SB202190 and SP600125, respectively. U, untreated; D, DMSO; F, FICZ; M, 3-MC; H, H_2O_2. Of note, L1-RTP caused by 3-MC was attenuated by SB (lane 8), but not by SP (lane 10). The arrowhead indicates the induction of L1-RTP. **c**. CREB is required for L1-RTP induced by 3-MC. Left panel: Western blot analysis detected efficient down-regulation of the endogenous protein by *CREB* siRNA. U, untreated; N, non-transfected; C, control siRNA; *CREB*, *CREB* siRNA. Right panel: PCR-based assay after the transfection of *CREB* siRNA. *CREB* siRNA attenuated L1-RTP induced by 3-MC (lane 9). **d**. Effects of MAPK inhibitors on L1-RTP induced by B[*a*]P. Reagents similar to those described in Fig. 2b were used. L1-RTP by B[*a*]P was attenuated by both SB (lane 8) and SP (lane 10).

8. Roles of MAPK on L1-RTP

It has been supposed that the increase of transposable elements coupled with evolution (7). Even in *Candida albicans*, an L1-like structure is present as a functional gene (55). On the other hand, the bHLH/PAS family, which has a variety of biological functions including the metabolism of xenobiotics, maintenance of the circadian rhythm, cellular responses to hypoxia, and neuronal differentiation (41,42), is also well conserved from lower species to mammals (56). Interestingly, *AhR* homologs are also present in the genomes of *Drosophila melanogaster* and *Caenorhabditis elegans* (56). Although no direct evidence on the functional relationship between these two biological phenomena has been claimed, our observation is the first to demonstrate the functional link of these biological events. Moreover, data suggest that MAPKs are involved in the bHLH/PAS-dependent L1-RTP. MAPKs are involved in cellular response to intracellular and extracellular stress (38,57), and it is plausible that MAPKs mediate various stresses in the induction of L1-RTP, resulting in genome shuffling. Random mutagenesis by L1-RTP may give emerging novel organisms to survive in altered environments.

It is important to clarify the roles of MAPKs in the induction of L1-RTP. At present, at least two functions of MAPKs can be postulated. As explained, environmental compounds activate MAPKs, by which the chromatin recruitment of ORF1 is induced as a necessary step in L1-RTP. ORF1 functions in *cis* with L1- mRNA and functions as a chaperon of L1-mRNA (24,25). Using MAPK inhibitors, we observed that L1-RTP was abrogated concomitantly with the reduced chromatin recruitment of ORF1. These observations suggest that MAPK activation drives the mobilization of ORF1 to chromatin, by which retroelements are translocated to chromtin.

Another possible role for MAPKs is related to the activity of the APOBEC family. It has been proposed that APOBEC family functions as innate restriction factors that suppress the activity of endogenous retroelements (58). Originally, it was postulated that the APOBEC family inhibits HIV-1 infection by editing C to T via deaminase activity (58). Vif, a gene product of HIV-1, degrades APOBEC proteins, causing infected cells to become permissive for HIV-1 infection (59). We previously showed that all members of the APOBEC family exhibit inhibitory activity toward L1-RTP (60). However, it was recently postulated that the APOBEC has dual activity (61) and inhibits the activity of RT (62). In *in vitro* experiments in which APOBEC3G were added to the reaction of RT in the synthesis of viral DNA, APOBEC interfered with elongation of the viral DNA (62). Interestingly, it has been shown that C/EBP-β bound APOBEC3G and attenuate the inhibitory activity of APOBEC3G (63). Moreover, it was demonstrated that the mutation of serine at 228 (S228), the phosphorylation of which is correlated with the cytoplasmic localization of the molecule (63), abolished both binding and inhibitory activity on APOBEC3G (64). Given that C/EBP-β is a substrate of p38

(38), a plausible model is that p38 augments the blocking activity of C/EBP-β on APOBEC3G via phosphorylation.

9. Further implications

Ataxia telangiectasis mutated (ATM), a phosphoinositide 3-kinase, has a functional link with L1-RTP (16). In an intriguing recent observation, the copy number of L1 increased in the brain tissues of patients with ATM (16). L1-RTP is consistently increased in the brain tissue of *ATM*-knock out mouse. Although these observations suggest that ATM functions as a negative regulator of L1-RTP, Gasior *et al.* originally reported that ATM was required for the induction of L1-RTP (65). Because of controversial observations regarding the role of ATM in L1-RTP, we focused on MAPKs in the current study.

Recent observations revealed that genome shuffling by L1-RTP in human somatic cells is a source of interindividual genomic heterogeneity (12,13). In addition, independent research groups reported that L1-RTP is frequently induced in tumors (19,20), suggesting the involvement of L1-RTP in the development of carcinogenesis. Importantly, L1 proteins are active on the retrotransposition of *Alu* (30,31), a non-autonomous retoroelement. On the other hand, it has been shown that *Alu* induces genomic instability via non-allelic homologous recombination (66). Thus, it is important to understand the activation mechanisms of L1. Our current observations support the idea that the chromatin recruitment of ORF1, which is controlled by cooperative regulation by members of the bHLH/PAS family and MAPKs, is a critical step in the regulation of L1-RTP. If this is the case, L1-RTP induction in the genome is selectively determined by cellular factors. Because AhR is a transcription factor that recognizes specific nucleotide element (36), carcinogens possibly induce L1-RTP in the genomes in the vicinity of the *cis*-element.

As observed in the analysis of L1-RTP by B[*a*]P and 3-MC, L1-RTP was not induced via the classical pathway controlled by both AhR and ARNT1. Our data suggest that L1-RTP is not necessarily induced by genotoxic activities of these compounds, further implying that L1-RTP is a novel type of genomic instability by which cellular cascades activated by environmental compounds lead to genome shuffling and generate stable phenotypes of the affected cells. The suppression of L1-RTP in somatic cells by targeting MAPK activity may be a novel strategy to protect the development of intractable diseases that include carcinogenesis.

10. Acknowledgments

We are grateful to Dr. Elena. T. Luning Prak (University of Pennsylvania Medical Center) for pEF06R. This work was supported in parts by a research grant for the Log-range Research Initiative (LRI) from Japan Chemical Industry Association (JCIA), and The Grant for National Center for Global Health and Medicine 21A-104) and a Grant-in-Aid for Research from the Ministry of Health, Labour, and Welfare of Japan (109156296). Mr. Noriyuki Okudaira is an applicant supported by Grant-in-Aid from the Tokyo Biochemical Research Foundation.

All authors declare that they have no conflict of interest for the current work.

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frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 31 May 2012
doi: 10.3389/fmicb.2012.00191

Modes of retrotransposition of long interspersed element-1 by environmental factors

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Approximately 42% of the human genome is composed of endogenous retroelements, and the major retroelement component, long interspersed element-1 (L1), comprises ~17% of the total genome. A single human cell has more than 5×10^5 copies of L1, 80~100 copies of which are competent for retrotransposition (RTP). Notably, L1 can induce RTP of other retroelements, such as *Alu* and *SVA*, and is believed to function as a driving force of evolution. Although L1-RTP during early embryogenesis has been highlighted in the literature, recent observations revealed that L1-RTP also occurs in somatic cells. However, little is known about how environmental factors induce L1-RTP. Here, we summarize our current understanding of the mechanism of L1-RTP in somatic cells. We have focused on the mode of L1-RTP that is dependent on the basic helix–loop–helix/per–arnt–sim (bHLH/PAS) family of transcription factors. Along with the proposed function of bHLH/PAS proteins in environmental adaptation, we discuss the functional linking of L1-RTP and bHLH/PAS proteins for environmental adaptation and evolution.

Keywords: LINE-1, environmental factors, retrotransposition, bHLH/PAS family

INTRODUCTION

Approximately half of the human genome (~45%) is composed of transposable elements, most of which are endogenous retroelements (~42%; Lander et al., 2001; Bannert and Kurth, 2004). Notably, humans have more endogenous retroelements than do mice (~37%) or fruit flies (~3.6%), implying a possible role for endogenous retroelements in evolution (Kazazian, 2004). In humans, ~8% of the genome comprises human endogenous retroviruses (HERV) that have gene structures similar to the long-terminal repeats (LTRs) of lentiviruses (Bannert and Kurth, 2004). By contrast, ~34% of retroelements are of non-LTR types, half of which are long interspersed element-1 (L1). A single human cell contains more than 5×10^5 copies of L1, 80~100 copies of which are competent for genome shuffling by retrotransposition (RTP; Brouha et al., 2003). Interestingly, ~10% of such RTP-competent L1 sequences are "hot" and account for more than 80% of the total RTP activity (Brouha et al., 2003).

L1 is actively expressed in embryonic stem cells (Georgiou et al., 2009), and L1-RTP occurs in oocytes and during early embryonic development (van den Hurk et al., 2007; Kano et al., 2009). L1-RTP is also coupled with neuronal cell differentiation (Muotri et al., 2005), supporting the hypothetical role of L1-RTP in the plasticity of nerve cells. Moreover, L1-RTP was shown to be a critical event at an early stage of cell division in the fertilized egg, although its exact role remains elusive (Vitullo et al., 2012). While these lines of evidence strongly suggest that L1-RTP is pivotal for early embryogenesis, recent observations indicate that L1-RTP also occurs in somatic cells. Interestingly, some studies suggest that the L1 copy number is increased in the human brain (Muotri et al., 2005; Baillie et al., 2011), and greater L1 activity was detected in patients with defective ataxia telangiectasia mutated (*ATM*) genes (Coufal et al., 2011). Moreover, new somatic inserts were identified in the tumors, suggesting increased activity therein (Iskow et al., 2010; Ting et al., 2011). These observations support the idea that somatic cells possess the machinery that is involved in the induction of L1-RTP, but little is known about the cellular factors required for L1-RTP. Recently, we found that L1-RTP was induced by environmental compounds that included carcinogens (Okudaira et al., 2010, 2011; Ishizaka et al., 2012). L1-RTP induction by these compounds was dependent on members of the basic helix–loop–helix/per–arnt–sim (bHLH/PAS) protein family, which has been proposed to be associated with the environmental adaptation of living organisms (Beischlag et al., 2008; McIntosh et al., 2010). Here, we provide an overview of our current understanding of the mechanism of L1-RTP coupled with bHLH/PAS proteins, and discuss the role of L1-RTP in relation to environmental adaptation and revolution.

L1 AND THE ROLES OF L1-ENCODED PROTEINS

L1 is ~6 kb in length and is composed of a 5′-untranslated region (UTR), two non-overlapping open reading frames (ORFs), and a 3′ UTR with a poly(A) tail (Goodier and Kazazian, 2008). L1 has evolved along a single, unbroken lineage for the past 40 million years (myrs) in primates, and five subfamilies of L1 (L1PA1-5) have developed within the past 25 myrs in hominoid primates (Lee et al., 2007). Interestingly, species-specific L1 subfamilies emerged in *Homo sapiens* and *Pan troglodytes* after their divergence 6 myrs ago. In humans, active L1 (Ta-1) arose from the Ta-0 subfamily

~4 myrs ago, and expanded as a dominant subfamily thereafter. Notably, it has also been shown that ~69% of L1-inserted loci are polymorphic for the presence or absence of the Ta-1 insert, and that ~90% of such loci possess Ta-1d inserts, the youngest subset of Ta-1, which arose ~1.4 myrs ago (Boissinot et al., 2000). Of note, Ta-1d accounts for approximately two-third of the Ta-1 subfamily, indicating that it has been selectively expanded during the evolution of *H. sapiens*. Intriguingly, humans have higher numbers of RTP-competent L1 sequences (80~100 copies) than do chimpanzees (~5 copies), although there are similar numbers of species-specific inserts (1200–2000 copies) in humans and chimpanzees (Lee et al., 2007).

L1 encodes two proteins: ORF1 and ORF2 (Goodier and Kazazian, 2008). ORF1 is a 40-kDa protein that acts on L1-mRNA *in cis* (Wei et al., 2001) and functions as its chaperone (Martin et al., 2005). ORF2 is a protein of about 150 kDa with both reverse transcription (RT; Mathias et al., 1991) and endonuclease (Feng et al., 1996) activities. ORF2 recognizes 5′-TTAAAA hexanucleotides in the genome and induces a nick between 3′-AA and TTTT in the complementary strand (Jurka, 1997; Gilbert et al., 2005). It has been proposed that first-strand DNA is synthesized by target site-primed RT, in which the poly(A) tail of L1 mRNA anneals to the poly-T stretch of nicked genomic DNA (Kazazian, 2004; Babushok and Kazazian, 2007; Goodier and Kazazian, 2008). ORF1 and ORF2 complete the entire process of L1-RTP and also transpose other elements, such as *Alu* and *SVA* (SINE–VNTR–Alus: short interspersed element, SINE; variable number of tandem repeats, VNTR; and Alu; Dewannieux et al., 2003; Wallace et al., 2008; Hancks and Kazazian, 2012; Raiz et al., 2012), indicating that L1 functions as a driving force of genome shuffling.

L1-RTP IN SOMATIC CELLS IS INDUCED BY ENVIRONMENTAL FACTORS

REPORTED INDUCERS

The environmental triggers, gamma irradiation (Farkash et al., 2006), and heavy metals (El-Sawy et al., 2005; Kale et al., 2005), such as mercury, cadmium, and nickel, have been shown to induce L1-RTP. Although it was proposed that L1-RTP induced by nickel occurs at the post-transcriptional level (El-Sawy et al., 2005), the precise mode of induction of L1-RTP remains to be clarified. Stribinskis and Ramos (2006) also reported that benzo[*a*]pyrene (B[*a*]P) induced L1-RTP. They found that B[*a*]P-induced L1-RTP depended on the aryl hydrocarbon receptor (AhR). Interestingly, however, it was shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), a well-known ligand of AhR (Beischlag et al., 2008; McIntosh et al., 2010), did not induce L1-RTP. In a proposed mechanism, ligand-bound AhR activates expression of metabolic enzymes encoded by cytochrome P450 (*CYP*) genes, which in turn convert B[*a*]P into a genotoxic compound that induces L1-RTP (Stribinskis and Ramos, 2006). By contrast, TCDD is not a genotoxic compound, and does not induce L1-RTP.

NEWLY IDENTIFIED ENVIRONMENTAL FACTORS AND NOVEL MODES OF L1-RTP

We recently discovered that 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct and a putative physiological AhR ligand (Wincent et al., 2009), induces L1-RTP (Okudaira et al., 2010). Experiments using short-interfering RNA (siRNA) along with an AhR inhibitor revealed that the L1-RTP induced by FICZ was not dependent on AhR, but required AhR nuclear translocator-1 (ARNT1; Hoffman et al., 1991). Chromatin recruitment of ligand-bound AhR is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997). Moreover, it was also shown that addition of FICZ initiated the molecular interaction between ARNT1 and ORF1, and promoted the recruitment of ORF1 to the chromatin-rich fraction (Okudaira et al., 2010). Given that there have been no reports indicating that ARNT1 by itself functions as a receptor for environmental compounds, it is likely that a novel cellular factor that functions as an FICZ receptor cooperates with ARNT1 in the induction of L1-RTP.

Further studies demonstrated that dimethylbenzoanthracene (DMBA), B[*a*]P, and 3-methylchoranthrene (3-MC) induced L1-RTP (Okudaira et al., 2011; Ishizaka et al., 2012). Interestingly, the results of siRNA-based experiments revealed that the induction of L1-RTP by these carcinogens depended on AhR. Moreover, the induction of L1-RTP by DMBA required ARNT1, whereas L1-RTP induced by B[*a*]P and 3-MC did not. Notably, *ARNT1* siRNA blocked mRNA expression of the CYP gene *CYP1A1*. Additionally, it has been ascertained that the expression of *CYP1A1* mRNA depends on a heterodimer of AhR and ARNT1 (AHRC, AhR complex). These observations indicate that *ARNT1* siRNA efficiently attenuated the biological function of the AHRC, confirming that the induction of L1-RTP by B[*a*]P and 3-MC was independent of ARNT1. Moreover, the expression of *CYP1A1* mRNA in response to environmental factors requires the chromatin recruitment of ligand-bound AhR, which is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997), lending support to the notion that a cellular factor, the function of which was similar to that of ARNT1, could contribute to chromatin recruitment of ORF1. Since AhR forms a complex with estrogen receptor α (ERα; Ohtake et al., 2003), we examined whether ERα was involved in induction of L1-RTP. Notably, experiments using *ERα* siRNA indicated that L1-RTP induced by both B[*a*]P and 3-MC depended on ERα (Ishizaka et al., 2012).

It has been shown that B[*a*]P increased the expression of L1 mRNA (Stribinskis and Ramos, 2006), whereas nickel chloride did not (El-Sawy et al., 2005). We also observed no apparent increase in L1 transcripts with FICZ (Okudaira et al., 2010). These observations suggest that the modes of L1-RTP triggered by environmental compounds are regulated differently at the transcriptional and post-transcriptional levels. It is conceivable that the cellular machinery that functions at the post-transcriptional level is regulated differently depending on the compound (**Table 1**). Although further study is required, the data support the idea that genotoxic carcinogens induce L1-RTP in an AhR-dependent manner. Moreover, the modes of L1-RTP induced by environmental compounds were different, suggesting that the integration sites of L1 differ depending on the trigger. We will discuss this possibility further later (**Figure 1**).

MITOGEN-ACTIVATED PROTEIN KINASES ARE REQUIRED FOR RTP INDUCED BY ENVIRONMENTAL COMPOUNDS

In mammalian cells, six groups of MAPKs – extracellular signal-regulated protein kinase1/2 (ERK1/2), ERK5, JNK, p38, ERK3/4,

Table 1 | Summary of cellular factors required for L1-RTP by environmental compounds.

Cellular factors	Inducers			
	FICZ	B[*a*]P	3-MC	DMBA
AhR	–	○	○	○
ARNT1	○	–	–	○
ERα	N.T.	○	○	–
MAPKs				
SB202190	○	○	○	–
SP600125	○	○	–	–

○, *dependent;* –, *independent; N.T., not tested.*
The induction of L1-RTP was examined by a PCR-based assay.

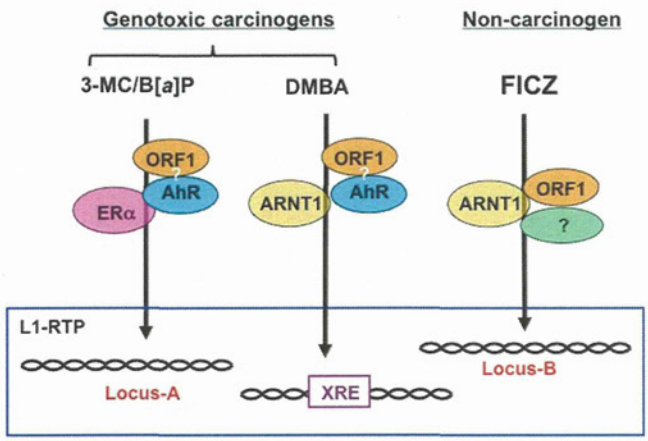

FIGURE 1 | Schematic modes of L1-RTP triggered by environmental compounds. Given that ORF1, which associates with retroelements, is recruited to the chromatin, regions of L1-RTP are likely determined by the binding partners of ORF1. As shown in **Table 1**, the induction of L1-RTP by environmental compounds depended on different sets of bHLH/PAS proteins. The induction of L1-RTP by DMBA depended on AhR and ARNT1, whereas that by 3-MC and B[*a*]P required AhR. Interestingly, L1-RTP by 3-MC and B[*a*]P did not require ARNT1, but depended on ERα. By contrast, FICZ-induced L1-RTP depending on ARNT1, whereas it did not require AhR. It is plausible that the regions where these compounds insert L1 are different: DMBA induces L1-RTP in the vicinity of XRE, whereas 3-MC and B[*a*]P induce L1-RTP in the region determined by AhR and ERα. The locus of L1-RTP by FICZ would differ from those determined by these genotoxic carcinogens. To prove this, it is necessary to identify the association of ORF1 and AhR and genome regions where L1 is inserted in response to each environmental compound.

and ERK7/8 – have been identified and shown to be activated by intracellular and extracellular stimuli (Cargnello and Roux, 2011). Among these MAPKs, the cellular signal cascades activated by ERK1/2, p38, and JNK have been well characterized because of the availability of inhibitors. PD98059, SB202190, and SP600125 are inhibitors of ERK1/2, p38, and JNK, respectively. To examine the involvement of MAPKs in the induction of L1-RTP, the effects of these inhibitors on the induction of L1-RTP have been analyzed. SB202190 blocked FICZ-induced L1-RTP, and siRNA specific for cyclic-AMP responsive element binding protein (*CREB*) mRNA efficiently attenuated FICZ-induced L1-RTP (Okudaira et al., 2010). Additionally, FICZ-induced phosphorylation of CREB in an ARNT1-dependent manner, and SB202190 blocked the FICZ-induced phosphorylation of CREB and chromatin recruitment of ORF1. These observations verified that p38 is required for the induction of L1-RTP by FICZ at the step of chromatin recruitment of ORF1. Further analysis on the mode of L1-RTP induced by 3-MC and B[*a*]P revealed that the L1-RTP induced by 3-MC was coupled to CREB phosphorylation, and that both *CREB* siRNA and SB202190 abrogated L1-RTP induction by the compound. The effects of MAPK inhibitors are summarized in **Table 1**. The next challenge is to determine how MAPKs and the phosphorylation of MAPK substrates are involved in the induction of L1-RTP. One plausible role of these molecules is driving the chromatin recruitment of ORF1 or retroelements in response to environmental compounds (Chiu and Greene, 2008). Several studies suggest that retroelements, such as Alu, are present in a cytoplasmic high-molecular weight complex due to the function of APOBEC3 proteins, an innate restriction molecule (Chiu and Greene, 2008).

FUNCTIONAL INTERACTION OF bHLH/PAS PROTEINS AND L1-RTP

BIOLOGY OF bHLH/PAS PROTEINS

The bHLH/PAS family is composed of numerous transcriptional factors with PAS domains consisting of approximately 275 amino acids. Family members have sequence homology to the clock gene *period* (*per*) from *Drosophila melanogaster*, which is involved in the control of circadian rhythms; the *arnt* gene in mammals, which is required for signaling pathways activated in response to dioxin or polyaromatic hydrocarbons; and the *single-minded* (*sim*) gene, a neurodevelopmental regulator in flies (Beischlag et al., 2008; McIntosh et al., 2010). It is interesting to note that the bHLH/PAS family is involved in these three apparently different biological responses. The PAS domain has two sub-domains, PAS-A and PAS-B, each of which comprises approximately 70 amino acids. The PAS-A domain functions as a binding site for other PAS-A domain-containing proteins (McIntosh et al., 2010). In contrast, the PAS-B domain is involved in interactions with other classes of proteins, such as heat shock protein 90 and small molecules. One of the best-characterized functions of bHLH/PAS proteins is in the response to environmental pollutants, such as polyaromatic hydrocarbons (PAH; Beischlag et al., 2008). When PAH binds to the PAS-B domain of AhR, a heterodimeric complex of ligand-bound AhR and ARNT1 (AHRC) is formed. AHRC is then recruited to chromatin, where it recognizes a xenobiotic responsive element (XRE; Probst et al., 1993) and activates the expression of genes encoding xenobiotic metabolic enzymes (Beischlag et al., 2008). Interestingly, the AHRC also activates expression of AhR repressor (AhRR), which has both bHLH and PAS-A domains, but not a PAS-B domain. It has been proposed that the expression of AhRR down-regulates the activity of the AHRC by a feedback mechanism (Mimura et al., 1997).

CHROMATIN RECRUITMENT OF ORF1 IS DEPENDENT ON bHLH/PAS PROTEINS

Experiments involving the forced expression of a plasmid DNA encoding *ORF1* cDNA revealed that ORF1 is present in cytoplasmic stress granules in ribonucleoprotein complexes (Hohjoh and Singer, 1996; Goodier et al., 2007). Given that ORF1 acts *in cis* on L1-mRNA, it is conceivable that ORF1 is recruited from the cytoplasm to chromatin. Consistently, we observed that FICZ-induced enrichment of ORF1 in the chromatin-rich fraction and also triggered the physical association of ORF1 and ARNT1 (Okudaira et al., 2010). Additional experiments revealed that carcinogen-induced L1-RTP was dependent on AhR (Stribinskis and Ramos, 2006; Okudaira et al., 2011). These observations led us to hypothesize that ORF1 is functionally associated with bHLH/PAS proteins, and that both bHLH/PAS proteins and MAPK are involved in the chromatin recruitment of ORF1.

The importance of bHLH/PAS proteins in L1-RTP suggests that L1-RTP in the genome induced by environmental compounds may be directed. As summarized in **Table 1**, environmental carcinogens induce L1-RTP in an AhR-dependent manner. By contrast, FICZ-induced L1-RTP was not dependent on AhR. Instead, it requires ARNT1 and an additional cellular factor. Interestingly, the dependence of carcinogen-induced L1-RTP on ARNT1 differed among compounds: L1-RTP induced by DMBA required ARNT1, whereas that induced by 3-MC and B[*a*]P depended on AhR and ERα. These observations suggest that DMBA induces L1-RTP in the vicinity of XRE, which the AHRC targets for induction of mRNA expression of genes, such as *CYP* genes. In contrast, 3-MC and B[*a*]P would induce L1-RTP in the genome region determined by AhR and ERα. Moreover, the region in which FICZ-induced L1-RTP occurs differs from that in which carcinogen-induced L1-RTP occurs (**Figure 1**). It is important to analyze the regions of the newly integrated L1 to obtain novel information on the roles of L1-RTP in carcinogen-induced genetic alternations.

L1-RTP IS LINKED TO THE DEVELOPMENT OF VARIOUS DISEASES

GENETIC ERRORS

L1-RTP accidentally disrupts structures of functional genes and gives rise to inborn errors (Goodier and Kazazian, 2008). Since the discovery of aberrant insertions of L1 in the gene encoding factor VIII in 2 of 240 sporadic hemophilia A patients (Kazazian et al., 1988), at least 20 genetic disorders have been identified as resulting from L1 insertional mutagenesis (Hancks and Kazazian, 2012). Including genetic alterations by *Alu* and *SVA*, 96 genetic disorders have been so far identified (Hancks and Kazazian, 2012). In addition, abnormal L1 insertion was detected in a patient with branchio-oto-renal syndrome, in which L1 disrupted the locus responsible for the disease (Morisada et al., 2010). Of note, a recent study of 18 unrelated patients with neurofibromatosis type 1 (*NF1*) identified insertions of 14 *Alu*, three L1, and one poly(T) stretch within the *NF1* gene, indicating the retrotransposon insertions account for ~0.4% of all *NF1* mutations (Wimmer et al., 2011). Importantly, inserted L1, which was responsible for *NF1* disruption, was Ta-1, which is the youngest subfamily of human L1 (Boissinot et al., 2000; Lee et al., 2007; Wimmer et al., 2011). Moreover, six different insertions were identified within the 1.5-kb region between exons 21 and 23. As the *NF1* gene is 280 kb in length, it was proposed that the insertions of endogenous retroelements into the *NF1* gene occurred non-randomly (Wimmer et al., 2011).

TUMORIGENESIS

During early studies of the alterations of cancer-related genes, aberrant L1 insertions in the c-*myc* and *APC* genes were found in breast and colon carcinoma, respectively (Morse et al., 1988; Miki et al., 1992). It was recently proposed that L1 functions as a natural mutagen for genetic alterations, and L1-RTP has been detected in lung carcinomas and pancreatic adenocarcinomas (Iskow et al., 2010; Ting et al., 2011). Interestingly, L1-RTP is associated with the expression of major satellite repeats and malignant tumor phenotypes (Ting et al., 2011). L1-RTP induces a variety of genetic alternations, including gene deletions, inversions, and inter/intra-chromosome translocations (Gilbert et al., 2002, 2005; Symer et al., 2002). Moreover, ORF2 induces DNA damage (Gasior et al., 2006). In addition to these direct effects of L1, the human genome is susceptible to genetic alterations by *Alu–Alu* non-allelic homologous recombination (NAHR; Belancio et al., 2010; Konkel and Batzer, 2010). For example, it was shown that *Alu–Alu* NAHR induced structural alterations in the tumor suppressor gene *BRCA1*, and the *MLL-1* and *Myb* genes, in cancers cells (Strout et al., 1998; Mazoyer, 2005; O'Neil et al., 2007). Notably, the frequency of deletion via *Alu–Alu* NAHR under conditions of p53 deficiency was 40–300 times higher than that observed in the presence of wild-type p53 (Gebow et al., 2000), suggesting the importance of *Alu–Alu* NAHR during multistep carcinogenesis (Hanel and Moll, 2012). Environmental stimuli possibly function as risk factors for carcinogenesis by modulating genetic alternations via L1-RTP.

Although there is no model system to evaluate the association of L1-RTP with tumorigenesis, we recently showed that L1-RTP was common in skin tumors induced by DMBA and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Balmain et al., 1984; Okudaira et al., 2011). Of 15 skin tumors, 13 were positive for L1-RTP. It has been proposed that DMBA induces *H-ras* activation (Nelson et al., 1992), whereas TPA promotes the growth of transformed cells in a manner depending on TPA susceptibility locus (*Psl*) that is located on chromosome 9 (Angel et al., 1997). Recently, glutathione *S*-transferase α4 was identified as a candidate *Psl* gene (Abel et al., 2010). Interestingly, it was recently reported that activation of the *H-ras* gene in normal cells activates an ATM-dependent growth-arrest signal (Bartkova et al., 2006; Di Micco et al., 2006). Since ATM-dependent signaling causes senescence, an additional genetic alteration is required for tumor development; otherwise, tumor growth would be prevented. It remained elusive to clarify how TPA-induced L1-RTP is linked with the functional modification of the *Psl* gene product and attenuation of the ATM-dependent growth-arrest signal. Although it remains unclear whether L1-RTP is really involved in tumorigenesis, DMBA/TPA-induced skin carcinogenesis would be a good animal model for clarification of the role of L1-RTP in tumor development.

AUTOIMMUNE DISEASES

Intriguingly, a positive link between L1-RTP and the development of autoimmune diseases was proposed recently (Crow, 2010). Originally, heterozygous mutations of *Trex1*, a gene that encodes the 3′ repair exonuclease 1, were detected in familial chilblain lupus and Aicardi–Goutières syndrome (Rice et al., 2007). Interestingly, *Trex1*-deficient mice provided evidence that Trex1 functions as a negative regulator of the interferon-regulatory DNA response (Stetson et al., 2008). Without Trex1 function, mice die due to severe autoimmunity. However, such lethal effects of Trex1 deficiency were attenuated in mice with *IRF3*, *IFNαR1*, and *RAG2* gene deficiencies (Stetson et al., 2008). Moreover, single-stranded DNA derived from endogenous retroelements accumulated in *Trex1*-deficient cells, and *Trex1* overexpression blocked RTP of L1 (Stetson et al., 2008). Consistent with these experimental data, Trex1 activity was reduced in the synovial fibroblasts of rheumatoid arthritis patients (Neidhart et al., 2010). These observations suggest that RTP-induced production of DNA derived from endogenous retroelements is a potential molecular mechanism for the development of autoimmune disorders.

FUTURE PERSPECTIVES

L1-RTP depends on three steps: transcription, RT, and integration. It is known that the expression of L1 is regulated by the methylation of CpG islands in the 5′ UTR region (Hata and Sakaki, 1997; Woodcock et al., 1997; Muotri et al., 2010). Moreover, the 5′ UTR of L1 contains a ubiquitously active antisense promoter that encodes siRNAs that effectively suppress L1-RTP (Yang and Kazazian, 2006). Although these reports indicate that L1 activity is regulated at the transcriptional level, our observations and those of others indicate that L1-RTP is also regulated post-transcriptionally. Environmental compounds induce the chromatin recruitment of ORF1 via bHLH/PAS proteins, suggesting that the functional coupling of L1-RTP and bHLH/PAS proteins is another pivotal step in the regulation of L1-RTP.

Since bHLH/PAS proteins are transcription factors that strictly recognize *cis* elements, it is plausible that the induction of L1-RTP in the genome depends on the selection of bHLH/PAS proteins by the individual compounds. DMBA-induced L1-RTP depends on both AhR and ARNT1, and it is likely that L1-RTP is induced in the vicinity of the XRE (**Figure 1**). Further study is required to identify a target locus for the induction of L1-RTP, which would provide novel information regarding the biological relevance of L1-RTP in somatic cells.

It is interesting to note that *Candida albicans* possesses a non-LTR-type retroelement with a structure similar to that of human L1 (Dong et al., 2009). This was shown to be functional in *Saccharomyces cerevisiae*, indicating that an endogenous retroelement similar to human L1 is functional in lower eukaryotes. Conversely, *AhR* homologs are present in the genomes of lower eukaryotes such as *D. melanogaster* and *Caenorhabditis elegans* (Hahn, 2002). Combined with our observation that L1-RTP and bHLH/PAS are linked functionally, these results suggest that genome shuffling by bHLH/PAS-dependent L1-RTP may facilitate adaptation to environmental changes. It is tempting to speculate that bHLH/PAS molecules recognize environmental pollutants and promote genome shuffling by RTP to generate novel cellular properties that can overcome changes in the environment (**Figure 2**). Such property of L1 might contribute to revolution.

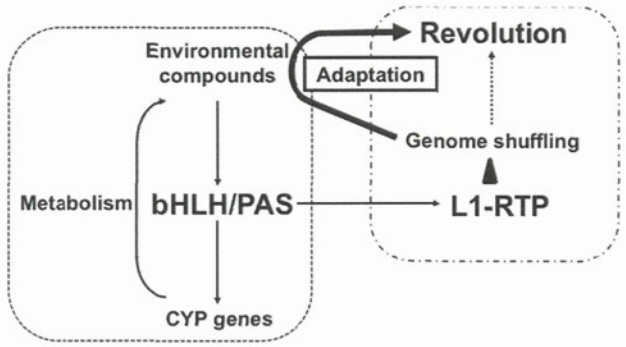

FIGURE 2 | Possible functional link between bHLH/PAS proteins and L1-RTP in environmental adaptation and evolution. As a well-characterized biological function, bHLH/PAS proteins of AhR and ARNT1 induce gene expression of *CYP* genes in response to various compounds. The induced CYP proteins metabolize the environmental compounds and detoxify them. By contrast, L1-RTP induces genome shuffling, and random mutagenesis increases the chance of the emergence of living organisms that possess novel characteristics. As shown here, bHLH/PAS proteins are involved in the induction of L1-RTP, indicating the functional link between these two well-conserved cellular activities. The novel properties can overcome the disadvantageous effects of environmental compounds, enabling living organisms to survive under the selective pressure in the altered environment and contributing to evolution.

After the divergence of humans and chimpanzees about 6 myrs ago, both L1s continued to propagate, and Ta-1d emerged as the major subfamily in *H. sapiens* (Boissinot et al., 2000). Together with our observation that L1-RTP is induced in somatic cells by environmental factors, this affords the opportunity to speculate about novel aspects of L1 biology. Since human- and chimpanzee-specific L1 copy numbers are similar (Lee et al., 2007), it is likely that L1-RTP in the germ cells of both species is strictly regulated. However, the observation that *H. sapiens* has more copies of active L1 than do chimpanzees suggests that "hot" L1 offers an unidentified advantage to human activity. Interestingly, L1-RTP was induced in the dentate gyrus of the hippocampus when mice performed voluntary exercise (Muotri et al., 2009), and some data suggest that L1 copy numbers are increased in the human hippocampus (Coufal et al., 2011). One plausible direction for future research on L1 would be to focus on its role in the central nervous system (Hancks and Kazazian, 2012).

ACKNOWLEDGMENTS

This work was supported in parts by a Grant-in-Aid for Research from the Ministry of Health, Labour, and Welfare of Japan (09156296), a research grant for the Log-range Research Initiative (LRI) from Japan Chemical Industry Association (JCIA) and a Grant-in-Aid from the Tokyo Biochemical Research Foundation. Mr. Noriyuki Okudaira is an applicant supported by the Grant-in-Aid from the Tokyo Biochemical Research Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 April 2012; paper pending published: 20 April 2012; accepted: 10 May 2012; published online: 31 May 2012.

Citation: Ishizaka Y, Okudaira N, Tamura M, Iijima K, Shimura M, Goto M and Okamura T (2012) Modes of retrotransposition of long interspersed element-1 by environmental factors. Front. Microbio. 3:191. doi: 10.3389/fmicb.2012.00191

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

Cancer Science
The official journal of the Japanese Cancer Association

Involvement of retrotransposition of long interspersed nucleotide element-1 in skin tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

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(Received April 21, 2011/Revised June 10, 2011; July 19, 2011/Accepted August 1, 2011/Accepted manuscript online August 9, 2011/Article first published online September 19, 2011)

Tumor development induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-characterized model of multistep carcinogenesis. DMBA mutates the *Ha-ras* gene, whereas TPA promotes the growth of transformed cells by activating cellular signaling molecules. It remains to be clarified how repeated TPA treatment endows transformed cells with autonomous cell growth. Long interspersed nucleotide element-1 (L1) is an endogenous retroelement, and 80–100 copies of L1 function as autonomous mobile elements. Although the L1 retrotransposition (RTP) has been found in various human tumors, implying the possible mobility of L1 during carcinogenesis, little is known about how L1-RTP arises in tumor cells, owing to a lack of experimental models. To dissect the mechanism of L1-RTP during carcinogenesis, we established a line of transgenic mice carrying human L1 and enhanced green fluorescent protein (hL1-EGFP mice) and subjected them to DMBA/TPA-induced skin tumorigenesis. Of 15 skin tumors examined, 13 were positive for L1-RTP; L1-RTP was not detected in normal skin tissues adjacent to the tumors. Moreover, nine L1-RTP-positive tumors were positive for activated *Ha-ras*, and immunohistochemical analysis revealed cells positive for both L1-RTP and phosphorylated Stat3, a marker of tumor cells. Additional *in vivo* experiments suggested that L1-RTP occurred during tumor promotion by TPA. This is the first report on the involvement of L1-RTP in chemical carcinogenesis. We propose hL1-EGFP mice as a versatile system for investigating the mode of L1-RTP in tumor development and discuss the possible role of L1-RTP in tumorigenesis. (*Cancer Sci* 2011; 102: 2000–2006)

Long interspersed nucleotide element-1 (L1) is the most abundant transposable element, comprising approximately 17% of the human genome.[1,2] A single human cell contains more than 5×10^5 copies of L1, and 80–100 copies are competent for retrotransposition (L1-RTP).[3] Long interspersed nucleotide element-1 has been identified in the c-*myc* and *APC* genes in breast carcinoma and colon carcinoma, respectively.[4,5] Recently, L1 insertion was found in the pericentric region of various human tumors, and it was suggested that the newly inserted L1 altered global gene expression in the tumor cells by modulating RNA expression from satellite repeats.[6] Moreover, L1-RTP induces a variety of genetic alterations, including gene deletions, inversions, and insertions,[7,8] further supporting the involvement of L1-RTP in carcinogenesis. However, the mechanism of L1-RTP induction during carcinogenesis is yet to be elucidated, owing to the lack of animal models in which the mobility of L1 could be monitored *in vivo*.

The development of skin tumors in response to treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-characterized model of multistep carcinogenesis.[9] A single dose of DMBA mutates the *Ha-ras* gene at codon 61 (A to T).[10] Repeated treatment with TPA supports the growth of transformed cells by activating inflammatory signaling molecules,[11] including epidermal growth factor receptor (EGFR),[12] ERK,[13] and signal transducer and activator of transcription 3 (Stat3).[14] It is well established that tumor susceptibility differs among mouse strains; for example, DBA/2 mice are sensitive to TPA-induced tumor promotion, whereas C57BL/6 mice are resistant.[15–19] Backcross experiments have identified several loci of TPA-susceptible genes,[19] but how TPA treatment enables transformed cells to grow autonomously is as yet unknown.

To identify factors that modulate L1-RTP during tumor promotion, we established a line of transgenic mice carrying human L1 as a transgene (hL1-EGFP mice) and subjected the mice to DMBA/TPA-induced tumorigenesis. Intriguingly, L1-RTP was frequently detected in skin tumors, but not in normal skin tissues adjacent to the tumors. The results of biochemical analyses and *in vivo* experiments involving brief exposure to the compounds suggested that L1-RTP occurred during tumor promotion directed by repeated TPA treatment. This is the first report on the induction of L1-RTP during chemical carcinogenesis. We conclude this report with a discussion of the possible role of L1-RTP in TPA-induced tumor promotion.

Materials and Methods

Cell lines and chemicals. HuH-7 cells (RCB1366; Riken BioResource Center Cell Bank, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FCS (Sigma, St. Louis, MO, USA). The transfection efficiency of this cell line was approximately 70%, as assessed by transient expression of plasmid DNA encoding EGFP and subsequent FACS analysis, carried out on day 2 post-transfection using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). We purchased TPA, DMBA, and the kinase inhibitors SB202190,[20] SP600125,[21] PD98,059,[22] and CL387,785[23] from Sigma.

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doi: 10.1111/j.1349-7006.2011.02060.x

Protease inhibitors were from Roche Diagnostics (Basel, Switzerland).

L1-RTP assays. Colony and PCR-based assays were carried out using pCEP4/L1*mneo*I/ColE1 (pL1-Neo^R)[24] and pER06R,[25] as described previously[26] (described in detail in Data S1). Both constructs contained human L1 with a CMV promoter (Fig. 1A). In the colony assay, each sample was assayed in quadruplicate, and the mean number of colonies and SD were calculated. Statistical analysis was carried out using the Mann–Whitney *U*-test. Each experiment was repeated more than twice. In the PCR-based assay, the relative intensity was calculated based on the signal intensities of amplified DNA derived from *EGFP* and *β-actin*.

Downregulation of endogenous protein expression by siRNAs. Three different siRNAs, synthesized by Applied Biosystems (Foster City, CA, USA), were prepared for each gene, and their functions were evaluated. The nucleotide sequences of the siRNAs specific for the aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator 1 (*ARNT1*) are given Table S1. In each experiment, two independent siRNAs that targeted different sequences were used. Silencer Negative Control siRNA #2 (Cat# AM4637; Applied Biosystems, Foster City, CA, USA) was used as a control.

Production of hL1-RTP-transgenic mice. An 8.8-kb *Not*I-*Apa*I fragment of pEF06R was microinjected into the pronuclei of fertilized eggs of BDF1 × C57BL/6 mice (Japan SLC, Hamamatsu, Japan), as described.[27] The injected DNA Japan fragment possessed the 5′-untranslated region of hL1, allowing expression of the L1 transgene under the control of its own promoter (Fig. 1A, arrow). Among 16 founder mice, #4 and #67 were selected as suitable strains (the procedures are described in detail in Data S1) and were backcrossed to C57BL/6 mice. Then N3 generations were subjected to further experiments.

DMBA/TPA-induced skin carcinogenesis. Seven mice (two from strain #4 and five from #67) were subjected to DMBA/TPA-induced skin carcinogenesis. After 7 days of treatment with DMBA (400 nmol in 200 μL acetone), treatment with TPA (17 nmol in 200 μL acetone) was initiated.[18] TPA was applied topically twice per week until tumors developed. We did not observe definite induction of L1-mRNA in skin tissues treated with TPA (R. Yanobu-Takanashi, data not shown). Tumor tissues and skin tissues were analyzed using a PCR-based assay to detect L1-RTP. The status of the *Ha-ras* gene was examined by PCR amplification followed by sequence analysis.[28] Histopathological analysis of skin tumors was carried out after staining with H&E (Biopathology Institute, Ohita, Japan).

Immunohistochemical analysis. When L1-RTP occurred, the cells became EGFP-positive (Fig. 1A, Data S1). Therefore, to characterize cells positive for L1-RTP, immunohistochemical analysis was carried out using an anti-EGFP antibody (αEGFP; Abcam, Cambridge, England, UK). An antibody specific for phosphorylated Stat3 (pStat3) (Cell Signaling Technology, Beverly, MA, USA) was used to identify tumor cells. The procedures are described in detail in the Data S1.

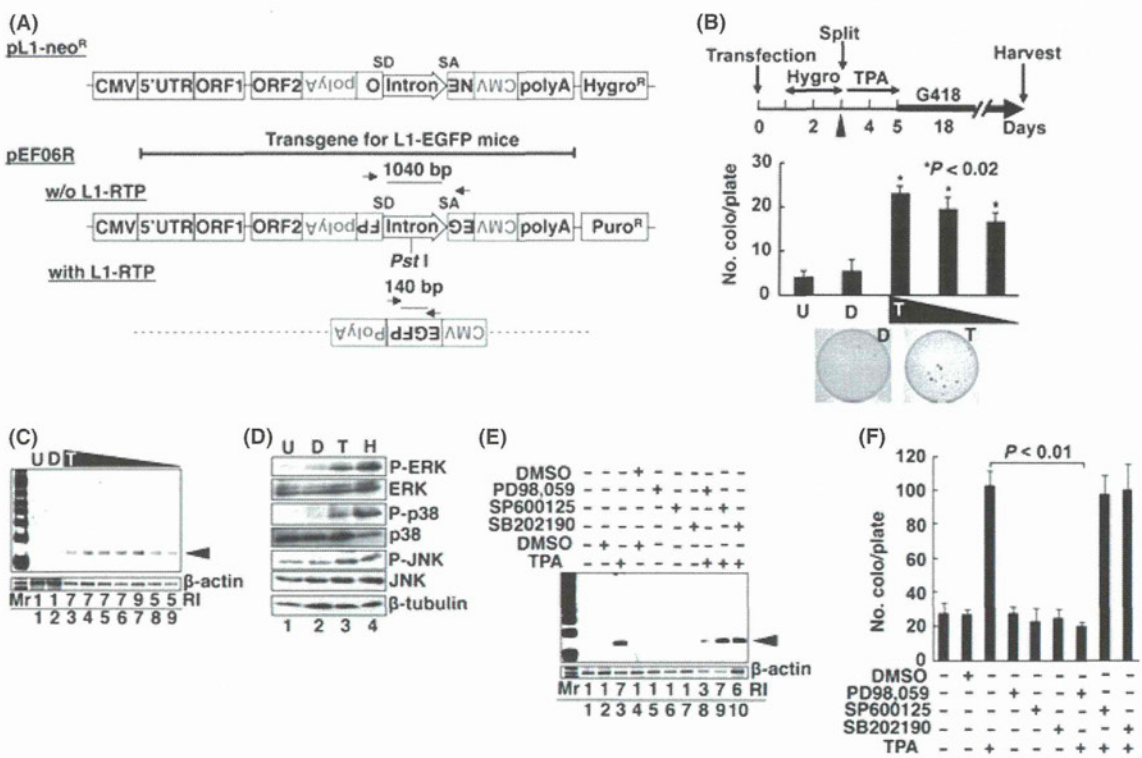

Fig. 1. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces long interspersed nucleotide element-1 retrotransposition (L1-RTP). (A) Schematics showing the constructs for detecting L1-RTP and the rationale for the PCR-based assay. In the PCR-based assay, a 1040-bp band was amplified without (w/o) L1-RTP, and a 140-bp band was amplified with L1-RTP. Arrows indicate the positions of the PCR-based assay primers. EGFP, enhanced green fluorescent protein; ORF, open reading frame; SA, splicing acceptor; SD, splicing donor; UTR, untranslated region. (B) Schematic of the protocol and colony assay results. TPA (10, 100, or 200 nM) was applied to approximately 1×10^5 cells, and G418 selection was carried out. Photographic images show G418-resistant colonies obtained with DMSO (D) or 200 nM TPA (T). Mean number of colonies + SD is shown. colo, colonies; U, untreated. (C) PCR-based assay of TPA-induced L1-RTP. Relative intensity (RI) was calculated based on the signal intensity of the 140-bp band (arrowhead), using *β-actin* as an internal control. Mr, marker. Lane 1, untreated (U); lane 2, DMSO (D); lanes 3–9, 200, 100, 10, 1, 0.1, 0.01, and 0.001 nM TPA, respectively. (D) Phosphorylation of MAPK substrates in response to TPA treatment (T). Cell extracts were prepared 30 min after treatment with 200 nM TPA (lane 3) or 1 mM H_2O_2 (lane 4). H, hydrogen peroxide as positive control. (E,F) Effects of MAPK inhibitors on TPA-induced L1-RTP, as assessed by PCR-based (E) and colony assays (F). PD98,058, SB202190, and SP600125 were applied at concentrations of 20, 1, and 100 μM, respectively. Mean number of colonies ± SD is shown.

Results

We first observed that TPA induced L1-RTP (Fig. 1B, $P < 0.02$). As assessed by the number of G418-resistant colonies, the frequency of TPA-induced L1-RTP was 10^{-4}–10^{-5}. A PCR-based assay confirmed TPA-induced L1-RTP by detecting a 140-bp band generated as a result of L1-RTP (Fig. 1C; for rationale see Fig. 1A).[26] No obvious cytotoxicity was detected in cells treated with TPA at the concentrations used in the L1-RTP experiments (Fig. S1A). Western blot analysis indicated that TPA induced the phosphorylation of ERK, p38 MAPK, and JNK (Fig. 1D, lane 3). Interestingly, the ERK inhibitor PD98,059[22] considerably attenuated TPA-induced L1-RTP (Fig. 1E, lane 8). In contrast, TPA-induced L1-RTP was not blocked by SB202190 or SP600125, which are inhibitors of p38 and JNK,[20,21] respectively (Fig. 1E, lanes 9, 10). The effects of these inhibitors on L1-RTP were confirmed in a colony assay (Fig. 1F, $P < 0.01$).

Similar to TPA, DMBA induced L1-RTP (Fig. 2A) at doses that did not cause cytotoxicity (Fig. S1B). DMBA is a bulky compound that is presumed to bind to AhR, eliciting cellular signals in concert with ARNT1, as reported for TCDD (dioxin).[29] To test whether these molecules were involved in the induction of L1-RTP, the effects of *AhR*- and *ARNT1*-specific siRNAs were examined. Using a previously reported method,[26] we first confirmed that each siRNA reduced endogenous protein expression to <10% of the control level (Fig. 2B, upper panels). With the siRNAs, DMBA-induced L1-RTP was completely abrogated (Fig. 2B, right panel, lanes 9, 12). In contrast, TPA-induced L1-RTP was not blocked by the siRNAs (Fig. 2C, lanes 11, 15). None of the MAPK inhibitors examined inhibited DMBA-induced L1-RTP (Fig. 2D, lanes 9, 11, 13). Additionally, CL387,785, an inhibitor of EGFR,[23] suppressed TPA-induced L1-RTP, but not L1-RTP induced by DMBA (Fig. 2E). These data indicate DMBA and TPA induce L1-RTP through different mechanisms.

To confirm the involvement of L1-RTP in tumorigenesis *in vivo*, we generated transgenic mice carrying human L1 as a transgene (hL1-EGFP mice). To clarify the role of L1 during tumorigenesis, we thought it important to select hL1-EGFP mice that had low or no background L1-RTP, but that displayed vigorous L1-RTP activity in response to external stimuli (Fig. S2). Of 16 original transgenic lines, we selected #4 and #67 (Fig. 3A) for use in further experiments. L1 transgene copy numbers, as estimated by Southern blotting and quantitative PCR, were approximately 10 and 20 for lines #4 and #67, respectively (Fig. S3, Table S2).

We then examined L1-RTP in DMBA/TPA-induced skin tumors in experiments carried out according to the reported protocol (Fig. 3B).[18] hL1-EGFP mice subjected to DMBA/TPA-induced skin carcinogenesis consisted of theoretically 87.5% of C57BL/6 background, which is known as a resistant strain[15–18] (see Materials and Methods). We used relatively high doses of DMBA (400 nmol) and TPA (17 nmol twice weekly) in order to ensure a substantial tumor response in the C57BL/6 background. From seven hL1-EGFP mice, 15 tumors were obtained 35 weeks after the initiation of TPA treatment (Fig. 3B, arrowheads). At least one tumor was formed per mouse, and the tumor

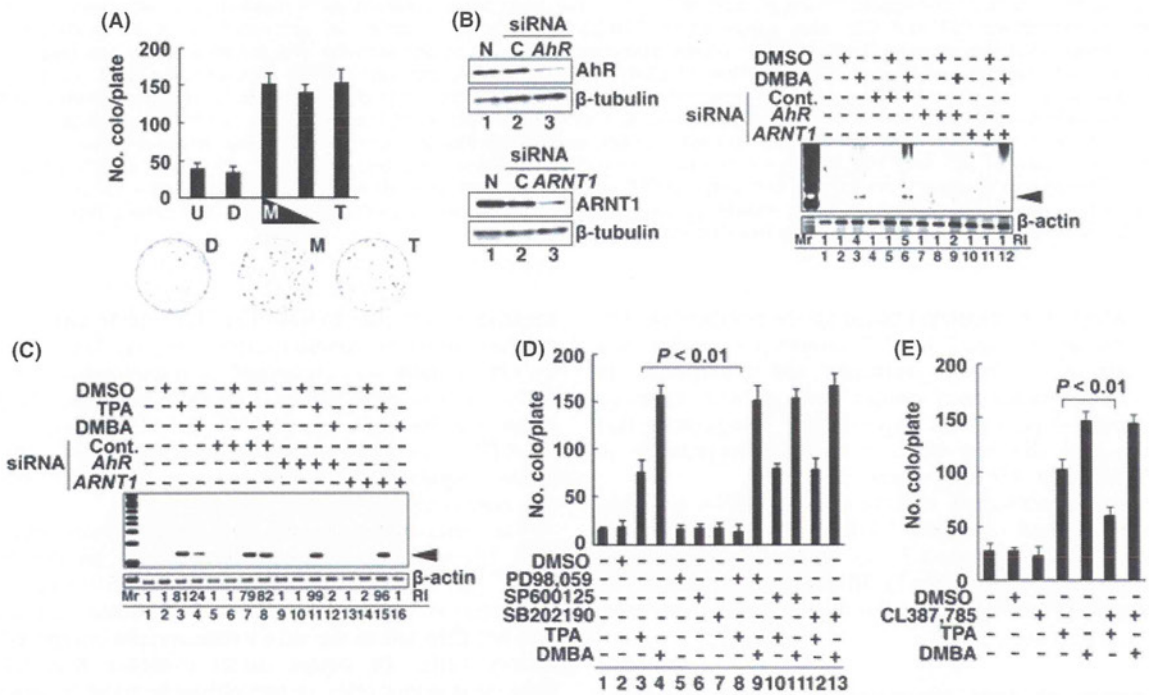

Fig. 2. 7,12-Dimethylbenz[a]anthracene (DMBA) induces long interspersed nucleotide element-1 retrotransposition (L1-RTP). (A) Colony assay of DMBA-induced L1-RTP. DMBA was used at concentrations of 1 and 10 nM. Photographic images show G418-resistant colonies obtained with DMSO (D) and 10 nM DMBA (M). Mean number of colonies (colo) + SD is shown. T, 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA); U, untreated. (B) Aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator (*ARNT1*) are both required for DMBA-induced L1-RTP. Upper panels, target siRNAs downregulated endogenous AhR and ARNT1 protein expression. Lane 1, control cells transfected with pEF06R (N); lane 2, cells cotransfected with control siRNA and pEF06R (C); lane 3, cells cotransfected with target siRNAs and pEF06R. Lower panel, PCR-based assay results. Data shown are representative of two independent siRNAs. Mr, marker; RI, relative intensity. (C) TPA-induced L1-RTP is not dependent on *AhR* or *ARNT1*. TPA-induced L1-RTP was examined under conditions of downregulated expression of endogenous *AhR* or *ARNT1*, as explained in (B). (D) Different cellular signaling pathways are involved in TPA- and DMBA-induced L1-RTP. A colony assay was carried out using a 1040-bp band amplified without (w/o) L1-RTP, and a 140-bp band amplified with L1-RTP. Mean number of colonies ± SD is shown. (E) Effect of epidermal growth factor receptor (EGFR) inhibition on TPA- and DMBA-induced L1-RTP. CL387,785 (100 nM), an inhibitor of EGFR, was added to the culture medium 60 min before the addition of 200 nM TPA or 1 nM DMBA. Mean number of colonies ± SD is shown. The effect of the inhibitor on TPA-induced L1-RTP was statistically significant ($P < 0.01$).

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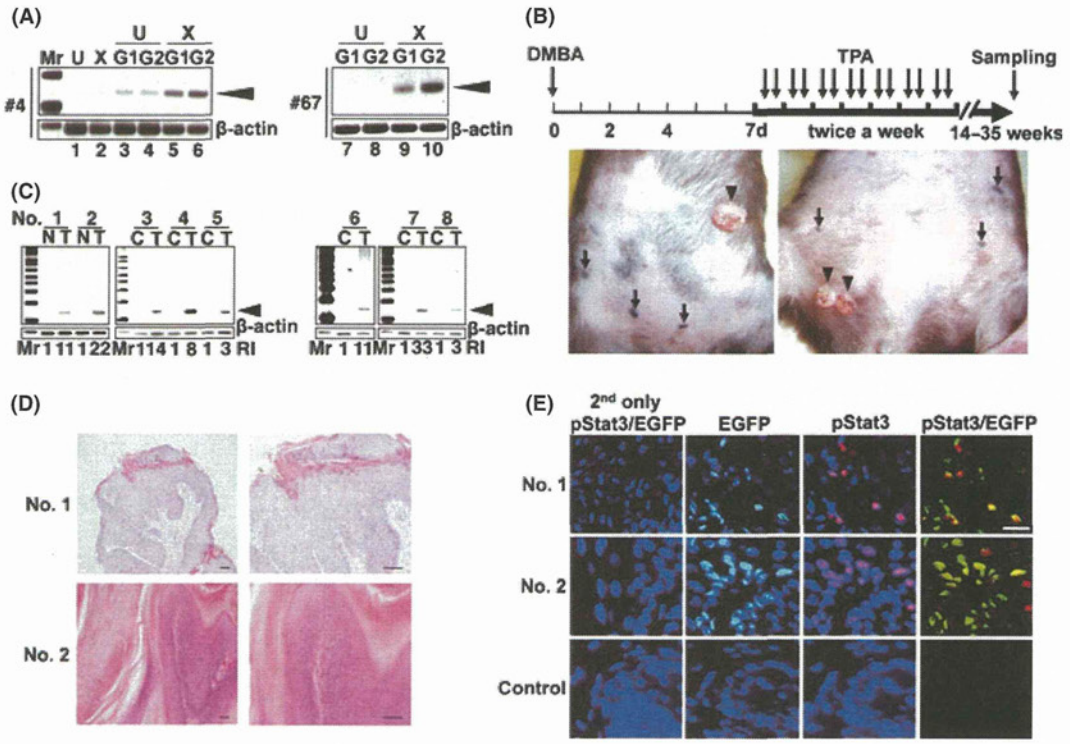

Fig. 3. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) is induced in 7,12-dimethylbenz[a]anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA)-induced skin tumors. (A) Generation of transgenic mice carrying human L1. L1-RTP induced in mouse embryonic fibroblasts of transgenic strains #4 and #67 after X-ray irradiation. Embryos were divided into two groups, and the pooled mouse embryonic fibroblasts (G1 and G2) were subjected to PCR-based assay. Mr, marker; U, untreated; X, X-ray treatment (4.5 Gy). (B) Experimental protocol and skin tumors. On day 7 after DMBA treatment, repeated treatment with TPA (twice a week) was begun. Skin tumors (arrowheads) and nevi (arrows) developed. (C) Induction of L1-RTP in skin tumors, but not normal skin or tail tissues. Each tumor (T) was enucleated and analyzed using the PCR-based assay. Control skin (N) and tail tissues (C) were also analyzed. RI, relative intensity. Tumors 1, 3, 5, and 6 were independent tumors derived from #4 mice; tumors 2, 4, 7, and 8 were obtained from #67 mice. (D) Histopathology of representative skin tumor lesions after staining with H&E. Upper panels, squamous cell papillomas formed in mouse strain #4 (No. 1). Lower panels, keratoacanthomas formed in #67 mice (No. 2). Tumor numbers correspond to those described in (C). Bar, 50 μm. (E) L1-RTP occurred in tumor cells. Immunohistochemical analyses were carried out using αEGFP (green) and αpStat3 (red) antibodies. Results for a squamous cell papilloma (No. 1, upper panels), a keratoacanthoma (No. 2, middle panels), and a control (lower panels) are shown. Left panels, results obtained using secondary antibody only. Nuclei were stained using Hoechst stain (blue). Bar, 20 μm.

multiplicity, which was calculated based on the numbers of subjected mice and tumors, was 2.3 ± 1.3 (tumors per mouse). In a study by Feith *et al.*, tumor incidence and multiplicity in C57BL/6 mice subjected to a similar protocol were approximately 95% and 6–7 per mouse, respectively,[18] suggesting that the hL1 transgene did not increase tumor susceptibility in DMBA/TPA-induced skin tumorigenesis.

Each tumor was enucleated, and the extracted DNA was subjected to a PCR-based assay for L1-RTP. As shown in Figure 3(C), L1-RTP was detected in 13 of the 15 independent tumors examined (see also Table 1). Histopathological analysis of seven samples positive for L1-RTP revealed that three were squamous cell papillomas (Fig. 3D, upper panel, No. 1) and another three were keratoacanthomas (Fig. 3D, lower panel, No. 2). One sample was composed of inflammatory cells. Interestingly, we also observed nevi (melanocyte hyperplasia, Fig. 3B, arrows), as has been reported.[30] The PCR-based assay detected L1-RTP in one of eight nevi examined (Fig. S4). The difference in the frequency of L1-RTP between tumors (13/15) and nevi was remarkable ($P < 0.01$; Table 1).

Table 1. Summary of long interspersed nucleotide element-1 retrotransposition in skin tumors and nevi induced by 7,12-dimethylbenz[a]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

Mouse lines	Tumors	Normal skin tissues	Nevi
#4	4/4†	0/6	0/1
#67	9/11	0/14	1/7
Total	13/15 (86.7)‡	0/20	1 (12.5)

†Positive number/number examined. ‡Number (%). $P < 0.001$, Fisher's exact test. Odds ratio (95% confidence interval) was 45.5.

The status of the *Ha-ras* gene in the tumors was also examined. Eleven of 13 tumors were positive for the A61T mutation.[28] Of these 11 tumors, nine were positive for L1-RTP. No mutations in codons 12 or 13 were detected. Activated *Ha-ras* was not detected in the skin tumor sample composed of inflammatory cells. To obtain direct evidence that L1-RTP was induced in tumor cells, immunohistochemical analyses were carried out for EGFP and pStat3, which were also detected in skin carcinogenesis.[14] As shown in Figure 3(E), EGFP and pStat3 were detected in the same tumor cells in a squamous cell papilloma (Fig. 3E, upper panels, No. 1) and a keratoacanthoma (Fig. 3E, middle panels, No. 2). In contrast, pStat3 was not detected in normal skin tissues (Fig. 3E, lower panels).

In the experimental protocol for DMBA/TPA-induced skin tumorigenesis,[9,18] DMBA was applied as a single dose, whereas TPA was applied repeatedly from day 7 after DMBA treatment. Given that both compounds can induce L1-RTP (Figs 1,2), we wanted to clarify which compound was responsible

for the induction of L1-RTP in the tumors. Cells were first treated with DMBA and subsequently transfected with pEF06R on day 7 (Fig. 4A, left panel). A PCR-based assay was carried out 2 days later. As shown in Figure 4(A), no induction of L1-RTP was observed in cells that had been treated with DMBA 7 days previously (right panel). Similar observations were made in *in vivo* experiments. We did not detect L1-RTP on day 7 after treatment with DMBA (Fig. 4B, lanes 7–9). In striking contrast, L1-RTP was detected after dual treatment with TPA (Fig. 4B, lanes 10–12). These data indicate that the effects of DMBA on the induction of L1-RTP had disappeared before the initial treatment with TPA, and that L1-RTP occurred in the tumors during repeated treatment with TPA.

Discussion

In the present work, the induction of L1-RTP was shown to accompany DMBA/TPA-induced skin tumorigenesis. Specifically, L1-RTP was detected in 13 of 15 skin tumors, but not in any of the 20 normal skin tissues examined. Of particular interest was the observation that, whereas both DMBA and TPA activated L1-RTP *in vitro*, the brief exposure of hL1-EGFP mice to these same compounds indicated that L1-RTP was induced by TPA *in vivo* (Fig. 4). Although several studies have identified L1 insertions in human tumors,[1,7,8] none have provided insight into the mode of L1-RTP. To the best of our knowledge, ours is the first report showing the induction of L1-RTP during the promotion step of carcinogenesis. Considering that TPA is known to induce cellular inflammation through a mechanism involving EGFR and ERK (Fig. 2D,E),[12,13] an eminently reasonable hypothesis of the mode of L1-RTP in human tumors is that environmental stimuli such as chronic inflammation can activate L1-RTP. Given that our hL1-EGFP mice had low background levels of spontaneous L1-RTP in mouse embryonic fibroblasts (Fig. 3A), but responded well to external stimuli at 4–5 weeks after birth, these mice offer a versatile system for elucidating the molecular mechanism of tumor promotion.

Immunohistochemical analysis revealed Stat3 phosphorylation in cells positive for EFGP (Fig. 3E), a marker of L1-RTP induction. It has been shown that Stat3 is involved in not only DMBA/TPA-induced skin carcinogenesis[14,31,32] but also in the induction of cellular transformation by oncogenes.[33,34] To exclude the possibility that L1-RTP was initiated secondarily to the activation of Stat3, L1-RTP was examined under the forced expression of a constitutively active form of Stat3 (Stat3-C).[35] A PCR-based assay for L1-RTP indicated that L1-RTP was not induced by Stat3-C (Fig. S5). Additionally, the forced expression of activated *Ha-ras* did not cause Stat3 to be phosphorylated (Fig. S6), as has been reported.[34] Together, these observations support the notion that L1-RTP is not induced as a consequence of cellular transformation. Instead, L1-RTP induction appears to be an upstream event of Stat3 activation. Importantly, it was shown that activation of the *Ha-ras* gene in normal cells activates a growth arrest signal that depends on ataxia telangiectasia mutated (ATM).[36,37] An additional genetic alteration is required for tumor development, otherwise ATM-dependent

Table 2. Summary of long interspersed nucleotide element-1 retrotransposition (L1-RTP) and activated *Ha-ras* in skin tumors and nevi induced by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

Mouse lines	Tumors				Nevi		
	No.	L1-RTP	*Ha-ras*†	L1-RTP + *Ha-ras*	No.	L1-RTP	*H-ras*
#4	4	4	3	3	1	0	0
#67	11	9	8	6	7	1	0
Total	15	13 (86.7)‡	11 (73.3)	9 (60.0)	8	1 (12.5)	0

†From A to T at the first letter of codon 61 of *Ha-ras*. ‡Frequency expressed as percentage.

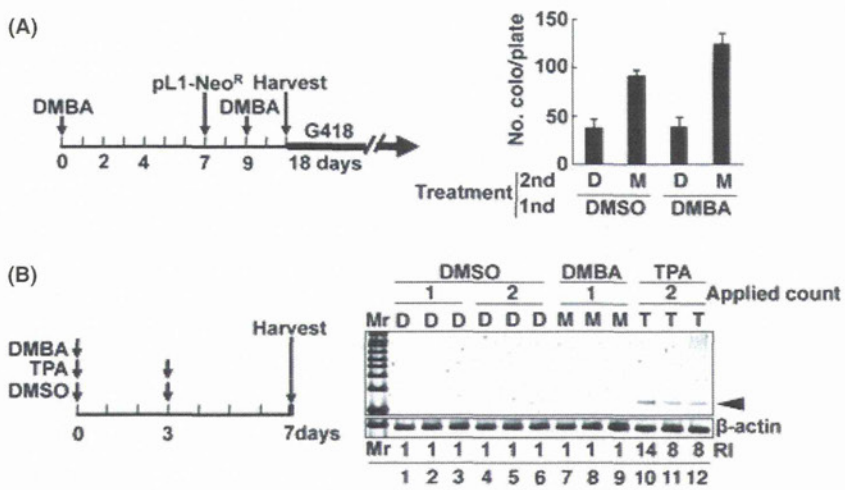

Fig. 4. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) occurred in skin tumors during repeated treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). (A) The effects of 7,12-dimethylbenz[*a*]anthracene (DMBA) on the induction of L1-RTP were transient. Left panel, experimental protocol used to examine the effects of DMBA. Right panel, colony (colo) assay results. HuH-7 cells were exposed to 1 nM DMBA and cultured for a further 7 days. They were then transfected with pL1-Neo^R and divided into two aliquots. One aliquot was directly subjected to G418 selection without exposure to DMBA. As a positive control, the second aliquot was treated with the same concentration of DMBA then subjected to G418 selection. Mean number of colonies ± SD is shown. (B) Induction of L1-RTP by TPA in mice carrying human L1 and enhanced green fluorescent protein (hL1-EGFP mice). L1-RTP was assayed in hL1-EGFP mice that had been treated with TPA (T) or DMBA (M). Skin tissues of three different portions were harvested on day 2 after treatment with TPA or day 7 after treatment with DMBA. DNA extracted from each sample was subjected to PCR-based analysis. Relative intensity (RI) was calculated based on the signal intensity of the 140-bp band (arrowhead), using *β-actin* as an internal control. D, DMSO. Note: TPA treatment induced skin inflammation, in contrast to the reaction of skin tissues after repetitive treatment during chemical carcinogenesis.

doi: 10.1111/j.1349-7006.2011.02060.x

signaling would cause senescence rather than tumor growth. In the current study, the activation of *Ha-ras* was also detected in skin tumors (Table 2); thus, one plausible explanation is that the genetic alteration caused by TPA-induced L1-RTP attenuates ATM-dependent growth arrest by co-activating Stat3.

In vitro experiments revealed that DMBA and TPA induced L1-RTP by different mechanisms: DMBA-induced L1-RTP was dependent on AhR; TPA-induced L1-RTP was not. AhR functions as a receptor for environmental pollutants including dioxin,[38] and ligand-bound AhR is recruited to chromatin depending on the nuclear localization signals of ARNT1.[39] A heterodimer of AhR and ARNT1 then induces gene expression through xenobiotic responsive elements.[38] Notably, we observed that L1-RTP was induced by other genotoxic carcinogens, such as 3-methylcholanthrene and benzo[*a*]pyrene (B[*a*]P, with a similar dependency on AhR (Fig. S7). In contrast, we recently reported that 6-formylindolo(3,2-*b*)carbazole (FICZ), a photoproduct of tryptophan, induced L1-RTP, but in this case, L1-RTP was AhR-independent.[26] One possibility is that AhR mediates carcinogenic activity of the genotoxic compounds by inducing L1-RTP. To date, no reports on the carcinogenicity of FICZ are available. A dependency of carcinogen-induced L1-RTP on AhR is consistent with a previous report in which B[*a*]P-induced skin tumorigenesis was shown to require intact *AhR* alleles.[40]

An important next step will be to clarify the roles of TPA-induced L1-RTP. Recently, the glutathione S-transferase α4 (*Gstα4*) gene was identified as a candidate gene targeted by TPA.[41] *Gstα4* is located in a region of chromosome 9 that was identified by a genetic approach based on mouse strains with different susceptibilities to tumor promotion by TPA.[19] Notably, DMBA-induced tumor development is enhanced in mice lacking the *Gstα4* gene.[41] These observations suggest that the *Gstα4* gene is a candidate target for TPA-induced L1-RTP. Our PCR-based analyses, however, detected no apparent structural alterations in the *Gstα4* gene in DMBA/TPA-induced skin tumors (Fig. S8). Further analysis is required to identify the gene(s) responsible for tumor promotion by TPA, which in turn will provide insight into the functions of non-genotoxic carcinogens in tumor promotion.

Acknowledgments

We are grateful to Drs. Elena T. Luning Prak (University of Pennsylvania Medical Center, Philadelphia, PA, USA), Gilbert Nicolas (University of Michigan Medical School, Ann Arbor, MI, USA), and Jacqueline F. Bromberg (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) for providing us with pEF06R, pCEP4/L1*mneo*I/ColE1, and Stat3-C/pBabe, respectively. We thank Drs. Takayoshi Koyama and Hideaki Kawada and Miss Yukiko Shimizu (National Center for Global Health and Medicine, Tokyo, Japan) for technical assistance. We are also grateful to Drs. Tetsuya Mizoue and Yi Siyan (National Center for Global Health and Medicine) for assisting with statistical analysis of the experimental data. This work was supported in part by a Grant-in-Aid for Research from the Ministry of Health, Labour, and Welfare of Japan (09156296), a grant from the National Center for Global Health and Medicine (21A-104), and a grant from the Long-range Research Initiative from the Japan Chemical Industry Association. Mr. Noriyuki Okudaira was supported by a Grant-in-Aid from the Tokyo Biochemical Research Foundation.

Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. No cytotoxicity was detected in cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[*a*]anthracene (DMBA).

Fig. S2. Selection of human long interspersed nucleotide element-1–enhanced green fluorescent protein (hL1-EGFP) mouse strains suitable for carcinogenesis experiments.

Fig. S3. Southern blot analysis of copy numbers of transgenes in #4 and #67 in mice carrying human long interspersed nucleotide element-1 and enhanced green fluorescent protein (hL1-EGFP).

Fig. S4. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) in nevi. Eight nevi samples were analyzed by PCR-based assay.

Fig. S5. Forced expression of constitutively activated form of Stat3 did not induce long interspersed nucleotide element-1 retrotransposition (L1-RTP).

Fig. S6. Forced expression of activated *H-ras* did not phosphorylate Stat3.

Fig. S7. Aryl hydrocarbon receptor (AhR) is required for long interspersed nucleotide element-1 retrotransposition (L1-RTP) by genotoxic carcinogens.

Fig. S8. No apparent abnormality of *Gsta4* gene in tumors induced by 7,12-dimethylbenz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA).

Table S1. Nucleotide sequences of siRNAs targeting aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator 1 (*ARNT1*).

Table S2. Quantitative PCR of copy numbers of transgene in #4 and #67 in mice carrying human long interspersed nucleotide element-1 and enhanced green fluorescent protein (L1-EGFP).

Data S1. Full details of experimental methods used in this work.

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doi: 10.1111/j.1349-7006.2011.02060.x