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Redox Regulation of a Novel *L1Md-A2* Retrotransposon in Vascular Smooth Muscle Cells*

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Activation and reintegration of retrotransposons into the genome is linked to several diseases in human and rodents, but mechanisms of gene activation remain largely unknown. Here we identify a novel gene of *L1Md-A2* lineage in vascular smooth muscle cells and show that environmental hydrocarbons enhance gene expression and activate monomer-driven transcription via a redox-sensitive mechanism. Site-directed mutagenesis and progressive deletion analyses identified two antioxidant/electrophile response-like elements (5′-GTGACTCGAGC-3′) within the A2/3 and A3 region. These elements mediated activation, with the A3 monomer playing an essential role in transactivation. This signaling pathway may contribute to gene instability during the course of atherogenesis.

Retrotransposons are mobile retroelements that utilize reverse transcriptase and RNA intermediates to relocate to new locations within the cellular genome. *L1*, a non-long terminal repeat (non-LTR)[1] retrotransposon, is the most characterized and abundant member of the *LINE-1* (long interspersed repetitive element) family in the mammalian genome (1). In mouse, *L1* is expressed at greater than 100,000 copies per genome and comprises 10% of genomic DNA (2). *L1Md* (*L1* in *Mus domesticus*) is the major mouse retrotransposon, with randomly truncated sequences toward the 5′ end resulting in high frequency repeats of extreme 3′ sequences (3). Unlike *L1s* in the human and rat, *L1Md* contains multiple copies of repeated sequences called monomers in their 5′ region (4). At least three subfamilies of *L1Md*, V, F, and A, have been identified in the mouse (5). The most ancient V subfamily lacks a 5′ monomer, whereas the monomers of the less actively transposing F subfamily and the youngest A subfamily have 206 and 208 bp, respectively (4, 6, 7). The A subfamily, together with the newly evolved G_F (8) and T_F (9) subfamilies, makes up a total of 3,000 actively transposing full-length elements within the mouse genome.

Monomers function as promoters, with increases in promoter activity as the number of monomers increase (10). Regardless of the number of monomers in an individual A- or F-type *L1Md*, monomers always contain two-thirds of full-length monomer at their most 5′ end tandem repeat, although their specific role in gene activation is unknown. Three A-type subfamily members have been described, *L1Md-A2* with 4 and 2/3, *L1Md-A13* with 2 and 2/3, and *L1Md-9* with 1 and 2/3 copies of monomers (4, 11). The structure of *L1* in the mammalian genome may be represented by the well characterized *L1Md-A2*, which consists of six to seven kb including a 5′ untranslated region (UTR), two open reading frames (ORFs) (1137-bp ORF1 encoding ribonucleoprotein (12, 13) and 3900-bp ORF2 encoding endonuclease and reverse transcriptase (14, 15)), a 3′ UTR, and a 3′ end poly(A) tail. A few bp target direct repeats are always found before and after the 5′ UTR and 3′ UTR, respectively, a feature consistent with active transposition into new locations. The regulation and subsequent expression of *L1* elements remains among the most critical unanswered questions concerning the structure and genomic organization of *L1*.

Factors known to activate retrotransposons include radiation (16, 17), chemical carcinogens (18, 19), and stress-related factors (20, 21). Retrotransposon activation may play a role in evolution of the genome (22), double strand break DNA repair (23), exon shuffling (24, 25), and diseases caused by insertional mutation in both human (26–29) and mice (30–34). A recent report (35) describing transcriptional interference by somatically active retrotransposons as epigenetic mediators of phenotypic variance among mammals raises important questions about their impact in development and progression of human disease. Previous studies in this laboratory (18) have shown that benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon carcinogen and atherogen, activates *L1Md* expression in mouse vascular smooth muscle cells. *L1Md* activation is also elicited by oxidative metabolites of the parent hydrocarbon, suggesting that modulation of redox homeostasis mediates epigenetic disruption of gene expression and possibly DNA damage (19).

Here we identify for the first time a novel A-type *L1Md* in mouse vascular smooth muscle cells that contains 3 and 2/3 copies of A-monomers within the 5′ regulatory region. PCR analysis of genomic DNA showed tissue-specific distribution of the gene, with strong amplification signals detected in vascular smooth muscle cells, and to a lesser extent mammary gland and kidney. Sequence alignment to *L1Md-A2* indicated that a full-length monomer equivalent (the entire 140-bp incomplete monomer A2/3 and the 68 bp from adjacent first full monomer A4) is absent in the most 5′ end tandem repeat of the regulatory region. This element, named *L1Md-A3.6*, preserved its promoter activity and was inducible by carcinogenic environmental hydrocarbons via a redox-sensitive mechanism involving ARE/EpRE-like sequences. Site-directed mutagenesis and progressive deletion analyses showed that: 1) promoter activity increases as the number of monomers increase, 2) the A2/3 monomer in genomic context may inhibit gene expression, 3) A1 alone lacks promoter activity but may assist in monomer-

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[1] The abbreviations used are: LTR, long terminal repeat; UTR, untranslated region; ORF, open reading frame; BaP, benzo(a)pyrene; nt, nucleotide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PDTC, pyrrolidine dithiocarbamate; RT, reverse transcriptase; Ahr, aryl hydrocarbon receptor.

driven transactivation, 4) mutation of both ARE/EpRE-like sites within A2/3 and A3 markedly reduce inducible activity, and 5) mutation at A3 alone significantly reduced BaP inducibility. Collectively, these data suggest that oxidative stress plays a significant role in the activation of a novel member of the A-type *L1Md* subfamily in vascular smooth muscle cells.

MATERIALS AND METHODS

Cell Culture and Genomic DNA Isolation—Aortic smooth muscle cells isolated from C57BL/6J mice were seeded at 100 cells/mm^2 in 10-cm dishes and maintained at 37 °C (5% CO_2) in Medium 199 supplemented with 10% fetal calf serum. At 85% confluence, genomic DNA was extracted using DNAzol genomic DNA isolation reagent (Molecular Research Center, Inc.). Briefly, 1 ml of DNAzol was added to lyse the cells. The cell lysate was passed through a pipette several times and collected into a 1.5-μl microtube. Genomic DNA was precipitated from the lysate by addition of 500 μl of 100% ethanol, mixed by inversion five to eight times, and incubated at room temperature for 3 min followed by centrifugation at 5,000 $\times$ *g* for 5 min at 25 °C. The DNA pellet was washed twice with 1 ml of 95% ethanol and solubilized in distilled water. For analyses of tissue distribution, genomic DNA from mouse aortic smooth muscle cells, mammary gland, liver, uterus, ovary, and kidney was extracted with a PUREGENE DNA isolation kit (Gentra) according to the manufacturer's specifications.

Polymerase Chain Reaction—Three sets of primers were designed using oligo 4.0 (National Biosciences) based on the published *L1Md-A2* sequence. Region 1 primer set (forward, 5′-CCCAACATAGAGTCCTGA-3′; reverse, 5′-AGTGGGCAGAGTATTCTC-3′) was designed to generate a 1114-bp PCR product from nucleotide (nt) 515 to 1628 of *L1Md-A2* and spanning the complete four and 2/3 A-monomers. Region 2 primer set (forward, 5′-GCCCTTCTGGACTTATCTCTTC-3′; reverse, 5′-TTG-CGGGTGTCAGGCGACTCAG-3′) was used to generate a 1008-bp fragment from nt 477 to 1484 of *L1Md-A2* covering 3 and 2/3 A-monomers. Region 3 primer set (forward, 5′-TGCCCACTTTCTCCCTACCT-3′; reverse, 5′-GCTTTTCCCCACTTTCTCCTC-3′) was designed to generate a 972-bp fragment from nt 4856 to 5827 of *L1Md-A2* ORF2 region. Each PCR reaction contained the following: 20 μM dNTPs, 200 pmol of each primer, 0.02 units/μl of *Taq* DNA polymerase (PerkinElmer Life Sciences), 1$\times$ $MgCl_2$ containing PCR buffer, and various amounts (800–1 ng) of genomic DNA. Amplification reactions were performed for 25 cycles, and each cycle consisted of a 30-s denaturation at 94 °C, 1-min annealing at 60 °C, and 2-min extension at 68 °C. For analyses of tissue distribution, 1 mg of genomic DNA was amplified in a 50-ml reaction containing 200 μM dNTPs, 500 pmol of primer (upper primer, 5′-AAA-AAGCTTCCCAACATAGAGTCCTGA-3′; lower primer, 5′-AAAAAGCT-TAGTGGGCAGAGTATTCTC-3′), 0.025 units/μl AmpliTaq Gold DNA polymerase (PerkinElmer Life Sciences), and 1$\times$ $MgCl_2$ containing PCR buffer. Amplification reactions were performed for 30 cycles, and each cycle consisted of a 45-s denaturation at 95 °C, 1-min annealing at 60 °C, and 1-min, 15-s extensions at 68 °C following initial denaturation at 95 °C for 5 min.

Plasmid Construction and Sequencing—pGB3.6–11 containing a full-length 5′ tandem repeat (designed to generate nt 515–1628 of *L1Md-A2*) was constructed by first cloning the PCR product generated from the *Hin*dIII restriction site attached to the 5′ end of Region 1 primer set M13002–18H (forward, 5′-AAAAGCTTGCCCTTCTGGACT-TATCTCTTC-3′; reverse, 5′-AAAAGCTTTTTGCGGGTGTCAGGCGACT-CAG-3′) into *Hin*dIII digested blunt end linearized PCR-TRAP vector (GenHunter Corp., Nashville, TN). After a successful clone was confirmed by a colony-PCR method using a primer set that flanks the cloning site of the PCR-TRAP vector, the insert fragment was retrieved by digestion of the plasmid with *Hin*dIII restriction enzyme and subcloned into pGL3 basis luciferase reporter vector (Promega). All plasmids with correct inserts were confirmed by sequencing from both ends to ensure that the correct sequence was cloned.

Transient Transfection Assay—Mouse vascular smooth muscle cells were seeded at 1 $\times$ 10^5 cells in 12-well plates and maintained at 37 °C (5% CO_2) in Medium 199 supplemented with 10% fetal calf serum. After seeding for 24 h, cells were transfected with *pGB3.6–11* or *pGB3.6–8*, plasmids containing the sense or antisense strand of Region 1 product, respectively, or mutant plasmids, for 2 h. Briefly, 2 μg of plasmid DNA and 0.5 μg of β-galactosidase (pcDNA/His/lacz) were mixed with 400 μl of serum-deprived OPTIMEM-1 medium. Then, 7.5 μl of TransFast transfection reagent (Promega) was added, vortexed immediately, and incubated at room temperature for 15 min. Growth medium was carefully removed, and 400 μl of TransFast reagent (Promega)/DNA mixture added to each well. Plates were returned to the incubator for 2 h. At the end of the incubation, transfected cells were recovered in 1 ml of Medium 199 supplemented with 10% serum for 24 h followed by challenge with BaP (0.3, 3, 30 μM), TCDD (0.1, 1, 10 nM), or H_2O_2 (25, 50, 100 μM) for 24 h. For antioxidant inhibition studies, cells were pretreated with 0.5 mM *N*-acetylcysteine or 0.01 μM PDTC for 3 or 6 h, respectively, before challenge with carcinogens. To harvest the cells, transfected cells were washed twice with phosphate-buffered saline, lysed in 175 μl of 1$\times$ reporter lysis buffer, and collected into a microtube. The suspension was then incubated at room temperature for 15 min followed by one cycle of quick freeze-thaw in liquid nitrogen and water at room temperature. The resulting cell lysate were collected after centrifugation at 12,000 $\times$ *g* for 1 min. For measurements of luciferase activity, 30 μl of cell lysate was mixed with 100 μl of luciferase assay reagent (Promega) in a 96-well plate and read at 420 mm in a plate reader. For β-galactosidase activity, 30 μl of cells lysate was mixed with 70 μl of substrate and incubated at room temperature for 5 min followed by addition of 100 μl of accelerate II buffer (Promega) and read in a plate reader.

Site-directed Mutagenesis and Deletion Analysis—Site-directed mutagenesis was performed using a GeneEditor *in vitro* site-directed mutagenesis system (Promega) according to manufacturer's specifications. Briefly, a 4-bp (boldface in the mutagenesis oligo) mutation was designed to generate mutations within the ARE/EpRE-like consensus sequence 5′-GTGACNNNAGC-3′ of *pGB3.6–11* using the mutagenesis oligo 5′-CCTTCCGCTCGACTC**TGCAG**TCGAGCCCCGGGCTA-3′. *Pst*I and *Xho*I restriction sites were engineered to facilitate screening of successful mutant clones. As a result, *pMut-12* plasmid containing both ARE/EpRE mutations, one each within A2/3 and A3, respectively, was generated. *pMut-4* plasmid contains an additional nonspecific mutation within A2 because of high sequence similarity among monomers. For deletion analyses, *pWtA2/3* and *pWtA321* plasmids were first recovered from *pGB3.6–11* by *Hin*dIII digestion followed by *Sac*II digestion to separate the A2/3 region from the A3, 2, and 1 monomers. A2/3 and A321 fragments were then blunt end cloned into *Hin*dIII digested pGL3 basic luciferase reporter vectors to give the *pWtA2/3* and *pWtA321* plasmids, respectively. Deletion plasmids *pMutA2/3* and *pMutA321* were generated from *pMut-12* using the same strategy. *Hin*dII/*Sac*II digested A321 fragment from pGB3.6–11 was then used as PCR template for pA32, pA21, and pA1. The deletion construct *pA32* was generated using upper primer 5′-AAAAAGCTTGTGCCTGCCCCAAT CCA-ATCGCGCGG-3′ and lower primer 5′-AAAAAGCTTTGTGTTCCACT-CAC CAGAGGTCTTAG-3′. Deletion plasmids *pA21* and *pA1* were generated from lower primer 5′-AAAAAGCTTTGTGTTCCACTCACTA-GAGGTCTTAG-3′ in conjunction with upper primers 5′-AAAAAGCT-TGTGCCTGCCCCAATCCAATCGCGCGG-3′ and 5′-AAAAAGCTTGC-GCCTACCCCAATCCAATCGCGTGG-3′, respectively. All mutations and deletions were confirmed by DNA sequencing prior to transfection.

Real-time RT-PCR—Total RNA was extracted using TRI reagent (Molecular Research Center, Inc.) from serum-stimulated mouse vascular smooth muscle cells treated with 3 μM BaP or an equivalent volume of Me_2SO for 8 h following growth arrest in 0.1% serum containing medium 199 for 48 h. Primers were designed using Primer Express Software (PerkinElmer Life Sciences). Location and sequences of primers were as follows: 5′ UTR (nt 307 to 383 of *L1Md-A2*) forward, 5′-ATTGAAGAGGGACAGAAAAACAAAG-3′ and reverse, 5′-ACTGTG-GTCAGGGCACAAATG-3′; ORF2 (nt 5896 to 5999 of *L1Md-A2*) forward, 5′-AATCGACAAATGGGACCTAATGA-3′ and reverse, 5′-GTA-AAGATCCTTTCCCAATCTGTTG-3′; internal control gene 18S (nt 1679 to 1748 of GenBank™ accession number X00686) forward, 5′-AGTCCCTGCCCTTTGTACACA-3′ and reverse, 5′-CGATCCGAGGGC-CTCACTA-3′. Real-time RT-PCR was performed using the ABI-PRISM 7700 sequence detection system (PerkinElmer Life Sciences). Two μg of genomic DNA was first reverse-transcribed to cDNA using TaqMan reverse transcription reagents (PerkinElmer Life Sciences) in a reaction volume of 100 μl containing 1$\times$ TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM reverse primers, 0.4 units of RNase inhibitor, and 1.25 units of MultiScribe reverse transcriptase under conditions of 25 °C for 10 min, 48 °C for 30 min, followed by 95 °C for 5 min in a thermocycler. Five μl of cDNA was then amplified in a total volume of 50 μl, containing 1$\times$ SYBR Green PCR Master Mix and 2.5 to 45 μM reverse and forward primer at 95 °C for 10 min followed by 40 cycles of 15-s denaturing at 95 °C and 1 min annealing at 95 °C in the ABI-PRISM 7700. All reactions were performed in triplicate.

Sequence Alignment—Sequences were aligned, and consensus sequences were determined using MacVector 7.0 (Oxford Molecular Group).

Accession Number—The *L1Md-A2* GenBank™ accession number is M13002, and for *L1Md-A3.6* it is AF502598.

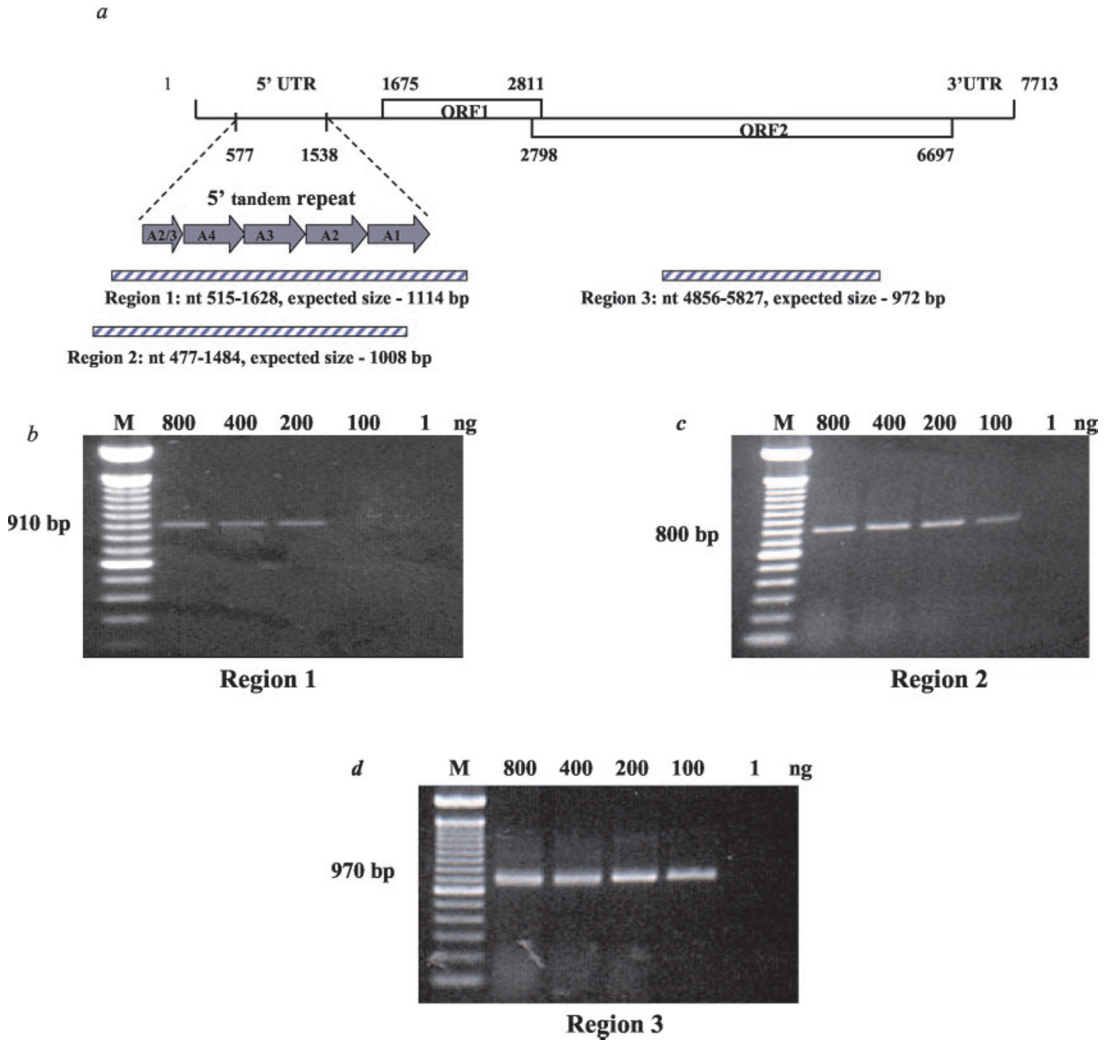

FIG. 1. **Genomic analysis of 5′ regulatory region of L1Md-A2 in vascular smooth muscle cells.** *a*, structure of *L1Md-A2* and primer location and size of expected PCR products. *b–d*, agarose gel analysis showing PCR products amplified for full (*b*) and partial (*c*) fragments of the 5′ tandem repeat. Notice that tandem repeat fragments lack ~200 bp in size, whereas the fragment for ORF2 region yielded the expected size (*d*). Vascular smooth muscle cells were seeded in a 10-cm plate at 100 cells/mm^2. At 85% confluence, genomic DNA was isolated, and various concentrations of DNA were amplified using M13002–18 and M13002–22 primers as described under "Materials and Methods." Amplicons were resolved on a 1.5% agarose gel.

RESULTS

Identification of a Novel L1Md-A2 in Vascular Smooth Muscle Cells—The full and partial 5′ regulatory region of *L1Md-A2* were amplified by PCR from genomic DNA based on GenBank™ published sequences. The ORF2 region of *L1Md-A2* was amplified as a reference control. The location of primers and size of expected products is shown in Fig. 1*a*. Amplicons from Regions 1 (Fig. 1*b*) and 2 (Fig. 1*c*), but not Region 3 (Fig. 1*d*), were ~200 bp shorter than expected. This finding suggests that *L1Md-A2* in murine vascular smooth muscle cells is missing one full monomer equivalent (208 bp), an interpretation consistent with evolutionary pressures known to give rise to genes truncated in their 5′ tandem repeat sequence. The 5′ end truncation of the gene was consistent with relative increases in efficiency of PCR amplification as the template region progressed to the 3′ end of the gene. To identify the missing sequence, a PCR product of Region 1 was regenerated with *Hin*dIII restriction sites at both 5′ ends to facilitate cloning. The resulting product was run on agarose gel to confirm the absence of ~200 bp before cloning into a *Hin*dIII digested PCR-TRAP vector for sequencing. Fig. 2*a* shows the complete sequence of the Region 1 PCR product. Direct sequence alignment of the Region 1 PCR product with the corresponding *L1Md-A2* sequence showed 100% homology at the 5′ and 3′ ends of the monomer (Fig. 2*b*). Interestingly, sequence alignment revealed that the first 208 bp spanning the region between A2/3 and A4 of the parent template were missing in the vascular smooth muscle gene, with only 2-, 1-, 1-, and 1-bp mismatches for the A4, A3, A2, and A1 monomers, respectively (Fig. 2*c*). These findings identified a novel A2 lineage *L1Md* retrotransposon that lacks a full-length monomer equivalent of 208 bp, specifically, 140 bp of A2/3 and 68 bp of 5′ A4 from *L1Md-A2*. Thus, the novel A-type transposon sequence was named *L1Md-A3.6*. Next, genomic DNA from various tissues was examined to evaluate the tissue specificity of this element. PCR analysis showed tissue-specific distribution of the gene, with strong amplification signals detected in vascular smooth muscle cells, and to a lesser extent mammary gland and kidney, but not in liver, uterus, or ovary.

BaP-induced L1Md-A3.6 Activation—Real-time RT-PCR is a high throughput technology that allows rapid and sensitive detection of gene expression (36, 37). Because families of retrotransposons are known to be conserved at the 3′ end and differ at their 5′ untranslated region (38), it was hypothesized that the newly identified *L1Md-A3.6* evolved from *L1Md-A2*. Therefore, real-time RT-PCR was performed to evaluate induc-

a

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  1  CCCAACATAG AGTCCTGAGT TTTTGAGTCT GTATCCAAGT TACAAATAAT
 51  TTTCTGGGGC CCGGATCTGG GGCACAAGTC CCTTCCGCTC GACTCGTGAC
101  TCGAGCCCCG GGCTACCTTG CCAGCAGAGT CTTGCCCAAC ACCTGCAAGG
151  GTCCACACAG GACTCCCCGC GGGACCCTAA GACCTCTGGT GAGTGGATCA
201  CAGTGCCTGC CCCAATCCAA TCGCGCGGAA CTCGAGACTG CGGTACATAG
251  GGAAGCAGGC TACCCGGGCC TGATCTGGGG CACAAGTCCC TTCCGCTCGA
301  CTCGTGACTC GAGCCCCGGG CTACCTTGCC AGCAGAGTCT TGCCCAACAC
351  CCGCAAGGGT CCACACGGGA CTCCCCACGG GACCCTAAGA CCTCTGGTGA
401  GTGGATCACA GTGCCTGCCC CAATCCAATC GCGCAGAACT TGAGACTGCG
451  GTACATAGGG AAGCAGGCTA CCCGGGCCTG ATCTGGGGCA CAAGTCCCTT
501  CCGCTCGACT CGAGACTCGA GCCCCGGGCT ACCTTGACAG CAGAGTCTTA
551  CCCAACACCC GCAAGGGCCC ACACGGGACT CCCCACGGGA CCCTAAGACC
601  TCTGGTGAGT GGAACACAGC GCCTACCCCA ATCCAATCGC GTGGAACTTG
651  AGACTGCGGT ACATAGGGAA GCAGGCTACC CGGGCTTGAT CTGGGGCACA
701  AACCCCTTCC ACTCCACTCG AGCCCCGGCT ACCTTGCCAG CTGAGTCGCC
751  TGACACCCGC AAGGGCCCAC ACAGGATTCC ACACATGATC CTAAGACCTC
801  TAGTGAGTGG AACACAACTT CTGCCAGGAG TCTGGTTCGA ACACCAGATA
851  TCTGGGTACC TGCCTTGCAA GAAGAGAGCT TGCCTGCAGA GAATACTCTG
901  CCCACT
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b

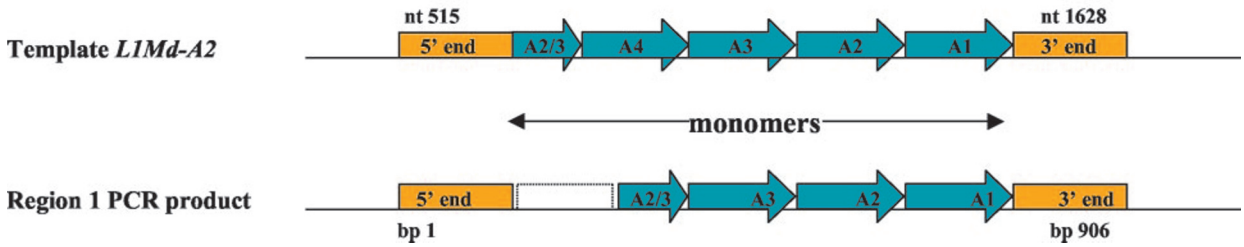

FIG. 2. **Identification of a novel retrotransposon in C57BL6J vascular smooth muscle cells.** *a*, complete sequence of Region 1 PCR product (*L1Md-A3.6*). *b*, summary map showing homologies between *L1Md-A2* and *L1Md-A3.6*. A 100% sequence homology was found for 5′ and 3′ end monomers between *L1Md-A2* and *L1Md-A3.6*. *c*, direct sequence alignment within monomers for *L1Md-A2* and *L1Md-A3.6*. The sequence is shown and numbered for monomer regions of *L1Md-A2* and *L1Md-A3.* Notice that the entire A2/3 monomer (1–140 nt) and the partial A4 monomer (1–68 nt) were deleted in *L1Md-A3.6*. The numbers of mismatched base pairs for each individual segment are summarized in the text.

ibility of *L1Md-A3.6* in response to BaP activation of ORF2 in *L1Md-A2*. A region containing DNA sequence upstream of monomers was used as control. The normalized amount of ORF2 for each treatment was determined by dividing the expression values for each treatment by the amount of corresponding endogenous reference 18S. The average of triplicate BaP-treated cultures was divided by the control average to give the relative amount of ORF2 in BaP-treated over Me_2SO control cells. The amplification profiles for both ORF2 and 18S, and the summary of -fold induction of the gene is shown in Fig. 3 and Fig. 4, *a* and *b*, respectively. As expected, no amplification was detected for the 5′ end tandem repeat promoter sequence (Fig. 4*b*). In contrast, BaP treatment significantly increased expression of *L1Md-A2* ORF2 region, confirming that *L1Md-A3.6* is a member of the *L1Md-A2* family with one monomer short in its 5′ tandem repeat region.

Inducibility of L1Md-A3.6—The mechanism of retrotransposon activation is unknown. To understand the regulation of retrotransposon activation in vascular smooth muscle cells by carcinogen treatment and to determine whether *L1Md* promoter activity is intact regardless of monomer shortening in the 5′ tandem repeat, *L1Md-A3.6* was subcloned into a luciferase reporter vector pGL3 basic vector and used in transient transfection assays. Fig. 5*a* shows the luciferase reporter activity of *L1Md-A3.6* in mouse vascular smooth muscle cells. *L1Md-A3.6* preserved its promoter function, such that luciferase activity was detected in both *pGB3.6–8* and *pGB3.6–11*, plasmids containing the *L1Md-A3.6* sequence in reverse and correct orientation, respectively. The promoter activity in *pGB3.6–8*, although considerably reduced, is consistent with a report showing that the retrotransposon promoter in antisense orientation is capable of disrupting neighboring genes (38). To further evaluate the inducibility of *L1Md-A3.6*, *pGB3.6–11* transfected cells were challenged with 0.3, 3, and 30 μM BaP or an equivalent volume of Me_2SO as vehicle control for 24 h. As shown in Fig. 5*b*, BaP treatment increased luciferase activity in a concentration-dependent manner relative to Me_2SO controls. When transfected cells were pre-treated for 3 h with *N*-acetylcysteine, an antioxidant precursor of cellular glutathione, marked inhibition of BaP-induced gene transcription was observed. Thus, an oxidative mechanism may mediate activation of the gene by the carcinogen. To test this hypothesis, the inhibitory effects of the antioxidant PDTC were examined. Pre-treatment of vascular smooth muscle cells with PDTC for 6 h also prevented activation of the gene (Fig. 5*b*). Oxidative stress is associated with retrotransposon activation in plants (20); however, to our knowledge, this is the first report showing redox-dependent activation of retrotransposons in mammalian cells.

To determine whether other carcinogenic hydrocarbons known to induce oxidative stress modulate *L1Md* transactivation profiles, the effects of TCDD (0.1–10 nM) were examined. Like BaP, TCDD induced concentration-dependent activation of the luciferase reporter, and this effect was antagonized by *N*-acetylcysteine (Fig. 5*c*). The response appears to be specific for carcinogenic hydrocarbons, because hydrogen peroxide (25–100 μM), a known inducer of oxidative stress, failed to induce *L1Md-A3.6* activation (Fig. 5*d*). Hydrogen peroxide was highly cytotoxic to vascular smooth muscle cells at higher concentrations, so higher peroxide concentrations could not be tested.

Role of ARE/EpRE in L1Md-A3.6 Activation—Overproduction of free radicals via cycles of reduction and oxidation between phenolic and quinone metabolites of BaP induces oxidative stress and plays a critical role in dysregulation of vascular

c

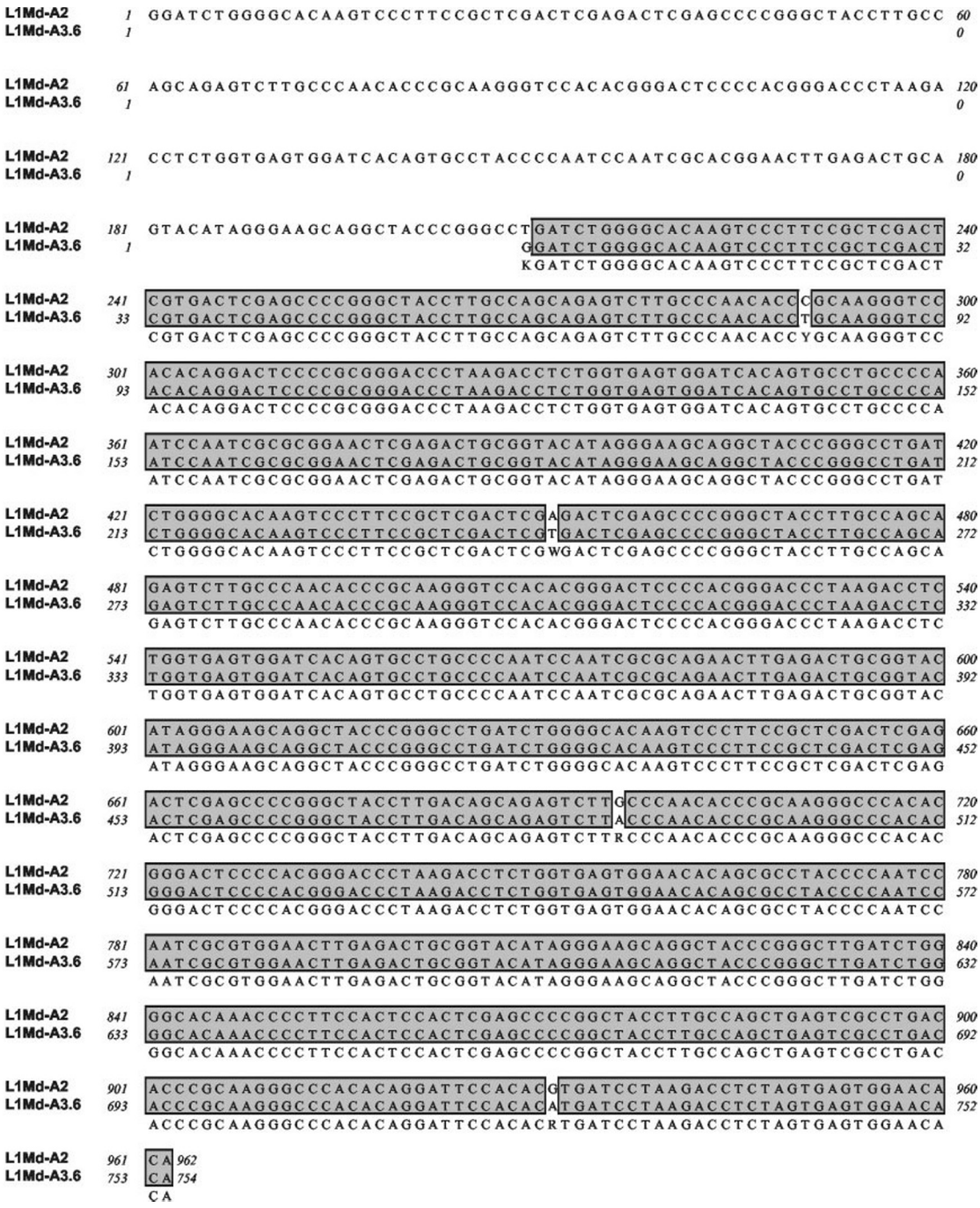

FIG. 2—*continued*

smooth muscle cells (39). A DNA sequence known as the ARE/EpRE (antioxidant/electrophile responsive element) mediates transcriptional activation of several genes, such as GSTA1 (40) and NAD(P)H:quinone oxidoreductase 1 (41). This element (5′-GTGACNNNGC-3′) is responsive to BaP in vascular smooth muscle cells (40) and therefore, may mediate transcriptional regulation of *L1Md-A3.6*. To test this hypothesis, we introduced a 4-bp mutation within the two ARE/EpRE-like sites (5′-GTGACNNN**A**GC-3′), one each within the A2/3 and A3 monomers of *L1Md-A3.6*, respectively. Mutation of G to T, T to G, G to C, and C to G were created within the ARE/EpRE-like sequence from the 5′ to 3′ end, respectively (Fig. 6*a*). *pMut-12* contains two perfect ARE/EpRE mutations, one each within A2/3 and A3 monomer, whereas *pMut-4* contains an additional nonspecific ARE/EpRE mutation within A2 monomer. Transient transfection of these mutants into vascular smooth muscle cells showed that the promoter activity of *L1Md-A3.6* was markedly inhibited by mutation of both A2/3 and A3 ARE/EpRE sites compared with wild type. Under both basal and inducible conditions, nonspecific mutation of A2 did not alter the inducibility of *L1Md-A3.6* promoter activity, suggesting that the ARE/EpRE-like elements play a critical role in activation of the gene.

In agreement with previous studies (10), the promoter activity of *L1Md-A3.6* increased as the number of monomers increases (compare *pA1*, *pA21*, and *pWtA321*). However, in vascular smooth muscle cells, this pattern only applied when the A2/3 monomer of *L1Md-A3.6* was removed from the full-length construct, as evidenced by significant differences in luciferase activity between *pWt321* and *pA21,* or the full-length wild type plasmid. Thus, inhibitory sequences may reside within the 62–171-bp region of A2/3, an interpretation consistent with the

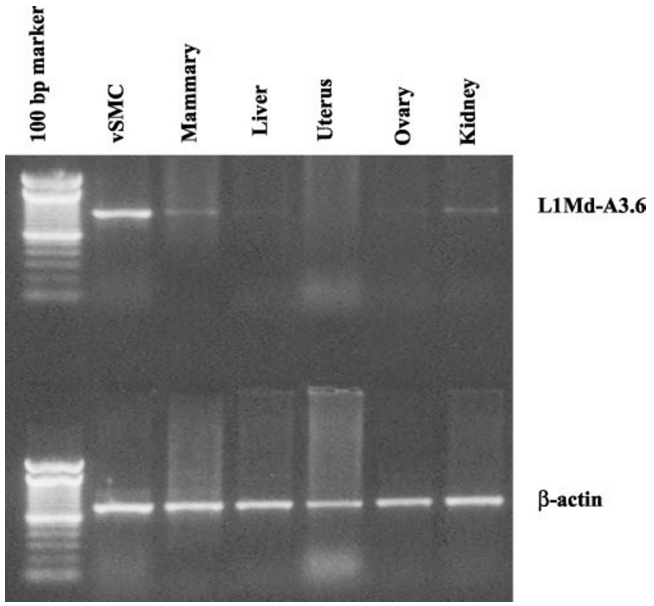

FIG. 3. **Tissue specific distribution for L1Md-A3.6 in the mouse.** Genomic DNA from vascular smooth muscle cells, mammary, liver, uterus, ovary, and kidney was amplified using *L1Md-A2*-specific primers as described under "Materials and Methods" and run on a 1.5% agarose gel.

finding that partial A2/3 deletion mutants were more active under constitutive conditions than wild type counterparts. Comparison of *pWtA321* and *pMutA321* showed that BaP inducible activity in *pGB3.6–11* is mediated by the ARE located within the A3 monomer. Monomer interactions are important for the regulation of *L1Md-A3.6* given that *pA1* completely lost promoter activity, whereas the *pA32* deletion construct containing a functional ARE showed markedly reduced activity compared with wild type. *pWtA2/3* was only modestly active, a pattern not influenced by mutational ablation of the ARE. Thus, site-directed mutagenesis and deletion analyses indicate that interactions between ARE/EpRE-like elements are involved in both basal and inducible activation of *L1Md-A3.6*, with the A3 monomer functioning as the major driving force for transcriptional activation of the gene.

Putative Model of L1Md-A3.6 Regulation—To gain further insight into functional differences between the ARE/EpRE-like sites within A2/3 and A3 monomers, the sequences of *L1Md-A3.6* were further evaluated. Fig. 7 shows the sequence alignment among monomers of *L1Md-A3.6*. Four base pairs at position 1, 82, 97, and 107 of A2/3 differed from corresponding nucleotides at 69, 150, 165, and 175 of A3. Monomer sequences encompassing nt 1–120 of A2/3 and the corresponding sequence on A3 were then submitted for transcriptional factor binding site analysis at www.cbrc.jp/research/db/TFSEARCH.html. The most significant finding was that a change of A to G at position 107 of A2/3 creates a Ttk 69 binding site. Ttk 69 is a binding site for a repressor protein Tramtrack69, which plays a role in asymmetric cell division during *Drosophila* eye development (42, 43) and may be involved in ARE signaling (44). Also noteworthy was the finding that changing of G to A at position 97 and A to G at position 107 in A2/3 disrupted NF-κB and USF binding sites, respectively, within A3.

DISCUSSION

The evidence presented here yields new insights into the expression and regulation of retrotransposon elements in mammalian cells. We have shown that mouse vascular smooth muscle cells posses a unique A type transposon, namely *L1Md-A3.6*, that is missing one full monomer equivalent in its 5′ tandem repeat region. Sequence alignment revealed that the missing sequence represents 140 bp of the A2/3 monomer and 1–68 bp of the A4 monomer. This transposon was also detected in mammary gland and kidney DNA, suggesting tissue-specific distribution may account for genomic context-specific differences in retrotransposition.

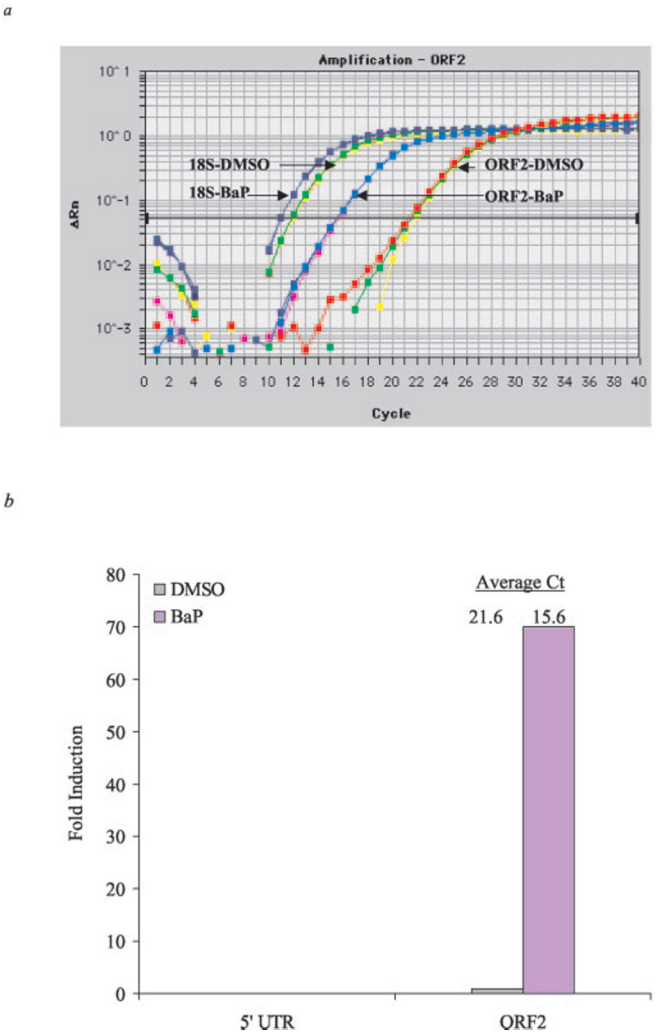

FIG. 4. **Amplification efficiency of the ORF2 region of L1Md-A2.** Real-time RT-PCR was performed as described under "Materials and Methods" using primer pairs as listed. One to 50 ng of chromosomal DNA-free total RNA was used for each standard curve. Five μl of 100-μl first strand cDNA generated from 2-μg total RNA from 8-h BaP- or Me_2SO-treated vascular smooth muscle cells were amplified with SYBR Green dye in ABI-PRISM 7700. International control gene 18S was amplified in the same plate. All reactions were performed in triplicate. *a*, real-time amplification plots of *L1Md-A2* ORF2 region extracted from BaP- and Me_2SO-treated vascular smooth muscle cells. Shown is the level of normalized reporter signal minus the baseline signal established in the first few cycles of PCR as a function of PCR cycle number. The *curve* on the *far left* corresponds to the highest amount of *L1Md-A2* ORF2 cDNA detected. The *horizontal line* of Rn is the threshold for detection. *b*, *bar graph* showing the average Ct value for each treatment and the expression level of *L1Md-A2* ORF2 in BaP-treated *versus* Me_2SO control cells. No amplification for 5′ UTR, upstream of the 5′ tandem repeat, was seen with real-time RT-PCR.

Retrotransposons are mobile DNA elements that propagate through transcription and reverse transcription and reintegration into new locations within the genome. Two major types of retrotransposons have been described, one containing LTRs that resemble retroviral pro-viruses *versus* non-LTRs having no terminal repeats but poly(A) tails (45). LTRs function as retroviruses and use reverse transcriptase and integrase en-

coded by *pol-like* open reading frame for their transposition. Non-LTRs were originally discovered in mammalian genomes (2) but have now been detected in a wide range of species from protozoa to fungi, plants, and animals. Non-LTRs usually contain two ORFs. ORF1, near the 5′ end of the element, encodes nucleic acid-binding proteins that form ribonucleoprotein particles during transposition (46), whereas ORF2 encodes for reverse transcriptase and endonuclease (47). In mammals, a major class of non-LTR retrotransposons consists of the highly repetitive *LINE*s (long interspersed repetitive elements), including the L1 family, the mouse-specific *L1Md* family, and the chicken-specific CR1 family (48). Insertion into chromosomal DNA probably occurs by a process termed target-primed reversed transcription; the endonuclease of ORF2 nicks a single strand of DNA, leaving a 3′-OH that serves as the primer for reverse transcription with the L1 RNA as template (49).

L1Md-A3.6 preserved its promoter function and was inducible by aromatic hydrocarbons that activate aryl hydrocarbon receptor (Ahr) signaling. Ahr is a ligand-activated transcription factor member of the basic helix-loop-helix family of transcription factors. Activation of Ahr signaling disrupts programs of gene expression and phenotypic control in vascular smooth muscle cells (39). Interestingly, *L1Md-A3.6* induction by aromatic hydrocarbons was abolished by two antioxidants, *N*-acetylcysteine and PDTC, a finding that suggests that oxidative mechanisms play a central role in regulation of the gene. Oxidative stress is associated with retrotransposon activation in plants (20), but to our knowledge this is the first report showing redox-dependent activation of retrotransposons in mammalian cells. The role of oxidative mechanisms in retrotransposon activation is complex, because peroxide treatment, a vascular smooth muscle cell oxidant, failed to transactivate the *L1Md-A3.6* promoter. Instead, the observed pattern of redox-dependent regulation is consistent with the ability of BaP and TCDD to activate functional cross-talk between ARE-binding proteins and Ahr in the regulation of mammalian gene expression (40, 50). Of note is the finding that hydrogen peroxide does not activate Ahr signaling in vascular smooth muscle cells (50).

ARE-binding proteins are redox-regulated transcription factors of the basic leucine zipper superfamily that "sense" alterations in cellular redox balance following environmental stress (44). Over production of free radicals via cycles of reduction and oxidation between phenolic and quinone intermediates in BaP-treated cells induces oxidative stress and activates ARE signaling in vascular smooth muscle cells (51). As shown for other BaP-responsive genes, AREs mediate transcriptional regulation of *L1Md-A3.6*. Mutation of two ARE/EpRE-like sites (5′-GTGACNNN**A**GC-3′), one each within the A2/3 and A3 monomers of *L1Md-A3.6*, respectively, markedly inhibited promoter activity. Hydrocarbon-inducible activity, however, was dependent upon the ARE sequence located within A3. Functional differences between the A2/3 and A3 AREs may be accounted for by genomic context-specific characteristics of protein binding and complex assembly. Together, the data presented here provide strong experimental support for the hypothesis that the A3 ARE/EpRE-like element plays a critical role in gene activation, particularly within the context of hydrocarbon inducibility.

Sequence analysis of *L1Md-A3.6* sequences identified several putative redox-regulated *cis*-acting elements, including c-Ets, NF-κB, USF, and Ttk 69, within the A2/3 and A3 monomers. Thus, interactions between proteins that recognize these sequences may dictate patterns of *L1Md-A3.6* transactivation and repression throughout the mouse genome. This interpretation is consistent with the ability of BTB proteins, such as

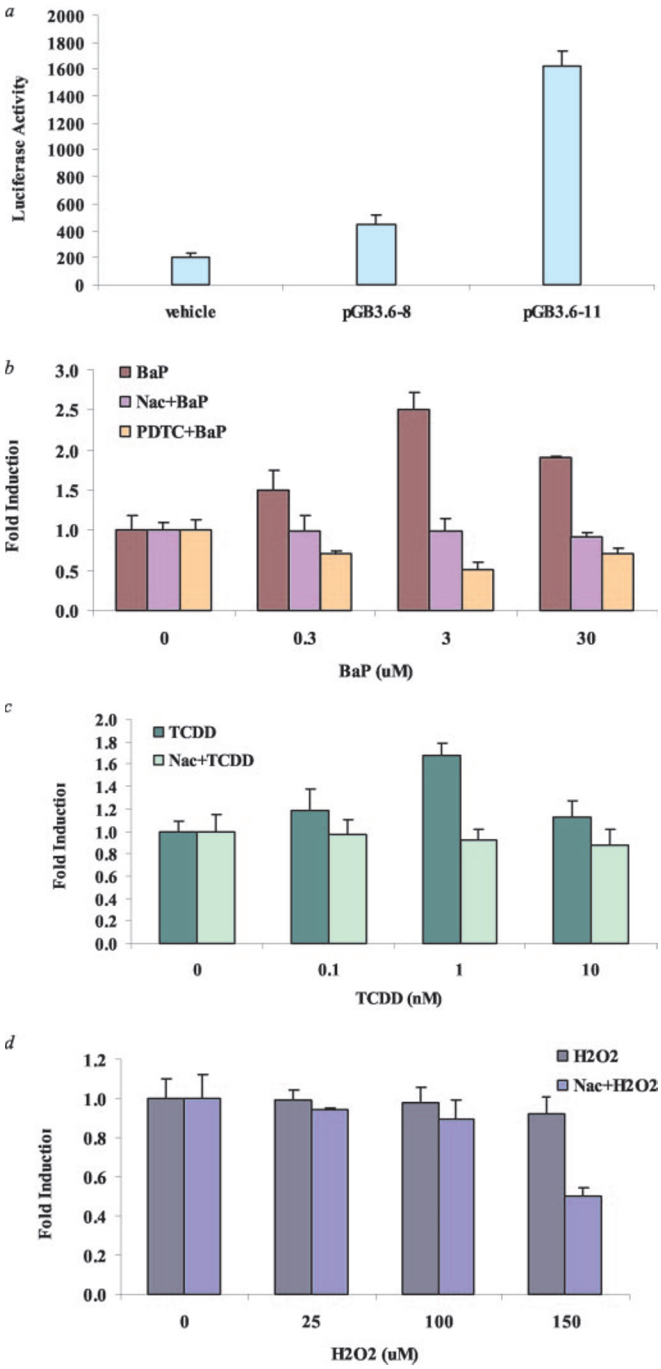

FIG. 5. **Inducibility of *L1Md-A3.6*.** *a*, transfection assay showing the promoter activity of *L1Md-A3.6* 5′ tandem repeat in vascular smooth muscle cells irrespective of sequence orientation as sense strand in pGB3.6–11 or antisense in pGB3.6–8. *b*, concentration-dependent activation of pGB3.6–11 reporter construct by BaP and the inhibitory effects of 0.5 mM *N*-acetylcysteine and 0.01 μM PDTC pre-treatment for 3 and 6 h, respectively. *c*, *L1Md-A3.6* in vascular smooth muscle cells was also induced by TCDD in a concentration-dependent manner. The response was inhibited by pretreatment with *N*-acetylcysteine. *d*, hydrogen peroxide did not activate *L1Md-A3.6* in vascular smooth muscle cells.

Tramtrack69, to interact with nrf2 and other Cap′N′Collar proteins in the regulation of ARE/EpRE-coupled transcription (44). Such protein-protein interactions may define critical regions within A2/3 and A3 that are regulated by environmental carcinogens that activate Ahr.

Propagation and integration of *LINE*s is believed to play a

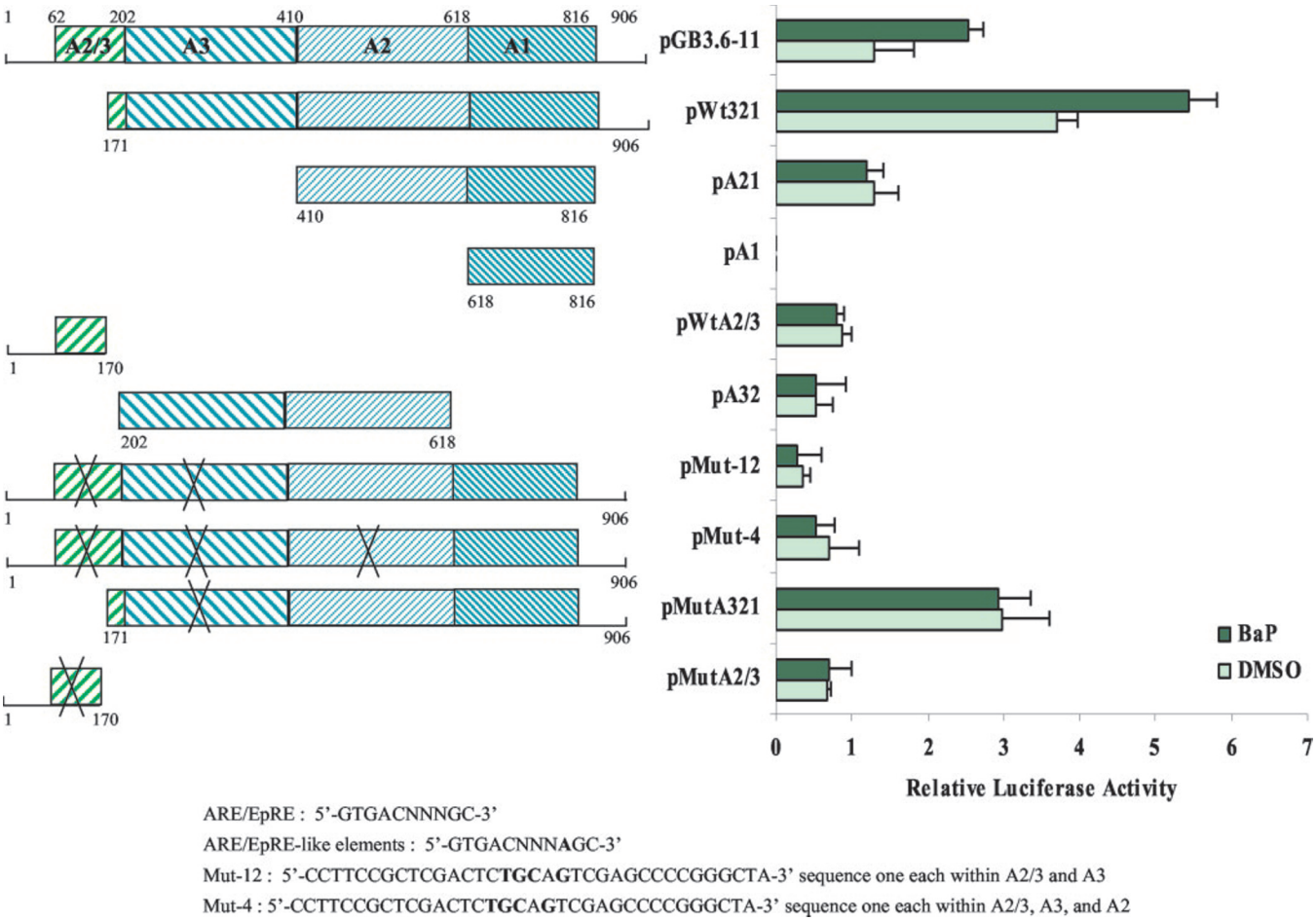

FIG. 6. **Influence of ARE/EpRE-like element on *L1Md-A3.6* transactivation.** Vascular smooth muscle cells were seeded in 24-well plates. At 80% confluence, cells were transfected with plasmids containing wild type *L1Md-A3.6*, two ARE/EpRE site mutations, two ARE/EpRE site mutations plus a nonspecific mutation, a deletion fragment containing A3 and A2 monomers, and a deletion fragment containing only A1 monomer, for 2 h. Transfected cells were recovered after 24 h and challenged with 3 μM BaP or an equivalent volume of Me_2SO for 24 h, before measurements of luciferase activity. A β-galactosidase plasmid was co-transfected as an internal control.

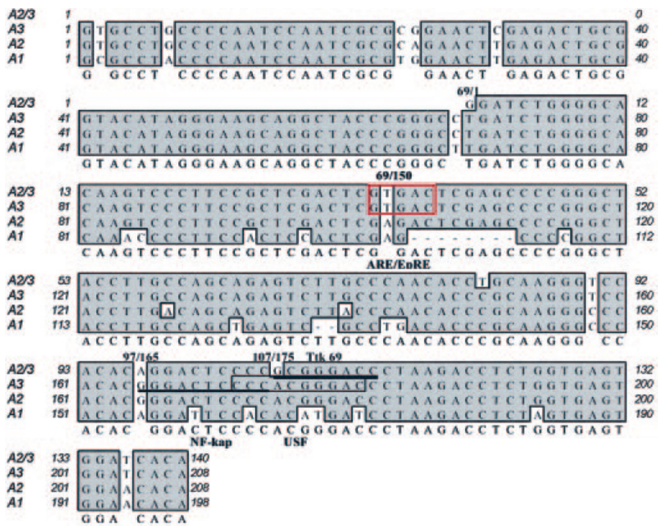

FIG. 7. **Putative carcinogen-responsive elements in L1Md-A3.6.** Monomer sequences of *L1Md-A3.6* were aligned with Mac Vector 7.0. The location of the consensus ARE/EpRE binding sequences in A2/3 and A3 is shown in the *box*.

major role in the pathogenesis of several human diseases, including hemophilia (52), colon cancer (53), and breast cancer (54). Alkylating esters of methanesulfonic acid (55), mitomycin C (56), and azacytidine (57) have all been shown to induce retrotransposition. Recent evidence has established an association between L1 retrotransposition and genomic instability as evidenced by element inversions, extra nucleotide insertions, exon deletions, chromosomal inversion, and flanking sequence co-mobilization (58, 59). Thus, epigenetic mechanisms involving *L1Md-A3.6* activation in vascular smooth muscle cells may give rise to genomic instability and vascular injury following environmental exposure to chemical carcinogens. This hypothesis is consistent with the ability of environmental hydrocarbon carcinogens to induce atherosclerotic lesions in laboratory animals and humans (for a review see Ref. 39). The distribution patterns of *L1Md-A3.6* suggest that genetic consequences resulting from activation of *L1Md-A3.6* transposon may be a generalized response in several mouse tissues.

In summary, evidence is presented here that environmental carcinogens activate a novel gene of *L1Md-A2* lineage in vascular smooth muscle cells. Gene regulation is mediated via a redox-sensitive mechanism involving monomer-regulated transcription by two ARE-like elements within the A2/3 and A3 region. The full monomer equivalent truncation model described here for *L1Md-A3.6* implicates monomer as a mechanism for molecular evolution of *L1Md-A*-type retrotransposons that either preserve or lose functional full-length retrotransposon activity, respectively.

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