An Alu Element from the K18 Gene Confers Position-independent Expression in Transgenic Mice*

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We have identified a 323-base pair fragment of the 5'-flanking sequence of the K18 gene, which confers position-independent and copy number-dependent expression on two heterologous transgenes. This fragment is composed primarily of an Alu repetitive element. Its activity in mice is correlated with its RNA polymerase III promoter activity and its orientation-dependent ability to inhibit potential transcriptional interference in a transfection assay. However, the activity of the Alu element is not correlated with its enhancer blocking activity, a characteristic of insulator elements. In addition, this Alu element did not block the suppressive effect of co-injecting mouse α satellite DNA with the transgene. This Alu element is likely responsible for at least part of the protective effects of the sequences flanking the K18. These results suggest that transcriptionally active Alu elements may eliminate transcriptional interference of neighboring genes. This Alu element is one component of the locus control region associated with the K18 gene. Other Alu repetitive elements may also function to define regulatory domains.

A position effect can result from recombination events that place a gene within a new chromosomal context. Position effects may result from the influence of regulatory elements of the flanking DNA, from repression by nearby heterochromatin (1–3), or possibly from transcriptional interference of one gene on another (4). In vertebrates, position effects are common in transgenic mice and after chromosomal translocations (3, 5, 6).

Locus control regions (LCR)3 are dominant acting, multi-component, regulatory regions that confer position-independent and copy number-dependent expression upon a transgene (7, 8). Enhancer and chromatin opening activities have been associated with the multiple elements of an LCR. For example, the CD2 and human β-globin locus LCRs can confer tissue-specific, position-independent expression even when the transgene is integrated near the centromere, a heterochromatic region (9, 10). Insulator elements associated with the 5’ end of the chicken β globin locus (11), the human T cell receptor locus (12), the gypsy transposon (13), ssc (14), and the Fab7 elements (15) are all capable of blocking the interaction between regulatory elements and a promoter (16). Some insulators can confer position-independent expression on a transgene when juxtaposed between the gene and the flanking sequences of the integration site (11, 14, 17, 18) and are thought to participate in the definition of the boundaries that define chromatin domains.

The human genome contains ~500,000 copies of an Alu sequence, which is a 281-bp short interspersed nucleotide repeat (19). Alu elements are found in primates and are grouped as distinct repetitive elements called B1 repeats are found in rodents. Most Alu and B1 elements contain functional RNA polymerase III (PolIII) promoters, as assayed by in vitro transcription. However, Alu transcripts are not as abundant in vivo as expected from their numbers in the genome, possibly because of specific repression by chromatin (21). Alu repeats can also contain enhancer or silencer elements in sequences surrounding the PolIII promoters (22, 23). However, evidence of a regulatory function for Alus in vivo is limited (24, 25).

Previously, we showed that the 2.5- and 3.5-kb regions, which flank the human keratin-18 (K18) gene conferred position-independent expression on K18 and two heterologous reporter genes in transgenic mice (24, 26, 27). Inactivation of an Alu promoter in the 5’-flanking region of the K18 gene suggested that transcription of the Alu element (Alu2) was involved in moderating position effects on the K18 gene (24). However, the subsequent discovery of the Lazarus regulatory element within the K18 5’-flanking region with both enhancer and cryptic promoter activity (28) complicated the evaluation of the regions of the K18 gene responsible for the cis-acting protective effects. Here, we show that the Alu2 fragment, without the Lazarus element, is sufficient to moderate position effects in transgenic mice. This activity is correlated with its PolIII transcriptional potential and the ability to inhibit potential transcriptional interference from a neighboring transcription unit.

MATERIALS AND METHODS

Recombinant DNA Constructions—Recombinant constructs were prepared by standard techniques (29). The metallothionein promoter-driven human growth hormone transgene (BSMtGH) was described previously (27). For plasmids nx and 2nx, the NsiI-XhoI fragment (bp 1457–2282) of the K18 gene (GenBank™ accession number AF179904), was inserted at the filled NolI and EcoRV sites of BSMtGH. Plasmids nxBI and 2nxBI were prepared by the same procedure but using the NsiI-XhoI K18 fragment excised from BSmBX (24). Plasmid nx23 was prepared by inserting a 3.6-kb fragment of K18 (bp 6457–1086) into the EcoRV site of plasmid nx. For plasmid 2int3, a 780-bp PvuII-EcoI fragment from intron3 of K18 (bp 4249–5035) was inserted at both the filled-in NolI and the EcoRV sites of BSMtGH. For the Alu2 plasmid
FIG. 1. Growth hormone transgene constructs. The names of the constructs are shown at the left. The mouse metallothionein-human growth hormone fusion gene (MtGH) used for each construction is shown at top with the hGH exons shown in solid black. An arrow indicates the direction of Mt transcription. The MtGH cassette is indicated as a line in each of subsequent transgene diagrams flanked on one or both sides by sequences derived from K18 gene. Alu elements are labeled and indicated by cross-hatched boxes. Arrows indicate the potential transcriptional direction of the Alu. Xb, XbaI; Bg, BglII; Ns, NsiI; Av, AvaII; Pst, PstI; Nd, NdeI; S, Smal; L, Lazarus 100 bp regulatory element (distinguished by vertical stripes).

FIG. 2. Southern blot analysis of direct-repeat deletions. A, diagram of 2nxdB transgene arrays with expected fragment sizes for full-length repeats or repeats with deletions of K18 flanking sequences. The products were amplified with primers incorporated restriction sites. The Alu2/4 hybrid (K18, bp 7989–8186) and through a filled XbaI site and the noncoding, 3′ end of the last exon of the K18 gene (AF179904, bp 6263–6462). PCR-amplified Alu2 and a shorter Alu4 (K18, bp 7902–8185) were generated by PCR using primer coded restriction sites. The Alu2/4 hybrid (K18, bp 7902–7987 and 2111–1878) were generated by PCR using 5′fu thermostable polymerase (Strategene, La Jolla) and internal hybrid primers. The products were amplified with primer incorporated XbaI, NsiI, and XhoI sites to facilitate directional cloning. All amplified fragments and junctions were verified by DNA sequencing.

Transgenic Mice—Transgenic mice were produced in the Burnham Institute Mouse Molecular Genetics Shared Service as described previously (37). Transgene fragments were prepared by digestion with NsiI and XhoI, agarose gel electrophoresis, and ion exchange resin or silica based purification. Mice were screened by dot-blots of tail DNA on Zeta probe nylon membrane (Bio-Rad) and hybridization with the hGH exon 3 probe (MtGH cassette; lane 5, Mt promoter; lane 6, Mt-1 promoter; lane 7, 5′-flanking region (nxdB fragment); lane 8, Mt promoter and AP DNA, respectively. The K18 exon 7 PCR product was prepared by Southern blotting to a series of plasmid standards (MtGH/HindIII) diluted in nontransgenic mouse DNA. Signals were analyzed on a phosphorimager (Bio-Rad). To confirm that equal amounts of DNA were loaded in each lane, the blots or replicate blots were rehybridized with a probe nylon membrane (Bio-Rad) and hybridization with the hGH exon 5 probe (MtGH cassette; lane 5, Mt promoter; lane 6, Mt-1 promoter; lane 7, 5′-flanking region (nxdB fragment); lane 8, Mt promoter and AP DNA, respectively. The K18 exon 7 PCR product was prepared by Southern blotting to a series of plasmid standards (MtGH/HindIII) diluted in nontransgenic mouse DNA. Signals were analyzed on a phosphorimager (Bio-Rad). To confirm that equal amounts of DNA were loaded in each lane, the blots or replicate blots were rehybridized with a probe nylon membrane (Bio-Rad) and hybridization with the hGH exon 5 probe (MtGH cassette; lane 5, Mt promoter; lane 6, Mt-1 promoter; lane 7, 5′-flanking region (nxdB fragment); lane 8, Mt promoter and AP DNA, respectively. The K18 exon 7 PCR product was prepared by Southern blotting to a series of plasmid standards (MtGH/HindIII) diluted in nontransgenic mouse DNA. Signals were analyzed on a phosphorimager (Bio-Rad). To confirm that equal amounts of DNA were loaded in each lane, the blots or replicate blots were rehybridized with a probe nylon membrane (Bio-Rad) and hybridization with the hGH exon 5 probe (MtGH cassette; lane 5, Mt promoter; lane 6, Mt-1 promoter; lane 7, 5′-flanking region (nxdB fragment); lane 8, Mt promoter and AP DNA, respectively. The K18 exon 7 PCR product was prepared by Southern blotting to a series of plasmid standards (MtGH/HindIII) diluted in nontransgenic mouse DNA. Signals were analyzed on a phosphorimager (Bio-Rad).
RESULTS

Direct Repeats of Terminal Flanking Sequences Are Deleted upon Integration—To identify smaller regions of the flanking sequences of the K18 gene responsible for facilitating transgene expression, transgenic mice were generated with a reporter gene composed of a mouse metallothionein enhancer-promoter and the human growth hormone gene (MtGH) (42) flanked by sequences from the K18 gene (Fig. 1). Southern blotting of NsiI-digested DNA from 2nxB mice (Fig. 2B) showed a weak band of ~4500 bp (fragment 1) and a predominant band of ~3700 bp (fragment 5) that corresponded to head to tail repeats of the full-length and partially deleted transgenes, respectively (Fig. 2A). The ~900-bp deletion corresponded to the loss of one of the two nx repeats between each MtGH cassette (Fig. 2A). This was confirmed by Southern blotting of Xbal-cut DNA with a probe for the K18 nx sequence (Fig. 2C, lanes 1–3), which revealed that most MtGH cassettes were separated by ~900 bp (fragment 6) rather than ~1700 bp (fragment 2) of DNA. Southern blotting of liver DNA from 2nx, and 2int3 mice (data not shown) indicated that whenever direct repeats of terminal flanking sequences were deleted, the lanes are numbered at the bottom. M, size marker. Alu2 transgenic mouse liver DNAs were digested with NsiI (lanes 1–6) or XbaI (lanes 8–13), C, hybridization of XbaI-digested liver DNA from Alu2 transgenic mice with the Alu2 probe. M, size marker.

**Fig. 3.** Southern blotting analysis of Alu2 transgene arrays. A, diagram of Alu2 transgene arrays showing expected fragment sizes. See legend to Fig. 2A for general description of transgene array diagrams. Gray bar with embedded cross-hatched box indicates K18 Alu2 sequence; arrows below the map indicate direction of Alu2 transcription. Fragments 2 and 3 are predicted for both head-tail and head-head repeats. B, x-ray film exposure of a Southern blot filter hybridized with the hGH probe. Individual mouse numbers are shown at the top of the filter, and the lanes are numbered at the bottom. M, size marker. Alu2 transgenic mouse liver DNAs were digested with NsiI (lanes 1–6) or XbaI (lanes 8–13). C, hybridization of XbaI-digested liver DNA from Alu2 transgenic mice with the Alu2 probe. M, size marker.
Position Effect Blocking by an Alu Element

TABLE I

| Mouse   | TG' | Copy | Heart | Brain | Intestine | Liver |
|---------|-----|------|-------|-------|-----------|-------|
|         |     |      | GH   | Mt-1  | GH        | Mt-1  |
| 1       | nx3 | ?    | 0.6  | 0.4   | 0.8       | 0.4   |
| 2       | nx3 | 4.3  | 8.1  | 19.4  | 10.6      | 16.9  |
| 3       | nx3 | 14.1 | 213.1| 32.2  | 53.6      | 21.5  |
| 4       | nx3 | 115.1| 425.6| 36.5  | 110.7     | 25.5  |
| 5       | Alu2| 0.7  | 12.3 | 0.4   | 5.1       | 0.4   |
| 6       | Alu2| 0.9  | 9.4  | 0.3   | 17.5      | 0.2   |
| 7       | Alu2| 1.8  | 19.8 | 17.4  | 24.8      | 57.5  |
| 8       | Alu2| 2.0  | 20.9 | 11.6  | 21.5      | 0.4   |
| 9       | Alu2| 5.1  | 31.0 | 25.6  | 23.1      | 12.5  |
| 10      | Alu2| 5.5  | 20.4 | 23.2  | 20.2      | 115.9 |
| 11      | Alu4| 1.0  | 15.4 | 4.0   | 37.6      | 0.4   |
| 12      | Alu4| 8.4  | 16.1 | 7.3   | 37.4      | 0.4   |
| 13      | Alu6| 0.3  | 25.7 | 1.3   | 38.7      | 0.4   |
| 14      | Alu6| 1   | 46.6 | 0.4   | 44.9      | 0.4   |
| 15      | Alu6| 2.8  | 18.0 | 6.9   | 35.9      | 0.4   |
| 16      | Alu6| 10.4 | 181.2| 44.4  | 36.8      | 144.0 |
| 17      | Alu6| 75   | 26.5 | 0.5   | 36.9      | 0.5   |
| 18      | 2nx | 3.0  | 22.8 | 24.1  | 21.8      | 166.7 |
| 19      | 2nx | 4.5  | 14.9 | 10.6  | 19.1      | 4.0   |
| 20      | nx  | 6.0  | 29.1 | 16.4  | 31.0      | 126.4 |
| 21      | nx  | 6.3  | 22.4 | 6.6   | 34.8      | 394.3 |
| 22      | 2nx | 8.9  | 31.5 | 3.3   | 32.7      | 1.0   |
| 23      | 2nx | 9.0  | 26.9 | 5.7   | 35.0      | 2.8   |
| 24      | 2nx | 15.1 | 4.5  | 5.2   | 22.6      | 2.4   |
| 25      | 2nx | 17.8 | 57.9 | 19.1  | 66.1      | 54.8  |
| 26      | 2nx | 17.8 | 19.9 | 7.9   | 17.7      | 289.5 |
| 27      | 2nx | 31.4 | 6.9  | 0.4   | 17.4      | 0.4   |
| 28      | 38  | 48.2 | 11.7 | 0.4   | 22.2      | 0.4   |
| 29      | 38  | 65.9 | 239.0| 24.4  | 19.4      | 298.5 |
| 30      | 38  | 66.7 | 12.8 | 0.4   | 18.7      | 0.4   |
| 31      | 38  | 133.5| 10.8 | 0.4   | 17.6      | 0.4   |
| 32      | 38  | 257.9| 16.0 | 10.9  | 27.5      | 0.5   |
| 33      | nxB | 0.5  | 7.1  | 1.0   | 18.0      | 0.2   |
| 34      | nxB | 1.8  | 9.6  | 2.2   | 17.9      | 2.0   |
| 35      | nxB | 2.2  | 12.0 | 2.1   | 17.8      | 4.2   |
| 36      | nxB | 2.5  | 11.0 | 0.4   | 19.8      | 0.4   |
| 37      | nxD | 19.0 | 9.0  | 0.4   | 17.9      | 0.4   |
| 38      | nxD | 19.6 | 34.2 | 1.3   | 27.4      | 0.4   |
| 39      | nxD | 80.1| 382.5| 11.6  | 13.7      | 35.5  |
| 40      | 2nt3| 1.5  | 20.5 | 11.5  | 1.4       | 17.7  |
| 41      | 2nt3| 1.5  | 10.5 | 0.4   | 18.9      | 0.4   |
| 42      | 2nt3| 5.5  | 6.9  | 0.4   | 19.5      | 0.4   |
| 43      | 2nt3| 14.1 | 15.0 | 2.6   | 22.2      | 0.4   |
| 44      | 2nt3| 35.0 | 10.5 | 0.4   | 19.1      | 0.4   |
| 45      | 2nt3| 54.7 | 259.2| 21.9  | 26.4      | 598.7 |
| 46      | 2nt3| 104.1| 416.7| 44.8  | 5.4       | 29.6  |
| 47      | 2nt3| 144.4| 9.3  | 8.4   | 19.2      | 0.4   |

Values represent attomoles RNA/μg total RNA; italicized bold face numbers represent the lower limit of the standard curve in a particular RNase protection run and are substituted values for samples in which hGH RNA was too low to measure, transgene copy number/cell, assuming 6 pg genomic DNA/cell.

correctly repeated sequences flanked the MtGH cassette one of the two flanking elements was deleted within the integrated transgene arrays.

Constructs Alu2, Alu4, and Alu6 also contained two copies of the test sequence but in inverted orientations (Fig. 1). Southern blotting of MtGH cassettes were largely intact (Fig. 3, A and B, lanes 8–13). An Alu2 probe detected a ~750-bp fragment (Fig. 3C, lanes 2–7, fragment 3), consistent with two copies of the Alu2 sequence between each MtGH cassette in multicopy transgene arrays. Mouse 5 and 6 had integrated one or fewer transgene copies (Table I).

RNA Expression in Transgenic Mice—RNase protection was used to measure the levels of transgene-derived hGH RNA and endogenous Mt-1 RNA in mouse tissues. The nx3' transgene that lacked 1.5 kb of Bgl II-flanking DNA was expressed similarly to the previously tested larger construct (27) (Table I and Fig. 4). The addition of single Alu2 elements to each end of the reporter gene resulted in strong expression, despite a generally low number of integrated copies (Table I). In brain, both nx3' and Alu2 resulted in copy number-dependent expression (Fig. 3A). In contrast the control 2nt3 and the Alu4 and Alu6 mice had low expression in brain (Fig. 4A). In heart, the relationship between hGH RNA, and copy number was less
strongly correlated in nx3’ and Alu2 mice and was not evident in other tissues (Table I). The large difference in transgene expression levels between the Alu2, Alu4, and Alu6 mice is of interest because the 281-bp Alu sequence found in all three is at least 85% identical. These results indicated that the 323-bp Alu2 fragment contains a substantial portion of the cis-acting protective activity of the larger K18 flanking sequences.

Because of their similar integrated structures, data from the 2nx and 2nxdB transgenic mice were grouped with data from the nx and nxdB mice, respectively. Levels of hGH RNA were too low to measure in more than 25% of the nx or nxdB mice (Table I). No relationship between hGH RNA and copy number was observed in either the nx or the nxdB transgenic mice (Table I). However, high expressing lines were more commonly found among nx mice than 2nt3, Alu4, Alu6, or nxdB animals (Fig. 4, B and C). The stimulatory activity of the nx fragment may be due to either the presence of the Alu2 embedded within it and/or the presence of the 100-bp Lazarus regulatory element present immediately downstream of Alu2 in the nx fragment (28). Comparison of the expression levels of the nx and nxdB transgenes supports the view that the PolIII promoter of the Alu2 may be more important than the Lazarus element for the stimulatory activity of the nx fragment (Fig. 4, B and C).

One potentially important difference between nx and Alu2 transgenes is the direct repeat orientation of the nx fragments and the inverted repeat orientation of the Alu sequences in Alu2, Alu4, and Alu6 genes.

Because high copy number transgenes in Drosophila are targets for heterochromatin formation and subsequent silencing (43), we considered whether the blocking of the position effect of the Alu2 element was an artifact of the generally low copy numbers of the Alu2 mice. Although the number of samples/group were small, the median expression levels for mice with less than 10 copies of the transgene were still much higher for the Alu2 mice than for the other groups including Alu4, Alu6, nxdB, and int3 mice.

Orientation-dependent Inhibition of Potential Transcriptional Interference by Alu2—Position-independent, copy number dependent expression of a transgene may involve several different activities. Transcriptional interference could inhibit expression of tandem arrays of integrated transgenes. Fragments of the flanking sequences of the K18 gene were tested by transient transfection for activities that might inhibit transcriptional interference. The K18 gene was positioned upstream of a neo transcription unit in one plasmid (Fig. 5A). Fragments of both the K18 3′-flanking sequence and the nx fragment were then inserted between the two genes in both possible orientations, and the transcriptional products of both genes were assessed by RNAse protection analysis of transiently transfected cells. Two of three fragments of the 3′-flanking sequences of the K18 gene resulted in increased expression of the downstream neo transcription unit (Fig. 5B). This effect was dependent upon the orientation of the 3A and 3C fragments. Only the orientations opposite to that found in association with the K18 gene were active. This orientation places the potential transcriptional direction of embedded Alu elements in the same orientation as that of the K18 and neo genes. The nx 5′-flanking sequence had a modest effect of increasing the neo gene RNA (Fig. 5). However, the half of the nx fragment that contained the Alu element was as active as the 3C fragment (note scale differences in Fig. 5, B and C), and this activity was also orientation-dependent (Fig. 5C, Alu-L). In contrast the remaining half of the nx fragment had no activity (Fig. 5C, N-Alu). When the Lazarus regulatory element was deleted from the Alu-L fragment, activity increased further (Fig. 5C, Alu2). Mutation of the B box of the Alu2 promoter abolished this activity (Fig. 5D, lanes 2 and 3).

Enhancer Blocking Activity of the Alu2 Fragment—Because
other DNA elements which confer integration site-independent expression on heterologous transgene have been correlated with the ability to block the effects of transcriptional enhancers when interposed between the enhancer and the promoter, we tested the enhancer blocking activity of the Alu2 element. To eliminate concerns about inactivating rather than simply blocking an enhancer element, a dual reporter plasmid was designed. The SV40 enhancer was interposed between the neo and puro resistance genes driven by near minimal promoters. Test fragments were inserted between the enhancer and promoter in both orientations (Fig. 6A). The plasmids were transfected into cultured cells and selected in either puromycin or G418. The insertion of the Alu2 fragment between the puromycin promoter and the enhancer resulted in greatly diminished but did not abolish transcriptional activity (Fig. 5). The Alu2 element contains both A and B box components of the PolIII promoter (Fig. 7) (24, 44). Because the Alu2B mutation contains an insertion of four bp in addition to the changes in the B-box, a second mutant form of the Alu2 B-box was constructed that altered the B-box without additional insertions (Fig. 7A, Alu2mB). The RNA polymerase III activity of K18 Alu elements and several different mutations were assessed in vitro and in the presence of a control plasmid containing the adenovirus VA1 gene. (Fig. 7B). The Alu2 element was transcribed efficiently in vitro (Fig. 7B, lane 3), whereas the Alu4 element was poorly transcribed. Both mutations of the B box of the Alu2 gene abolished transcription (Fig. 7B, lanes 5 and 7). Deletion of the A box element, greatly diminished but did not abolish activity, as expected (44).

A previous analysis of the Alu2 promoter identified four half-sites that defined two functional hormone response-binding sites capable of conferring retinoic acid responsiveness on a promoter. The mHRE3 mutation of the Alu2 promoter was more effective than the control fragment (Fig. 6C and additional data not shown). However, mutation of the B-box had no effect on the activity of the Alu2 fragment, and a second Alu fragment from the 3′-flanking sequence of the K18 gene was similarly active (Fig. 6C, Alu2mB and Alu4). Thus, although the Alu fragments appear to have enhancer blocking activity, the orientation independence, lack of effect of the B box mutation, and similar activity of the Alu4 fragment distinguished this activity from the transcriptional interference inhibitory activity (Fig. 5) and protective effects in transgenic mice (see below).

**Transcriptional Activity of Alu Elements in Vitro**—The Alu2 element contains both A and B box components of the PolIII promoter (Fig. 7) (24, 44). Because the Alu2B mutation contains an insertion of four bp in addition to the changes in the B-box, a second mutant form of the Alu2 B-box was constructed that altered the B-box without additional insertions (Fig. 7A, Alu2mB). The RNA polymerase III activity of K18 Alu elements and several different mutations were assessed in vitro and in the presence of a control plasmid containing the adenovirus VA1 gene. (Fig. 7B). The Alu2 element was transcribed efficiently in vitro (Fig. 7B, lane 3), whereas the Alu4 element was poorly transcribed. Both mutations of the B box of the Alu2 gene abolished transcription (Fig. 7B, lanes 5 and 7). Deletion of the A box element, greatly diminished but did not abolish activity, as expected (44).

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reported to nearly abolish the hormone responsiveness of the element (45). In our hands this mutation greatly diminished the PolIII activity of the Alu2 element \textit{in vitro} (Fig. 7B, lane 8). Thus this mutation did not permit us to discriminate between potential effects of the HRE elements and PolIII transcription of the Alu2.

To localize the biological activity of the Alu2 farther, the Alu4 promoter was substituted with the Alu2 promoter to create the A2/4 element and its reciprocal swap of the Alu4 promoter to the body of the Alu2 gene (A4/2). As expected, the transcriptional activity of the hybrid gene containing the Alu2 promoter had similar activity to the Alu2 element (Fig. 7B, lane 9). The transcription of the adenovirus VA1 gene appeared to be influenced by competition with other PolIII genes. However, doubling the amount of Alu2 plasmid still resulted in increased Alu2 product (lane 12). Thus, the standard amount of Alu2 plasmid did not saturate the system. These results confirmed the effectiveness of both B-box mutations and the low activity of the Alu4 promoter, perhaps because of an inefficient A box element.

Alu2 Facilitates Transgenic Expression of Alkaline Phosphatase—To further assess the potential importance of the transcriptional competence of Alu2 on facilitating transgene expression, a second set of transgenic animals were prepared.
utilizing the human placental alkaline phosphatase cDNA as a reporter gene. The same Mt-1 promoter was used to drive the reporter gene and inverted repeat copies of the Alu2, Alu2mB, Alu4, or reciprocal hybrids of Alu2 and Alu4 (A2/4 and A4/2) flanked the reporter gene (Fig. 8A). In addition, the MtAP and A2 transgenes were also co-injected with mouse satellite DNA to determine whether repetitive DNA might direct the transgene integration events into repressive chromatin environments. The resulting transgenic mice were nearly all single copy integration events except for those co-injected with satellite DNA (Table II). Southern blot analysis revealed rearrangements or loss of restriction enzyme sites in six of the transgenic lines (Fig. 8, B and C, and Table II). Southern analysis of BamHI-cut DNA and probes specific for the distal portions of the Mt-1 promoter and the 3′ noncoding region confirmed the retention of these regions (data not shown). Furthermore, re-probing with the Alu2 fragment (data not shown) and genomic PCR confirmed that each transgene was flanked by the expected Alu fragment except for one (Fig. 8D, line A2/4-29), which lacked the Alu2/4 hybrid proximal to the Mt-1 promoter but retained the copy flanking the 3′ RNA processing region.

Expression of the heat stable, human alkaline phosphatase was found in F1 animals of both MtAP transgenic lines that lacked additional flanking sequences (Table II). Expression was highest in brain and very low to moderate in liver. This pattern of expression differs from both the endogenous Mt-1 gene (Table I) and MtGH transgenes. Expression in brain was lower than heart or liver for MtGH transgenes (Table I). However, brain was the highest expressing tissue with MtAP transgenes. Two independent A2 transgenic lines had very high expression in brain and strong expression in all other examined tissues. However, this effect of the Alu2 was abolished by mutation of the B-box as three independent, unarranged, mouse lines expressed undetectable or only trace amounts of AP in brain and other tissues (Table II, A2mB). One informative A4/2 transgenic line failed to express the reporter gene in adult tissues. This result is consistent with the low PolII transcriptional activity of this element (Fig. 7). Although the number of informative transgenic lines was low, these results support the view that the Alu2 element facilitates expression of the MtAP transgene in multiple tissues and particularly in liver. Furthermore, the B box element of the Alu2 is necessary for the protective effect.

Alu2 Does Not Protect Transgene Expression from Inhibition by Satellite DNA—The co-injection of satellite DNA with the MtAP transgene might be expected to increase the frequency of integration into heterochromatic satellite DNA by homologous recombination because of the abundance of the endogenous target. Co-injection with the cloned satellite DNA resulted in increased number of integrated genes in four out of five informative transgenic lines (Table II). Expression of the MtAP gene in two transgenic lines was generally weak except for brain expression in one line. All three A2 transgenic lines co-injected with satellite DNA had little or no AP activity in multiple organs. These results suggest that co-injected satellite DNA results in inhibition of transgene expression, but Alu2 sequences are not effective in protecting against such inhibition.

**DISCUSSION**

**Potential Mechanisms of Blocking by the Alu2 Fragment—** Copy number-dependent expression in transgenic mice requires that expression be integration site-independent and implies that each copy of a transgene in a tandem array functions independently. It is remarkable that two copies of the 323-bp Alu2 fragment retained much of the position effect blocking activity contained in the larger 5′- and 3′-flanking regions of K18 albeit in fewer tissues than the larger fragments. The activity of the Alu2 element in transgenic mice is correlated with the inhibition of presumptive transcriptional interference in an orientation-dependent manner and with its Pol13 transcriptional activity in vitro. Although the orientation-dependence of the Alu2 observed in the transcriptional interference assay was not directly tested in transgenic mice, the orientation used was expected to block inhibitory effects of transcription converging on the transgene from either direction. In addition, flanking the transgene with one Alu2, embedded within the larger nx fragment, did not ensure efficient expression of the MtGH transgene (Fig. 4) perhaps because only one side of the tandemly inserted gene arrays would be protected. The inhibition of potential transcriptional interference may be a key component of the copy number-dependent behavior of the K18 gene. Other Alu transcription units in the 3′-flanking...
The activity of Alu2 that best correlates with its effect on transgene promoter activity and blocking position effects in transgenic mice involves a 70-kb Alu2 fragment oriented divergent from other elements that define regulatory domains of euchromatic chromosomes. The locus control regions of both human β-globin and CD2 are composed of multiple discrete elements and act to establish or maintain open chromatin domains. They contain multiple DNase-hypersensitive regions and may not necessarily include enhancer blocking activities. The transgenic behavior of various K18 transgenes implies an LCR-like activity because its expression is independent of over 47 integration sites. The Alu2 region is DNase-hypersensitive in expressing tissues (47). However, none of the K18 transgene integration sites have been confirmed to be in centromeric heterochromatin, and it remains to be tested if the K18 gene can withstand the inhibition observed in this study by co-injected satellite DNA. One potential use of the K18 gene and its protective flanking sequences would be to facilitate stable gene expression.

Locus control regions can ensure expression of a transgene even when integrated into centromeric locations (9). The failure of Alu2 to facilitate expression when co-injected with cloned satellite DNA suggests that one flanking copy of the Alu2 sequence is not sufficient to ensure expression in all chromosomal contexts and thus does not constitute an LCR by itself. However, multimerization of insulator elements increases activity (48). Additional copies of active Alu elements may be more effective.

Table II

| Construct | Line | Copy* | Liver | Spleen | Brain | Heart | Kidney | Lung |
|-----------|------|-------|-------|--------|-------|-------|--------|------|
| MtAP      | 10   | 1     | 413   | 17     | 3,330 | 108   | 15     | 159  |
| MtAP      | 12   | 1     | 10    | 70     | 12,026| 689   | 58     | 189  |
| A2        | 4    | 1     | 1,258 | 325    | 21,700| 2,903 | 448    | 187  |
| A2        | 14   | 2     | 3,166 | 995    | 22,400| 855   | 286    | 436  |
| A2mB      | 26   | 1     | 0     | 0      | 70    | 0     | 32     | 0    |
| A2mB      | 27   | 1     | 0     | 0      | 80    | 14    | 0      | 0    |
| A2mB      | 31   | 1     | 19    | 12     | 2,560 | 156   | 14     | 36   |
| A4/2      | 17   | 1     | 0     | 0      | 0     | 0     | 0      | 0    |
| MtAP + Sat| 8    | 8     | 0     | 46     | 5378  | 40    | 0      | 13   |
| MtAP + Sat| 15   | 3     | 23    | 31     | 834   | 883   | 54     | 68   |
| A2 + Sat  | 3    | 2     | 0     | 0      | 0     | 0     | 0      | 0    |
| A2 + Sat  | 5    | 8     | 0     | 0      | 0     | 0     | 0      | 0    |
| A2 + Sat  | 10   | 1     | 0     | 0      | 703   | 19    | 0      | 0    |
| Transgene rearrangements | | | | | | | | |
| A4        | 23   | 1     | 0     | 0      | 0     | 23    | 13     | 0    |
| A2/4      | 29   | 1     | 0     | 0      | 0     | 0     | 0      | 0    |
| A4/2      | 5    | 1     | 0     | 0      | 0     | 0     | 114    | 0    |
| A2mB      | 2    | 1     | 0     | 0      | 0     | 0     | 0      | 0    |
|MtAP + Sat | 18   | 1     | 0     | 0      | 0     | 0     | 0      | 0    |
| A2 + Sat  | 18   | 1     | 0     | 0      | 0     | 0     | 0      | 0    |

* Gene copy number determined by dot blot and Southern blot hybridization.

Comparison with LCRs, Boundaries, and Insulators—The characteristics of the Alu2 fragment are both similar and divergent from other elements that define regulatory domains of euchromatic chromosomes. The locus control regions of both human β-globin and CD2 are composed of multiple discrete elements and act to establish or maintain open chromatin domains. They contain multiple DNase-hypersensitive regions and may not necessarily include enhancer blocking activities. The transgenic behavior of various K18 transgenes implies an LCR-like activity because its expression is independent of over 47 integration sites. The Alu2 region is DNase-hypersensitive in expressing tissues (47). However, none of the K18 transgene integration sites have been confirmed to be in centromeric heterochromatin, and it remains to be tested if the K18 gene can withstand the inhibition observed in this study by co-injected satellite DNA. One potential use of the K18 gene and its protective flanking sequences would be to facilitate stable gene expression.

Locus control regions can ensure expression of a transgene even when integrated into centromeric locations (9). The failure of Alu2 to facilitate expression when co-injected with cloned satellite DNA suggests that one flanking copy of the Alu2 sequence is not sufficient to ensure expression in all chromosomal contexts and thus does not constitute an LCR by itself. However, multimerization of insulator elements increases activity (48). Additional copies of active Alu elements may be more effective.

The Alu2 element has enhancer blocking activity. However, the lack of correspondence between Alu insulator activity, PolIII promoter activity and blocking position effects in transgenic mice suggests that the activity of Alu2 in transgenic constructions is not mediated only by blocking enhancers neighboring the integration site. Furthermore, the enhancer blocking activity detected in our assay assesses only relatively short range interactions, whereas Drosophila insulators can act over considerable genomic distances. It remains possible that the K18 flanking sequences may contain boundary element activity, which requires cooperation between Alu2 and additional elements within the context of the K18 gene.

The activity of Alu2 that best correlates with its effect on heterologous transgenes is the apparent inhibition of transcriptional interference. Both the orientation dependence and PolIII promoter dependence distinguish this activity from the en-
hancer blocking activity. As transcriptional interference is assessed in the absence of stable integration, it seems unlikely that the Alu2 is acting through a chromatin structure-mediated process. Interference of expression from a closely linked transcription unit in mammalian cells is well known (4, 49). Perhaps active Alu transcription units provide an additional mechanism of terminating transcription and thus alleviating interference from a neighboring, upstream transcription unit.

A Function for Alu and Other Short Interspersed Nucleotide Repeat Elements—Our results with the Alu2 element suggest that a subset of Alu elements may participate in defining transcriptionally permissive regulatory domains. Current characteristics of LCRs, insulators, and boundary elements from multiple organisms do not appear to be compatible with a simple single model (16). Perhaps active Alu elements may cooperate with chromatin opening activity and insulator activity in defining functional regulatory domains. The mouse K18 gene contains a single B1 repeat upstream within 50 bp of the location of the Alu2 upstream of the human K18 promoter. It also contains two B1 repeats downstream in positions similar to those of the more numerous Alu elements flanking the human gene (50). Because the human Alu and mouse B1 elements expanded independently, the very similar locations of these repetitive elements may reflect functional selection.

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