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Regulation of Retrotransposition of Long Interspersed Element-1 by Mitogen-Activated Protein Kinases

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1. Introduction

Our genome contains a higher amount of endogenous retroelements (～42 %) than mouse (～37 %) or fruit fly (～3.6 %) (1-3). Long interspersed element-1 (L1) is the most abundant of transposable elements, comprising ～17% of the genome (1-4). L1 is an autonomous endogenous retroelement that has evolved in a single, unbroken lineage for the past 40 million years in primates (5). A single human cell has more than $5x10^5$ copies of L1 (2,4), and most of them are functionally defective (6). However, 80 to 100 copies of L1 are competent for retrotransposition (L1-RTP) (7), and approximately 10 % of these are highly active for "copy and paste" (7). L1 is actively expressed in embryonal stem cells (8) and L1-RTP is induced in oocytes or early embryonic development (9-11). L1-RTP occurring in germ lines would function an intrinsic factor responsible for allelic variants among individuals (12,13). However, aberrant L1-RTP alternates critical gene structures, leading to the development of inborn errors (14). At the moment, at least 17 genetic diseases have been reported as sporadic cases of inheritable disorders caused by aberrant insertion of L1 (14). On the other hand, recent observations suggest that L1-RTP occurs in somatic cells. Strikingly, it was shown that copy numbers of L1 is increased in human brain tissues (15,16). Aberrant L1 insertions have been detected in c-*myc* gene and the *APC* gene in breast carcinoma and colon carcinoma, respectively (17,18). Moreover recent analysis demonstrated that L1 is frequently mobilized in human lung cancers and pancreatic carcinomas (19,20). These observations indicate that it is important to understand the mode of L1-RTP, but little is known about the cellular factors for the induction of L1-RTP in somatic cells. We herein summarize our current understanding of L1-RTP induction, with an emphasis on mitogen-activated protein kinases (MAPKs), which are activated by environmental compounds, and we discuss their roles in genome shuffling.

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2. Biology of L1-RTP

L1, a non-long terminal repeat (non-LTR)-type endogenous retroelement, encodes two proteins: open reading frames 1 and 2 (ORF1 and 2) (3). ORF1 is a cytoplasmic 40 kDa protein that is present within ribonucleoprotein complexes (21-23). ORF1 associates in *cis* with L1-mRNA (24) and functions as a chaperone of L1-mRNA (25). ORF2 is a protein of about 150 kDa with dual activities as reverse transcriptase (RT) (26) and an endonuclease (27). ORF2 recognizes the 5′-TTAAAA hexanucleotide in the genome and induces a nick between 3′-AA and TTTT in the complementary strand (28,29). It has been proposed that the first-strand DNA is synthesized by target site-primed reverse transcription (3,29). ORF1 and 2 complete the entire process of L1-RTP and are competent for the induction of retrotransposition of *Alu*, a non-autonomous retroelements (30, 31).

3. Reported triggers of L1-RTP

As to the environmental factors that induce L1-RTP in somatic cells, Farkash *et al*. reported that gamma irradiation at 4.5 Gy induced L1-RTP (32). Independently, Deininger's group reported that heavy metals of such as mercury, cadmium and nickel also induced L1-RTP (33,34). They also reported that nickel-induced L1-RTP is induced by a post-transcriptional mechanism (34). As to an environmental carcinogen, Stribinskis and Ramos found that benzo[*a*]pyrene (B[*a*]P) induced L1-RTP (35). An extensive analysis revealed that aryl hydrocarbon receptor (AhR), which serves as a receptor for such environmental pollutants as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (36), was required for the B[*a*]P-induced L1-RTP (35). Because TCDD, a non-genotoxic hydrocarbon carcinogen, did not induce L1-RTP, it was proposed that as one of the its mechanisms an AhR-dependent cellular response converts B[*a*]P into an active genotoxic compound, which in turn induces L1-RTP (35). Although the exact modes of L1-RTP are unclear, these studies inspired us to investigate the possibility that various environmental compounds can induce L1-RTP.

4. Induction of L1-RTP by an environmental compound and identification of p38 as a pivotal cellular factor

First, we found that 6-formylindolo[3,2-*b*]carbazole (FICZ), a tryptophan photoproduct, induced L1-RTP (37). FICZ is highly active, and even picomolar comncentratiom of the compound induced L1-RTP. In mammalian cells, six groups of MAPKs, namely extracellular signal-regulated protein kinase (ERK)1/2, ERK5, JNK, p38, ERK3/4 and ERK7/8, are identified, and are activated by intracellular and extracellular stimuli (38). Among these, cellular signal cascades of ERK1/2, p38 and JNK have been well characterized, because of the availability of inhibitors, including PD98,059, SB202190 and SP600125, respectively. Using these MAPK inhibitors, we found that FICZ-induced L1-RTP was dependent on p38 (37). Interestingly, the compound induced phosphorylation of cyclic-AMP responsive element binding protein (CREB), and the down-regulation of endogenous CREB by short interference RNA (siRNA) attenuated the induction of L1-RTP by FICZ. Moreover, a transfection-back experiment of cDNA that encoded a siRNA-resistant CREB restored the induction of L1-RTP. These data indicate that the induction of L1-RTP by FICZ depended on p38-CREB-dependent signaling. Intriguingly, L1-RTP by FICZ was not dependent on AhR, although FICZ is a candidate physiological ligand of AhR (39). In contrast, L1-RTP by FICZ was dependent on AhR nuclear translocator 1 (ARNT1), a binding partner of AhR (40).

AhR and ARNT1 are members of the basic helix-loop-helix/per-arnt-sim (bHLH/PAS) family, which are transcription factors involved in a variety of biological functions (41). Recently, it was shown that the bHLH/PAS family is functionally linked with environmental adaptation of living organisms (42). When AhR binds environmental compounds, it forms a heterodimer with ARNT1, which is recruited from the cytoplasm to chromatin and recognizes a xenobiotic responsive element (XRE) (36). It has been shown that the chromatin recruitment of ligand-bound AhR depends on the nuclear localization signal of ARNT1 (43), but there are no reports showing that ARNT1 functions as a receptor for environmental compounds. A cellular factor that cooperates with ARNT1 in FICZ-induced L1-RTP has yet to be identified.

5. MAPKs required for L1-RTP by FICZ

To explore the involvement of MAPKs in L1-RTP, we extended our experiments to explore whether environmental carcinogens induce L1-RTP. In two-stage chemical carcinogenesis, it has been shown that skin tumors develop by treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (44). DMBA functions as an initiator and activates *H-ras* gene, whereas TPA functions as a tumor promoter thorough non-genotoxic effects (45). However, how TPA induces tumor progression remains to be clarified. We first analyzed whether L1-RTP is involved under skin carcinogenesis. When transgenic mice harboring human L1 as a transgene (hL1-EGFP mouse) were subjected to DMBA/TPA-induced skin carcinogenesis, L1-RTP was frequently observed in the DMBA/TPA-induced skin tumors (46). Interestingly, i*n vitro* experiments revealed that both DMBA and TPA were active for the induction of L1-RTP. On the other hand, *in vivo* experiments, in which hL1-EGFP mice were transiently treated with DMBA or TPA suggested that L1-RTP in the skin tumors was attributable to the effects of the repeated treatment with TPA. Notably, we observed that the mode of L1-RTP by DMBA and TPA was different. DMBA-induced L1-RTP was dependent on both AhR and ARNT1, whereas TPA-induced L1-RTP required neither protein. Instead, it depended on ERK1/2 and epidermal growth factor receptor (EGFR). Since Balmain *et al* (44) originally reported on DMBA/TPA-induced two-stage carcinogenesis, a major issue of cancer research is to clarify the mechanism of the TPA-induced tumor promotion. Using genetically-engineered mice, it has been proven that TPA-induced tumor promotion depends on ERK1/2 and EGFR (47,48). Interestingly, TPA-induced L1-RTP was shown to be dependent on these molecules, suggesting that the genome shuffling by L1-RTP is linked with the mode of TPA-dependent tumor promotion.

6. MAPKs are involved in the induction of L1-RTP by carcinogens

Given that environmental compounds seemed to induce L1-RTP by involving different cellular proteins, we investigated other carcinogens such as B[*a*]P and 3-methylcholanthrene (3-MC). Consistent with a previous report (35), B[*a*]P induced L1-RTP in an AhR-dependent manner (46). Additionally, 3-MC also induced L1-RTP in an AhR-dependent manner (46). However, we found that the L1-RTP was induced even when siRNA against *ARNT1* was transfected into the cell (Fig. 1b, lanes 9 and 18). The siRNA clearly suppressed the mRNA expression of *CYP1A1* (Fig. 1c, lanes 11 and 12), indicating that the siRNA effectively abrogated the function of endogenous ARNT1 protein. These data support the idea that ARNT1 is dispensable for the induction of L1-RTP by these compounds. Because it has been

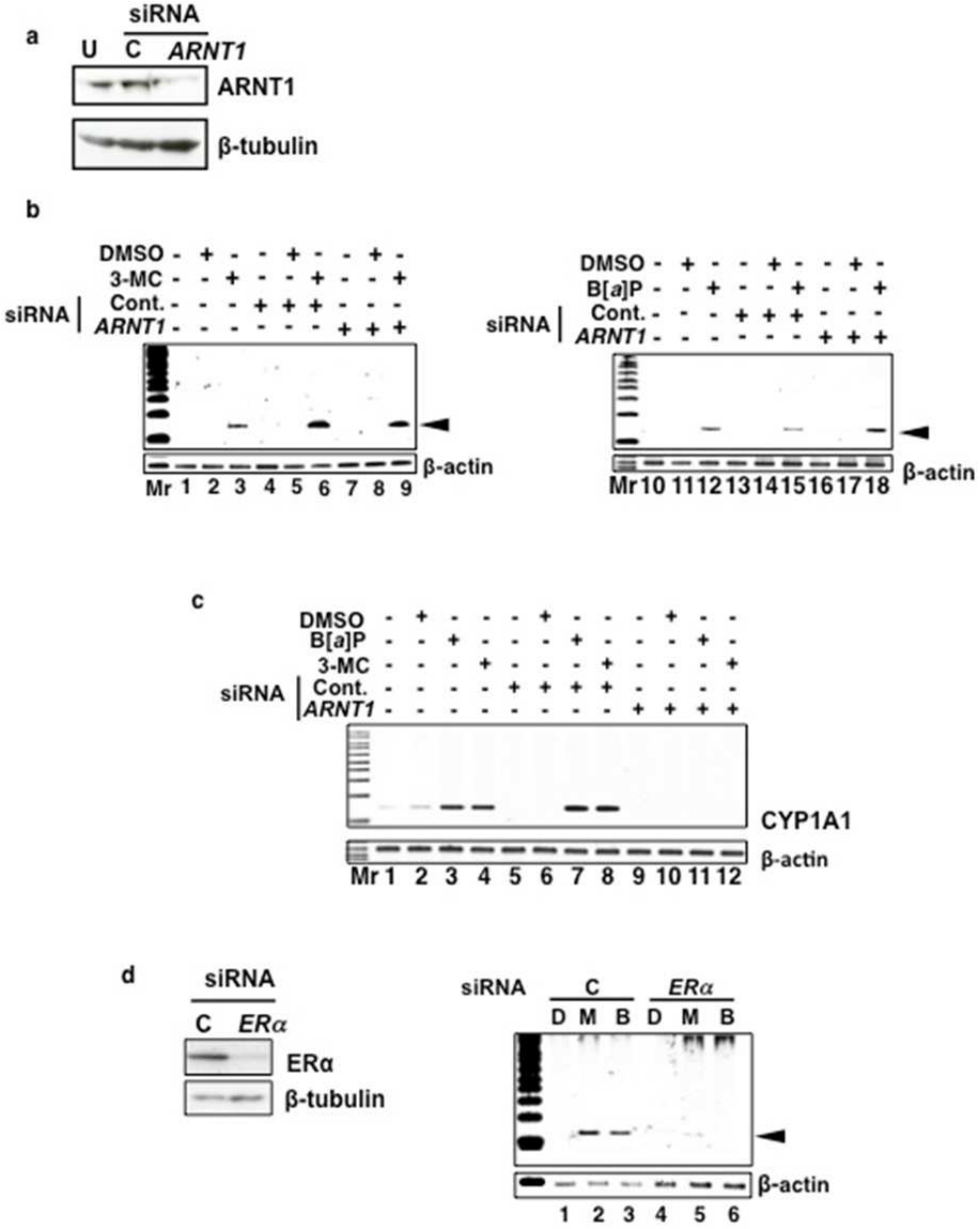

Fig. 1. B[*a*]P and 3-MC induced L1-RTP depending on AhR and ERα, but not on ARNT1. An L1-RTP assay was performed according to the procedures described (37,46). Briefly, HuH-7 cells from a human hepatoma cell line were transfected with pEF06R on day 0, then treated with 0.5 μg/mL puromycin for two days (days 1-3). The cells were then trypsinized and

replated for treatment with the compounds. Two days after the addition of 3 μM B[*a*]P or 1 μM 3-MC, the cells were harvested and their DNA extracted. No cytotoxicity was caused by 3 μM B[*a*]P or 1 μM 3-MC (data not shown). For the PCR-based assay, a spliced form of *EGFP* cDNA (140 bp in length) was amplified by PCR with primers specific for the separated exons of *EGFP* cDNA. The amplified DNA was then loaded onto an agarose gel and detected after staining with SYBR Green. As an internal control, the same samples were used as templates for the amplification of β-actin. **a**. Effects of *ARNT1* siRNA on the down-regulation of endogenous ARNT1. Western blot analysis was performed on day 2 after the transfection of *ARNT1* siRNA. U, untreated; C, control siRNA; *ARNT1*, *ARNT1* siRNA. **b**. L1-RTP caused by B[*a*]P and 3-MC was independent of ARNT1. The PCR-based assay of the effects of *ARNT1* siRNA is shown. HuH-7 cells were transfected with pEF06R on day 0 and then selected from days 1-3. On day 3, the cells were trypsinized, replated, and further transfected with control (Cont.) or *ARNT1* siRNA. On day 4, the cells were again divided into three groups and treated with DMSO, 3-MC (left panel), or B[*a*]P (right panel). After two days, DNA was extracted and subjected to a PCR-based assay. The arrowhead indicates the PCR-amplified band corresponding to the induction of L1-RTP. **c**. *ARNT1* siRNA effectively blocked the mRNA expression of *CYP1A1*, which was induced by the compounds. HuH-7 cells were first transfected with control or *ARNT1* siRNAs. On day 2 after transfection, the cells were trypsinized, replated, and treated with B[*a*]P or 3-MC. RT-PCR analysis was performed on day 2 after the addition of the compounds. **d**. ERα is required for the induction of L1-RTP by B[*a*]P or 3-MC. In this experiment, MCF-7 cells from a human breast carcinoma cell line were used. Using a similar experimental protocol, the effect of *ERα* siRNA on the induction of L1-RTP was examined. As an internal control, β-actin was amplified.

Cellular factors		Inducers				
		FICZ	B[*a*]P	3-MC	DMBA	TPA
AhR		-	○	○	○	-
ARNT1		○	-	-	○	-
ERα		N.T.	○	○	-	-
MAPK	SB202190	○	○	○	-	-
	SP600125	-	○	○	-	-
	PD98,059	N.T.	N.T.	N.T.	-	○

○, dependent; -, independent; N.T., not tested.
The induction of L1-RTP was examined by a PCR-based assay (see legend for Fig. 1).

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

shown that AhR forms a complex with estrogen receptor α (ERα) (49), we further tested the involvement of ERα in the induction of L1-RTP. Interestingly, the transfection of *ERα* siRNA attenuated L1-RTP induced by these compounds (Fig. 1d, lanes 5 and 6). In addition, we found that CREB was definitely phorphorylated (Fig. 2a, lane 4), and checked the effects of MAPK inhibitors on the induction of L1-RTP by 3-MC. As shown in Fig. 2b, SB202190 attenuated the induction of L1-RTP (lane 8), whereas SP600125 did not (lane 10). To further

identify a candidate substrate of p38, we examined the effects of *CREB* siRNA. The transfection of *CREB* siRNA abrogated the induction of L1-RTP by 3-MC (Fig. 2c). These data suggest that L1-RTP by 3-MC is induced by the cooperative function of AhR and ERα depending on a signal cascade involving the p38-CREB pathway. Our data also indicate that the induction of L1-RTP by B[*a*]P is dependent on p38 and JNK (Fig. 2d, lanes 8 and 10).

Although further study is required, our current understanding is that various environmental compounds induce L1-RTP by combinations of the bHLH/PAS family and MAPKs (Table 1). L1-RTP was differentially induced by FICZ, DMBA, B[*a*]P, 3-MC and TPA. Most of the compounds examined, with the exception of DMBA, depended on MAPKs. Moreover, the L1-RTP by carcinogens depended on AhR, whereas FICZ did not. It is important to collect more information about chemical compounds active in the induction of L1-RTP and to elucidate the involvement of MAPKs.

It has been proposed that L1-RTP is controlled at the transcriptional and post-transcriptional levels. *In vitro* experiments revealed that that the expression of L1 is tightly regulated by methytlation of CpG in the region of 5′-LTR. In normal somatic cells, the 5′-LTR of L1 is methylated at CpG (50,51), but it is hypomethylated in transformed cells (52). It has been consistently reported that treatment with B[*a*]P induced the hypomethylation of CpG in HeLa cells (53). Moreover, it was reported that L1-5′UTR has a ubiquitously active antisense promoter that encodes small interfering RNAs, which effectively suppressed the retrotransposition of L1 (54). These observations indicate that epigenetic alternation of the 5′-UTR was proposed as the activation mode of L1-UTR by the compound. However, the following *in vitro* experiments suggested the presence of another regulatory system of L1-RTP. A reporter construct was transfected into cultured cells, and treatment with the compounds increased the frequency of L1-RTP. Because the reporter construct (*e.g.*, pEF06R, which carries *EGFP* cDNA as a reporter gene) contained a potent CMV promoter (32,34), L1-mRNA was strongly expressed when it was transfected into cultured cells. Even under such conditions, remarkable effects on the induction of L1-RTP were detectable by additing inducers such as FICZ, B[*a*]P, and 3-MC (37,46). Data indicate the presence of an additional regulatory system in which cellular proteins regulate the induction of L1-RTP. One possible mode of regulation is the chromatin recruitment of ORF1.

7. The chromatin recruitment of ORF1 is MAPK-dependent

Because it has been postulated that ORF1 is present in the cytoplasm (21-23) and carcinogen-induced L1-RTP was dependent on AhR, it is plausible that ORF1 is functionally associated with the bHLH/PAS family. To prove this, we evaluated the association of ORF1 and AhR by an immunoprecipitation followed by Western blot analysis with a polyclonal antibody to human ORF1. Intriguingly, ORF1 and AhR were associated even under normal conditions (Okudaira N, submitted). More importantly, we detected that recruitment of ORF1 into the chromatin-rich fraction was coupled with L1-RTP. As reported, chromatin recruitment of ORF1 was induced by FICZ in a MAPK-dependent manner. It is interesting that the chromatin recruitment of ORF1 was induced by FICZ, although FICZ-induced L1-RTP was not dependent on AhR (37). Interestingly, ARNT1 was associated with ORF1 when FICZ was added to the culture medium (37). Although the precise role of the MAPK is unclear, these data suggest that the chromatin recruitment of ORF1 is the important regulatory step in L1-RTP, where at least p38 is involved as a crucial cellular factor.

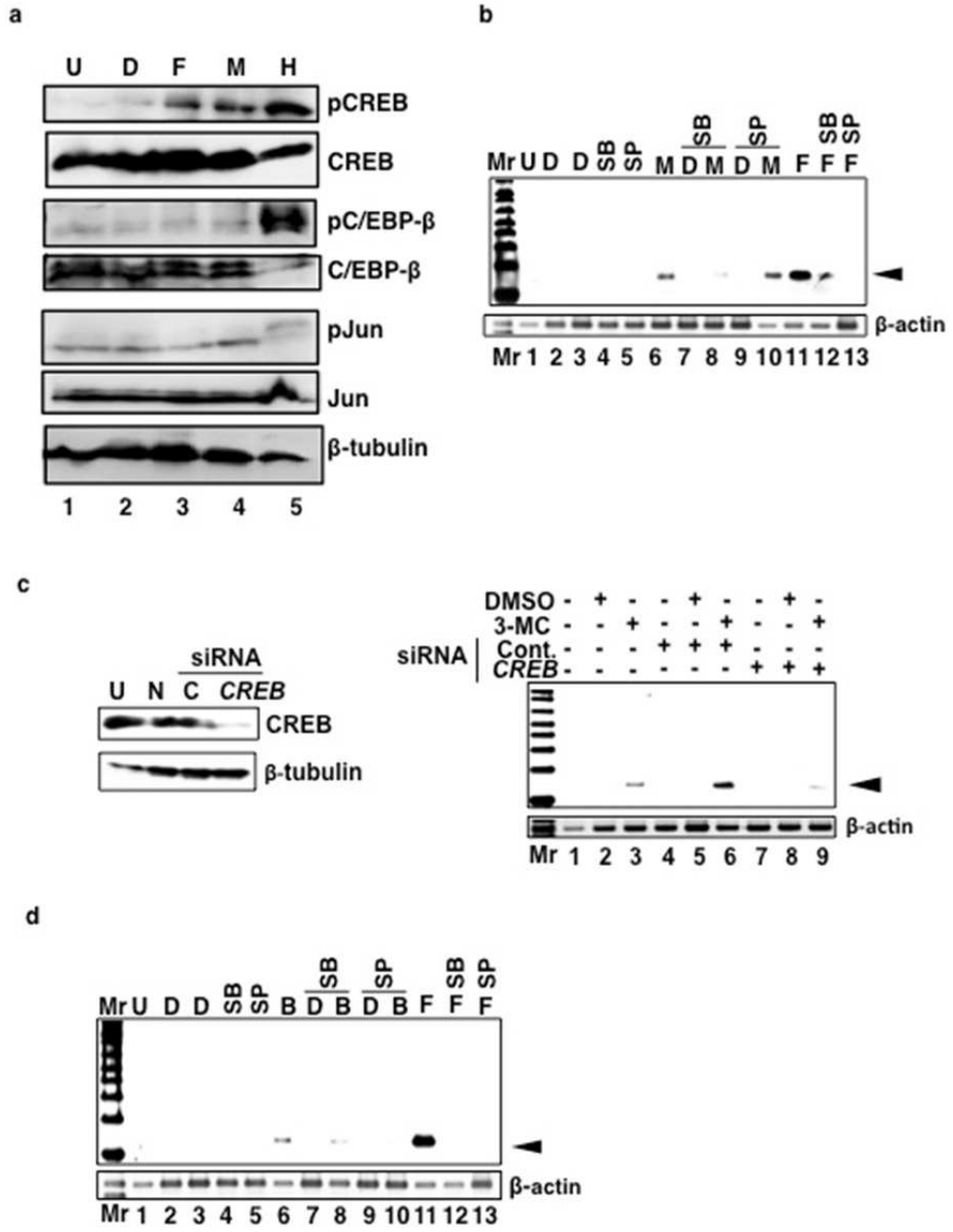

Fig. 2. MAPK is required for the induction of L1-RTP by B[*a*]P or 3-MC. **a**. Phosphorylation of MAPK substrates induced by 3-MC. HuH-7 cells were analyzed on day 2 after the addition of the compound. U, untreated; D, dimethylsulfoxide (DMSO); F, FICZ; M, 3-MC; H, H_2O_2. **b**. Effects of MAPK inhibitors on L1-RTP induced by 3-MC. SB and SP are

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.

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

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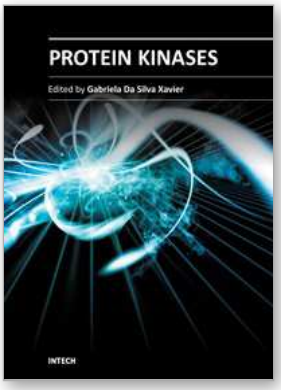

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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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