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ORIGINAL ARTICLE

Suggestive evidence for chromosomal localization of non-coding RNA from imprinted *LIT1*

Kazuhiro Murakami · Mitsuo Oshimura · Hiroyuki Kugoh

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Abstract The non-coding RNA *LIT1/KCNQ1OT1*, itself the product of an imprinted gene, is involved in *cis*-limited silencing within an imprinted cluster on human chromosome 11p15.5. Although the locus serves as an imprinting center, the mechanism of transcriptional regulation is not clear. To help understand the function of the *LIT1* non-coding RNA, we used fluorescence in situ hybridization (FISH) to examine the sub-cellular localization of *LIT1* RNA molecules. *LIT1* RNA signals were observed in most of the interphase human lymphoblast and fibroblast cells. The RNA also appeared to accumulate on neighboring regions of chromatin containing the *SLC22A18/IMPT1* and *CDKN1C/p57KIP2* genes, as shown by high-resolution fiber RNA FISH and modified RNA TRAP (tagging and recovery of associated proteins) methods. These results suggest that *LIT1* RNA stably localizes to a specific chromatin region and plays an important role in the transcriptional silencing of the imprinting domain.

Keywords Chromosome immunoprecipitation · Fluorescence in situ hybridization · Genomic imprinting · *KCNQ1OT1* · *LIT1* · Non-coding RNA · RNA TRAP

Introduction

Genomic imprinting, a phenomenon whereby alleles of some mammalian genes are expressed differentially depending on their parental origin, is critical to mammalian development (Delaval and Feil 2004; Surani et al. 1986). Functional analyses have identified several features associated with imprinted genes and regions, including allele-specific DNA methylation, histone modification and asynchronous replication timing. However, the precise molecular mechanism of gene regulation at imprinted loci remains to be clarified.

LIT1/KCNQ1OT1 is expressed exclusively from the paternal allele, an effect that is attributed to the presence of an antisense RNA that recognizes *KCNQ1/KvLQT1* (Fig. 1a). The human *LIT1* transcription unit lies within the 11p15.5 imprinted gene cluster; the *LIT1* transcription product is reportedly greater than 60 kb and functions as non-coding RNA (Mitsuya et al. 1999). Aberrations of *LIT1* expression, such as those caused by loss of imprinting (LOI), have been frequently observed in Beckwith–Wiedemann syndrome (BWS) and colorectal cancer (Lee et al. 1999; Tanaka et al. 2001). In addition, loss of maternal-specific methylation at the *LIT1* locus in BWS and several cancers correlates with abnormal imprinting status of *CDKN1C* (Bliek et al. 2001; Nakano et al. 2006; Scelfo et al. 2002; Schwienbacher et al. 2000). We have recently shown that loss of CpG and histone H3 methylation at a differentially methylated region (DMR)-*LIT1* leads to a reduction of *CDKN1C* expression in esophageal cancer (Soejima et al. 2004).

We previously reported that targeted deletion of the paternally DMR at the human *LIT1* locus causes loss of the expression of *LIT1* and the silencing of several genes, including *KCNQ1*, *KCNQ1DN* and *CDKN1C* (Horike et al. 2000). Similarly, targeted deletion of the imprinting control center (ICR) located in intron 10 of *Kcnq1* in mice results in the de-repression of six maternally expressed genes, including *Phlda2/Tssc3*, *Slc22a18/Impt1* and *Cdkn1c/p57kip2* (Fitzpatrick et al. 2002). These protein-coding

K. Murakami · M. Oshimura · H. Kugoh (✉)
Department of Biomedical Science, Graduate School of Medical Science, Tottori University, 86 Nishicho, Yonago 683-8503, Japan
e-mail: kugoh@grape.med.tottori-u.ac.jp

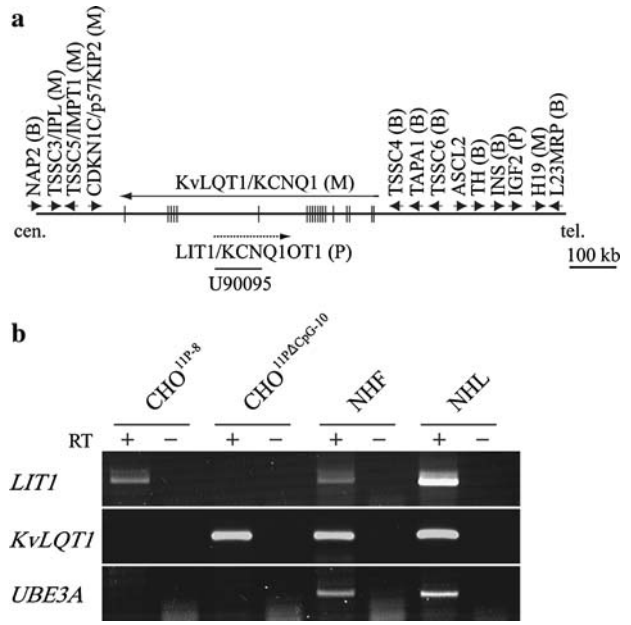

Fig. 1 Schematic representation of the imprinted gene cluster at human chromosome 11p15.5. and expression of *LIT1*, *KCNQ1* and *UBE3A* in CHO11P-8, CHO11P$^{\Delta CpG}$-10, normal human fibroblasts (*NHF*) and lymphoblasts (*NHL*). **a** The deduced transcriptional orientations are shown with *arrowheads* and *arrows*. *P* Paternally expressed genes, *M* maternally expressed genes, *B* biallelically expressed genes, *cen*. centromere, *tel*. telomere. **b** *LIT1* was detected by reverse transcriptase(RT)-PCR in CHO11P-8, NHF and NHL, but was absent in CHO11P$^{\Delta CpG}$-10. In contrast, maternally expressed *KCNQ1* was reactivated in CHO11P$^{\Delta CpG}$-10 cells. NHF and NHL express all three genes, including *UBE3A*

genes are all positioned towards the centromere relative to *LIT1* (Fig. 1a). These data suggest that *LIT1* is required for silencing of these genes and may contribute in *cis* to the formation of the imprinting center (IC) that is thought to bring about coordinate imprinting in the 11p15.5 region. Recent results using an episome-based vector system pointed to the possibility that mouse *Lit1* RNA plays a critical role in silencing at the IC of the imprinted gene cluster and showed that the transcript length is important for the degree of silencing (Kanduri et al. 2006). Another study suggested that full-length *Lit1* RNA is necessary for maintaining the imprinting status through functional analysis of a truncated *Lit1* transcript constructed via insertion of a transcriptional stop signal (Mancini-Dinardo et al. 2006). However, how *LIT1* RNA actually affects transcriptional regulation within the sub-chromosomal domain of these genes remains to be elucidated.

To understand the functional role of the *LIT1* non-coding RNA, we used DNA/RNA fluorescence in situ hybridization (FISH) to examine the sub-cellular localization of *LIT1* RNA molecules (Parra and Windle 1993). We also developed a modified RNA tagging and recovery of associated proteins (TRAP) method to determine the precise region over which *LIT1* RNA spreads (Carter et al. 2002). We report here that *LIT1* RNA stably co-localizes with its own gene region through the cell cycle and accumulates at least on the *SLC22A18/IMPT1* and *CDKN1C/p57KIP2* regions, which lie outside of the *LIT1* RNA transcriptional domain. This provides evidence that *LIT1* RNA may play a significant role in modulating transcription at a specific chromosome domain such as IC.

Materials and methods

RNA extraction and reverse transcriptase-PCR analysis

Total RNA was prepared using RNeasy columns in accordance with the manufacturer's instructions (Qiagen, Valencia, CA) and was treated with RNase-free DNase I (TaKaRa). First-strand cDNA synthesis was carried out with an oligo $(dT)_{15}$ primer and M-MLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) in accordance with standard procedures.

Primer sequences and PCR conditions are available upon request. Products were visualized by electrophoresis on 2% agarose gels.

RNA/DNA fluorescence in situ hybridization

RNA FISH was carried out as described in a previously published protocol with several modifications (Herzing et al. 2002; Van Raamsdonk and Tilghman 2001). Cells were seeded in in a Lab-Tek chamber slide (Nalge Nunc Int, Rochester, NY) and fixed for 20 min at room temperature with 4% paraformaldehyde after treatment with 0.075 *M* KCl. After being washed with phosphate buffered saline (PBS), cells were permeabilized with 0.1% pepsin in 0.01 *M* HCl for 10 min and post-fixed for 5 min at room temperature with 1% paraformaldehyde. Cells were dehydrated through an ethanol series and then air dried at room temperature. We used the U90095 P1-derived artificial chromosome (PAC) genomic probe and the 5′-*LIT1* 10-kb genomic probe for *LIT1* RNA FISH, and the cosmid probe for *UBE3A* RNA FISH. The probes were prepared as follows: labeled with biotin-16-dUTP (Roche, Basel, Switzerland) by nick-translation (Roche), purified by ethanol precipitation, dissolved in 20 µl formamide, mixed and denatured. Labeled probes and hybridization solution [bovine serum albumin (Roche):10× SSC:50% dextran sulfate (Sigma), 1:2:2] were mixed 1:1, dropped onto the slide, covered with Parafilm and incubated at 37°C for 15 h in a humidified chamber.

After hybridization, the slide was washed sequentially at 37°C in 50% formamide/2× SSC, 2× SSC and 1× SSC for

5 min each time and once in 4× SSC for 5 min; the slide was then incubated in 4× SSC with 1% BlockAce (Dainippon Pharmaceutical Co, Osaka, Japan) containing 3 μg/ml fluorescein isothiocyanate (FITC)–avidin (Vector Laboratories, Burlingame, CA) for 60 min at 37°C. The slide was then washed sequentially for 5 min in each of 4× SSC, 4× SSC containing 0.05% Triton X-100, and 4× SSC. The slide was mounted in antifade solution [1% diazabicyclooctane (Sigma) in glycerol with 10% PBS] containing 1 μg/ml 4′, 6′-diamidino-2-phenylindole (Sigma) and 1 mg/ml *p*-phenylenediamine (Sigma). Digital images of RNA signals were acquired with a Nikon fluorescence microscope and CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan).

For DNA FISH, the cover slide was removed in 4× SSC and the sample treated with 0.1 mg/ml RNase (Sigma) for 1 h at 37°C, denatured with a mixture of 70% formamide and 2× SSC at 72°C for 2 min and finally quenched in ice-cold 70% ethanol for 2 min. The sample was dehydrated and hybridized with a digoxigenin-labeled probe following which the slide was washed as above and immersed in a mixture of 1.6 μg/ml anti-digoxigenin-rhodamine (Roche), 4× SSC and 1% BlockAce for 45 min at 37°C. After the slide was washed, detection and capture of DNA signals were carried out as described above for RNA FISH. Images of selected RNA and DNA signals were merged and analyzed using ARGUS imaging software (Hamamatsu Photonics).

High-resolution fiber RNA FISH

Fiber FISH analysis was performed largely as previously described with some modifications (Parra and Windle 1993). Two hundred cells in 10 μl PBS were placed on the end of a slide. After drying, 5 μl of cell lysis buffer was applied [0.5% sodium dodecyl sulphate (SDS)/50 m*M* EDTA/200 m*M* Tris–HCl]. The slide was incubated for 5 min at room temperature and then placed on an angle to release stretching DNA from interphase cell nuclei. These bundles were dried and fixed for 20 min at room temperature with 4% paraformaldehyde and dehydrated through an ethanol series. The LIT1 transcripts were detected by RNA FISH as described above.

Modified RNA TRAP method

A modified RNA TRAP method was used as follows (Carter et al. 2002). RNA in situ hybridization was first performed; that is, biotin-labeled probes were hybridized to single-strand RNA on chromatin. Then, applying the chromosome immunoprecipitation (ChIP) method, we collected the biotin-labeled probe/single-strand RNA complex and associated chromatin using anti-biotin antibodies. Finally, genomic PCR was carried out to analyze the chromatin region in which the RNA had accumulated.

Cells (5×10^5) were spread on a petri dish for 2 days and then fixed in 1% formaldehyde for 10 min at 37°C. The reaction was stopped by adding 100 μl 1.5 *M* glycine for 10 min at room temperature. After the cells had been washed with PBS, permeabilization and post-fixation were performed as described above and biotin-labeled probe hybridization was carried out in the petri dish. The dish was incubated at 37°C for 15 h in a humidified chamber, and post-hybridization washing were carried out as described above. Cells were scraped from the petri dish and spun down at 10,000 *g* for 3 min, resuspended in 300 μl of a mixture of 1% SDS, 10 m*M* EDTA, 50 m*M* Tris–HCl (pH 8.0) and 1× protease inhibitor, and sonicated for 90 s on ice (nine 10-s bursts with 60 s between bursts) using a Bronson Sonifire (output 3; duty cycle 40). This procedure yielded chromatin fragments that had an average length of 500 bp each. The crude chromatin was centrifuged at 13,000 *g* for 10 min, the supernatant containing the soluble chromatin was collected, and 2.7 ml of a mixture of 0.01% SDS, 1.1% Triton-X, 1.2 m*M* EDTA (pH 8.0), 20 m*M* Tris–HCl (pH 8.0), 150 m*M* NaCl, and 1× protease inhibitor was added; 10% of the soluble chromatin was set aside as the input and the suspension was pre-cleared with 60 μl of salmon sperm DNA/protein G agarose in a 50% slurry for 60 min at 4°C with agitation. The supernatant was then collected and 10 μl mouse monoclonal anti-biotin antibody was added (Sigma). The solution was incubated overnight at 4°C with rotation. To collect the antibody/biotin/RNA/chromatin complex, we used G agarose beads (Upstate Technology), followed by washing and elution. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation in accordance with the standard ChIP protocol described elsewhere (Soejima et al. 2004).

Genomic PCR analysis was performed for detecting *LIT1* RNA-accumulated chromatin regions. Primer sequences and PCR conditions are available upon request. Products were visualized by electrophoresis on 2% agarose gels.

Results

Visualization of the *LIT1* RNA molecule by FISH

To better understand the profile of the *LIT1* non-coding RNA, we attempted FISH to examine the sub-cellular localization of *LIT1* RNA molecules in Chinese hamster ovary (CHO) hybrid cells. As shown in Fig. 1b, human

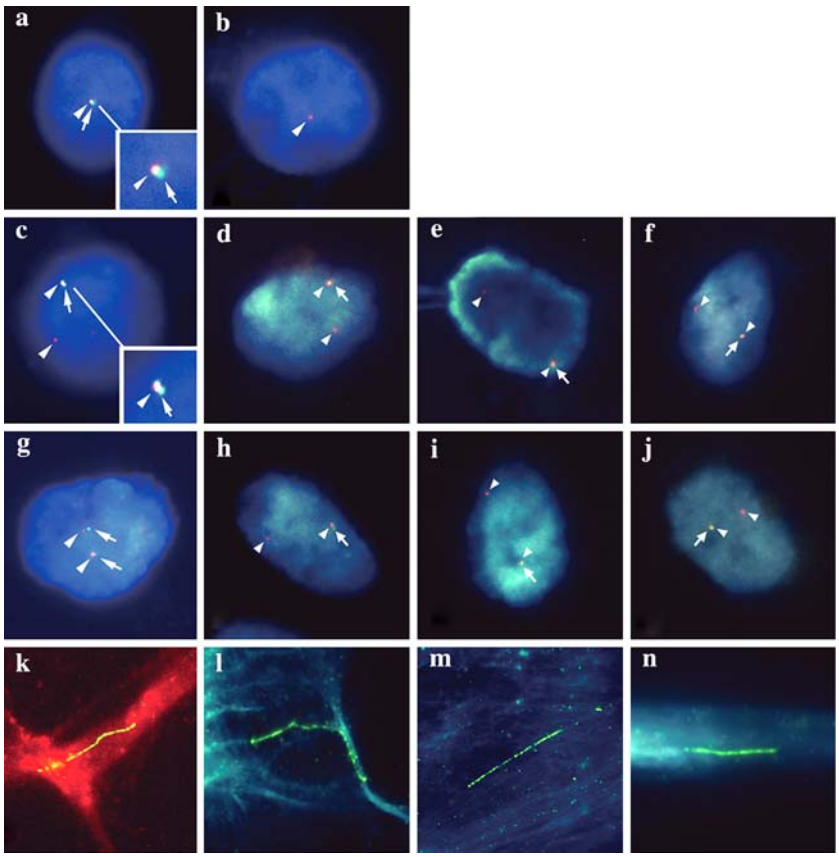

Fig. 2 Expression analysis and sub-cellular localization of *LIT1* and *UBE3A* RNA. Cytogenetic analysis of *LIT1* localization by RNA/DNA fluorescence in situ hybridization (FISH). RNA signals are shown in *green* (*arrow*) and DNA signals are shown in *red* (*arrowhead*). Interphase nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). **a**–**f** *LIT1* RNA/DNA FISH. *LIT1* RNA and DNA signals are detected in CHO11P-8, while only the *LIT1* DNA signal is detected in CHO11P$^{\Delta CpG}$-10 (**a** CHO11P-8, **b** CHO11PΔCpG-10). Monoallelic expression of *LIT1* is observed in normal human fibroblast (NHF) cell lines. The RNA signal was detected in the vicinity of its DNA signals (**c** NHF, **d** NHF2, **e** NTI-4, **f** TIG-1-20). *LIT1* RNA stably localizes to the region containing the *LIT1* gene in CHO11P-8 and NHFs. **g**–**j** *UBE3A* RNA/DNA FISH. *UBE3A* RNA and DNA are detected in normal human fibroblast cell lines (**g** NHF, **h** NHF2, **I** NTI-4, **j** TIG-1-20). **k**–**n** High-resolution fiber FISH. Chromatin fibers were counterstained with propidium iodide (PI) or DAPI. Coating of the chromatin fiber with the *LIT1* RNA is visualized by high-resolution fiber FISH in normal human fibroblast cell lines (**k** NHF, **l** NHF2, **m** NTI-4, **n** TIG-1-20). *LIT1* RNA signal was detected along with chromatin fibers, but not *UBE3A*. Results with the NHF are representative of results from normal human lymphoblast cell lines (date not shown)

LIT1 transcripts, but not from the maternally expressed imprinted *KCNQ1*, were detectable in CHO hybrid cells that carried a paternal copy of human chromosome 11 (CHO11P-8) (Fig. 1b). In contrast, CHO11P$^{\Delta CpG}$ -10 hybrid cells, which contain a modified paternal human chromosome 11 that lacks the *LIT1* CpG island, expressed *KCNQ1*, presumably due to a loss of *LIT1* function. These results are consistent with the findings of a previous study (Horike et al. 2000).

We used a U90095 PAC that includes the whole sequence of the *LIT1* transcript and which in our case consisted of the majority of intron sequences of *KCNQ1* as a FISH probe. We used the same probe in both RNA and DNA FISH. However, because cells were subjected to hybridization under non-denaturing conditions such that cellular DNA was not accessible, we could detect only primary transcripts by RNA FISH (Clemson et al. 1996; Van Raamsdonk and Tilghman 2001). We performed RNA FISH followed by DNA FISH on the same samples (RNA/DNA FISH). The RNA/DNA FISH shows that the *LIT1* RNA signal co-localized with DNA in these CHO hybrid interphase nuclei. We detected a single *LIT1* RNA signal in the nuclei of CHO11P-8 hybrid cells; in contrast, the RNA signal was not detected in CHO11P$^{\Delta CpG}$ cells (Fig. 2a, b). These results coincide with data from the RT-PCR (Fig. 1b). The *LIT1* RNA signal was detected only in nuclei as a single spot, even when PAC (approximately 100 kb in length) or the 10-kb *LIT1* 5'-genomic DNA fragment was used as a probe (data not shown). This signal disappeared when the samples were treated with RNase A prior to probe hybridization (data not shown). These results indicate that the U90095 PAC probe was exclusively able to detect *LIT1* transcripts but not *KCNQ1* transcripts.

Table 1 Frequency distribution (%) of the number of spots per cell for *LIT1* and *UBE3A*

Imprinting gene	Cell lines[a]	Number of RNA signals				
		Zero	One	Two	Three	Four
LIT1	NHL	3	89	7	1	0
	NHF	2	96	2	0	0
	NHF2	9	83	8	0	0
	NTI-4	6	84	9	1	0
	TIG-1-20	6	82	12	0	0
UBE3A	NHL	35	43	20	2	0
	NHF	41	39	15	4	1
	NHF2	39	38	21	2	0
	NTI-4	39	43	17	1	0
	TIG-1-20	46	35	18	1	0

One hundred interphase cells were analyzed

[a] NHL, Normal human lymphoblast cell line; NHF, NHF2, NTI-4, TIG-1-20, normal human fibroblast cell lines

LIT1 RNA stably co-localizes with its own gene region through the cell cycle

We next performed RNA/DNA FISH on normal human lymphoblasts (NHF) and normal human fibroblasts (NHF, NHF2, NTI-4, TIG-1-20) to confirm the physiological state of *LIT1* RNA in human cells (Fig. 2c–f; Table 1). *LIT1* RNA signals were observed in almost all interphase nuclei of the lymphoblast cell line (97% of interphase nuclei) and of four fibroblast cell lines (98, 91, 94, 94% of interphase nuclei; Table 1). In these cells, *LIT1* RNA spatially coincided with the *LIT1* DNA locus. The distribution of the RNA in both human lymphoblast and fibroblast cell lines was similar to that found in the CHO hybrid cell. Intriguingly, the RNA signal was detectable in most nuclei, despite the fact that cell cycle was not synchronized.

To evaluate this distinctive feature of non-coding transcript *LIT1*, we analyzed the RNA FISH signal of *UBE3A*, an imprinted gene known to exhibit exclusively maternal expression. In contrast to *LIT1*, the *UBE3A* transcript is translated into protein. The *UBE3A* RNA was visible in only 65% of the lymphoblast cell line and in only 59, 61, 61, 54% of the four fibroblast cell lines, respectively (Fig. 2g–j; Table 1).

Using DNA/RNA FISH, we detected intronless non-coding *LIT1* RNA signals in most nuclei, which is in sharp contrast to *UBE3A* RNA signals, and did not detect *LIT1* RNA signals in the cytoplasm. These results strongly suggest that *LIT1* transcripts stably co-localize with its own gene region as non-coding RNA throughout the cell cycle and, in contrast to *UBE3A* transcripts, exhibit tissue-specific imprinted expression (Kagotani et al. 2002). These results therefore support the idea that *LIT1* acts as a functional RNA in the nucleus (Kanduri et al. 2006; Mancini-Dinardo et al. 2006) and that the co-localization of the *LIT1* RNA molecule to the *LIT1* locus may represent mature and functional RNA molecules, rather than simply nascent transcripts.

LIT1 RNA accumulates on chromatin

To date, nine different non-coding RNAs associated with epigenetic gene regulation (including genomic imprinting and *X* chromosome inactivation) at single gene loci, subchromosomal regions or chromosomal regions have been found in mice and humans (Heard and Disteche 2006; Jong et al. 1999; Lee et al. 2000; Lin et al. 2003; Rougeulle et al. 1998; Sleutels et al. 2002; Wroe et al. 2000). Of these, the non-coding *XIST* RNA has been particularly well characterized. *XIST* RNA is known to act as a regulatory molecule in *X* chromosome inactivation, which accomplishes dosage compensation in mammals. *XIST* RNA coats and spreads along the *X* chromosome, recruits the silencing complexes, including histone methyltransferase and the polycomb group proteins Eed/Ezh2/Suz12, and eventually establishes *X* chromosome inactivation by inducing heterochromatin formation and DNA methylation (Heard and Disteche 2006).

It has recently been suggested that gene regulation by *Lit1* RNA is accompanied by histone modifications (mono-, di-, triMe-H3K9 and triMe-H3K27) and that DNA methylation in specific regions and the existence of the *Lit1* RNA is important for these regulatory systems (Kanduri et al. 2006; Soejima et al. 2004). Together with our previous our experimental data (Horike et al. 2000; Mitsuya et al. 1999), our findings suggest that *LIT1* transcripts may act by coating the subchromosomal region in a way that resembles X chromosome inactivation.

To test this theory, we used high-resolution fiber FISH, which allows the visualization of small DNA elements on subchromosomal regions, to analyze *LIT1* transcript localization (Parra and Windle 1993). Interestingly, the accumulation of *LIT1* RNA signals, but not *UBE3A* signals, was observed on chromatin fibers prepared from a normal human fibroblast cell line (Fig. 2k–n; data not shown). Our results suggest that prolonged association of *LIT1* RNA with chromatin may be significant for transcriptional silencing of the imprinted gene clusters.

Identification of the *LIT1* RNA spread region by modified RNA TRAP analysis

To determine the precise region over which *LIT1* RNA spreads, we performed DNA FISH after fiber RNA FISH. However, we did not detect any DNA signal. The fiber DNA was very weak, and the RNA signal on the DNA fiber was detected throughout many complex processes that involved washing by FISH. As a result, fiber DNA was lost due to these additional DNA FISH processes, i.e. denaturing and washing. We then developed a modified RNA TRAP analysis combined with RNA FISH and ChIP analysis to study the single strand RNA–DNA interactions on chromatin in situ (Carter et al. 2002). Following RNA in situ hybridization using a biotin-labeled U90095 PAC probe, crosslinked chromatin was isolated by immunoprecipitation with an antibody recognizing biotin. The DNA sample prepared from these complexes was analyzed in a number of imprinted gene regions by PCR analysis to determine whether the DNA sample contained regions outside of the *LIT1* domain. We confirmed the expression profiles (RT-PCR and RNA FISH) of some imprinted genes containing *LIT1* in normal human fibroblast cell lines (Table 1; Fig. 3a). We then attempted the modified RNA TRAP method. Intriguingly, PCR products showing the presence of *LIT1* RNA were found on *CDKN1C* and *IMPT1*, both of which lie outside of the *LIT1* RNA transcriptional domain in normal human fibroblasts. The *LIT1* RNA was not detected on *IGF*, indicating that the distal border lies between the *LIT1* and the *IGF2* (Fig. 3b, c). However, the accumulation of *LIT1* RNA on *IPL* region was not reproducible. We are not able to identify the proximal boundary region at this time, and the results suggest that a better method is required for the identification of the proximal boundary region.

These results suggest that *LIT1* transcripts may regulate gene expression by coating the subchromosomal region in a way that resembles *X* chromosome inactivation by *XIST* RNA. The unique behavior of *LIT1* RNA may contribute to the establishment of parental-specific expression within an imprinted cluster. Thus, functional non-coding RNAs may play important roles in modulating transcription at specific chromatin domains (Horike et al. 2000; Kanduri et al. 2006; Mancini-Dinardo et al. 2006).

Discussion

In an attempt to elucidate the regulatory mechanism of the human 11p15.5 imprinting domain by *LIT1* non-coding RNA, we performed *LIT1* DNA/RNA FISH, fiber RNA FISH and the modified RNA TRAP method using CHO

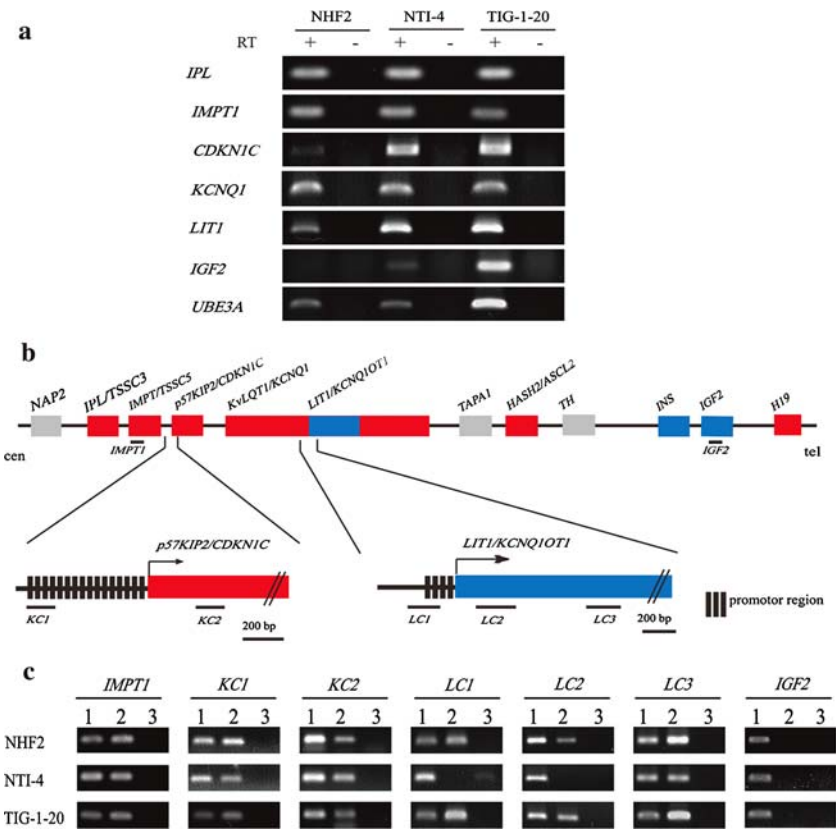

Fig. 3 Analysis of regions in which *LIT1* RNA accumulates using a modified RNA tagging and recovery of associated proteins (TRAP) method. **a** Expression analysis of imprinted genes in NHFs by reverse transcriptase-PCR. **b** Schematic representation of the primers used for the modified RNA TRAP method. *Red boxes* indicate maternally expressed genes and *blue boxes* indicate paternally expressed genes. **c** Accumulation of *LIT1* RNAs on the *IMPT1* region, KC1, KC2 and LC1 on the *CDKN1C* region and LC2, LC3 on the *LIT1* and *IGF2* regions. PCR was performed using DNAs extracted from input (*lane 1*), antibody (*lane 2*) and no antibody (*lane 3*) preparations. Modified RNA TRAP analysis was performed independently three times. Primer sequences and PCR conditions are available upon request

hybrid, NHL and NHF cells. Intriguingly, we observed that *LIT1* RNA stably accumulated on its own gene region and that the *SLC22A18/IMPT1* and *CDKN1C/p57KIP2* regions, which lie outside of the *LIT1* RNA transcriptional domain, accumulated through the cell cycle. These data suggest that *LIT1* RNA may play a significant role in modulating transcription at specific chromosome domains, such as IC.

Kagotani et al. (2002) reported that they detected RNA signals in some imprinted and non-imprinted genes that contain *UBE3A* in more than 80% of the interphase nuclei of human lymphoblastoid cells and mouse embryo fibroblast cells. We currently cannot explain this large discrepancy in the frequency of *UBE3A* RNA signal reported by Kagotani et al. and our data, but this question will be solved in future investigations carried out on control of the cell cycle.

To detect the *UBE3A* RNA signal, we used a cosmid probe (cosmid 16) that consists of the majority of intron sequences on *UBE3A-ATS* (Meguro et al. 2001; Runte et al. 2001). Therefore, it dominantly detected *UBE3A* transcripts in human fibroblast cell lines but not *UBE3A-ATS* transcripts. A PAC probe (U90095) that contains intron sequences on *KvLQT1*, exclusively detected *LIT1* transcripts in our study. Furthermore, if a cosmid probe were to detect *UBE3A-ATS* nascent RNA, U90095 should be also be detected at nascent RNA of antisense *KvLQT1*. However, U90095 detected only *LIT1* transcripts.

The *LIT1* RNA was detected at the LC1 and LC2 regions in NHF2 and TIG-1-20 cells, but not in NTI-4 cells. It is possible that this accumulation to genes of the *LIT1* RNA may significantly affect the expression of a specific region rather than the overall length of a gene for transcriptional regulation. Another possible mechanism is the different strength of the coating in genes. Further analysis of *LIT1* region in various cells is required to resolve the issue.

The well-known non-coding RNA, *Xist*, triggers XCI by accumulation along the whole *X* chromosome. The triggering of XCI is accompanied by DNA methylation and histone modification. The polycomb complex (PRC), BRCA1 and HP1 are also associated with the inactive *X* chromosome. These factors are essential for XCI activation. It has recently been suggested that DNA methylation and histone modification are also important for the *LIT1* RNA regulatory mechanisms. By analogy with *Xist* RNA, it is feasible that the XCI and *LIT1* RNA regulatory mechanisms share same factors. We are currently attempting to isolate these factors that interact with the *LIT1* RNA accumulated regions.

The *Lit1* RNA has been reported to regulate placenta-specific imprinted genes expression, including *Osbpl5*, *Tssc4*, *Cd81* and *ASCL2*, in mammalian early development. It has also been suggested that the timing of *LIT1* RNA expression is important for the proper initiation of these genes (Annabelle Lewis et al. 2006; Kelly Green et al. 2007). We used differentiated human fibroblasts for the RNA TRAP methods. One interesting result was that the *LIT1* RNA was detected in only the ubiquitously imprinted gene regions but not in placenta-specific imprinting gene regions (date not shown). Future studies of *LIT1* RNA function should focus on whether *LIT1* RNA accumulates in placenta-specific imprinted gene regions during the early development stages that establish the placental specific imprinting.

The mechanism by which *LIT1* RNA accumulation contributes to the transcriptional silencing of imprinted gene clusters is still unknown. Therefore, it will be important to focus future studies on functional sequences within *LIT1* RNA and on proteins that bind to non-coding RNAs in order to better understand how non-coding RNAs bring about epigenetic gene regulation at imprinted gene clusters.

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