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Nickel Stimulates L1 Retrotransposition by a Post-transcriptional Mechanism

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Abstract

Sequence studies of the human genome demonstrate that almost half of the DNA is derived from mobile elements. Most of the current retrotransposition activity arises from L1 and the L1-dependent, non-autonomous elements, such as Alu, contributing to a significant amount of genetic mutation and genomic instability. We present data demonstrating that nickel chloride, but not cobalt chloride, is able to stimulate L1 retrotransposition about 2.5-fold. Our data suggest that the stimulation occurs at a post-transcriptional level, possibly during the integration process. The effect of nickel on the cell is highly complex, limiting the determination of the exact mechanism of this stimulation. The observed stimulation of L1 retrotransposition is not due to a general increase in L1 transcription or an increase in the number of genomic nicks caused by nickel, but more likely caused by a decrease in DNA repair activities that influence the downstream events of retrotransposition. Our observations demonstrate the influence of environmental toxicants on human retroelement activity. We present an additional mechanism for heavy-metal carcinogenesis, where DNA damage through mobile element activation must be considered when dealing with genomic damage/instability in response to environmental agents.

Keywords

retroelement; nickel; genetic instability; L1; DNA repair

Introduction

Almost half of the human genome is composed of mobile elements.[1] The vast majority of these elements are dead “fossils” of past activity. Estimates indicate the existence of several hundred active elements, primarily from the LINE-1 (L1) and its non-autonomous counterparts, still capable of active transposition and currently contributing to genetic damage and disease.[2] Due to its damaging potential, the retrotransposition activity of mobile elements is limited by the cell. It is well established that methylation of mobile elements suppresses their expression,[3–5] and alterations of their expression leads to increased genetic instability with deleterious effects.[6] Thus, factors affecting mobile-element activity can induce genetic events, causing a disease state. There are a number of examples of environmental stresses that influence the

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expression and rate of transposition for an assortment of mobile elements in various organisms.[7] In particular, benzo(a)pyrene has been reported to increase expression of the mouse L1Md LINE in vascular smooth muscle cells.[8]

Retroelement activity (autonomous and non-autonomous) in humans is dictated primarily by the L1 elements.[9–11] L1 elements are long interspersed repeated elements (LINEs) that belong to the class I autonomous, non-long interspersed repeated retrotransposons. LINEs amplify by the reverse transcription of their RNA that integrates into a new genomic location, in a process known as retrotransposition. The human genome contains more than 500,000 copies of L1, comprising approximately 17% of its mass.[1] Most of the existing L1 inserts are truncated,[12] or mutated, rendering them defective, with probably less than 100 elements capable of retrotransposition.[13] Approximately 0.1% of human germ-line disease is caused by insertion of these elements,[14,15] with a similar rate for the non-autonomous Alu elements.[16] Recently, a report of a systematic analysis of the L1 and the L1-dependent retroelements (Alu and SVA) lists 40 insertional events into genes that generated disease-causing mutations.[2] L1 elements encode two open reading frames (ORF1 and ORF2) that are required for its retrotransposition using a target-primed reverse transcription.[17] Two full-length L1 elements (L1.2[18] and LRE-2[19]) were the basis for the creation of a selectable system in which a retroposition-activated reporter gene (Figure 1(a)) is used to detect L1 amplification events in culture.[20,21]

L1 expression is detected at the highest levels in germ cells, but also in distinct somatic cell types of steroidogenic tissues, vascular endothelial cells, and differentiating neuronal cells.[22–24] L1 expression also appears to be generally higher in tumors.[25–29] Although expression does not necessarily demonstrate that the full retrotransposition process occurs, these studies suggest that germ cells, specific somatic cells, and a broad range of tumors may be impacted primarily by retrotransposition. The full retrotransposition process occurs readily in several tissue culture cells,[20,30–32] demonstrating that both germ and somatic cells are capable of supporting this activity. In addition to transcriptional regulation,[33] it is likely that post-transcriptional regulation, involving transport or integration of the L1 RNA, may alter the rate of retrotransposition. Post-transcriptional regulation is supported by the finding that cells deficient in non-homologous end joining lose their dependence on the L1-encoded endonuclease for integration.[31] Closer inspection of the inserts revealed examples of L1 integration into sites demonstrating the use of genomic nicks that were not generated directly by the L1 endonuclease.[34] These data suggest that the L1 insert may play a role in repairing a double-strand breaks, or at least take advantage of the breaks in the DNA. All of these data support the possibility that agents that damage DNA, alter the cell cycle, or alter DNA-repair capacity in cells can potentially alter retrotransposition rates and cause genetic instability.

We demonstrated previously that exposure to the particulate form of cadmium, mercury and nickel stimulate L1 retrotransposition in a dose-dependent manner, in culture using both stable cell lines and transient transfections.[35] Heavy metals, such as nickel and cadmium, are well-known carcinogens in humans and animal models.[36–40] Although heavy metals are known to be DNA-damaging agents,[41] the molecular mechanisms by which they induce cancer are ill-defined. Heavy metals can have a multitude of effects on the cellular homeostasis, leading to a large array of repercussions. Some of the known effects include DNA breaks, through the induction of reactive oxygen species (ROS),[42–44] alterations in gene expression,[45–47] and the enhancement of mutagenic properties of other DNA-damaging agents, such as UV light, due to the inhibition of DNA-repair enzymes.[48,49] Understanding how heavy metals influence L1 retrotransposition may be important in understanding the extent of the toxic and carcinogenic effects of heavy metals on cellular homeostasis.

Results

Heavy metals have differential effects on retrotransposition

HeLa cells were transfected with the JM101/L1.3ΔCMV plasmid[50] containing the full-length human L1.3 modified as a reporter system for retrotransposition (Figure 1(a)). This plasmid has been engineered to express the neomycin-resistance (neo^R) gene only after expression of its RNA, splicing and then integration of a copy of the spliced RNA through retrotransposition.[51] Transiently transfected cells were exposed to different doses of $NiCl_2$ (0–250 μM), and $CoCl_2$ (0–150 μM) for two days prior to G418 selection to obtain neo^R colonies. An unrelated plasmid containing a functional neo^R gene (pIRES2-EGFP) was transfected in parallel as a control to evaluate the influence of the metal toxicity on both transfection efficiency and colony formation. The average number of colonies with no treatment was used as the 100% value for both the toxicity and L1 activity (Figure 1(b) and (c)). A correction factor was utilized to compensate for the toxic effects of the heavy metals (evidenced as an overall loss of colony formation) when comparing the L1 retrotransposition to the control at different doses.[35] L1 retrotransposition increased with $NiCl_2$ dose, as reflected by the number of neo^R colonies, peaking at about 2.5-fold at 150 μM $NiCl_2$ in comparison to the untreated (0 μM $NiCl_2$) control. This effect was not seen in cells exposed to $CoCl_2$, which showed only decreased retrotransposition as cobalt dosage increased. The results shown are the average of three independent experiments done in triplicate, and the stimulation of retrotransposition by $NiCl_2$ at 100 μM and 150 μM were significant at the $p < 0.01$ level using Student's paired *t*-test. At higher doses, the retrotransposition rate drops precipitously, probably due to cellular toxicity.

Nickel does not increase L1 transcription

The first step of L1 retrotransposition is the transcription of RNA. To determine the effects of nickel on the L1 promoter, we utilized a reporter vector where the L1.3 promoter drives transcription of the firefly luciferase gene (Figure 2(a)). Cells were treated with 150 μM $NiCl_2$ (dose with highest L1 response) and with 100 μM $CoCl_2$ (highest dose with over 10% cell survival) for comparison. We co-transfected a constant amount of vector containing the *Renilla* luciferase gene under the control of the CMV promoter to standardize between samples. Treatment with 100 μM $CoCl_2$ had no effect; whereas L1 promoter activity decreased almost 50% with 150 μM $NiCl_2$ treatment (Figure 2(b)). This inhibition by nickel suggests that stimulation of retrotransposition activity is post-transcriptional, and that the post-transcriptional influence may be stronger than seen in order to compensate for lower L1 transcription. To corroborate our observation, RNA from nickel-treated and untreated cells was quantified by Northern blot analysis. Evaluation of full-length L1 RNA, using the neomycin expression as an internal control for transfection, corroborates our observations that treatment with 150 μM $NiCl_2$ does not increase L1 expression (Figure 2(c)).

The effect of $NiCl_2$ on L1 retrotransposition was also determined using a vector (JM102/L1.3) lacking the endogenous L1 promoter (5′UTR) but instead driven by the CMV promoter, which did not show the same decrease as the L1 promoter in the reporter-gene assay (Figure 2(b)). The rate of L1 retrotransposition was stimulated 2.1-fold at 150 μM $NiCl_2$ (see Supplementary Data, Figure 1s); confirming that any stimulation of L1 retrotransposition by nickel is not controlled by the L1 promoter.

Nickel and the cell-cycle

Exposure to heavy metals causes multiple alterations in the regulation and signaling of cells.[52] At moderate doses, some cells show mitogenic activation,[53] while toxicity occurs at higher doses. To determine the influence of nickel and cobalt treatments on the proliferation rate of the HeLa cells in our studies, cells were exposed to different doses of the heavy metals in the

presence of bromodeoxyuridine (BrdU). The extent of BrdU incorporation by replication was measured after 24 h. No significant effect of nickel or cobalt on proliferation was observed until levels of toxicity increased, causing decreased proliferation (Figure 3(a)). Therefore, the proliferation rates are unlikely to contribute to the increased L1 retrotransposition rate observed after exposure to $NiCl_2$.

As a further test of the possible influence of nickel on HeLa cell proliferation, we assessed changes in cell-cycle distribution that might suggest alterations of cell-cycle progression influencing the retrotransposition. HeLa cells were exposed to various doses of $NiCl_2$ for 24 h, and subsequently stained with propidium iodide to determine the DNA content by fluorescent-activated cell sorter (FACS) analysis (Figure 3(b)). No significant difference of the cell-cycle distribution after nickel exposure at levels below the cytotoxic doses was observed.

Nickel stimulation of L1 is independent of an increase in DNA breaks

L1 elements have been reported to be able to insert into endogenous nicks in the genome,[31] thus "repairing" DNA lesions. It is well-established that heavy metals, including nickel chloride, cause DNA nicking.[54,55] We hypothesized that the increase in L1 activity may be a reflection of having a higher number of nicks in the DNA for its insertion. COMET evaluations of $NiCl_2$-treated HeLa cells confirmed that under our experimental conditions, DNA nicking occurs in a dose-dependent manner, as expected (data not shown). Although there is a correlation between the ability of nickel to create nicks in the DNA and its stimulation of retrotransposition, exposure to nickel causes DNA breaks and has a vast array of other effects on the cells, such as inhibition of DNA repair processes.[56]

Due to the complexity of the effects, we evaluated an alternative substance as a source for DNA nicks. Paraquat (1,1′-dimethyl-4′,4′-bipyridilium dichloride/dimethylsulfate) is an herbicide known to continuously generate reactive oxygen species, which keeps a steady-state level of DNA breaks.[57] Paraquat is not known to inhibit DNA repair processes. We treated L1-transfected cells with different doses of Paraquat dichloride (0–50 μM) in the same manner as described for $NiCl_2$ above. Paraquat does not stimulate retrotransposition (Figure 1(d)), even though it creates significant levels of nicks detected by the COMET assay (data not shown). This observation suggests that nicks alone are not sufficient to stimulate retrotransposition, and that some other influence of the nickel on the cells is required for the L1 stimulation observed.

Magnesium reverses the L1 stimulation by nickel

Nickel is thought to influence a number of cellular processes, such as DNA repair and chromatin structure, by competing with magnesium required by various proteins.[49,56] Previous experiments show that several of the nickel effects, both *in vitro* and *in vivo*, can be reversed by the supplementation of magnesium.[58,59] We tested the ability of magnesium to reverse the influences of nickel on L1 activity. The L1 retrotransposition assay was performed on cells treated with 150 μM $NiCl_2$ supplemented with 150 μM $MgCl_2$ or 1 mM $MgCl_2$. Increasing levels of magnesium resulted in an almost complete block of the nickel stimulation on L1 retrotransposition (Figure 4). Treatment with up to 1 mM $MgCl_2$ alone did not affect L1 retrotransposition. It seems unlikely that magnesium would interfere with the generation of reactive oxygen species by nickel. Instead, a more plausible scenario is that the reversal of L1 stimulation is due to the ability of higher concentrations of magnesium to overcome the inhibition of magnesium-dependent enzymatic activities by $NiCl_2$, such as DNA repair.

L1 utilizes the same insertion sites in the presence of heavy metals

The stimulation of L1 retrotransposition induced by nickel appears to be independent of an increase in DNA nicks. However, a previous report demonstrated that an endonuclease-deficient L1 element inserted into atypical target sequences provided by endogenous nicks in a DNA repair-deficient cell line.[31] Using the same recovery system, we analyzed the pre and post-integration sites of L1 inserts from cells treated with 150 μM $NiCl_2$ or 100 μM $CoCl_2$. These doses were selected because they represent the dose with the highest level of L1 stimulation by $NiCl_2$ and the highest dose of cobalt that would still yield a reasonable number (~25/T75 flask) of neo^R colonies to attempt recovery. Recovered L1 inserts from untreated cells were used as control. All except one of the recovered clones (24 nickel, 20 of 21 cobalt and all 30 controls) contained L1 inserts with the typical A-tail. In addition, full sequence analysis of sample clones revealed that the inserts presented the usual hallmark target-site duplications (see Supplementary Data, Table 1s). Evaluation of the integration sites demonstrated that the majority of the L1 elements inserted into a site closely resembling the consensus sequence and no significant difference was observed between the treated and untreated samples (Figure 5). The distribution of L1 insert length of the nickel-treated cells included more of the shorter inserts than the recovered clones of the control (see Supplementary Data, Figure 3s). This would indicate that the L1 stimulation by nickel is not due to the generation of longer inserts. Notably, the L1 inserts recovered from the cobalt-treated cells tended to be longer than the control inserts. Because the treatment with cobalt did not stimulate L1 retrotransposition activity, interpretation of this observation is complex.

Discussion

Amplification of LINE elements occurs through an RNA intermediate *via* a process termed retrotransposition. In the first step, L1 is transcribed into RNA using an unusual internal RNA polymerase II promoter in its 5′UTR.[60] The bicistronic L1 RNA is thought to be transported to the cytoplasm, where both open reading frames are translated. Next, the L1-encoded proteins are likely to bind to the RNA to form a cytoplasmic RNP. This leads to a *cis*-preference of the proteins for the RNA that encoded them.[61] The L1 RNP must reach the nucleus for retrotransposition. The details of the subsequent steps of the L1 retrotransposition mechanism are not well understood. L1 elements are thought to integrate by a coupled reverse transcription/integration process previously referred to as target-primed reverse transcription (TPRT, Figure 6).[62] Initially, the L1-encoded endonuclease cleaves one strand of DNA with preference for its consensus target site (3′-AA/TTTT-5′), producing a 3′OH at the nick.[63] The L1 RNA base-pairs at the nick and the reverse transcriptase use the free 3′OH to prime reverse transcription. Interruption of the reverse transcription of the LINE RNA or internal priming of the second strand leads to a variety of 5′ truncated LINEs. The other strand of the genomic DNA is then cleaved near the target site by an unknown mechanism to produce a staggered break. The cDNA inserts into the break by an unknown mechanism, followed by removal of the RNA and completion of the cDNA synthesis, producing a complete insertion flanked by the target-site duplication.

The influence of nickel on the rate of L1 retrotransposition may occur at any of these steps. Our data obtained by using the endogenous L1 promoter on a reporter gene and the retroposition assays using a CMV-driven L1 plasmid suggest that the regulation is not at the level of transcription. In the reporter assay, the L1 promoter is almost 50% weaker in the presence of nickel, which would predict a potential reduction of the retrotransposition rate. Direct evaluation by Northern blot analysis demonstrates that nickel exposure does not increase L1 transcript levels. This demonstrates that the stimulatory effect must be at one of the post-transcriptional steps. Because the L1 retrotransposition reporter system involves expression from a transiently transfected plasmid, the assay should not be influenced by factors such as

methylation or chromatin structure. There are extensive data suggesting that these chromatin-level regulatory events may be important with L1 elements.[4,64,65] However, we have previously assessed the effect of several heavy metals on cell lines with a stable integration of the reporter plasmid.[35] Our data demonstrate that both assays yield equivalent results. Furthermore, nickel(II) has been reported to influence methylation and gene expression.[46,66] Thus, in addition to the influence of nickel on downstream steps of the retrotransposition process as demonstrated in these studies, we cannot rule out that nickel could alter expression from chromosomal sites not reflected by our stable cell-line assay.

Nickel salts can have a stimulatory influence on T-cell proliferation at modest doses.[67] In addition to previous observations,[8] we have data that suggest that L1 retrotransposition may be sensitive to changes in cell proliferation rate (Supplementary Data, Figure 2s). However, in HeLa cells, both the BrdU incorporation studies to measure proliferation rates and the FACS analysis of the cell-cycle distribution suggest that the proliferation of the cells is not affected significantly at the doses of nickel that lead to the stimulation of L1 retrotransposition. However, at higher doses of nickel, the cell-cycle is perturbed, and L1 retrotransposition drops sharply. These higher doses have been shown to have general toxic effects on cell growth and colony formation as evidenced by the control.

A previous report suggests that under certain conditions, L1 elements are able to take advantage of endogenous nicks in the genome, bypassing their requirement for the L1 endonuclease activity.[31] The increased nicks from nickel treatment are likely the result of a combination of DNA-damaging processes in conjunction with peroxides,[44] along with inhibition of the DNA repair processes that would increase the steady-state level of DNA nicks.[49] Thus, one possible mechanism for nickel's stimulation of L1 activity is through more frequent use of these increased nicks in the DNA. However, our data showing that Paraquat-induced DNA nicks do not increase L1 activity, as well as the observation that the insertion sites are still consistent with the use of the L1 endonuclease, suggests that L1 retrotransposition may be due to the inhibition effects of nickel on DNA repair enzymes rather than to an increase of DNA breaks caused by ROS.

Nickel, cadmium and cobalt have been reported to inhibit DNA repair processes, but different metals influence these processes at different steps.[49,52] Nickel and cadmium inhibit both base-excision and nucleotide-excision repair, while cobalt is more associated with inhibition of nucleotide-excision repair. Specifically, nickel and cadmium affect the ability to recognize the DNA damage, i.e. the initial step of nucleotide excision repair.[68] On the other hand, both the incision and the polymerization of repair patches are affected by cobalt.[69] There are several intermediates in the proposed L1 retrotransposition mechanism (Figure 6) that may be recognized by specific proteins in the repair apparatus and result in removal of the L1 intermediates. Thus, repair surveillance might be a critical part in keeping the retrotransposition rate minimized, suggesting that inhibition of DNA repair may be one of the ways nickel affects L1 retrotransposition.

Although mobile elements are responsible for a wide range of genetic defects,[16,70] there are few data on environmental influences that may accelerate the contribution of mobile elements to human genetic instability. Our finding that nickel can significantly stimulate retrotransposition is particularly relevant to the possible role of chronic exposures in both germline and somatic disease. Chronic exposures to heavy metals, like nickel, and other toxicants, through workplace and environmental exposure, may specifically increase the damage caused by retrotransposition over a long period of time. Accumulation of this damage may contribute to initiation and/or progression of cancer, as well as other diseases of chronic exposure. It is well established that although many heavy metals are not mutagenic on the standard bacterial assays, they are carcinogenic. Multiple models of how heavy metals lead to

cancer have been proposed. Because L1 transcription has been observed in differentiated cells,[24] our data suggest that genetic damage caused through the stimulation of mobile elements present in the genome needs to be considered. Thus, in addition to changing the level of germ-line genetic disease in exposed individuals, increased mobile element insertions may be another contributing factor by which heavy-metal exposure induces genetic instability in somatic cells, leading to cancer initiation or progression, aging, or other diseases associated with chronic exposure to carcinogenic/toxic compounds. Overall, these data suggest that damage caused by retrotransposition of mobile elements may need to be considered when developing mechanistic models for genetic damage associated with environmental exposures.

Materials and Methods

Plasmids

JM101/L1.3ΔCMV and JM102/L1.3 used in the retrotransposition assay[50] were a kind gift from Dr John Moran. Both plasmids contain a full-length L1 element with its two open reading frames driven by the endogenous L1 pol II promoter or the CMV promoter respectively, and both utilize an SV40 polyadenylation to aid transcription termination (schematic shown in Figure 1(a)). pIRES2-EGFP (Clontech) contains a neomycin-resistance cassette and was used in parallel in the retrotransposition assays as a combined control for transfection and cytotoxicity. The plasmid SynL1_neo (S. L. Gasior *et al*., unpublished results) was derived from JM101/L1.3K7i (a gift from Dr Moran) and used for recovery of LINE inserts. SynL1_neo was a multi-step construction. The NotI to Bst11071 fragment from pJM102/L1.3 was inserted into the same digest of L1.3cepK7i,[71] which we called L1_CMV_rec. This construct does not contain the L1.3 5′ UTR. We removed the episomal portion of pJM102/L1.3 by digestion with SgrAI and then with ExoIII timed to not digest the amp^R or L1 portions of vector. This DNA was then treated with mung bean nuclease and ligated. This vector is termed L1_CMV_epi-. We then constructed L1_CMV_rec_epi-by inserting into L1_CMV_rec digested with NruI and ApaI the same digest fragment containing the epi-deletion from CMV_L1_epi-. Synthetic L1 (synL1) was designed to remove all canonical polyadenylation sites within the L1-RP sequence in ORF1 and ORF2, and included non-synonymous changes to create a different set of restriction sites (sequence available upon request). The synL1 was synthesized by Bionexus (Oakland, CA) and included flanking unique NotI and EcoRI restriction sites. SynL1 was digested with EcoRI, blunted with mung bean nuclease and then digested with BstEII and inserted into L1_CMV_rec_epi- digested with BstEII and Bst11071. We call this construct SynL1_neo. To quantify the effects on L1 transcription, the full-length L1 promoter (base 1 to 910 of L1.3 PDB code L19088) was cloned into the HindIII site of the pGL3-Basic vector (Promega) upstream of the firefly luciferase gene (pFLIP-cor). The pRL-CMV vector (Promega), containing a *Renilla* luciferase gene driven by the CMV promoter, was used as the transfection control plasmid. All plasmid DNA was purified by alkaline lysis and twice purified by cesium chloride buoyant-density centrifugation. Final evaluation of the DNA quality was performed from the visual assessment of ethidium bromide-stained agarose gel electrophoresed aliquots.

Transfection and selection of cells

HeLa cells (ATCC CCL2) were grown in a humidified, 5% CO_2 incubator at 37 °C in Earl's minimal essential medium (EMEM). EMEM was supplemented with 10% (v/v) fetal bovine serum. HeLa cells were seeded in T-75 flasks at a density of 1.5×10^5 cells/flask and grown for 20 h prior to transfection. Cells were transfected with Lipofectamine Plus (InVitrogen) for 3 h using 1 μg of either the L1 or 0.3 μg of the neomycin control plasmid (pIRES2-EGFP) with 18 μl of the plus reagent and 12 μl of Lipofectamine, following the manufacturer's protocol. Following removal of transfection cocktail, the cells were treated with various doses of metals, as shown in Results, in EMEM plus 10% serum for 48 h. Treatment was removed and the

medium containing G418 (Fisher) at 400 μg/ml was added. After 14 days, the medium was aspirated, the cells washed in phosphate-buffered saline (PBS), and then the colonies were fixed and stained for 30 min with crystal violet (0.2% (w/v) crystal violet in 5% (v/v) acetic acid, 2.5% (v/v) isopropanol). The retrotransposition efficiency was then determined as the number of visible neo^R-resistant colonies using an Oxford Optronics ColCount colony counter.

Northern blot analysis

RNA extraction and poly(A) selection was performed as described.[33] Total RNA was extracted from four 75 cm^2 cell-culture flasks using the recommended protocol for the TRIzol reagent (Invitrogen). The PolyATract mRNA Isolation System III (Promega) was used to select polyadenylated RNA species following the manufacturer's protocol. Poly(A) selected from the four flasks was pooled, and the precipitated RNA was resuspended in 30 μl of RNase-free water and fractionated in a formaldehyde/1% (w/v) agarose gel. RNA was transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) by capillary transfer overnight at room temperature in a standard 5 × sodium chloride/sodium citrate (SSC) solution. The RNA was cross-linked to the membrane using a UV lamp (GS Gene linker, BioRad) and pre-hybridized in 30% (v/v) formamide, Denhardt's solution, 1% (w/v) SDS, 1 M NaCl, 100 μg/ml of salmon sperm DNA, 100 μg/ml of yeast tRNA at 60 °C for at least 6 h. DNA template for the probe was produced by PCR with the primers that amplified the 3′ region of the neomycin gene (forward: 5′-CGACCCAACACC-CGTGCG-3′ ; reverse: 5″-AGGACGAGGCAGCGCGGC-3′. PCR products were fractionated on a 1% low-melting agarose gel, excised, and purified using a QIAquick gel extraction kit (QIAGEN). Random labeling was performed using the Megaprime DNA-labeling system (Amersham Biosciences) following the manufacturer's recommended protocol (Ambion). Hybridization with the randomly labeled probe (final concentration of 4×10^6–8×10^6 cpm/ml) was carried out overnight in the same solution at 60 °C. Multiple ten-minute washes were performed at high stringency (0.1 × SSC, 0.1% SDS) at 60 °C. The results of the Northern blot assays were quantified on a Typhoon Phosphorimager (Amersham Biosciences) using the ImageQuant software.

Recovery of L1 inserts

To recover L1 insertion events, the G418-resistant colonies obtained from the treated and untreated transfections of HeLa cells with SynL1neo were allowed to grow until easily visible by eye. The cells were then trypsinized, pooled together and seeded on a 100 mm × 15 mm dish. The cells were grown to confluency under G418 selection. The cells obtained were utilized for DNA extraction using the DNA Easy kit (Qiagen) following the manufacturer's recommended protocol. Following a previously described protocol,[31,71] 200 μg of extracted DNA was digested overnight at 37 °C with 200 units of HindIII and then heat-inactivated at 65 °C for 20 min. The digested DNA was diluted in a volume of 1000 μl containing phage T4 ligase buffer and 1200 units of T4 ligase and incubated overnight at room temperature. After ligation, the sample was concentrated using a Microcon YM-50 filter (Amicon), washed once with 500 μl of distilled water and finally concentrated to a final volume of approximately 20 μl. Electrocompetent *Escherichia coli* EP10MAX (BioRad) were incubated with 2 μl of the sample in a 0.4 cm cuvette (BioRad) and pulsed using a MicroPulser power source (BioRad) at the manufacturer's preset conditions for bacteria. Bacteria were plated on LB plates containing 50 μg/ml of kanamycin. Plasmid DNA was obtained from individual bacterial colonies using the Wizard Plus SV miniprep purification system (Promega). Size of L1 inserts were initially analyzed by restriction site mapping. Samples were sent for sequencing to the Translational Genomics Research Institute (TGen) Tempe, AZ. DNA Star software was utilized for sequence analysis. Sequences are available upon request.

BrdU incorporation

Cell proliferation was assayed with a BrdU ELISA kit (Roche). HeLa cells were cultured in 96-well plates at the same density as for the retrotransposition assay and labeled with BrdU for the last 2 h of the 48 h treatment at the appropriate doses of heavy metals. Cells were treated as recommended by the manufacturer's protocols. Chemiluminescence was measured on an Orion microplate luminometer with the Simplicity computer program and exported into a Microsoft Excel worksheet for analysis.

Flow cytometric analysis of the cell-cycle

HeLa cells treated as in the retrotransposition assay were fixed with 70% (v/v) ethanol following treatment with heavy metals for 24 h. Cells were suspended in a 69 μM propidium iodide, 38 mM sodium citrate solution for 45 min. Cells were separated by filtration through nylon mesh with 70 μm pore size. Flow cytometry was carried out on a Becton Dickinson flow cytometer and 50,000 events were collected. Data including debris/aggregate gating and the percentage G1/S/G2 was analyzed.

L1 promoter assay

HeLa cells, 300,000 per T75 flask, were co-transfected with 2 μg of pFLIP-cor, or pGL3-Basic (Promega) along with pRL-CMV (Promega) as an internal control, treated with heavy metals at various concentrations for 24 h and harvested for luciferase activity following the manufacturer's recommended protocol. Firefly and *Renilla* luciferase activities were measured using a Dual Luciferase kit (Promega) and an Orion microplate luminometer. The files generated on Simplicity Software were exported into Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

LINE	long interspersed repeated element
UTR	untranslated region
ORF	open reading frame
IARC	International Agency for Research on Cancer
Neo^R	neomycin resistance
BrdU	bromodeoxyuridine
FACS	fluorescence-activated cell sorter
RNP	ribonucleoprotein complex
ROS	reactive oxygen species

TPRT target-primed reverse transcription

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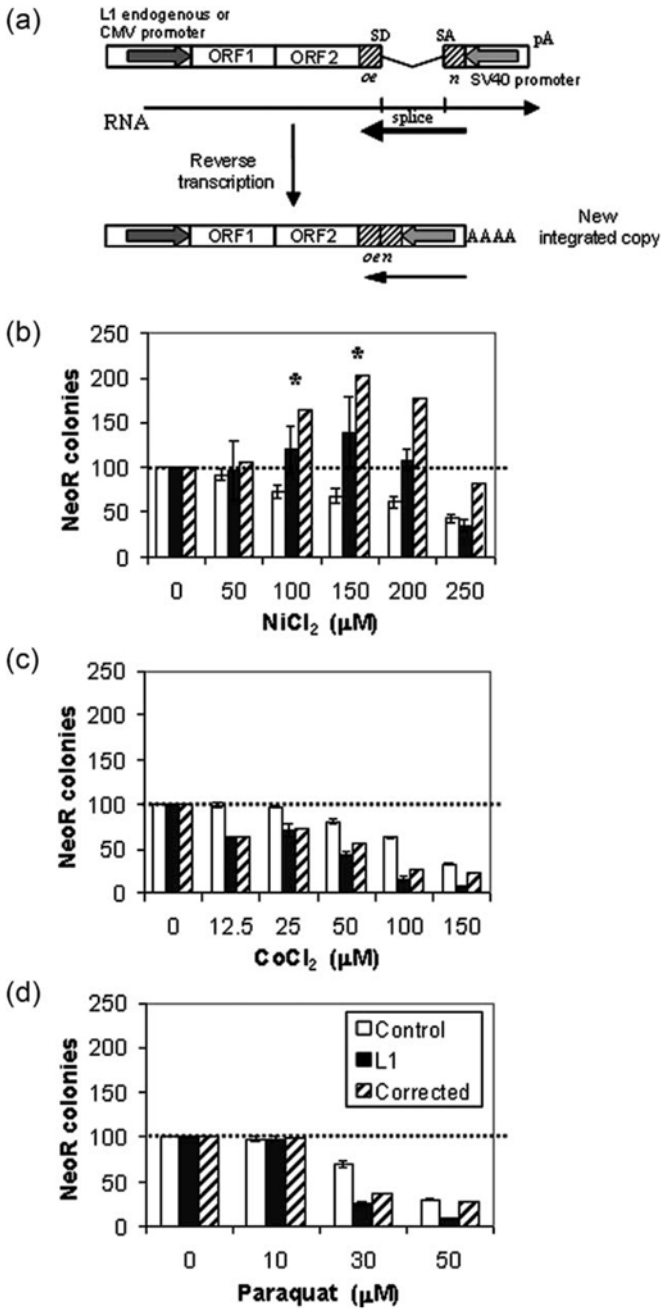

Figure 1.

(a) Schematic of the L1 assay system. Top: RNA transcription is driven by the internal L1 promoter located in the 5′ untranslated region (UTR) or the CMV promoter. The construct contains the SV40 promoter in the 3′UTR in the "reverse" direction that will transcribe a *neo* gene containing a "forward" intron that blocks proper expression of the neomycin resistance. The intron interrupting the neomycin-resistance gene will be removed by splicing from RNA generated from the L1 or CMV promoter. In the L1 retrotransposition process, the RNA is reverse transcribed, followed by integration of the DNA into the genome. Bottom: The new L1 copy contains a functional *neo* gene. Only newly integrated copies that retrotransposed from the spliced L1 RNA will present neomycin resistance. Promoter and transcription

orientations are indicated by black arrows. SD, splice donor; SA, splice acceptor; pA, SV40 polyadenylation signal. The *neo* gene in the opposite orientation relative to the L1 gene is shown as a hatched box. RNA is represented by thin lines with arrows to show the direction of transcription. Note that the Figure is not drawn to proportion. (b)Effect of $NiCl_2$, (c) $CoCl_2$ and (d) Paraquat dichloride on L1 retrotransposition activity in tissue culture. Neo^R colonies from separate L1 transfections (black bar) treated with different doses of nickel chloride, cobalt chloride, or Paraquat dichloride are shown. An unrelated plasmid encoding neomycin-resistance was used as a transfection and toxicity control (open bar). The data are also shown adjusted for toxicity (hatched bar). Three independent assays in triplicate (n=9) were performed in HeLa cells and error bars indicate standard deviations. The treatment with 100 μM and 150 μM nickel showed a statistically significant difference from no treatment (Student's t-test $p<0.01$(*)). Nickel stimulates L1 retrotransposition in a dose-dependent manner around 2.5-fold, but cobalt and Paraquat have no stimulatory effect.

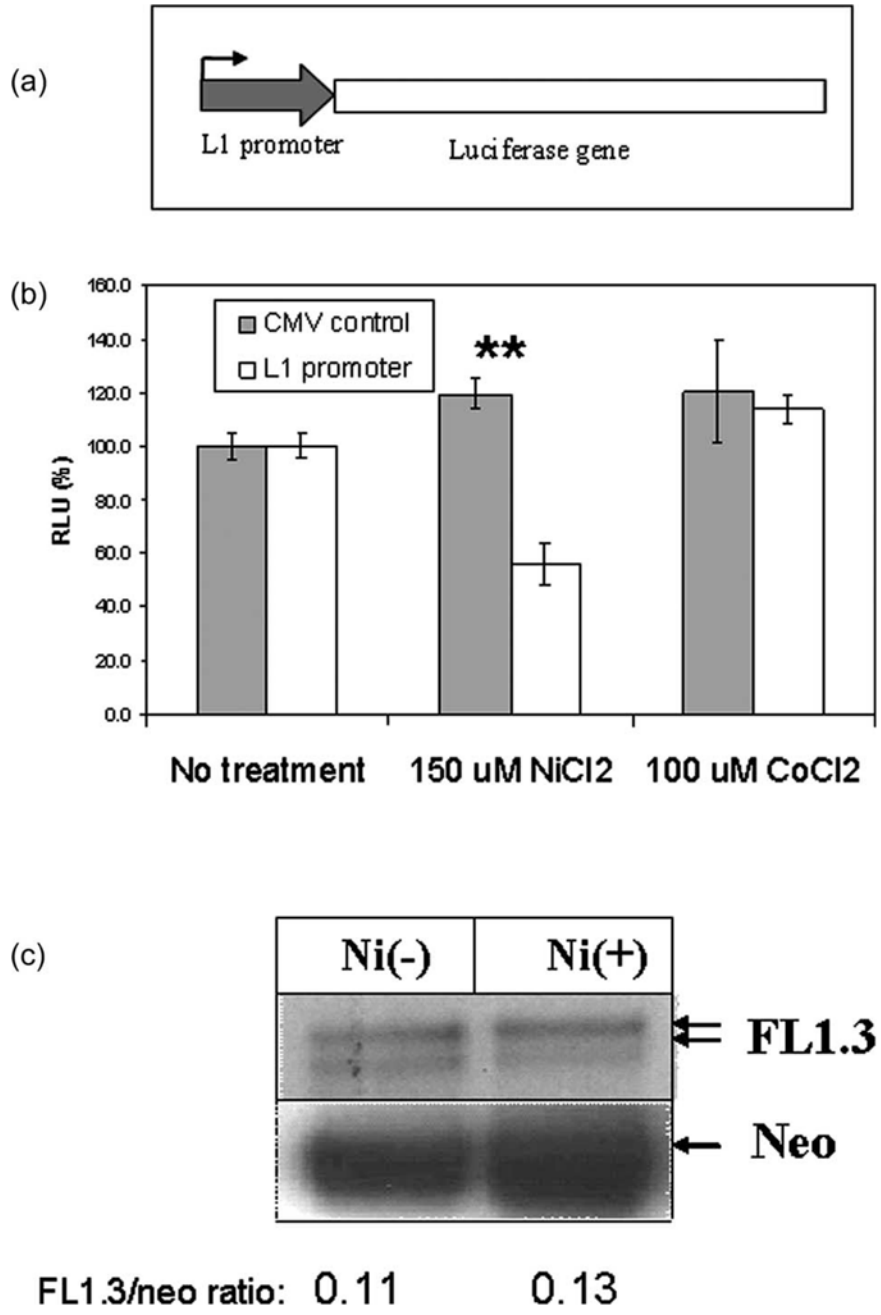

Figure 2.

Effect of $NiCl_2$ on L1 promoter activity. (a) Schematic of the construct containing the L1.3 promoter (5′UTR sequence) cloned in front of the firefly luciferase gene. The L1 promoter is an internal promoter and transcription start is indicated by an arrow. (b) HeLa cells were transiently transfected with the L1 promoter or CMV-luciferase plasmid together with a plasmid expressing *Renilla* luciferase (pRL-CMV) used as a transfection control to which all results were normalized and expressed as percentage relative light units (RLU). The no-treatment control was used as 100%. Luciferase activity decreased about twofold when cells were treated with 150 μM $NiCl_2$ (** Student's paired *t*-test $p < 0.00001$ relative to no treatment control). (c) Effect of treatment with $NiCl_2$ on L1 RNA levels. Evaluation of L1 expression

levels was performed by Northern blot analysis of poly(A)-selected RNA from NIH 3T3 cells transiently transfected with the L1.3 Neo expression vector after treatment with 150 μM $NiCl_2$ (Ni(+)). Untreated, transiently transfected cells were used as control (Ni(−)). Full-length L1.3 (FL1.3) and neomycin (Neo) mRNAs were detected by hybridization with randomly labeled Neo probe. Neomycin expression was used as an internal control to correct for transfection and loading variation. The ratio of the full-length L1 transcript/neo control transcript for treated and untreated cells is indicated.

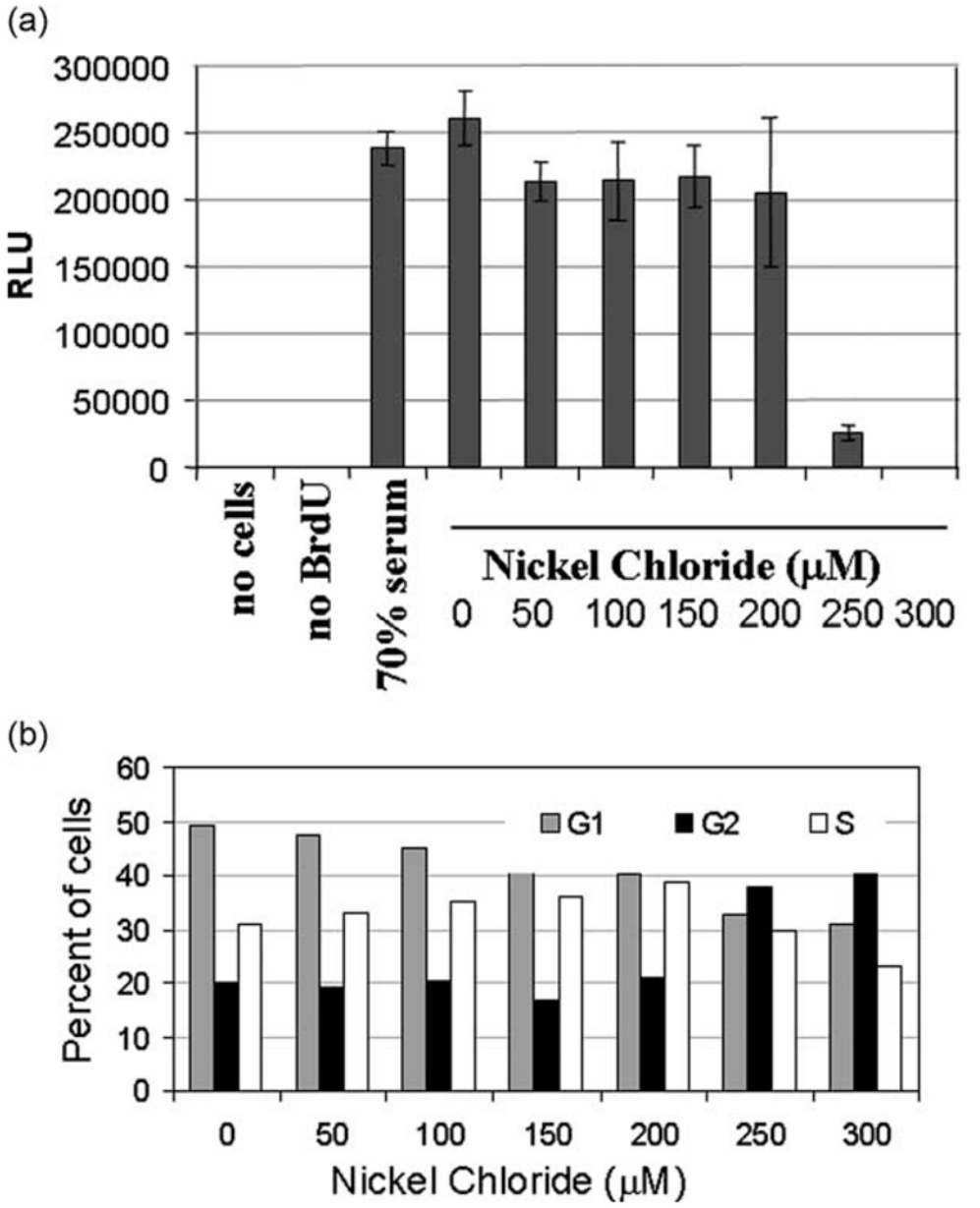

Figure 3.

(a) Evaluation of the effect of $NiCl_2$ and $CoCl_2$ on HeLa cell proliferation using the bromodeoxyuridine (BrdU) assay. Incorporation of BrdU was measured as relative light units (RLU) in response to various doses of $NiCl_2$ (0, no treatment; 1, 50 μM; 2, 100 μM; 3, 150 μM; 4, 200 μM; 5, 250 μM; and 6, 300 μM) or $CoCl_2$ (0, no treatment; 1, 12.5 μM; 2, 25 μM; 3, 50 μM; 4, 100 μM; and 5, 150 μM) as well as several control conditions (open bars) to evaluate whether cellular proliferation occurs in response to the heavy-metal treatments utilized. The no-treatment data were used to define 100% or baseline proliferation. Bars represent the averages of BrdU incorporation normalized relative to 100%, with the standard deviation shown as error bars. No significant increase in cell proliferation was seen in response to nickel or cobalt. (b) Cell-cycle distribution in response to $NiCl_2$. HeLa cells were exposed to various doses of $NiCl_2$, stained with propidium iodide and the cell-cycle was measured using

fluorescence-activated cell sorting (FACS). Populations of cells in different stages of the cell-cycle (G1, G2 and S) are shown in the graph, together with the control. No effect on cell-cycle distribution in response to nickel was observed at doses below the cytotoxic threshold.

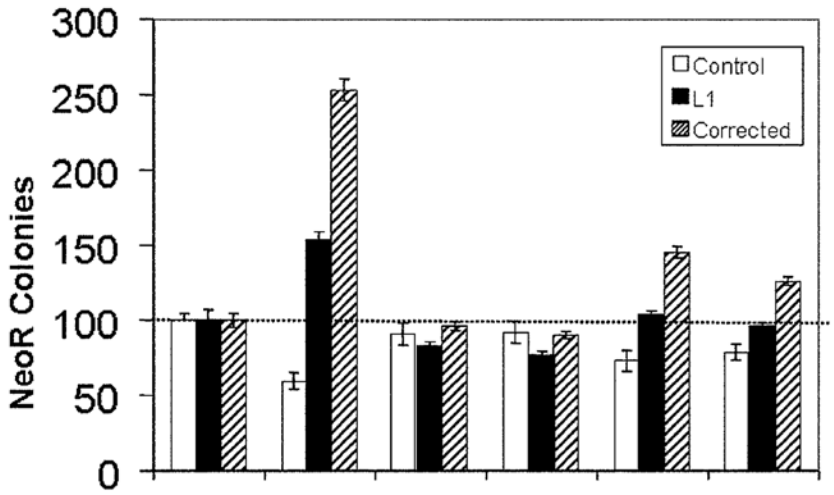

Figure 4.

Effect of the presence of added $MgCl_2$ on the $NiCl_2$ retrotransposition activity. The L1 retrotransposition activity was evaluated after treatment for 48 h with: 0 or medium control; 150 μM $NiCl_2$ (positive control); 150 μM $MgCl_2$ and 1 mM $MgCl_2$ (negative controls); 150 μM $NiCl_2$+150 μM $MgCl_2$; 150 μM $MgCl_2$+1 mM $MgCl_2$. Neo^R colonies from separate L1 transfections are shown.

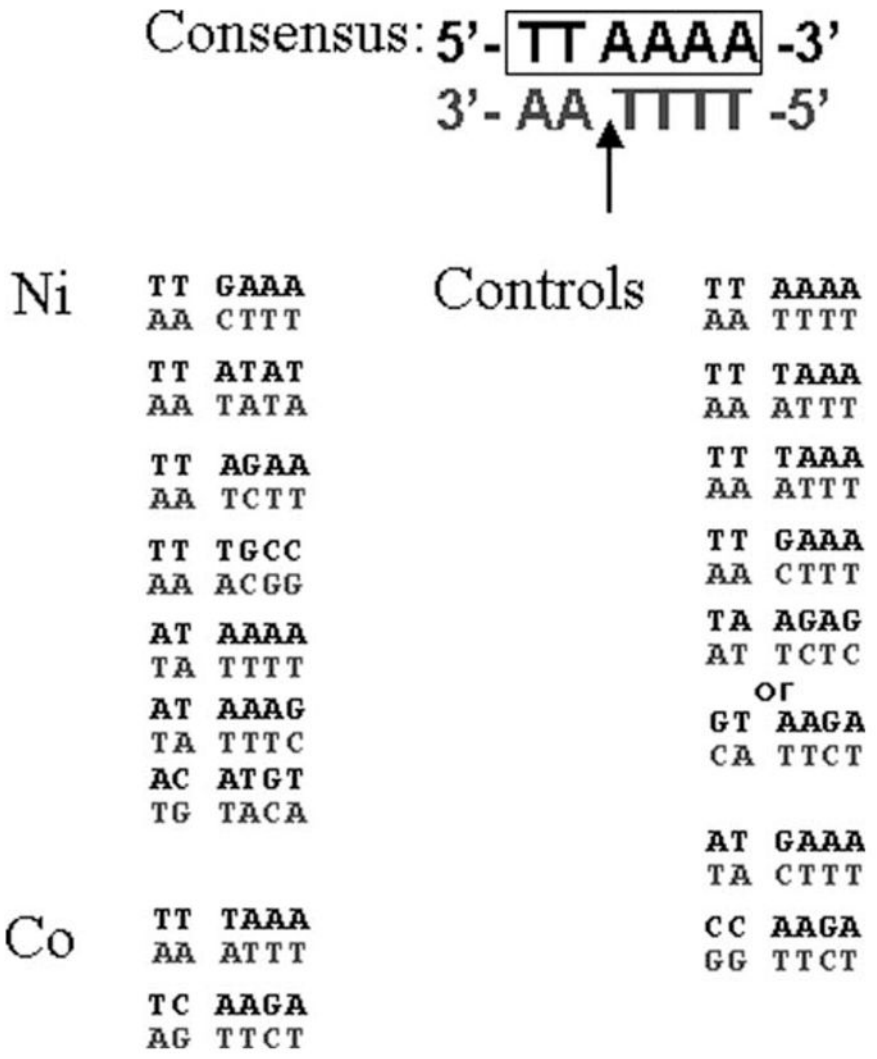

Figure 5.

Endonuclease cleavage sites from L1 inserts. L1 inserts were recovered from the transiently transfected cells treated with 150 μM $NiCl_2$ or 100 μM $CoCl_2$ and from untreated cells (control). Comparison of the sequences of recovered L1 inserts and predicted pre-integration sites retrieved from the human genome database allowed for the characterization of the insertion site. The consensus for the L1 endonuclease site is shown in the box at the top. Note that the first cleavage (indicated by an arrow) occurs on the opposite strand shown in gray.

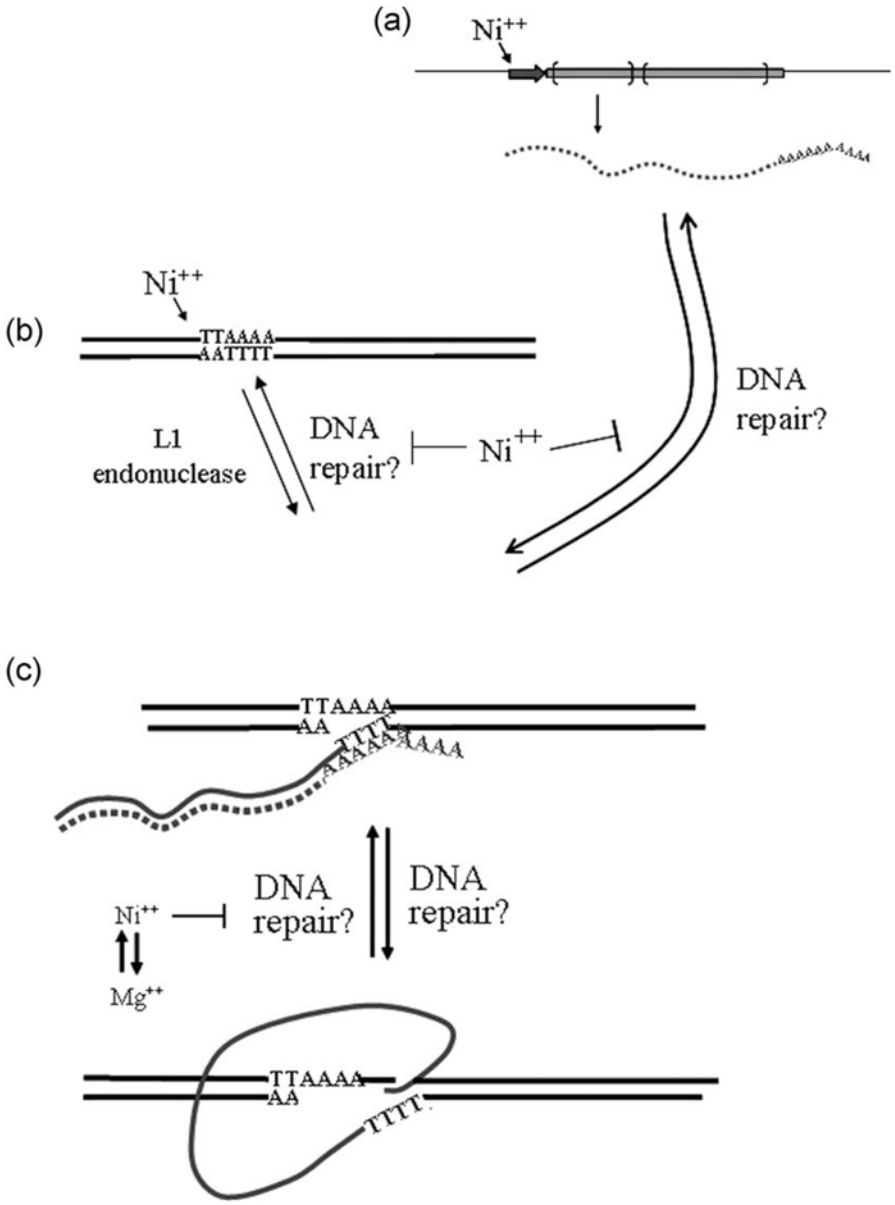

Figure 6.

Model depicting the steps where nickel can potentially affect L1 retrotransposition intermediates. (a) Transcription: the first step in the retrotransposition process is the formation of a polyadenylated L1 transcript (broken line) from an L1 locus. It seems likely that increased expression of an L1 locus will result in a greater retrotransposition rate, although direct studies correlating transcript level and retrotransposition have not been carried out. Although heavy metals affect expression of many genes, L1 transcription is not increased by nickel exposure. (b) Generation of DNA nicks: it has been proposed that the L1 RNA, complexed with ORF2 protein that has endonuclease and reverse transcriptase activities, migrates to the genome where the endonuclease cleaves at the consensus 5′-TTAAAA-3′/3′-AA↑TTTT-5′, as shown. The T bases then prime reverse transcription of the RNA using the reverse transcriptase activity as shown. The generation of DNA nicks by nickel oxidation could potentially increase available priming sites for the L1 RNA. However, our data do not support this hypothesis. DNA repair

processes are likely to be involved in repairing the DNA breaks generated by the L1 endonuclease preventing the L1 retrotransposition process. Inhibition of DNA repair enzymes at this step could favor the L1 insertion. (c) Reverse transcription, cDNA generation and integration. Recognition of the L1 complex during integration by the DNA repair machinery could result in its removal (a) and inhibition of the retrotransposition rate. In addition, completing the retrotransposition requires a second nick, caused by an unknown source, and linkage of the 3′ end of the cDNA to the chromosome. The cell must then complete second-strand synthesis and ligate the gaps. At least some of these steps must involve endogenous cellular activities. Potential nickel inhibition of DNA repair enzymes or other cellular proteins involved in this step that require Mg^{2+} could alter the equilibrium favoring the generation of new L1 inserts. Our data favor this hypothesis, where the increase in L1 activity is due to the nickel effect on cellular enzymes, in particular those involved in DNA repair.