. These techniques were done as described (4).

RNA Preparation and Analysis. Nuclear and cytoplasmic RNAs were prepared after Nonidet P-40 lysis, as described (12). Polyadenylylated RNAs were isolated on oligo(dT)-cellulose, as described (13). RNA blot experiments were performed as described (4). For S1 mapping assays, 40 μg of RNA was ethanol-precipitated; half of the preparation was analyzed by RNA blotting, and the remaining 20 μg was hybridized at 53°C with the labeled *Eco*RI–*Xho* I restriction fragment of pFV1 DNA, treated with S1 nuclease, and electrophoresed according to standard procedures (13). Size determinations were made relative to ^{32}P-end-labeled molecular weight markers (*Hin*fI-digested pAT153). Nuclear transcription assays were done as described (4).

RESULTS

Mouse Genes Related in Sequence to Rat pIL Genes Are Expressed in the Same Growth- and Transformation-Dependent Manner. RNA blot hybridization of polyadenylylated cytoplasmic RNAs from mouse BALB/c 3T3 fibroblasts with labeled pIL2 and pIL8 rat cDNA probes revealed discrete bands of 2 kilobases (kb) and 1.4 kb, respectively. These mouse pIL genes exhibit the same dependence on the growth and transformation states as their rat counterparts. In either BALB/c 3T3 or C127 cells, their levels increased 5- to 10-fold during the transition from a quiescent to a proliferative state, reaching maximal values during the G_2 phase of the first cycle (Fig. 1*A*). These mRNAs also increased at the same ratio after transformation by the *ras* oncogene and by polyoma virus (Fig. 1*B*). A cDNA library representative of the polyadenylylated cytoplasmic RNAs of BALB/c 3T3 mouse cells was searched for sequences homologous to the rat pIL2 and pIL8 cDNAs. Two positive clones, thereafter designated mouse pIL2 (542 nt) and mouse pIL8 (450 nt), were isolated and further characterized.

Both Mouse pIL8 and pIL2 mRNAs Contain a Repetitive Sequence of the B1 Family. Southern hybridization of mouse genomic DNA with the labeled pIL8 cDNA resulted in a smear of unresolved hybridization fragments, indicating that the mouse pIL8 cDNA clone contained a repeated element (data not shown). Establishment of its nucleotide sequence (Fig. 2*A*) and then computer searches in sequence data banks demonstrated the presence of a B1 repetitive element (20, 21).

Such a repeated sequence could not be evidenced in the original pIL2 cDNA. However, because the cDNA clone was incomplete (542 nt as compared with 2 kb for the mRNA), a B1 element could still be found in the complete transcript. We thus used primers with sequences from the B1 element and

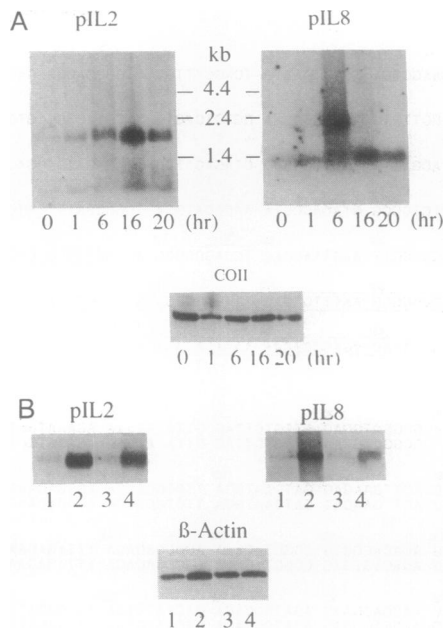


FIG. 1. Growth- and transformation-dependent expression of mouse pIL genes. (*A*) RNA blot analysis of mouse pIL transcripts after serum stimulation. Fetal calf serum (20%) was added at time 0 to quiescent BALB/c 3T3 cultures maintained for 36 hr in serum-depleted medium. Cells were harvested at the indicated time (hr). Cytoplasmic RNA was prepared, and 20 μg was hybridized with ^{32}P-labeled DNA fragments corresponding to the nonrepetitive region of the rat pIL2 or pIL8 cDNA. Control hybridization was done with a probe for the growth-independent mitochondrial cytochrome oxydase subunit II mRNA (COII) (16). (*B*) Increase in mouse pIL transcript level upon transformation by *ras* or polyoma virus. RNA blots were prepared, as described, from exponentially growing cell cultures Lanes: 1, BALB/c 3T3; 2, BALB/c 3T3–*ras* (17); 3, C127; 4, SCOP T1, a polyoma virus-transformed derivative of C127 (18). Hybridizations were done with ^{32}P-labeled rat pIL2 and pIL8 cDNA fragments deprived of their repetitive sequences and, as a control, with a β-actin probe (4).

from the known pIL2 sequence to search for a PCR product amplified from the reverse transcripts of BALB/c 3T3 mRNAs. The nucleotide sequence of the resulting 240-nt DNA fragment confirmed the presence of a B1 element in the mouse pIL2 cDNA (Fig. 2*B*). The pIL2 clone contained an open reading frame that could be translated into a protein sequence 90% homologous to that encoded by the rat pIL2 cDNA (4). No sequence with a significant degree of similarity could be found in data banks.

B1 Elements Are Present in a Complex Family of Growth and Transformation-Dependent Transcripts. This B1 repetitive sequence appears, in fact, to be common to a variety of mRNAs with the same growth-dependent pattern of expression. RNA blot hybridization of cytoplasmic polyadenylylated RNAs with a probe corresponding only to the repetitive element produced a smear of unresolved RNA molecules with sizes ranging from <1 to >5 kb, the intensity of which increased at least 10-fold after serum stimulation (Fig. 3). A variety of B1-containing transcripts appear, therefore, to be regulated in the same way as the pIL mRNAs.

A Fragment of Murine pIL8 cDNA that Includes B1 Sequence Is Sufficient for Growth- and Transformation-Dependent Expression of a Reporter Gene in Mouse Cells. The *Rsa* I–*Eco*RI fragment of the mouse pIL8 cDNA (230 nt), which includes the B1 repeat (136 nt) (Fig. 4) was inserted into the 3′ noncoding region of the rabbit β-globin expression vector pEP1 (9). The resulting plasmid (pFV0) and, as a control, pEP1 DNA were separately introduced into BALB/c 3T3 mouse fibroblasts by cotransfection with neo-

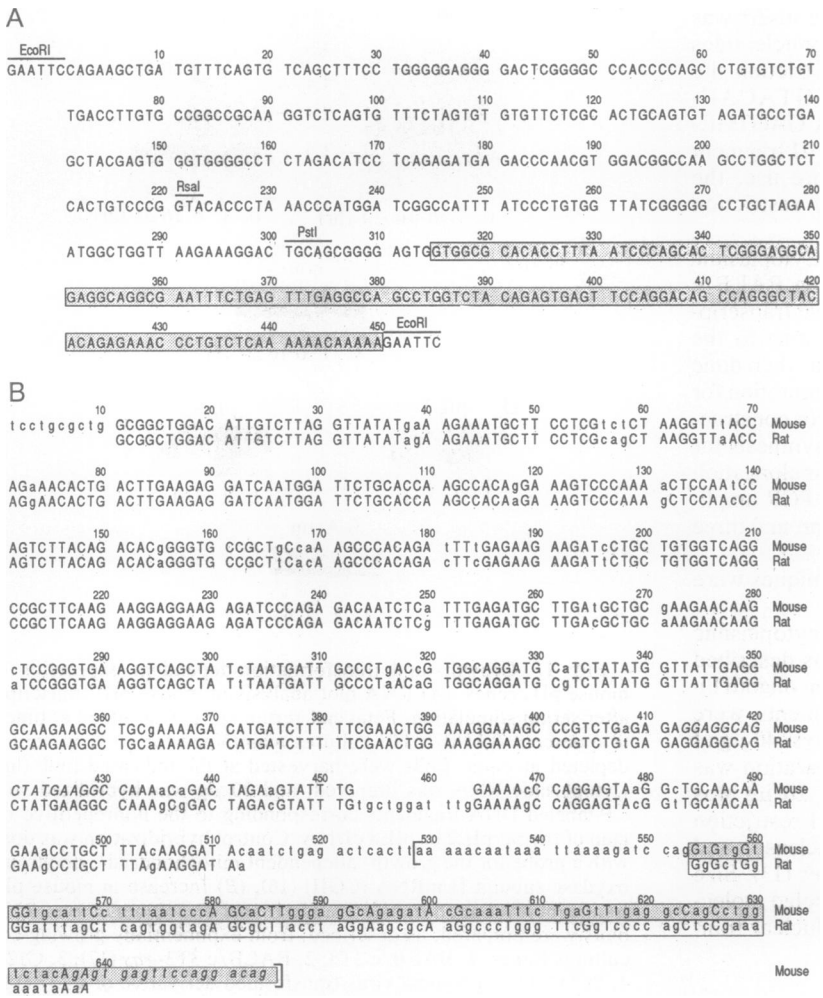


FIG. 2. Both mouse pIL2 and pIL8 mRNAs contain a B1 repetitive element. B1 and ID repetitive elements are indicated as boxed sequences. (*A*) Sequence of pIL8 cDNA; positions of the restriction sites used for cloning pEP1 and pFV1 derivatives are indicated above sequence. (*B*) Sequence comparison between rat (4) and mouse pIL2 cDNA inserts. Nucleotide differences between mouse and rat sequences are indicated in lowercase letters. Most of the mouse pIL2 sequence was established on the original cDNA insert. The sequence located between brackets has been obtained after PCR amplification using oligonucleotides (italic letters) within the original pIL2 cDNA and the B1 element. Note the A+T-rich region, immediately upstream of the latter, a likely initiation site for synthesis of the original pIL2 cDNA.

mycin-resistant DNA. Three resistant cell lines established in each case from colonies grown in G418 medium were designated BFV0-1 to 0-3 and BEP1-1 to 1-3, respectively. Three additional cell lines, BFV2-1 to 2-3, were isolated after transfer under the same conditions of plasmid pFV2. Cytoplasmic RNA was extracted from serum-starved cells and after subsequent stimulation of growth. This RNA was analyzed by S1 mapping for transcripts of β-globin (BEP1 cells) and β-globin with the B1 element (BFV0 and BFV2 cells). In each case, the probe protected a fragment of the expected size—namely, 360 nt for BFV0 and BFV2 and 36 nt for BEP1. Levels of cytoplasmic β-globin transcripts were the same in arrested and growing BEP1 cell lines. In BFV0 and BFV2 cell lines, these levels were low during serum starvation and increased after serum addition to reach 5- to 10-fold higher values within 16–20 hr after stimulation (Fig. 5*A*). As previously observed for a β-globin–ID construct in rat cells, the accumulation of β-globin–B1 RNA increased after oncogenic transformation with the c-Ha-*ras* oncogene of BFV0 and BFV2 but did not increase in BEP1 cells (Fig. 5*B*).

The presence of the 230-nt fragment containing the B1 repeat thus appears as sufficient to change the expression

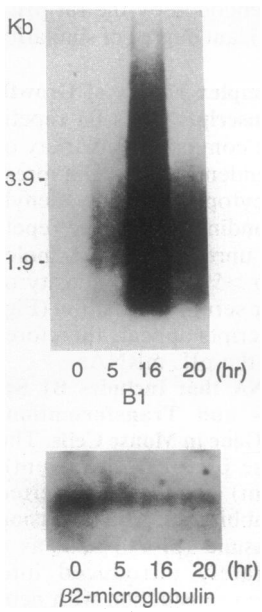


FIG. 3. B1 sequences are present in a variety of growth-regulated mRNAs. (*Upper*) RNA blot analysis of B1-containing transcripts after serum stimulation. Cytoplasmic RNAs were prepared after serum starvation and after further stimulation as described in the legend of Fig. 1. The samples (20 μg per lane) were hybridized with the *Rsa* I–*Eco*RI fragment of mouse pIL8 cDNA, which contains the B1 sequence. (*Lower*) As a control, the same blot was subsequently hybridized with plasmid pβ2m2 (β2-microglobulin).

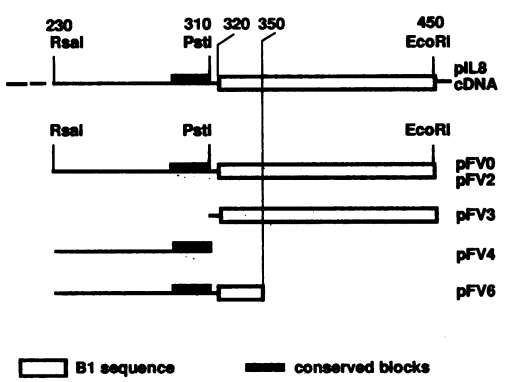


FIG. 4. Summary of constructs with subfragments of mouse pIL8 cDNA. Numbers refer to the nucleotide sequence of pIL8 cDNA.

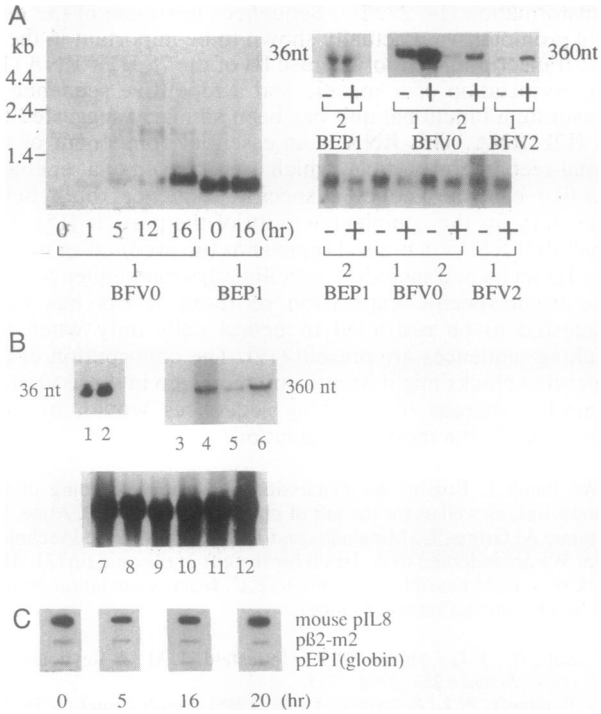


FIG. 5. Growth- and transformation-dependent expression of globin–B1 chimaeric transcripts in mouse fibroblasts. (*A*) Growth-dependent expression. Cells of the indicated lines (see text) were harvested after either serum starvation or serum starvation and subsequent stimulation, as described in the Fig. 1 legend. (*Left*) Cytoplasmic RNAs of BEP1-1 and BFV0-1 cells were prepared at the indicated times and analyzed by RNA blotting with labeled pEP1 DNA. (*Right*) Cytoplasmic RNA was prepared at time 0 (−) and 16 hr after addition of serum (+) and was analyzed for globin-related transcripts by S1 mapping assay (*Upper*) and RNA blotting with the growth-independent mitochondrial cytochrome oxidase subunit II mRNA probe (*Lower*). (*B*) Transformation-dependent expression. (*Upper*) Cytoplasmic RNAs were prepared from exponentially growing cells. Lanes: 1, BEP1-1 (globin DNA); 2, *ras*-transformed BEP1-1 derivative; 3 and 5, BFV0-2 and BFV2-1 (transfected with pFV0 and pFV2, respectively); 4 and 6, *ras*-transformed derivatives of BFV0-2 and BFV2-1, respectively. (*Lower*) The samples (20 μg per lane) were analyzed by S1 mapping for β-globin-related transcripts: lanes 7–12, β-actin mRNA was used as a control on the same RNAs (see Fig. 1 legend). (*C*) Nuclear run-on transcription assay. BFV0-2 cells were harvested at the indicated time (hr) after serum stimulation, and run-on incorporation of [α^{32}P]UMP was done. Equal amounts of ^{32}P-labeled transcription products were hybridized to filters containing the following DNAs: plasmid pβ2m2 (β_2-microglobulin), β-globin (pEP1) DNA (which detects transcription of the transfected β-globin–B1 gene), and the mouse pIL8 cDNA insert. Note the stronger signal produced by the latter, which probably corresponds to a variety of B1 transcripts (for which transcription does not seem to be regulated).

pattern of the gene. However, when the same experiments were done by using nuclear instead of cytoplasmic RNA, no variation of the amount of β-globin–B1 transcripts could be evidenced. This result suggested that, as for the rat pIL genes, expression is not regulated at a transcriptional level. This hypothesis was actually confirmed by run-on analysis with β-globin–B1-containing cells; no significant variation in β-globin–B1 transcript levels was seen on stimulation of growth-arrested cells (Fig. 5*C*).

Are the Repetitive ID or B1 Sequences Sufficient for the Growth-Dependent Expression of pIL Genes? In these experiments, as in our previous work, the DNA fragment that conferred growth- and transformation-dependence on β-globin expression was significantly larger than the repetitive block itself (230 nt as compared with 136 nt for B1, 121 nt as compared with 85 nt for ID), leaving open the possibility that other sequences might be important in the observed regulation. This hypothesis gained strength when we noticed that, in all the mouse and rat pIL cDNAs sequenced so far, conserved nucleotide blocks were present within these flanking sequences, at similar positions relative to the repetitive block (Fig. 6). The next step was to examine mRNA sequences stored in the European Molecular Biology Laboratory and GenBank libraries that contain either B1 or ID repeats. No sequence even loosely related with that of the pIL flanking sequence could be evidenced in the vicinity of the repeats, indicating that the blocks identify only a subset of B1 or ID families.

To test the respective roles of the flanking sequences and of the repetitive elements, we constructed β-globin reporter genes carrying various combinations of the repetitive element and of the flanking sequences. These plasmids were tested in growth-arrested and serum-stimulated cells as in the previous experiments (Fig. 7). Deleting the whole B1 element plus 10 nt of the flanking sequence, up to the *Pst* I site (plasmid pFV4, Fig. 4) abolished growth-dependent regulation. The same was true of a construct (plasmid pFV6) containing the whole flanking region and only 32 nt of the B1 sequence. On the other hand, the complete B1 element without the flanking sequence (plasmid pFV3) could not confer the growth-dependent regulation. An ID element identical in sequence to that in the rat pIL genes but cloned from a BC1 brain transcript (11) and also devoid of the flanking sequence was equally ineffective in rat cells. Two components in the pIL genes are, thus, necessary for their growth-dependent expression—the repetitive elements and their flanking region.

DISCUSSION

Transcribed regions that include a repetitive sequence of the B1 family appear to play a critical role in regulating the expression of a group of genes in mouse cells (pIL genes). This effect is similar to the one exerted in rat fibroblasts by sequences that include an element of the ID repeats (4). The mouse and rat pIL mRNAs exhibit the same pattern of expression: low level in growth-arrested normal cells, maximal induction during the G_2 period of the cycle, and increased expression in transformed cells. These regulations are operated at a posttranscriptional level, and the critical sequence elements were identified by their effect on transfected β-globin reporter genes. These elements include distinct repetitive elements in the rat and in the mouse, in both instances tRNA-derived *Alu*-like sequences with polymerase III promoter activity (22, 23). A crucial role is also played by flanking sequences, which are similar in the two species and appear to be specific for RNAs submitted to the same growth-dependent regulation.

TTGGAAAACCCAGGAGTAAGGCTGCAACAAGAAACCTGCTTTACAAGGATACAATCTGAGACTCACTTAAAAACAATAAATTAAAAGATCCAGGTGTGGTGGTGCATT mouse pIL2

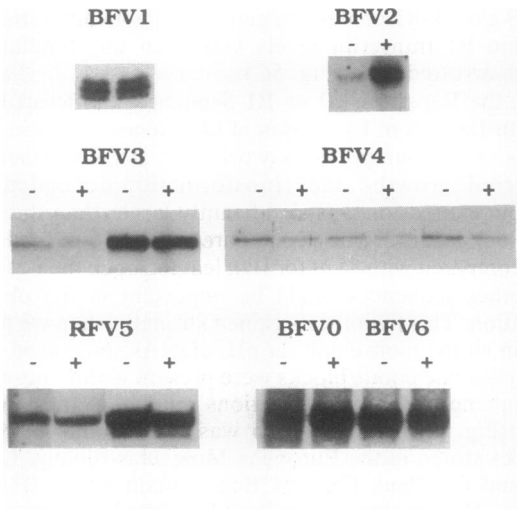
TGTGGTTATCGGGGGCCTGCTAGAAATGGCTGGTTAAGAAAGGACTGCAGCGGGGAGTGGTGGCGCACACCTT mouse pIL8

TGTGCTGGATTTGGAAAAGCCAGGAGTACGGTTGCAACAAGAAGCCTGCTTTAGAAGGATAAGGGCTGGGGATTTA rat pIL2

TAAAGAAAAAAAACCCGGAAGGCAGGTGGGCTGTCAAGTTTAGGGGAAGAATCATGCCTGTTTAGAAATGGCCGGGCTGGGGATTTA rat pIL6

TAGAAAGCAAACAAAAACCCAAAGGGTGCGTGGGTGGACGGGTATTTAAGAACCTGTTTAGAAACAGACTGGGTTGGGGATTTA rat pIL8

FIG. 6. Nucleotide sequence flanking the B1 or ID repetitive elements in mouse and rat pIL transcripts. Part of the sequences of rat pIL2 (4), pIL6, and pIL8 and of mouse pIL2 and pIL8 cDNAs. Boldface type identifies nucleotide blocks common to several sequences; the beginning of the repetitive region is boxed.



Cell line	Species	Repetitive sequence	Flanking sequence
BFV0	mouse	B1	yes
BFV1	mouse	-	no
BFV2	mouse	B1	yes
BFV3	mouse	B1	no
BFV4	mouse	-	yes
RFV5	rat	ID	no
BFV6	mouse	truncated B1	yes

FIG. 7. Growth-dependent expression requires both the repetitive element and flanking sequences. (*Upper*) Cells were harvested either after 36 hr in serum-free medium (−) or after an additional 16-hr stimulation in complete medium (+). For BFV1, BFV2, BFV3, BFV4, and RFV5 cells, one to three clonal isolates were analyzed by S1 nuclease mapping under the same conditions as in the experiment of Fig. 5. BFV0 and BFV6 are pools of neomycin-resistant cells grown after transfection of neomycin-resistant DNA and of either pFV0 or pFV6 DNA. Cytoplasmic RNAs were analyzed by RNA blot hybridization. (*Lower*) Table of cell line characteristics.

In both rat and mouse cells, repetitive elements thus participate in the regulation of expression of a family of genes but as parts of more complex regulatory sequences that also include regions with a lesser degree of repetitivity, similar between the different genes of the family and conserved between the two species. These sequences are present neither in the brain-specific BC1 RNA (11) nor in ID-containing genes that are not expressed in a growth-dependent manner, such as pyruvate kinase in cultured hepatocytes (ref. 24; A. Kahn, personal communication).

The function of the repetitive block may be only to provide a promoter for the synthesis by RNA polymerase III of a transcript that would itself be involved in the processing of polymerase II-transcribed RNAs. The truncated and inefficient B1 element in plasmid pFV6 includes only one of the two conserved boxes of the polymerase III promoter. Preliminary results (data not shown) suggest, however, that expression of β-globin–B1 chimaeras is not growth-dependent in rat cells, nor is expression of the equivalent ID constructs growth-dependent in mouse cells. Such a specificity would indicate that recognition by polymerase III is not the only function of the repeat.

Repeated sequences would, thus, appear as modular components in a variety of structures, the effects of which specified in each case by flanking sequences, modify the expression of entire sets of genes. ID, B1, and other *Alu*-like RNAs, such as the mouse B2, were reported to accumulate in a tissue-specific manner [ID in BC1 transcripts in brain (1)] and to be expressed as a function of cell differentiation (25), of embryonic development (26), and of cell proliferation and transformation (14, 27, 28). Sequences upstream of the minimal promoter were actually shown to be important in the *in vivo* transcription by polymerase III of the 7S H2K RNA (14, 26). Association of a specific and a repetitive sequence to constitute a functional unit has been similarly suggested for 7S H2L RNA. This RNA is an essential component of the signal-recognition particle, which is composed of a repetitive *Alu*-like element and of a specific sequence, both being necessary for the complete activity of the particle (15). The small BC1 RNAs in brain (1) appear as the products of mainly one ID locus and include a specific adjacent sequence (11). The tissue-specific expression of these RNAs has been suggested to be restricted to neural cells only when the flanking sequences are present (19). The contribution of the repetitive blocks might be at a common step in these various controls, whereas the flanking sequences would, in each case, specify the mode of regulation.

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